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***Labelling techniques of
biomolecules for
targeted radiotherapy***

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FOREWORD

Radiotherapy using open radioactive sources has become an important and promising mode of treatment of cancer patients, particularly in conjunction with other modalities like surgery and chemotherapy. For this purpose therapeutic radiopharmaceuticals are designed to deliver high doses of radiation to selected target organs or tissues with an aim of minimizing unwanted radiation to surrounding healthy tissue.

Several radionuclides have been developed and tagged into appropriate carriers for targeted radiotherapy. As radionuclide carriers, chelating chemical agents, monoclonal antibodies, peptides, biodegradable particles, colloids, etc., have been investigated as radiopharmaceuticals for therapeutic nuclear medicine.

In spite of the potential of targeted radiotherapy to treat a wide range of malignant conditions, the routine clinical use has been hampered by the lack of suitable radionuclide carrier molecules which would adequately concentrate these radionuclides in target tissues of interest. Much higher and specific uptake of the radiopharmaceutical in the target tissue is needed for effective therapeutic applications than is possible with most of the diagnostic agents currently available. Following the recommendations of a group of experts in 1997, and in view of the realization that higher levels of targeting for therapeutic agents would be possible using biomolecules such as monoclonal antibodies, peptides and other receptor avid molecules as carriers of the radionuclide, in 1998 the International Atomic Energy Agency (IAEA) launched a Co-ordinated Research Project (CRP) on Labelling Techniques of Biomolecules for Targeted Radiotherapy. The CRP aimed at developing and optimizing existing procedures for radiolabelling of monoclonal antibodies and small peptides with therapeutic radionuclides, such as ^{90}Y , ^{188}Re and others.

With the participation of 15 scientists from selected research institutes in Argentina, Austria, Brazil, Cuba, Greece, Finland, Hungary, India, Italy, Mexico, Pakistan, China, Romania, Thailand and Uruguay several laboratory procedures and protocols for the radiolabelling and quality control of monoclonal antibodies and peptides were optimized. Moreover, the CRP provided the participants an opportunity to study the feasibility of using bioactive molecules for developing radiopharmaceuticals and studying its biological efficacy in tumour models. The first part of the report includes standardized labelling and quality control protocols used and validated during the CRP; the second part includes reports of each individual research agreement and contract holder.

The IAEA wishes to thank all the participants in the CRP for their valuable work and scientific contributions. The IAEA officers responsible for this publication were H. Vera Ruiz and D.V.S. Narasimhan of the Physical and Chemical Sciences Division.

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CONTENTS

INTRODUCTION.....	1
ACHIEVEMENTS OF THE CRP	2
SCOPE OF THE CRP AND TECHNICAL OBSERVATIONS	3
REMARKS ON METHODOLOGIES FOR RADIOLABELLING AND QUALITY CONTROL OF SELECTED BIOMOLECULES	14
POTENTIAL AREAS OF FUTURE RESEARCH	16
COLLABORATIVE ACTIVITIES	17
TECHNICAL CONCLUSIONS OF THE CRP.....	17
PUBLICATIONS AND PRESENTATIONS ORIGINATING FROM THE CRP	18
BIBLIOGRAPHY	25
COUNTRY REPORTS	
Optimization of biomolecules labelling with Rhenium-188 using direct and indirect methods.....	31
<i>S.G. de Castiglia, J. Crudo, E. Obenaus, M. Edreira, E. D'orio</i>	
Labelling of peptides with $^{188}\text{Re}/^{99}\text{Tc}^{\text{m}}$ and monoclonal antibodies with $^{90}\text{Y}/^{111}\text{In}$	45
<i>B. Linkowski Faintuch, C.P.G. Silva, N.P.S. Pereira, E.B. Araujo</i>	
Labelling technique of biomolecules for target radiotherapy	65
<i>Hongsheng Bai, Jin Xiaohai, Cheng Zhen, Jia Bing Fan Hongqiang, Lu Weiwei</i>	
Evaluation of biomolecules for metabolic radiotherapy	73
<i>G.J. Pimentel, M. Miranda, W. Quesada, H.E. Garay, O. Reyes, J. Vazquez, L.J. Cruz, I. Sanchez</i>	
Development of radioactively labelled cancer seeking biomolecules for targeted therapy	85
<i>A.D. Varvarigou, S.C. Archimandritis, M. Fani, P. Bouziotis, S. Vranjez, S. Xanthopoulos, A. Leonti, Th. Sourlingas, M. Sotiropoulou</i>	
Preparation, quality control and animal testing of Re-188 labelled compounds	99
<i>G.A. Jánoki, L. Körösi, L. Balogh, G. Andócs, K. Bodó, J. Láng, A. Polyák, R. Király, Domokos</i>	
Development of radioactively labelled cancer seeking biomolecules for targeted therapy ...	107
<i>M.R.I. Pillai, M. Venkatesh, S. Banerjee, G. Samuel, K. Kothari, A. Dash, P.R. Unni, A. Korde, K. Bapat, U. Pandey, T. Dash, S. Chakraborty, A. Mukherjee, H.D. Sharma</i>	
Labelled peptides through the DOTA chelating agent for diagnosis and treatment of receptor positive tumours	123
<i>M. Chinol</i>	
Labelled biomolecules for targeted radiotherapy: Biodistribution in mice with induced malignant tumours.....	129
<i>C. Arteaga de Murphy, G. Ferro-Flores, M. Pedraza-López, E.M. Molina-Trinidad, E. Murphy-Stack, S. Hernández-Gutiérrez</i>	

Labelled biomolecules with ^{153}Sm , ^{188}Re , and ^{90}Y for targeted radiotherapy.....	139
<i>A. Mushtaq, S. Pervez, M. Khalid</i>	
Development of ^{188}Re radiolabelling procedures of peptides and monoclonal antibodies.....	155
<i>V.V. Lungu, G. Mihailescu, D. Niculae</i>	
Radiolabelling of monoclonal antibodies for radiotherapy.....	165
<i>J. Sangsuriyan, C. Wardwilai, W. Iamsam-Ang, T. Ngamprayad, P. Picha, M. Dangpraset</i>	
Labelling and control of biomolecules with ^{188}Re and ^{153}Sm	183
<i>H.S. Balter, E.S. Verdera, G. Rodríguez, P. Oliver, B. Souto, L. Mallo, A. Robles</i>	
LIST OF PARTICIPANTS	197

INTRODUCTION

Malignant tumour disease accounts for approximately one third of deaths worldwide. Gastrointestinal adenocarcinomas, prostate and breast cancers are among the most frequently appearing tumours. Radiotherapy is an essential mode of treatment of all cancer patients either alone or in conjunction with other modalities like surgery and chemotherapy. In most cases radiotherapy is given using external radiation sources. It is also possible to administer radiotherapy by specifically localizing radioisotopes emitting particulate radiation in the tumour tissue. This targeted therapy has proved to have several advantages over external beam therapy, notably the possibility of selectively delivering higher radiation doses to the targeted tumour cells and treating multiple metastases. Procedures for therapy of thyroid carcinoma and hyper-thyroidism using radioiodine (^{131}I) introduced about five decades ago, have stood the test of time and are still widely used the world over. In addition to the therapeutic nuclides of the first generation ^{131}I , ^{89}Sr , ^{32}P , ^{90}Y , etc., which are still widely utilized and accepted by the medical community, many other beta emitting radionuclides with relatively short half-lives such as ^{153}Sm , ^{186}Re , ^{188}Re , ^{166}Ho , ^{165}Dy , etc. have also been recently made available for therapy and used with promising good results.

In spite of the potential of targeted radiotherapy to treat a wide range of malignant conditions, routine clinical use is mostly confined to therapy of thyroid carcinoma, hyperthyroidism, metastatic bone pain and synovectomy. In most of the cases, the limitation is obviously not the availability of suitable radionuclides but rather the lack of suitable carrier molecules that would adequately concentrate these radionuclides in target tissues of interest. Much higher and specific uptake of the radiopharmaceutical in the target tissue is needed for effective therapeutic applications than is possible with most of the diagnostic agents currently available. It is expected that higher levels of targeting needed for therapeutic agents would be possible using biomolecules such as monoclonal antibodies, peptides and other receptor avid molecules as carriers of the radionuclide.

During the last decade considerable efforts were directed towards stable binding of many radionuclides to monoclonals using a variety of bifunctional chelating agents (BFCA). Many antibody based agents have now been cleared for human diagnostic use. Radiolabelled antibodies to treat successfully hematological malignancies have been reported. Attempts were also made to pre-target tumours with other substrates and accelerated clearance of circulating antibodies to achieve the necessary specific concentration. Based on the experience gained with diagnostic agents, it can be expected that monoclonal antibody based therapeutic radiopharmaceuticals will soon be available for regular clinical use.

Moeover, the introduction of small peptides as carrier molecules for radionuclides has opened up new possibilities for development of diagnostic agents as evidenced by the use of ^{111}In -octreotide. Efforts are underway to extend the use of peptides as carriers for other therapeutic radionuclides such as ^{188}Re , ^{90}Y , ^{153}Sm , etc. The initial successful attempts to use Auger electrons of ^{111}In for therapeutic effect by high dose administration of ^{111}In octreotide has provided a strong impetus for the search, development and use of more accessible and perhaps more economical radionuclides like ^{153}Sm , ^{90}Y , ^{177}Lu , etc. coupled with peptides and monoclonal antibody carriers. The incorporation of beta emitters into a variety of bifunctional chelates of bioactive peptides including octreotide and VIP analogues is vigorously pursued at many leading laboratories. These efforts are expected to result in the development of several new radiopharmaceuticals for the radiotherapy of a number of malignancies.

Based on the above considerations, the scope of the Co-ordinated Research Project (CRP) has focused on the synthesis of the required BFCAs for MoAbs and peptide labelling, development and optimization of radiolabelling techniques with beta emitting radioisotopes, including quality control procedures and evaluation of the labelled products in *in vitro* and *in vivo* models. Biomolecule carriers that were considered for investigation included somatostatin analogues like lanreotide, Tyr-octreotide, ior-P1394 and vasointestinal peptide (VIP), as well as anti-CEA monoclonal antibody. Using either the bifunctional chelate approach or direct reductive exposure of -SH groups, labelling of these biomolecules with therapeutic radionuclides was pursued. Through these attempts it was expected to identify one or two agents with high radiochemical purity and labelling efficiency for further evaluation. Promising agents would be selected for further investigation using biodistribution studies in animals and uptake in tumour bearing animals.

Fifteen selected research institutes from Argentina, Austria, Brazil, Cuba, Greece, Finland, Hungary, India, Italy, Mexico, Pakistan, Peoples Republic of China, Romania, Thailand and Uruguay with recognized expertise in the field of therapeutic radionuclides and radiopharmaceutical research were selected to participate in the CRP. The first research co-ordination meeting to plan the work and define the research protocols to be investigated was held in Milan, Italy in July 1998. At this meeting it was decided to concentrate the efforts on two radionuclides, ^{188}Re and ^{90}Y , which are produced by generator systems and result in carrier free products; lanreotide was selected as a model peptide. Synthesis of the bifunctional chelating agent DOTA was also recommended for the labelling of biomolecules with ^{90}Y . The development of the corresponding coupling techniques, optimization of protocols for direct and indirect labelling of lanreotide and anti-carcino embrionic antigen (CEA) monoclonal antibody and *in vitro* evaluation in cell lines and membrane receptors were also included in the work plan for the following period. IgG labelled with ^{131}I and ^{125}I and $^{99}\text{Tc}^{\text{m}}$ was also included in the work plan for the initial investigation and method evaluation.

The second RCM to review the progress and discuss future work was held in Mumbai, India in February 2000. In view of the progress reported at this meeting, it was recommended that in the remaining period the CRP should concentrate on the testing of *in vitro* bioactivity of the labelled ^{188}Re lanreotide, confirm the results reported on the direct labelling method for ^{188}Re -lanreotide and continue the investigations to label DOTA conjugated peptides with ^{90}Y . The third and final RCM was held in Budapest, Hungary in October 2001 to assess the progress and achievements of the CRP and draft the final report.

A list of general references to guide the reader into recent research work done in the field is given in Section VIII.

ACHIEVEMENTS OF THE CRP

- The CRP provided an opportunity for the participants to study the feasibility of using bioactive molecules for developing radiopharmaceuticals and studying its biological efficacy in tumour models.
- Most of the participants achieved the development of technology for the preparation of a generator, synthesis of bifunctional chelating agents and radiolabelling of the biomolecules with several radionuclides.

- Radiolabelling of lanreotide with ^{90}Y through bifunctional chelating agent was also achieved. The procedure developed is suitable for making clinical doses of ^{90}Y labelled lanreotide.
- Radiolabelling of peptides, in particular lanreotide, in microgram quantities with ^{188}Re was done during the course of the CRP. Radiolabelling studies are significant in that they provided opportunities to develop radiotracers in nanomolar concentration of ligands/ peptides. The studies carried out during the CRP also culminated in the development of directly labelled ^{188}Re -lanreotide. The labelling technique developed could be extended to several other biomolecules.
- The participants developed several quality control techniques for radionuclides and for labelled peptides, which will be useful for evaluating peptide-based radiopharmaceuticals.
- The participants during the course of the CRP developed *in vitro* and *in vivo* biological techniques. These techniques are essential and the knowledge accumulated is relevant for studying the biological efficacy of receptor-based radiopharmaceuticals.
- Most of the participants generated good quality scientific data, which were presented in meetings and published in journals.
- Interactive co-operation and collaboration was established between several participants. Both information and material exchange were established between participants. The collaborations established between the groups during the course of the CRP are expected to continue and benefit the participants.

SCOPE OF THE CRP AND TECHNICAL OBSERVATIONS

Radionuclides

Rhenium-188

^{188}Re is an attractive radionuclide for use in targeted radiotherapy due to its favourable physical characteristics. Another advantage is its availability through a generator system. Production of the parent (^{188}W) involves a double neutron capture reaction and requires a very high neutron flux in the order of 10^{15} n/s/cm² to get good yield. The major problem with the production of $^{188}\text{W}/^{188}\text{Re}$ generator is therefore the availability of high specific activity ^{188}W .

Two batches of $^{188}\text{W}/^{188}\text{Re}$ generator from MAP Medical Technologies Oy, Finland were made available by the IAEA to all participants for laboratory use and animal studies. Evaluation of generator parameters was done until six months after calibration to assure good quality of the eluates for their use in radiolabelling studies. The results indicated that elution yield was in the order of 70-80% and elution profile showed the maximum of activity (88% of total activity) in the first three mL. Thus the activity concentration could be at least 10 mCi/mL, which is sufficient for peptide labelling. The pH was according to specifications. Chromatography in saline and methylethylketone (MEK) as solvents on Whatman N° 1 showed radiochemical purity higher than 99%. The level of radionuclidic impurities, as determined by gamma ray spectrometry, was in all cases below the limits allowed for this type of generators. The presence of ^{188}W ranged from 5.2 to $9.6 \times 10^{-3}\%$. Chemical controls showed that levels of Al^{3+} were in all cases lower than 5 ppm. Moreover, no toxic effects were observed in the studies conducted in animals.

Also, some participants used $^{188}\text{W}/^{188}\text{Re}$ generators from Oak Ridge National Laboratory, USA. The elution volumes were generally higher thus requiring a further step of concentration.

^{90}Sr - ^{90}Y generator

^{90}Y was selected as the radionuclide of choice for labelling biomolecules through a chelating agent. Commercial source of ^{90}Y is expensive and hence efforts are to be directed towards developing ^{90}Sr - ^{90}Y generator for local production of ^{90}Y . One generator concept was developed in India based on the supported liquid membrane technique. The generator consists of a glass cell comprising of two compartments, namely feed and receiver, of capacity 5 mL each, separated by a polytetrafluoroethylene membrane impregnated with 2-ethylhexyl-2-ethylhexyl phosphonic acid (KSM-17). A suitable aliquot of high level waste depleted of actinides-lanthanides and ^{137}Cs in 0.5 M HNO_3 (up to 100 mCi, 3.7 GBq) was diluted to 5 mL, adjusted to pH 1-2 and used as feed. 5 mL of 1 M HCl/HNO_3 was used in the receiver compartment. Both the feed and receiver compartments were constantly stirred with small teflon coated magnetic stirrer to enable quick and effective transport of ^{90}Y across the membrane. After 4 hours (h) the solution containing yttrium chloride/nitrate was drawn from the receiving compartment and used.

Solvent extraction/absorption of ^{90}Y from ^{90}Sr

An alternate method is the separation of ^{90}Sr and ^{90}Y by solvent extraction followed by ion exchange chromatography. This separation is based on an initial solvent extraction of ^{90}Y from dilute nitric acid solution of the ^{90}Sr - ^{90}Y mixture using D_2EHPA /dodecane. Further purification of ^{90}Y was carried out using ion exchange technique based on the absorption of the product on a column containing Dowex-50 \times 12 and selective elution of ^{90}Y in 0.6M sodium acetate buffer solution at pH 5.57. Based on the experimental data, a process was demonstrated in which the recovery of ^{90}Y obtained is higher than 75%. The radionuclidic purity of the ^{90}Y obtained proved to be satisfactory for medical application. The details of the procedure are available in the country report.

The most important criteria for a ^{90}Sr - ^{90}Y generator are the quality control tests to ensure the absence of ^{90}Sr in the eluate. Analytical methods developed for measuring ^{90}Sr breakthrough are discussed in the quality control section.

Holmium-166

^{166}Ho can be prepared in high specific activity by irradiating natural Ho in a medium to high flux reactor. Typically, 6 mg of natural Ho_2O_3 powder (100% ^{165}Ho , 65 barns) was weighed and sealed in a quartz ampoule and irradiated at a thermal neutron flux of 3×10^{13} n/cm²/s for seven days and cooled for 6 h. Irradiated Ho_2O_3 powder was dissolved in 5 mL of 0.1 M HCl by gentle warming. The resultant solution was evaporated to near dryness and reconstituted in 10 mL of double distilled water.

The gamma ray spectrum of ^{166}Ho did not show the presence of any radionuclidic impurity. Major gamma peak observed was at 81 keV (6%), photopeak of ^{166}Ho . Minor peaks of ^{166}Ho at 1379 keV (0.9%), 1581 keV (0.18%) and 1662 keV (0.12%) were also notable.

Lutetium-177

^{177}Lu is one of the isotopes offering very good potential for targeted therapy due to the possibility of making the isotope in high specific activity (reaction cross-section 2100 barns). Lu also has a very favourable chemistry and could be used with chelating agents that are suitable for Y. A typical procedure was given: 1.2 mg of enriched Lu_2O_3 powder (60.7% ^{176}Lu) was dissolved in 1M HCl on gentle warming. The resulting solution was evaporated to near dryness and reconstituted in 1.2 mL of double distilled water. 10 μg of the solution (10 μg of Lu_2O_3) was taken in a quartz ampoule and carefully evaporated to dryness. The target was irradiated in a medium to high flux reactor for a period of seven days.

Around 20-22 mCi of ^{177}Lu activity was obtained at 6 h after EOB from 10 μg of enriched Lu_2O_3 powder irradiated for 7 days, corresponding to a specific activity of 2000-2200 Ci/g. Theoretical calculations show that 7 day irradiation at 1.8×10^{13} n/cm²/s flux would yield ~1800 Ci/g of ^{177}Lu activity using enriched Lu_2O_3 target. On the other hand, by using natural Lu target the specific activity of ^{177}Lu would be ~100 Ci/g for 7 day irradiation at the same flux. The radionuclidic purity of ^{177}Lu was ~100% as obtained by analysing the γ ray spectrum. It is worthwhile to note that there is a possibility for the formation of $^{177\text{m}}\text{Lu}$ ($T_{1/2} = 160.5$ d) on thermal neutron bombardment of Lu_2O_3 target. However, the γ ray spectrum did not show any significant peak (at 128 keV, 153 keV, 228 keV, 378 keV, 414 keV, 418 keV) corresponding to $^{177\text{m}}\text{Lu}$. This is expected as radioactivity since $^{177\text{m}}\text{Lu}$ produced will not be insignificant after a 7 day irradiation owing to its comparatively low cross-section ($\sigma=7$ barns) and long half-life ($t_{1/2}=161$ days) for its formation.

Gallium-67

^{67}Ga is a useful cyclotron produced radioisotope in nuclear medicine. Its physical half-life of 3.26 days and various γ radiations following electron capture (EC) reactions are suitable for tracer studies. The important oxidation state is Ga(III). Its co-ordination chemistry is similar to that of Fe (III). Several chelates for ^{67}Ga have been developed. Peptides conjugated to DOTA or DTPA chelators can be labelled with ^{67}Ga with promising binding to receptors.

Technetium-99m

$^{99}\text{Tc}^{\text{m}}$ is the most popular radionuclide for clinical imaging because it has ideal nuclear properties, i.e. a single photon energy of 140 Kev, a half-life of 6 h. It is readily available from a $^{99}\text{Mo}/^{99}\text{Tc}^{\text{m}}$ generator. Some participants labelled biomolecules with $^{99}\text{Tc}^{\text{m}}$ in order to establish a comparison between this radionuclide and ^{188}Re . Because of the similar chemistry of these two radionuclides, participants used their experience to label peptides (lanreotide, Tyr-octreotide, ior-P1394, N₄-Lys-biotin) and antibodies (IgG, iorCEA1, scFv).

Thallium-201

^{201}Tl is an Auger electron emitting radioisotope. In this case, if the carrying biomolecule is labelled within the tumour cell, the labelled species can be useful for radiotherapeutic applications.

Thallium-201 was used by the participant from Greece to label human polyclonal immunoglobulin (IgG, sandoglobulin).

In this case, in a commercially available thallos chloride ($^{201}\text{TlCl}$) solution of 0.1–0.5 mL, a solution of H_2SO_4 5M (1.0 mL) was added followed by a fresh solution of KBrO_3 5% w/v (0.2 ml). The mixture was allowed to stand at room temperature for 15 minutes (min) before the addition of 50 μL 4M NaCl solution. The $^{201}\text{Tl}^{+++}$ so formed, was extracted by adding 1.5 mL butyl acetate. The content was mixed for 20 min at room temperature. The organic phase was separated from the water phase and was evaporated in a water bath by adding a small volume of water. $^{201}\text{Tl}^{+++}$ was received as an aqueous solution ready to use for labelling.

Other radioisotopes like Iodine-125 and Iodine-131 were used as non-carrier added preparations in high radioactive concentration. Receptor studies, molecule characterization, and method development experiments were conducted to gain experience which eventually can be used in replacing them with therapeutic radioisotopes.

Indium-111

^{111}In is a radionuclide available from commercial sources and which shows chemical similarities with ^{90}Y widely used in radiotherapy but lacks gamma photons. The same biomolecules could be labelled with this radionuclide to develop new radiotracers and follow the *in vivo* behaviour in view of replacement with ^{90}Y . For example, in the development of new somatostatin analogues DOTATOC, the affinity for somatostatin receptors and the *in vivo* stability of molecule was tested with ^{111}In prior to the use with ^{90}Y . Dosimetric studies are necessary in the planning of radiotherapeutic trials with new radioligands. With these ^{111}In studies we can get information on the pharmacokinetics and biodistribution of the tracer and also to calculate the residence times in the various organs. These parameters are essential for planning the therapeutic doses to be administered.

Another application of this radionuclide could be the possibility to perform radiotherapy due of its Auger electrons if the ^{111}In labelled molecule is internalized within the tumour cell.

In the frame of this CRP, four participants used this radionuclide for labelling somatostatin analogues and biotin derivatives.

Samarium-153

^{153}Sm is a radiolanthanide, which possesses excellent physical characteristics for radioimmunotherapy. It is a beta-emitter with a half-life of 1.95 days. In addition, it emits 103 keV gamma rays (28%) that are suitable for gamma camera detection.

It can be produced in the reactor by neutron irradiation of enriched ^{152}Sm . For that, Samarium oxide, isotopically enriched in ^{152}Sm ($^{152}\text{Sm}_2\text{O}_3$), can be irradiated. Under these conditions, the specific activity of the ^{153}Sm obtained can be very high depending on the irradiation time and the neutron flux of the reactor. The activated oxide is then dissolved in a solution of 6M HCl , to produce Samarium chloride ($^{153}\text{SmCl}_3$). For the labelling, $^{153}\text{SmCl}_3$ is diluted to the desired volume with ultra pure water and the stock solution is ready for further use.

The ^{153}Sm produced under these conditions is suitable for the labelling of MoAbs, biotin-DTPA and biotin-EDTA molecules as well as DOTA-lanreotide.

For labelling biotin and Lanreotide good results could be obtained without reaching high specific activities. For DOTA-Lanreotide, extra purification steps are needed. The specific activity of the labelled product must be as high as possible. Specific activities lower than 100mCi/mg did not give so good results.

^{153}Sm was used by eight of the participating countries, either by irradiating in their own reactors (Argentina, China, Greece, India, Mexico, Pakistan and Thailand), or within the frames of IAEA or the ARCAL XV programme, Peru provided ^{153}Sm to Uruguay.

Chelating agents and biomolecules

Synthesis of bifunctional chelating agents

A) DOTA (1,4,7,10-tetraazacyclododecane-N,N',N'',N''') tetraacetic acid)

1,4,7,10-Tetraazacyclododecane-N, N', N'', N''') tetraacetic acid (DOTA) was synthesized in four steps. The synthetic route involved the use of inexpensive starting materials.

Step I: Triethylene tetramine (3.3g, 0.027 moles) was tosylated using p-toluene sulphonyl chloride (26.3 g, 0.14 moles) in acetonitrile (125 mL) in the presence of triethylamine (16 mL, 0.115 moles). The reaction involved a portion wise addition of p-toluene sulphonyl chloride to a magnetically stirred solution of triethylene tetramine and triethyl amine at room temperature under N_2 circulation for 1 h. Stirring was continued for 5 h while monitoring the progress of the reaction on a silica gel TLC in methanol: chloroform: ammonium hydroxide (5:94.5:0.5 v/v/v) (R_f 0.8). The solvent was removed under vacuum to yield a white semisolid. The workup involves addition of DDW and extraction using 3×25 mL of chloroform after saturation with brine. The pooled organic extracts were dried over sodium sulphate and dried under vacuum to give the tetratosylated product as a white solid (19.8g, 97%).

Step II: Cyclization of the tetratosylate was effected by condensation with 1,2-dibromoethane in dimethyl formamide as solvent and K_2CO_3 as base, to yield the tetratosylated cyclen. In a typical reaction, a magnetically stirred mixture of tetratosylated triethylene tetramine (10 g, 0.013 moles) and anhydrous K_2CO_3 (4.5g, 0.032 moles) in freshly distilled dry DMF (70 mL), under N_2 atmosphere, was stirred for 1.5 h when a white precipitate was observed. To this was added dropwise 2.4 g (0.013 moles) of 1,2-dibromoethane in 30 mL of distilled and dry DMF. The progress of the reaction was monitored by silica gel TLC in methanol: chloroform: ammonium hydroxide (3:96.5:0.5 v/v/v) (R_f 0.6). The reaction was continued for 24 h following which DMF was removed by vacuum distillation. The workup involves addition of DDW (50 mL) and extraction using 5×25 mL of chloroform, and subsequent removal of chloroform to give the product (8 g, 78%).

Step III: Following the cyclization reaction, detosylation is effected using conc. H_2SO_4 to yield 1,4,7,10-tetraazacyclododecane (cyclen), the key synthon. 9.3 g (0.012 moles) of tetratosylated cyclen along with 35 mL of concentrated H_2SO_4 was heated to 110°C for 24-28 h. The reaction was monitored by TLC using chloroform: methanol: 3:97 (v/v) (R_f 0.2). The reaction mixture was allowed to cool to room temperature and the pH adjusted to ~ 12 under cooling (0-5°C) and extracted with 6×25 mL of dichloromethane after saturation with brine. The pooled organic extracts were washed with 3×25 mL brine and solvent removed to yield 0.69g (33%) of cyclen.

Step IV: A mixture of cyclen (0.24g, 0.0014 moles) and chloroacetic acid (0.6g, 0.006 moles), in 25 mL of DDW, is stirred for 10 min, resulting in a clear solution. The pH of the solution is then adjusted to 9.5-10 by dropwise addition of 5N NaOH. The resultant solution is heated to 50-60°C. The progress of the reaction is indicated by decrease in the pH of the solution, which is maintained at approximately 10 after attaining room temperature. After 30 h of reaction, silica gel TLC using ammonium hydroxide: methanol (40:60 v/v) shows completion of the reaction (R_f 0.8). On attaining room temperature, the pH of the solution is adjusted to 2 using concentrated HCl. The reaction mixture is concentrated to dryness under vacuum to yield the crude product. The purification of DOTA was carried out by dissolving the solid in 0.1% TFA in water and loaded on a silica gel column. The first solvent used was 150 mL of 0.1% TFA in H₂O. The second solvent used was 150 mL of 0.1% TFA in acetonitrile. The solvent from the fractions collected was removed by vacuum distillation to give the pure product (500 mg).

In another procedure, cyclen 1,2-disulphate (2g, 0.005 moles) was converted to DOTA via a one step reaction involving the liberation of cyclen in alkaline medium and its subsequent conversion to DOTA with chloroacetic acid (2.3g, 0.023 moles), following the same procedure as above. After a similar workup the yield of DOTA was 90%.

All the reaction conditions were standardized and the intermediates characterized with the help of TLC in suitable solvents. The intermediates and final products were characterized by FT-IR and high resolution ¹H-NMR spectroscopy.

¹H-NMR, D₂O, (δ ppm) 2.44, 2.67 (16 H, broad singlets, -CH₂ of cyclen) 3.12 (8H, singlet, -CH₂COOH)

B) 5-hydroxy-3,7-diazanonan-1,9-dithiol (DAHPEs)

To a stirred solution of 2-hydroxy-1,3-diaminopropane (4g, 0.044moles) in 30mL dry toluene was added a mixture of ethylene sulphide (5.87g, 0.097 moles) in 20mL of dry toluene under reflux and nitrogen flushing, over a period of 3h. The refluxing was continued for a period of 40 h, following which silica gel TLC using NH₄OH : CH₃OH (6:94 v/v) indicated the completion of the reaction (product $R_f \sim 0.6$). Toluene was removed under vacuum distillation when a white residue results. The residue was washed with a mixture of methanol and dichloromethane (1:1) 5x10mL. The pooled organic extracts were concentrated to yield a viscous liquid, which subsequently solidified to a pale white solid (5.2g, 57%) on storage. The final product was characterized by FT-IR, high resolution ¹H-NMR spectroscopy and mass spectroscopy.

IR (KBr, ν cm⁻¹): 3364(-NH), 2960 (-SH); ¹H-NMR (CD₃OD, δ ppm): 2.64-2.71 [2H dd -NHCH_AH_B(CHOH)], 2.79-2.93 [6H m -NHCH_AH_B(CHOH)] and -NHCH₂CH₂SH, 3.16 (4H t, J = 5.4 Hz, -CH₂SH), 3.65-3.80 (1H m, -CHOH); Mass Spectra (EI) m/z: 210(M⁺)

C) DOTA-lanreotide (Mauritius)

The synthesis of Mauritius was carried out in a three-step reaction using Lanreotide (Pichem) and locally synthesized DOTA.

Step I: The reaction in the first step involved the protection of the free amino residue of lysine in Lanreotide (LAN) with ditert butyl dicarbonate (BOC-anhydride) to yield BOC-LAN. BOC-anhydride (2.2 equivalents, 2.1 mg), at 0°C was added to a stirred solution of 5

mg lanreotide (0.0045mM) in 0.1mL dioxane, 0.05mL DDW. The pH of the solution was checked and found to be ~8. To ensure alkalinity, 0.01 mL 1M sodium hydroxide solution was added when pH was found to be 10. The resultant solution was stirred at room temperature for 30 min. Dioxane was removed under a slow stream of nitrogen when a white residue was observed.

Step II: In the second step, the peptide bond formation is effected via the formation of the N-hydroxy succinimide ester of DOTA in the presence of DCC as the condensing agent. The LANBOC-DOTA conjugate is formed at the β -naphthyl amino residue.

To the residue obtained in Step I, 1 mL DDW and 1 mL DMF, N-hydroxysuccinimide (5.1 mg), dicyclohexyl carbodiimide (7 mg) and DOTA (6 mg) were added. The pH of the solution was maintained at ~9 by the addition of 0.02 mL of 1M NaOH. The reaction was kept stirred for 16 h at room temperature following which turbidity was observed. The solvent was removed under a slow stream of nitrogen gas. The progress of the reaction at this stage was checked by silica gel TLC in NH₄OH: methanol (40: 60 v/v) (R_f-0.8).

Step III: The final step involved de-blocking of the BOC protected NH₂ group to yield the conjugated product LAN-DOTA. The deprotection was carried out by stirring the product obtained from Step II using trifluoroacetic acid (0.13 mL) in methylene chloride (1.5 mL) at room temperature for 30 min. The solvent was removed under nitrogen.

- **Purification of lanreotide-DOTA (LANDOTA):** Purification of the final product was effected on preparative TLC using NH₄OH: methanol (40:60 v/v) (R_f of LAN-DOTA – 0.7, R_f of lanreotide – 0.3). The desired zone was scrapped off and extracted in methanol to obtain the pure product.
- **Characterization:** It was observed that the UV spectra of the synthesized sample of Lanreotide coupled DOTA compared well with that of the authentic sample obtained from Pichem, Austria. Samples for recording NMR were prepared by dissolving 3 mg of the peptides in 540 μ L H₂O and 160 μ L of D₂O. ¹H-NMR spectra was recorded in a Varian Unity Plus 600 MHz FT-NMR spectrometer operating at a ¹H resonance frequency of 600 MHz. Preliminary information towards sequence specific ¹H resonance assignments were attempted using standard methodology reported by Wuthrich. The coupling of a carboxyl group in DOTA with the free amino group of the β -naphthyl alanine moiety is observable through the changes in the ¹H-NMR signals at the site of the alanine moiety.

CHANGES OBSERVED IN THE NMR SPECTRA OF LANREOTIDE AND LAN-DOTA

Changes observed at	DOTA	Lanreotide	Lanreotide-DOTA
-NH ₂ – (Ala)	-	8.26 <i>d</i>	8.37 <i>d</i>
-NH – (Cys)	-	8.37 <i>d</i>	8.47 <i>d</i>
-C _{α} -H (Ala)	-	4.4 <i>t</i>	5.0 <i>broad t</i>
-C _{β} -CH ₂ -naphthyl (Ala)	-	3.938 <i>t</i>	4.08-4.17 <i>m</i>
-CH ₂ COOH (DOTA)	3.12 <i>s</i>	-	3.208 <i>broad s</i>
LanNHCO-CH ₂ DOTA	3.12 <i>s</i>	-	4.012 <i>t</i>
Ring CH ₂ -DOTA	2.44-2.67 <i>broad s</i>	-	5 –3.852 <i>m</i>

D) ior-P1394

The labelling method through an intrinsic linear chelating amino acidic sequence seems to be an attractive alternative for directly ¹⁸⁸Re labelled peptide.

The ior-P1394 was synthesized adding at the *N*-terminal the $^{99}\text{Tc}^{\text{m}}$ -chelating sequence (AGGG β A). A group of four amino acids Ala-Gly-Gly-Gly was chosen as chelating moiety, in order to supply a chelating configuration type N_4 through their NH_2 groups. Furthermore, an additional amino acid β -Ala was inserted as a spacer between the chelating moiety and the primary peptide. This amino acid has the purpose of minimizing any possible steric hindrance resulting from the $^{99}\text{Tc}^{\text{m}}$ chelation.

The peptide was synthesized using the Boc/Bzl strategy and the “tea bag method” on 200 mg of MBHA resin (substitution level 1 mM/g) each. The Boc group was used for N^α -protection. The side chain protecting groups used were 4-methoxybenzyl (Mob) for Cys, benzyl (Bzl) for Asp; Formyl (For) for Trp, dichlorobenzyl (Cl_2 -Bzl) for Tyr, and chlorobenzylloxycarbonyl (Cl-Z) for Lys. Cleavage of the Boc group was carried out with 37.5% TFA in DCM during 30 min. The TFA salt was neutralized with 5% DIEA in DCM three times for 2 min each. The amino acids were coupled using DIPCDI and the completion of the reaction was monitored by ninhydrine test. Side chain deprotection and cleavage from the resin were performed following the “low-high” HF procedure with HF-DMS-*p*-cresol (25:65:10) during 2 h at 0° and with HF-DMS-anisole-thiocresol (79.8:10:10:0.2) during 1 h at 0° , respectively. The peptide was extracted with 30% Hac in water and lyophilized.

Oxidation of the I groups was carried out with 20% DMSO in water. The peptide was dissolved in Hac- H_2O (1:19) at 0.2 mM and pH was adjusted to 6 with ammonium hydroxide solution (25% in water). DMSO was added to achieve the desirable concentration. The completion of the oxidation reaction was monitored by Ellman test.

Radiolabelling

(1) Gallium- 67 labelling with DOTA lanreotide (MAURITIUS)

Dissolve 5 mg of DOTA lanreotide in 10 μl of absolute ethanol and 10 μl of acetic acid. Dilute the solution with 580 μl dionized water (purified in a Chelex 100 column) and dispense 10 and 20 μl aliquots into 1 mL of plastic Eppendorf reaction vials. Store this solution in -20°C or -70°C .

(2) Labelling

Add 10 μl of ^{67}Ga chloride solution to 10 or 20 μl of 0.5 mM DOTA lanreotide into plastic vial. Add sufficient amount of 0.1M ammonium acetate buffer (pH 6.0) to the same Eppendorf vial to reach pH 5.0. With continuous stirring, incubate the solution in boiling water bath for 15 to 20 min.

On Gelman ITLC SG sheet with mobile phase of 20 mM EDTA pH (5–6) radiolabelled peptides remains at the origin ($R_f = 0$) and the free ^{67}Ga -chloride moves with the solvent front as ^{67}Ga -EDTA complex ($R_f = 1$).

Reverse phase C_{18} HPLC with 0.1% TFA and 0–90% acetonitrile gradient –shortly before injection dilute 1 μl of the reaction mixture with an EDTA solution –radiolabelled peptide has slightly altered elution time (cf UV trace) and free ^{67}Ga is eluted with the solvent front as an EDTA complex.

Biomolecules

Different biomolecules – antibody, antibody fragments, biotin and peptides, were investigated in this CRP as carrier molecules for different radionuclides.

Argentina, Cuba, China, Greece, Mexico, Romania and Uruguay worked on labelling antibodies such as anti-CEA, human IgG, 83D4, 147f and fragments with different radionuclides such as ^{153}Sm , ^{188}Re , ^{90}Y , $^{201}\text{Tl}^{+3}$, ^{111}In , ^{125}I , $^{99}\text{Tc}^{\text{m}}$. Some of these carrier molecules were locally produced, i.e. ior-P1394, ior-CEA1 and fragments in Cuba, and 83D4MoAb and fragments in Uruguay.

The observation that somatostatin receptors are over expressed on most of tumours has made somatostatin analogues favourable target molecules. The focus was put on lanreotide, which was labelled with ^{153}Sm , ^{188}Re , ^{90}Y and $^{99}\text{Tc}^{\text{m}}$. For that, 10 mg of lanreotide from PiChem Co., Austria was supplied by the IAEA to each participant. Other somatostatin analogues (octreotide, tyr-octreotide) were also investigated, as well as an RGD peptide labelled with ^{111}In , ^{188}Re and/or ^{90}Y . In the case of RGD peptide, the structure of the complex with oxorhenium was elucidated by NMR spectroscopy with the use of two dimensional techniques.

Labelling methodologies, either direct or indirect, were investigated, and the influence of pH, temperature, molar ratio of reactants, etc. were evaluated. Radiochemical quality control procedures were established and good labelling yields were achieved without purification steps in many cases. *In vitro* binding studies and biodistribution in normal and tumour bearing animals were also conducted.

Some radiolabelled analogues were evaluated in patients with tumours, expressing somatostatin receptors. In particular, tyr- octreotide through the HYNIC chelator was labelled with $^{99}\text{Tc}^{\text{m}}$ (Argentina) and administered in twenty five patients in comparison with ^{111}In -tyr-octreotide.

Several participants investigated biotin derivatives. Biotin was conjugated with various chelating agents for labelling with different radionuclides. Some groups used biotin-DTPA and EB1(EDTA biotin) for labelling with ^{153}Sm , and studied biodistribution alone in pre-targeting systems in animal models.

Other groups made their own conjugation with MAG_3 and $\text{N}_4\text{-Lys}$ chelators for the labelling with the ^{188}Re and $^{99}\text{Tc}^{\text{m}}$. One patient was studied in Uruguay using the 3 steps pre-targeting approach. $^{99}\text{Tc}^{\text{m}}$ - $\text{N}_4\text{-Lys}$ -biotin was administered in the third step.

Quality control methods

Radionuclidic purity

Yttrium-90

As ^{90}Sr is a bone seeker, the limit set for its level in ^{90}Y preparations to be used in humans is $<2 \mu\text{Ci}$. Hence, estimation of ^{90}Sr contamination in ^{90}Y is an important aspect. The ^{90}Sr contamination in ^{90}Y can be estimated by spiking the ^{90}Sr solution feed solution with trace levels of $^{85/89}\text{Sr}$. The ^{90}Y separated can be subjected to gamma ray spectroscopy to identify presence of the spiked isotopes.

Chromatographic methods for the separation of ^{90}Sr and ^{90}Y acetates could also be carried out. In paper electrophoresis, (30 cm Whatman No.1 paper, 0.03 M NaCl and 0.15 g/L sodium citrate, 500 V, 2 h) the $^{85/89/90}\text{Sr}^{+2}$ moves towards the cathode while $^{90}\text{Y}^{+3}$ moiety moved towards anode. Both, Sr and Y need to be in the form of acetate.

The radionuclidic purity of ^{90}Y (in acetate form) can be estimated by paper chromatographic technique on Whatman No. 1 or ITLC-SG develop in 0.9% NaCl, ^{90}Sr migrates to solvent front with Rf 0.8-1.0, while ^{90}Y (as ^{90}Y acetate) remains at origin with Rf 0.0-0.1. However, an accurate determination of Sr-90 breakthrough should be done by Doering's method, which permits the determination of ^{90}Sr -in ^{90}Y .

Radiochemical purity

The estimation of the radiochemical purity of labelled biomolecules was carried out by the standard chromatography techniques like ITLC or paper chromatography, and by HPLC.

ITLC-SG sheet was developed mostly in 0.9% NaCl and methyl ethyl ketone to obtain the percentage of free perrhenate or pertechnetate. The labelled transchelating agent remained at the origin in MEK and migrates with the solvent front in NaCl 0.9%. For checking colloid formation in labelled peptides ethanol-chloride acid 0.01N 90:10 was used as solvent on AlugramTM RP-18W/UV₂₅₄ strips.

HPLC chromatography was done using reverse phase HPLC with appropriate gradient systems containing water/acetonitrile mixtures with TFA or acetic acid as ion pairing agent. Labelled IgG and MoAb with different radionuclides were checked by HPLC using a gel permeation column.

In vitro stability studies

In vitro stability of the labelled lanreotide was studied by means of quality control methods described below.

- **Challenge with cysteine:** *in vitro* stability of labelled biomolecules was checked by cysteine challenge (molar ratios cysteine: biomolecule 0.5 to 500) in 0.4M phosphate buffer, pH 7 at 37°C for 1 h. Most of the biomolecules showed good stability in respect to transchelation.
- **Stability in serum:** stability in the presence of human serum was done for selected biomolecules by incubating them in increasing serum concentration at 37°C during 24 h in 5% CO₂ atmosphere as well as at atmospheric conditions. ^{90}Y -DOTA-lanreotide showed good stability even after 24 h incubation in serum.

Evaluation of radiolabelled biomolecules

In vitro binding assays

Binding studies were done using cell lines (AR 42J and A 431), which express somatostatin receptors. The binding characteristics of the newly synthesized somatostatin analogues were investigated by competition binding assay using unlabelled somatostatin as well as somatostatin analog peptides as inhibiting agents. A typical protocol used for the binding studies is given below.

A431 (Epidermoid mammary carcinoma) cell line expressing somatostatin receptors was used for *in vitro* cell binding studies. The cells were maintained in DMEM with 10% Fetal Calf Serum in 5% CO₂/95% air at 37°C. Adherent cells were passaged with trypsin-EDTA solution after confluency was reached. Before being used in binding experiments, the cells were washed with 50 mM Tris HCl buffer (pH 7.5) and resuspended in 50 mM Tris HCl buffer (pH 7.5) containing 5 mM MgCl₂, 1 mM CaCl₂ and 0.1 M NaCl. Binding was studied by incubating 10⁵ cells with 0.3 µg/tube of ⁹⁰Y-DOTA-lanreotide for 30 min at 37°C. In order to determine the specificity of binding of ⁹⁰Y-DOTA-lanreotide to the receptors, cells were incubated with 0.3 µg of ⁹⁰Y-DOTA-Lanreotide along with cold lanreotide of concentrations 25 µg and 100 µg, respectively. Reaction was also carried out using ⁹⁰Y-DOTA. After incubation, reaction mixture was diluted 1:10 with assay buffer at 4°C and centrifuged at 4000 rpm for 10 min. The pellet was washed twice with buffer and the pellet as well as the supernatant were counted using NaI(Tl) scintillation counter. Cell binding was determined from the above data.

In the case of ⁹⁰Y-DOTA-Lanreotide the binding challenging to A431 cell line expressing somatostatin receptors showed acceptable binding, which was inhibited when increasing amounts of cold lanreotide were added (India).

The binding of labelled antibodies was mainly analysed by challenging cells from different cancer types (Colo 205, A-431, LNCaP), as well as their specific antigens *in vitro* (CEA, PSA, Tn). The results show that the labelled antibodies maintain their capability of recognition to the antigen.

In vitro analysis included use of immunoradiometric or Elisa methods based on immobilization of antigen to the solid phase.

Receptor binding studies using rat brain cortex membrane

Binding characteristics of the newly synthesized somatostatin analogue(s) were investigated by a competition binding assay using rat brain cortex membrane as a source of somatostatin receptors (1) and [¹²⁵I-Tyr³]-octreotide as a specific ligand.

Rat cortex membrane binding studies were also carried out as per the protocol recommended in the 2nd RCM held in Mumbai. Some modifications in the procedure were incorporated by Uruguay in order to overcome the use of ultracentrifuge and improve the separation step in the binding assays.

The protocol used for the preparation of cortex membrane from adult rat brains is, as follows:

The dissected cortex (2) are immediately placed in ice cold Hank's balanced salt solution (HBSS) pH 7.5, rinsed twice with cold HBSS, cut into small pieces and minced with two surgical blades in 10 mL fresh HBSS on ice. The fine, uniform cell aggregate suspension is then transferred into two sterile 50 mL test tubes and diluted with 40 ml ice cold HBSS. The tubes are centrifuged at 500 g for 10 min at 4°C and the supernatant is removed and placed in ice bath. The pellet is re-suspended in 20 mL homogenization buffer (25 mM Tris-buffer pH 7.5), the homogenate is centrifuged as above and the pellet homogenized in the same way for three more times, saving the supernatant after each centrifugation. The combined supernatants are then centrifuged at 48,000 g for 45 min at 4°C. Supernatant is discarded and the pellet is washed twice with 50 mM Tris buffer (pH 7.5) containing 5 mM

MgCl₂, 20 mg/L bacitracin, 0.25M PMSF, 100,000 KIU/L aprotinin and 1000 i.u./ml Rnase inhibitor.

The final pellet is resuspended in 5 mL of the washing buffer, separated into 50 µL aliquots (40 µg protein), frozen immediately and stored at –80°C.

A typical binding study is, as follows:

- 20000 cpm of [¹²⁵I-Tyr³]-octreotide, 40 µg of cortex membranes and the new analogue(s) (1 µM to 1 pM) are incubated at room temperature for 30 min. The membranes are isolated by rapid filtration and the activity assayed in a well counter. For each data point, triplicates are performed, averaged and data analysed by a competition-curve analysis. Binding curves and IC₅₀ for displacement of [¹²⁵I-Tyr³]-octreotide binding by the unlabelled new analogue(s) are calculated using a computer program (MUNSON, P.J., RODBARD, D., Ligand: A Versatile Computerized Approach for Characterization of Ligand Binding Systems. *Anal Biochem* 107; 220-239, 1980.).
- Non-specific binding is defined as the amount of activity bound to the membranes in the presence of 1 µM unlabelled analogue(s).

In vivo biodistribution studies

Candidate therapeutic radiopharmaceuticals should show high and long lasting uptake in the targeted tumour tissue and low uptake and fast excretion from the normal organs/tissues. The above requirements can be determined in well planned experiments in animals bearing the relevant tumours containing cells carrying the target receptor or antigen. The biodistribution and *in vivo* stability of ¹⁸⁸Re lanreotide and ⁹⁰Y-DOTA–lanreotide were determined in normal mice and rats. Some of the data are presented in the individual reports of the participants.

The kinetic data collected during these experiments are useful to calculate radiation dose delivery to normal and target tissue.

Therapeutic efficacy study

Therapeutic efficacy of the compound can ultimately be determined in a direct way in the relevant animal tumour model. Depending upon the experimental model, efficacy of the therapeutic radiopharmaceutical can be determined by the direct measurement of the tumour volume or by the assessment of animal survival as compared to non-treated control group. Dose escalation and body weight measurements of experimental animal are also advised. These biodistribution results are promising for undertaking clinical studies.

REMARKS ON METHODOLOGIES FOR RADIOLABELLING AND QUALITY CONTROL OF SELECTED BIOMOLECULES

Labelling of lanreotide with rhenium–188 by direct method

The labelling of lanreotide with ¹⁸⁸Re by direct method can be performed by two technical approaches: (1) pre–reduction of perrhenate with reducing agents and attachment to the lanreotide, and (2) reduction of lanreotide and ¹⁸⁸Re are made simultaneously.

Lanreotide solution (in water or in buffers)

- Reducing agents: stannous chloride, stannous fluoride or stannous tartrate
- Transchelating agents: hydroxyethylene diphosphonate (HEDP), citric acid
- Buffer: tartrate or phthalate pH 5.6, acetate buffer pH 5.0, bicarbonate pH 9.0.
- Stabilizers: ascorbic acid, gentisic acid.

General procedure

Lanreotide solution and the reducing agent solution is added to a vial including the transchelating and stabilizer agents with a final pH (1.0–5.5). Freshly eluted ^{188}Re from the $^{188}\text{W}/^{188}\text{Re}$ generator in 0.9% saline solution is added. The vial is heated in a water bath at 100°C for 1 to 2 h.

Examples of this procedure

1) 18 mg of HEDP is dissolved in 0.2 mL of 0.5 M bicarbonate buffer and the volume made up to 1 mL with saline. 20 μL of stannous chloride solution (2.1 mg in concentrated HCl) is added to the HEDP solution followed by addition of 20 μL (10 μg , 0.05 μM) of ReO_4^- . The reaction mixture is purged with nitrogen for 10 min. The pH of the reaction mixture is adjusted to 2.0 and was reacted with 250 μg (0.025 μM) of lanreotide in a boiling water bath for 90 min.

2) 5.3 mg of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ in 1 mL of nitrogenated 0.1M citrate buffer pH 5, 1.4 mg of gentisic acid, 2 mg of tartaric acid, pH 5.5 and 60 μg of Lanreotide in 16 μL of acetate buffer are added to 1 mL of a saline solution of perrhenate (10 mCi) fresh eluted in a nitrogen atmosphere. The vial is sealed and heated for 45 min in boiling water.

3) Add to a vial 350 μg of lanreotide in 0.35 mL, 2.5 mL of tartrate/phthalate buffer solution pH 5.6 and 3.5 mg of stannous chloride solution in 0.1N HCl. Allow the mixture to stand at room temperature for 4 h with agitation. Then the vial can be frozen at -30°C until use. For labelling add to the vial at room temperature 73 to 370 MBq of ^{188}Re fresh eluted from $^{188}\text{W}/^{188}\text{Re}$ generator. Then the mixture is heated in a water bath for 1 h.

Observation: the whole procedure must be done in a nitrogen atmosphere to avoid reoxidation of perrhenate.

Quality control

Radiochemical purity of ^{188}Re lanreotide can be performed by ITLC–SG (Gelman Science, Inc.) and HPLC using a reverse phase column in a gradient solvent.

ITLC chromatography

Solvent	R_f peptide	R_f $^{188}\text{ReO}_4$	R_f ReO_2
Saline 0.9%	0.0	1.0	0.0
Ethanol 10% HCl 0.001N	1.0	1.0	0.0

HPLC chromatography

Reverse phase C18 column

Solvent system

System A: acetonitrile, solvent B: water TFA 0.1%

Gradient: 0–3 min 100% B, linear increase of eluent A to 50% from 3–13 min, 13–18 min 50% A, 18–20 min linear increase of eluent A to 70%

Flow rate: 1mL/min.

Labelling conjugated peptide with ^{90}Y

Reagents

- DOTA-peptide dissolved in ultra pure water at a concentration 1 $\mu\text{g}/\mu\text{l}$
- Buffer solution with gentisic acid made by mixing 0.4 M sodium acetate with 30 mg/ml gentisic acid
- ^{90}Y in 0.04 M HCl (usually 1mCi/ μl concentration).

Place in an acid washed glass vial (conical better) 30-50 μg DOTA-peptide in an equal volume of solution 2. Then add to it the $^{90}\text{YCl}_3$ (usually 1mCi/ μg of peptide); if necessary, adjust the pH to 5.0-6.0 with sodium hydroxide. Mix well by vortexing and incubate for 30-60 min in boiling water in a thermostatic bath. Cool the contents to room temperature. DTPA can be added to remove uncomplexed ^{90}Y . Then remove a small aliquot for quality control, placing it in an Eppendorf tube.

Quality control

Sep Pak: Add to the Eppendorf tube equal volume of 50 mM DOTA solution and then apply to a Sep Pak cartridge pre-conditioned with 2 mL methanol. Pass 2 mL of acetate buffer collecting the eluate (it contains free ^{90}Y or ^{90}Y -DTPA), and add 2 mL of methanol collecting again (^{90}Y -DOTA-peptide). Count separately the two fractions and calculate the labelling yield.

ITLC: In 0.004 M EDTA, pH 4.0. $R_f = 0$ for ^{90}Y -DOTA-peptide and 1.0 for free ^{90}Y .

HPLC: RP C_{18} , Mobile phase: Solvent A: 0.1% TFA in H_2O , solvent B: $\text{H}_2\text{O}/\text{CH}_3\text{CN}$ (20/80), linear gradient 0% to 100% over 20 min.

POTENTIAL AREAS OF FUTURE RESEARCH

- Continue with clinical studies using ^{188}Re -lanreotide
- Continue clinical research with the biomolecules investigated under the project, radiolabelled with the radionuclides also used in the CRP
- Start work with new peptides like DOTA-Lys⁸-Vasotocin labelled with radionuclides using bifunctional chelators
- Study biomolecule labelling with radionuclides like ^{177}Lu
- Work with *in vivo* generators like ^{166}Dy - ^{166}Ho or ^{212}Pb - ^{212}Bi for labelling of several molecules
- Explore the possibility for regional research reactors to produce ^{188}W of sufficient specific activity and further develop a gel type generator system to overcome the problem of the availability of economical ^{188}Re for research and applications.

COLLABORATIVE ACTIVITIES

The general scope of the CRP focused on the optimization of labelling, quality control, as well as *in vitro* and *in vivo* evaluation of biomolecules based on therapeutic radionuclides.

In order to accomplish the overall work plan, as decided by the CRP participants, collaborative activities were agreed upon.

The expert from Italy provided important advice to some of the contract holders. A close collaboration between the groups in Argentina and Italy on the development of peptides and biotin derivatives radiolabelled with ^{188}Re and ^{90}Y was carried out. This exchange was implemented by the scientific staging in both institutes by investigators involved in the CRP. The result was presented in scientific meetings and included in this report.

There was also a close collaboration between Uruguay and Italy on the development of $^{99\text{m}}\text{Tc}$ - N_4Lys -biotin compound. The clinical use of a 3 step pretargeting approach has increased the scientists' and clinicians' interest in developing new radiolabelled biomolecules and their clinical applications.

Co-operation between the participants enabled the transfer of materials, information and expertise, e.g. Argentina with Uruguay, Cuba with Mexico and Greece in providing the IOR-CEA antibody, Greece with Uruguay in the synthesis of chelating agents.

National interdisciplinary collaboration was also established or improved.

As a result closer relationships and future technical co-operation projects were agreed between them.

TECHNICAL CONCLUSIONS OF THE CRP

- Several isotopes were used during the course of the CRP. These included ^{90}Y , ^{188}Re , ^{201}Tl , ^{166}Ho , ^{153}Sm and ^{177}Lu for therapeutic applications. $^{131/125}\text{I}$, ^{111}In , ^{67}Ga and $^{99\text{m}}\text{Tc}$ were also used by some of the participants as diagnostic/control techniques.
- ^{188}W - ^{188}Re generator was made available to all the participants. The generator was purchased by the IAEA from MAP Technologies and distributed to all the participants. Part of the ^{188}W used was provided free of charge by Oak Ridge National Laboratory, USA. The elution efficiency, radionuclidic purity and radiochemical purity of the eluted $^{188}\text{ReO}_4^-$ were appropriate for labelling studies. ^{188}Re obtained from the generator was used for radiolabelling lanreotide, monoclonal antibodies and other biomolecules such as IgG and ior-1394 (the peptide developed in Cuba). The participants got the opportunity to use the generator and get familiarized with labelling techniques using generator eluted ^{188}Re .
- ^{90}Y was used by several participants. The source of ^{90}Y was either from commercial sources or from a locally produced generator, as in India and Thailand. A ^{90}Sr - ^{90}Y generator based on supported liquid membrane was developed by India. This generator could be loaded with up to 100 mCi (3.7 GBq) of ^{90}Sr . 60-70 mCi of ^{90}Y could be eluted from this generator. Separation of ^{90}Y from ^{90}Sr - ^{90}Y mixture by solvent extraction and further purification by ion exchange chromatography on Dowex 50x12 was also developed. The method could be used in a centralized radiopharmacy for getting ^{90}Y in acetate form for labelling studies.

- Quality control techniques to ensure absence of radionuclidic impurity, especially ^{90}Sr , will have to be instituted for the eluted ^{90}Y . Gamma ray spectroscopy using $^{85/89}\text{Sr}$ spike, paper electrophoresis and paper chromatography are some of the quality control techniques, which can be availed for the above purpose. The above quality control techniques need to be validated before using ^{90}Y in clinical studies.
- The participants tried several other isotopes. ^{201}Tl as Tl(+3) was proposed as an Auger electron therapy radionuclide. ^{153}Sm and ^{166}Ho were used by some of the participants. However, the specific activity of these radionuclides will not be optimal for labelling peptides. Radiolabelling studies with $^{125/131}\text{I}$ were also carried out by several participants who obtained high Radiolabelling yields. The isotope ^{177}Lu could offer specific activity adequate for radiolabelling of peptides. The longer half-life of ^{177}Lu might be advantageous in situations matching the biological half-life of the peptides.
- The selected peptide, lanreotide, was used by all the participants and labelled with ^{90}Y through the DOTA chelator and ^{188}Re . Use of other biomolecules such as monoclonal antibodies and other peptides such as ior-P1394 (the peptide developed by Cuba), oxytocin was also described by some of the participants.
- The peptide, lanreotide, was directly labelled with generator eluted $^{188}\text{ReO}_4^-$. High radiolabelling yields could be obtained using the protocols developed by the participants. The influence of reducing agents and secondary ligands/stabilizing agents were thoroughly investigated by several participants. The biological efficacy of the labelled peptide needs to be established.
- Modification of lanreotide by conjugation with MAG_3 was successfully developed. The MAG_3 -lanreotide gave high radiolabelling yields with ^{188}Re .
- Radiolabelling of DOTA-lanreotide with $^{90}\text{YCl}_3$ at pH 5-6 was carried out by several groups. The radiolabelling yield was generally high. However, purification by SepPak separation was performed whenever necessary.
- Cell binding studies using different cell lines expressing somatostatin receptors were performed by several participants to evaluate the biological efficacy of ^{90}Y -DOTA-lanreotide. Cell binding and subsequent inhibition on addition of cold lanreotide were shown thereby indicating the biological activity of the labelled peptide. Binding studies with rat brain cortex membrane were also done on the newly synthesized somatostatin analogues. The results obtained for ^{188}Re -lanreotide in these assays suggest that the labelled peptide by direct as well as indirect methods was able to bind the brain cortex membrane receptors of rats.
- Biodistribution studies in normal mice, tumour induced mice/nude mice were done by several participants. The studies demonstrated the preferential uptake of radiolabelled peptides in the tumour.

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COUNTRY REPORTS

OPTIMIZATION OF BIOMOLECULES LABELLING WITH RHENIUM-188 USING DIRECT AND INDIRECT METHODS

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Abstract

Active tetrafluorophenol-MAG₃-¹⁸⁸Re ester, obtained from S-benzoyl-MAG₃, is useful for the preconjugate radiolabelling of a variety of biomolecules. We report the optimization of polyclonal IgG labelling by ¹⁸⁸Re using S-benzoyl-MAG₃ as a model for labelling monoclonal antibodies. We examined the *in vitro* stability of the labelled protein and its localization and excretion in mice with induced focal inflammation. Stability in serum was greater than 85.5% after 24 h. Biodistribution and imaging studies following administration to mice showed mainly renal and hepatic excretion and high IT/NT ratios (4.5 and 4.6) at 24 and 48 h, respectively. Likewise, the monoclonal antibody 14f7 was labelled with ¹⁸⁸Re using this technique and the same controls were carried out with the labelled protein but in mice bearing a tumour. Tumour uptake increased in 24 h from 3.9 to 8.8% ID/gr and stood constant since then. On the other hand, a direct labelling method was studied and lanreotide-¹⁸⁸Re was obtained with almost 100% of radiochemical purity. Lanreotide was also labelled with ¹¹¹In and ⁹⁰Y through DOTA chelator, showing mainly renal excretion when administered to rats. Finally DOTA-TOC was labelled with ⁹⁰Y and data showed that a lower mass is needed in order to label it with the same amount of activity than DOTA-lanreotide.

1. INTRODUCTION

The utility of radiolabelled monoclonal antibodies (MoAb) and small peptides in various therapeutic applications has increased interest in radiolabelling procedures, in particular for the high energy beta emitters ⁹⁰Y, ¹⁸⁶Re and ¹⁸⁸Re. ¹⁸⁸Re has several advantageous properties. It can be obtained carrier-free, relatively cheaply and on demand from an in-house ¹⁸⁸W/¹⁸⁸Re generator (¹⁸⁸W T_{1/2}=69.4 h; ¹⁸⁸Re T_{1/2}=17 h) [1][2], and it decays by emission of high energy beta particles (E_{max}=2.11 MeV) suitable for radiotherapy, followed by emission of 155 keV gamma photons in 15.88% abundance.

As with ⁹⁹Tc^m [3] there are two methods of labelling antibodies with rhenium: the direct method via endogenous or generated thiol groups [4] and the indirect method via chelators such as N₂S₂ [5], PN₂S [6], hydrazinonicotinyl (HYNIC) derivatives [7] and S-benzoyl-mercaptoacetyltriglycine (S-benzoyl-MAG₃) [8,9]. Using the indirect method both preconjugate and postconjugate approaches may be employed [10].

S-benzoyl-MAG₃ was used extensively in nuclear medicine because it is easy to synthesize, has a long shelf-life, and its rhenium complex is stable, both *in vitro* and *in vivo*. In the preconjugate approach, radiolabelled S-benzoyl-MAG₃ is chemically activated by esterification (producing ¹⁸⁸Re-MAG₃-activated ester) and this is coupled to the free amino groups of antibodies (polyclonal or monoclonal) [11][12], peptides or biotin derivatives, maintaining their biological activity.

The aim of the present study was to optimize procedures for labelling biomolecules with ¹⁸⁸Re using direct and indirect methods and to label somatostatin analogues with ¹¹¹In and ⁹⁰Y through DOTA chelator. Quality assurance tests were performed on the products at each step and on the final radiolabelled biomolecules. Stability studies in saline and human serum

were also performed and the biodistribution of the labelled biomolecules was evaluated in animal models.

2. MATERIALS AND METHODS

2.1. Labelling of s-benzoyl-MAG₃ with ¹⁸⁸Re

A saline solution of ¹⁸⁸ReO₄⁻ (approximately 370 MBq) was freshly eluted from a ¹⁸⁸W/¹⁸⁸Re generator (MAP Medical Technologies, Finland) and evaporated to dryness. The influence of stannous chloride dihydrate (SnCl₂·2H₂O) (range 6-5000 µg) and S-benzoyl-MAG₃ (kindly provided by CGM Nuclear, Chile) (range 100-2600 µg), on the efficiency with which S-benzoyl-MAG₃ was labelled by ¹⁸⁸Re was studied. For example in one experiment, 1,5 mg of SnCl₂·2H₂O (250µl of a 6mg/ml soln. in 0.1M citrate buffer pH=5.5) and 750µg of S-benzoyl-MAG₃ (250µl of a 3 mg/ml soln. in acetonitrile:water (6:4 v/v)) were added to the rhenium in a nitrogen atmosphere, the vial was sealed and heated for 30 min at 90 °C. The ¹⁸⁸Re-MAG₃ was obtained.

HPLC was employed to check the labelling: a Waters 600 HPLC with radiometric and UV detectors and a Deltapak C18 column. The gradient was: solvent A: acetonitrile, solvent B: water TFA 0.1%, gradient: 0-3min 100% B at 1ml/min, linear increase of eluent A to 50% from 3-13 min at 1ml/min, 13-18 min 50% A at 1ml/min, 18-20 min linear increase of eluent A to 70% at 1ml/min.

2.2. Synthesis of ¹⁸⁸Re- MAG₃-TFP ester

After optimization of the above reaction, the product (¹⁸⁸Re-MAG₃) was allowed to cool and molar ratios of 2,3,5,6-tetrafluorophenol (TFP) to MAG₃ in the range 7:1 to 70:1 were tried and the optimal yield obtained. For example, 74.5 µmol of TFP (125µl of a 100 mg/ml soln. in CH₃CN:H₂O, 9:1) and 265 µmol (50 mg) of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) were added and the mixture agitated at room temperature for 40 min. The ¹⁸⁸Re-MAG₃-TFP ester was subsequently purified with Sep-Pack C-18 Waters cartridge. The cartridge was washed successively with 5 mL ethanol and 5 mL water. The reaction mixture was loaded and the cartridge washed with 5 mL deionized water and two fractions of 1 mL acetonitrile. The majority of the eluted ester was collected in the first acetonitrile fraction. The solvent was evaporated under nitrogen at room temperature.

2.3. Labelling of biomolecules using the labelled active ester

2.3.1. Preparation of ¹⁸⁸Re- MAG₃-IgG and ¹⁸⁸Re- MAG₃-14f7

The active ester and IgG were conjugated at molar ratios of 60:1, 90:1, 100:1 and 300:1. Thus different amounts of IgG (10 mg/ml in CO₃HNa 0.1M pH=10) were added to the active C18 purified ester and the reaction mixture incubated 15 min at room temperature. In the case of labelling the 14f7 the molar ratios active ester to MoAb were 37:1,100:1, 110:1, 145:1 and 300:1.

2.3.2. Purification and quality control of labelled IgG and 14f7

Quality control of the labelled IgG and monoclonal antibody was carried out by HPLC using a Protein Pack SW-300 (Waters) column and phosphate buffer 0.02M pH=7.2 as eluent at 1ml/min. The product was purified by HPLC using the same column and buffer as for

quality control. 1 ml fractions were collected and the fraction containing the labelled protein was used subsequently for the *in vitro* and *in vivo* studies.

2.4. Stability in saline and serum

Aliquots (100 μ l) of $^{188}\text{Re-MAG}_3$ and purified $^{188}\text{Re-MAG}_3\text{-IgG}$ or $^{188}\text{Re-MAG}_3\text{-14f7}$ were incubated in the saline solution and human serum (1:3 v/v, labelled product : human serum) for 24 h, respectively, at room temperature. The incubated mixture was analysed by HPLC using the same system as for quality control and the results were also checked with instant thin layer chromatography (ITLC SG, Gelman Instruments Company) run in sodium citrate 0.1M.

2.5. Experimental animals

2.5.1. Testing $^{188}\text{Re-MAG}_3\text{-IgG}$ in NIH mice

Eight to twelve-week old inbred NIH mice from the National Atomic Energy Commission facility, with average weight of 25 g, were kept (5-10 animals/cage) with water and food ad libitum in accordance with the Guide for the Care and Use of Laboratory Animals. Focal inflammation was induced by injecting 40 μ l of turpentine in the right thigh. The animals were left for 48 h, following which normal and inflamed mice were then injected intravenously (lateral tail vein) with 100 μ Ci of $^{188}\text{Re-MAG}_3\text{-IgG}$. Whole body images were obtained 4, 24 and 48 h after injection, using a gamma camera equipped with a medium-energy collimator. 500,000 counts were obtained in a 128 x 128 matrix.

The biodistribution in normal NIH mice, and those with induced focal inflammation, was determined at 4, 24, 48 and 120 h post i.v. injection of 10 μ Ci of labelled IgG. To do this, three animals per group were sacrificed by cervical dislocation at each time. Blood samples were taken and organs of interest [including inflamed thigh (IT) and normal thigh (NT)] were resected, rinsed, dried and placed into pre-weighed tubes. The activity of all samples was counted together with appropriate dilutions of the labelled IgG standard and results were expressed as percentage of the ID per tissue gram (%ID/g). The total injected dose (ID) was calculated by comparison with a standard.

2.5.2. Testing $^{188}\text{Re-MAG}_3\text{-14f7}$ in mice bearing a tumour

BC57 mice were injected i.v. with 10 μ Ci of labelled MoAb and biodistributions were carried out at 2, 24 and 48 h in the same manner as that described with labelled IgG.

2.6. Labelling and quality control of $^{188}\text{Re-Lanreotide}$ via a direct method

2.6.1. Labelling of lanreotide with ^{188}Re

Lanreotide was labelled with ^{188}Re via a direct method using $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ in order to reduce both the radionuclide and peptide. The influence of stannous chloride dihydrate and peptide mass on the labelling efficiency was studied. As an example: 5.3 mg of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ in 1 mL of nitrogenated 0.1M citrate buffer pH=5.0, 1.4 mg of gentisic acid and 60 μ g of lanreotide in 16 μ l of acetate buffer were added to 1 mL of a saline solution of perrhenate (10

mCi) freshly eluted and in a nitrogen atmosphere. The vial was sealed and heated during 45' at 97°C.

2.6.2. *In vitro* studies

A cysteine challenge was carried out with the labelled peptide and six cysteine solutions of different concentrations from 83 mM to 0.83 mM. 90 µl of the labelled peptide (0.2 mM) were added to each of the cysteine solutions. Samples were incubated 30' at 37°C. ITLC were performed in saline.

Serum stability was determined after 4 and 24 h incubation of the labelled peptide in human serum at 37°C. After precipitation of proteins with acetonitrile the incubation mixture was analysed by HPLC.

2.6.3. *Biodistributions*

Biodistributions were performed in NIH normal mice at 1, 4 and 24 h after the injection of 10 µCi of labelled peptide.

2.7. Labelling and quality controls of peptides with ¹¹¹In or ⁹⁰Y

2.7.1. ¹¹¹In-DOTA-lanreotide: *in vitro* and *in vivo* assays

2.7.1.1. Radiolabelling

Same volumes of ¹¹¹In Chloride (100µCi) and sodium acetate 0.1M were mixed in order to obtain ¹¹¹In acetate. A solution of lanreotide-DOTA (30 µg in 60 µl sodium acetate 0.4M) was added. The vial was sealed and heated at 95°C during 30'.

2.7.1.2. Serum stability

Serum stability was determined after 4 and 24 h incubation of the labelled peptide in human serum at 37°C. After precipitation of proteins with acetonitrile the incubation mixture was analysed by HPLC.

2.7.1.3. Biodistributions in normal rats

Normal Wistar rats were injected i.v. with 0.37 MBq of ¹¹¹In labelled peptide. Animals were sacrificed after 4h and samples of different organs obtained and counted and results were expressed as%ID/gr.

2.7.2. *Labelling of DOTA-Lanreotide and DOTA-TOC with ⁹⁰Y*

⁹⁰Y used for radiolabelling was obtained from Nordion as a solution and under the advice of Dr. Marco Chinol from IEO (Milan, Italy). Solutions of gentisic acid in sodium acetate 0.4M pH=5 and DOTA-Lanreotide or DOTA-TOC were added to the ⁹⁰Y chloride. The final solution was heated at 90°C during 25'. We tried different amounts of the two peptide complexes per mCi of ⁹⁰Y and stability studies at 24h and room temperature were performed with the labelled peptides. Reverse phase HPLC was employed to check the labelling.

3. RESULTS

3.1. Labelling of MAG_3 with ^{188}Re

This was a solid phase reduction of perrhenate with stannous ion, with citrate as transfer ligand. Yields of more than 95% were obtained when the ratio of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ to ^{188}Re activity was greater than $125 \mu\text{g}/\text{mCi}$ as shown in Fig. 1. In this case nearly quantitative yields were obtained when the ratio of S-benzoyl- MAG_3 to ^{188}Re activity exceeded $15 \mu\text{g}/\mu\text{Ci}$ as shown in Fig. 2. Specific activities up to $253 \text{ mCi}/\text{mg}$ MAG_3 were obtained.

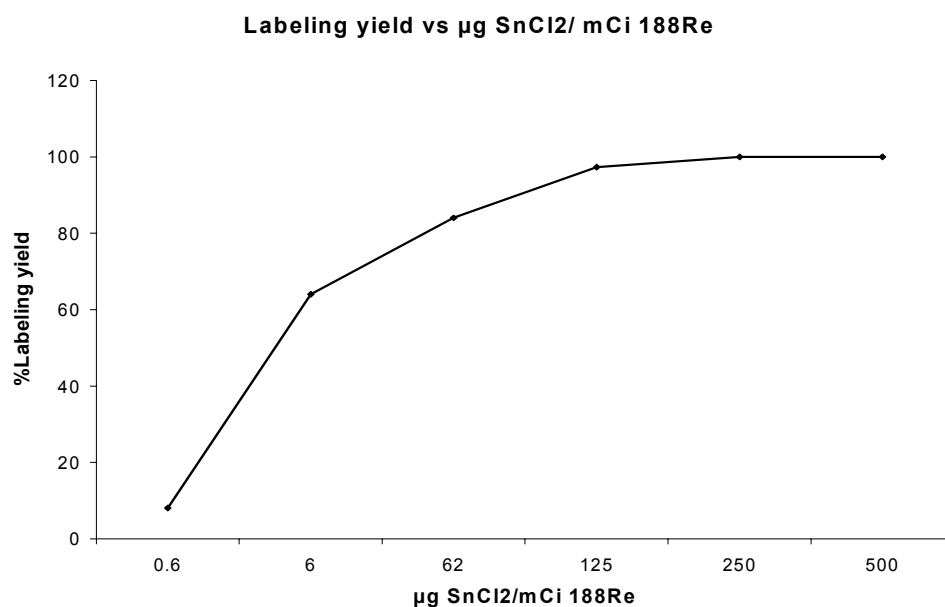


FIG. 1. Influence of SnCl_2 amount/ $\text{mCi } ^{188}\text{Re}$ on labelling yield.

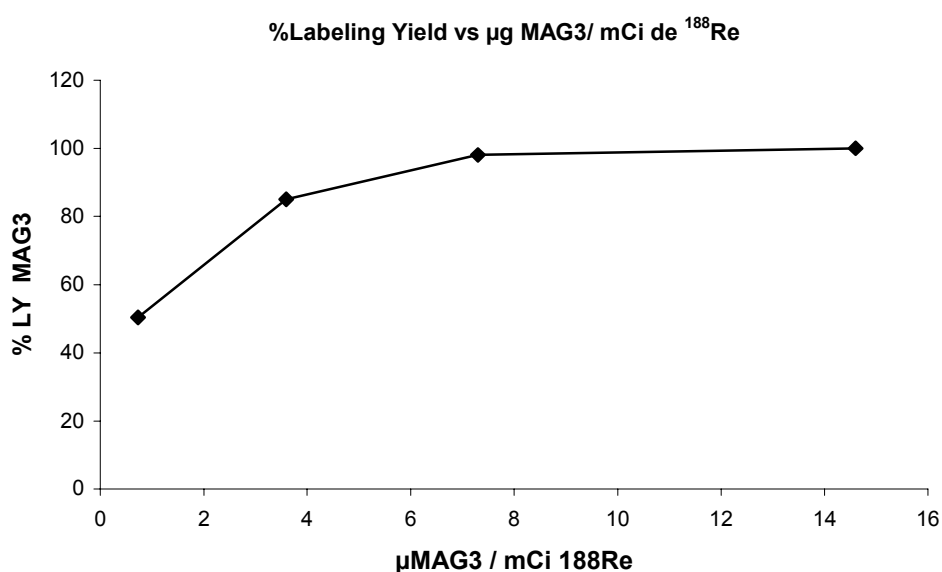


FIG. 2. Influence of MAG_3 amount/ $\text{mCi } ^{188}\text{Re}$ on labelling yield.

3.2. Esterification of $^{188}\text{Re-MAG}_3$ with TFP

The best esterification yield was obtained when the molar ratio of TFP to $^{188}\text{Re-MAG}_3$ was 35:1 in which case up to 75% of the activity was recovered after purification. The ester was stable in acetonitrile at room temperature for 24 h.

3.3. Conjugation of biomolecules with $^{188}\text{Re-MAG}_3\text{-TFP}$

3.3.1. Conjugation of IgG with the labelled ester

Fig. 3 shows the various molar ratios of purified radiolabelled ester and IgG studied. The maximum efficiency (82.8%) was obtained when the molar ratio of ester to protein was 300:1, in which case the specific activity of labelled IgG was 1.65 mCi/mg IgG. Protein aggregates were not detected on subsequent HPLC when purified $^{188}\text{Re-MAG}_3\text{-TFP}$ was used.

3.3.2. Conjugation of 14f7 with the labelled ester

The recovery of Sep-Pack C-18 purification for $^{188}\text{Re-MAG}_3\text{-TFP}$ was 75% of the total activity. The maximum efficiency for the conjugation (46.9%) was obtained when the molar ratio of ester to protein was 110:1 as shown in Fig. 4, in which case the specific activity of labelled 14f7 was 90.3 MBq/mg (2.44 mCi/mg MoAb).

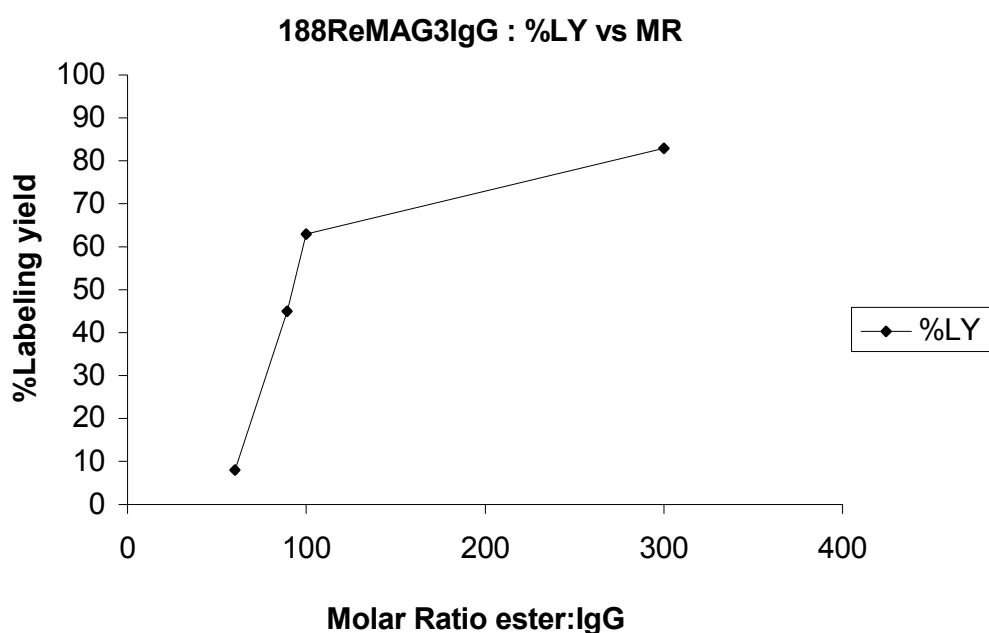


FIG. 3. Effect of mole ratios on the complexation of active ester and IgG.

3.3.4. Quality controls

The HPLC retention times were 1.88, 11.55 and 15.59 min for $^{188}\text{ReO}_4^-$, $^{188}\text{Re-MAG}_3$ and $^{188}\text{Re-MAG}_3\text{-TFP}$ respectively, using a Deltapack C18 reverse phase column. The HPLC chromatograms of the labelled IgG or 14f7 using a Protein Pak column had a retention time of 7.73 min for the labelled protein, 12.43 min for the labelled ester, and 13.2 min for $^{188}\text{ReO}_4^-$.

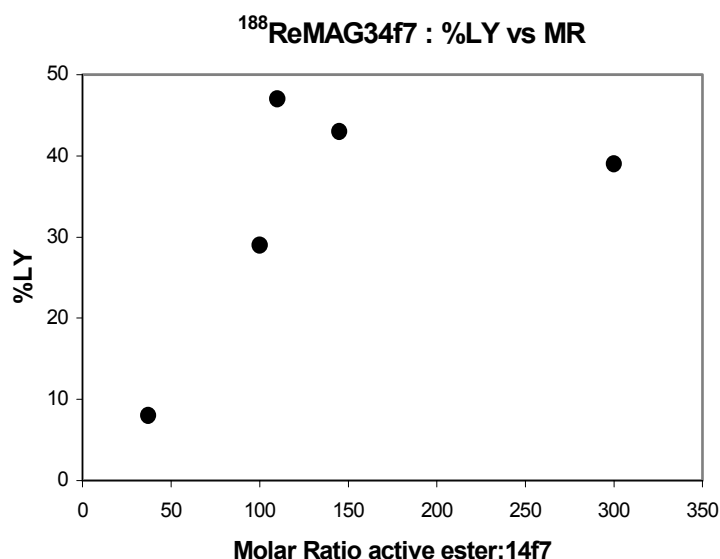


FIG. 4. Effect of mole ratios on the complexation of active ester and MoAb.

3.4. Stability in saline and serum

The results of *in vitro* stability assays of $^{188}\text{Re-MAG}_3\text{-IgG}$ and $^{188}\text{Re-MAG}_3$ are shown in Fig. 5. Over 96% of $^{188}\text{Re-MAG}_3$ activity was recovered after incubation for 24 h in saline and serum at room temperature. Similarly 96.2% and 85.5% of the activity remained bound to IgG-MAG₃ after incubation in saline and serum, respectively. ITLC data were consistent with these findings. HPLC analysis after incubation of labelled IgG in saline demonstrated that 3.8% of the radioactivity was associated with either ^{188}Re or $^{188}\text{ReMAG}_3$. When $^{188}\text{Re-MAG}_3\text{-IgG}$ was incubated with human serum, 14.3% of the radioactivity was associated with serum proteins. In the case of MoAb, 80.0% and 70.3% of the activity remained bound to 14f7MoAb-MAG₃ after incubation in saline and serum, respectively.

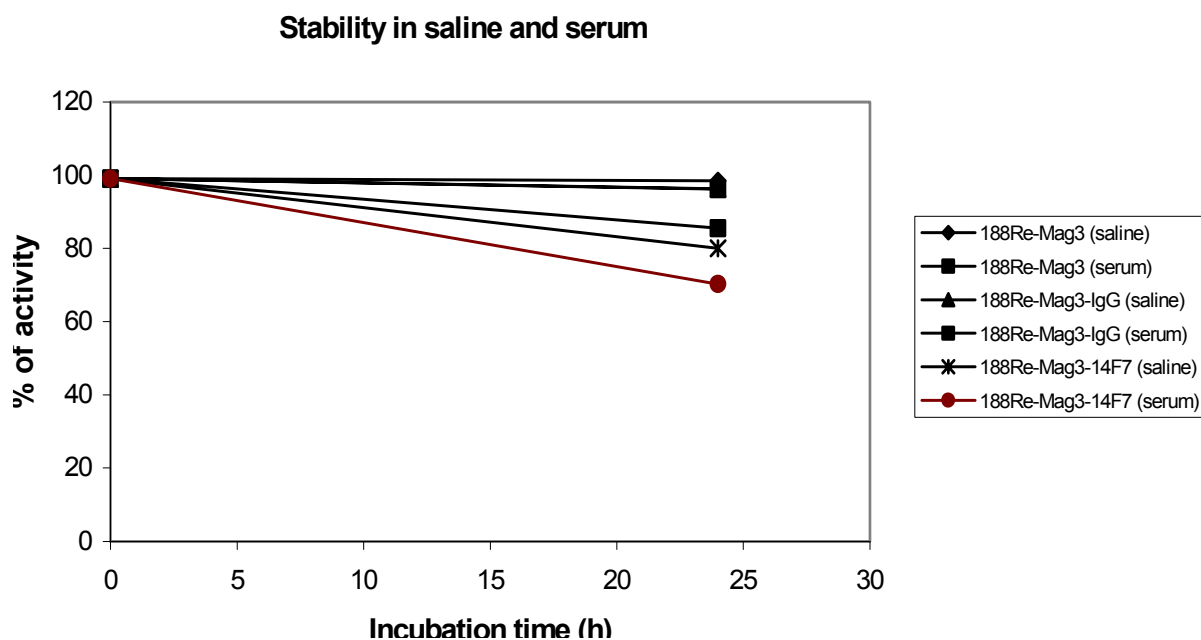


FIG. 5. Stability in saline and serum at 24h of labelled MAG₃ and labelled proteins.

3.5. Biodistributions

3.5.1. Animal studies of $^{188}\text{Re-MAG}_3\text{-IgG}$

The biological distribution of ^{188}Re -labelled IgG was similar in control mice and in those with focal inflammation (Fig. 6) with no significant differences in uptake for any organ. The uptake of the radiolabelled IgG to liver and kidney was similar and much higher than in other organs, indicating combined hepatic uptake and renal excretion. Clearance rates for liver and kidney were similar as for blood (Fig. 7). IT and NT had slower clearances than other organs. Uptake differences between IT and NT were nearly constant over time so the IT/NT ratios were similar at all times.

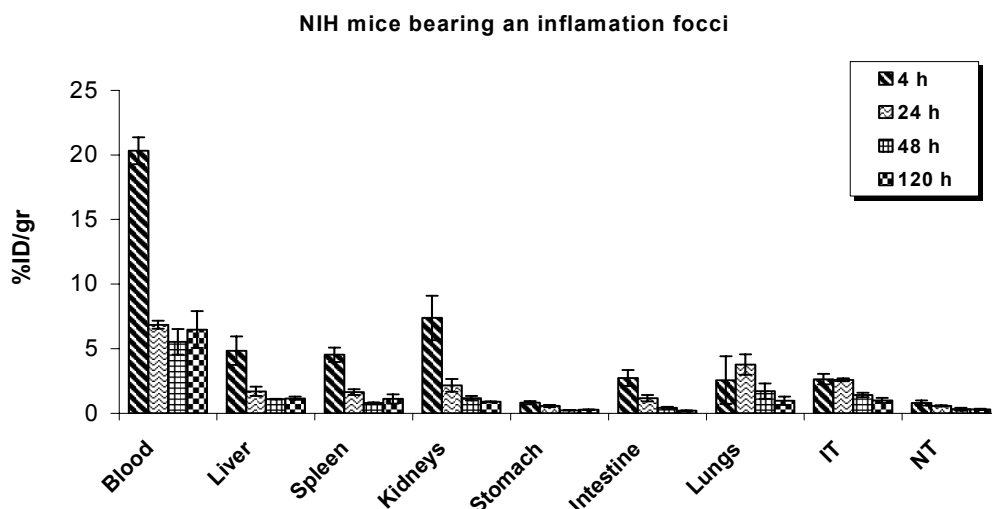


FIG. 6. Biodistributions in mice bearing inflammation foci at 4,24,48,120 h.

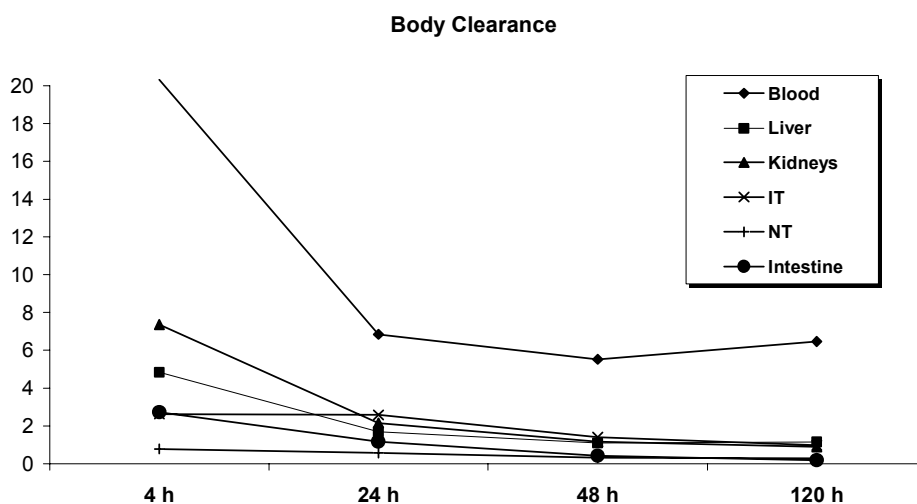


FIG. 7. Body clearance in inflamed mice.

3.5.2. Biodistribution of $^{188}\text{Re-MAG}_3\text{-14f7}$ in mice bearing a tumour

Tumour uptake of $^{188}\text{Re-MAG}_3\text{-14f7}$ increased in 24 h from 3.9 to 8.8% ID/gr and stood constant since then. Meanwhile, non-target organs uptake decreased with time. Due to monoclonal antibody clearance, highest tumour to non-target organ ratios and thus, lower background radioactivity, were found at 48 h.

Biodistribution ^{188}Re -MAG3-14f7

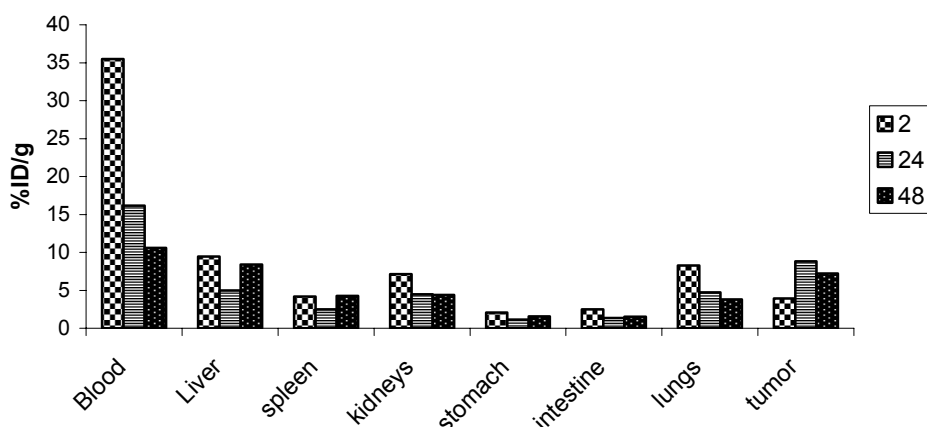


FIG. 8. Biodistribution in mice bearing a tumour at 2, 24 and 48 h.

Body clearance

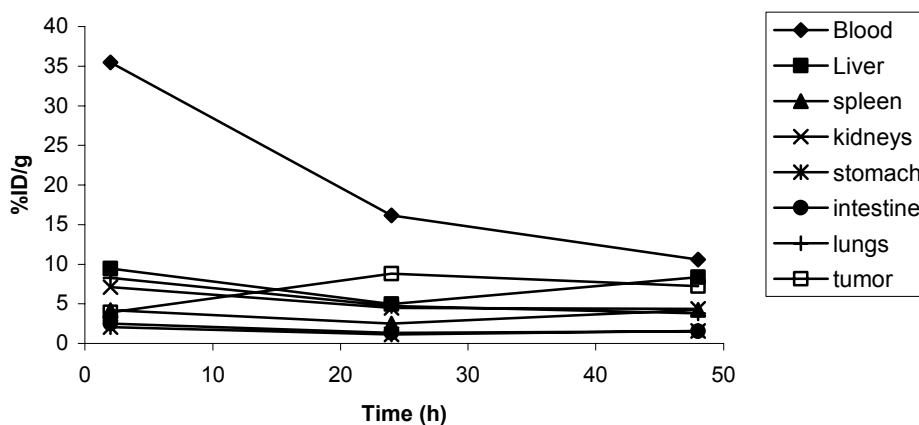


FIG. 9. Body clearance of labelled MoAb in mice bearing a tumour.

3.6. Labelling and quality control of lanreotide with ^{188}Re via a direct method

3.6.1. Radiolabelling

Different experiments were carried out in order to obtain the highest specific activity. Decreasing amounts of peptide per mCi of ^{188}Re were used. The formation of a single derivative of labelled lanreotide was influenced by the experimental conditions applied, namely by the amount of peptide and reducing agent, the reaction time and the temperature. We found that $6\mu\text{g}$ of lanreotide and 0.53 mg of SnCl_2 per mCi are needed in order to obtain one peak at the HPLC and 100% of labelling yield as shown in Fig. 10. In this case the specific activity rised $166\mu\text{Ci}/\mu\text{g}$. This product was instable at room temperature after 24h and all the rhenium was in the form of perrhenate.

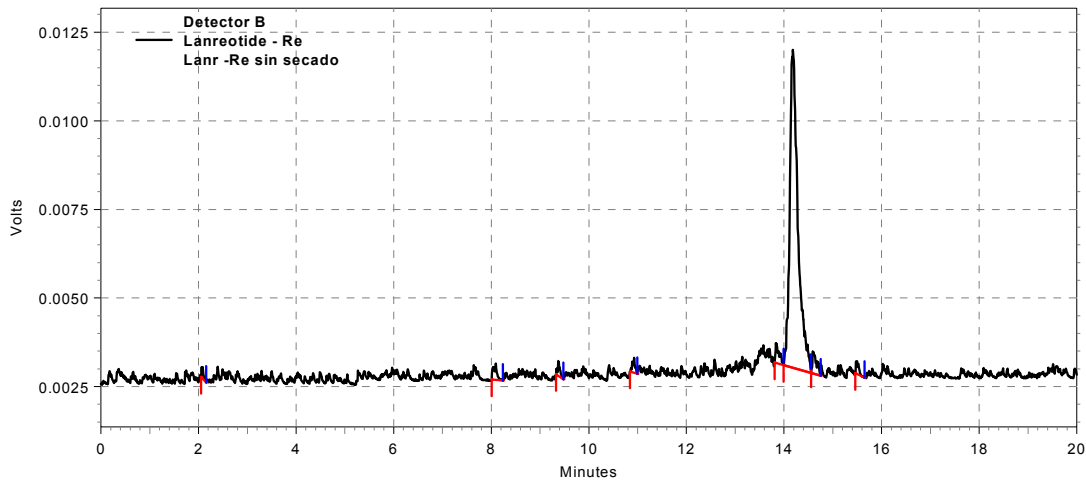


FIG. 10. Reverse phase C18 HPLC radiochromatogram of ^{188}Re -lanreotide.

An alternative approach was carried out using different reducing agents as secondary reducing agent, in order to improve the reduction properties of stannous chloride. Oxalate, tartrate and ascorbate at pH=5.0 were tried as secondary reducing agent in combination with gentisic acid. The best stability after 24 h at room temperature was obtain when 2.0 mg of tartrate (10 mg/mL) pH=5 was used as shown in HPLC chromatograms (Fig. 11). Fig. 12 shows the HPLC profiles of the labelled peptide when ascorbate was used as a secondary reducing agent. ITLC was also performed in saline and acidified ethanol. Results showed 3% of colloid.

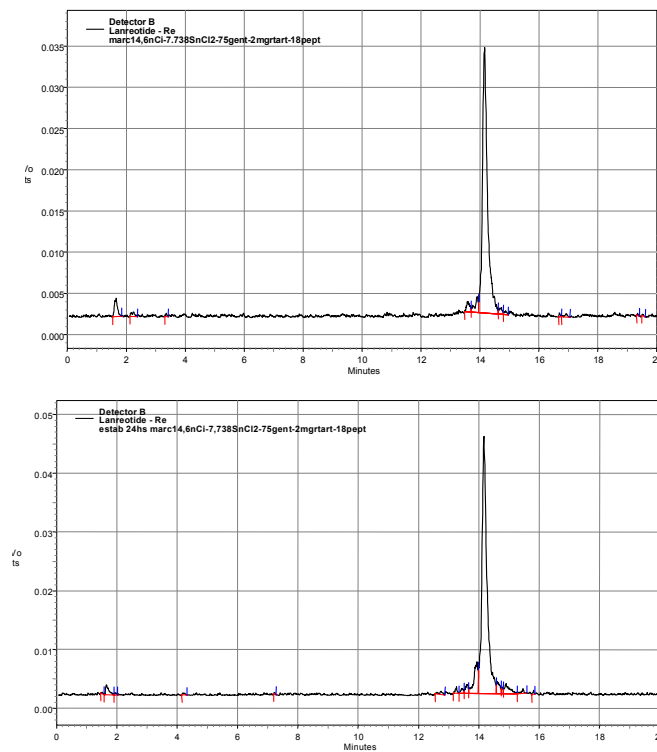


FIG. 11. Reverse phase C18 HPLC radiochromatograms of ^{188}Re -Lanreotide at 0h (left) and 24 h (right) at room temperature using tartrate.

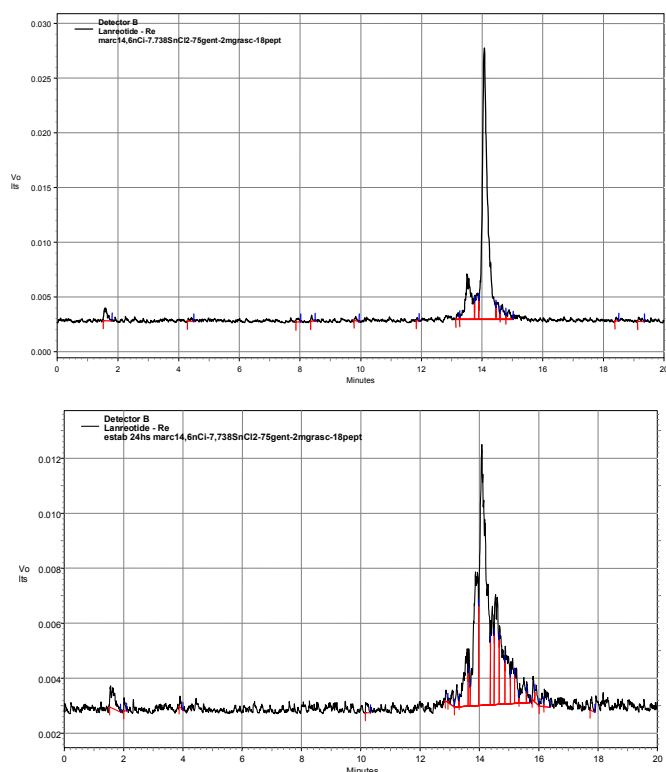


FIG. 12. Reverse phase C18 HPLC radiochromatograms of ^{188}Re -Lanreotide at 0 h (left) and 24 h (right) at room temperature using ascorbate.

3.6.2. *In vitro* studies

There was no displacement of ^{188}Re from the labelled peptide at the different cysteine concentrations studied. The ^{188}Re -lanreotide was stable after 4 h incubation in serum at 37°C . After 24 h, 95% of ^{188}Re remained bound to the peptide.

3.6.3. Biodistribution of ^{188}Re -lanreotide in mice

Fig. 13 shows the results of *in vivo* evaluation of the labelled peptide. The biodistribution data showed a tendency towards hepatobiliary elimination of the labelled peptide.

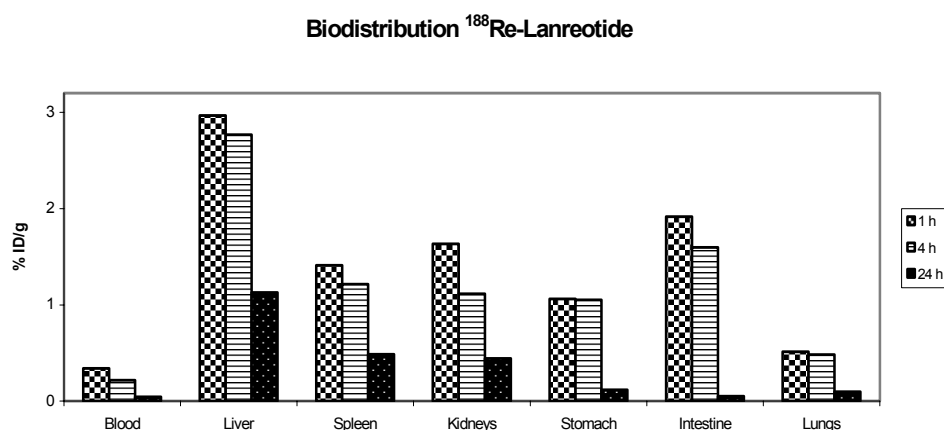


FIG. 13. Biodistribution in Balb/c mice of ^{188}Re -Lanreotide at 1, 4 and 24 h.

3.7. Labelling and quality control of peptides with ^{111}In or ^{90}Y

3.7.1. ^{111}In -DOTA-Lanreotide

3.7.1.1. Radiolabelling

The radiochemical purity of labelled peptide was higher than 98% when using peptide amounts higher than $30\mu\text{g}$. Fig. 14 shows the HPLC profile.

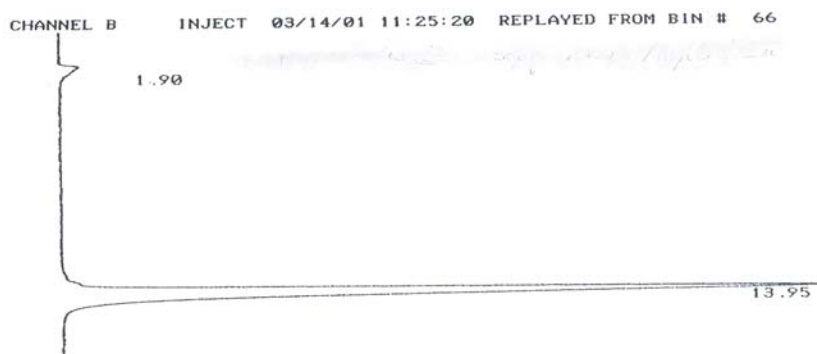


FIG. 14. Reverse phase HPLC radiochromatogram of ^{111}In -DOTA-lanreotide.

3.7.1.2. Serum stability

HPLC analysis after 4h incubation in human serum showed no degradation of the radiolabelled peptide after precipitation with acetonitrile. After 24h incubation the chromatogram showed 95% of labelled peptide.

3.7.1.3. Biodistribution

Fig. 15 shows biodistribution results at 4 h with ^{111}In -DOTA-lanreotide. The labelled peptide showed a tendency towards renal excretion and lower levels of activity in liver and intestine.

3.7.2. Labelling DOTA-TOC and DOTA-lanreotide with ^{90}Y

A radiochemical purity of 100% was obtained when using higher amounts than $8\mu\text{g}$ of DOTA-TOC per mCi of ^{90}Y as it shown in Fig. 16.

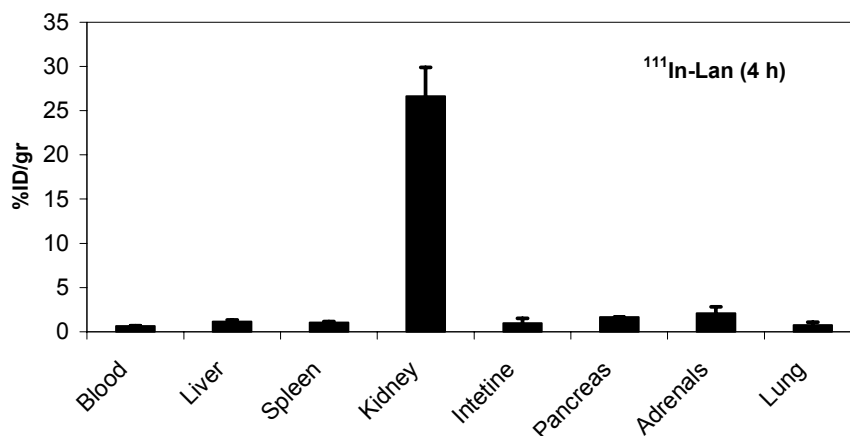


FIG. 15. Biodistribution in normal rats of ^{111}In -DOTA-Lanreotide at 4h.

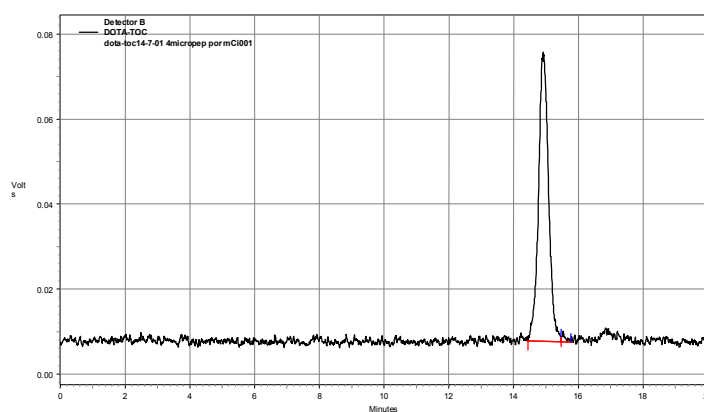


FIG. 16. Reverse phase HPLC radiochromatogram of ^{90}Y -DOTA-lanreotide.

We tried different amounts of Lanreotide-DOTA in order to obtain a good labelling yield. It was necessary to increase the peptide mass 70 fold over that required for the DOTA-TOC. Fig. 17 shows the difference in retention times of the two-labelled peptide. ^{90}Y -DOTA-Lanreotide has a higher retention time than the ^{90}Y -DOTA-TOC.

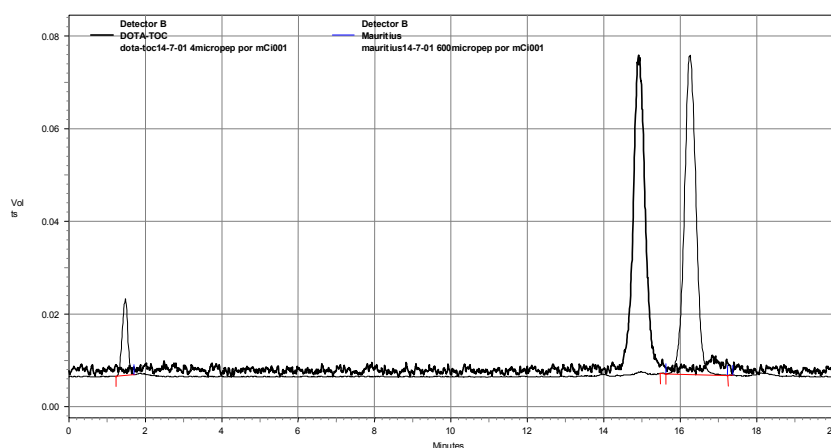


FIG. 17. Reverse phase HPLC radiochromatograms of ^{90}Y -DOTA-Lanreotide ($R_t=16.5'$) and ^{90}Y -DOTA-TOC ($R_t=15'$).

4. DISCUSSION AND CONCLUSIONS

S-Benzoyl-MAG₃ was used because it is more stable to oxidation than MAG₃ with its free thiol group. As reported in Materials and Methods, for efficient S-benzoyl-MAG₃ labelling, the SnCl₂·2H₂O, S-benzoyl-MAG₃ and the $^{188}\text{ReO}_4^-$ mixture had to be heated for 30 min at 90° C. At this temperature, S-benzoyl-MAG₃ loses the benzoyl protecting group and the reduced rhenium is complexed. However at this temperature ^{188}Re labelling of MAG₃-IgG or MAG₃-MoAb is not feasible because of protein denaturalization and consequent loss of immunoreactivity.

We therefore optimized the preconjugate approach in which radiolabelled MAG₃ was first chemically activated to obtain an active ester, following which the ester was coupled to free amino groups on the protein. Comparing the results of our experiments when labelling biomolecules with ^{188}Re and $^{99}\text{Tc}^m$, we conclude that to achieve an effective reduction of perrhenate it was necessary to increase the stannous chloride mass 620 fold over that required for the same radioactivity of $^{99}\text{Tc}^m$.

The indirect method of labelling antibodies using a ^{188}Re -labelled active ester of MAG_3 produced ^{188}Re - MAG_3 -IgG of high *in vitro* stability and favourable uptake at sites of focal inflammation. Likewise, application of this optimized labelling procedure to anti-melanoma MoAb, produced labelled antibody with high specific activity (2.44 mCi/mg MoAb) showing that the procedure reported here can be extended to the ^{188}Re labelling of other antibodies.

We efficiently labelled lanreotide with ^{188}Re via a direct method and clinical studies in patients have started.

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LABELLING OF PEPTIDES WITH $^{188}\text{Re}/^{99}\text{Tc}^{\text{m}}$ AND MONOCLONAL ANTIBODIES WITH $^{90}\text{Y}/^{111}\text{In}$

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Abstract

Radiopharmaceuticals derived from oncophilic small molecular weight peptides have attracted attention as vehicles to deliver radioactivity to tumour cells for external imaging and targeted radiotherapy [1,2]. The aim of the present work was the study of labelling of biomolecules, specifically peptides or antibodies with beta emitter radioisotopes such as ^{188}Re . The direct labelling of two somatostatin analogues, Octreotide and Lanreotide, with ^{188}Re and $^{99}\text{Tc}^{\text{m}}$ was done, including optimization of this labelling and biological studies. The radiochemical yield was greater than 95%. ^{188}Re -lanreotide gave better radiochemical yields than ^{188}Re -octreotide and was stable for 24 h. Similar results were obtained with $^{99}\text{Tc}^{\text{m}}$ -octreotide and $^{99}\text{Tc}^{\text{m}}$ -lanreotide. However, a difference in mass ratio (reducing agent/peptide) was observed, being much greater with ^{188}Re . $^{99}\text{Tc}^{\text{m}}$ -labelled peptides were localized in the gastrointestinal tract or in the kidneys and blood clearance wasn't observed during 6 h. Biodistribution of ^{188}Re -peptides showed similar results as obtained with $^{99}\text{Tc}^{\text{m}}$ -peptides. The indirect method was used to label DOTATOC with ^{188}Re and $^{99}\text{Tc}^{\text{m}}$ and competition binding assays were done with the labelled product. The results showed a radiochemical yield in the range of 92 to 94%. We observed better uptake of $^{99}\text{Tc}^{\text{m}}$ -DOTATOC in AR42J cell membranes than in brain cortex membranes. The results obtained with ^{188}Re -DOTATOC were inconclusive. Labelling of monoclonal antibody for prostate cancer cells (J591) with ^{111}In and ^{90}Y , and immunoreactivity of the product was also studied. The radiolabelling yield for huJ591 with ^{111}In and ^{90}Y was higher than 90%. An average of five DOTA molecules could be randomly conjugated to J591, yielding specific activities of 280 MBq $^{111}\text{In}/\text{mg}$ DOTA-J591 and 360 MBq $^{90}\text{Y}/\text{mg}$ DOTA-J591, with little apparent loss of immunoreactivity.

1. INTRODUCTION

The major radiopharmaceuticals used in nuclear medicine are guided to diagnostic procedures. There are very few agents for cancer radiotherapy commercially available. It is necessary to obtain results that confirm that treatment with therapeutic radiopharmaceuticals had effect and cured disease over the period of many years.

Over the years, the development of radioimmunotherapeutics has generally been performed using radionuclides such as ^{111}In and ^{90}Y [3].

For cancer radiotherapy, beta emitters are used. Yttrium-90 and rhenium-188 have been determined to be among the best nuclides for radiation therapy [4]. Their half-lives are long enough to permit accumulation in target tissues, and they possess strong beta emission capable of delivering high radiation doses to tissues.

For radioimmunotherapy, rhenium-188 is a very good radionuclide with advantages over other therapeutic radionuclides, including the fact that it is carrier-free, and can be obtained cost-effectively and on demand from an in house $^{188}\text{W}/^{188}\text{Re}$ generator [5]. ^{188}Re decays by emission of a relatively high-energy beta particle (2.27 MeV), followed by gamma emission (155 keV, 15%) that can be used to monitor the biodistribution by scintigraphy.

Biomolecules (antibodies, peptides) have generally been considered particularly attractive as selective carriers of therapeutic agents due to their unique *in vitro* specificity and high affinity for their antigen.

Peptides have shown advantages over antibodies with respect to tumour-to-nontumour ratios. Lower-molecular weight agents generally provide better target-to-nontarget ratios, due to their rapid background clearance [6].

Covell, et al. (1986) [7] pointed out that the body residence time for antibodies is much longer than that for fragments of antibodies, which may be an advantage when delivering a sufficient quantity of antibody to the tumour site in order to perform radioimmunotherapy, especially in the case of solid tumours.

Somatostatin is a peptide hormone consisting of 14 amino acids. It is present in the hypothalamus, the cerebral cortex, the brain stem, the gastrointestinal tract and the pancreas. Various tumours contain high numbers of somatostatin receptors, which enable *in vivo* localization of the primary tumour and its metastases by scintigraphy with radiolabelled somatostatin analogue peptides [8,9].

Lanreotide [D-βNal-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr.NH₂] as octreotide [H₂N-D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr(ol)] are synthetic octapeptide analogues of the native hormone somatostatin, produced by the hypothalamus and pancreas¹⁰. They are cyclized via cysteine bridge.

Many studies reported octreotide labelled with various radionuclides like ¹³¹I, ¹¹¹In, ¹⁸⁸Re and ⁹⁹Tc^m [8, 11, 12]. But with lanreotide we can find only some studies made with radioisotope ⁹⁰Y by indirect method conjugated with the bifunctional chelator DOTA [10]. There are few studies reported about the labelling of lanreotide with ¹⁸⁸Re and with ⁹⁹Tc^m.

There are two methods to label peptides with ¹⁸⁸Re or ⁹⁹Tc^m, namely the direct and indirect method. There are advantages and disadvantages associated with each of these methods.

The direct method of labelling peptides containing a cyclized cysteine bridge gives some advantages, because it is simple, rapid and efficient and does not require commercially unavailable agents, such as the bifunctional chelating agents (BFCAs) [2].

But the reduction of cysteine bridge can inactivate the peptide. On the other side the indirect method is very complex, lengthy, with insufficient labelling yields [13].

Prostate cancer is the most frequently diagnosed cancer and the second most common cause of cancer mortality in United States males. Monoclonal antibodies for prostate specific membrane antigen (PMSA) were labelled with ¹¹¹In and ⁹⁰Y for the evaluation of their diagnostic and therapeutic potential [14].

Objectives

- In the first part of the research project, studies were developed for the labelling of peptides with ¹⁸⁸Re by direct method, the optimization of this method by varying some parameters such as mass of ligand, reductor and peptide ratio, incubation time and stability of the labelling. We made the same studies for labelling peptide by direct method using as the radioisotope technetium-99m, and also biodistribution and stability *in vitro* studies of the labelled product (Results shown in the 2nd Co-ordination Meeting in Mumbai, India).
- As the second part, indirect labelling of conjugated peptide (HYNIC-TOC) with rhenium-188 and technetium-99m was made, followed by preparation of rat brain cortex cell

membranes and cell binding assays of the labelled products. These studies were done in St. Bartholomew's Hospital, London with Dr. Stephen Mather.

- As the third part, we studied the labelling of monoclonal antibody for prostate cancer (mAbs PCas) with ^{111}In and ^{90}Y . These studies were carried out in The New York Presbyterian Hospital, Cornell University with Dr. Shankar Vallabhajosula.

- As the final part, we completed the studies of direct labelling of peptides with ^{188}Re , specifically on standardization of labelling protocols, cysteine challenge and biodistribution studies in animals.

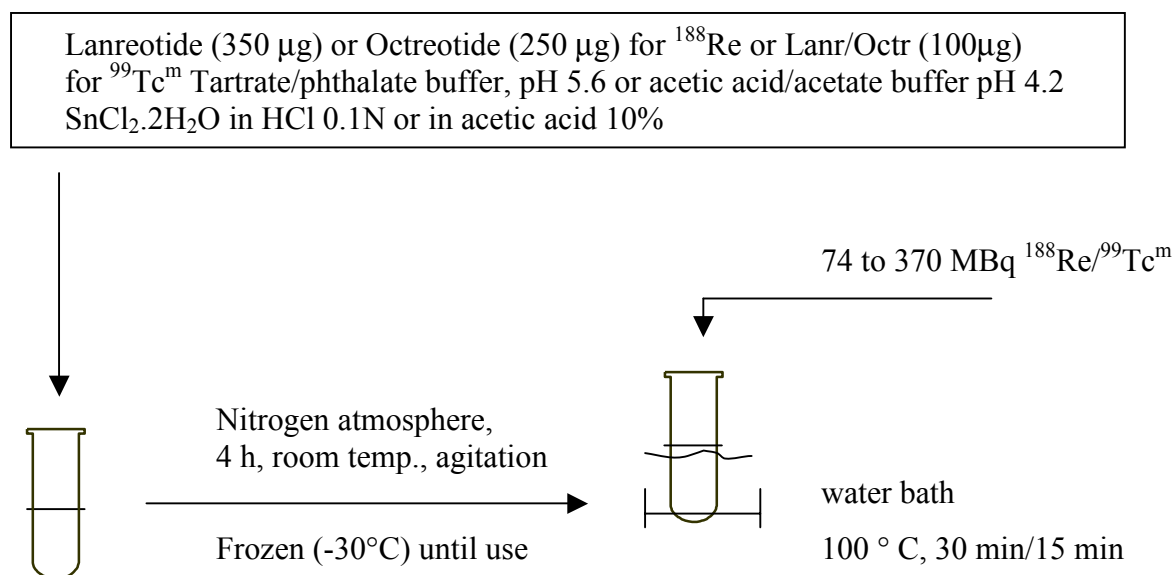
2. MATERIALS AND METHODS

2.1. Studies of peptide labelling with ^{188}Re and $^{99}\text{Tc}^{\text{m}}$ by direct method [11]

Materials: Lanreotide 8-mer (from PiChem-Austria) and $^{188}\text{W}/^{188}\text{Re}$ generator (MAP Medical Technologis Oy Finland) were provided by IAEA, Octreotide was provided by Novartis, Brazil and the $^{99}\text{Mo}/^{99}\text{Tc}^{\text{m}}$ generator by the Radiopharmacy Center of IPEN/CNEN-SP, Brazil.

Labelling

The direct labelling of peptide (octreotide and lanreotide) with ^{188}Re and $^{99}\text{Tc}^{\text{m}}$ isotopes was achieved through three main steps: (1) Preparation of the peptide, (2) Labelling and quality control, (3) Optimization of the labelling as indicated in the diagram below.



Radiochemical control

After labelling the ^{188}Re -peptide passed through a $0.22\ \mu\text{m}$ filter (Millipore) and was analysed for radiolabelled yield using thin layer chromatography (TLC) and reverse phase step-gradient separation on C_{18} -SepPak columns (Millipore Inc.). For $^{99}\text{Tc}^{\text{m}}$ -peptide it was not necessary the step of filtration.

Optimization of radiolabelling

It was achieved by varying some parameters as mass of peptide (100 µg to 500 µg and 10,50,100 µg), stannous chloride/peptide mass ratio (3.0, 3.5, 4.0 and 0.4, 0.6, 0.8, 1),

stability of radiochemical purity of the complex (24 h and 6 h), reaction time (1,2,3 h and 30, 60 min) for ^{188}Re and $^{99}\text{Tc}^m$ studies, respectively.

Cysteine challenge studies with $^{99}\text{Tc}^m$ and ^{188}Re -peptides

The bond strength of the radioisotope $^{99}\text{Tc}^m$ or ^{188}Re peptide complexes was evaluated by displacement of bound radionuclide with cysteine [15]. Known amounts of $^{99}\text{Tc}^m$ or ^{188}Re -lanreotide and $^{99}\text{Tc}^m$ - or ^{188}Re -octreotide were challenged over a range of cysteine concentrations (0.1mM – 100mM) at 37 °C for 1 h. The percent $^{99}\text{Tc}^m$ displaced by cysteine was determined by TLC using 0.9% saline as a mobile phase, and the amount of displaced radiolabel found at the solvent front determined.

Biodistribution studies with octreotide and lanreotide labelled by direct method with ^{188}Re and $^{99}\text{Tc}^m$

The biodistribution studies were performed in groups of three animals by intravenous injection of 0.1 mL of radiolabelled peptides with 500 μCi of $^{99}\text{Tc}^m/^{188}\text{Re}$. The animals were sacrificed at determined times after injection and selected organs were removed, weighed and the radioactivity was determined with a well-type gamma counter. Radioactivity was also measured in a sample of blood. The results were expressed as% of injected dose per gram of tissue.

Dynamic imaging studies were performed at the first 10 min after the administration of the drug and static imaging after 15 min, 2, 4 and 6 h post injection for $^{99}\text{Tc}^m$ -peptides.

2.2. Studies of peptide labelling with ^{188}Re and $^{99}\text{Tc}^m$ by indirect method (conducted at St. Bartholomew's Hospital, London)

Materials: Tyr³-octreotide (TOC) was purchased from Bachem Ltd., Saffron Walden, UK. The hydrazinonicotinyl (HYNIC) was synthesized and protected (BOC-HYNIC) in the Imperial Cancer Research Foundation.

Preparation of HYNIC-TOC conjugate

Five micromoles 6-BOC-hydrazinopyridine-3-carboxylic acid (BOC-HYNIC), 6 μmol O-(7-azobenzotriazolyl)-1,1,2,2-tetramethyl-uronium hexafluorophosphate, and 20 μmol diisopropylethylamine in 300 μL dimethylformamide were allowed to react for 15 min at room temperature. Sixty microliters of this solution were added to 1 μmol [Lys⁵-BOC]-protected TOC (Bachem) in a mixture of 20 μL dimethylformamide (DMF): 5 μL water and allowed to react for 1 h. The resulting solution was purified on a SepPak column, and the peptide was deprotected with trifluoroacetic acid containing 2% thianisole and purified on HPLC.

Purification in HPLC [16]

A solvent module 125 (Beckman Coulter, Inc., Fullerton, CA) with a 166 nm ultraviolet detector (Beckman) and radiometric detection was used for reverse-phase high-performance liquid chromatography (HPLC) analysis and preparation.

An Ultrasphere ODS 5- μm system (Beckman), 4.6 \times 250 mm column, 1 mL/min flow rate, and 220-nm ultraviolet detection, was used with the following solvent systems:

acetonitrile (ACN):0.1% trifluoroacetic acid:water (0-3 min, 0% ACN; 3-10 min, 0-40% ACN; 10-20 min, 40% ACN; 20-23 min, 40-70% ACN; 26-27 min, 70-100% ACN).

Radiolabelling

Labelling of HYNIC-TOC using (EDDA) as coligand

Ten micrograms HYNIC-TOC were incubated with 0.5 mL EDDA solution and 0.5 mL was added of $^{99}\text{Tc}^{\text{m}}\text{O}_4^-$ solution (500 MBq) or ^{188}Re solution (238 MBq), and 5-10 μL or 80 μL tin(II) solution (10 mg $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ /10 mL or 10 mg/1,6 mL) nitrogen-purged 0.1 N HCl for 60 min at room temperature or with heating for 30 min in water bath (70 °C) for $^{99}\text{Tc}^{\text{m}}$ and ^{188}Re , respectively.

Radiochemical control

The radiochemical control was done using HPLC as described by Decristoforo & Mather, 1999 [16].

Somatostatin receptor binding assays done for DOTA-TOC labelled with technetium and rhenium

Receptor binding studies were performed in order to determine the receptor affinity of the radiolabelled peptides.

The binding affinity of peptide conjugates (^{188}Re -HYNIC-TOC and $^{99}\text{Tc}^{\text{m}}$ -HYNIC-TOC) was tested in a competition assay using rat pancreatic tumour cell (AR42J) membranes and rat brain cortex membranes as a source for somatostatin receptors.

Preparation of cell membranes AR42J

AR42J cells growing in suspension were spun down at 2000xg (3000rpm) in a Beckman GS-6R centrifuge for 20 min, resuspended in 20 ml of cold HEPES buffer (20mM, pH 7.3) and homogenized for 10s using and Ultraturrax T8 Netzgerat homogenizer (IKA Labortechnik) on the highest setting. The sample was centrifuged (2000 xg (3000 rpm) for 20 min), the supernatant was removed, the pellet was resuspended in 20 mL of HEPES buffer solution and homogenized again. This procedure was repeated twice.

The final cell membrane pellet was resuspended in HEPES buffer and homogenized for 10 s. The protein concentration was determined using the Bio-Rad DC Protein Assay, and the membranes were then aliquotted into 2 ml Cryo Tubes each containing 2.5 mg of protein in 0.5 mL of sample, i.e. sufficient membranes for two binding assays. The tubes were frozen immediately in an acetone/dry ice bath and stored at -70 °C until required.

Preparation of rat brain cortex membrane

Tissue membranes were prepared using a modification of the method of Raynor & Reisine, 1989 described by Kolan, et al., 1996 [17] and the IAEA, 2000 [18].

Competition binding assays

Millipore Multiscreen Filtration System plates with glass fiber filters (Millipore MAFB NOB 10) were prepared the day before the assay was performed using polyethyleneimine aqueous solution.

A stock solution of competitor peptide was prepared and sequential dilutions were made. The radioligand was also diluted so the radioactivity corresponded to approximately 50000 cpm per 50 μL of solution. The competitor solutions were added to the wells of the

assay plate prepared one day before, followed by the addition of radioligand followed by the cell membranes. The plate was incubated at room temperature using a Thermolyne Maxi-Mix Shakes at low speed for 2 h. Once the incubation was finished, the plate was filtered, washed and dried in the oven at 60-70 °C for about 20 min. The filters were punched out and placed into tubes and counted in a gamma counter.

Inhibitory concentration of 50% (IC₅₀) values were calculated using non-linear regression with version 5.0 Origin software (Microcal, Northampton, MA). The specific binding of the ⁹⁹Tc^m-HYNIC-TOC and ¹⁸⁸Re-HYNIC-TOC was determined by competition against unmodified TOC in a similar assay.

2.3. Studies of monoclonal antibodies for prostate cancer with ¹¹¹In and ⁹⁰Y

Murine monoclonal antibody mAbs J591 for prostate cancer cells was conjugated to the bifunctional chelate DOTA and then labelled with ¹¹¹In or ⁹⁰Y.

Antibody conjugation

HuJ591 antibody was modified with DOTA by a method analogous to that used by Lewis [19]. The main steps are:

- a) Concentration of the antibody
- b) Washing with 1% DTPA solution
- c) Changing of the antibody buffer
- d) Development of an active ester of DOTA
- e) Conjugation of the ester of DOTA with J591
- f) Purification and concentration of DOTA-J591.

¹¹¹In and ⁹⁰Y labelling of DOTA conjugate – Radiolabelling of DOTA-J591 with ¹¹¹In was achieved by adding the radionuclide to the ammonium acetate-buffered DOTA-J591. The mixture was allowed to react at 37° C for 20 min and then washed in a column with 5 ml of 1% HAS-PBS before the main ¹¹¹In-DOTA-J591 fraction was eluted with 3 ml of 1% HAS-PBS. A similar procedure was used for radiolabelling with ⁹⁰Y, but an incubation time of 5 min was used, and the labelling mixture included 50mM ascorbic acid.

Quality control of radiolabelling – Free ¹¹¹In in the radiolabelled DOTA-J591 preparations was determined using the ITLC method with a silica gel-impregnated glass fiber support and a mobile phase of 1% DTPA (pH 5.5).

Immunoreactivity studies – The immunoreactivity of ¹¹¹In and ⁹⁰Y-labelled Mab preparations was assessed by the method of Lindmo. et al., 1994 [20], which extrapolates the binding of the radiolabelled antibody at an infinite excess antigen. Briefly, six test solutions were prepared (in duplicate) and contained 20000 cpm of the radiolabelled antibody, and increasing amounts of membranes were prepared from LNCaP cells in a total test volume of 250 µl of PBS. The solutions were incubated at 37° C for 45 min prior to being filtered through a glass membrane filter and washed with ice-cold 10 mM Tris-0.9% buffer. Filters were counted in a gamma counter with standards representing the total radioactivity added. Data were then plotted as the reciprocal of the substrate concentration (X axis) against the reciprocal of the fraction bound (Y axis). The data were then analysed according to a least squares linear regression method using Origin software. The Y intercept gave the reciprocal of the immunoreactive fraction. A similar method using intact or permeated LNCaP cells and centrifugational isolation of the cells gave the same results.

3. EXPERIMENTAL RESULTS

Studies of peptide labelling with ^{188}Re and $^{99}\text{Tc}^m$ by direct method

Labelling efficiency determined by thin-layer chromatography when using 0.9% saline as a mobile phase showed that the unbound radioactivity migrated with the solvent front, while the bound radioactivity remained at the origin (Fig. 1).

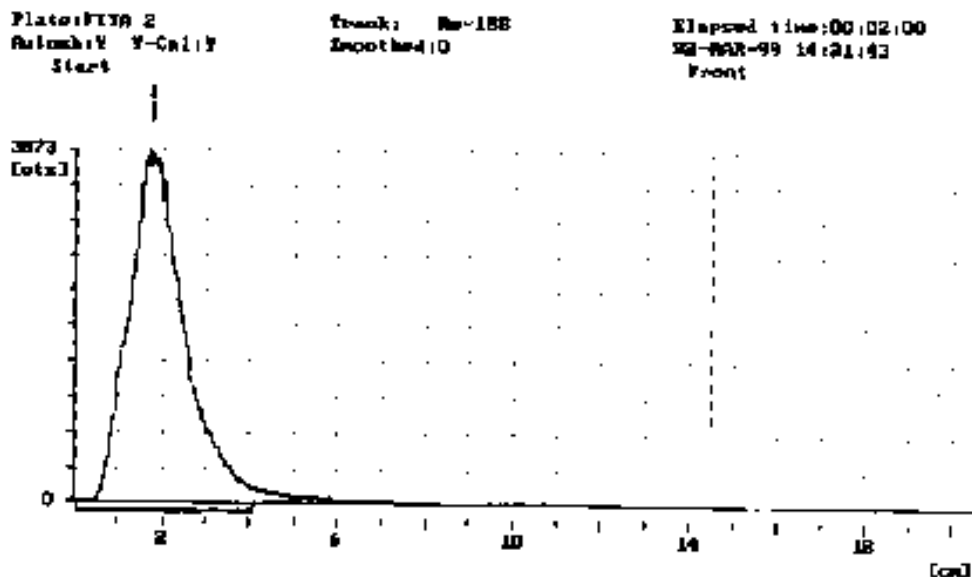


FIG. 1 Radiochromatogram of ^{188}Re -Lanreotide in saline 0.9%.

The amount of colloids formed in the peptide solution was determined using 85% ethanol as a mobile phase. The radiolabelled peptides moved to the solvent front and colloids remained at the origin (Fig. 2).

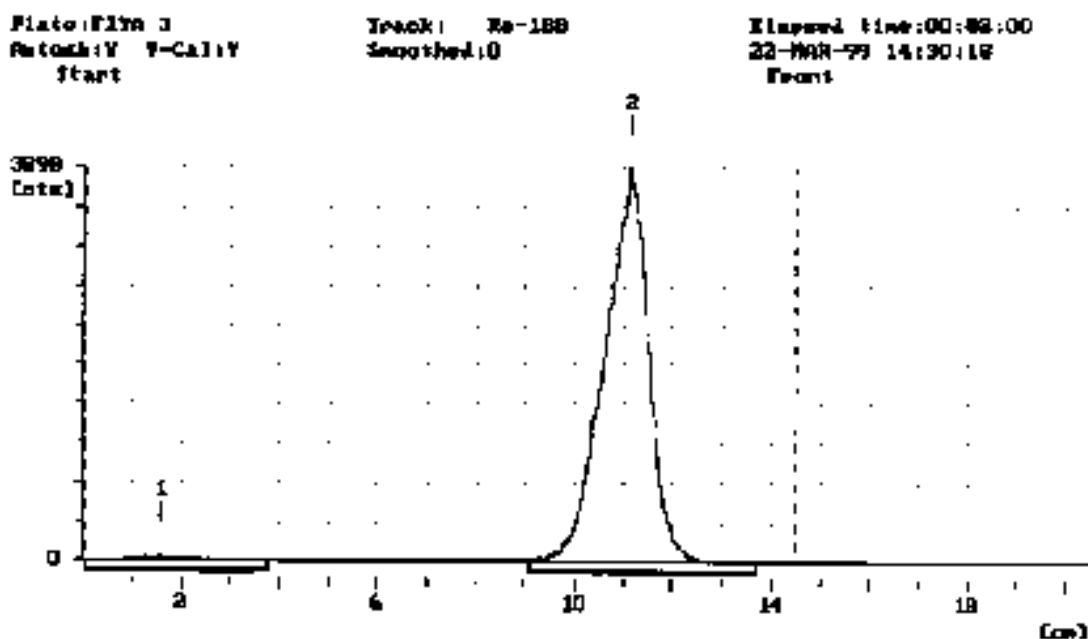


FIG. 2 Radiochromatogram of ^{188}Re -Lanreotide in ethanol 85% as solvent.

During radiochemical analysis made by C₁₈ SepPak the radioactivity eluting with 10% ethanol was assumed to be unbound ¹⁸⁸Re, that eluting in 85% ethanol to be peptide bound, and that resident on the cartridge to be radiocolloid (Fig. 3).

Labelling efficiencies were generally greater than 95% as determined by TLC until 2 h after labelling. (Figs 4, 5 and 6). Reverse phase chromatography using C₁₈ SepPak confirmed the high labelling efficiency (Fig. 3).

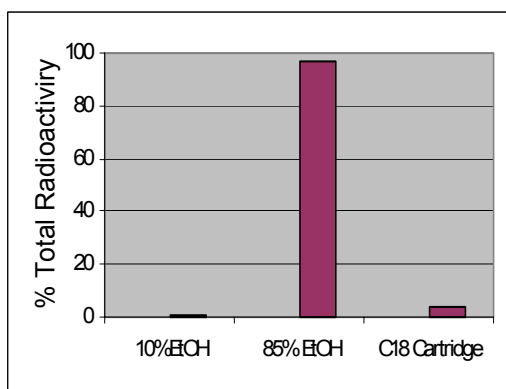


FIG. 3. Radiochemical analysis C₁₈ SepPak.

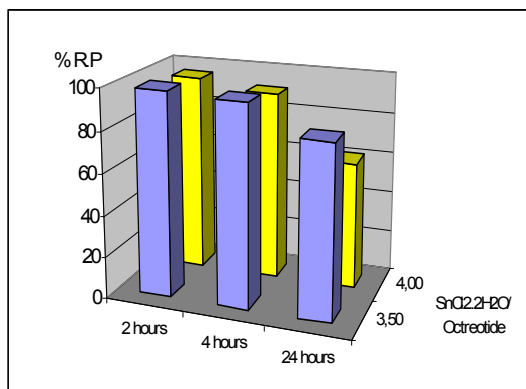


FIG. 4. Labelling efficacy of ¹⁸⁸Re-octreotide in tartrate/phthalate buffer.

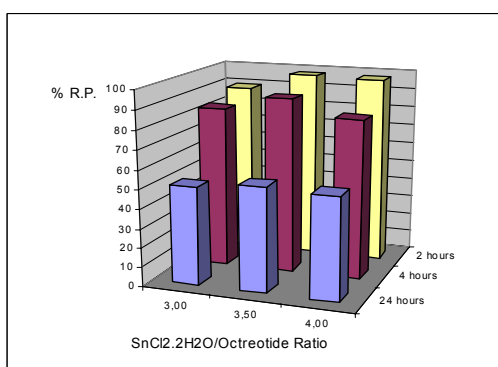


FIG. 5. Labelling efficacy of ¹⁸⁸Re octreotide in acid/acetate buffer.

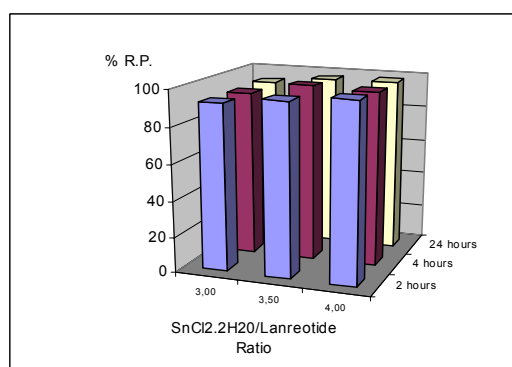


FIG. 6. Labelling efficacy of ¹⁸⁸Re-lanreotide.

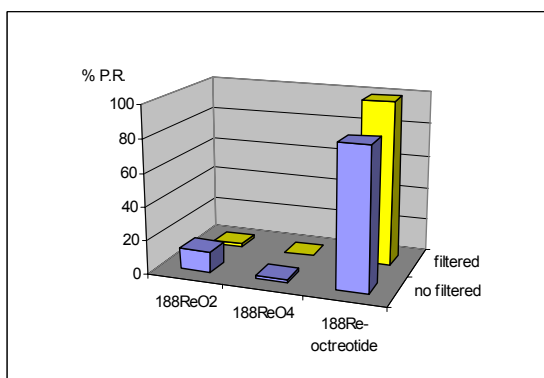


FIG. 7. Amount of radiocolloid after 2h for ¹⁸⁸Re-octreotide.

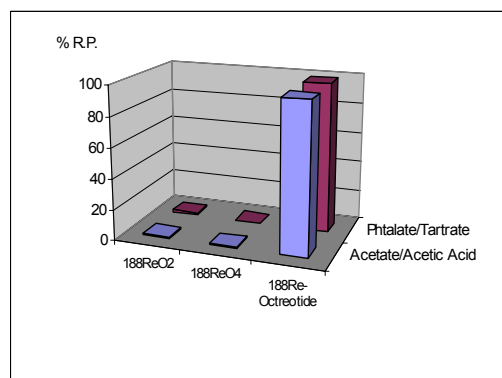


FIG. 8. Analysis of ¹⁸⁸Re-octreotide in different buffer solutions.

The amount of radiocolloid determined by ITLC with 85% acidified ethanol as the mobile phase was greater when we didn't make a purification by using filter with membrane 0.22 μm . When we made the filtration much activity was retained in the filter (Fig. 7).

For ^{188}Re -Lanreotide we obtained high labelling efficiency using 350 μg of the ligand lanreotide and for ^{188}Re -Octreotide a mass of 250 μg only was sufficient for good labelling in both buffers.

The reaction time was 2 h for both complexes and the best ratio between mass of stannous chloride and peptide was 3.5 for ^{188}Re -Octreotide and 4.0 for ^{188}Re -Lanreotide (Figs 4, 6). With the ratio of 3.0 we observed that the amount of stannous chloride was insufficient to reduce all the rhenium so we observed a larger amount of free perrhenate (Fig. 5).

The radiochemical purity of ^{188}Re -Lanreotide was $98.76\pm 0.87\%$ and the product stayed stable for 24 h (Fig. 6).

^{188}Re -Octreotide presented $97.14\pm 1.2\%$ purity in tartrate/phthalate buffer. In acetic acid/acetate buffer, findings were similarly close to 97% (Fig. 8).

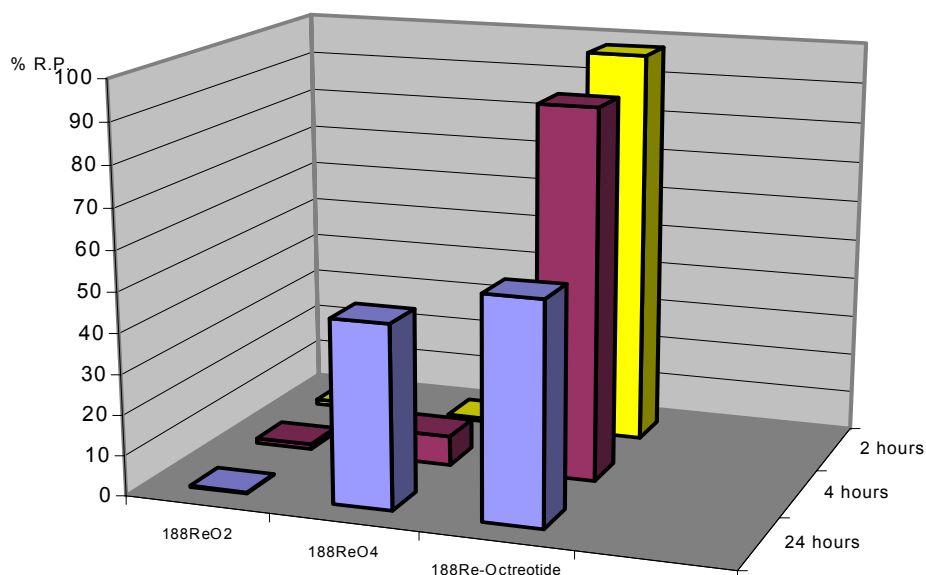


FIG. 9. Labelling stability of ^{188}Re -octreotide in acetate/acetic acid buffer.

However, ^{188}Re -octreotide stayed stable for 4 h and with 24 h, the stability fell by about 50% (Fig. 9).

When we tried to label a very small mass of ligand such as 10 μg and also 100 μg with a mass ratio of stannous chloride to peptide in the range of 10 to 50 to label with ^{188}Re , very small radiolabelled yield was achieved.

When octreotide was labelled with $^{99}\text{Tc}^{\text{m}}$ it was observed that when the mass ratio between the reducing agent and the peptide was low, the yield obtained was low. The best finding was for a ratio of one (1.0) (Table I).

TABLE I. RADIOCHEMICAL PURITY OF $^{99}\text{Tc}^{\text{m}}$ -OCTREOTIDE VARYING THE MASS RATIO OF $\text{SnCl}_2 \cdot \text{H}_2\text{O}$ /OCTREOTIDE

m $\text{SnCl}_2 \cdot \text{H}_2\text{O}$ / m octreotide	% $^{99}\text{Tc}^{\text{m}}\text{O}_4$	% $^{99}\text{Tc}^{\text{m}}\text{O}_2$	% $^{99}\text{Tc}^{\text{m}}$ -octreotide
0.4	91.04	2.9	6.06
0.6	30.52	1.88	67.60
0.8	48.91	0.98	50.11
1.0	3.48	1.38	95.14

The study of the labelling of lanreotide with $^{99}\text{Tc}^{\text{m}}$ was based on the results obtained with $^{99}\text{Tc}^{\text{m}}$ -octreotide. So, we observed that the best ratio between $\text{mSnCl}_2 \cdot \text{H}_2\text{O}$ /m lanreotide was 1.0 for a mass of lanreotide and a mass of stannous chloride above 50 μg (Table II).

TABLE II. RADIOCHEMICAL PURITY OF $^{99}\text{Tc}^{\text{m}}$ -LANREOTIDE

lanreotide m (μg)	$\text{SnCl}_2 \cdot \text{H}_2\text{O}$ m (μg)	ratio	% $^{99}\text{Tc}^{\text{m}}\text{O}_4$	% $^{99}\text{Tc}^{\text{m}}\text{O}_2$	% $^{99}\text{Tc}^{\text{m}}$ - lanreotide
10	30	3	93.65	1.05	5.30
20	20	1	44.67	1.30	53.96
50	50	1	0.60	1.51	97.89
100	100	1	0.18	3.09	96.73

Both, $^{99}\text{Tc}^{\text{m}}$ -octreotide and $^{99}\text{Tc}^{\text{m}}$ -lanreotide were radiochemically stable for 6 h (Table III). However, $^{99}\text{Tc}^{\text{m}}$ -octreotide had a loss in the radiochemical purity of 5.6%.

TABLE III. RADIOCHEMICAL STABILITY

Time (h)	$^{99}\text{Tc}^{\text{m}}$ -octreotide	$^{99}\text{Tc}^{\text{m}}$ -lanreotide
t=0	97.59	96.25
t=2	92.10	97.86
t=4	91.89	98.35
t=6	91.24	96.73

Cysteine challenge

Analysis by TLC-aluminium saline of $^{99}\text{Tc}^{\text{m}}/^{188}\text{Re}$ -labelled peptides incubated in solutions containing cysteine invariably showed migration. Since the aim of this measurement was to determine instability in face of cysteine transchelation and not oxidation to $^{99}\text{Tc}^{\text{m}}\text{O}_4^-$ / $^{188}\text{ReO}_4^-$, it was important to ensure that migrating activity was due to labelled cysteine and not due to pertechnetate or perrhenate which also migrate in this system and with an R_f value close to that for labelled cysteine.

Transchelation to cysteine was studied at five molar ratios. We can observe that $^{99}\text{Tc}^{\text{m}}$ -octreotide was relatively more susceptible to Cys challenge than $^{99}\text{Tc}^{\text{m}}$ -lanreotide (Fig. 10).

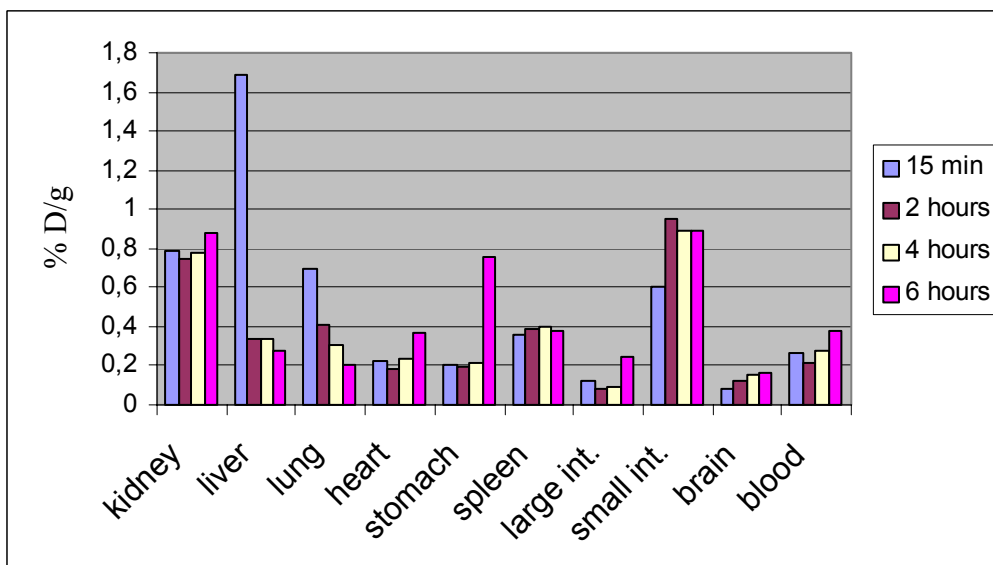


FIG. 10. Percent dissociation of $^{99}\text{Tc}^m$ from lanreotide and octreotide vs. cysteine concentration during incubation for 1 h at 37 °C.

The results of Cys challenge for ^{188}Re -peptides revealed a relatively high ^{188}Re -chelation strength for both the peptides (Fig. 11). The CD_{50} value (concentration of Cys required to displace 50% of peptide bound ^{188}Re) for ^{188}Re -octreotide was about 10 mM and for ^{188}Re -lanreotide was about 60 mM.

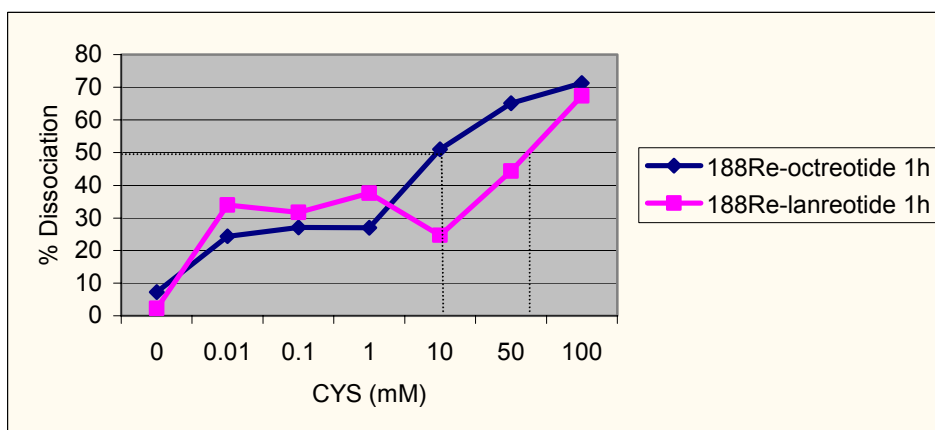


FIG. 11. Percent dissociation of ^{188}Re from lanreotide and octreotide vs. cysteine concentration during incubation for 1 h at 37 °C.

Biodistribution with $^{99}\text{Tc}^m$ -labelled peptides

The biodistribution of $^{99}\text{Tc}^m$ -lanreotide and $^{99}\text{Tc}^m$ -octreotide was evaluated in normal Wistar rats by dynamic imaging techniques during the first 10 min after injection, and by static imaging at 15 min, 1, 2 and 4 h post injection, and also by invasive method (organ removal).

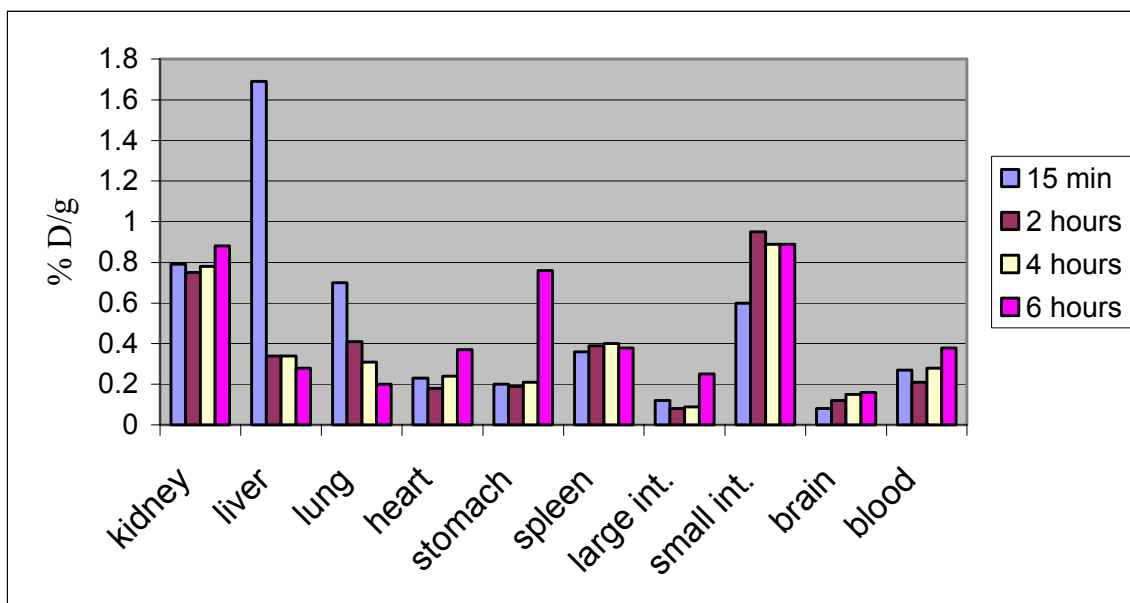


FIG. 12. Biodistribution of ⁹⁹Tc^m-lanreotide in normal rats.

⁹⁹Tc^m-labelled peptides were localized in the gastrointestinal tract or in the kidney (Figs 12,13). For ⁹⁹Tc^m-octreotide the uptake by the kidneys increased until 4 h p.i. with decrease after this time (Fig. 13). The small intestine had also a great uptake. With ⁹⁹Tc^m-lanreotide the greater uptake of the drug was by the liver at the first 15 min p.i., and we did not observe blood clearance during 6 h (Fig. 12).

Other organs presenting uptake were kidney and stomach.

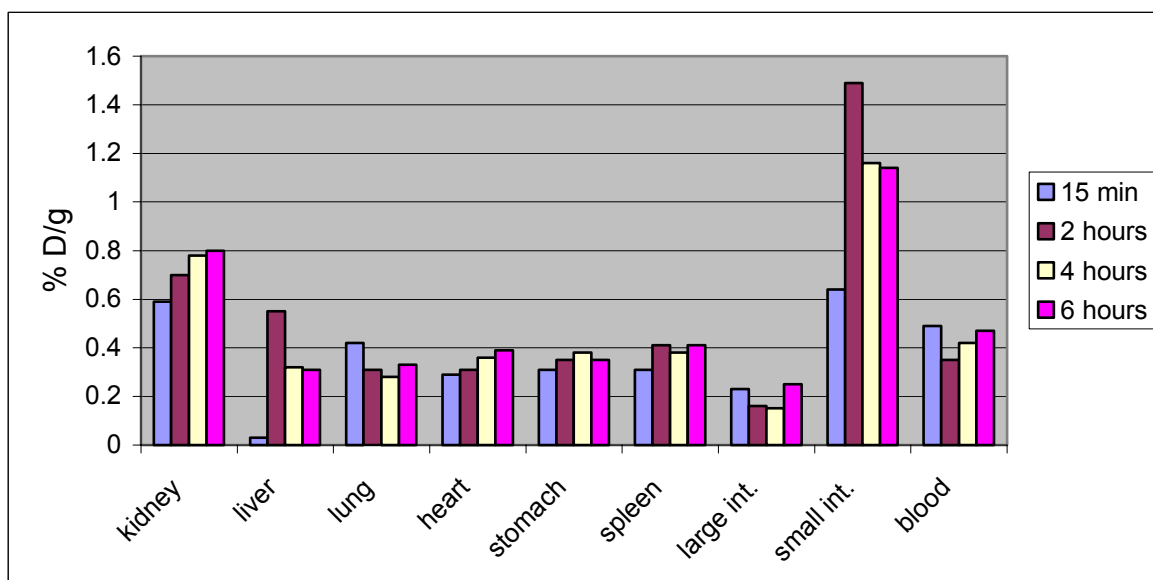


FIG. 13. Biodistribution of ⁹⁹Tc^m-octreotide in normal rats.

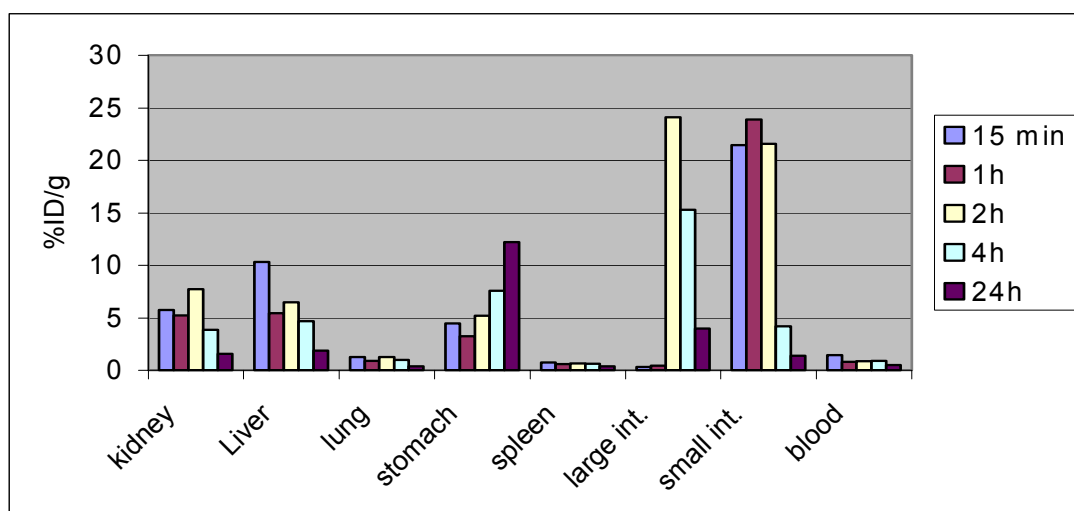


FIG. 14. Biodistribution of ¹⁸⁸Re-octreotide in normal mice.

Biodistribution of ¹⁸⁸Re-peptides was evaluated in swiss mice by invasive method at different times after injection. Results were similar to those obtained with ⁹⁹Tc^m. For ¹⁸⁸Re-octreotide, the highest uptake observed was by the intestine followed by the liver, kidneys and stomach (Fig. 14). With ¹⁸⁸Re-lanreotide the largest uptake observed was by the small intestine followed by the liver in the first 15 min, post-injection (Fig. 15).

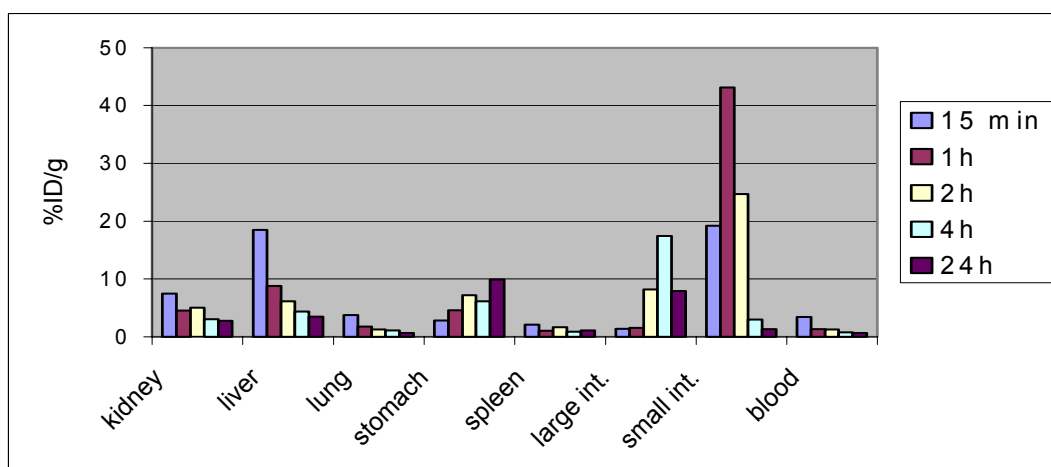


FIG. 15. Biodistribution of ¹⁸⁸Re-lanreotide in normal mice.

Studies of peptide labelling with ¹⁸⁸Re and ⁹⁹Tc^m by indirect method

The radiochemical purity of the ¹⁸⁸Re-HYNIC-TOC and ⁹⁹Tc^m-HYNIC-TOC was assessed by means of thin layer chromatography and high performance liquid chromatography in two different solvents. The results showed a radiochemical yield in the range of 92 to 94%.

Receptor binding to the AR42J rat pancreatic tumour cell membranes and rat brain cortex cell membranes was determined in the absence and presence of saturation.

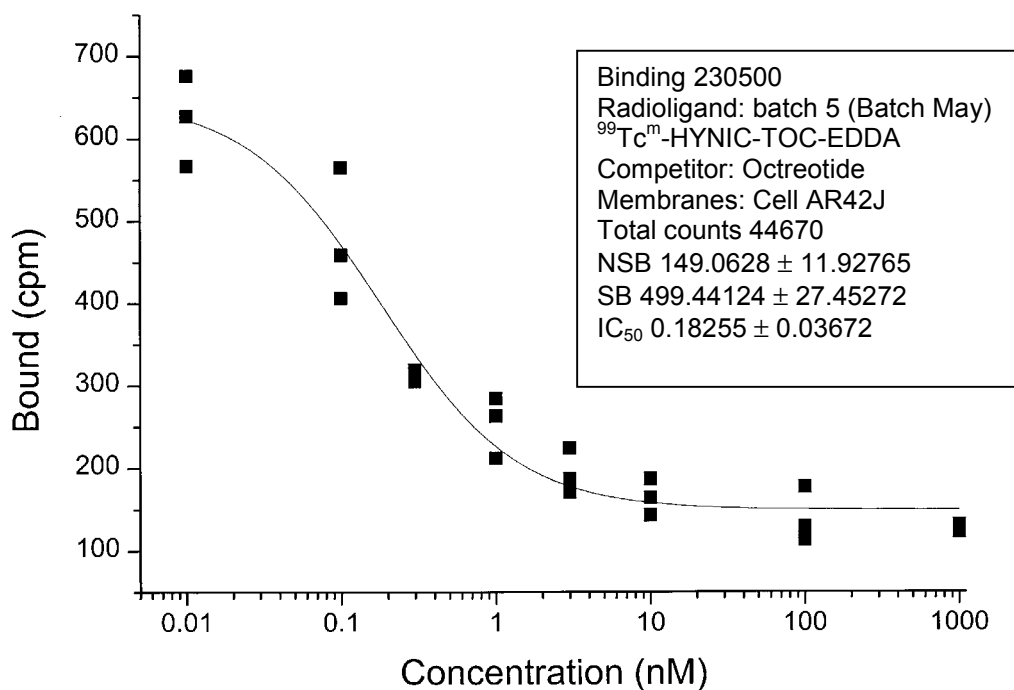


FIG. 16. Receptor binding of $^{99}\text{Tc}^m\text{-HYNIC-TOC-EDDA}$ on cell AR42J.

Concentration of somatostatin to determine the total and non-specific binding at increasing concentrations of $^{99}\text{Tc}^m\text{-HYNIC-TOC}$. The specific binding was determined as the difference between total and non-specific binding.

In Figs 16 and 17 we have the results of competition assays of $^{99}\text{Tc}^m\text{-HYNIC-TOC}$ using rat pancreatic tumour cell (AR42J) and rat brain cortex membranes, respectively.

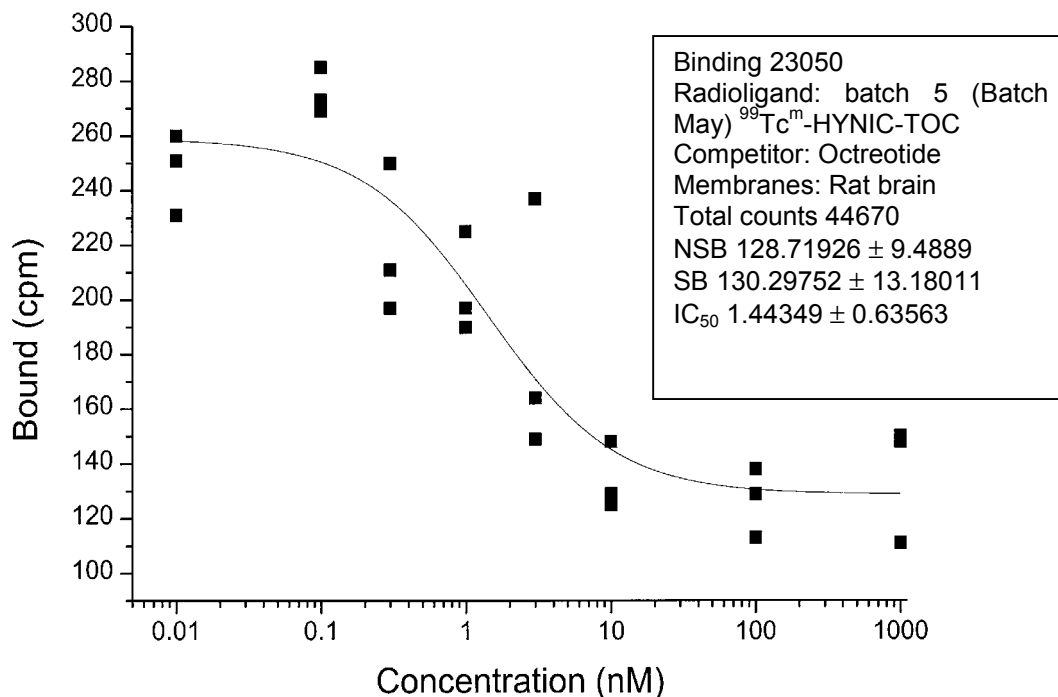


FIG. 17. Receptor binding of $^{99}\text{Tc}^m\text{-HYNIC-TOC}$ on rat brain cell membranes.

The receptor binding properties are summarized in Table IV.

TABLE IV. RECEPTOR BINDING PROPERTIES

Assay system	IC ₅₀	NSB	SB
AR42J	0.18	149.06	499.44
Brain cortex cells	1.44	128.71	130.29

NSB (non-specific binding)

SB (specific binding).

Studies of monoclonal antibodies for prostate cancer with ¹¹¹In and ⁹⁰Y

The radiolabelling yield for huJ591 with ¹¹¹In and ⁹⁰Y was higher than 90%. An average of five DOTA molecules could be randomly conjugated to J591. Specific activities of 280 MBq ¹¹¹In/mg DOTA-J591 and 360 MBq ⁹⁰Y/mg DOTA-J591 were achieved, with little apparent loss of immunoreactivity as can be observed in Figs 18 and 19.

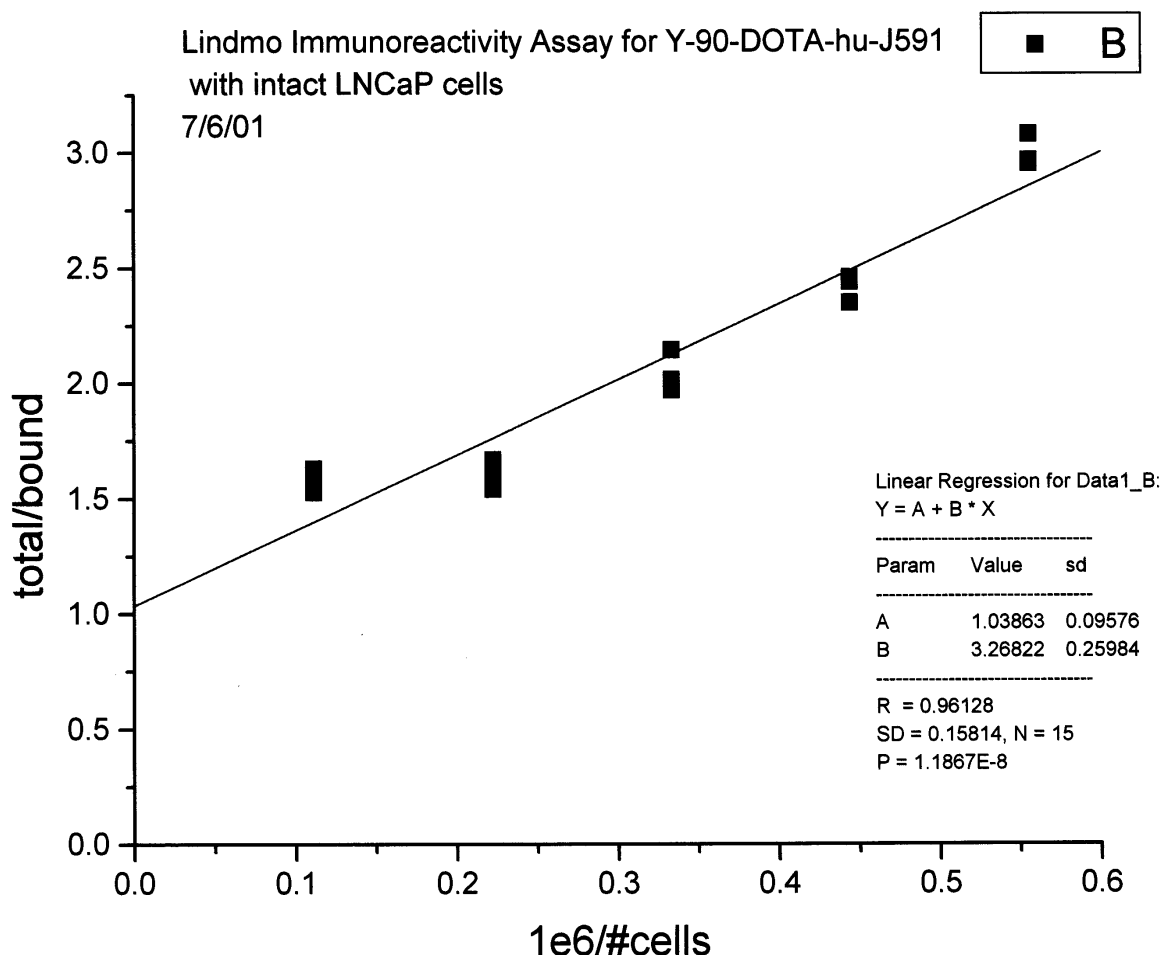


FIG. 18. Lindmo immunoreactivity for ⁹⁰Y-DOTA- hu-J591.

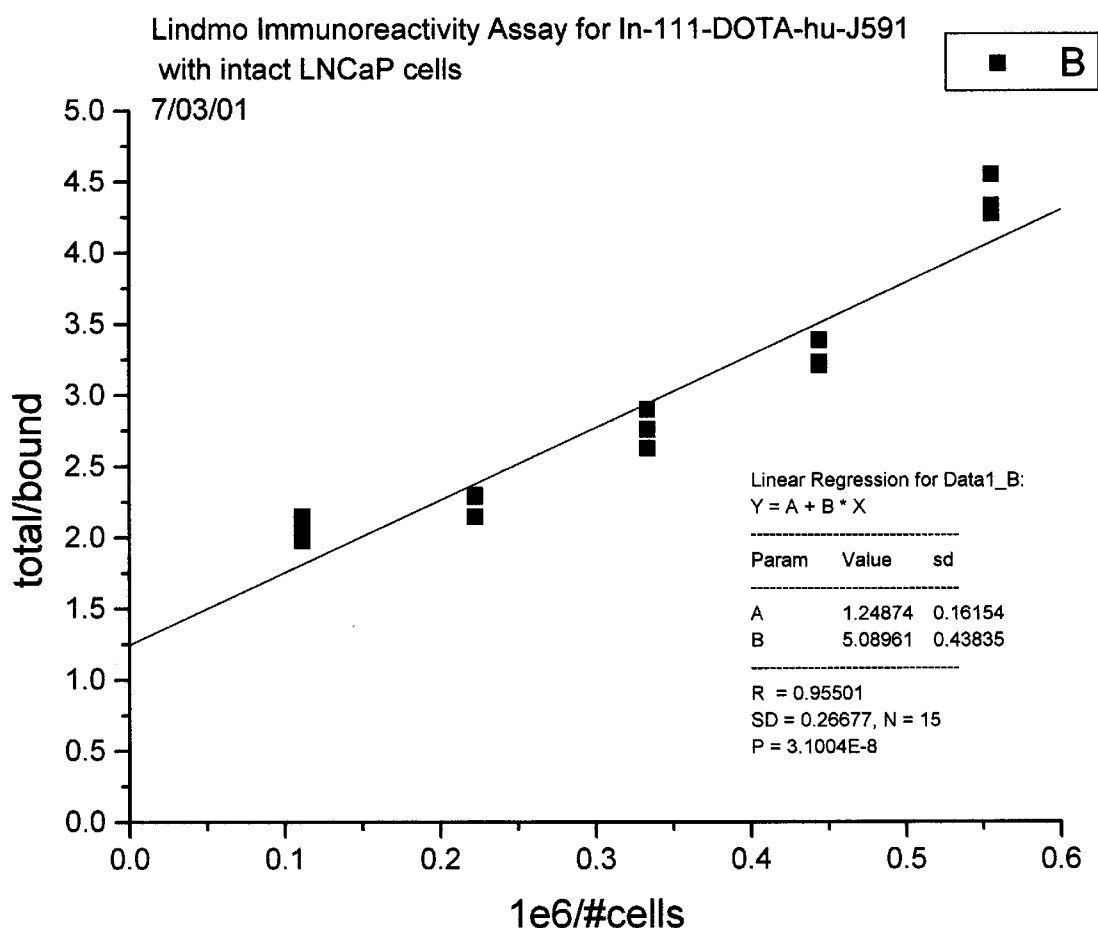


FIG. 19. Lindmo immunoreactivity for ^{111}In -DOTA- hu-J591.

4. DISCUSSION

Small peptides radiolabelled with $^{99}\text{Tc}^{\text{m}}$ or ^{188}Re are being currently investigated as potential radiopharmaceuticals, mainly for cancer diagnosis and therapy [5, 21].

Rhenium chemistry is very similar to technetium chemistry due to the periodic table relationship between the two elements [22] and as such, rhenium also forms a stable anionic species ReO_4 (perrhenate) and will not bind to organic ligands without reduction to a lower oxidation state.

Direct labelling of peptide with rhenium isotope can be done using the same procedures as used for direct labelling with $^{99}\text{Tc}^{\text{m}}$ but the labelling conditions for rhenium must be more energetic because of a lower redox potential and therefore a greater tendency to reoxidize. So the reaction time is longer and the concentration of Sn (II) is higher.

The involvement of tin in protein labelling as a tin-protein intermediate was suggested by Rhodes [23], for a “pretinning” procedure, when the reduction of native disulfide bridges was carried out by incubating the protein with stannous ions. This procedure is, however, limited to labelling only those peptides, which contain cyclized cysteines in their structure. This procedure can also be a limitation because it uses very small mass of ligand, with the formation of the intermediate compound Sn-tartrate labelled with ^{188}Re .

It is known that in direct protein labelling with ^{188}Re the best labelling yields are achieved within a relatively narrow pH range: 4-5.5. A pH lower than 4 compromises the immunoreactivity of the peptides, and a higher pH contributes to reoxidation of $^{188}\text{Re(V)}$ back to perrhenate [24]. A final pH value of 4 was here used for labelling in tartrate/phthalate buffer, but in acetate/acetic acid buffer for ^{188}Re -Octreotide the final pH was 3.0. For $^{99}\text{Tc}^{\text{m}}$ -labelling peptides the final pH was 5.0.

The assay of cysteine-challenge *in vitro* ranks different $^{99}\text{Tc}^{\text{m}}/^{188}\text{Re}$ labelling methods according to the stability of the label towards transchelation to cysteine and to determine whether it is possible to predict the relative radioactivity levels in organs and blood in normal animals.

The challenge assay is dependent on various factors, which could be broadly categorized as non-molecular and molecular factors. The non-molecular factors are pH, temperature, and other labelling conditions including buffers. Among molecular factors are the nature of amino acids involved in the formation of the complex [25]. Peptides labelled with $^{99}\text{Tc}^{\text{m}}$ show different responses to the cysteine challenge assay. Some $^{99}\text{Tc}^{\text{m}}$ -peptides appear to undergo rearrangement over time, presumably to thermodynamically stable states, which increases resistance to displacement with cysteine, while other peptides show no change over time [15]. Thakur [2] showed that $^{99}\text{Tc}^{\text{m}}$ -sandostatin and $^{99}\text{Tc}^{\text{m}}$ -RC-160 when submitted to cysteine challenge with a 100-fold molar excess of cysteine suffered less than 10% of loss of radioactivity by transchelation. In our experiments with $^{99}\text{Tc}^{\text{m}}$ -peptides, less than 20% was transchelated but for ^{188}Re -peptides the transchelation reached nearly 70% in 1 h of incubation.

The maximum concentration of cysteine one expects the peptide to be exposed *in vivo* is 1 mM [26]. So the high molar ratio demonstrated to cause displacement also suggests that the binding strength of $^{99}\text{Tc}^{\text{m}}$ -peptides is very high, but not for ^{188}Re -peptides.

In biodistribution studies of $^{99}\text{Tc}^{\text{m}}$ -labelled peptides, main organ uptake by $^{99}\text{Tc}^{\text{m}}$ -lanreotide was observed in the liver, being cleared to small intestines, and for $^{99}\text{Tc}^{\text{m}}$ -octreotide, main uptake was by small intestine, cleared to kidneys. The difference between both complexes is due the different structure (amino acid sequence) that influences the lipophilicity and biodistribution. In addition to lipophilicity, the stability of the complex also seems to be an important factor in influencing biodistribution. Some $^{99}\text{Tc}^{\text{m}}$ -labelled peptides have a potential ability to pass the blood-brain barrier¹⁶ and have been suggested as imaging agents for brain tumours. So, a significant uptake of $^{99}\text{Tc}^{\text{m}}$ -lanreotide by brain was observed but was insufficient for observing by imaging methods because the study was made in whole body.

The peptide lanreotide is very similar to another peptide fully studied that is Vapreotide (RC-160) with differences in the extremes of the molecule. As ^{188}Re -RC-160 was studied in treatment of small cell lung carcinoma¹¹ maybe this can explain some uptake of $^{99}\text{Tc}^{\text{m}}$ -lanreotide by the lung. ^{188}Re -peptides showed similar biodistribution as $^{99}\text{Tc}^{\text{m}}$ -peptides.

Since the direct labelling technique can be applied only for cyclized peptides, many studies are performing the indirect method using a bifunctional chelating agent, so that it can be used to label any peptide of biomedical interest. Nevertheless, the method requires a long synthesis and so far has resulted in low labelling yields.

Somatostatin receptors are hyperexpressed on many malignant tumours. Somatostatin and related analogues have been demonstrated to bind to tumours that are well differentiated and/or have neuroendocrine features [27].

Receptor-binding, low molecular weight peptides provide an excellent basis for the design of specific-binding radiopharmaceuticals. A major challenge was to label such molecules with radioisotopes without loss of receptor binding affinity or specificity.

Octreotide binds with high affinity to SSTR2 and SSTR5, to a lesser extent to SSTR3, whereas it does not bind to SSTR1 and to SSTR4. Because the subtype 3 receptor is also expressed on most adenocarcinomas, lanreotide scintigraphy and/or radionuclide therapy would have a broader field of application [28]. However, comparative studies in SSTR2 receptor-positive tumour-bearing rats indicated that internalization, as well as tumour localization by radiolabelled lanreotide was lower than that for the octreotide analogs [29].

Hydrazinonicotinamide conjugate to Tyr3-octreotide was labelled with $^{99}\text{Tc}^m$ and ^{188}Re with a high specific activity. EDDA was used as coligand and showed that it is a good coligand. The binding assay is a very important step to evaluate the specificity of the radiopharmaceutical. We observed better uptake of the product in AR42J cell membranes than in brain cortex membranes probably because there are fewer receptors in the latter. Binding to receptors with ^{188}Re -HYNIC-TOC was inconclusive.

5. CONCLUSIONS

The direct labelling of peptides with rhenium-188 isotope can be done using the same general procedures as used for the direct labelling with $^{99}\text{Tc}^m$. The labelling was simple and efficient, although the reaction time for ^{188}Re -labelled peptides was much longer (2 h) than for $^{99}\text{Tc}^m$ -labelled peptides (30 min).

^{188}Re -lanreotide gave a better radiochemical yield than ^{188}Re -octreotide and remained stable for 24 h, but the mass of lanreotide had to be greater than that of octreotide, and the ratio between the mass of stannous chloride and lanreotide was also greater than that used for labelling octreotide.

As the products labelled with ^{188}Re can be obtained with a high radiochemical purity, they may be used for *in vivo* studies to evaluate their efficacy in radioimmunotherapy similarly to $^{99}\text{Tc}^m$ agents in radiodiagnosis of cancer.

$^{99}\text{Tc}^m$ -HYNIC-TOC had good *in vitro* and *in vivo* properties as a high affinity molecule for SSTR2 receptors.

J591 is a monoclonal antibody that may allow targeting of viable PMSA expressing tissue with diagnostic and therapeutic metallic radionuclides.

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LABELLING TECHNIQUE OF BIOMOLECULES FOR TARGET RADIOTHERAPY

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Abstract

Labelling techniques were developed for the preparation of biomolecules (DOTA-IgG, DOTA-lanreotide, anti-hepatoma antibody fragment, lanreotide) with radionuclides such as ^{90}Y , ^{153}Sm and ^{188}Re . The labelling yield and radiochemical purity of these labelling biomolecules were determined by PC, ITLC and Sep-Pak C18 cartridge. The stability *in vitro* and bio-behaviour in normal rats were also evaluated. The experimental results showed that labelling efficiency of biomolecules (DOTA-IgG and DOTA-lanreotide) with ^{90}Y and ^{153}Sm is more than 95% and had good stability *in vitro*, but the labelling efficiency of biomolecules (anti-hepatoma antibody fragment and lanreotide) with ^{188}Re via directly labelling technique is at range of 88%~95% and stability *in vitro* was less.

1. INTRODUCTION

Increased effort was made to label monoclonal antibody and peptide with radionuclides because of their potential role in the radioimmunotherapy and radiodiagnosis of tumour.

For some years monoclonal antibody to tumours' antigenic sites were labelled with emit gamma rays, permitting detection of disease. Recently, people took efforts to investigate labelling monoclonal antibody with radionuclides emitting beta rays for therapy of tumour and obtained some promising results.

Somatostatin is a cyclic disulfide-containing peptide hormone of 14 amino acids It exists in the hypothalamus, the cerebral cortex, the brain stem, gastrointestinal tract and pancreas, and exerts an inhibitory effect on several cell functions such as secretion of peptide hormones and growth factors. The clinic value of Somatostatin is limited due to its very short half-life *in vivo*. In recent years much attention are drawn to the development radiolabelled somatostatin analogues (such as octreotide, RC-160 and lanreotide) with radionuclides (example for ^{188}Re , $^{131/125/123}\text{I}$, $^{99}\text{Tc}^m$, ^{111}In , ^{90}Y , etc.) for a variety of diagnostic applications as well as for therapy of malignant tumours. Lanreotide (D- β -Nal-Cys-Trp-D-Trp-Lys-Val_Cys-Thr-NH₂) is a new somatostatin analogue. It can bind to human somatostatin receptor (hSSTR) subtype 2 through 5 with high affinity and to hSSTR subtype 1 with low affinity. Virgolini, I., et al, investigated biodistribution, safety and radiation absorbed dose of ^{111}In -DOTA-lanreotide in 1998 and these experimental results were promising.

2. MATERIALS AND METHODS

2.1. Preparation of DOTA-IgG with ^{90}Y

Lanreotide and DOTA-lanreotide (provided by IAEA, HPLC grade). Stannous chloride (HPLC grade), acetate, sodium acetate, concentrated hydroxyl chloride, ethanol (analysis grade, Beijing Chemical Reagent Co.) and 0.9% sodium chloride solution (Sijiazhuang the Fourth Pharmacy Factory). $^{188}\text{WO}_4^-$ solution (USA, Oak Bridge); $^{188}\text{W}/^{188}\text{Re}$ generator (China Institute of Atomic Energy and Finland); $^{90}\text{Sr}/^{90}\text{Y}$ solution (Russia); $^{90}\text{Sr}/^{90}\text{Y}$ generator

(China Institute of Atomic Energy); enriched Sm_2O_3 (Russia, Sm-152 is more than 95%). Anti-hepatoma antibody fragment (provided by the 4th People Military Hospital).

2.1.1. Conjugation of DOTA with antibody (IgG)

The conjugation of DOTA with antibody went through two steps: the first step was activation of DOTA; and the second step was reaction of DOTA-NHS with antibody and purification of DOTA-IgG by Sephadex G50 column.

2.1.2. Preparation of DOTA-NHS

DOTA (0.435g) and NHS (0.115g) were dissolved with 5 mL of dry DMSO in the 20 mL of vial, 3 mL of solution containing DCC (0.213 g) was added to the vial, mixed and stirred. The mixture was continued at room temperature overnight. The precipitated dicyclohexylurea was removed by filtration and washed with hot DMSO, and DMSO in the filtered solution was removed by distillation.

2.1.3. Preparation and purification of DOTA-IgG

According to different mole ratio of DOTA/IgG, DOTA-NHS (solid) was added to IgG solution (13.6 mg/mL) in the vial, mixed by vortex and incubated for 2 h at room temperature and 37°C, respectively. After conjugation, DOTA was removed from DOTA-IgG by the Sephadex G50 column and 0.85mol/l NaAc solution as elution agent. We did plenty of experiments to determine the optimal conjugation condition of DOTA with IgG.

2.1.4. Labelling DOTA-IgG with ^{90}Y

DOTA-IgG is dissolved with metal free water to make 24mg/mL DOTA-IgG solution 40 μL of $^{90}\text{YCl}_3$ solution (6-8mCi) was added to 100 μL of 0.3mol/L sodium acetate (pH~5.5) buffer solution in the vial, the mixture was mixed carefully by Vortex. Then we added 100 μL of DOTA- IgG into above the vial and mixed again by Vortex, and the reaction mixture incubated in the water bath (37°C) for 2 h.

2.2. Preparation of lanreotide with ^{188}Re

2.2.1. Preparation of ^{188}Re - citrate /tartate (^{188}Re -citrate/tartatic acid)

1 mL of tartate solution (0.15mol/l) and 1 mL of citrate solution (0.015mol/l) are added to 10 mL of vial, mixes with 0.2 mL SnCl_2 solution (10 mg/mL), 1 mL of $^{188}\text{ReO}_4^-$ solution (20mCi/mL) is added. The mixture reacts for 45 min at 60°C and pH 2-3. The pH value of mixture is adjusted to 5-7 with 0.5mol/L sodium acetate solution. The labelling efficiency is determined by paper chromatography using acetone and 0.9% sodium chloride solution as mobile phase.

2.2.2. Preparation of ^{188}Re -lanreotide

0.02 mL of lanreotide solution (0.4mg/mL) and 0.05mL stannous chloride (10 mg/mL) are added to the vial together. The mixture incubates for 20 min at room temperature, then 0.2 mL of ^{188}Re -CT/TT (37MBq) is added, mixes and reacts for 40 min at 60°C. After cooling, pH value of the mixture is adjusted to 5-6 with 0.5 mol/L sodium acetate solution.

2.2.3. Preparation of DOTA-lanreotide with ^{153}Sm

DOTA-lanreotide is dissolved with metal free water to make 4mg/mL DOTA-lanreotide solution. 40 μL of samarium chloride solution (6-8mCi, Sm about 10 μg) is added to 100 μL of 0.3 mol/L sodium acetate (pH~5.5) buffer solution in the vial, the mixture is mixed carefully by Vortex. Then, 100 μL of DOTA-kanreotide is added into above vial, and mixed again by Vortex, the reaction mixture incubated in the water bath (90~100°C) for 1 h.

2.3. Preparation of anti-hepatoma monoclonal antibody fragment with ^{188}Re

2.3.1. Reduction of anti-hepatoma monoclonal antibody fragment

0.45 mg of anti-hepatoma monoclonal antibody fragment is dissolved in 0.02 mol/L phosphonic buffer solution (PBS) to make 8 g/L solution, then added NaHSO_3 solution (mole ratio of NaHSO_3 /antibody=2500:1), reaction volume is 100 μL , the mixture is mixed by Vortex and incubated for 1.5 h at room temperature.

2.3.2. Reduction of ^{188}Re

40 μL of tartate (0.15mol/l)-citrate solution (0.015mol/l) is added to 10 mL of vial, mixed with 12 μL SnCl_2 solution (10 mg/mL) and 10 μL $^{188}\text{ReO}_4^-$ solution (37-148 GBq/L), reaction volume is 100 μL . The mixture reacts for 45 min at 60°C and pH 2-3.

2.3.3. Labelling antibody

6 μL of ascorbic acid (6 mg/ml) is added to reduced antibody (mole ratio of ascorbic acid and antibody=750:1), then added reduced ^{188}Re solution. The mixture is mixed carefully by Vortex and incubated for 2 h in 37°C water bath.

2.3.4. Quality control

Thin layer chromatography (TLC) is used to monitor the quality of labelling yield. In the TLC studies, TLC-SG (Gelman Sciences Inc. Ann Arbor, Michigan) chromatography paper is cut into 1.5 \times 15cm strip and activated by heating for 30 min at 110°C according to manufacture's instructions. After heating, the strips are stored dry at room temperature until use. 0.005 mL portion of sample is spotted at 2 cm from lower end of the TLC paper and dried in air. The strips are developed in the different developing systems until the solvent reaches up to 12 cm of strip. After drying, the strip is cut into 1 cm piece and radioactivity is measured in a NaI(Tl) well detector.

2.3.5. Animal test

Biodistribution of labelling biomolecules is performed in male Kunming white mice (weight:20 \pm 2g). 20 μCi of labelling biomolecules in 0.1 ml volume is injected though tail vein and these mice are sacrificed at specific time intervals. The tissues and organs are excised, weighed and counted in a NaI(Tl) well detector. The uptake of activity in different organs is calculated as percentage injected dose per gram organ.

3. RESULTS AND DISCUSSION

3.1. Labelling DOTA-IgG with ^{90}Y

20 mCi of $^{90}\text{Sr}/^{90}\text{Y}$ generator was prepared, ^{90}Y solution was obtained from the generator with 0.003 mol/L EDTA solution. The elution efficiency, elution curve and ^{90}Sr breakthrough were elevated, the prepared ^{90}Y solution reached to the demand of medical use. ^{90}Sr breakthrough is less than 10^{-5} , and elution efficiency is more than 75%. Based on different distribution coefficients of metal element in the Dowex 50×8 cation exchange resin, ^{90}Y solution is purified with resin column using different concentration of H_2SO_4 and HCl solution as elution agent. The purified ^{90}Y solution could promote labelling efficiency of labelling antibody with ^{90}Y . Optimal conjugation of DOTA with IgG is listed in Table I. The conjugation efficiency was determined by paper chromatography and is 40%-50% under the optimal conjugation condition.

TABLE I. OPTIMAL CONJUGATION CONDITION OF ANTIBODY AND DOTA

Mole ratio of DOTA/IgG	Temperature	Reaction time	H	Concentration of antibody
5/1	37°C	2 h	8.0-8.5	13.6 mg/ml

In order to obtain optimal labelling condition of DOTA-IgG with ^{90}Y , the mole ratio of DOTA-IgG/ ^{90}Y , effect of pH, reaction time, mole ratio and temperature on labelling efficiency were studied. Experimental results showed that labelling efficiency was more than 95% under optimal condition of mole ratio of DOTA-IgG/ ^{90}Y : 50/1, pH: 7.5-8.5, reaction temperature: 3h and reaction temperature: 37°C.

TABLE II. DEVELOPMENT SYSTEM AND RF VALUE OF ^{90}Y -DOTA-IgG AND $^{90}\text{YCl}_3$

Developing system	^{90}Y -DOTA-IgG	$^{90}\text{YCl}_3$	^{90}Y -DOTA
System 1	0.0	0.9-1.0	0.9-1.0
System 2	0.0	0.0	0.9-1.0

Developing system 1: NH_4OH : H_2O =2ml:40ml+2g NH_4Cl +10ml 0.03mol/L EDTA solution
Developing system 2: NH_4OH : H_2O =2ml: 40ml+2g NH_4Cl .

The stability of ^{90}Y -DOTA-IgG was investigated by adding 100 μl of ^{90}Y -DOTA-IgG to 4 ml of 0.9% sodium chloride solution, 4 ml of 1.0% BSA solution and 4 ml of 0.03 mol/l DTPA solution, respectively, incubated for seven days. The radiochemical purity of ^{90}Y -DOTA-IgG was determined daily with HPLC and paper chromatography. The experiment results showed that the radiochemical purity was more than 98% when ^{90}Y -DOTA-IgG was incubated in three solutions for seven days. This showed that ^{90}Y -DOTA-IgG was very stable *in vitro*.

Animal test results of ^{90}Y -DOTA-IgG are given listed in Table III in normal rats at 1hr, 12hr, 24 hr, 24hr and 54hr post-injection. The experimental results showed that the uptake of ^{90}Y -DOTA-IgG was 2.54% I.D./g in the bone at 48hr post-injection; the uptake of ^{90}Y -DOTA-IgG in the liver was 3.40% I.D./g at 48hr post-injection, and uptake of ^{90}Y -DOTA-IgG in the blood was 20.40% I.D./g, 7.6% I.D./g and 4.6% I.D./g at 1hr, 24hr and 48hr post-injection, respectively. This showed that ^{90}Y -DOTA-IgG was very stable *in vivo* and the blood clearance of ^{90}Y -DOTA-IgG was slow. The urine sample of ^{90}Y -DOTA-IgG was

analysed by HPLC, the experimental result was shown that ^{90}Y -DOTA-IgG in the urine existed in form of ^{90}Y -DOTA.

3.2. Labelling lanreotide directly with ^{188}Re

The optimal labelling condition is that 10 μg of Lanreotide reacts with 0.4 mL of ^{188}Re -CT/TT solution for 40min at pH 2-3 and 60 $^{\circ}\text{C}$, the labelling yield is at range of 88%-94%. The determination of labelling yield and RP of ^{188}Re -Lanreotide is performed with two developing systems respectively. The TLC method is used widely for measurement of labelling yield of radiolabelled peptide, there is three components (unbound ^{188}Re , ^{188}Re -Lanreotide and radiocolloid) in the labelling mixture. The R_f value of those components in different developing system is listed in the Table IV. The labelling yield is at range of 88%-94%.

TABLE III. ANIMAL TEST OF ^{90}Y -DOTA-IgG IN NORMAL MICE AT DIFFERENT TIME POST-INJECTION (n=3); UPTAKE OF ^{90}Y -DOTA-IgG(% I.D/g)

Organ	1 h	12 h	24 h	48 h
Blood	20.40	15.80	7.6	4.6
Heart	3.0	1.8	1.0	0.6
Lung	2.8	1.5	1.3	1.1
Liver	13.4	10.34	8.65	3.4
Spleen	2.4	2.3	1.60	1.20
Kidney	7.3	7.80	5.40	2.0
Muscle	0.8	0.30	0.12	0.10
Bone	0.3	1.15	1.81	2.54

TABLE IV. R_f VALUE OF COMPONENT IN TWO KINDS OF MOBILE PHASE

Component mobile phase	$^{188}\text{ReO}_4^-$	$^{188}\text{ReO}_2$	^{188}Re -lanreotide
85% acidic ethanol (pH3.5)	0.8-1.0	0.0-0.1	0.8-1.0
0.9% sodium chloride	0.8-1.0	0.0-0.1	0.0-0.1

For Sep-Pak C_{18} cartridge method, Sep-Pak C_{18} cartridge is used for labelling yield and purification of ^{188}Re -lanreotide. Each cartridge is washed with 10 ml of ethanol followed by 10 ml of 0.001 mol/L HCl solution. Aliquots of 0.1 ml sample is loaded onto the cartridge, unbound peptide ($^{188}\text{ReO}_4^-$ or ^{188}Re -ct/tt) is eluted with 0.001 mol/L HCl solution, ^{188}Re -lanreotide is eluted with 80% aqueous ethanol solution, but radio-colloid is kept on the cartridge. The labelling yield is at range of 88%-94%. Though labelling yield is 88%-94%, ^{188}Re -lanreotide must be purified before stability study in order to get correct evaluation about stability *in vitro*. 0.1 mL of purified ^{188}Re -lanreotide is added to one vial contained 1 mL of normal saline solution and other vial contained 1 mL mixed solution of normal saline and 1 mg ascorbic acid. Then two vials are incubated for 24 h at 37 $^{\circ}\text{C}$ and radiochemical purity (RP) is tested with TLC at specific time intervals. The results are given in Fig. 1. It is demonstrated that the RP of ^{188}Re -lanreotide is more than 95% within 2.5 h without ascorbic acid, but RP keeps no change for 6 h with ascorbic acid. This shows that ^{188}Re -lanreotide is unstable *in vitro* and ascorbic acid promotes stability of ^{188}Re -lanreotide. The biological

behaviour of ^{188}Re -lanreotide is given in Table V. Biodistribution studies with ^{188}Re -lanreotide shows about 2% ID/g in the blood at 3 h post-injection, ^{188}Re -lanreotide is eliminated rapidly from the blood and concentrates in the lung and intestine. The uptake of thyroid increases with time of post-injection increasing. This indicates that dissociation of ^{188}Re -Lanreotide occurs. Uptake of adrenal gland is 3.05% ID/g at 3 h post-injection and uptake of muscle is very low.

TABLE V. BIODISTRIBUTION *IN VIVO* OF ^{188}RE -LANREOTIDE IN RAT, n=3% ID/g

Tissue	Time/h				
	0.5	1	3	6	24
Blood	3.52%	2.11%	1.65%	0.86%	0.28%
Liver	10.12%	6.76%	3.48%	1.58%	0.98%
Spleen	3.18%	4.50%	2.15%	1.25%	0.56%
Lung	2.18%	3.58%	1.91%	1.03%	0.37%
Kidney	1.54%	2.52%	1.19%	0.86%	0.46%
Heart	1.45%	1.09%	0.86%	0.53%	0.13%
Muscle	0.64%	0.76%	0.38%	0.15%	0.07%
Intestine	2.42%	4.58%	5.69%	2.89%	1.32%
Thyroid	0.87%	1.52%	4.95%	2.45%	0.65%
Adrenal gland	0.68%	2.87%	3.02%	2.53%	1.87%

Labelling DOTA-lanreotide

The optimal labelling condition is pH 7~10, mole ratio of DOTA-lanreotide/Sm>50:1, reaction time: 1 h and reaction temperature: 80~100° C. The labelling efficiency is more than 97%. The labelling efficiency is determined with TLC, the development agent is NH₃: EDTA(0.4mol/L) :NH₄Cl =2ml: 30ml:0.5g, Rf value of ^{153}Sm is 0.8~1.0, Rf value of Sm-DOTA-lanreotide is 0.0. ^{153}Sm -DOTA-lanreotide is incubated in 0.9% sodium chloride solution for seven days, the radiochemical purity is determined every day. The radiochemical purity is more than 95% for seven days, this shows that ^{153}Sm -DOTA-lanreotide is stable *in vitro*. The biological behaviour of ^{153}Sm -DOTA-lanreotide is given in Table VI. Biodistribution studies with Sm-153-DOTA-lanreotide show about 0.84%I.D/g in the blood at 3 h post injection, ^{153}Sm -DOTA-lanreotide is eliminated rapidly from the blood and concentrates in the lungs and intestine. Uptake of adrenal gland is 2.05% ID/g at 3 h post injection. The uptake in the liver is very low; this shows that labelling compound is stable *in vivo*.

Labelling anti-hepatoma monoclonal antibody fragment with ^{188}Re

^{188}Re anti-hepatoma monoclonal antibody is stored for 8 h in 0.9% sodium chloride solution at 37°C, the radiochemical purity is more than 95%. The animal test is listed in Table VIII. The results show that ^{188}Re anti-hepatoma monoclonal antibody is eliminated slowly in blood, and has high uptake in liver, kidney and spleen.

TABLE VI. BIODISTRIBUTION *IN VIVO* OF ^{153}Sm DOTA-LANREOTIDE IN RAT, n=3%ID/g

Tissue	Time/h				
	0.5	1	3	6	24
Blood	3.15%	1.67%	0.84%	0.53%	0.21%
Liver	0.23%	0.35%	0.44%	0.20%	0.15%
Lung	0.78%	1.56%	2.65%	2.78%	2.03%
Spleen	0.24%	0.54%	0.34%	0.23%	0.15%
Kidney	1.45%	3.45%	2.78%	0.89%	0.78%
Heart	0.54%	0.24%	0.28%	0.25%	0.18%
Muscle	0.21%	0.18%	0.15%	0.10%	0.11%
Bone	0.34%	0.56%	0.87%	0.54%	0.34%
Intestine	1.87%	2.78%	4.67%	3.45%	2.34%
Adrenal gland	0.68%	1.26%	2.05%	1.89%	1.78%

TABLE VII. RF VALUE OF LABELLING COMPOUNDS IN DIFFERENT DEVELOPING SYSTEMS

Developing agent	$^{188}\text{ReO}_2$	$^{188}\text{ReO}_4^-$	$^{188}\text{Re-Tart/Cit}$	$^{188}\text{Re-Mcab}$
Acetone	0.0	0.9~1.0	0.9~1.0	0.0
0.9%NaCl	0.0	0.9~1.0	0.0	0.0
Water: ethanol: $\text{NH}_3=5:2:1$	0.0	0.9~1.0	0.9~1.0	0.5~1.0

TABLE VIII. BIODISTRIBUTION *IN VIVO* OF ^{188}Re -ANTI-HEPATOMA MONOCLONAL ANTIBODY FRAGMENT Y IN RAT, n=3%ID/g

Tissue	Time/h		
	24	48	72
Blood	0.41	0.13	0.06
Liver	1.68	0.66	0.66
Spleen	1.30	0.33	0.23
Lung	0.59	0.29	0.30
Kidney	17.91	4.06	1.92
Heart	0.25	0.1	0.06
Muscle	0.08	0.04	0.02
Intestine	0.59	0.13	0.12
Thyroid	0.86	0.29	0.12

EVALUATION OF BIOMOLECULES FOR METABOLIC RADIOTHERAPY

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Abstract

We evaluated several biomolecules as radiotherapeutical agents, such as the antibodies ior-CEA1 and an anti-CEA fragment scFv, as well as the peptides ior-P1394, lanreotide and DOTA-lanreotide (MAURITIUS). The first three agents were synthesized and obtained by our group and the others were supplied by the IAEA. The synthesis of ior-P1394, containing a peptide sequence AGGG β A as chelator for ^{188}Re is described. Except MAURITIUS, all other agents were first evaluated with $^{99}\text{Tc}^m$, attaining a yield of >90%. Likewise the ^{188}Re evaluation exhibited yields of >90% labelling efficiency without major effects on *in vitro* stability for all agents, except the scFv, which was not evaluated with ^{188}Re . For DOTA-lanreotide, it was only obtained up to 83% labelling efficiency with ^{90}Y .

1. INTRODUCTION

The science of the bioconjugates is a research activity of increasing importance. This field has reached a fast development, in which are harmonized two important molecular functions: the chemistry and the biological functions [1].

The prospect of localizing or treating neoplastic diseases using specific antibodies labelled with radioactive isotopes was the goal of many researchers during the last three decades. Recent success in this area was largely due to genetic and molecular techniques that now permit production of a large number of suitable monoclonal antibodies directed against specific epitopes individually of specific tumour. Specificity of these monoclonal antibodies for an associated tumour antigen was employed in the technique of immunoscintigraphy, which is now a routine diagnostic procedure for the management of cancer patients [2, 3, 4]. For over 15 years labelled monoclonal antibodies (MAbs) have shown their efficiency in the diagnosis of neoplastic diseases through a relative high sensitivity and specificity. That is, the radioimmunoscintigraphy began to establish a new technique, opening the doors to radioimmunotherapy as immediate goal.

Recently there has been a significant growth of radiotherapy branch of clinical nuclear medicine with the introduction of a number of new radionuclides and radiopharmaceuticals for the treatment of metastatic bone pain, neuroendocrine and other tumours. Achieving the molecular recognizing units (peptides) is of considerable importance in this field [5, 6].

The advantage of the peptides over monoclonal antibodies is in their pharmacokinetic properties and biodistribution. The transport of these agents to the exact lesion focus contributes, in a crucial manner, to an efficient diagnosis and therapy, with significant reduction of secondary dose to the normal organs [7].

In the framework of the CRP we developed and evaluated several biomolecules as radiotherapeutical agents, such as the antibodies ior-CEA1 and an anti-CEA fragment scFv, as well as the peptides ior-P1394, lanreotide and DOTA-lanreotide (MAURITIUS). Our group developed the ior-CEA1, anti-CEA fragment scFv and ior-P1394. Lanreotide and DOTA-lanreotide were supplied by the IAEA.

2. MATERIALS AND METHODS

2.1. Generator

The Re-generator was supplied by the IAEA from MAP Medical Technologies Oy, Finland, with a total activity of 3,7 GBq.

2.2. Biomolecule

Monoclonal antibody (MAb) ior-CEA1: This is a murine IgG-1 antibody secreted by clone K3/J5. This IgG is highly specific against protein epitope on cell-bound CEA and it is included in the gold 1 group according to Hedin's classification [8].

Anti-CEA fragment scFv: The genes encoding for the heavy (VH) and light (VL) variable regions of the anti-CEA specific mouse MAb ior-CEA1 were individually amplified by reverse transcription-polymerase chain reaction (RT-PCR) starting from hybridoma RNA. Overlapping PCR [9] was used then to construct the VH-linker-VL scFv, using the linker structure suggested by Chaudhary, et al. [10]. Finally, the scFv gene was cloned in an appropriate vector and expressed in a recombinant strain of the methylotrophic yeast *Pichia pastoris*. In this model, the biologically active scFv containing a hexahistidine tag at its C-terminus is secreted to the culture supernatant at a concentration of approximately 1.2 g/l.

Synthesis of ior-P1394: The peptide was synthesized using the Boc/Bzl strategy and the "tea bag method" [11] on 200 mg of MBHA resin (substitution level 1 mM/g) each. The Boc group was used for N^α -protection. The side chain protecting groups used were 4-methoxybenzyl (Mob) for Cys; benzyl (Bzl) for Asp; Formyl (For) for Trp; dichlorobenzyl (Cl₂-Bzl) for Tyr and chlorobenzylloxycarbonyl (Cl-Z) for Lys. Cleavage of the Boc group was carried out with 37.5% TFA in DCM during 30 min. The TFA salt was neutralized with 5% DIEA in DCM three times for 2 min each. The amino acids were coupled using DIPCDI and the completion of the reaction was monitored by ninhydrine test [12]. Side-chain deprotection and cleavage from the resin were performed following the "Low-High" HF procedure [13] with HF-DMS-*p*-cresol (25:65:10) during 2 h at 0° and with HF-DMS-anisole-thiocresol (79.8:10:10:0.2) during 1 h at 0°, respectively. The peptide was extracted with 30% HAc in water and lyophilized.

Oxidation of thiol groups was carried out with 20% DMSO in water [14]. The peptide was dissolved in HAc-H₂O (1:19) at 0.2 mM and pH was adjusted to 6 with ammonium hydroxide solution (25% in water). DMSO was added to achieve the desirable concentration. The completion of the oxidation reaction was monitored by Ellman test [15].

Lanreotide: (D) β NaI-Cys-Trp-D-Trp-Lys-Val-Cys-Thr-NH₂. It is a somatostatin analogue. It can bind to the human somatostatin receptor subtypes with high affinity. It was obtained commercially from the firm piCHEM, Austria.

DOTA-Lanreotide (MAURITIUS): [α -DOTA-(D) β NaI, ϵ -Boc-Lys]-lanreotide is a somatostatin analogue, which was provided by the IAEA and supplied gently by Dr. Virgolini's group from Austria.

2.3. Labelling with $^{99}\text{Tc}^{\text{m}}$

By concept of work algorithm, we evaluated first each molecule with $^{99}\text{Tc}^{\text{m}}$, except MAURITIUS, i.e. ior-CEA1 [16], scFv [17], ior-P1394 [18] and lanreotide.

Ior-CEA1: The MAb ior-CEA1 was labelled according to the Schwarz method modified as reported [19]. Briefly, this procedure was performed using 1 mg of 2-mercaptoethanol-reduced protein and 5 mCi of $^{99}\text{Tc}^{\text{m}}\text{O}_4$, reported previously elsewhere [16].

ScFv: The recombinant protein has great potential for improved tumour penetration, better blood clearance, and reduced immunogenicity [20-21]. In this work we evaluated a novel one-step method for direct $^{99}\text{Tc}^{\text{m}}$ labelling of an anti carcinoembryonic antigen (CEA) recombinant scFv fragment, using a C-termini hexa-histidine tag as labelling site. Labelling: $^{99}\text{Tc}^{\text{m}}$ can directly bind to proteins via histidine residues [17]. Briefly, purified scFv (1 mg/mL in phosphate buffer) was purged with nitrogen for 10 min. The purged protein solution was mixed 1:1 with 20 mM tartrate, 80 mM phthalate buffer, pH 5.6, containing stannous chloride, to a final concentration of 0.01 mM stannous ions, and ca. 20 μM protein. The labelling reaction was accomplished by addition of 1-3 mCi of $^{99}\text{Tc}^{\text{m}}\text{O}_4^-$ in a volume of 20 μL , and incubation at room temperature for 20 min.

Ior-P1394: We have synthesized of a new analogue of somatostatin derived from the Vapreotide, containing an amino acidic sequence (AGGG β A) as $^{99}\text{Tc}^{\text{m}}$ -chelating moiety [18]. Labelling: Aliquots of 20 μL sodium tartrate of 50 mg/mL, dissolved in 0.5 M NaHCO_3 , 0.25 M NH_4AcO and 0,175 M NH_4OH pH 9.2, were added to 50 μg of peptide (1 mg/mL), followed by 10 μL of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$, containing the correspond amounts. All solutions were previously gassed under nitrogen atmosphere. After the pH adjustment, approximately 1 mCi (37 MBq) of $^{99}\text{Tc}^{\text{m}}\text{O}_4^-$ was added to the reaction mixture and allowed to incubate during 1 h at room temperature.

2.4. Labelling with Re-188

Re-labelling was performed with the biomolecules: ior-CEA1, ior-P1394 and lanreotide, testing different weak ligands, such as MDP, tartrate, gluconate, citrate, phthalate/tartrate. The influence of pH, amount of ligand, amount of Sn and reaction time on labelling yield were also investigated.

Once the weak ligand was selected, an experimental design was carried out, using as independent variables: amounts of SnF_2 (2–8 mM); pH (1.5–5); citric acid (10–170 mM) and reaction time (1–3h). Labelling in case of protein: aliquots of 250 μg of previously reduced ior-CEA1 (1 mg/mL) were added to 160 μL of citric acid and 40 μL SnF_2 followed by 20 μL of ReO_4^- . After the pH adjustment, the reaction mixture was left to react for 3 h at room temperature. In case of peptides: aliquots of 50 μL citric acid were added to 50 μg of the peptide (1 mg/mL), followed by 20 μL of ReO_4^- and 20 μL of SnF_2 , containing the corresponding concentrations. After the pH adjustment, the reaction mixture was left to react for 1-2 h at 90°C. No previous reduction of the disulphide bridge was necessary. All solutions were previously gassed under nitrogen atmosphere.

2.5. Labelling with ^{90}Y

DOTA-lanreotide (MAURITIUS) was labelled with carrier-free ^{90}Y obtained from CENTIS, in Havana.. Briefly, ^{90}Y stock solution (in 0.04 M HCl) was adjusted to pH6.5. MAURITIUS was dissolved in 0.2 M sodium acetate buffer at a concentration of 1 $\mu\text{g}/\mu\text{L}$, pH

6.5. Then, 1 mCi ^{90}Y was added and allowed to react at 90°C for 0.5-1.5 h. The reaction was quenched by addition of 20 μL of 0.025M DTPA.

2.6. Quality control

After radiolabelling, the resulting product was tested by Instant Thin Layer Chromatography (ITLC) (Gelman Instruments, USA), AlugramTM RP-18W/UV₂₅₄ (Macherey-Nagel, Germany), Sep-pak RP-C18 cartridge (Waters & Assoc., Milford, Mass., USA).

ITLC was performed on 10×150 mm silica gel-impregnated glass using Methyl ethyl ketone (MEK) as moving phase for the determination of $^{188}\text{ReO}_4^-$. It was allowed to migrate for 120 mm on the strips and air-dried and cut in 1.0 cm sections, which were then measured in a gamma scintillation counter (1272 Clinigamma, LKB Wallac, Sweden). AlugramTM RP-18W/UV₂₅₄ strips, using EtOH:saline 1:1 as moving phase, were also employed to determine radiocolloids or aggregates.

Sep-pak RP-C18 cartridge was used in order to purify the preparation. It was activated with 5 mL of absolute ethanol and 5 ml of 0.1 mM HCl. Samples were applied and eluted with 5 mL of 0.1 mM HCl, removing the hydrophilic species, such as $^{188}\text{ReO}_4^-$, ^{188}Re -citrate or tartrate, etc. A second elution with EtOH was used to remove the labelled peptide and the radiocolloids or aggregates remained in the column. The radioactivities in the eluted fractions and Sep-pak RP-C18 cartridge were measured in a well counter under similar geometric conditions.

In case of Y-90 labelling, quality controls were performed in paper chromatography (in Whatman No. 3), using saline as mobile phase. Aliquots were spotted on chromatographic paper and allowed to run 10-12 cm from origin. ^{90}Y -labelled peptide showed Rf values ~ 0.0 , while ^{90}Y -DTPA, ^{90}Y -DOTA exhibited 1.

2.7. Transchelation challenge test

The samples were submitted to two different challenge media, serum and cysteine solution, in order to test the “*in vitro* stability”. In serum: 10 μg of the labelled antibody is added to 1 mL of fresh human serum. After mild shaking the samples were incubated for 60 min at 37°C and analysed by paper and ITLC chromatography, as well as by electrophoresis when considered necessary. In cysteine: a set of standard solutions was prepared starting from a fresh 10 mg/mL cysteine (sigma) solution in 100 mM phosphate buffer pH 7.0, considering 5 different cysteine:biomolecule molar ratios (500:1, 50:1, 5:1, 0.5:1, and 0:1). These mixtures were incubated for 60 min at 37°C and analysed by Sep-pak RP-C18 cartridge and a paper chromatographic system [16], whose mobile phase was phosphate buffer pH = 7.2.

2.8. Biodistribution study

Balb/C female mice weighing 18–25 g were obtained from CENPALAB (Havana). About 100 μL of labelled molecule, corresponding to ~ 3.7 MBq (100 μCi), was injected intraperitoneally. The animals were sacrificed 4 and 24 h later administration was done by cervical dislocation. Radioactivity in each organ was counted in a gamma counter and recorded as percentage of injected dose/g tissue.

3. RESULTS

Table I summarizes the most relevant results of the $^{99}\text{Tc}^{\text{m}}$ -evaluation of each molecule.

TABLE I. RELEVANT RESULTS OF THE $^{99}\text{Tc}^{\text{m}}$ EVALUATION FOR DIFFERENT BIOMOLECULES

	$^{99}\text{Tc}^{\text{m}}$ -ior-CEA1	$^{99}\text{Tc}^{\text{m}}$ -scFv	$^{99}\text{Tc}^{\text{m}}$ -ior-P1394	$^{99}\text{Tc}^{\text{m}}$ -lanreotide
Labelling	>95%	>95%	~ 92%	>93%
Principal impurities	$^{99}\text{Tc}^{\text{m}}\text{O}_4^-$	$^{99}\text{Tc}^{\text{m}}\text{O}_4^-$	Colloid/aggregates	$^{99}\text{Tc}^{\text{m}}\text{O}_4^-$
Stability in 100 mM Cysteine (% dissociation)	5%	12%	10%	7.3%
Biological reactivity	+++	++	n.d.	+++
Biodistribution	Normal behaviour for protein	Uptake in bone marrow*	Higher uptake in somatostatin receptor-positive tissues	n.d.

n.d.= not determined

*Note: In this work we observed that the scFv showed a high uptake in bone marrow, perhaps associated to a cross-reaction with NCA-95 antigen expressed in granulocytes. Therefore our group decided to enhance the biological characteristic of the molecule, before applying it in the target therapy.

3.1. ^{188}Re -ior-CEA1

The radiolabelled products obtained through MDP route showed great instability, as well as precipitation formation. The experimental design established that citric acid as well as pH played a significant role in antibody labelling. The best results for the ior-CEA1 were obtained with citric acid as weak ligand, 2.6 mM SnF_2 and pH 3. Fig.1 shows the labelling yields, at different citric acid concentrations in the system. As it can be observed, the best results were attained in a concentration range of 10-32 mM citric acid and between 2-3 reaction h. Afterwards the labelling yield decreases until values <80% at 24 h.

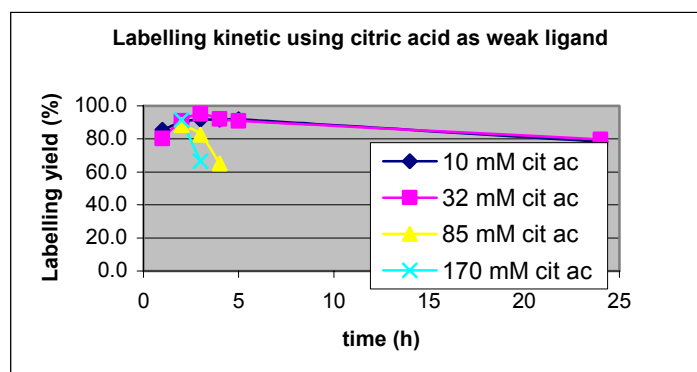


FIG. 1. Yield of ^{188}Re -ior-CEA1 with 2.6 mM SnF_2 and pH 3. ($n=3$).

In order to enhance the label stability of $^{188}\text{Re-iorCEA1}$ at 24 h, 100 μL of 2% BSA and 10 μL of 3% ascorbic acid (AA) were added, which preserved the reoxidation and colloid formation of ^{188}Re . These additives were added once the labelling reaction was completed, i.e. after 3h. From Fig. 2, the influence of the additives in the stability can be observed. If the pH is increased, stability is affected.

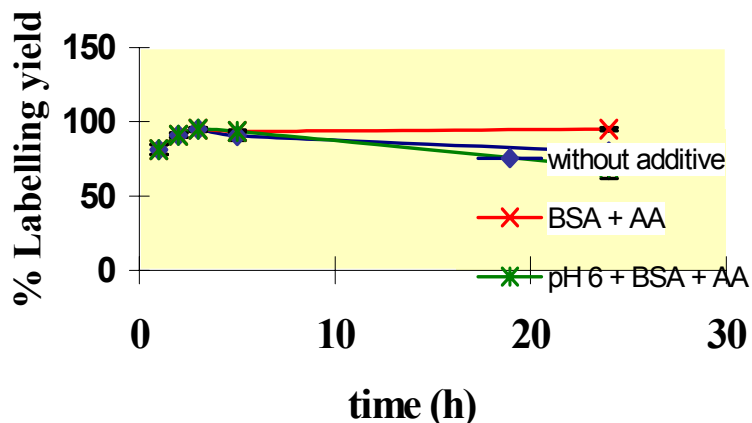


FIG. 2. Labelling yield of $^{188}\text{Re-iorCEA1}$ without and with additive of ascorbic acid and BSA, whose final concentration was 0.03% and 0.5% respectively.

In vitro stability studies of $^{188}\text{Re-ior-CEA1}$ in challenge media of cysteine at 37°C are shown in Fig. 3. The activity associated with protein decreases with the increment of cysteine concentration. Nevertheless, addition of AA and BSA guarantees a minimal transchelation.

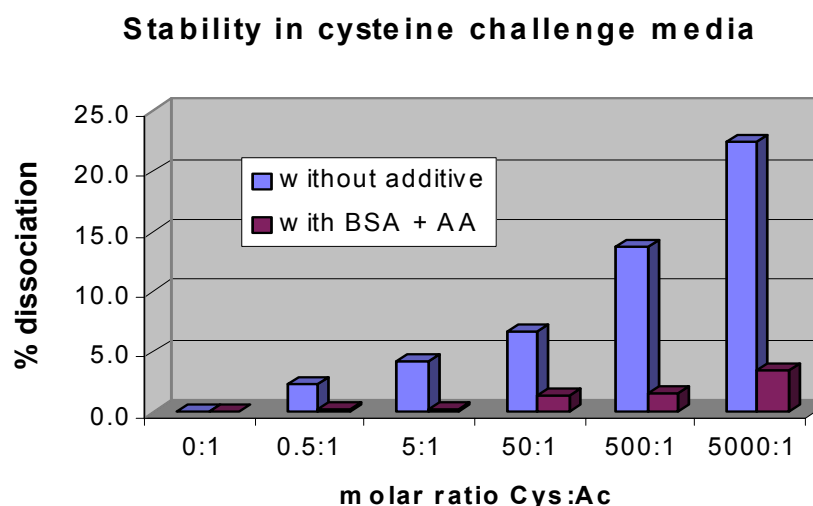


FIG. 3. *In vitro* stability of $^{188}\text{Re-ior-CEA1}$ at 37°C in cysteine challenge media during 1 h.

The results obtained in the biodistribution of $^{188}\text{Re-iorCEA1}$ suggested that there is no significant accumulation in critical organs. The excreting organs behaved as normal (Fig. 4).

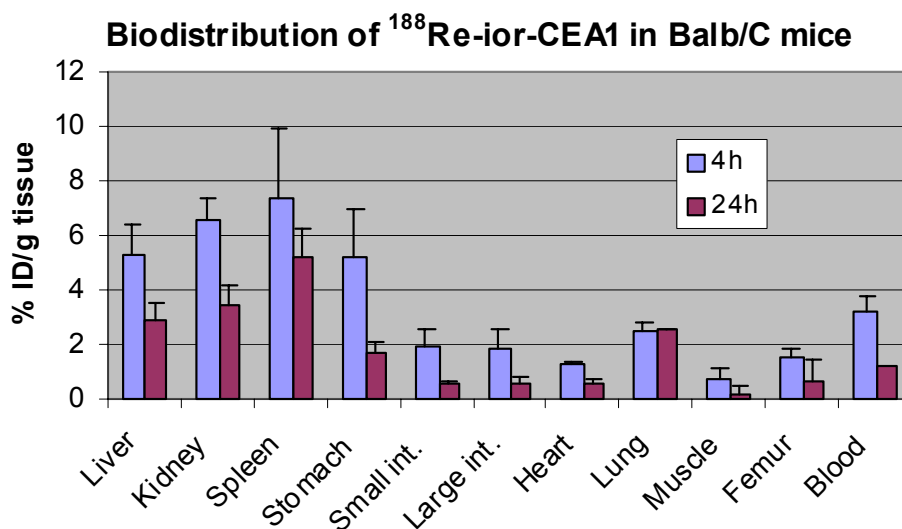


FIG. 4. Biodistribution of Re-188-ior CEA1 in BALB/C mice at 4 h and 24 h.

The radioactivity uptake in the different organs is relatively low and similar to those obtained with ⁹⁹Tc^m-iorCEA1[16], except the uptake in spleen, which is slightly higher.

3.2. ¹⁸⁸Re-ior-P1394 and ¹⁸⁸Re-lanreotide

Fig. 5 shows the results obtained with different ligands, fixing the rest of the parameters in the plane centre. The best results were attained with citric acid for both peptides: ior-P1394 and lanreotide.

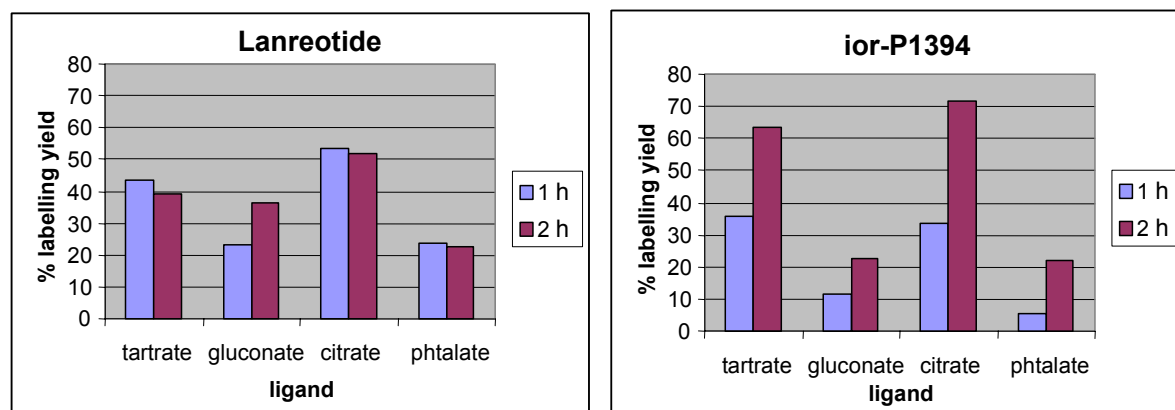


FIG. 5. Labelling yield of ¹⁸⁸Re-ior-P1394 and ¹⁸⁸Re-Lanreotide using different weak ligands.

As a result of the experimental design, it was concluded that all independent variables influence positively the labelling yield. The variable that had highest influence in the system was the SnF₂ amount, followed by pH. The influence of the weak ligand concentration in the system was minimal, in contrast to the antibody labelling. As optimal, the following parameters were fixed: 50 µg peptide, 4 mM SnF₂, 80 mM citric acid, pH 3 and 1 h reaction

time. Under these conditions a labelling yield of >95% was obtained for lanreotide and ~90% for ior-P1394.

^{188}Re -ior-P1394 exhibited a relatively higher colloid/aggregate formation ~ 9%, while ^{188}Re -lanreotide showed values under 2%. Likewise ^{188}Re -ior-P1394 showed a certain instability post labelling, obtaining until 15% of dissociation at 6 h at room temperature, without challenge media. We could not investigate the reason for this instability because we had problems with the data reproducibility. However, the percentage of dissociation at 6 h was significantly lower for ^{188}Re -lanreotide (<5%). The results of the cysteine challenge are shown in Fig. 6. At a low Cys: pep molar ratio, the complex ^{188}Re -peptide exhibited relatively low dissociation, around 12%. However, using higher cysteine concentrations (molar ratios of 1:500) dissociation occurred in ~42%.

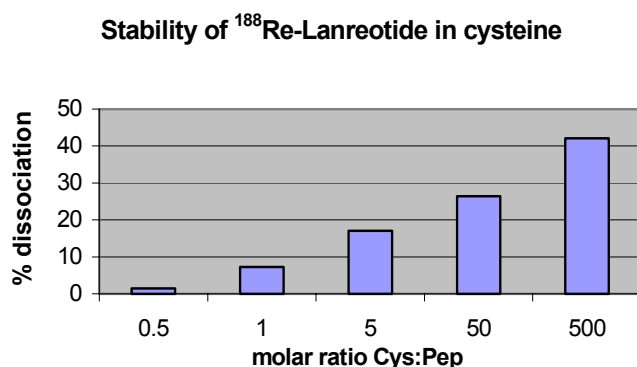


FIG. 6. *In vitro* stability of ^{188}Re -Lanreotide at 37 °C in cysteine challenge media during 1 h.

3.3. ^{90}Y -DOTA-lanreotide

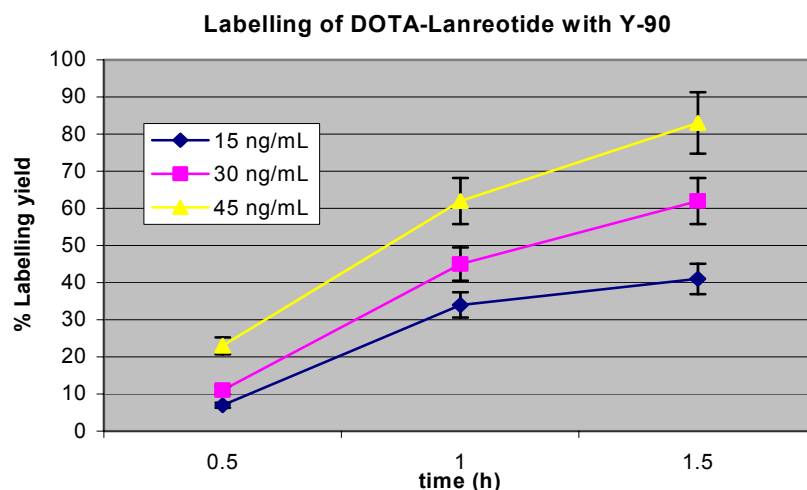


FIG. 7. Labelling yield of ^{90}Y -DOTA-lanreotide, varying the peptide concentration at different times.

The labelling efficiency of ^{90}Y -DOTA-lanreotide was studied through an experimental design type 3². Fig. 7 shows the results varying the peptide concentration at different reaction time. As it can be observed at higher concentration and longer time, better yields were obtained. This work could not be continued due to problem of isotope availability.

4. DISCUSSION

Radiolabelling of iorCEA1 is accomplished by direct method using 2-mercaptoethanol as reducing protein agent. Two different weak chelating agents were employed, which had been reported in other published works before [23, 24]. All experiments carried out with MDP showed low labelling yields and high precipitate formation, even using SnF₂, which has better redox properties. The best labelling yields were attained with low citric acid concentration, as well as 2.6 mM SnF₂ at pH 3.

As it can be observed from Fig 1, under the conditions specified earlier, relatively high labelling yields could be achieved, within 1 h incubation (>80%). Experiments in which a transfer ligand was formed “*in situ*” during the labelling reaction showed better results than those where Re weak chelate had been already obtained.

¹⁸⁸Re-iorCEA1 was extremely stable when challenged with cysteine, if pre-protected with the addition of AA and BSA. At 5000:1 Cys: MAb molar ratio only a dissociation of 3,5% was observed. Inclusively, without adding such additive, the dissociation was <5% at low cysteine concentrations (Fig. 3). These results were better than those obtained by John, et al. [24] at similar or lower cysteine concentration, suggesting the formation of a more stable complex.

The biodistribution in BALB/C mice was similar as ⁹⁹Tc^m-iorCEA1, except for spleen, where uptake was relative high. This could be explained as due to the binding of the Fc portion of the labelled murine Ab with the cytoplasmatic membrane of the red blood cells (RBCs), which could harm RBC, product of the radiolysis of the strong β emission from ¹⁸⁸Re. Although this phenomenon could compromise its application in the radiotherapy, it could not be observed in humans for this antibody because it is a murine protein.

On the other hand, in order to enhance the pharmacokinetic parameters of ior-CEA1 to be used in the metabolic therapy, our group developed a single chain fragment of this protein through genetic engineering techniques. These fragments are particularly attractive due to their small size (ca. 25-27 kD).

The results obtained in the ⁹⁹Tc^m labelling indicate that the scFv was adequately labelled and its biological activity was conserved. The cysteine challenge demonstrated that dissociation by transchelation to cysteines at a very high concentration of competitor occurred in a similar fashion for the intact MAb and the scFv, even though in the latter the binding for ⁹⁹Tc^m was made through histidine nitrogen atoms and not by thiol group. Nevertheless, although the chemical characteristics of the molecule accomplished with the established requirements to be used in the radiotherapy, the “*in vivo*” experiments attained a high uptake in bone marrow, perhaps associated to a cross-reaction with NCA-95 antigen expressed in granulocytes. Therefore, our group decided to enhance the biological characteristic of the molecule before continuing its evaluation for the target therapy.

During the last years many peptides were investigated for their potential to improve the receptor images. Their smaller size conducts to expect a more rapid clearance from the circulation and to be less immunogenic than labelled proteins, maintaining in most of them similar affinity level for their receptors. So, given the physical ¹⁸⁸Re characteristic, the ¹⁸⁸Re - labelled peptides attained a great sympathy for target therapy.

The labelling method through an intrinsic linear chelating amino acidic sequence seems to be an attractive alternative for directly ¹⁸⁸Re-labelled peptide. Thakur and collaborators showed recent developments in the use of these radioactive agents [25, 26].

We have synthesized a peptide adding at the *N*-terminal the $^{99}\text{Tc}^{\text{m}}$ (^{188}Re) -chelating sequence (AGGG β A). A group of four amino acids Ala-Gly-Gly-Gly was chosen as chelating moiety, in order to supply a chelating configuration type N_4 , through their NH_2 groups. The proposed structure of ^{188}Re -ior-P1394 is given in Fig. 8. Furthermore, an additional amino acid β -Ala was inserted as a spacer between the chelating moiety and the primary peptide. This amino acid has the purpose of minimizing any possible steric hindrance resulting from the $^{99}\text{Tc}^{\text{m}}$ (^{188}Re)-chelation. The cycle peptide was obtained with high yield using the DMSO method for disulphide bond formation. No oligomeric population was observed during the oxidation reaction. This fact was determinant for the high yield of the final product. The resulting peptide has an expected molecular weight of 1444.9, which was determined experimentally. It showed good agreement with the theoretical average masses calculated for each peptide, taking into account the modifications contained within their structures.

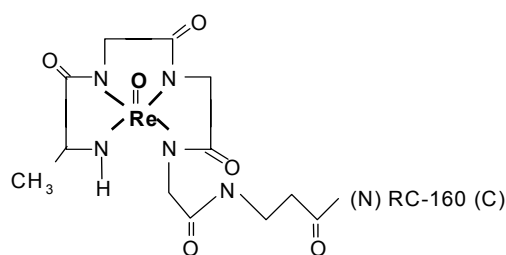


FIG. 8. Proposed structure of ^{188}Re -ior-P1394.

The radiolabelling of ior-P1394 with Re-188 is a simple and single-step procedure with a high efficiency ($\sim 90\%$). The best results were obtained at pH 3, in a concentration of 80 mM citric acid and 4 mM SnF_2 . Under these conditions, it can be also observed the presence of colloids/aggregates relatively high ($\sim 9\%$). We had not time to investigate the character of these aggregates, neither its cause. We mind that the formation of these aggregates could be related with the molecule polarity and its isoelectric point. However, although this work requires more investigation, the results are sufficiently encouraging to suggest that the Intrinsic Linear Chelating Amino Acidic Sequence was a good alternative for the peptide direct labelling with Re-188.

Likewise the ^{188}Re -Lanreotide was obtained through direct method with high labelling yields ($>95\%$). Using Ellman's test [15] for the sulphidryl group determination, we could prove that once lanreotide is in contact with Sn ions, the sulphidryl bridge is broken without requiring heating. This suggests that the co-ordinate binding can occur through thiol groups.

The cysteine challenge demonstrated that dissociation by transchelation to cysteines at a low concentration of competitor occurred relatively low. However, at high concentrations the complex ^{188}Re -peptide exhibited dissociation $\sim 42\%$, suggesting the formation of a less stable complex than formed with $^{99}\text{Tc}^{\text{m}}$, whose values were around 18%.

The highest labelling efficiency for ^{90}Y -dOTA-lanreotide was around 83%. In this experiment both the peptide concentration and reaction time played a significant role, with a positive tendency for higher peptide concentrations and reaction times. This experiment could not be continued, because no isotope was available at the time.

5. CONCLUSIONS

From the evaluation of ior-CEA1, scFv, ior-P1394, lanreotide and DOTA-lanreotide, the following can be concluded:

- The chosen labelling method for ior-CEA1 with ^{188}Re attained high radiochemical purity of >95%, exhibiting high stability for 24 h with the addition of 2% BSA and 3% AA. Its biodistribution showed relatively higher uptake in spleen, a phenomenon that could compromise its application in radiotherapy.
- Although the evaluation of scFv with $^{99}\text{Tc}^{\text{m}}$ attained high labelling yields and good stability, biodistribution studies showed a high uptake in bone marrow, perhaps related to a cross-reaction with NCA-95 antigen expressed in granulocytes. Therefore, it was decided to enhance the biological characteristics of the molecule before its application in the target therapy.
- Ior-P1394 and lanreotide were evaluated with ^{188}Re , obtaining yields of ~90% and >95%, respectively. Although ^{188}Re -labelling of ior-P1394 exhibited a relatively higher colloid formation, our results were sufficiently encouraging to suggest that the strategy of using an amino acidic sequence (AGGG β A) as isotope-chelating moiety for the direct labelling was correct. Likewise ^{188}Re -Lanreotide exhibited good stability at least for 6 h.
- ^{90}Y -labelling method with DOTA-lanreotide showed yields up to 83%. The parameters with a high influence in the system were peptide concentration and reaction time.

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DEVELOPMENT OF RADIOACTIVELY LABELLED CANCER SEEKING BIOMOLECULES FOR TARGETED RADIOTHERAPY

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Abstract

In radioimmunotherapy (RIT) potentially therapeutic radionuclides are transported to the desired site of action by target-selective vehicles, such as monoclonal antibodies (MoAbs) and peptides. The labelling of MoAbs with radionuclides suitable to be used in the field of therapy was investigated. For this purpose we used ^{153}Sm and ^{201}Tl . For the labelling of the antibodies we used the dicyclic anhydride of diethylenetriaminepentaacetic acid (cDTPAa) as intermediate chelating agent. Radiochemical purity was confirmed with radiochemical techniques (HPLC, ITLC-SG, paper electrophoresis, SDS-PAGE). The factors which were proven to affect labelling were mainly the pH and concentration of the antibody, both during labelling and formation of the immunoconjugate. Labelling with ^{153}Sm was performed at a pH range of 5-7, for 60 min at room temperature. The labelling yield was higher than 90%. As far as labelling with ^{201}Tl is concerned, low concentrations of antibody and immunoconjugate were used. Labelling is performed at pH 6-7, for 60 min at room temperature. The labelling yield reached a maximum of 40%. After purification the labelled antibodies were biologically evaluated in normal Swiss mice. For the ^{201}Tl labelled antibody, high uptake in muscle and kidneys was observed. For ^{153}Sm -antiCEA, slow blood clearance was observed, while the liver and muscles showed high uptake. Intravenous injection in nude mice transplanted with colon cancer cells afforded imaging of the experimental tumour on a γ camera. Work on peptide labelling was initially carried out on non-radioactive nuclides, so as to optimize labelling conditions and purification techniques. Thus, the peptide lanreotide was labelled with both "cold" $^{185/187}\text{Re}$ and "hot" ^{188}Re , affording one major product. DOTA-lanreotide was labelled with "cold" ^{152}Sm and "hot" ^{153}Sm . HPLC data showed the formation of one major product, the yield of which depended on the Sm to peptide molar ratio. In all cases, the labelled peptides can easily be isolated and characterized. Experiments are still in progress.

1. INTRODUCTION

Monoclonal antibodies (MoAbs) have attracted considerable attention due to their potential to serve as selective carriers of radionuclides to specific *in vivo* antigens for the development of agents for tumour imaging (RIS: radioimmunoscintigraphy) and for tumour therapy (RIT: radioimmunotherapy) [1]. The majority of the studies of RIT used ^{131}I as the radionuclide for antibody labelling. However, ^{131}I is far from ideal because of its tendency to deiodinate *in vivo* and its high irradiation of normal tissues due to its long half-life and medium energy γ emission [2]. Other potential therapeutic radionuclides that were studied include short-lived α emitters, like ^{212}Bi and ^{211}At and β^- emitters, like ^{67}Cu , ^{109}Pd , ^{47}Sc and ^{90}Y [3]. At this stage, therapy is largely experimental but progress is being made in developing useful clinical agents [4].

The over expression of peptide receptors on human tumour cells as compared to normal tissues and cells has provided the basis for the successful use of radiolabelled somatostatin (SST) analogues for tumour targeting [5, 6]. Recently, a novel human somatostatin receptor-recognizing ligand, DOTA-lanreotide, was developed. This compound binds to all known hSSTR as well as to a variety of primary tumour cells, which have been shown to express the one or the other hSSTR subtype [7].

Successful radiolabelling of MoAbs depends on a number of factors including the chemical characteristics of the nuclide used and the techniques employed for its incorporation into the protein. The most common method for labelling of antibodies with radiometals is by the use of strong chelating groups covalently attached to antibodies so that they may be radiolabelled with the metallic radionuclides in order to result in high stability *in vivo* [2]. As far as ^{153}Sm is concerned, it is already known that the cation of $^{153}\text{Sm}^{3+}$ has good chelation properties with polyaminopolycarboxylic acids, like ethylene-diamine-tetraacetic acid (EDTA) or diethylene-triamine-pentaacetic acid (DTPA) [8,9]. DOTA is also one of the more effective chelators for yttrium and the lanthanides, known to form thermodynamically stable chelates with most transition metals, lanthanides and even some of the alkaline earth metals.

Samarium-153 (^{153}Sm) is a radiolanthanide which has not yet been widely used, but which possesses excellent physical characteristics for radioimmunotherapy [10]. It is a beta-emitter with a half-life ($t_{1/2}$) of 1.95 days. In addition it emits a 130 keV gamma ray (28%) that is suitable for gamma camera detection. It can be produced by neutron activation of enriched ^{152}Sm .

Rhenium-186 and ^{188}Re have been identified as important radionuclides with therapeutic potential. ^{188}Re is available in high specific activities as $^{188}\text{ReO}_4^-$ from $^{188}\text{W}/^{188}\text{Re}$ generators. ^{188}Re , which has a higher beta-particle energy and short half-life ($t_{1/2} = 17\text{h}$) is generally more suitable for preparing radiopharmaceuticals that target larger tumours and have reasonably fast clearance from the blood and other non-target tissues.

Two general approaches are being employed for the synthesis of labelled site-specific radiopharmaceuticals:

1. direct labelling of biomolecules and
2. metalation of biomolecules via a covalently appended chelating framework.

For our study of monoclonal antibodies, we chose DTPA as the intermediate chelating agent, which is linked to the antibodies via the bicyclic anhydride (cDTPAa) [11,12]. As far as we know, the studies of labelling antibodies with ^{153}Sm are limited [13-15]. The main purpose of this study was to investigate the factors, which possibly affects labelling, so as to find those which lead to a labelled product obtained in high yield and with high radiochemical purity. The next step was the radiobiological evaluation of the labelled antibodies in normal mice and in nude mice transplanted with cancer cells. A second purpose was to use the method for labelling of antibodies with ^{153}Sm for other radionuclides in the +3 oxidation state. For this purpose we worked with ^{201}Tl , which is already known in nuclear medicine as an imaging agent for the myocardium. Tl-201 emits γ -rays suitable for imaging and has a half-life of 3.04 days. It also emits Auger electrons (22.7–77 keV) and conversion electrons (1.6–167 keV). These properties make Tl-201 attractive for use as a radiotherapeutic agent, too. For the labelling of antibodies with ^{201}Tl with the developed method, ^{201}Tl must be oxidized from the +1 to the +3 oxidation state.

In our studies of peptides, we labelled lanreotide with ^{188}Re directly, taking advantage of the sulphur and nitrogen atoms found within the peptide molecule, which act as donor atoms, complexing the radiometal and forming a stable radiometal chelate. We have also labelled the peptide with ^{153}Sm via the bifunctional chelator DOTA, which holds the radiometal with high stability under physiological conditions. This stability is essential to avoid metal ion dissociation and excessive radiation damage to nontarget cells. The study of the complexation of DOTA-lanreotide was performed with ^{153}Sm which is readily available,

and at a low cost, from the “Demokritos” Nuclear Reactor, so that a work protocol could be set up with respect to the radiolabelling of biomolecules with trivalent metal ions in general. Our work on the labelling of peptides with radionuclides was initiated with the labelling of the very same peptides with the non-radioactive nuclides ^{152}Sm and $^{185/187}\text{Re}$, so as to facilitate the study of the complexation of the peptides with these isotopes.

2. $^{153}\text{Sm}/^{201}\text{Tl}$ – LABELLED MONOCLONAL ANTIBODIES

2.1. ^{153}Sm –labelled monoclonal antibodies

2.1.1. ^{153}Sm

Samarium-153 was produced in the “Demokritos” reactor by neutron irradiation of ^{152}Sm .

Samarium oxide, isotopically enriched in Sm-152 ($^{152}\text{Sm}_2\text{O}_3$), was irradiated for 5 to 6 h, by a neutron flux of 4.81×10^{13} neutrons/cm². Sec. Specific activity of the produced Sm-153 was 18 – 32 mCi/mg. The activated oxide was dissolved in a solution of HCl 6M, to produce Samarium chloride ($^{153}\text{SmCl}_3$). For the labelling $^{153}\text{SmCl}_3$ was diluted to the appropriate volume with ultra pure water, to produce a stock solution.

2.1.2. Abs

The study was initiated on the human polyclonal immunoglobulin “Sandoglobuline”, commercially available. It was applied thereafter on the monoclonal antibody anti-CEA, kindly offered by Cuba, which selectively localizes in colon cancer.

2.1.3. ^{153}Sm – DTPA

Labelling of DTPA with ^{153}Sm was initially investigated for comparison. DTPA was dissolved in acetate buffer 0.1M, pH=5.6 in a concentration of 1.0 mg/ml. The pH of the final solution was 5.2. For the labelling 5–10 μl of the solution of $^{153}\text{SmCl}_3$ (150–300 μCi) was added to the solution of DTPA. The final pH was adjusted in a pH range 5.0–5.5, if it is necessary, with a dilute solution of NaOH. The solution was stirred gently and was incubated at room temperature for 60 min. Labelling was studied by ITLC-SG.

Labelling of DTPA was evaluated by instant thin layer chromatography on silica gel (ITLC-SG) using $\text{NH}_4\text{OH} : \text{H}_2\text{O}$ 1: 25, as solvent system. R_f values were 0.0 for $^{153}\text{SmCl}_3$ and 0.9 – 1.0 for ^{153}Sm – DTPA.

2.1.4. Immunoconjugate Ab – DTPA

For the preparation of the immunoconjugate, the bicyclic anhydride of DTPA was added in solid form to the solution which contains the antibody in phosphate buffer 0.1M, NaCl 0.1M, pH=8.0. The molar ratio of Ab to DTPA was 1:10. The mixture was allowed to stand at room temperature for 30 min. Separation of the Ab–DTPA from free DTPA was achieved by gel filtration in a Bio Gel P30 column, eluted with acetate buffer 0.1M, pH=5.6. Fractions were collected and their absorbance at 280 nm was measured for the determination and isolation of those which contain the immunoconjugate. The fractions were pooled together and aliquoted to 0.5 or 1.0 ml fractions and frozen.

Gel filtration led to the isolation of the immunoconjugate Ab–DTPA from any excess of the ligand, as presented in Fig. 1.

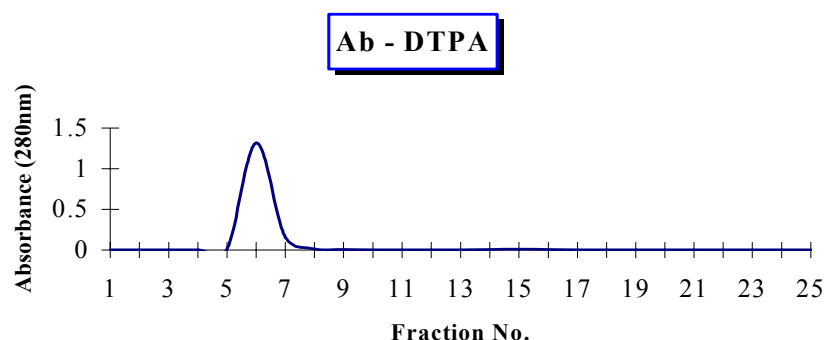


FIG. 1. Ab–DTPA elution profile.

2.1.5. ^{153}Sm –DTPA – Ab

For the labelling of the antibody a small volume of $^{153}\text{SmCl}_3$ (5 – 15 μl) was added to the solution which contained the Ab–DTPA in acetate buffer 0.1M, pH = 5.6. The mixture was allowed to stand at room temperature for 60 min. The labelled antibody was then purified by gel filtration, eluted with acetate buffer. During gel filtration fractions were collected and their absorbance at 280 nm was measured for the determination of those which contain the antibody, and then their radioactivity was measured in an isotope counter. The labelling yield can be calculated from the ratio of the radioactivity of fractions that contain Ab–DTPA to the total radioactivity. The results from a representative experiment are shown in the Fig. 2.

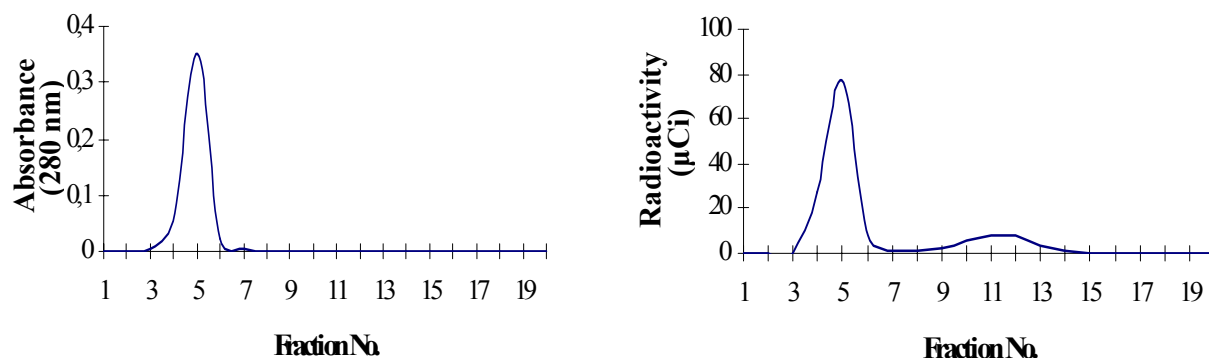


FIG. 2. AB-DTPA elution profile

The radiochemical purity was confirmed with a combination of radiochemical techniques as HPLC, ITLC-SG and SDS-PAGE.

The purified antibodies were analysed by size exclusion HPLC (column: TSK 2000 SW, solvent: phosphate buffer 0.1M, Na_2SO_4 0.1M, NaN_3 0.05% w/v, pH=6.7, flow rate 1ml/min), compared with unlabelled antibodies. Retention time with the UV detector was 5.98 min while with the γ -detector was 6.33 min. Instant thin layer chromatography was performed on silica gel

using acetate buffer 0.1M, pH=5.6 as mobile phase. The radiochromatographs were studied with electronic autoradiography. R_f values were 0.9 – 1.0 for $^{153}\text{SmCl}_3$ and 0.0 for $^{153}\text{Sm} - \text{DTPA} - \text{Ab}$.

2.1.6. Factors affecting labelling

We studied many factors that may affect labelling. Table I shows the most important of these.

Antibody concentration effect: In order to see if antibody concentration affects labelling, we used antibody in two different concentrations, 3mg/ml and 15mg/ml, for the preparation of the immunoconjugate. As we can see from the results in all cases using the same quantity of immunoconjugate, higher labelling yields were obtained using Ab–DTPA produced from the antibody with a concentration of 15mg/ml, compared to those produced from the 3mg/ml preparation.

TABLE I. FACTORS AFFECTING LABELLING

Ab–DTPA (mg)	C_{Ab} (mg/ml)	^{153}Sm (μCi)	Incubation time	% yield
Antibody concentration effect				
0.5	3	155	60 min	20
0.5	15	140	60 min	35
1.0	3	145	60 min	45
1.0	15	175	60 min	91
Incubation time effect				
1.0	15	180	30 min	75
1.0	15	175	60 min	91
1.0	15	195	18 h	87
Immunoconjugate concentration effect				
0.3	15	250	60 min	30
0.5	15	140	60 min	35
1.0	15	175	60 min	91
2.0	15	280	60 min	93

Incubation time effect: The labelling was studied for incubation time periods of 30 min, 60 min and overnight at room temperature, for the same concentration of Ab and Ab – DTPA. At 30 min the labelling yield was 75%, while at 60 min it became 91% and remained practically the same up to 18 h later.

Immunoconjugate concentration effect: Finally we studied the effect of immunoconjugate concentration on the labelling yield. Using different quantities of Ab-DTPA, starting from the same concentration of antibody (15mg/ml) and using the same labelling conditions, we observed that the yield increases significantly by increasing the concentration of Ab–DTPA. The yield reached a maximum of 93% using 2 mg Ab–DTPA.

pH: Labelling can be achieved at a pH range from 5.0 to 7.0.

2.1.7. Tissue distribution in Swiss mice

Biodistribution of the labelled anti-CEA was evaluated in normal Swiss mice, after intravenous injection into the tail vein. Animals were sacrificed by ether anesthesia in compliance with European instructions and Hellenic law. Comparatively, the $^{153}\text{SmCl}_3$ solution used for labelling and a solution of the complex $^{153}\text{Sm} - \text{DTPA}$ were also biologically evaluated in the same way. The comparative biodistribution results 4 h post injection are presented in Fig. 3.

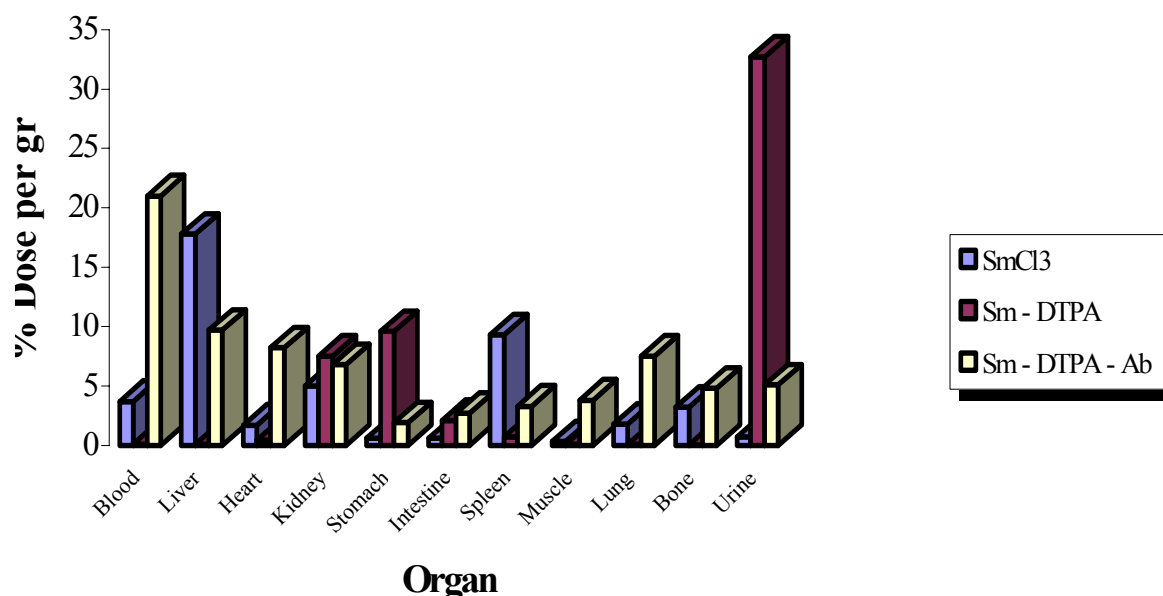


FIG. 3. Comparative biodistribution results in mice 4 h p.i.

For this interval the labelled antibody had slow blood clearance, high liver uptake, while no specific accumulation in any organ was observed. On the other hand $^{153}\text{SmCl}_3$ was mainly localized in the liver and spleen, while $^{153}\text{Sm}-\text{DTPA}$ was excreted through the kidneys to the urine.

2.1.8. Cancer cell binding assay

Cell binding assay was performed using the epithelial-like Colo 205 (CCL 222 of the American Type Culture Collection) isolated from human colon adenocarcinoma. The cancer cells of the Colo 205 were grown as monolayers in RPMI medium supplemented with 10% fetal bovine serum at 37°C and 5%CO₂. Subculturing of the Colo 205 monolayers was performed after trypsinization (0.25% trypsin in EBSS) using a 1:2 split ratio every 2–3 days. For the binding assay, the cells were harvested, washed twice with PBS and resuspended in PBS containing 1% BSA at a cell density of 10⁶ cells/100 µl. All the work, except the incubations, which were performed at room temperature for one h, was carried out in ice. Four dilutions of the labelled monoclonal antibody were used. The incubation mixtures consisted of 10⁶ cells plus labelled antibody at concentrations of 325, 162, 110 and 81 nM in PBS containing 1% BSA. All samples were assayed in duplicate.

After incubation the samples were centrifuged. The resulting supernatants were separately collected from each Eppendorf and placed into tubes for counting. The pellets were resuspended in 300 µl PBS and transferred onto 0.22 µm filters (Millipore), which were

placed into the wells of a manifold filtration apparatus (12 wells/apparatus). The samples were vacuum dried onto the filters. The filters were washed four times with PBS and dried in the same manner as that described above. In continuation, the filters were placed into counting tubes and the radioactivity of the filters as well as that of the first supernatants were measured in a gamma counter (Minaxi autogamma 5000 series, Packard). The results were assessed using the Scatchard analysis. Scatchard analysis of the data obtained from the binding assay experiments of the ^{153}Sm -DTPA-antiCEA to the binding sites of the Colo 205 human adenocarcinoma revealed an apparent K_d of 2.0 nM. (Fig. 4). This finding is indicative of a high affinity amongst the binding sites of the colon cancer cell line.

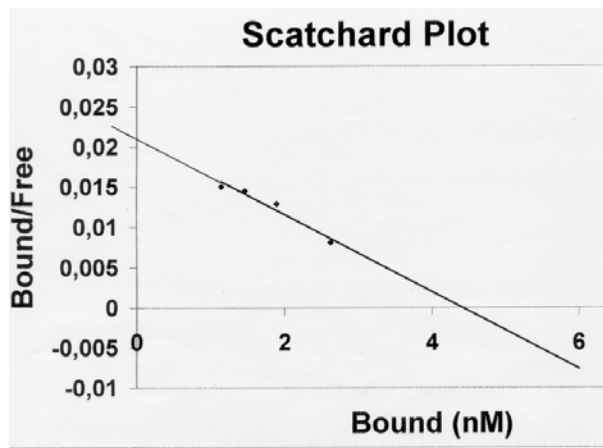


FIG. 4. Scatchard plot.

2.1.9. γ -camera imaging of nude mice

Nude mice were transplanted with colon cancer cells, subcutaneously. After 10 – 15 days the tumour had been developed, as verified by histochemical techniques. The labelled anti-CEA was intravenously injected into the tail vein. The animals were sacrificed at 4 h post injection. As we can see in the image from the γ -camera, Fig. 5, the experimental tumour can be clearly visualized.

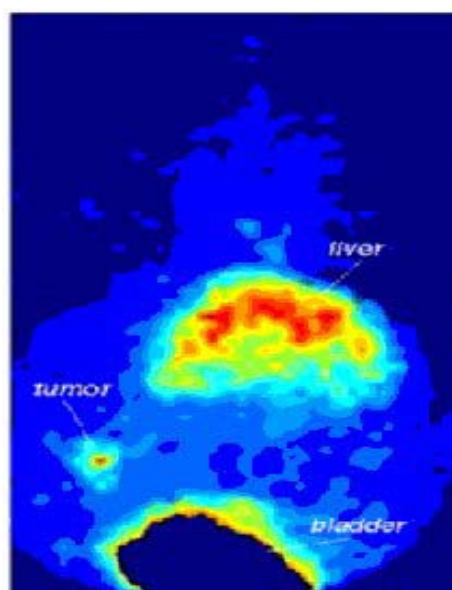


FIG. 5. Scintigram of a nude mouse, 4 h p.i.

2.2. ^{201}Tl – Labelled monoclonal antibodies

2.2.1. Preparation of ^{201}Tl for labelling of antibodies

2.2.1.1. Preparation of ^{201}Tl

Oxidation of $^{201}\text{Tl}^+$ to $^{201}\text{Tl}^{+++}$

To a solution of $^{201}\text{TlCl}$ (0.1 – 0.5 ml) a solution of H_2SO_4 5M (1.0 ml) was added followed by a fresh solution of KBrO_3 5% w/v (0.2 ml). The mixture was allowed to stand at room temperature for 15 min before the addition of a solution of NaCl 4M (50 μl).

Extraction of $^{201}\text{Tl}^{+++}$

$^{201}\text{Tl}^{3+}$ was extracted with the addition of 1.5 ml butyl acetate. The mixture was mixed for 20 min at room temperature. The organic phase was separated from the water phase and was evaporated in a water bath, by adding a small volume of water. ^{201}Tl was received as an aqueous solution ready to use for labelling.

2.2.1.2. Radiochemical control

$^{201}\text{Tl}^{+++}$ was checked by low voltage electrophoresis in nitrocellulose acetate strips (200V, 30min), in comparison with the solution of $^{201}\text{Tl}^{1+}$. $^{201}\text{Tl}^{3+}$ migrates to the negative electrode, while $^{201}\text{Tl}^{1+}$ stays at the origin, as shown in Fig 6.

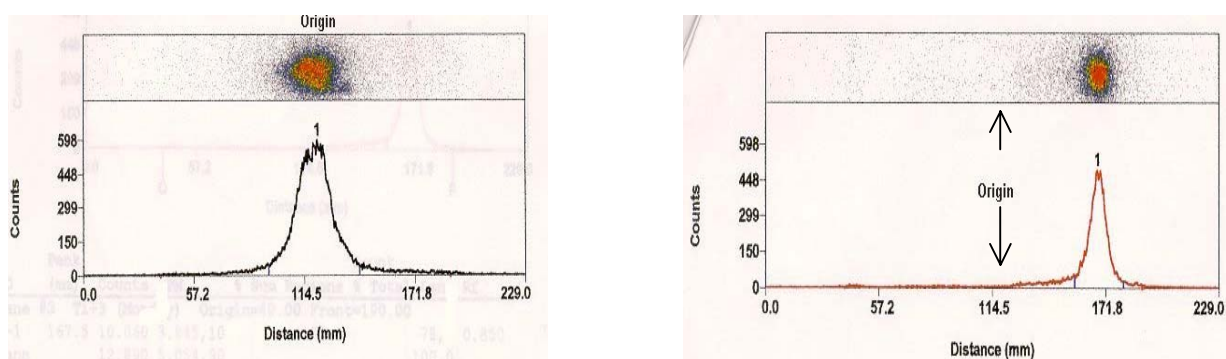


FIG. 6. Electrophoresis of $^{201}\text{Tl}^{1+}$ and $^{201}\text{Tl}^{3+}$.

2.2.2. ^{201}Tl – DTPA – Ab

For the labelling of antibodies with ^{201}Tl we used human polyclonal immunoglobulin (Sandoglobuline). The antibody was conjugated with DTPA by the bicyclic anhydride of DTPA. The immunoconjugate was produced as is described for the labelling of antibodies with ^{153}Sm .

Labelling was performed by the addition of the solution of $^{201}\text{Tl}^{3+}$ to the solution, which contained the immunoconjugate Ab – DTPA in NaCl 0.9% w/v. The mixture was allowed to stand at room temperature for 60 min. The labelled antibody was then purified by gel filtration in a Bio Gel P30 column, eluted with saline.

The radiochemical purity was confirmed with low voltage electrophoresis in nitrocellulose acetate strips (200V, 30min), in the same way as in the case of the $^{201}\text{Tl}^{3+}$ control. The ^{201}Tl -DTPA-Ab remains at the origin, while $^{201}\text{Tl}^{3+}$ migrates to the negative electrode.

2.2.3. Biodistribution studies

The labelled antibody was biologically evaluated in normal Swiss mice after intravenous injection into the tail vein. Comparatively, the $^{201}\text{Tl}^{3+}$ solution was biologically evaluated in the same way. Table II shows the comparative biodistribution results 4 h post injection.

$^{201}\text{Tl}^{3+}$ is rapidly excreted through the kidneys and as expected there is a high accumulation in the heart and muscle. From the results of the biodistribution of ^{201}Tl -IgG one can conclude that the radioimmunoconjugate shows good *in vivo* stability (lower stomach values), low accumulation in liver and lower renal excretion.

TABLE II. COMPARATIVE BIODISTRIBUTION RESULTS 4 H P.I

Organ	Tl(III)	Tl – DTPA - IgG
	% dose per gram	
Blood	0.56 ± 0.1	1.24 ± 0.1
Heart	6.24 ± 0.2	4.12 ± 0.3
Lungs	4.03 ± 0.4	2.89 ± 0.5
Liver	3.65 ± 0.1	2.73 ± 0.7
Kidneys	58.4 ± 3.2	38.56 ± 5.2
Spleen	6.93 ± 0.5	4.06 ± 0.7
Stomach	5.24 ± 0.3	3.12 ± 0.4
Intestine	7.07 ± 0.9	3.70 ± 0.8
Muscle	5.39 ± 0.7	2.47 ± 0.8
Urine (% Dose/organ)	4.36 ± 0.8	2.94 ± 1.2

3. $^{153}\text{Sm}/^{188}\text{Re}$ -LABELLED PEPTIDES

3.1. $^{185/187}\text{Re}/^{188}\text{Re}$ -lanreotide

3.1.1. Preparation of $^{185/187}\text{Re}$ -lanreotide

To a suspension of $\text{ReOCl}_3[\text{P}(\text{C}_6\text{H}_5)_3]_2$ (1.899 mg, 2.28×10^{-3} mmol) and CH_3COONa (1.869 mg) in MeOH (2 mL) a solution of Lanreotide (2,5 mg, 2.28×10^{-3} mmol) in MeOH (1 mL) was added under stirring. The mixture was stirred at R.T. overnight, while its initial yellowish color has disappeared, leaving a clear, colorless solution. HPLC analysis (C_{18} column, acetonitrile-0.1% TFA gradient elution system: 0-60% acetonitrile over 20 min)

showed the formation of one main product, with a retention time of 18 min. The formation of another product, with a retention time of 15 min, was also noted.

3.1.2. Preparation of ^{188}Re -lanreotide

The ^{188}Re eluate is acquired from a $^{188}\text{W}/^{188}\text{Re}$ generator. 5.0 mL eluate was acquired. 2.0 mL of the eluate was added to a mixture of reducing agents (SnCl₂: 1.6 mg, ascorbic acid: 2.46 mg, citric acid: 16 mg). The resulting mixture is left for 2 h at room temperature. The 2.0 mL mixture containing the reduced rhenium-188 is added to 3 mg of a mixture of SnCl₂, sodium gluconate and sodium bicarbonate (this mixture is prepared by adding 1 g sodium gluconate, 2 g sodium bicarbonate and 15 mg SnCl₂ to an empty vial, and shaking well). The resulting mixture is left for 15 min. A solution of the peptide is prepared (1 mg peptide/mL). 0.4 mL ^{188}Re -gluconate solution was added to 0.5 mL peptide solution (containing 0.5 mg peptide). The resulting mixture is heated at 90°C for 60 min. The volume of the mixture was brought to 2 mL with saline.

HPLC studies of ^{188}Re -Lanreotide showed the formation of one major product, with a retention time of 17.5 min. The formation of this product was influenced by the reaction time and the temperature. Complex formation is accelerated by increasing the temperature. By heating the reaction mixture at 90°C for 60 min, a yield of approximately 90% was obtained.

Biodistribution studies in normal Swiss mice showed that the radiolabelled biomolecule presented fast blood clearance and practically complete hepatobiliary elimination. Urine elimination was negligible.

In vitro cell binding studies were not satisfactory, and are currently being repeated. Tumour uptake was evaluated in female BALB/c nude mice, 5 weeks old. The experimentally induced gastrointestinal tumour is clearly delineated 2 h after intravenous injection of the radiolabelled peptide. PANC-1 cells are currently being grown and will soon be implanted into nude mice so as to see whether this Re-188 derivative shows specificity for this type of cancer.

3.2. $^{152}\text{Sm}/^{153}\text{Sm}$ -DOTA-lanreotide

3.2.1. Preparation of ^{152}Sm -DOTA-lanreotide

A stock solution of samarium chloride was prepared by dissolving 366 mg SmCl₃.6H₂O (FW 366) in 1000 µL (1 mL) ultra pure water. The final concentration of the SmCl₃ solution is 1000 nmol/ 1000 µL. The DOTA-lanreotide conjugate was dissolved in freshly prepared 0.2 M ammonium acetate buffer (metal-free: pH 7.00). The concentration of the final solution was 1000 µg/ 1000 µL. Reaction ratios of 0.5:1, 1:1 and 2:1 SmCl₃:Peptide were used, and the complexing reaction was allowed to proceed for 60 min at 90°C. An aliquot was then removed and its purity assessed by HPLC (C₁₈ column, same as for Rhenium-lanreotide analysis). HPLC analysis of the DOTA-lanreotide conjugate afforded one main peak at 16.00 min (84%), with two shoulders on either side of this peak, at 15.10 min (0.89%) and 17.17 min (4.04%). DOTA-lanreotide, when labelled with “cold” SmCl₃, gave practically the same elution profile in all three cases, as can be seen below.

The HPLC profile of the “neat” peptide conjugate differs from that of ^{152}Sm -DOTA-lanreotide, possibly because of the formation of the samarium-peptide complex, via the

DOTA linker. The UV profile remains practically unchanged even when the molar ratio of SmCl_3 : peptide changes significantly (Table III).

TABLE III. HPLC DATA OF DOTA-LANREOTIDE AND ^{152}Sm -DOTA-LANREOTIDE

Product under evaluation	$t_r(\text{UV})$ (retention time)	%
DOTA-lanreotide	16.00	84.00
SmCl_3 : DOTA-lanreotide 0.5 : 1	15.77	41.88
SmCl_3 : DOTA-lanreotide 1 : 1	15.87	40.63
SmCl_3 : DOTA-lanreotide 2 : 1	15.82	41.00

3.2.2. Preparation of ^{153}Sm -DOTA-lanreotide

A stock solution of ^{153}Sm was prepared as described above (Part A). DOTA-Lanreotide was dissolved in ammonium acetate buffer (see: Synthesis of ^{152}Sm -DOTA-Lanreotide). The pH of the final solution was 6.9. For the labelling with ^{153}Sm , a variety of volumes of $^{153}\text{SmCl}_3$ solutions were added to the DOTA-Lanreotide solution (radioactivity range 25 – 200 μCi). The solutions were incubated at 90 °C for 60 min. Labelling efficiency was studied by HPLC (column and gradient elution system same as above). The results are shown in Fig. 7. Gradual increase of the added radioactivity leads to a marked decrease in the labelling yield for peptide samples of the same concentration.

The above findings help us reach an initial conclusion that labelling efficiency of the DOTA-lanreotide conjugate depends on the ^{153}Sm to peptide molar ratio. Further radiochemical and radiobiological studies are in progress.

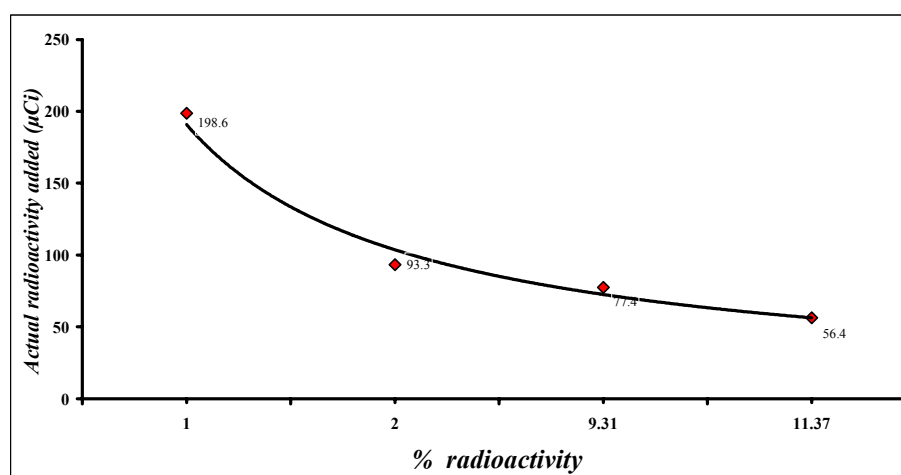


FIG. 7. Labelling efficiency of DOTA-lanreotide.

Biomolecules were labelled with a variety of nuclides and were evaluated in terms of radiochemical purity and radiobiological performance. Monoclonal antibodies were successfully labelled with the radionuclides ^{153}Sm and ^{201}Tl to be used in combined Radioimmunoscinigraphy and Radioimmunotherapy. Labelling can be performed via a chelating agent, like DTPA, which forms a stable complex with the radionuclides. The radionuclide must be in the +3 oxidation state. For this purpose ^{201}Tl must be oxidized before use from +1 to +3. It is possible that the developed method can generally be applied to radionuclides in the +3 oxidation state. The concentration of antibody during the formation of immunoconjugate Ab–DTPA and concentration of Ab–DTPA during labelling significantly affect the labelling yield. Other factors like incubation time, pH and radioactivity also play an important role on the labelling yield. The methodology we developed results in a labelled antibody, purified and in high yield. Radiobiological studies advocated for possible successful *in vivo* use of the radiolabelled MoAbs. Intravenous injection of the ^{153}Sm –anti CEA in nude mice transplanted with colon cancer cells afforded imaging of the experimental tumour on a γ camera. The SSTR specific peptide lanreotide and the DOTA-lanreotide conjugate were both labelled with radioactive and non-radioactive isotopes of the same element and were radiochemically evaluated. Lanreotide was directly labelled with $^{185/187}\text{Re}$ and ^{188}Re , while ^{152}Sm and ^{153}Sm labelled the same peptide via the bifunctional chelating agent DOTA, resulting in a stable complex, as is expected. ^{188}Re lanreotide was evaluated *in vitro* and *in vivo*, and will continue to be evaluated with other cancer cell lines, while the same assays are currently in progress for the DOTA-lanreotide complex.

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PREPARATION, QUALITY CONTROL AND ANIMAL TESTING OF Re-188 LABELLED COMPOUNDS

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Abstract

Rhenium-188 is an attractive therapeutic radioisotope obtained by elution from tungsten 188/rhenium 188-generator system. We used this eluate to label HEDP and lanreotide to prepare radiopharmaceuticals for experimental use. The results of our studies showed that using methods worked out under the framework of the CRP allowed us to prepare ^{188}Re -HEDP and ^{188}Re -lanreotide with high yield and favourable biodistribution in animals.

In animal studies ^{188}Re -HEDP was successfully used for localizing osteosarcoma in dog.

1. INTRODUCTION

The fundamentals of successful radionuclide therapy is sufficient uptake and prolonged retention of radiopharmaceuticals in the target tumouric tissues. The pharmacokinetic properties of therapeutic radiopharmaceuticals can be assessed with a low dose tracer study before the single or multiple application of the therapeutic dose.

Radiation is delivered selectively by appropriate radiopharmaceuticals taking the advantage of short-range β particles or ultra short-path-length of α particles or Anger electron emitting radionuclides.

The advantages and limitations of radionuclide therapy are listed in Table I.

TABLE I. ADVANTAGES AND LIMITATIONS OF RADIONUCLIDE THERAPY

Advantages	Limitations
Specificity, radiation dose limited to target	Isolation of patients
Efficacy and low toxicity	Storage of radioactive waste
Systemic or locaregional application	Availability, as licenced therapeutical radiopharmaceuticals and isolation beds in hospital.
Excellent palliation	Need for dosimetry calculation
Limited long-term consequences	Multidisciplinary approach required
Pre-assessment of uptake and retention (in repeated tracer study)	Mechanism poorly understood
Multiple treatments possible	High cost of some newer forms
New indications emerging	

The use of beta-emitting radioisotopes labelled compounds for the treatment of solid tumours and palliative treatment of skeletal bone pain, especially for treatment of multiple lesions has developed as an important alternative modality to more traditional therapeutic regimens.

In a recent programme we studied and used the tungsten-188/rhenium-188 generator system, which provides high levels of carrier-free ^{188}Re for the preparation of various therapeutical agents.

The long useful shelf-life (up to one year) and high activity (500-800 mCi) of these generators and the high daily yield of perrhenate-188 and appropriate radionuclidic properties (2,1 MeV β ; 155 KeV γ) make this isotope an attractive candidate for wiely use for bone palliation and other therapeutic application. During our experimental work rhenium-188 labelled hydroxyethylidene-diphosphonate (HEDP), ^{188}Re -dimercaptosuccinic acid (DMSA) and ^{188}Re labelled somatostatin peptide analogues (lanreotide and DOTA-lanreotide) were prepared and evaluated in radiochemical purity and biological study. For tracer study Ga-67 labelled DOTA-lanreotide and for therapeutic purpose ^{131}I , labelled-lanreotide were also prepared in limited quantity.

2. MATERIAL AND METHODS

All chemicals and reagents were of analytical grade. Perrhenic acid (65-70% in water, 99,9% purity) and gentrisic acid (99%) were purchased from Aldrich Chemical Company. The HEDP (>97%) was obtained from Fluka AG: Lanreotide and DOTA lanreotide was supplied by pi-CHEM (Austria). Alumine-based Tungsten-188/Rhenium-188 generator was purchased from Oak Ridge National Laboratory (USA). Generator set-up, elution and daily quality control were done according to the manufacturer's instructions. At the end of each elution the generator was purged with nitrogen gas to maintain the column in a dry state between elutions.

Ga-67 chloride solution was obtained from MDS Nordion Inc. (Canada).

3. PREPARATION OF ^{188}Re -HEDP

At aseptic condition 166 mg of HDP and 60 mg of gentisic acid were dissolved in 10 ml physiological saline. The solution was stirred during the addition of 77 mg of stannous chloride dihydrate to provide a clear colorless solution (pH~1). One ml aliquots of this solution were then dispensed using a steril syringe by elution through a steril 0.22 μm Millipore filter into steril dose vials. The vials were then filled with sterile nitrogen gas to yield on inert atmosphere. Carrier perrhenic solution was prepared (20 μl perrhenic acid solution was mixed with 2ml of sterile saline, purged with nitrogen, placed in dark and coole storage and 20 μl volume from this solution was added to eluate). To predispedned vial 1-2 ml of ^{188}Re perrhenate, 20 μl of carrier rhenium was added and the mixture was then heated at 96-100°C for 30 min. After cooling 2 ml of sodium acetate buffer added. Quality control consisted of TLC analyses of the neutralized solution using Gelman ITLC-SG plates developed in saline and acetone.

Preparation of ¹⁸⁸Re labelled-lanreotide

Reducing solution was prepared by using stannous chloride dyhydrate (1,6 mg) ascorbic acid (2,46 mg) citric acid (16 mg) and ¹⁸⁸Re perrhenate in saline (200-700 MBq) was added. After stirring at room temperature for 2 h, 0,4 ml sample was added to 0,1 ml of lanreotide (100 µg, in 0,1µ acetate puffer) and mixture was heated at 90-100°C for 60 min. After cooling neutralizing stabilizing puffer solution was added. Radiochemical purity was determined as indicated above. HT-29 (human colon adenocarcinoma) cells were evaluated to bind ¹⁸⁸Re-lanreotide. During assay 5,10 and 50 ng/ml labelled lanreotide was incubated with 1,5 and 10x10⁶ cell and incubated at 37°C for 1 h.

Biodistribution studies

¹⁸⁸Re-HEDP biodistribution studies were performed in nude mice bearing HT-29 human colon adenocarcinoma xenograft, adult Beagle dog and dog with osteosarcoma. Images were acquired with a Nucline X-Ring (Mediso Ltd., Hungary) digital gamma camera (HEGP collimator, 75KeV and 156 KeV energy with 10% window width) for pharmacokinetic study 20 MBq/bodyweight kg was injected intravenously.

Experimental results

The formulation we used for Re-188 labelling of HEDP yielded a labelling efficiency of 90% to 98%. If ¹⁸⁸Re-HEDP solution was stored at room temperature it slow radiochemical decomposition (1-2%/hrs) was shown. The biodistribution of ¹⁸⁸Re-HEDP when injected intravenously to dogs showed significant bone uptake and excretion throughout the kidneys. Distribution of compounds when injected into dog with osteosarcoma showed skeletal distribution and very significant uptake in the primer lesion. Lesion delineation and uptake was compared to scan taken by using ⁹⁹Tc^m-MDP. Comparison of the distribution of two compounds are seen in Fig. 1. To know the biodistribution and pharmacokinetic of ¹⁸⁸Re perrhenate in separated experiment 20 MBq/Kg eluate was injected. Perrhenate accumulated in thyroid, salivary glands and in the gastric mucosa as seen Fig. 2.

The labelling efficiency of lanreotide with ¹⁸⁸Re ranged between 85-95% before store aliquots were mixed 1:1 with pro injection Human Serum Albumin 20% solution and ascorbic acid for injection. The results of the binding assay are shown in Table II.

Biodistribution of ¹⁸⁸Re lanreotide in nude mice bearing HT-29 human tumour xenograft is shown in Fig. 3. Scans up to 3 h showing fast biliary and renal extraction with very moderate uptake in tumour.

TABLE II. ¹⁸⁸RE -LANREOTIDE BINDING TO HT-29 CELLS

No. of HT-29 cells	Concentration of labelled lanreotide		
	5 ng/ml	10 ng/ml	50 ng/ml
10×10 ⁶	44.6%	34.9%	32.6%
5×10 ⁶	32.6%	27.3%	22.35
1×10 ⁶	25,6%	26.4%	19.2%

4. DISCUSSION

Key requirement for routine preparation of ^{188}Re labelled radiopharmaceuticals for clinical use is the availability of simple and efficient method for concentration of generator eluate. Because of the daily clinical need for developing cost-effective alternatives for the treatment of bone pain in our work we have concentrate the preparation work of ^{188}Re -HEDP and ^{188}Re (V)-DMSA which also was prepared in limited experiment. System used for concentration of the rhenium generator saline eluant was not effective and therefore we had to concentrate eluate by heating.

^{188}Re -HEDP prepared in our laboratory showed high labelling yield and localized in skeletal tissue as well as in osteosarcoma lesion. The lesion pharmacokinetic was favourable and allowed us to calculate absorbed dose. Preliminary result of labelling lanreotide with rhenium was promising. Working out eluate concentration method and optimal conditions for labelling were still needed.

By using experiences gained during the CRP we successfully labelled HEDP and lanreotide, which are going to be the basic therapeutical radiopharmaceuticals in the future. The chemical research work done will allow us to prepare kit formulation for ^{188}Re isotope labelling also.



$^{99}\text{Tc}^m\text{-MDP}$



Re-188-HEDP

FIG. 1. Distribution and tumour localization in dog with osteosarcoma.

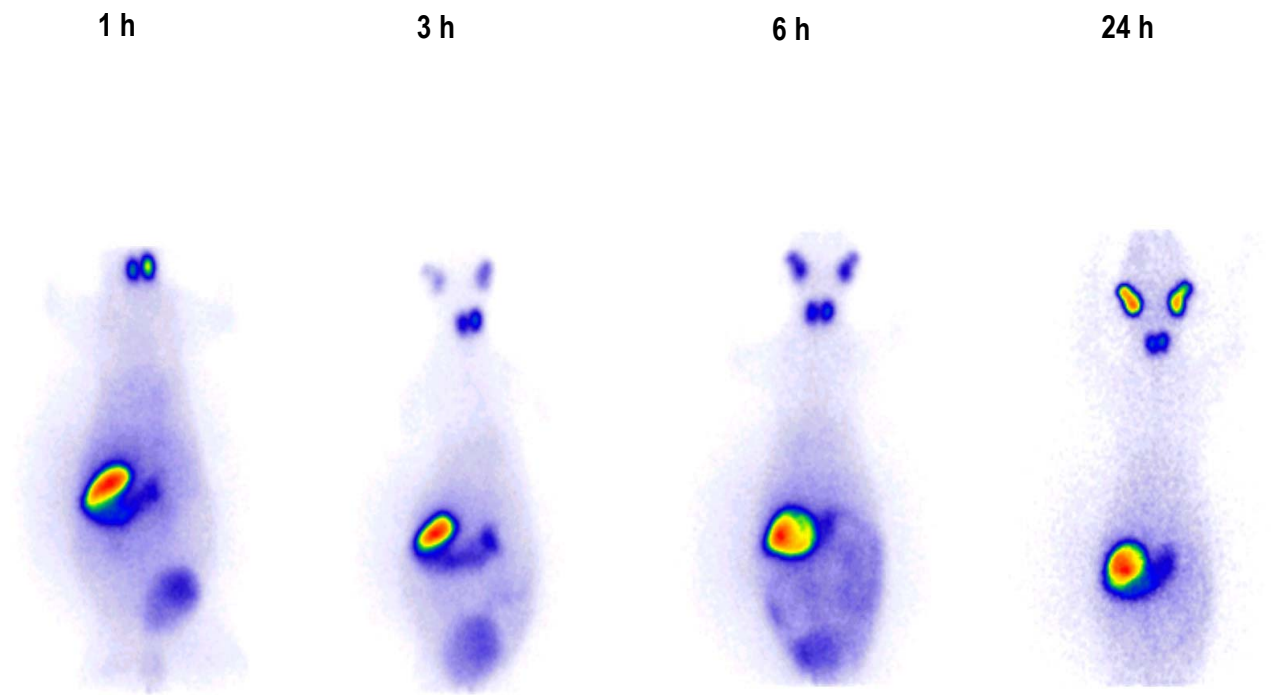


FIG. 2. ^{188}Re perrhenate biodistribution in healthy Beagle dog static images.

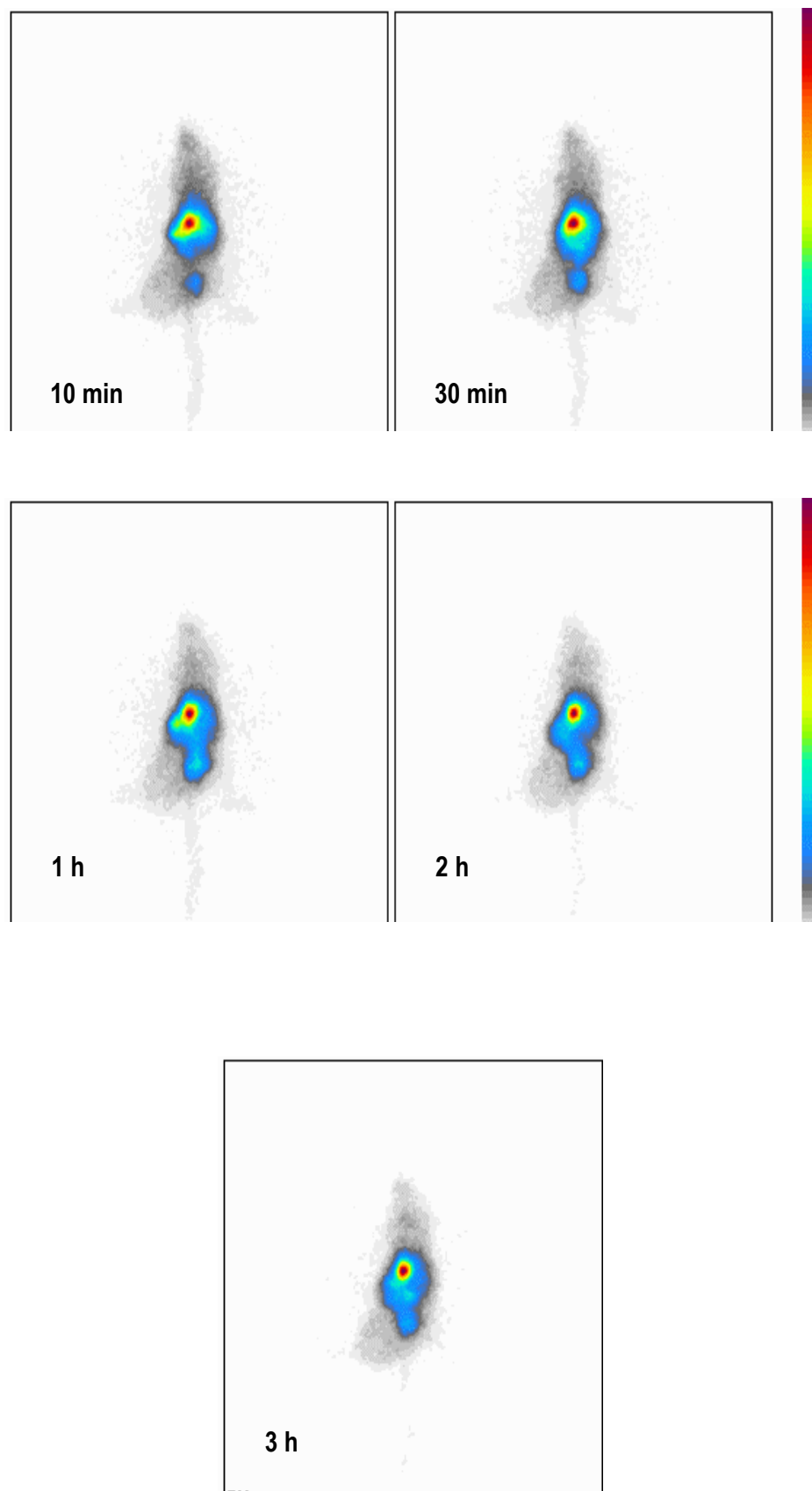


FIG. 3. Biodistribution of ^{188}Re -lanreotide in nude mice bearing HT-29 human tumour xenograft.

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DEVELOPMENT OF RADIOACTIVELY LABELLED CANCER SEEKING BIOMOLECULES FOR TARGETED THERAPY

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Abstract

Several radioisotopes such as ^{90}Y , ^{186}Re , ^{166}Ho , ^{177}Lu , were produced and processed in our laboratory for evaluation as potential isotopes for therapy. ^{90}Y was sourced from ^{90}Sr in the form of a generator based on a supported liquid membrane separating technique as well as by solvent extraction /adsorption procedure. The other isotopes were obtained by irradiation of the natural/enriched targets. Among the various isotopes studies for possible therapeutic use, ^{177}Lu was identified as an ideal candidate for labelling of peptides owing to its high specific activity. Bifunctional chelating agents for complexing with these isotopes were synthesized and characterized. Radiolabelling procedures were standardized to obtain maximum complexation. Standard quality control techniques such as PC, TLC, HPLC have been utilized to characterize the radiolabelled species. Lanreotide, a somatostatin analogue was coupled to DOTA in a three-step procedure and purified by preparative TLC. The radiolabelling of the peptide conjugate with ^{90}Y and its subsequent purification with Sep-Pak column was optimized. The direct labelling of lanreotide with ^{186}Re as well as ^{125}I were also accomplished. The *in vivo* bioefficacy studies of the radioiodinated lanreotide as well as ^{90}Y -DOTA-lanreotide were carried out in mice bearing melanoma. *In vitro* cell binding studies of ^{90}Y -DOTA-lanreotide with A431 cell lines were also carried out.

1. INTRODUCTION

Somatostatin receptors are known to be expressed by most of the neuroendocrine tumours and therefore radiolabelled somatostatin or its analogues are expected to exhibit binding affinity towards these tumours. Lanreotide, an octapeptide, has been identified as a somatostatin analogue bearing affinity to most of the sub-types of human somatostatin receptors. In connection with our efforts on the "Development of radioactively labelled cancer seeking biomolecules for targeted therapy" we have explored the potential of several therapeutic radioisotopes. Among these, two of the isotopes viz ^{188}Re and ^{90}Y were available directly from the generator whereas the others such as ^{186}Re , ^{166}Ho , ^{177}Lu were obtained from the irradiation of natural or enriched targets and subsequent radiochemical processing of the irradiated targets in our laboratory. ^{90}Y has been reported to be a suitable therapeutic radionuclide for labelling of peptides through a BFCA. In the present study, the radiolabelling yields of ^{90}Y with DOTA-lanreotide under optimized conditions were found to be >70%. However, due to the logistic considerations, ^{177}Lu ($t_{1/2}$ 6.7d, β_{max} 0.5 MeV, γ 208 keV) was identified as an ideal therapeutic radioisotope. 10 μg of enriched Lu irradiated at a flux of 10^{14} n/cm²/sec for 28 d yield 112 mCi. This corresponds to a specific activity of 1.94×10^4 Ci/g. Therefore 2 μg of ^{177}Lu will yield a therapeutic dose of ~20 mCi. While several methods have been reported for the preparation of the radiolabelled peptide conjugates, the major thrust of the present work was to effect the radiolabelling via the use of a bifunctional chelating agent. In this respect, DOTA was found to be an ideal BFCA for coupling with lanreotide. The complexation yields of DOTA with ^{177}Lu were >99%. at a Lu concentration of 5 $\mu\text{g}/\text{mL}$ and 40 μg of DOTA for 3 h at room temperature.

2. MATERIALS

95% D₂EHPA was obtained from BDH (Poole, England). Dowex-50×12, 200-400 mesh was from Sigma Chemical Company, USA, dodecane from Transware Chemicals, Handelsgesellschaft, Hamburg, Germany. All other reagents used were obtained from E. Merck

(Mumbai, India) or BDH (Poole, England) and were of analytical quality. PTFE membranes with pore size 0.45 μm and thickness 160 μm were purchased from M/s. Sartorius, Germany. Rhenium metal for irradiation (Spectroscopic grade, >99.99% pure, natural abundances of Re-185 and Re-187 are 37.07 and 62.93%, respectively) was obtained from Johnson Matthey Company. Enriched lutetium oxide (60.7% ^{176}Lu) was a gift from the IAEA and holmium oxide (Spectroscopic grade >99.99% pure) was from American Potash Inc. All other enriched targets were obtained from Isoflex, USA.

The chemicals used in the synthesis of the BFCAs (DOTA, DAHPES) and the peptide conjugates were obtained from Aldrich Chemical Co., MO, USA and Fluka Chemical Company, USA. All the inorganic salts were either from M/s. Sarabhai M. Chemicals or M/s. S.D. Fine Chemicals, India. The solvents were from M/s. Merck (India). Lanreotide was obtained as a gift from the IAEA.

Na^{125}I was obtained from Du PONT, Canada, chloramine T from Sigma Chemical Co, USA, HEDP from BRIT, Navi Mumbai, India, and SepPak C18 cartridge from WATERS, India.

$\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ was obtained from Sigma Chemical Company. Flexible silica gel plates (7.5 x 2.5 cm, coating thickness, 0.25 mm) were from J.T. Baker Chemical Company and Whatman 3 chromatography was used for paper chromatography and electrophoresis. 2-ethylhexyl-2-ethylhexylphosphonic acid (KSM-17) was synthesized locally according to a reported method. All reagents were made in metal ion free triple distilled water. All radioactive tracers used ^{90}Y , $^{85+89}\text{Sr}$, ^{90}Sr - ^{90}Y were available at the Radiopharmaceuticals Division of this Centre. Borosilicate glass column of dimension 15 cm x 0.4 cm (i.d), provided with a sintered disc at the bottom suitable to hold 2-5 ml of ion exchanger (Dowex 50 x 12, 200-400 mesh) were used.

^{90}Sr - ^{90}Y generator used in these studies was developed by the Process Development Division of Bhabha Atomic Research Centre. $^{90}\text{SrCl}_2$ was obtained from the reprocessed fuel effluents in one of our laboratories. ^{90}Y was assayed either by using a NaI(Tl) solid scintillation counter (Star Electricals, Bombay, India) to measure the Bremsstrahlung radiation or by using a liquid scintillation counter (Model - Tricarb 2100TR - Packard Instrument Co., USA). All radioactive measurements were made using a NaI(Tl) counter.

^1H -NMR spectra of the ligands were recorded using a Varian VXR 300S spectrometer, using CDCl_3 as the solvent and tetramethyl silane (TMS) as the internal reference. ^1H -NMR spectra of the peptide and their coupled derivative with BFCA were recorded in a Varian Unity Plus 600 MHz FT-NMR spectrometer operating at a ^1H resonance frequency of 600 MHz. These samples were prepared by dissolving 3 mg of the respective peptides in 540 μL H_2O and 160 μL of D_2O . FT-IR spectra were recorded using KBr pellets on Jasco FT/IR-420 spectrometer. Elemental analysis was carried out in Elemental Analyser EA 1110. UV spectra were recorded in a JASCO V-530 spectrophotometer as methanolic solutions. Mass spectra were recorded on Shimadzu QP 1000 spectrometer operating at 70 eV using direct insertion probe.

Radionuclidic purity of $^{186/188}\text{Re}$, ^{166}Ho , ^{177}Lu , were ascertained from the respective γ ray spectrum of an appropriately diluted sample using a HPGe detector coupled to a 4 K MCA system. ^{152}Eu standard source obtained from Amersham International was used for Efficiency vs. energy calibration of the detector.

Cell lines A 431, melanoma, were obtained from CRI, Mumbai, India and propagated in the Microbiology laboratory of the Division.

3. METHODS

3.1. Production and processing of the radioisotopes

^{90}Y was prepared by two independent methods, which are described below.

3.1.1. $^{90}\text{Sr}/^{90}\text{Y}$ generator

The $^{90}\text{Sr}/^{90}\text{Y}$ generator has been developed at the Process Development Division, BARC. The generator is based on supported liquid membrane technique. The generator consists of a glass cell comprising of two compartments, namely feed and receiver compartments, of capacity 5 mL each, separated by a Polytetrafluoroethylene membrane impregnated with 2-ethylhexyl-2-ethylhexyl phosphonic acid (KSM-17). High level waste depleted of actinides-lanthanides and ^{137}Cs was in 0.5 M HNO_3 . A suitable aliquot of this solution (upto 100 mCi, 3.7 GBq) was diluted to 5 mL, adjusted to pH 1-2 and used as the feed. 5 mL of 1 M HCl/HNO_3 was used in the receiver compartment. Both the feed and receiver compartments were constantly stirred with small teflon coated magnetic stirrer to enable quick and effective transport of ^{90}Y across the membrane. After 4 h, the solution containing yttrium chloride/nitrate was drawn from the receiving compartment and used.

Quality control of ^{90}Y

As ^{90}Sr is a bone seeker, the limit set for its level in ^{90}Y preparations to be used in humans is $<2 \mu\text{Ci}$. In order to ascertain the movement of Sr^{+2} across the membrane from the feed to the receiver compartment, a small aliquot of $^{85/89}\text{Sr}$ chloride was added to the feed compartment along with the ^{90}Sr chloride. Gamma ray spectrum of the ^{90}Y chloride solution in the receiver compartment was taken. Breakthrough of $^{85/89}\text{Sr}$ into the receiver compartment was checked by gamma ray spectrometry. The feed spiked with $^{85/89}\text{Sr}^{+2}$ did not show any breakthrough of Sr into the receiver compartment at all up to 72 h.

The transport of Sr^{+2} ions across the membrane was also ascertained by addition of carrier strontium at a concentration of 5 mg/ml in the feed compartment. Samples from the receiver compartment were drawn at regular intervals and the Sr^{+2} concentration was determined by ICP-AES method. Even after 12 h of operation of generator, only 0.0008% of Sr^{+2} was estimated to have been transported. Since the operation of the generator is expected to be 4-6 h with 1 M acid, the above breakthrough would be far below the accepted limit.

Chromatographic methods for the separation of ^{90}Sr and ^{90}Y were also carried out. In paper electrophoresis, (30 cm Whatman no. 1 paper, 0.03 M NaCl and 0.15 g/L sodium citrate, 500 V, 2 h) the $^{85/89/90}\text{Sr}^{+2}$ moved towards the cathode while $^{90}\text{Y}^{+3}$ moiety moved towards anode as depicted in Fig. 1. Both Sr and Y were used in the form of acetate. In paper chromatography using saline as the mobile phase, $^{90/85/89}\text{Sr}$ moved with the solvent front while ^{90}Y stayed at the point of application when both Sr^{+2} and Y^{+3} were in the form of acetate (Fig. 2).

As a regular practice, an aliquot of ^{90}Y was taken and the decay of ^{90}Y was followed using liquid scintillation counting. The ^{90}Y samples counted after passage of 30 days did not show any activity significantly different from the background. The measured activity matched

very well with the estimates as expected from the 64.1 h half life of ^{90}Y . Consistent results that matched with the calculated values assured the high quality of ^{90}Y obtained and the absence of ^{90}Sr breakthrough. At present, this generator has been scaled up to 100 mCi of ^{90}Sr to give ~ 70 mCi of ^{90}Y .

3.1.2. Solvent extraction/absorption of ^{90}Y from ^{90}Sr

The above generator developed could be used in hospitals for elution of ^{90}Y . A method has been developed for the separation of ^{90}Y as yttrium acetate from ^{90}Sr - ^{90}Y mixture, which can be directly used to prepare the radioconjugate without further chemical manipulations. This separation is based on an initial solvent extraction of ^{90}Y from dilute nitric acid solution of the ^{90}Sr - ^{90}Y mixture using D_2EHPA /dodecane. Further purification of ^{90}Y have been carried out using ion exchange technique based on the absorption of the product on a column containing Dowex-50 x12 and selective elution of ^{90}Y in 0.6M sodium acetate buffer solution at pH 5.57. Based on the experimental data, a process has been demonstrated in which the recovery of ^{90}Y obtained is higher than 75%. The radionuclidic purity of the ^{90}Y obtained has been proved to be satisfactory for medical application. The details of the procedure are given below.

^{90}Sr obtained from the stock solution is $\text{Sr}(\text{NO}_3)_2$ in 2M HNO_3 . 20 mCi of this solution was taken in a 50 mL beaker and 1 mCi of $^{85+89}\text{Sr}$ as $\text{Sr}(\text{NO}_3)_2$ was added to it. The resulting solution was evaporated to dryness, taken up in 10 mL of 0.1M HNO_3 and transferred to a 50 mL separating funnel containing 10 mL of 1.0M D_2EHPA in dodecane. The phases were mixed by agitating for 10 min and allowed to separate. The aqueous phase was separated and kept for subsequent extraction. The organic phase containing ^{90}Y was washed three times with 20 ml of 0.1M HNO_3 for removing any residual ^{90}Sr in the organic phase. ^{90}Y from the organic phase was back extracted by shaking with 10 mL of 5M HNO_3 for 5 min. The strip solution was evaporated to dryness, cooled and taken up in 0.1M HNO_3 which is the feed for the removal of trace amount of any residual ^{90}Sr by ion exchange chromatography.

Cation exchange resin Dowex-50 \times 12 (200-400 mesh) in hydrogen form was prepared and conditioned with 0.1M HNO_3 . The ^{90}Y rich strip solution in 0.1M HNO_3 was then passed through the ion exchange column to remove residual ^{90}Sr if present, phosphate and other anionic impurities generated (due to radiolysis of D_2EHPA and dodecane) during the previous solvent extraction step. The column was then washed with DDW till the pH of the effluent becomes neutral. The absorbed ^{90}Y was then eluted with 0.6M acetate buffer at pH 5.57. Fig. 3 depicts the flow sheet diagram for the separation of ^{90}Y from ^{90}Sr in a highly radiochemically pure form.

In order to standardize the optimum pH at which ^{90}Y could be desorbed from the resin phase, the elution characteristic of ^{90}Y on Dowex- 50x12 (200-400 mesh) was studied using 0.6 M acetate buffer at different pH and the summary of the results are given in Table I.

TABLE I. ELUTION OF ^{90}Y FROM DOWEX-50

pH	% elution of ^{90}Y
5.23	13
5.57	78
5.9	18
30% methanol+0.6 M NaAc	11

Eluent: 0.6 M acetate buffer at different pH.

The breakthrough curve of Sr^{2+} and Y^{3+} on Dowex – 50×12 at 0.1 M HNO_3 was studied. The breakthrough capacity and saturation capacity of Sr^{2+} were found to be 4.8 and 7.3 meq/g, and that of Y^{3+} , 5.1 and 9.2 meq /g, respectively. Therefore about 4.5 meq of Sr/g of the exchanger can be loaded without any breakthrough being observed. Since the amount of ^{90}Sr present in the feed after the solvent extraction step is extremely low, total breakthrough of the column will never be reached and no extra precaution is needed during the loading of the activity in the column.

Following the procedure outlined, a number of batches of ^{90}Y were processed. At the beginning of the experiment ^{90}Sr - ^{90}Y was spiked with ^{85}Sr to follow the path of ^{90}Sr in the process. ^{90}Y obtained by this method was checked in a HPGe detector coupled to a 4K analyser which does not show any peak corresponding to ^{85}Sr spiked. Further, the measurement of half-life of the separated species (i.e. ^{90}Y) was also carried out which followed the $T_{1/2}$ of ^{90}Y (64.1 h). The solvent extraction followed by cation exchange chromatography can be used as a good technique for the preparation of ^{90}Y for therapeutic applications in a radiopharmaceutical company.

$^{186/188}\text{Re}$ production

5 mg of natural rhenium was irradiated in the Dhruva reactor for seven days at a flux of 3×10^{13} n/cm²/sec and dissolved in 5mL of HNO_3 . Radionuclidic estimation of $^{186/188}\text{Re}$ was estimated by gamma ray spectroscopy (HPGe detector coupled to a 4 K analyser).

Radiochemical processing

3 mL of rhenium solution (3mg) was evaporated to dryness and treated with 1 ml of 25% ammonia solution. Excess ammonia was removed by heating. The residue was dissolved in 5 mL of 5 M sodium hydroxide. The rhenium was extracted into 5 mL of methyl ethyl ketone. Extraction was repeated with an equal volume of MEK and both the fractions were pooled together. MEK was removed by gentle heating and the residue was dissolved in 5 mL of normal saline. Radiochemical purity was determined by thin layer chromatography using acetone as solvent. An aliquot of the sample (37-74 KBq) was counted to estimate the percentage of ^{188}Re and ^{186}Re in the samples besides any other radionuclidic impurities, using 155 and 137 keV γ photopeaks for ^{188}Re and ^{186}Re , respectively.

^{188}Re

Two $^{188}\text{W}/^{188}\text{Re}$ generators, 100 mCi and 46 mCi, were obtained from MAP Medical Technologies, Finland. The generators were eluted 25 times with an average elution yield of ~60%. The ^{188}Re eluted was used for the complexation of lanreotide, DMSA, tetraphosphonates, EC and DAHPES. The radiochemical purity of the $^{188}\text{ReO}_4^-$ was determined by thin layer chromatography in acetone as solvent ($R_f = 0.9-1.0$). The RC-purity of the sodium perrhenate solution thus obtained was >99%. Radionuclidic purity of the eluted ^{188}Re was estimated by gamma ray spectrometry.

^{166}Ho

6 mg of natural Ho_2O_3 powder (100% ^{165}Ho) was weighed and sealed in a quartz ampoule and irradiated at a thermal neutron flux of 3×10^{13} n/cm²/s for seven days and cooled for 6 h. Irradiated Ho_2O_3 powder was dissolved in 5 mL of 0.1 M HCl by gentle warming.

The resultant solution was evaporated to near dryness and reconstituted in 10 mL of double distilled water.

Radioactivity assay of high activity samples was carried out by measuring the ionization current obtained when an aliquot of the batch was placed inside a precalibrated well-type ion chamber. 500-600 mCi of ^{166}Ho activity was recovered after radiochemical processing corresponding to a specific activity of 100-120 Ci/g. By irradiating natural Ho at a neutron flux of $>10^{14}$ to saturation activity one could get a specific activity of >1000 Ci/g.

The gamma ray spectrum of ^{166}Ho did not show the presence of any radionuclidic impurity. The major gamma peak observed was at 81 keV (6%), photopeak of ^{166}Ho . Minor peaks of ^{166}Ho at 1379 keV (0.9%), 1581 keV (0.18%) and 1662 keV (0.12%) are also notable.

^{177}Lu

1.2 mg of enriched Lu_2O_3 powder (60.7% ^{176}Lu) was dissolved in 1M HCl on gentle warming. The resulting solution was evaporated to near dryness and reconstituted in 1.2 mL of double distilled water. 10 μL of the solution (10 μg of Lu_2O_3 was taken in a quartz ampoule and carefully evaporated to dryness. The ampoule was subsequently flame sealed and irradiated in Dhruva reactor, BARC, India, at a flux of 3×10^{13} n/cm²/s for a period of seven days.

The irradiated ampoule was cut inside a lead-shielded plant and the content was dissolved in 1 M HCl by gentle warming. The resultant solution was evaporated to near dryness and reconstituted in 1 mL of double distilled water.

Radioactive concentration was measured using an HPGe detector linked to a multi channel analyser following efficiency calibration with a standard ^{152}Eu source. Radioactivity assay was also carried out by measuring the ionization current obtained when an aliquot of the batch was placed inside a pre-calibrated well-type ion chamber.

Around 20-22 mCi of ^{177}Lu activity was obtained at 6 h post EOB from 10 μg of enriched Lu_2O_3 powder irradiated for 7 days, corresponding to a specific activity of 2000-2200 Ci/g. Theoretical calculations show that 7 day irradiation at 1.8×10^{13} n/cm²/s flux would yield ~ 1800 Ci/g of ^{177}Lu activity using enriched Lu_2O_3 target. On the other hand, by using natural Lu target the specific activity of ^{177}Lu will be ~ 100 Ci/g for seven day irradiation at the same flux. The radionuclidic purity of ^{177}Lu was $\sim 100\%$ as obtained by analysing the γ ray spectrum. It is worthwhile to note that there is a possibility of the formation of $^{177\text{m}}\text{Lu}$ ($T_{1/2} = 160.5$ d) on thermal neutron bombardment of Lu_2O_3 target. However, the γ ray spectrum did not show any significant peak (at 128 keV, 153 keV, 228 keV, 378 keV, 414 keV, 418 keV) corresponding to $^{177\text{m}}\text{Lu}$. This is expected as the radioactivity due to $^{177\text{m}}\text{Lu}$ produced will not be insignificant on 7 d irradiation owing to its long half-life and comparatively low cross section ($\sigma = 7$ barns) and long $t_{1/2}$ (161 days) for its formation.

3.2. Comparative evaluation of the radioisotopes

A comparative evaluation of the radioisotopes in terms of their theoretical, obtainable specific activity is given in Table II. In case of ^{186}Re , the presence of the other radionuclide ^{188}Re [^{187}Re (n, γ) ^{188}Re , $t_{1/2} = 17$ h], is eliminated following a protocol of four days cooling

after irradiation. From the results it can be seen that ^{177}Lu , owing to its high cross-section, is one of the isotopes which can be prepared by neutron activation in a relatively high flux reactor at sufficiently high specific activity for peptide labelling studies. Lu is also reported to have very high equilibrium constant with some of the bifunctional chelating agents such as DOTA.

TABLE II. POSSIBLE RADIOISOTOPES FOR THERAPY

Isotope	Source	Specific activity (theoretical)
^{188}Re	$^{188}\text{W} / ^{188}\text{Re}$ generator	$9.80 \times 10^5 \text{ Ci / g}$
^{90}Y	$^{90}\text{Sr} / ^{90}\text{Y}$ generator	$5.44 \times 10^5 \text{ Ci / g}$
^{90}Y	$^{89}\text{Y}(\text{n},\gamma) ^{90}\text{Y}$; 100%, $\sigma = 1.28 \text{ b}$	30Ci /g
^{186}Re	$^{185}\text{Re}(\text{n},\gamma) ^{186}\text{Re}$; 37%, $\sigma = 106 \text{ b}$	345 Ci/g
^{186}Re	$^{185}\text{Re}(\text{n},\gamma) ^{186}\text{Re}$; 98% (<i>enriched</i>)	$>9.14 \times 10^2 \text{ Ci / g}$
^{166}Ho	$^{165}\text{Ho}(\text{n},\gamma) ^{166}\text{Ho}$; 100%, $\sigma = 66 \text{ b}$	$>6.50 \times 10^2 \text{ Ci / g}$
^{153}Sm	$^{152}\text{Sm}(\text{n},\gamma) ^{153}\text{Sm}$; 100%, $\sigma = 210 \text{ b}$	$>2.23 \times 10^3 \text{ Ci/g}$
^{177}Lu	$^{176}\text{Lu}(\text{n},\gamma) ^{177}\text{Lu}$; 2.6%, $\sigma = 2100 \text{ b}$	$>8.34 \times 10^2 \text{ Ci / g}$
^{177}Lu	$^{176}\text{Lu}(\text{n},\gamma) ^{177}\text{Lu}$; 60.7% (<i>enriched</i>)	$>1.94 \times 10^4 \text{ Ci / g}$

Irradiation at $>10^{14}$ neutron flux to saturation activity

Synthesis

Bifunctional chelating agents

DOTA (1,4,7,10-tetraazacyclododecane-N,N',N'',N''' tetraacetic acid) 1,4,7,10-Tetraazacyclododecane-N,N',N'',N'''tetraacetic acid (DOTA) was synthesized in four steps (Fig. 4). The synthetic route involved the use of inexpensive starting materials.

Step I: Triethylene tetramine (3.3g, 0.027 moles) was tosylated using p-toluene sulphonyl chloride (26.3 g, 0.14 moles) in acetonitrile (125 mL) in the presence of triethylamine (16 mL, 0.115 moles). The reaction involved a portion wise addition of p-toluene sulphonyl chloride to a magnetically stirred solution of triethylene tetramine and triethyl amine at room temperature under N_2 circulation for one hour. The stirring is continued for 5 h while monitoring the progress of the reaction on a silica gel TLC in methanol : chloroform : ammonium hydroxide (5:94.5:0.5 v/v/v) (R_f 0.8). The solvent is removed under vacuum to yield a white semisolid. The work up involves addition of DDW and extraction using 3 x 25 mL of chloroform, after saturation with brine. The pooled organic extracts were dried over sodium sulphate and dried under *vacuo* to give the tetratosylated product as a white solid (19.8g, 97%).

Step II: Cyclization of the tetratosylate was effected by condensation with 1,2-dibromoethane in dimethyl formamide as the solvent and K_2CO_3 as the base, to yield the tetratosylated cyclen. In a typical reaction, a magnetically stirred mixture of tetratosylated triethylene tetramine (10 g, 0.013 moles) and anhydrous K_2CO_3 (4.5g, 0.032 moles) in freshly distilled dry DMF (70 mL), under N_2 atmosphere, was stirred for 1.5 h when a white precipitate was observed. To this was added dropwise 2.4 g (0.013 moles) of 1,2-

dibromoethane in 30 mL of distilled and dry DMF. The progress of the reaction was monitored by silica gel TLC in methanol:chloroform:ammonium hydroxide (3:96.5:0.5 v/v/v) (R_f 0.6). The reaction was continued for 24 h following which DMF was removed by vacuum distillation. The work-up involved addition of DDW (50 mL) and extraction using 5×25 mL of chloroform, and subsequent removal of chloroform to give the product (8 g, 78%).

Step III: Following the cyclization reaction, detosylation is effected using Conc. H_2SO_4 to yield 1,4,7,10-tetrazacyclododecane (cyclen), the key synthon. 9.3 g (0.012 moles) of tetraosylated cyclen along with 35 mL of concentrated H_2SO_4 was heated to 110°C for 24–28 h. The reaction was monitored by TLC using chloroform: methanol: 3:97 (v/v) (R_f 0.2). The reaction mixture was allowed to cool at room temperature and the pH adjusted to ~12 under cooling (0–5°C) and extracted with 6×25 mL of dichloromethane after saturation with brine. The pooled organic extracts were washed with 3×25 mL brine and solvent removed to yield 0.69g (33%) of cyclen.

Step IV: A mixture of cyclen (0.24g, 0.0014 moles) and chloroacetic acid (0.6g, 0.006 moles), in 25 mL of DDW is stirred for 10 min following which a clear solution results. The pH of the solution is then adjusted to 9.5–10 by dropwise addition of 5N NaOH. The resultant solution is heated to 50–60°C. The progress of the reaction is indicated by decrease in the pH of the solution, which is maintained at approximately 10 after attaining room temperature. After 30 h of reaction, silica gel TLC using ammonium hydroxide:methanol (40:60 v/v) shows completion of the reaction (R_f 0.8). On attaining room temperature, the pH of the solution is adjusted to 2 using concentrated HCl. The reaction mixture is concentrated to dryness under vacuum to yield the crude product. The purification of DOTA was carried out by dissolving the solid in 0.1% TFA in water and loaded on the column. The first solvent used was 100 mL of 0.1% TFA in H_2O and 50 mL of 1% TFA in H_2O . The second solvent used was 100 mL of 0.1% TFA in acetonitrile and 50 mL of 1% TFA in acetonitrile. The solvent from the fractions collected were removed by vacuum distillation to give the pure product (500 mg).

In another procedure, cyclen 1,2-disulphate (2g, 0.005 moles) is converted to DOTA via a one step reaction involving the liberation of cyclen in alkaline medium and its subsequent conversion to DOTA with chloroacetic acid (2.3g, 0.023 moles) following the same procedure as above. After a similar work-up the yield of DOTA was 90%.

All the reaction conditions were standardized and the intermediates characterized with the help of TLC in suitable solvents. The intermediates and final products were characterized by FT-IR and high resolution 1H -NMR spectroscopy.

1H -NMR, D_2O , δ ppm) 2.44, 2.67 (16 H, broad singlets, $-CH_2$ of cyclen) 3.12 (8H, singlet, $-CH_2COOH$)

5-hydroxy-3,7-diazanonan-1,9-dithiol (DAHPEs)

To a stirred solution of 2-hydroxy-1,3-diaminopropane (4g, 0.044 moles) in 30 mL dry toluene was added to a mixture of ethylene sulphide (5.87g, 0.097 moles) in 20 mL of dry toluene under reflux and nitrogen flushing over a period of 3 h. The refluxing was continued for a period of 40 h, following which silica gel TLC using NH_4OH : CH_3OH (6:94 v/v) indicated the completion of the reaction (product R_f ~ 0.6). Toluene was removed under vacuum distillation when there was resulting white residue. The residue was washed with a mixture of methanol and dichloromethane (1:1) 5×10 mL. The pooled organic extracts were

concentrated to yield a viscous liquid, which subsequently solidified to a pale white solid (5.2g, 57%) on storage. The final product was characterized by FT-IR, high-resolution $^1\text{H-NMR}$ spectroscopy and mass spectroscopy.

IR (KBr, $\nu\text{ cm}^{-1}$): 3364(-NH), 2960 (-SH); $^1\text{H-NMR}$ (CD_3OD , δ ppm): 2.64-2.71 [2H dd - $\text{NHCH}_\text{A}\text{H}_\text{B}(\text{CHOH})$], 2.79-2.93 [6H m- $\text{NHCH}_\text{A}\text{H}_\text{B}(\text{CHOH})$] and - $\text{NHCH}_2\text{CH}_2\text{SH}$, 3.16 (4H t, $J = 5.4\text{ Hz}$, - CH_2SH), 3.65-3.80 (1H m, - CHOH); Mass Spectra (EI) m/z : 210(M^+)

DOTA-lanreotide (Mauritius)

The synthesis of Mauritius was carried out in a three-step reaction (Fig. 5) using commercially supplied lanreotide and locally synthesized DOTA.

Step I: The reaction in the first step involved the protection of the free amino residue of lysine in Lanreotide (LAN) with di-tert butyl dicarbonate (BOC-anhydride) to yield BOC-LAN. To a stirred solution of 5 mg lanreotide (0.0045mM) in 0.1mL dioxane, 0.05mL DDW, was added BOC-anhydride (2.2 equivalents, 2.1 mg), at 0°C . The pH of the solution was checked and found to be ~ 8 . To ensure alkalinity 0.01 mL 1M sodium hydroxide solution was added when pH was found to be 10. The resultant solution is stirred at room temperature for 30 min. Dioxane was removed under a slow stream of nitrogen when a white residue was observed.

Step II: In the second step, the peptide bond formation is effected via the formation of the N-hydroxy succinimide ester of DOTA in the presence of DCC as the condensing agent. The LANBOC-DOTA conjugate is formed at the β -naphthyl amino residue.

To the residue obtained in Step I, 1 mL DDW and 1 mL DMF were added to N-hydroxysuccinimide (5.1 mg), dicyclohexyl carbodiimide (7 mg) and DOTA (6 mg). The pH of the solution was maintained at ~ 9 by adding 0.02 mL of 1M NaOH. The reaction was kept stirred for 16 h at room temperature following which turbidity was observed. The solvent was removed under a slow stream of nitrogen gas. The progress of the reaction at this stage was checked by silica-gel TLC in $\text{NH}_4\text{OH} : \text{methanol}$ (40:60 v/v) (R_f -0.8).

Step III: The final step involved de-blocking of the BOC protected NH_2 group to yield the conjugated product LAN-DOTA. The deprotection was carried out by stirring the product obtained from Step II using trifluoroacetic acid (0.13 mL) in methylene chloride (1.5 mL) at room temperature for 30 min. The solvent was removed under nitrogen.

Purification of lanreotide-DOTA (LANDOTA)

Purification of the final product was effected on preparative TLC using NH_4OH : methanol (40:60 v/v) (R_f of LAN-DOTA-0.7, R_f of lanreotide-0.3). The desired zone was scrapped off and extracted in methanol to obtain the pure product.

Characterization

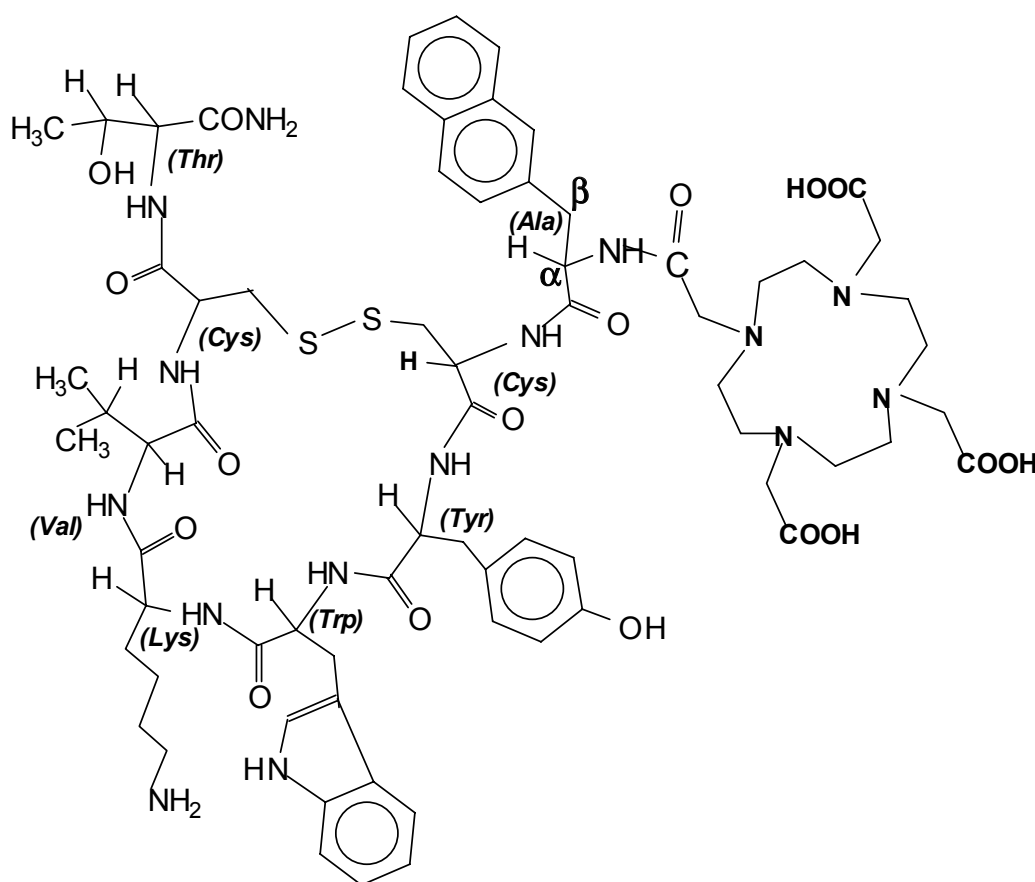
The UV spectra is given in Fig. 6. It is observed that the spectra of the synthesized sample of lanreotide coupled DOTA compared well with that of the authentic sample obtained from Pichem, Finland.

Samples for recording NMR were prepared by dissolving 3 mg of the respective peptides in 540 μL H_2O and 160 μL of D_2O . ^1H -NMR spectra was recorded in a Varian Unity Plus 600 MHz FT-NMR spectrometer operating at a ^1H resonance frequency of 600 MHz. Preliminary information towards sequence specific ^1H resonance assignments were attempted using standard methodology reported by Wuthrich.

The coupling of a carboxyl group in DOTA with the free amino group of the β -naphthyl alanine moiety is observable through the changes in the ^1H -NMR signals at the site of the alanine moiety (Table III).

TABLE III. CHANGES OBSERVED IN THE NMR SPECTRA OF LANREOTIDE AND LAN-DOTA

Changes observed at	DOTA	Lanreotide	Lanreotide-DOTA
$-\text{NH}_2$ - (Ala)	-	8.26 <i>d</i>	8.37 <i>d</i>
$-\text{NH}$ - (Cys)	-	8.37 <i>d</i>	8.47 <i>d</i>
$-\text{C}_{\alpha}\text{-H}$ (Ala)	-	4.4 <i>t</i>	5.0 <i>broad t</i>
$-\text{C}_{\beta}\text{-CH}_2\text{-naphthyl}$ (Ala)	-	3.938 <i>t</i>	4.08-4.17 <i>m</i>
$-\text{CH}_2\text{COOH}$ (DOTA)	3.12 <i>s</i>	-	3.208 <i>broad s</i>
LanNHCO- CH_2DOTA	3.12 <i>s</i>	-	4.012 <i>t</i>
Ring $\text{CH}_2\text{-DOTA}$	2.44-2.67 <i>broad s</i>	-	3.675 -3.852 <i>m</i>



LANREOTIDE-DOTA

3.3. Radiolabelling BFCAs/ligands and characterization of the complexes

⁹⁰Y-DOTA

The pH of ⁹⁰YCl₃ was adjusted to ~6.0 using 1M NaOH. To ~500 μL (~37 MBq) of pH adjusted ⁹⁰YCl₃ added 10 μL of DOTA (1mg/ml in 0.4 M sodium acetate buffer). 100 μL of 0.4 M sodium acetate buffer is added and incubated for 30 min at 60°C. The reaction was also carried out at room temperature to study the reaction kinetics with increase in temperature. Carrier-added studies were also done using 0.2 μg of carrier ⁸⁹Y (equivalent to ~110 mCi ⁹⁰Y). Quality control was performed by paper chromatography using Pyridine:Ethanol:Water (1:2:4) as the developing system. In this solvent system, ⁹⁰Y acetate remained at the point of application (R_f=0) while ⁹⁰Y-DOTA moved along the solvent front (R_f=0.9-1.0).

Radiolabelling yields >90% could be achieved with 10 μg of DOTA using trace levels of ⁹⁰Y (37 MBq). However, on addition of carrier 0.2 μg (equivalent to 110 mCi of ⁹⁰Y), >95% complexation could be achieved only with 100 μg of DOTA. The time required for complexation was found to be temperature dependant. It was observed that the reaction was slow at room temperature. But when the reaction was carried out at 60°C, the time required for complexation was drastically reduced. At 0.2 μg of carrier ⁸⁹Y (equivalent to 110 mCi of ⁹⁰Y) and 100 μg of DOTA, the complexation was complete within 15 min at 60°C (Fig. 7).

¹⁷⁷Lu-DOTA

Radiolabelling of DOTA with ¹⁷⁷Lu was carried out by dissolving the ligand in 0.5 M NaHCO₃ buffer (pH 9) and adding ¹⁷⁷LuCl₃ solution into it. The reaction mixture was incubated at room temperature after adjusting the volume to 1 mL by adding normal saline. Reaction pH was maintained at 8.5 to 9 (pH of bicarbonate buffer).

Complexation yield was ascertained by paper chromatography in 0.9% saline as well as paper electrophoresis technique. In paper chromatography using 0.9% saline as the solvent, it was observed that ¹⁷⁷Lu-DOTA complex moved towards the solvent front (R_f=1.0), while uncomplexed ¹⁷⁷Lu under identical conditions remained at the point of spotting (R_f=0).

In order to obtain maximum complexation yield using minimum possible amount of DOTA, several experiments were carried out by varying – such as ligand concentration (10-50 μg/mL), ¹⁷⁷Lu concentration (2-20 μg/mL) and reaction time. Results are shown on Table IV.

Paper electrophoresis was carried out using 0.025 M phosphate buffer (pH 7.4) as electrolyte and applying a potential gradient of 10 V/cm for 1 h. It was observed that ¹⁷⁷Lu-DOTA complex moved towards the anode indicating that the complex is negatively charged. On the other hand, ¹⁷⁷LuCl₃ did not show any movement from the point of application under identical conditions.

Complexation yield >90% was achieved using 30 μg/mL of DOTA and 5 μg/mL of ¹⁷⁷Lu at pH ~9. Complex was found to retain its stability up to 7 days at room temperature.

TABLE IV. OPTIMIZATION OF ^{177}Lu -DOTA

^{177}Lu	[DOTA] ($\mu\text{g/mL}$)	% Complexation		
		1 h at RT	3 h at RT	24 h at RT
2 $\mu\text{g/mL}$	10	7.21	14.23	17.07
	20	29.25	48.38	49.22
	30	44.53	79.08	70.90
	40	61.01	88.34	84.21
	50	89.66	-	92.21
	10	7.77	12.17	10.11
5 $\mu\text{g/mL}$	20	12.06	9.18	10.24
	30	76.05	91.57	90.49
	40	86.96	99.07	98.92
	50	90.69	99.10	99.22

 ^{166}Ho -DOTA

Complexation studies using ^{166}Ho and ^{177}Lu was carried out identically. Results of the complexation studies with ^{177}Lu are summarized in the Table 5.

TABLE V. COMPLEXATION YIELD OF ^{166}Ho WITH DIFFERENT CONCENTRATIONS OF DOTA

[DOTA] ($\mu\text{g/mL}$)	% Complexation		
	1 h at RT	3 h at RT	24 h at RT
50	49.37	59.68	62.90
70	80.60	97.05	97.43
80	97.07	98.21	97.88

^{166}Ho : 10 $\mu\text{g/mL}$, pH ~ 9, incubation at RT

3.4. Direct radiolabelling of lanreotide $^{186/188}\text{Re}$ -lanreotide

18 mg of hydroxyethylene diphosphonate (HEDP) was dissolved in 0.2 mL of 0.5 M bicarbonate buffer and the volume was made to 1 mL with saline. 20 μL of stannous chloride solution (2.1 mg in concentrated HCl) was added to the HEDP solution followed by addition of 20 μL (10 μg , 0.05 μM) of ReO_4^- . The reaction mixture was purged with nitrogen for 10 min. The pH of the reaction mixture was adjusted to 2 and was reacted with 250 μg (0.25 μM) of lanreotide in a boiling water bath for 90 min. The complex formed was characterized by TLC, paper chromatography and HPLC. TLC was carried out using acetone and saline as two different solvent systems, While paper chromatography was carried out in ethanol: ammonia: water (2: 1: 5).

HPLC was carried out using an analytical reverse phase column (Keytech, C 18, 250×4.6 mm). The mobile phase consisted of a gradient system, with solvent A corresponding to water with 0.1% trifluoroacetic acid and solvent B corresponding to acetonitrile with 0.1% trifluoroacetic acid. The mobile phase started with 90% A at time $t = 0$ followed by a linear gradient from 10%B to 90%B from 0 to 25 min. The flow rate of the mobile phase was 1 mL/min.

Lanreotide was labelled with ^{186}Re by ligand exchange method using HEDP as exchanging ligand. Lanreotide was reacted with ^{186}Re -HEDP in acidic medium at 100°C for 90 min. The labelling yield determined by chromatography technique was found to be 65%. Composition of radiochemical impurities such as reduced hydrolyzed ^{186}Re , unreacted ^{186}Re -HEDP and free $^{186}\text{ReO}_4^-$ associated with labelled peptide could be estimated by using combination of different chromatography techniques. In TLC acetone ^{186}Re -peptide, ^{186}Re -HEDP and reduced hydrolyzed ^{186}Re remained at the point of spotting while free perrhenate migrated with the solvent front giving the estimate of free perrhenate. In TLC saline reduced hydrolyzed rhenium and ^{186}Re -lanreotide remained at the point of spotting and ^{186}Re -HEDP migrated with the solvent front indicating the yield of ^{186}Re -HEDP associated with the hydrolyzed peptide. Composition of reduced hydrolyzed species could be obtained from PC results. In PC ethanol: ammonia: water (2: 1: 5) ^{186}Re -HEDP, ^{186}Re lanreotide and free perrhenate migrated with solvent front and reduced hydrolyzed ^{186}Re remained at the point of spotting. Composition of each species associated with ^{186}Re lanreotide is given in Table VI.

TABLE VI. COMPOSITION OF RADIOCHEMICAL SPECIES PRESENT ALONG WITH ^{186}RE -LANREOTIDE

% of ^{186}Re -lanreotide	% ^{186}Re -HEDP	% $^{186}\text{ReO}_4^-$	% $^{186}\text{ReO}_2$
65 ± 4	22 ± 3	7 ± 2	6 ± 3

% yields determined by TLC and PC; ($n=3$)

HPLC pattern of ^{186}Re -lanreotide is depicted in Fig. 8. ~62% of the injected activity was eluted out as ^{186}Re -lanreotide with retention time 15 min. Retention time for Re-HEDP and $^{186}\text{ReO}_4^-$ was 3 min. The amount of total activity retained on the column was calculated by subtracting the eluted activity from the total activity injected in the column. Activity retained on the column was found to be ~7%. Stability of the complex with time was studied up to 24 h. The complex was found to be stable for this period.

^{125}I -lanreotide

Lanreotide was radioiodinated using chloramineT as the oxidizing agent. 30 μL of 0.5 M PO_4^{3-} , pH 7.5, was added to 2 μg of lanreotide. ~500 μCi of Na^{125}I was added followed by 10 μg of chloramin T in 10 μL of 0.05 M PO_4^{3-} buffer, pH 7.5. After a reaction time of 90 sec, the reaction mixture was diluted to 0.5 mL with 0.05 M PO_4^{3-} buffer.

Purification

A Sep Pak column was used for purification. The column was initially washed with 1 mL of 0.05 M PO_4^{3-} . 0.2 mL of the reaction mixture was loaded on to the column. The column was initially eluted with 1 mL of 0.05 M PO_4^{3-} buffer and later eluted with 2 mL of ethanol. The ethanol was separately collected. Both the reaction mixture as well as the purified fraction were subjected to paper electrophoresis in phosphate buffer, pH 7.5 for 75 min at 10 volts/cm (Fig. 9). The labelling yield was 95% and the radiochemical purity was

>98%. The compound was used for biodistribution studies in mice bearing melanoma tumours.

3.5. Radiolabelling of lanreotide-DOTA

⁹⁰Y-DOTA-lanreotide

To 500 μ L of ⁹⁰YCl₃ (37 MBq) was added ~450 μ L of 1 M NaOH to adjust the pH to ~6. To 500 μ L of pH adjusted ⁹⁰YCl₃ (37 MBq) was added 100 μ L of gentisic acid (5 mg/ml in 0.4 M sodium acetate) and 50 μ g of DOTA-Lanreotide. The reaction mixture was incubated for 30 min at 90°C.

The purification of ⁹⁰Y-DOTA-lanreotide was carried out using a Sep-Pak C18 column. An aliquot of the ⁹⁰Y-DOTA-lanreotide was reacted with 100 μ g of DOTA. This was then passed through a Sep-Pak C18 column pre-conditioned with 2 mL of methanol. The free yttrium complexed with DOTA was eluted out with 2 ml of 1 M sodium acetate buffer while ⁹⁰Y-DOTA-Lanreotide was eluted out using 2 ml of methanol. The radiolabelling yield was calculated as the percent radioactivity associated with the methanol fraction and was found to be ~ 70% (Table VII). The methanol was removed by nitrogen purging and ⁹⁰Y-DOTA-lanreotide was reconstituted in buffer and used for biodistribution as well as in vitro cell binding studies.

TABLE VII. SEP-PAK PURIFICATION OF ⁹⁰Y-DOTA-LANREOTIDE

Fraction (2 mL)	% Radioactivity
Acetate (free ⁹⁰ Y)	27.9±4.5
Methanol (⁹⁰ Y-lanreotide)	72.1±4.5

n=3

4. BIOLOGICAL EVALUATION

4.1.1. *In vivo* biodistribution in mice models

4.1.1.1. ¹²⁵I-Lanreotide

Melanoma cells are found to express somatostatin receptors. Therefore, in order to determine the localization of ¹²⁵I-lanreotide in tumours expressing somatostatin receptors, biodistribution studies were carried out in mice bearing melanoma. Melanoma was induced in C57BL6 mice by injected ~ 0⁶ cells/animal of melanoma cells. Tumours developed ~15 days after the induction. ~150 kBq of ¹²⁵I-lanreotide in a volume of 0.1 mL was injected through the tail vein. Mice were sacrificed at 3 h and 24 h. Blood (~1 mL) was drawn from heart and the tissues and organs were excised following sacrifice. The radioactivity associated with each specimen was measured in a NaI (TI) flat type counter and the activity distribution in the various organs and tumour was estimated. Radioactivity in the blood and muscle was estimated by assuming blood and muscle weight as 7% and 40% of total body weight, respectively. It was observed that (Table 8) ~2.0% ID/g was resident in the tumour at 3 h p.i. There was some amount of activity present in major organs such as liver, intestine and kidney. The activity in the tumour was reduced to ~0.4% ID/g of tumour while 75% of radioactivity was cleared through excreta at 24 h p.i. Most of the radioactivity in all the organs also drastically decreased.

TABLE VIII. BIODISTRIBUTION OF ¹²⁵I-LANREOTIDE IN C57BL6 MICE BEARING MELANOMA

Tissue/organ	%ID/g 3 h	%ID/g 24 h
Blood	1.34±0.05	0.14±0.04
Liver	19.26±3.76	3.40±0.40
GIT	26.5±2.34	1.17±0.07
Kidney	5.82±0.64	1.57±0.16
Stomach	9.76±1.21	1.64±0.6
Heart	1.54±0.69	0.37±0.19
Lungs	3.7±0.04	3.77±0.91
Femurs	2.76±0.72	0.55±0.07
Muscle	0.09±0.01	0.02±0.0
Spleen	4.05±0.53	1.16±0.01
Excreta	5.75±2.87	75.4±4.11
Tumour	2.03±0.32	0.39±0.04

(n=3)

4.1.1.2. ⁹⁰Y-DOTA-lanreotide

Biodistribution studies of ⁹⁰Y-DOTA-lanreotide were carried out in mice bearing melanoma. The results are shown in Table IX. At 24 h p.i., ~1.3% injected dose was observed to be in the tumour with small amounts of activity in the liver, kidney, intestine and femur.

TABLE IV. BIODISTRIBUTION OF ⁹⁰Y-DOTA-LANREOTIDE IN C57BL6 MICE BEARING MELANOMA

Tissue/Organ	%ID/g 24 h	Tissue/Organ	%ID/g 24 h
Blood	0.0	Lungs	5.59±0.71
Liver	6.39±0.31	Femurs	6.89±2.41
GIT	1.03±0.31	Muscle	0.0
Kidney	17.74±3.26	Spleen	3.44±2.24
Stomach	0.98±0.67	Excreta	78.7±4.2
Heart	0.0	Tumour	1.30±0.1

4.1.2. *In vitro* studie s- cell labelling

4.1.2.1 ⁹⁰Y-DOTA-lanreotide

A431 (Epidermoid mammary carcinoma) cell line expressing somatostatin receptors was used for *in vitro* cell binding studies. The cell line was obtained from the Cancer Research Institute, Mumbai. The cells were maintained in DMEM with 10% Fetal Calf Serum in 5% CO₂/95% air at 37 °C. Adherent cells were passaged with trypsin-EDTA solution after

confluency was reached. Before being used in binding experiments, the cells were washed with 50 mM Tris HCl buffer (pH 7.5) and resuspended in 50 mM Tris HCl buffer (pH 7.5) containing 5 mM MgCl₂, 1 mM CaCl₂ and 0.1 M NaCl. Binding was studied by incubating 10⁵ cells with 0.3 µg/tube of ⁹⁰Y-DOTA-lanreotide for 30 min at 37 °C. In order to determine the specificity of binding of ⁹⁰Y-DOTA-lanreotide to the receptors, cells were incubated with 0.3 µg of ⁹⁰Y-DOTA-lanreotide along with cold lanreotide of concentrations 25 µg and 100 µg, respectively. Reaction was also carried out using ⁹⁰Y-DOTA. After incubation, reaction mixture was diluted 1:10 with assay buffer at 4°C and centrifuged at 4000 rpm for 10 min. The pellet was washed twice with buffer and the pellet as well as the supernatant were counted using NaI(Tl) scintillation counter. Cell binding was determined as the % of total activity bound to the pellet. The results are tabulated in Table X.

14.5% binding was achieved with ⁹⁰Y-DOTA-lanreotide while binding decreased to 13% and 8.6% on addition of cold lanreotide. ⁹⁰Y-DOTA showed ~4.4% binding. Decrease in binding on addition of cold lanreotide showed the binding specificity of ⁹⁰Y-DOTA-lanreotide to somatostatin receptors expressed by A431 cells.

TABLE X. *IN VITRO* CELL BINDING STUDIES OF ⁹⁰Y-DOTA-LANREOTIDE IN A431 CELL LINE

Compound	% Binding
⁹⁰ Y-DOTA-Lanreotide	14.5 ± 0.3
⁹⁰ Y-DOTA-Lanreotide + 25 µg cold Lanreotide	13.3 ± 0.7
⁹⁰ Y-DOTA-Lanreotide + 100 µg cold Lanreotide	8.63 ± 0.4
⁹⁰ Y-DOTA	4.4 ± 0.6

LABELLED PEPTIDES THROUGH THE DOTA CHELATING AGENT FOR DIAGNOSIS AND TREATMENT OF RECEPTOR POSITIVE TUMOURS

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Abstract

Under the framework of the CRP, I studied the chemistry involved with chelating agent DOTA (1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid) which is known to form highly stable complexes with trivalent radiometals such as ^{111}In and ^{90}Y and could be used to tag these radionuclides to tumour seeking biomolecules such as biotin and peptides. In particular, the coupling reaction and labelling with ^{90}Y for targeted therapy were optimized and two radiolabelled DOTA-conjugated peptides were tested in pre-clinical settings.

1. INTRODUCTION

Cells express on their cellular membranes a variety of receptor proteins with high affinity for regulatory peptides, including somatostatin [1]. Somatostatin receptors (SSTR) are membrane glycoproteins with five different subtypes cloned that are distributed in a large variety of tissues throughout the body. The overexpression of SSTR, especially type 2 (SSTR2), in different tumours — classically defined as neuroendocrine — has been exploited for the *in vivo* localization of these tumours and their metastases using ^{111}In labelled somatostatin analogue scintigraphy [2]. Ongoing multicenter clinical trials, using high doses of ^{111}In -DTPA-octreotide (Octreoscan®) in patients with neuroendocrine tumours [3], have yielded approximately 15% of objective responses, with a 66% overall response rate. These results may be ascribed to the Auger and conversion electrons emitted by ^{111}In . However, ^{90}Y seems more suitable for therapeutic use because of its energetic β particles ($E_{\text{max}} = 2.27 \text{ MeV}$) and the long range ($R_{95} = 5.94 \text{ mm}$), which allow “cross fire” irradiation.

We have developed a new somatostatin analogue containing the metal chelator DOTA, [DOTA-D-Phe¹-Tyr³]octreotide, named DOTATOC, which has shown favourable characteristics for therapeutic use: high affinity for somatostatin subtype receptors SSTR2 and SSTR5, high hydrophilicity and ease of labelling and stability with ^{111}In and with ^{90}Y [4].

In fact, for trivalent metallic radionuclides such as ^{111}In and ^{90}Y , the 12 membered ring macrocyclic chelating agent DOTA has shown enhanced thermodynamic and kinetic stability relative to an open-chain analogue. The metal complexes are remarkably stable, especially with yttrium, under physiological conditions and conjugation reactions have been optimized for the linkage of DOTA to monoclonal antibodies and peptides [5].

In order to extend this strategy to other tumours not expressing SSTR, I focused on another natural peptide named oxytocin (OT), which is secreted by the neurohypophysis and binds to several tumours through specific membrane receptors (OTR).

OTR has been described in a high percentage of primary breast cancers and in tumours arising in other organs such as brain and endometrium, where OT is known to exert a physiological function [6]. Moreover, *in vitro* and *in vivo* experiments have shown that in breast and endometrial carcinomas, in neuroblastomas and in glioblastomas both OT and synthetic analogues play a biological role by inhibiting cell growth via OTR and the cAMP-PKA pathway [7].

Following the same pathway used for DOTATOC, I initially planned to link DOTA to the α amino group of position one in OT. However, this peptide is very sensitive to substitutions in this position resulting in loss of binding activity to OTR. I therefore focused on Lys⁸-oxytocin (Lys⁸-OT), alternatively known as Lys⁸-vasotocin (LVT), a nonapeptide which retains a high affinity for OTR and where the leucine in position 8 of OT is substituted with lysine. The ϵ amino group of this amino acid offers a safe binding site. In fact, it was used by others to produce a fluorescent tracer (Fluo-OT), which selectively binds to OTR and retains OT activity in *in vitro* and *in vivo* assays [8].

The present study describes the experimental development and properties of DOTA coupled to LVT and [¹¹¹In]-DOTA-LVT, and the selective binding of this novel radioactive ligand to OTR+ tumour cells, both *in vitro* and *in vivo*.

2. PLAN OF THE EXPERIMENTS

In order to prove an efficient binding of the radioactive OT analogue to OTR positive tumour cells, a series of successive experimental steps was planned. The chelating agent DOTA was initially linked to LVT and proved that this compound (DOTA-LVT) still retained affinity for OTR. Therefore, the compound was radiolabelled with ¹¹¹In and the specificity and intensity of the binding of the radioactive compound [¹¹¹In]-DOTA-LVT to OTR+ cells and tumours, both *in vitro* and *in vivo*, were tested.

2.1. Conjugation of DOTA to LVT

The carboxyl group of DOTA was activated by means of a carbodiimide reagent [9]. Briefly, DOTA was dissolved in anhydrous DMSO at 80°C and the solution was allowed to cool under an argon atmosphere. A solution of N-hydroxy-2,5-pyrrolidinedione (NHS) in DMSO was added dropwise to a stirred solution of DOTA, followed by the dropwise addition of N,N'-dicyclohexylcarbodiimide (DCC) in DMSO. The molar ratio between DOTA:NHS:DCC was 1:1.4:0.8. The mixture was allowed to react overnight with stirring and then filtered to separate the byproduct dicyclohexylurea. The conjugation between DOTA and LVT was carried out at a molar ratio of 50:1 by adding an adequate volume of the DOTA-activated ester solution to the LVT dissolved in 0.1M phosphate buffer (pH 8.0). After overnight reaction the conjugate was purified by means of a reverse phase column in a FPLC system coupled with a UV detector and a radiodetector. A linear gradient method was applied using a solution of distilled water with 0.1% TFA (solvent A) and methanol (solvent B). The eluents were delivered at a flow of 4 ml/min starting from 0% of solvent A to 100% of solvent B in 37 ml. Two peaks corresponding to the LVT conjugates with DOTA were evident in the UV profile. The retention volume was 8.4 ml for the first compound (A) and 10.0 ml for the second (B), whereas unconjugated LVT eluted at 7.0 ml in the same conditions. An integrated fraction collector performed the recovery of each compound. Each peak was analysed subsequently by MALDI-TOF mass spectrometry.

2.2. Affinity studies

To determine LVT and DOTA-LVT affinity constants, heterologous competition experiments were performed on membranes prepared from monkey kidney COS7 cells transiently transfected with the human OTR cDNA. Briefly, electroporated cells were homogenized in a Dounce glass potter, washed twice, and resuspended in binding buffer (50mM Tris-HCl, 5mM MgCl, pH 7.4). 5-10 μ g of membrane proteins were incubated with a fixed concentration of [³H]-OT (1-2 nM) for 30 min at 30°C in the presence of increasing

concentration of unlabelled peptides. Non-specific binding was determined in the presence of 1 mM OT. Bound and free radioactivity were separated by filtration over Whatman GF/C filters pre-soaked in 10 mg/ml BSA. Binding isotherms were analysed with the iterative curve-fitting program LIGAND.

2.3. Radiolabelling of DOTA-LVT (peak A) with [¹¹¹In]

To prepare [¹¹¹In]-DOTA-LVT, 3.7 MBq of [¹¹¹In]Cl₃ that is diluted in acetate buffer (0.1 M, pH 5.5), was added to 0.07 μmol of fraction obtained from peak A. The solution was heated for 25 min at 80°C, and the labelling yield was checked by FPLC as above.

2.4. Binding studies on OTR+ and OTR-tumour cells

Human breast carcinoma (MCF7), glioblastoma (MOG-U-V-W) cell lines are OTR+, while colon carcinoma (HT29) cells represent the OTR- control.

All the cells were grown as monolayers in RPMI medium with 10% fetal calf serum, in 25 cm² T flasks in a 5% CO₂ humidified atmosphere, at 37°C. Binding experiments were performed on intact cells. Briefly, 20×10⁵ cells, suspended in 100 μl of culture medium were incubated for 30 min at 4°C in the presence of 1 μM [¹¹¹In]-DOTA-LVT. The cell suspension was then centrifuged for 5 min at 2000 g and the pellet was washed and re-suspended in fresh medium. Centrifugation and cell washing were repeated twice. The entity of the radiolabelling was evaluated measuring the radioactivity bound to cells (cpm/10⁵ cells) by a Packard auto-gamma counter.

Specificity of the binding was determined by evaluating the radioactive displacement. Briefly, the cell suspension was incubated for 5 min in the presence of 100 μM and 1 mM of LVT or OT, or in the presence of unrelated peptides, followed by 20 min of incubation with 1 μM [¹¹¹In]-DOTA-LVT. Cells were then centrifuged and washed twice as reported above. The activity of two different agonists and of the unrelated peptides to compete with the radioligand was evaluated measuring the radioactivity bound to cells. All experiments were performed in triplicate. Statistical analysis was carried out by ANOVA. Cut off for significance was 0.05.

2.5. *In vivo* studies

To determine the entity and specificity of receptor mediated uptake of [¹¹¹In]-DOTA-LVT in tumours in comparison with the non-specific compound [¹²⁵I]-DOTA-TOC, an experimental model of Balb/c mice growing OTR+ and SSTR2- tumour was used. A total of eight mice were injected subcutaneously with mammary carcinoma cells. Twenty days later, when the growing tumour reached the size of about 2 cm in diameter, the animals were injected intraperitoneally with a mixture of 1.1 MBq of [¹¹¹In]-DOTA-LVT and 74 kBq of [¹²⁵I]-DOTA-TOC. The animals were divided into groups and sacrificed at 3 and 24 h post-injection. Tumour, blood, liver, kidney and brain were removed and weighted. Radioactivity was measured in a gamma ray detector with a well counter geometry together with standards of the injection mixture at two different time points to calculate the contribution of each isotope: (a) immediately after the removal of the tissues; and (b) after 2 weeks, corresponding to five physical half-lives of ¹¹¹In. Activity was expressed as a percentage of injected doses/mg of tissue and ratios of uptake between tumour and brain vs. blood were calculated.

3. SUMMARY OF THE RESULTS

The conjugation between DOTA and LVT was complete as verified by the absence of unconjugated LVT in the reaction mixture. Radiolabelling yields were >95% for [¹¹¹In]-DOTA-LVT. After HPLC purification, the new conjugate showed hundred-fold decreased affinity for OTR compared to unconjugated LVT by means of heterologous competition experiments on membranes. In particular, LVT inhibited [³H]-OT binding with high affinity ($K_i = 1.87 \pm 0.41$ nM) whereas DOTA-LVT was able to compete [³H]-OT binding with a calculated K_i of 238 ± 52 nM.

However, DOTA-LVT was radiolabelled with ¹¹¹In and binding experiments showed specific radiolabelling in all the OTR positive cell lines and negligible binding in OTR negative ones. Specificity of the binding was proved by displacement with cold radioligands. In OTR positive cells, more than 90% of the specific ¹¹¹In-DOTA-LVT binding was displaced by the two agonists OT and LVT.

The results of the biodistribution study in mice bearing OTR+ tumours showed that neither ¹¹¹In-DOTA-LVT nor ¹²⁵I-DOTA-TOC accumulated in the brain, an organ where OTRs are known to be expressed.

As expected, kidney uptake was rather high, although lower for ¹¹¹In-DOTA-LVT than for ¹²⁵I-DOTA-TOC, especially at 24 h.

The tumour to blood uptake ratios for both radiolabelled peptides are given in Table I.

TABLE I. TUMOUR TO BLOOD RATIOS

	3h		24h	
	¹¹¹ In-DOTALVT	¹²⁵ I-DOTATOC	¹¹¹ In-DOTALVT	¹²⁵ I-DOTATOC
	2.7	0.96	2.8	4.86
	4.1	1.85	4.4	2.07
	1.2	0.81	9.1	2.13
	N.A.	0.49	12.6	1.88
mean ± s.d.	2.7 ± 1.4	1.02 ± 0.58	7.2 ± 4.5	2.7 ± 1.4

These experiments showed that ¹¹¹In activity (specific peptide) in tumour was remarkably higher than ¹²⁵I (non-specific peptide) demonstrating the receptor mediated binding of ¹¹¹In-DOTA-LVT to OTR expressing tumours.

4. CONCLUSIONS

The use of radiolabelled peptides in tumour therapy is gaining increasing acceptance. While therapeutic clinical trials with ⁹⁰Y labelled somatostatin analogue DOTATOC have shown objective therapeutic responses, the development of a new radioligand such as DOTA-LVT still requires further improvements in order to overcome the loss of affinity for OTR of the conjugate.

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LABELLED BIOMOLECULES FOR TARGETED RADIOTHERAPY: BIODISTRIBUTION IN MICE WITH INDUCED MALIGNANT TUMOURS

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Abstract

Antibodies and antibody fragments react against the cell membrane receptors or associated-antigens and can be ^{188}Re -labelled and ^{153}Sm -labelled as potential radioimmunotherapy (RIT) radiopharmaceuticals. A variety of malignant tumours over-express somatostatin receptors, proteins that can be targeted with radiolabelled biomolecules, like the lanreotide peptide. The direct labelling methods described here are easy to perform from a freeze-dried kit, are reliable and can be carried out in a hospital radiopharmacy. ^{188}Re -lanreotide, thus prepared, shows affinity to HeLa cells and to the SiHa cells, both from a human cervical cancer, to 293 cells from human embryonic renal carcinoma and to B16F cells of murine melanoma, implanted in mice. ^{188}Re -lanreotide standardized tumour uptake value SUV was 1.5 for the cervix cancer, 4.8 for the kidney tumour, and 0.8 for the melanoma metastases. Many of the neuroendocrine tumours which over-express somatostatin receptors are of epidermoid origin and the 4 induced tumours studied have many epidermoid characteristics. The cell origin might be the common factor to all these cancer cells, which bind ^{188}Re -lanreotide. In conclusion: easy labelling methods with ^{188}Re and ^{153}Sm for biomolecules used for targeted therapy have been developed. ^{188}Re -lanreotide binds to 4 different cancer cells implanted into mice.

1. INTRODUCTION

Antibodies and antibody fragments react against the cell membrane associated-antigen or receptors and on occasion are internalized into the malignant cell. These biomolecules can be ^{188}Re -labelled and ^{153}Sm -labelled, as potential radioimmunotherapy (RIT) radiopharmaceuticals. The pharmacokinetics of antibodies has been studied in animal models and its specificity can be increased using the biotin-avidin pre-targeting strategy in which, an antibody and radiopharmaceutical are administered separately, bringing about a high tumour/non-tumour ratio [1-4].

A variety of malignant tumours over-express somatostatin receptors, proteins that can be targeted with radiolabelled biomolecules. The radionuclides used for the detection and treatment of the malignant cells were gamma and beta emitters, such as ^{188}Re and ^{153}Sm . The most used biomolecules are small molecules like somatostatin analogue peptides.

Since rhenium is a transition metal of the Mn and Tc family the radioisotope ^{188}Re has been used as a biomolecule labelling agent. Its physical half-life is 16.9 h, it decays by a $E_{\beta\text{max}}$ 2.12 MeV beta radiation, adequate for therapy, and emits a gamma radiation of 155 keV, which allows for imaging. The somatostatin analogue peptides labelled with beta emitters have been successfully used for tumour localization and targeted radiotherapy. Labelled octreotide and vapreotide RC 160 appear in the medical literature quite often. Another somatostatin receptor analogue peptide, a long-acting cyclic octapeptide, β -(2-naphthyl)-D-Ala-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-amine, β -naphthyl-peptide or lanreotide can inhibit, *in vitro* and *in vivo*, growth of the human small cell lung carcinoma [5]. Lanreotide has been ^{90}Y and ^{111}In labelled, via bifunctional chelators, and used for treatment of somatostatin receptor expressing tumours [6-7]. It has also been ^{188}Re labelled by direct methods [8]. For the direct labelling method a reducing agent is used to reduce rhenium to a lower oxidation state and, reduce the peptide's cysteine disulphide bridge. The metal-coordinated compound is easily formed by competing with a weak ligand like hydroxyethylidenediphosphonate, HEDP [9].

Labelled antibodies show, in general, prolonged circulation and non-specific accumulation in metabolic organs and their biodistribution and elimination routes play a relevant role in the tumour uptake. In order to minimize radiation toxicity it is desirable to have a fast renal excretion or low lipophilicity. The tumour/non tumour radioactive rate could be improved using hydrophilic compounds like ^{153}Sm -DTPA-bis-Biotin with the avidin-biotin strategy [10].

^{153}Sm -samarium is a versatile radionuclide with a physical half life of 46.8 h, a 103 keV gamma emission (23%) for scintigraphic imaging, a therapeutic $E_{\beta\text{max}}$ 0.8 MeV beta emission and can be produced with high specific activity in a fairly low flux nuclear reactor. Due to its radionuclidic properties it is easily handled from the point of view of radiation protection.

The aim of this research was to review our labelling methods for several biomolecules. The methods were designed, considering the need for freeze-dried kits or easy to label methods in a hospital radiopharmacy, and the main advantages offered by ^{188}Re and ^{153}Sm . Reagents' molar concentration for each biomolecule were determined as were the labelling conditions and the radiochemical purity. The biodistribution in control mice and in malignant tumour-induced laboratory animals was studied for each labelled compound and the radiopharmacokinetic parameters were determined.

2. MATERIALS AND METHODS

2.1. Direct ^{188}Re -antibody labelling

The proteins were: polyclonal IgG (*Sandoglobulin*); murine monoclonal (MoAb)IgG1 *ior cea 1* against carcinoembryonic antigen (CEA), (CIMAB, Cuba), (MoAb); biotinylated MoAb (MoAb-biotin); MoAb fragments ($\text{F(ab}')_2$) and biotinylated MoAb fragments ($\text{F(ab}')_2$ -biotin). (2) The labelling method is basically as follows: 2-mercapto-ethanol is used to reduce the disulphide groups and stannous chloride and HEDP are added in molar concentrations specific for each molecule as well as pH and incubation time and temperature. Succinimidyl-6-(biotinamide) hexanoate (*PierceCo.*) was used to prepare the biotinilated compounds [1-4].

^{153}Sm -DTPA-bis-biotin labelling method

DTPA-bis-biotin (*Sigma Chemical Co.*). $^{153}\text{SmCl}_3$ was added to the solution of diethylenetriaminepentaacetic acid covalently conjugated to biocytin in bicarbonate buffer at pH 8.0 [10].

2.2. Direct labelling of ^{188}Re -lanreotide

β -(2-naphthyl)-D-Ala-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-amine, β -naphthyl-peptide or lanreotide (*PiChem*, Austria) was rhenium labelled by a modified direct method [8] using stannous chloride to reduce both rhenium (^{188}W - ^{188}Re generator, MAP Medical Technologies Oy, Finland) to a reactive species, and to reduce the peptide's disulphide bridge for subsequent chelation of the metal with HEDP, as the weak competing ligand [9]. The reaction was carried out at pH 1.7 and 90 min incubation in a boiling water bath (92°C). Citrate buffer pH 7.0 (2.9% sodium citrate, 0.1% EDTA) was used to raise the pH to 6.0 [11].

Quality control

The radiochemical purity of the labelled biomolecules was determined by ITLC, HPLC chromatography and SepPak cartridges.

ITLC-SG systems

On 1×10 cm strips (Gelman Sciences, Inc. Ann Arbor, Mich.) the samples were inserted into the mobile phases: saline (0.9% NaCl), acid ethanol (10% HCl 0.3 M). Free perrhenate migrates with the front in the solvents, R_f 1.0. Reduced-hydrolyzed rhenium stays at the origin or seeding point, R_f 0.0.

HPLC

The columns with photodiode array detector and Millenium software were used. The collected fractions (0.5 mL) were counted in a well-type scintillation detector and cpm were plotted against time to obtain a radiochromatogram. The column used for molecular size exclusion was a ProteinPak 125 SW gel filtration column (Waters) and a mobile phase 0.1 M phosphate pH 7.4, at a rate of 1.5 mL/min. For reverse phase HPLC with gradient system, a C-18 μ BondaPak 3.9×300 mm column (Waters) with photodiode array detector was used. The mobile phase was A: 0.1% trifluoroacetic acid-H₂O/CH₃CN (1:3) and B: 0.1% TFA-H₂O with a gradient 0-15 min 30-50% A and 15-40 min 50-100% A at a 1.3 mL/min rate. The amount of reduced hydrolyzed rhenium in β -naphthyl-peptide, or column bound radioactivity, was calculated by comparison of recovered activity from the HPLC column with a standard.

SEP-PAK cartridges

Activation

The cartridges were activated with 10 mL absolute ethanol, followed by 10 mL 0.001 N HCl and dried with 10 mL air.

Purification

0.1 mL of labelled biomolecule was placed on top of the cartridge and 10 mL of 0.001N HCl pH 3 were passed to separate free perrhenate. Acidified ethanol (10% 0.01N HCl) was passed to collect the labelled biomolecules. The two fractions plus the cartridge (reduced-hydrolysed radionuclide) were counted in a well type scintillation detector.

Analytical methods

Several of the following methods were used to characterize some of the biomolecules: IR spectroscopy, thermogravimetric analysis TGA, scattering electronic microscopy SEM and elemental dispersion analysis by X-rays EDA.

Theoretical analysis

This was used to visualize the complex and the peptide molecule. Geometric analysis was done by energetic principles, using the MM method of FF and *ab initio* calculations by DFT were useful for explaining the metal-ligand interactions and their conformational properties.

Cell lines

Cell lines were kept frozen, cultured and prepared for injection in the molecular laboratory of the Universidad Panamericana. All cell lines were plated on 10 cm diameter

dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum and antibiotics: 100 units/mL penicillin and 100 µ/mL streptomycin and incubated at 37°C under 5% CO₂ atmosphere and 100% humidity. Semi-confluent cells were detached using trypsin/EDTA and counted in a Neubauer chamber.

HeLa cells

These are cells of epithelial origin and originally derived from an epidermoid human carcinoma of the uterine cervix. They contain the human papilloma virus HPV18 DNA which is integrated into the cellular genome and has been shown to harbour transcriptionally active oncogenic sequence E6 and E7. HPV produces different proteins which are tumour specific antigens and therefore the HeLa cell cancer model provides tools to examine the molecular mechanisms of cervical cancer [2, 10]. Also, allelotype analysis of cervical carcinomas has been performed to localize tumour suppressor loci. The 3p losses are common to cervical, lung, clear cell renal, and nasopharyngeal carcinomas [12].

SiHa cells

These are squamous human cervix cancer cells established from fragments of a primary tissue sample from a patient after surgery. The cell line presents typical desmosomes at the cell junctions and abundant tonofilaments in the cytoplasm. The immortal and tumorigenic cell line is reported to contain 1-2 copies per cell of an integrated human papilloma virus type 16 genome (HPV-16).

Human renal carcinoma 293 cell line

This is an epithelial cell line of primary human embryonal kidney transformed by sheared human adenovirus type 5 (Ad 5) DNA. The cells express an unusual cell surface receptor for vitronectin composed of the integrin beta-1 sub-unit and the vitronectin receptor alfa V sub-unit.

Melanoma B16F cells

These cells are from a C57BL/6 mouse melanoma, tumour. They are epithelial like, produce melanin and are validated by isoenzymes. These cells are tumorigenic and metastasize in syngeneic animals.

Histopathological studies

Solid tumours, from the back of the mice, were studied. The excised tumours were embedded in paraffin blocks, dehydrated and eosin-hematoxilin stained.

Animal subjects

Tumour uptake, biodistribution and radiopharmacokinetics were determined according to the rules and regulations of our Institute for the handling and care of experimental animals.

The rodents used for all the experiments were a) female Balb/c 25 g mice, b) female, athymic, 20 g mice; and c) female B₁₅C₅₇, 25 g mice. The athymic mice were kept in micro-isolators, handled under an airflow cabin and fed irradiated PICO LAB PMI feed and water *ad libitum*. The other mice were kept in cages at constant temperature and humidity and were

fed standard PMI feed and water *ad libitum*. The animals were injected with the tumour cell line (one million cells suspended in 0.2 mL DMEM) in the right flank, close to the axillary nodes. A smear of all the cell lines injected was taken to pathology for identification.

Biodistribution studies

These were undertaken after a period of time when the tumour in the back of the mice was visible and well formed (7-55 days). The rodents (3 per time point) were injected in the tail vein 1.85 MBq (50 μ Ci) in 0.1 mL of the radiopharmaceutical and CO₂ sacrificed after a determined interval 0.083, 0.25, 0.5, 1, 2, 24 and 48 h. A surgical midline incision was made and blood was drawn into a disposable syringe and approximately 1 mL was placed into a test tube. Then the organs of interest were extracted. Each organ (heart, lung, liver, gall bladder, spleen, stomach, intestines, left kidney, muscle, bone and tumour) were saline rinsed, paper blotted, placed into pre-weighed test tubes and the activity was determined with a well type scintillation detector (Canberra). The mean cpm were used to obtain accumulation in percentage injected activity per gram of tissue% IA/g.

Tumour uptake

The standardized tumour uptake value SUV was calculated as follows [13]:

$$SUV = \frac{\text{Decay corrected activity (MBq) in the tumour / mass of tumour (g)}}{\text{Total injected activity / rat weight (g)}}$$

3. RESULTS

Labelled antibodies

¹⁸⁸Re-polyclonal IgG (¹⁸⁸Re-IgG); ¹⁸⁸Re-murine anti-CEA monoclonal antibody, (¹⁸⁸Re-MoAb); biotinylated ¹⁸⁸Re-MoAb (¹⁸⁸Re-MoAb-biotin); ¹⁸⁸Re-MoAb fragments (¹⁸⁸Re-F(ab')₂) and biotinylated ¹⁸⁸Re-MoAb fragments (¹⁸⁸Re-F(ab')₂-biotin) were produced with specific activities of 1.3 \pm 0.2 GBq/mg from freeze-dry kits. The radiochemical purity was >95% and the immunoreactivity was similar to that of ⁹⁹Tc^m radioconjugates. Blood clearance in female Balb/c mice is rapid during the first 5 h after administration and 48 h later the% activity injected/g is less than 3% for all the compounds. Renal clearance is the same for biotinylated MoAb and fragments. Biodistribution of biotinylated and non-biotinylated immunoconjugates was not significant statistically, p>0.5, and the elimination was mainly hepatointestinal and urinary. Injected avidin as a "chase" increased liver accumulation, lowered blood concentration and the accumulated activity in all the organs. The bone marrow absorbed dose, extrapolated to humans, went from 0.06mGy/MBq to 0.02 mGy/MBq for the labelled antibody and for the fragments and, after avidin was injected, the values decreased to 0.013 mGy/MBq-0.005 mGy/MBq respectively (2,4).

¹⁵³Sm-DTPA-bis-Biotin

The labelling efficiency was >98%, two and 72 h after labelling at pH 8.0 and with a molar ratio DTPA-bis-biotin/Sm from 2 to 4. Spectroscopic and thermal studies show that the complex is formed by 3 carboxylate anions which neutralize the 3 positive charges of the metal and the ligands: carboxylic groups bound to the methylene of the secondary amide

groups and the amide function itself completely shield the core. The 2 biotin moieties are free for avidin binding. This complex is stable for three days at 18-25°C. Blood clearance is biexponential and 3 h after the administration of the radiopharmaceutical 88-96% of the injected activity is eliminated in the urine of Balb/c mice. There was no concentration in any other organ (10).

¹⁸⁸Re-lanreotide

The radiopharmaceutical was prepared with a labelling efficiency $>96 \pm 2\%$ and 90 min incubation period at 92°C and a final pH 6. Stability was 90% 24 h after preparation and no radiolytic degradation was observed. Without HEDP in the preparation the labelling efficiency is 10%. The biologically active part of the molecules is free, that is the Tyr-D-Trp-Lys-Val union is not affected by the labelling process and can bind to the tumour receptors. The metal is co-ordinated to five donor atoms: an oxygen, two Cys sulphurs and two nitrogen atoms from Cys and Thr. This fact indicates that the rhenium-188 complex is probably an oxo-rhenium(V) N₂S₂ penta-coordinated complex, [¹⁸⁸ReO-lanreotide]. A model of the molecular structure of both the peptide and the ¹⁸⁸Re-β-naphthyl-peptide was generated and can be seen that the two amino acids (Trp and Lys) which are common to somatostatin are free to bind with the somatostatin receptors [11].

Radiopharmacokinetic Balb/c mice parameters indicated that ¹⁸⁸Re-lanreotide is rapidly distributed in all the tissues with a fast distribution constant $\alpha=2.1$ 1/h; and a slow or total elimination constant $\beta=0.28$ 1/h; elimination from the central compartment constant $k_{10}=0.91$ 1/h. The apparent volume of distribution was 21.5 mL and the radio-peptide remained in the body for a mean residence time MRT = 2.49 h. Total clearance is high Cl = 19.7 mL/h. The high activity initially seen in the gallbladder decreases 24 h later and is found in the faeces. The remaining organs had low radioactivity. The biodistribution in athymic mice followed the same pattern except for tumour uptake. There was $6.2\% \pm 2.9\%$ IA/g tumour in the athymic mice. The tumour/muscle ratio in athymic mice, 24 h after radiopeptide injection, was 7.1 and tumour/blood ratio was 3.3 [11].

The histopathological study showed that the HeLa cells used are of human epidermoid origin, positive for cytokeratins and vimentin, and negative for chromogranin. Berruti, et al. mentioned that advanced prostate cancer patients had above normal range chromogranin-A levels and after receiving lanreotide (30 mg×4) showed a decreased plasma concentration [14].

In the human cervix cancer tumour biopsy (SiHa cells) a neoplastic process is identified. It is constituted by flat cells with big nuclei of vacuolated aspect with keratin granules and scarce cytoplasm of pale eosinophilic colour. Many mitoses are identified. The tumour rests on a fibrous stroma and it is surrounded by abundant lymphocytic exudates. The diagnosis was malignant tumour invading soft tissue compatible with epidermoid origin human carcinoma of the uterine cervix. The tumour uptake was 7.4% IA/g tissue at 3 h after injection, which rapidly decreases at 24 h P.I. The tumour/blood ratio is shown in Fig. 1.

The induced human renal tumour in athymic mice showed many epithelial, neoplastic cells with round, pleomorphic and hyperchromatic nuclei. The diagnosis was epithelial tumour of renal cell characteristics. The renal tumour bearing mice presented a similar biodistribution as in control mice except that the kidney had a high ¹⁸⁸Re-lanreotide uptake (24.6% IA/g). The kidney/blood ratio was 34, two hours after injection. Biodistribution is shown in Fig. 2. SUV was 4.8.

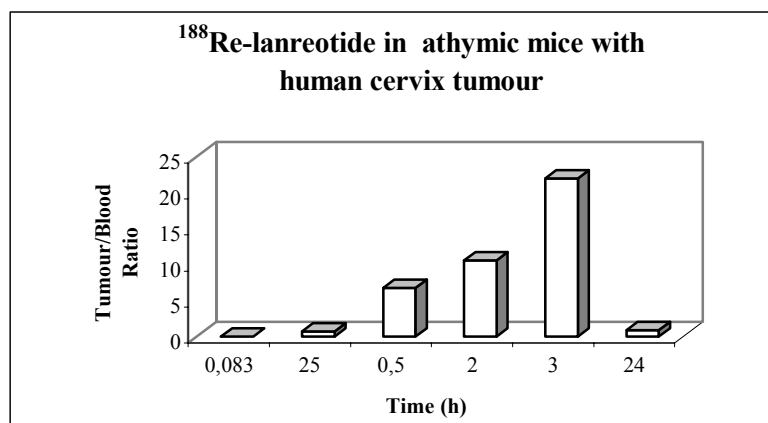


FIG. 1. Tumour/blood ratio of ^{188}Re -lanreotide in athymic female mice with induced human cervix tumour from 0.083 to 24 h ($n=3/\text{time point}$).

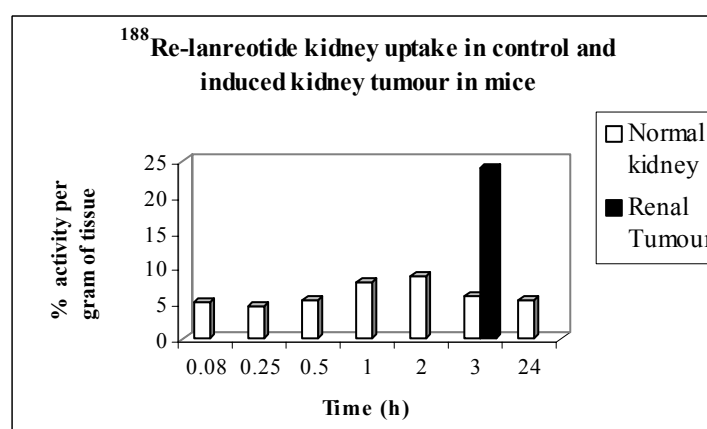


FIG. 2. Uptake, in% injected activity per gram of tissue, of ^{188}Re -lanreotide in control athymic mice and in athymic mice with an induced human renal tumour; $n=3$ per time point.

The B₁₅C₅₇ mice with the induced melanoma tumour presented, after 10 days, a large flank tumour with adjacent surviving epidermis. In the dermis, hypodermis and soft tissue there was a neoplastic process constituted by proliferation of round, fusiform and polyhedral cells with large hyperchromatic nuclei. In some nuclei there was a prominent nucleolus. The cytoplasm of many cells contained melanin deposits. The tumour destroyed skeletal muscle, its borders are infiltrating and there was invasion of blood vessels. There were multiple black metastases (0.05-0.2 mm in diameter) in the thoracic and peritoneal cavities especially next to the large intestine. Diagnosis: subcutaneous, soft tissue malignant melanoma. Biodistribution is shown in Fig. 3.

4. DISCUSSION AND CONCLUSIONS

MoAb-based radiotherapy of cancer has become very useful because it is an efficient and relatively safe therapy for radiosensitive, and readily accessible neoplasms, like non-Hodgkin lymphoma. Tumour response depends mainly on dose rate, cumulative radiation dose and tumour radiosensitivity. In radiosensitive lymphomas an uptake of 0.001-0.01% of the specific monoclonal activity injected/g of tissue delivers <15 Gy which produces anti-tumour response. A dose of 50 Gy or more is considered necessary for therapeutic response of adenocarcinomas. MoAb and chemotherapy are successful in treating solid tumours. (15).

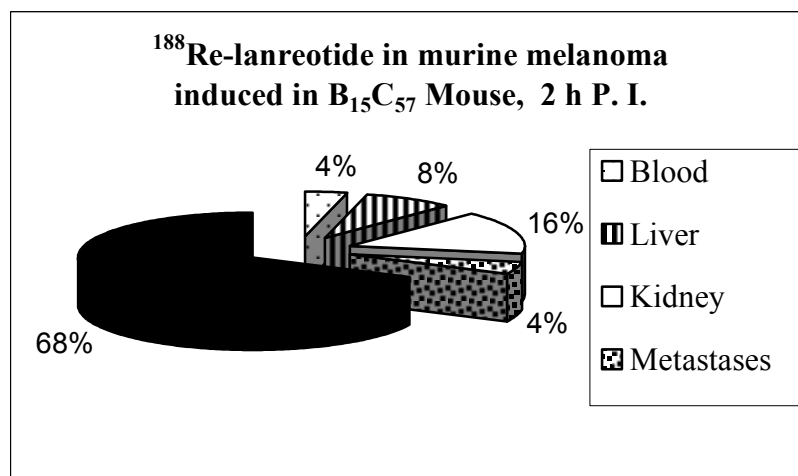


FIG. 3. ¹⁸⁸Re-lanreotide uptake (%IA/g) in murine melanoma induced in B₁₅C₅₇ female mice, two hours post administration, via I.V.

Unlabelled somatostatin analogues also have therapeutic effects. Lanreotide was administered intramuscularly (30 mg) every 14 days for two months to patients with prostatic cancer. The tumour growth was stabilized in 57% of the patients and the lanreotide treatment was very well tolerated and did not cause major toxicity [14]. In another study lanreotide was given: 30 mg/every 14 days for seven months together with octreotide (0.1 mg thrice daily for 17 months) to 35 patients with neuroendocrine tumours also with good response. Somatostatin analogues have been considered an efficient treatment and the authors recommend it as a first-line therapy [16].

The therapeutic effect of somatostatin analogues is increased when labelled with beta emitters (receptor-targeted radionuclide therapy).

A tumour dose >10 Gy/GBq ⁹⁰Y-DOTA-lanreotide produced stable tumour disease in roughly 35% of the patients with neuroendocrine tumour lesions and did not produce chronic severe hematological toxicity, or modification of renal or liver function values (17). This is in agreement with the data that the maximum activity tolerated by the kidneys is 20-25 Gy meaning the renal tolerated dose (TD) having a 5% probability of late toxicity within five years (TD 5/5). [18].

In pharmacokinetic animal research it is important to recognize the high variability in processes due to genetic, environmental, pathophysiologic and quantitative inter-individual differences [19]

Animal models suffer these factors but are useful in extrapolating results to humans. It has been reported that similar biokinetics make the animal model a suitable model for somatostatin analogue therapy evaluation. The fact was that carcinoid tumour material from a patient, who underwent surgery, was cultured and the cells were injected into nude mice. Biokinetics of ¹¹¹In-DTPA-D-Phe-octreotide were studied in mice at different time intervals and in the patient by sequential scintigraphy and in both the biokinetics was similar (20). It has also been reported that the metabolic properties of ¹¹¹In-DTPA-octreotide are similar in rat and man (21). Therefore, dosimetric estimates in the chosen animal model could give a close estimate in humans in order to maintain a low TD.

¹⁵³Sm-DTPA-bis-biotin is a biomolecule with tumour affinity and with the avidin-biotin strategy could be a very promising agent for targeted therapy. Work with hepatoma induced Wistar rats is in progress.

Most of the human tumours seem to over-express one or more of the five distinct somatostatin receptor subtypes and many of the neuroendocrine tumours, which over-express somatostatin receptors are of epidermoid origin. ^{188}Re -lanreotide, thus prepared, shows affinity to HeLa cells and to the SiHa cells, both from a human cervical cancer but with different HPV type (18 and 16 respectively) and to human 293 cells from human embryonic kidney and murine melanoma tumours, implanted in mice, all of which have many epidermoid characteristics. The cell origin might be the common factor to all these cancer cells, which bind ^{188}Re -lanreotide.

In conclusion, the four induced tumours tested incorporate the labelled peptide with a tumour standardized uptake value SUV from 0.8 to 4.8. The biomolecule labelling methods described here are easy to perform from a freeze-dried kit, are reliable and can be carried out in a hospital radiopharmacy.

ACKNOWLEDGEMENT

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LABELLED BIOMOLECULES WITH ^{153}Sm , ^{188}Re , AND ^{90}Y FOR TARGETED RADIOTHERAPY

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Abstract

A somatostatin analogue Lanreotide was labelled with ^{188}Re , $^{99}\text{Tc}^{\text{m}}$ and ^{131}I . Labelling was accomplished by reduction of cysteine bridge, which provided sulfhydryl groups for chelation with $^{188}\text{Re}/^{99}\text{Tc}^{\text{m}}$. Stannous chloride was used as reducing agent, while tartrate acted as transchelating agent. The lower redox potential of ReO_4^- than TcO_4^- required the addition of excess SnCl_2 and medium-chelating agent for stabilizing the excess of SnCl_2 in solution. ITLC and HPLC techniques employed for monitoring the labelling yield revealed >95% labelling efficiency. Radioiodination of lanreotide was carried out by Chloramine-T and iodogen methods. The radiolabelling yield varied between 40-80%. Chloramine-T method was found more suitable than Iodogen method, because ~25% of the initial iodine activity was adsorbed on Iodogen coating. Avidin-biotin system is widely used in medical research, especially in pretargeted radioimmunoimaging and therapy. Biotinyl-hydrazine-EDTA (EDTA-B₁) was labelled with ^{153}Sm . A molar ratio of 15:1 (EDTA-B₁: Sm) resulted in >90% labelling yield. TLC and HPLC techniques were used to determine the labelling and binding efficiency. The ^{153}Sm labelled EDTA-B₁ was found to bind to avidin, thereby demonstrating retention of its biological activity. For the labelling of EDTA-B₁ with ^{188}Re , SnCl_2 and heptagluconate were used as reducing and transchelating agents. Studies relating to development of $^{90}\text{Sr} \rightarrow ^{90}\text{Y}$ generator using various inorganic adsorbents were also carried out.

1. INTRODUCTION

Like monoclonal antibodies, peptides are also receptor-specific. Somatostatin has been demonstrated to exhibit a wide spectrum of biological and oncological actions, and the clinical potential of the peptide has been appreciated for several years. Various studies showed the inhibitory effects of somatostatin on a wide range of tumours [1]. However, therapeutic application of somatostatin was limited by its multiple action and the very short half-life in the circulation. Therefore, analogues were synthesized, which were more potent and long acting than somatostatin itself [2-4]. Radiolabelled receptor-specific biomolecules can detect primary sites, identify occult metastatic lesions, guide surgical intervention, stage tumours, predict efficacy of certain therapeutic agents or, when labelled with suitable radionuclides, are useful radiotherapeutic agents. Radiolabelled peptides evaluated for various applications were reviewed by Thakur, et al. [5]. ^{111}In -DTPA-Octreotide has been shown to detect a variety of neuroendocrine tumours with high specificity and sensitivity [6-8]. Although this agent has been a valuable tool in diagnostic imaging, it suffers from at least one major drawback – the cost. The impetus generated by these results have prompted investigator to label peptides with such radionuclides as $^{99}\text{Tc}^{\text{m}}$, ^{123}I , ^{18}F , ^{67}Cu , ^{67}Ga , $^{186,188}\text{Re}$ and ^{90}Y .

To solve the problems of the low level and slow uptake in the tumour and slow clearance from the blood of radiolabelled antibody in the tumour imaging and therapy, improvements were made with pretargeting techniques that, through administering antibodies and chloramines separately, provided an alternative way to get highly selective accumulation of radioactivity in tumours with simultaneous reduction of background radioactivity in non-tumour tissue [9,10]. Avidin or streptavidin and biotin were used for this purpose because of the dissociation constant of the avidin-biotin complex is of the order of 10^{-15} M [11] and for, practical purposes, the binding of biotin to avidin can be regarded as an irreversible process. Radiolabelling of biotin, avidin or streptavidin is done either directly to the protein (radioiodination) or by covalently linking a chelating agent, which in turn can bind a suitable

radionuclide [12]. Various radiobiotin labelled with ^{131}I , ^{18}F , ^{111}In , $^{99}\text{Tc}^{\text{m}}$, and ^{90}Y have been evaluated *in vitro* and *in vivo* [13-17].

2. MATERIALS

Lanreotide (8-mer, Cys2-7,cyclo) was a product of piCHEM Austria, while $^{188}\text{W}/^{188}\text{Re}$ was fabricated by MAP Finland. Both peptide and generator were supplied by the IAEA. The $^{99}\text{Tc}^{\text{m}}$ -pertechnetate was obtained from a $^{99}\text{Mo}/^{99}\text{Tc}^{\text{m}}$ radionuclide generator Pakgen, PINSTECH, Pakistan. The generator contains fission produced molybdenum-99 adsorbed on alumina. $^{99}\text{Tc}^{\text{m}}$ may be eluted aseptically using saline as an eluent. Iodine-131 in carbonate buffer (pH=8.5-9) was obtained from neutron irradiated TeO_2 via dry distillation method. ^{85}Sr and ^{90}Y tracers were prepared by irradiation of $\text{Sr}(\text{NO}_3)_2$ and Y_2O_3 targets in the core of the Pakistan Research Reactor-I. Most of the chemicals, such as anhydrous/hydrated SnCl_2 , tartaric acid, ascorbic acid, dichloromethane, were purchased from E. Merck, Germany. Iodogen (1,3,4,6-tetrachloro-3a,6a-diphenylglycouril) was a product of Pierce Chemical Company, Rockford, IL. Biotin hydrazide, EDTA-dianhydride and chloramine-T were obtained from Sigma and Aldrich.

3. METHODS

Lanreotide is a cyclic peptide, hence direct labelling method was studied to label the peptide with ^{188}Re and $^{99}\text{Tc}^{\text{m}}$. To determine the labelling yield various factors like pH, reducing agent, temperature, incubation time, etc. were evaluated.

3.1. Labelling of lanreotide with ^{188}Re

3.1.1. Reduction of dicystein bond with 2-ME

- (a) 25 μg lanreotide (0.25 mL, pH \sim 7)
- (b) Incubation of lanreotide with 2-ME at different molar ratio of 2-ME: lanreotide. 1000: 1 to 5000: 1 at 22 ± 2 °C for 30 min.
- (c) Addition of SnCl_2 solution (2.5 mg – 10mg) and tartaric acid (2mg – 10 mg).
- (d) Mixing of $\text{Na}^{188}\text{ReO}_4$ (1-1.5 mL)
- (e) Monitoring of pH (1.5-3)
- (f) Agitation or incubation in boiling water for 30 min to 1 h.

3.1.2. Reduction of dicystein bond with ascorbic acid

- (a) 25 μg lanreotide (0.25 mL pH \sim 7)
- (b) Incubation/agitation with ascorbic acid (20 – 60 mg) at room temperature of 30 min to 1 h
- (c) Addition of SnCl_2 solution (2.5 – 5 mg) and tartaric acid (10-15 mg)
- (d) Mixing of $\text{Na}^{188}\text{ReO}_4$ (1-1.5 mL)
- (e) Monitoring of pH (1.5 – 3)
- (f) Incubation in boiling water for 1 h.

3.1.3. Reduction of dicystein bond with stannous chloride

- (a) 25 or 50 μg lanreotide (0.25 or 0.5 mL, pH \sim 7).
- (b) Agitation of lanreotide with tin tartrate [SnCl_2 2.5 – 5 mg/tartaric acid 7-15 mg] after addition of ^{188}Re for 15 min.

- (c) Addition of Na¹⁸⁸ReO₄ (1-1.5 mL)
- (d) pH was 2±0.2
- (e) Incubation in boiling water for 1 h.

The amount of radioactivity of ¹⁸⁸Re added varied from 111-370 MBq (3-10 mCi).

3.1.4. Radiochemical analysis

To determine the amount of unbound ¹⁸⁸Re, the sample was chromatographed on ITLC-SG (Gelman Sciences Inc, USA) using 0.9% saline as a mobile phase. Unbound ¹⁸⁸Re migrated with the solvent front. The radioactivity was determined by cutting the strips (1.5×10 cm) into 1 cm pieces and counting in a well type gamma counter. Sometimes 2π scanner (Berthold Germany) was used for scanning the radioactivity on strips. The amount of radiocolloid was determined using 85% acidified ethanol (pH 3.5) to develop the ITLC strip. In this system, colloidal material was retained at the origin.

For reverse phase analysis, C₁₈ cartridges (SepPak, Millipore Inc, USA) were used as reverse-phase adsorbents to evaluate the binding of the radionuclide to the lanreotide. Each cartridge was washed with 100% ethanol followed by 0.001 N HCl. Aliquots of 100 μL of the test sample were loaded onto the cartridge and the unbound material eluted with 10 mL of 0.001 N HCl. The cartridge was then serially eluted with a graded series of acidified ethanol solutions (10 mL of each). The radioactivity in each eluant fraction (0.001N HCl; ethanol solutions of 20,40,60,80, and 100% ethanol) was determined by gamma counting.

Conditions for analytical reverse-phase HPLC analysis are given below:-

Instrument: Hitachi L-6200 Intelligent pump, L-4200 UV-VIS detector. Eluted activity was monitored on Line using a NaI probe (raytest-Steffi), collected fractions were measured by well type gamma counter.

Column: RP-18 (5μm) Lichrosorb 25×0.45 cm

Gradient: Continuous gradient of acetonitrile (10-90%) and 0.1% aqueous trifluoro-acetic acid

Flow: 1mL/min

Inj. Vol: 20 μL

3.1.5. Cysteine challenge

Cysteine solutions were prepared fresh in phosphate buffer saline (PBS) and the pH adjusted to 7.4 with 1.0 M NaOH. Aliquots of 0.5 mL were diluted into separate vials to result in six dilutions. One vial contained only saline. To each vial was added 100 μL of ¹⁸⁸Re-Lanreotide. Samples were incubated for 45 min at 37°C. At the end of incubation period each sample was spotted on 1 TLC strip and chromatographed in PBS, pH 7.4. The chromatogram was developed, each strip was cut in half, and the radioactivity in each half determined. The amount of displacement was expressed as the percentage of total radioactivity associated with the solvent front. ¹⁸⁸Re displaced by cysteine migrated near the solvent front.

3.1.6. Stability of ¹⁸⁸Re-lanreotide

Radiolabelled peptide was kept at room temperature up to 24 h and the stability was checked by HPLC. Pre and post labelling addition of ascorbic acid effect was noted.

3.2. Labelling of lanreotide with $^{99}\text{Tc}^{\text{m}}$

Lanreotide in acetate buffer was reduced by addition of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ + tartaric acid with incubation at room temperature for certain period and a required radioactivity of $^{99}\text{Tc}^{\text{m}}$ was added. Finally the mixture was kept for 30 min in a boiling water bath. Various parameters, such as the mass ratio of peptide and stannous chloride, concentration of $^{99}\text{Tc}^{\text{m}}$ and incubation period for the reduction of the peptide were studied. After incubation in a boiling water bath, the reaction mixture was cooled and aliquots were taken for radiochemical analysis.

Radiochemical analysis of $^{99}\text{Tc}^{\text{m}}$ -lanreotide was based on similar techniques as mentioned in Sections 3.1.4 and 3.1.5 for ^{188}Re -lanreotide.

3.3. Labelling of lanreotide with ^{131}I

The most widely used radioiodination technique is that of Hunter and Greenwood. It was studied to label lanreotide with ^{131}I . Chloramine-T and iodogen were used as oxidizing agents to form some electrophilic iodine species.

3.3.1. Chloramine-T radioiodination

Radioiodination of lanreotide was performed by the method of Hunter and Greenwood with the modification that dilution with distilled water be used to replace meta bisulphite in order to inhibit competitive reactions with chemically sensitive peptide. The lanreotide (10 μg in 50 μL water), 50 μL of phosphate buffer (pH 6.8) and 10 μL of ~ 55 MBq no-carrier-added Na^{131}I were added to a small fusion tube. The reaction was initiated by the addition of 10 μg of chloramines-T (10 μL of phosphate buffer) and was allowed to proceed at room temperature for 5 min with occasional agitation. Reactions were terminated prior to purification by adding 0.5-1 mL of distilled water.

3.3.2. Iodogen radioiodination

Iodogen was dissolved in chloroform at a concentration of 0.1 mg/mL and plated onto the bottom of a test tube by evaporating the aliquot with a stream of nitrogen gas. To this tube lanreotide (10 μg in 50 μL water), 50 μL of phosphate buffer (pH 6.8) and 10 μL of ~ 55 MBq no-carrier-added Na^{131}I were added. The tube was mixed gently and allowed to stand at a room temperature for 30 min. The reaction mixture was then filtered through a glass wool plug to remove any flakes of iodogen that were dislodged from the walls of the test tube. An aliquot of phosphate buffer (0.5-1 mL) was added to rinse the test tube.

3.3.3. Radiochemical analysis of ^{131}I -lanreotide

The labelling yield and radiochemical purity were determined by paper chromatography. For chromatography method the reaction product was spotted on the Whatman No.1 paper and developed in a mixture of $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{OH}$, $\text{CH}_3\text{CH}_2\text{OH}$ and NH_4OH (v/v/v = 5:2:1). The R_f value of free ^{131}I is 0.5-0.6, while ^{131}I -lanreotide moves with solvent front (0.9-1.0). The purity of labelled peptide was also determined by using the already mentioned HPLC conditions in section 3.1.4.

3.3.4. Purification of ^{131}I -lanreotide

Purification was performed by using a SepPak C_{18} reversed phase extraction cartridge which was prewashed with 5 mL 70% ethanol and subsequently activated with 5 mL

2-propanol. After application of the sample, the cartridge was washed successively with 5 mM ammonium acetate, distilled water, 0.5 M acetic acid (5 mL, each) and finally radioiodinated lanreotide was eluted with 5 mL 96% ethanol. The radioactivities in the fractions eluted from SepPak C₁₈ were measured in a dose calibrator under similar geometric conditions. Removal of free ¹³¹I from labelled Lanreotide was also achieved by passing the reaction mixture through a very small column of anion exchange resin AG 1-X8, 100-200 mesh.

3.3.5. Stability of ¹³¹I-lanreotide

For comparative purposes purified solutions of ¹³¹I-lanreotide containing 10 mg ascorbic acid and without ascorbic acid were monitored by HPLC for a period of five days.

3.4. Preparation of biotinyl-hydrazino-EDTA (EDTA-B₁)

Biotinyl-hydrazino-EDTA was prepared by Virzi, et al. method [18]. To a stirred solution of biotin hydrazide (50 mg, 0.194 mmol) in 20 mL of DMF/CHCl₃ (1:1, v/v) at 65 °C was added 20 mL of DMF/CHCl₃ (1:1, v/v) containing EDTA bicyclic anhydride (65.0 mg, 0.254 mmol). The mixture was stirred for 30 min, chloramine by the addition of 0.5 mL of water and stirred for additional 10 min. The reaction mixture was poured into 300 mL of CH₂Cl₂ and then refluxed briefly (1-2 min), cooled to room temperature, and the white solid was filtered, immediately washed with CH₃CN, dried, redissolved in 1 mL of 0.2 M sodium bicarbonate pH 8.5, and passed through a 1.4×120 cm gel filtration column of P-2 (Bio-Rad) with water as eluent. The quantitation of biotin and EDTA was carried out, the compound decomposed at 196-199°C.

3.4.1. Labelling of biotinyl-hydrazino-EDTA with ¹⁵³Sm

Solutions of EDTA-B₁ (100 µg /mL) in 1 M sodium acetate, pH 6.0 were labelled with ¹⁵³Sm at a specific activity of 6300 MBq/mg. Different molar ratio of EDTA-B₁ and ¹⁵³Sm were used to determine the labelling yield of EDTA-B₁ with ¹⁵³Sm. Effect of temperature and incubation time on labelling yield was also determined.

3.4.2. Quality control of ¹⁵³Sm-EDTA-B₁

Paper chromatography was used to assess the quantities of labelled compound and ¹⁵³SmCl₃. Whatman 3 MM paper strips were used as support, whereas ammonia:methanol:water (0.2:2:4) was employed as a solvent system. Free ¹⁵³SmCl₃ stayed at the origin and ¹⁵³Sm-EDTA-B₁ migrated with the solvent front. Binding of ¹⁵³Sm-EDTA-B₁ was determined by incubation for 15 min of an aliquot of the 10 µL ¹⁵³Sm-EDTA-B₁ with 10 µL of a solution of avidin in PBS (>15 fold excess) and by analysis of the bound fraction by means of TLC. Thin layer chromatography was carried out on silica gel-impregnated glass fibre sheets (Gelman Sciences Inc., ITLC-SG) with 0.1 M sodium citrate as eluent. Samples of 1-5 µL were loaded on 12×1.5 cm strips, and after elution for 5 min the strips were scanned by 2π scanner, (Berthold, Germany).

For HPLC analyses a 25 cm Lichrosorb 10 RP 18 column was used. For determination of radiolabelled species, the eluent consisted of a 95:5 mixture of EtOH and 0.01 M sodium phosphate buffer (pH 6) solution (eluent A). A was gradually replaced by a 9:1 mixture of MeOH and water (eluent B). The gradient was, as follows: 5 min 100% eluent A at 1 mL/min; linear increase of B to 70% during 15 min at 1 mL/min; 5 min 70% eluent B at 1 mL/min.

3.5. Labelling of biotinyl-hydrazino-EDTA (EDTA-B₁) with ¹⁸⁸Re

In order to label a biotin derivative with ¹⁸⁸Re, stannous ion was used to reduce perrhenate to lower oxidation state and weak chelating agent glucoheptonate as stabilizer and transchelating agent. Following formulation was employed to determine the labelling yield of EDTA-B₁ with ¹⁸⁸Re. EDTA-B₁(100 µg) in 0,5 mL of acetate buffer (pH 6), 200 mg sodium glucoheptonate, 10 mg gentisic acid, 3 mg anhydrous SnCl₂ and 0.5 mL Na¹⁸⁸ReO₄ (185 MBq) were mixed thoroughly. The reaction vial was evacuated before it was kept in boiling water bath for one hour.

3.5.1. Quality control of ¹⁸⁸Re-EDTA-B₁

To determine the amount of unbound ¹⁸⁸Re, the sample was chromatographed on ITLC-SG (Gelman Sciences Inc., USA) using 0.9% saline as a mobile phase. Unbound ¹⁸⁸Re migrated with the solvent front. The radioactivity was determined by cutting the strips (1.5× 10 cm) into 1cm pieces and counting in a well type gamma counter. Sometimes 2π scanner (Berthold, Germany) was used for scanning the radioactivity on strips. The amount of radiocolloid was determined using 85% acidified ethanol (pH 3.5) to develop the ITLC strip. In this system, colloidal material was retained at the origin. Details of HPLC technique for the analyses of different species generated during labelling procedure are already described in Section 3.4.2.

3.6. Studies related to development of ⁹⁰Sr → ⁹⁰Y generator

3.6.1. Adsorbents

The following adsorbents were studied:

- Aluminum oxide-90 active basic, particle size 70-230 mesh ASTM (Merck)
- Silica gel 70-230 mesh, 60 Angstrom for column chromatography (Aldrich)
- SnO₂ (hydrous) was prepared by the reaction of high purity tin metal with hot 35% W/W HNO₃. The precipitate obtained was insoluble in dilute solutions of H₂SO₄ and HCl a property typical of the material called β-stannic acid. This precipitate was filtered and washed thoroughly with distilled water until the pH of washings was about 5-6. The product was air dried at 30 °C to constant weight.
- SnO₂ powder (Merck)
- MnO₂ (hydrous) was prepared by adding a hot solution (~90 °C) of manganese sulphate (71.4 g /L) to a potassium permanganate solution (21.3 g/L). The resulting manganese dioxide was washed with distilled water, dried at 60 °C and sieved. The fraction of 200-400 mesh was used in the experiments.
- Cellulose for column chromatography (Merck)
- Charcoal activated, 0.5 – 0.8 mm particle size (BDH)
- Hydrous zirconium oxide (HZO) ion exchange crystals, 100-200 mesh (Bio-Rad)
- Titanium molybdate (TIM) was prepared by mixing an aqueous solution of TiCl₄ with a solution of ammonium molybdate with stirring. Precipitate was filtered, washed and dried. Dried TIM was ground and sieved. 100-200 mesh TIM was used.
- ⁸⁵Sr and ⁹⁰Y tracers were prepared by irradiation of Sr(NO₃)₂ and Y₂O₃ targets in the core of Pakistan Research Reactor – I.

3.6.2. Batch method

Distribution coefficients (K_d) were measured by means of a batch equilibrium method using ^{85}Sr and ^{90}Y as radiotracers at room temperature. 100 mg adsorbent was added to 6 mL solution containing the tracer. The pH of the solutions was adjusted to the desired value by adding HCl or NaOH solution. After one hour of continuous agitation, the solutions were centrifuged and the activities were measured by means of a well type gamma counter, and the distribution coefficient was calculated.

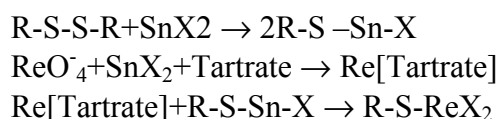
3.6.3. Column method

1-2 gm of adsorbents such as Al_2O_3 , SnO_2 , Silica gel, HZO were placed in glass columns. Adsorbents were treated with appropriate solvents before loading of an equilibrium mixture of ^{90}Sr and ^{90}Y (μCi level). Different eluants and their compositions were tried to separate ^{90}Y from ^{90}Sr .

4. RESULTS

4.1. ^{188}Re -lanreotide

Lanreotide is a cyclic peptide (D- β -Nal-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH₂), hence direct labelling with $^{99}\text{Tc}^m$ and $^{188}\text{Re}/^{186}\text{Re}$ is applicable. In this method the dicystein bond is reduced with stannous chloride. The sulphahydryls resulted by the reduction serve as strong chelating groups for reduced ^{188}Re . Tartrate serves as transchelating agent. The involvement of tin in protein labelling as tin-protein intermediate was suggested by Rhodes [18] for "pretinning" procedure when the reduction of native disulfide bridges was carried out by incubating the protein with stannous ions. Direct labelling of peptides with ^{188}Re involves, in brief: (1) application of a reducing agent to cleave disulfide bonds in order to expose cysteines residues on the peptide; (2) reduction of ^{188}Re to the required reduced states with the reducing agent, mainly stannous chloride, in the presence of an appropriate complexing agent with simultaneous complexation of reduced ^{188}Re ; and (3) transchelation of reduced ^{188}Re from the intermediate complex to the cysteine residues on the peptide.



Paik, et al. [19] proved the existence of both high-and-low-affinity binding sites by measuring the amount of protein labelling in the presence of varying concentration of DTPA, a strong chelator of $^{99}\text{Tc}^m$ metal ions. They titrated the free sulfide groups, and as a result, suggested that the high affinity binding was related to the presence of these groups. They also suggested that the pretinning procedure was increasing the number of high affinity binding sites, i.e. the number of reactive sulfides.

A typical elution profile of ^{188}Re -lanreotide from an analytical HPLC at 30 min post labelling is shown in Fig.1. The overall RP-HPLC elution did not change at 24 h post-labelling. Step gradient elutions of ^{188}Re -lanreotide on C_{18} cartridges were used to confirm the results obtained by analytical HPLC (Fig. 2). Results from ITLC with saline or ethanol as the mobile phases also indicated a high radiolabelling yield. Less than 1% of the ^{188}Re was found to migrate in saline with the solvent front. The amount of radiocolloid was examined by ITLC using 85% acidified ethanol (pH 3.5) as a mobile phase and was frequently less than 4%. The

stability of the Re-peptide bond was evaluated by cysteine challenge studies (Fig. 3). The amount of cysteine required to displace 50% of the ^{188}Re from the radiolabelled peptide was approximately 40 mM.

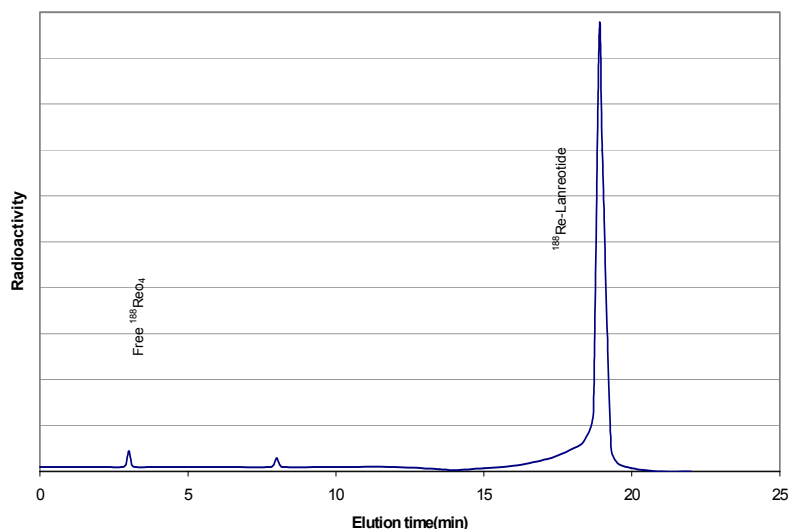


FIG. 1. Elution profile of ^{188}Re -lanreotide at 30 min post labelling from a C_{18} RP-HPLC column.

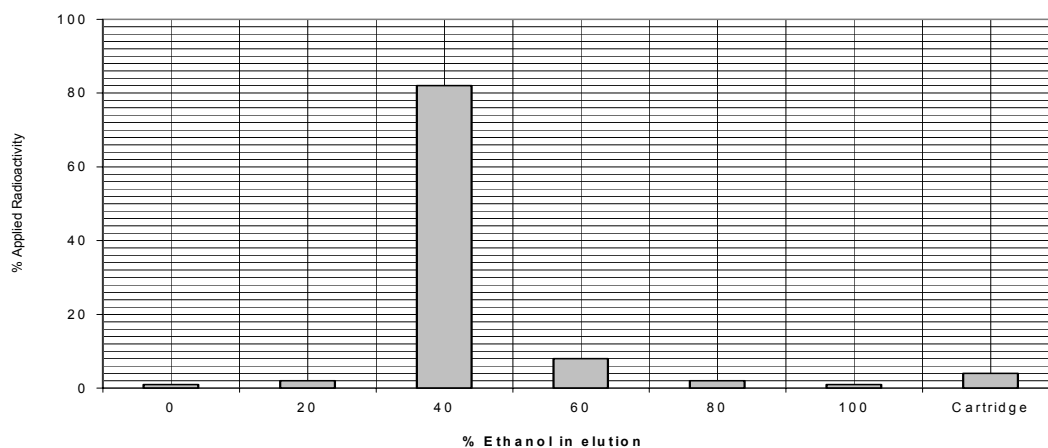


FIG. 2. Elution profile of ^{188}Re -lanreotide from a C_{18} cartridge using a step gradient of acidified ethanol.

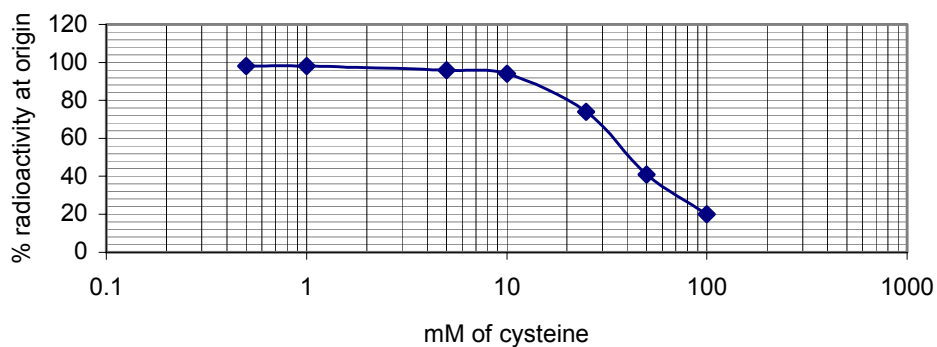


FIG. 3. Displacement of ^{188}Re from ^{188}Re -lanreotide using cysteine as a challenge test.

Once radiolabelled the preparations were reasonably stable for periods of time up to 24 h post-labelling. The order of addition of the ascorbic acid solution in the labelling procedure was found to be critical. Addition of ascorbic acid (40 mg) after labelling may result in stabilization [20]. In our case, the effect of adding ascorbic acid could not be studied because preparations were found to be stable up to 24 h without addition of ascorbic acid. However, when the same amount of ascorbic acid was added prior to addition of the ^{188}Re , lanreotide was not effectively labelled.

Labelling of lanreotide with ^{188}Re varied from 30-70% when dicysteine bridge was reduced with 2-ME. Similarly, when reduction was carried out with ascorbic acid the labelling yield varied from 20-50%.

4.2. $^{99}\text{Tc}^{\text{m}}$ -lanreotide

Based on the results of initial experiments 50 μg peptide was used to determine the labelling of lanreotide with $^{99}\text{Tc}^{\text{m}}$ lanreotide (50 μg) was dissolved in one mL acetate/acetic acid (pH 2.8) buffer. Stannous chloride/tartaric acid solution (100 μL) containing 50 μg $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ and 32 μg tartaric acid were added and incubated for various time intervals at room temperature. After a certain period of incubation, freshly eluted pertechnetate solution (37-74 MBq/mL) was added and vortexed. Finally the reaction vial was kept in a boiling water bath for 30 min. After a hr incubation period the labelling yield was 15%, which gradually increased with increase in incubation period. A 5 h incubation period resulted in 80% labelling of lanreotide with $^{99}\text{Tc}^{\text{m}}$. To get a labelling yield of >97% the reaction vial containing peptide and stannous tartrate in acetate buffer was kept overnight (~18h) at room temperature. In each experiment the vial containing the reaction mixture was sealed under vacuum.

The mass ratio of peptide and stannous chloride had a significant effect on the labelling of the peptide with $^{99}\text{Tc}^{\text{m}}$. The best results obtained were near the 1:1 mass ratio of peptide and stannous chloride. When the amount of stannous chloride was doubled, the labelling yield was decreased to 30%. Similarly the labelling yield was also decreased when the mass ratio of peptide and stannous chloride were 1:0.5 or 1:0.75.

The final preparation contained 100 μg Lanreotide, in 1mL acetate/acetic acid buffer (pH 2.8), stannous chloride (100 μg), tartaric acid (64 μg). After overnight incubation at room temperature, 1mL freshly eluted $^{99}\text{Tc}^{\text{m}}$ (12 mCi/444 MBq) was added to the reaction vial and finally, the vial was heated in a boiling water bath for 30 min. A typical elution profile of $^{99}\text{Tc}^{\text{m}}$ -Lanreotide from an analytical HPLC at 30 min post labelling is shown in Fig.4. No change in the elution profile of $^{99}\text{Tc}^{\text{m}}$ -lanreotide was observed at 6 h post labelling when analytical RP-HPLC profiles revealed one primary peak, which accounted for approximately 95% of the radiolabelled material. Step gradient elutions of $^{99}\text{Tc}^{\text{m}}$ -lanreotide on C_{18} cartridges were used to confirm the results obtained by analytical HPLC (Fig. 5). The results from ITLC with saline or acidified ethanol as the mobile phases also indicated a high radiolabelling yield. The amount of radiocolloid was determined by ITLC-SG using acidified ethanol (85%, pH=3.5) as the mobile phase and were frequently less than 1%; less than 2% of the $^{99}\text{Tc}^{\text{m}}$ was found to migrate in saline with the solvent front. The stability of the $^{99}\text{Tc}^{\text{m}}$ peptide bond was evaluated by cysteine challenge tests. The amount of cysteine required to displace 50% of the $^{99}\text{Tc}^{\text{m}}$ from the labelled peptide was approximately 50 mM (Fig. 6).

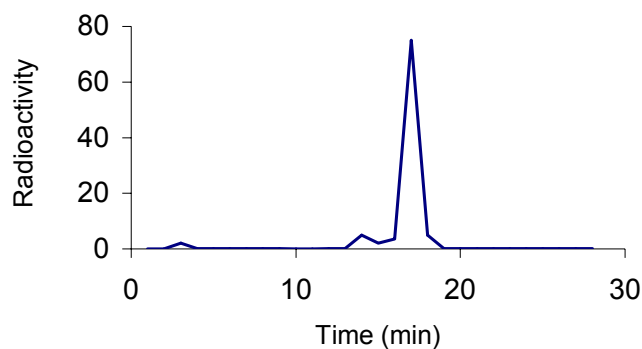


FIG. 4. Elution profile of $^{99}\text{Tc}^m$ -lanreotide at 30 min post labelling from a C_{18} RP-HPLC column.

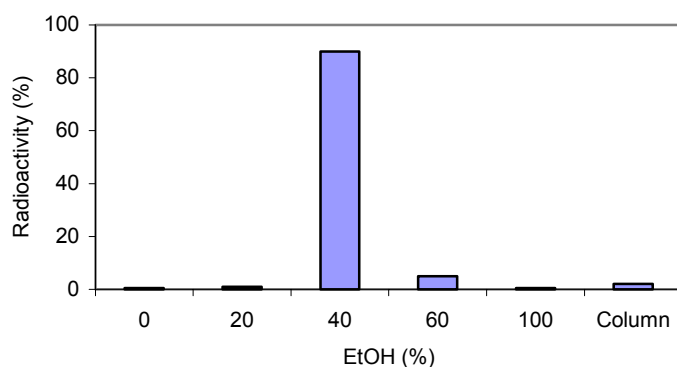


FIG 5. Elution profile of $^{99}\text{Tc}^m$ -lanreotide from a C_{18} cartridge using a step gradient of acidified ethanol.

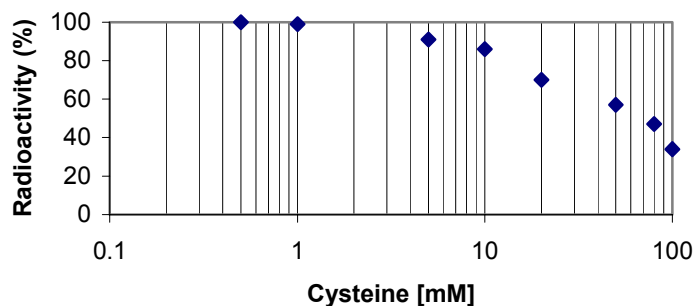


FIG. 6. Displacement of $^{99}\text{Tc}^m$ from $^{99}\text{Tc}^m$ -lanreotide using cysteine as a challenge test.

4.3. ^{131}I -lanreotide

The radiolabelling yield varied between 40-80% using chloramines-T or iodogen method. However ~25% of the initial iodine activity was adsorbed on iodogen coating. Hence further studies were carried out with the product obtained by chloramines-T method. A typical elution profile of ^{131}I -Lanreotide from an analytical HPLC at 20 min post purification is shown in Fig. 7. Step gradient elutions of ^{131}I -Lanreotide on C_{18} cartridges were used to confirm the results obtained by analytical HPLC (Fig. 8). The radiolabelled ^{131}I -Lanreotide very rapidly degraded. To slow the rate of degradation ascorbic acid was added. Comparative

stability with and without ascorbic acid of iodine-131 labelled lanreotide is shown in Fig. 9. The addition of ascorbic acid resulted in a preparations with >90% stability at 24 h post purification. Purification of radiolabelled peptide using anion exchange resin reduces free ^{131}I <2%.

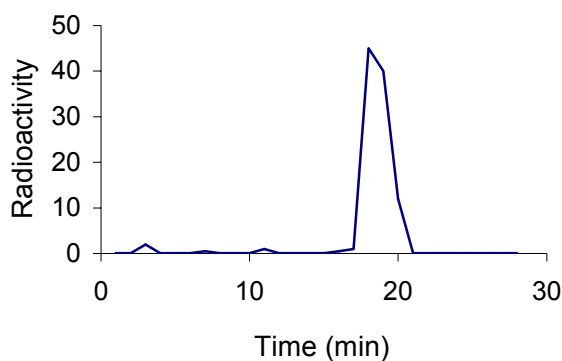


FIG. 7. Elution profile of ^{131}I -lanreotide at 20 min post purification from a C_{18} RP-HPLC column.

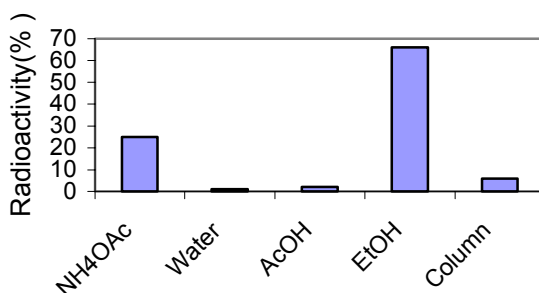


FIG. 8. SepPak C_{18} elution pattern after radioiodination of lanreotide.

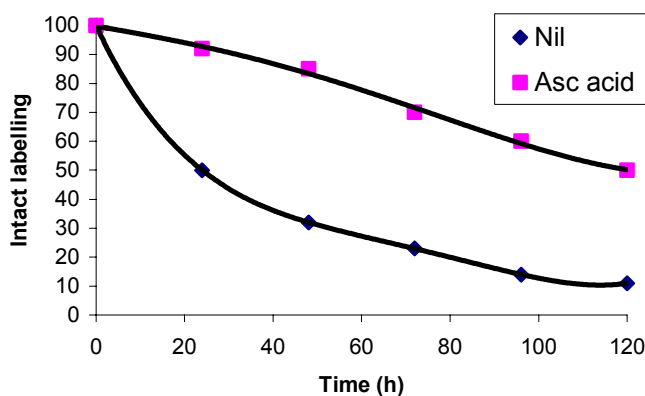


FIG. 9. Comparative stability with and without ascorbic acid of ^{131}I -lanreotide.

4.4. ^{153}Sm -EDTA- B_1

To achieve a labelling efficiency of >90% a molar ratio of 1:15 (EDTA- B_1 :Sm) was essential. The effect of molar ratio of EDTA- B_1 :Sm is shown in Fig. 10. Labelling of EDTA- B_1 with ^{153}Sm was quicker in boiling water than at ambient temperature (Fig. 11). After 30

min incubation in boiling water the labelling yield reached up to 96%, whereas same labelling efficiency was noted after 1 h incubation at room temperature. A typical elution profile of $^{153}\text{Sm-EDTA-B}_1$ from an analytical HPLC is shown in Fig.12. The labelling yield determined by paper chromatography and HPLC was in good agreement. Binding of $^{153}\text{Sm-EDTA-B}_1$ to avidin was determined by TLC. R_f values were 0.0-0.3 for the labelled biotin-avidin complex and 0.7-1.0 for the labelled unbound conjugate. The binding capacity of $^{153}\text{Sm-EDTA-B}_1$ to avidin ranged from 50 to 65%.

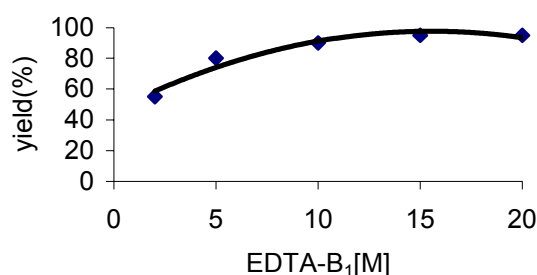


FIG. 10. Complexation of $^{153}\text{Sm-EDTA-B}_1$ with different molar ratios of EDTA-B1 and ^{153}Sm .

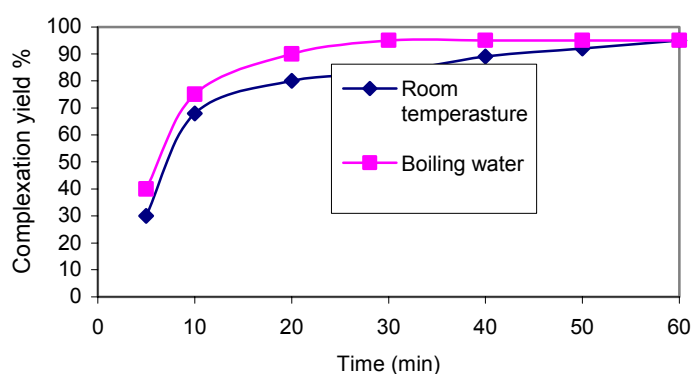


FIG. 11. Rate of formation of $^{153}\text{Sm-EDTA-B}_1$.

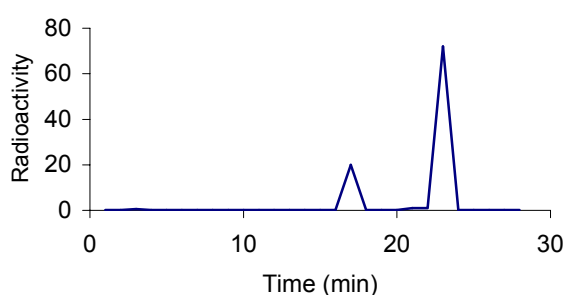


FIG. 12. Elution profile of $^{153}\text{Sm-EDTA-B}_1$ from a C_{18} RP-HPLC column.

4.5. $^{188}\text{Re-EDTA-B}_1$

The results from ITLC with saline or acidified ethanol as the mobile phases indicated a high radiolabelling yield (95%). The amount of radiocolloid was <4%, and amount of free ^{188}Re was <0.5%. A typical elution profile showed two distinct peaks, amounting 80% and 20% (Fig. 13). The binding capacity of labelled biotin to avidin was 70-80%.

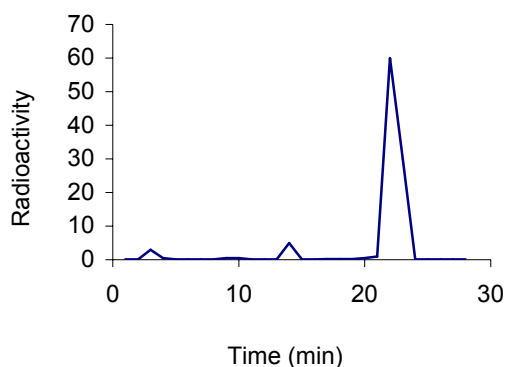


FIG. 13. Elution profile of $^{188}\text{Re-EDTA-B}_1$ from a C_{18} RP-HPLC column.

4.6. Development of $^{90}\text{Sr} \rightarrow ^{90}\text{Y}$ generator

The distribution coefficients of Sr and Y as a function of pH on various adsorbents (exchangers) are presented in Table I. In most of the cases Y is more firmly adsorbed than the Sr. In basic media Al_2O_3 , silica gel, SnO_2 (hydrous) and TIM (titanium molybdate) show significant K_d values for Sr, along with Y. Therefore in column experiments selective desorption of ^{90}Y was tried by using different concentrations of eluants. These attempts were unsuccessful to elute pure ^{90}Y from ^{90}Sr . Further investigations are warranted to make a successful generator for the separation of ^{90}Y from its parent ^{90}Sr .

TABLE I. DISTRIBUTION COEFFICIENTS (K_D VALUES ML/G) OF SR AND Y ON DIFFERENT ADSORBENTS

pH→ Adsorbent	1	2	4	6	7	8	9	10
Al_2O_3 basic	-	-	1300	1500	1800	2000	3000	-
	-	1	1751	261	219	213	260	-
Silica gel	6	7	9	9	8	-	140	300
	-	1	2	40	54	57	53	-
SnO_2	6	8	8	9	11	9	12	-
	-	1	7	8	9	9	9	-
SnO_2 (hydrous)	5	7	8	9	7	-	115	300
	-	2	96	40	50	45	50	-
MnO_2	2	1	2	-	-	-	-	-
	1	16	174	213	213	213	213	470
Cellulose	-	-	-	-	-	-	-	-
	4	4	4	15	28	28	146	478
Charcoal	1	5	6	-	5	-	6	-
	4	3	15	20	27	20	10	-
HZO	1	4	10	-	9	20	-	-
	2	2	49	50	50	50	50	50
TIM	4	4	5	6	5	-	19	110
	1	5	40	43	60	50	98	178

K_d values of "Sr" first row

K_d values of "Y" second row

5. CONCLUSIONS

Lanreotide was labelled with $^{188}\text{Re}/^{99}\text{Tc}^{\text{m}}$ using direct labelling method. Labelling was accomplished by reduction of the cysteine bridge, which provided sulfhydryl groups for chelation with $^{188}\text{Re}/^{99}\text{Tc}^{\text{m}}$. Stannous chloride was used as reducing agent, while tartrate acted as transchelating agent. More than 95% labelling efficiency was confirmed by RP-HPLC, ITLC-SG and C_{18} cartridge analysis. For labelling of lanreotide with ^{131}I chloramines-T method was found superior over iodogen method. Simple procedures for labelling of EDTA- B_1 with ^{153}Sm and ^{188}Re were developed. Both radiolabelled EDTA- B_1 showed high binding capacity to avidin. Receptor binding studies are essential to evaluate the binding affinity of these radiolabelled compounds. Further experiments are warranted for the development of $^{90}\text{Sr}\rightarrow^{90}\text{Y}$ generator based on high radiation resistant inorganic adsorbents.

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DEVELOPMENT OF ^{188}Re RADIOLABELLING PROCEDURES OF PEPTIDES AND MONOCLONAL ANTIBODIES

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Abstract

In this report are described ^{188}Re radiolabelling methods of anti-CEA Mab and lanreotide 8mer using stannous ions from stannous chloride and stannous tartrate as reducing agents for ^{188}Re -perrhenate. Radiolabelling was done with 2220-2775 MBq (60-75 mCi) with a high labelling efficiency. *In vivo* stability and tumour affinity were evaluated using tumour bearing rats. A high binding affinity was observed from studies regarding the binding of ^{188}Re -anti CEA-Mab to CEA molecules and the binding of ^{188}Re -lanreotide to the somatostatin receptors.

1. INTRODUCTION

The high specificity of antibodies for antigens and the peptides for receptors determine the new candidate molecules for radiolabelling and new cancer radiotherapy radiopharmaceuticals obtained. We selected the following biomolecules for ^{188}Re labelling in this study.

- Anti-carcinoembryonic antigen monoclonal antibody (anti-CEA Mab), which reacts specifically with human carcinoembryonic antigen (CEA) from several types of malignant tissues, including colorectal, lung and breast tumours
- Lanreotide 8 mer, somatostatin analogue which reacts specifically with somatostatin receptors.

The goals of this project were:

- ^{188}Re biomolecules labelling with a high labelling efficiency
- quality control of labelled biomolecules
- studies *in vivo* regarding post-labelling stability and biospecificity of biomolecules using bearing tumour rats
- studies *in vitro* regarding the strength of Re to peptide bound
- binding of ^{188}Re anti-CEA Mab to CEA and binding of ^{188}Re -lanreotide to somatostatin receptors.

2. MATERIALS AND METHODS

Studies were performed regarding the kinetics of biomolecule radiolabelling process, focusing on: stannous ions concentration, biomolecule/ Sn^{2+} massic ratio, temperature and time incubation, antioxidant excipients and free radical scavengers. Most of the results were report in the 1st RCM in 1998 in Milan, Italy and in the 2nd RCM in 2000 in Mumbai, India. The reaction conditions for high efficiency labelling were selected and optimized taking account the obtained results and the IAEA recommendations.

In this report we present the labelling methods of biomolecules in our optimization. High quality chemical and biological reagents were used: Lanreotide 8mer, cys2-7, cyclo and DOTA-lanreotide (from piChem Research and Development, Austria); anti-human carcinoembryonic antigen (from Sigma), ELISA, CEA Serozyme (from Biochem. Immunosystem, Italy), ^{188}Re carrier free eluted from a ^{188}W - ^{188}Re generator (3700 MBq; 100

mCi) with normal saline (10 mL) according to instructions from MAP Medical Technologies Oy, Finland.

2.1. ¹⁸⁸Re-radiolabelling of lanreotide 8mer

The preparation of lanreotide-Sn(II) in lyophilized form was carried out in aseptic conditions. The following procedure was developed: 200 µg lanreotide dissolved in 1 mL 0.05 M Na tartrate and 0.9 mL of 0.01 M stannous tartrate (1 mg in stannous ions) was bubbled 15 min with N₂ gas. As conditioner excipient was added 10 mg myo-inositol. The final volume was 2 mL and 2.7-3 pH. The vials were sealed after lyophilization under inert gas (argon) and stored at 2-8°C. The lyophilized lanreotide-Sn(II) samples were labelled by adding 2220-2590 MBq (60-70 mCi) in 4-5 mL ¹⁸⁸Re-perrhenate solution and 90°C incubation for 45 min. 1 mL of 0.1 M Na ascorbate was added by Millipore filter after cooling to room temperature; a 5-6 final pH is reached.

2.2. ¹⁸⁸Re-radiolabelling of DOTA-lanreotide

DOTA-lanreotide (MAURITIUS) bioconjugate for indirect radiolabelling method was used. To 100 µg DOTA-lanreotide in 0.3 mL of 0.1 M CH₃-COONa was added 1.33 mg stannous ions in 1 mL tartrate buffer pH 4.5 under bubble nitrogen gas. The final solution pH was adjusted to 4.5. and the solution stabilized 30 min under bubble nitrogen. Vials containing 2 mL solution were freeze-dried. The ¹⁸⁸Re radiolabelling of Sn(II)-DOTA lanreotide by 4-5 mL adding of ¹⁸⁸Re-perrhenate (2220-2590 MBq; 60-70 mCi) followed by 1 hour incubation at 90 °C was done.

2.3. ¹⁸⁸Re-radiolabelling of antiCEA-Mab

Samples of 100 µg antiCEA-Mab in H₂O and 400 µg ascorbic acid in 200 µL water were incubated for 2 h to 22°C for the prerreduction of the –S-S- bounds to –SH groups. 800 µL stannous chloride dihydrate solution (0.2 g SnCl₂·2H₂O and 2g citric acid in 100 mL water) was added and the pH was adjusted to 5 with 0.1 M sodium citrate, followed by 30 min incubation at 22°C, in N₂ atmosphere. Then, the solution pH was adjusted to 5.5 with 1M Na tartrate. Vials containing 2mL samples were lyophilized and then reconstituted with 2405-2701 MBq (65-73 mCi) in 5 mL ¹⁸⁸Re perrhenate for ¹⁸⁸Re-radiolabelling, 3 h incubation time at 37°C.

3. EXPERIMENTAL RESULTS

After the powder sample reconstitution and radiolabelling with 4-6 mL ¹⁸⁸Re-perrhenate (2220-2590 MBq; 60-70 mCi) *in vitro* and *in vivo* analyses were achieved for each product characterization.

3.1. Radiochemical analyses

The reducing yield of perrhenate to stannous ions concentration was established by Whatman 1 paper chromatography in acetone solvent. Between 0.6-1 mg concentrations range (Fig. 1) there is the maximum of reducing yield. The concentrations of this above range are used in the labelled products preparation.

For radiochemical purity of final labelled products by Whatman 1 paper chromatography method various solvents has been used (Table I).

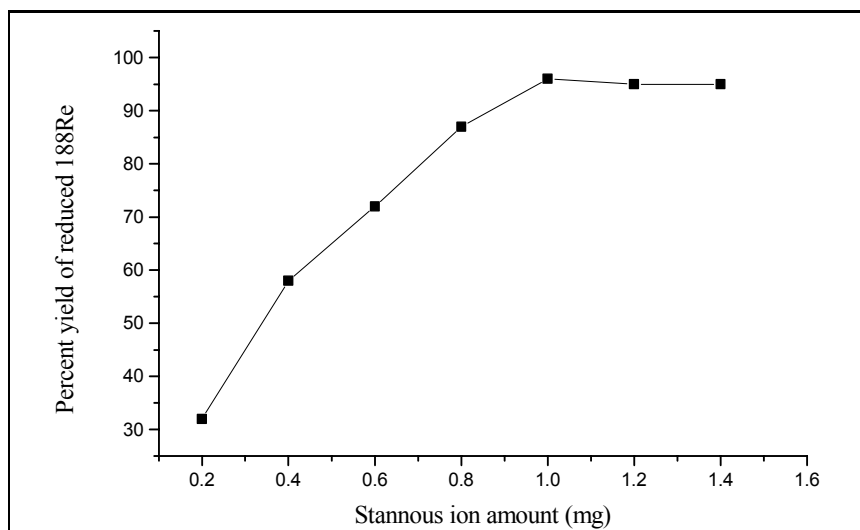


FIG. 1. Effect of stannous ions quantity on the reducing yield of ¹⁸⁸Re-perrhenate.

TABLE I. CHROMATOGRAPHIC SOLVENTS AND R_F ESTABLISHMENT

Solvent	R _f				
	ReO ₄ ⁻	Re (Red)	Re-Lanreotide	Re-DOTA-Lanreotide	Re-Anti-CEA
85% ethanol pH 3.5	0.61-0.72	0.00-0.10	0.80-1.00	0.80-1.00	-
Acetone	0.80-1.00	0.00-0.15	0.00-0.15	0.00-0.15	0.00-0.10
Et-ol:NH ₃ :H ₂ O (2:1:5)	0.70-0.80	0.00-0.05	0.80-0.95	0.80-0.95	0.80-1.00

The radiochemical purity has been calculated by subtraction, using two solvents for the same sample. Also we used the Sephadex G-25 gel chromatography method coupled with spectrometric and radiometric methods for each fraction (Figs 2 and 3).

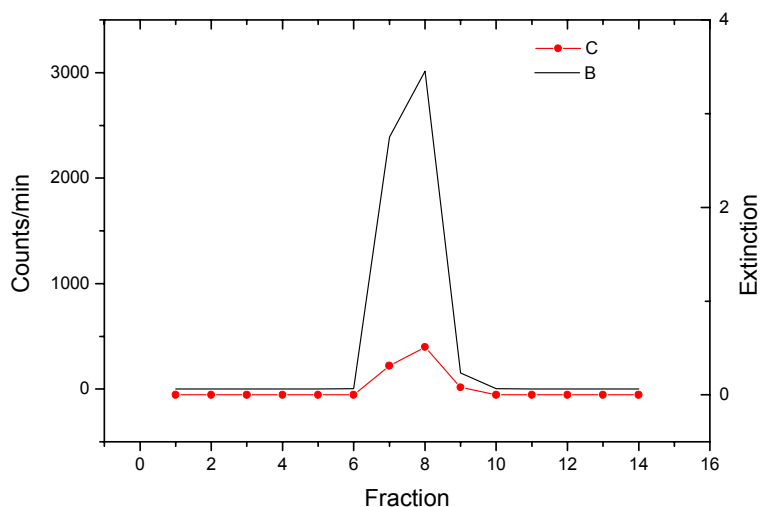


FIG. 2. The radioactive (— B, $\times 10^4$ counts) and 280 nm UV absorption (—●— C) measurements of gel chromatography ¹⁸⁸Re-antiCEA fractions.

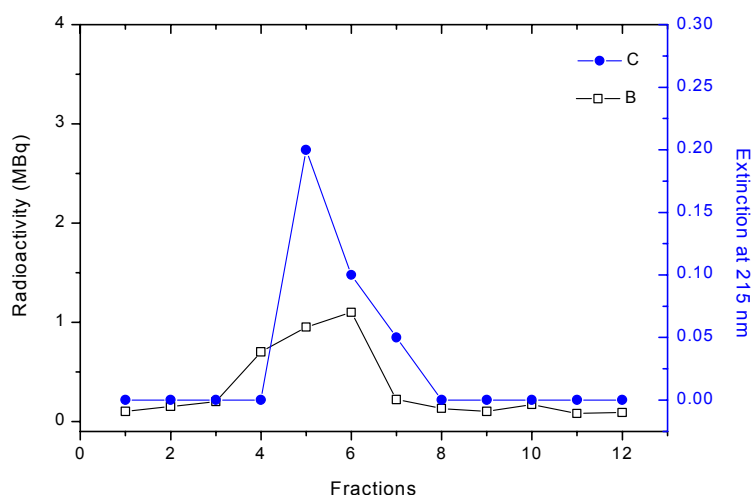


FIG. 3. The radioactive (—●—C) and 215 nm UV absorption (—□—B) measurements of gel chromatography fractions.

Table II presents the typical parameters for each product in powder form and the radiochemical purities for the labelled products.

TABLE II. THE MAIN PARAMETERS OF LYOPHILIZED AND LABELLED PRODUCTS

Product	Sn ²⁺ (mg)	Biomolecule (μg)	Labelling pH	Incubation time (min)	Final pH	Radiochemical purity
¹⁸⁸ Re-lanreotide	1 (as tartrate)	200	3.5-4	45	5 – 6	94 – 97
¹⁸⁸ Re-DOTA-lanreotide	1.33 (as tartrate)	100	4.5	60	5 – 5.5	85 – 98
¹⁸⁸ Re-antiCEA-mab	0.8 (as chloride)	100	5.5	180	5.5 – 6	94 – 98

3.2. Cysteine displacement of ¹⁸⁸Re from ¹⁸⁸Re-lanreotide and ¹⁸⁸Re-DOTA-lanreotide

In vitro stability of ¹⁸⁸Re-peptide has been studied in presence of cysteine (concentration range: 0.1-100 mM [4]). Fresh solutions of cysteine were prepared in phosphate buffer, pH = 7.4. Aliquots of 100 μL cysteine solution with increasing concentrations were mixed with 100 μL (37 MBq) ¹⁸⁸Re-peptides and incubated for 1 hour at 37°C. After incubation the samples were chromatographed in phosphate buffer pH 7.4. Rhenium displaced by cysteine, migrated with the R_f = 0.9-1. Fig. 4 shows that starting with 10 mM cysteine concentration the percent of ¹⁸⁸Re suddenly increases.

3.3. Receptor binding studies using rat brain cortex membrane

The biospecificity of ¹⁸⁸Re-lanreotide biomolecules for expressive somatostatin receptors was tested using the methods described in the research references [1–3] and the recommendations of IAEA Vienna.

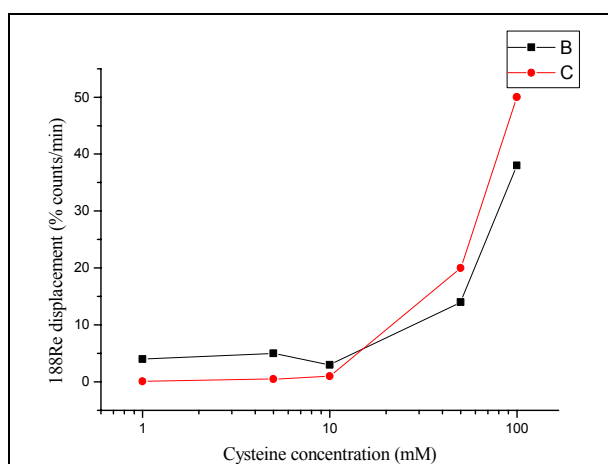


FIG. 4. Displacement percentage of ^{188}Re from ^{188}Re lanreotide (C) and ^{188}Re -DOTA-lanreotide (—B) using increasing concentrations of cysteine.

Receptor binding assays were performed in triplicate test tubes using cortex membrane equivalent to 40 μg protein in 50 μL Tris buffer pH 7.5 containing 5 mM MgCl_2 , 20 mg/L bacitracin, 0.25 M PMSF, 100,000 KIU/L aprotinin and 1000 i.u./mL Rnase inhibitor. 150 μL solution containing unlabelled lanreotide with concentration between 10^{-12} to 10^{-3} M and 22,000 cpm ^{188}Re -lanreotide traces was added to each tube. The obtained samples were gentle mixed and incubated for 45 min to 22 $^\circ\text{C}$. After incubation the samples were rapid filtered by Whatman GF/C filters preliminary treated with 0.1% BSA in 0.9% NaCl. After separation the filters were washed with 1 mL 0.2% BSA in 50 mM Tris buffer 7.5 pH. The radioactivity of the filters and filtrates was counted using gamma spectroscaler counter. Samples without receptors were prepared in the same time only with 150 μL lanreotide solution (22,000 cpm) for determining the percent of radioactivity retained in the filters, which is proved to be 1.2%. The binding to receptors of ^{188}Re -lanreotide and ^{188}Re -DOTA lanreotide are given in Fig. 5. Maximum binding of ^{188}Re -lanreotide to receptors is in 10^{-11} - 10^{-8} mol/L range. For ^{188}Re -DOTA-lanreotide there is not a distinguishable binding level.

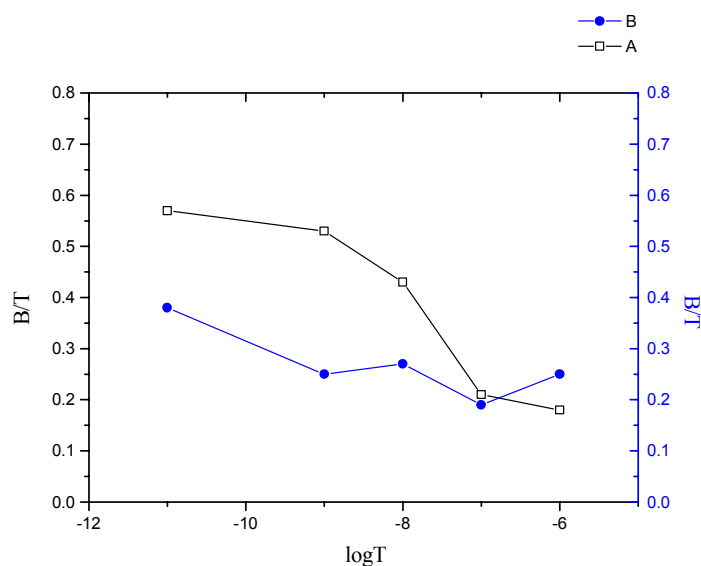


FIG. 5. Binding curves of ^{188}Re -lanreotide (A) and ^{188}Re -DOTA-lanreotide (B); T is the added lanreotide concentration in mol/L; B is the concentration of ^{188}Re -lanreotide bounded to the somatostatin receptors.

3.4. Evaluation of immunoreactivity of ^{188}Re -antiCEA Mab

The immunoreactivity of ^{188}Re -antiCEA Mab was evaluated using the ELISA CEA Serozyme kit. This kit is used for the assessment of carcinoembryonic antigen (CEA) from human ser. Theoretically, the method follows the binding of CEA with two carcinoembryonic antibody enzyme (antiCEA^E) and fluorescein labelled (antiCEA^{FITC}) in *sandwich* complex (Ab^E-Ag-Ab^{FITC}). Our experiments investigate the competition binding of ^{188}Re -antiCEA-Mab in antiCEA^E-CEA-antiCEA^{FITC} *sandwich* complex to CEA increasing concentrations. The method was modified and adapted in next manner: to each tube of 100 μL CEA standard solution with 0.5; 13; 33; 67; ng/mL concentrations were added: 100 μL antiCEA^{FITC}; 100 μL antiCEA^E and 10 μL ^{188}Re -antiCEA (10,000 counter/min) The samples obtained are incubated at 37°C, 30 min time, for immunological reaction. 200 μL of separation reagent (antifluorescein goat antiserum bonded to the magnetizable particles in Tris buffer pH 7.5 with 0.5% BSA) were added after and incubated 10 min at 37°C. After incubation the samples are separated on magnetic support 5 min time and solid complex is washed with TritonX-100 aqueous solution. 300 μL solution of phenolphthalein monophosphate (PMP) as specific enzyme substrate were added to each tube with solid complex. After enzymatic incubation at 37°C and 20 min the color development and reaction is stopped with NaOH solution 11 pH. The samples were analysed spectrometric (550 nm) and radiometric measurements. Fig. 6 and Fig. 7 show that for an increasing amount of CEA the extinction and radioactivity of the same sample rise.

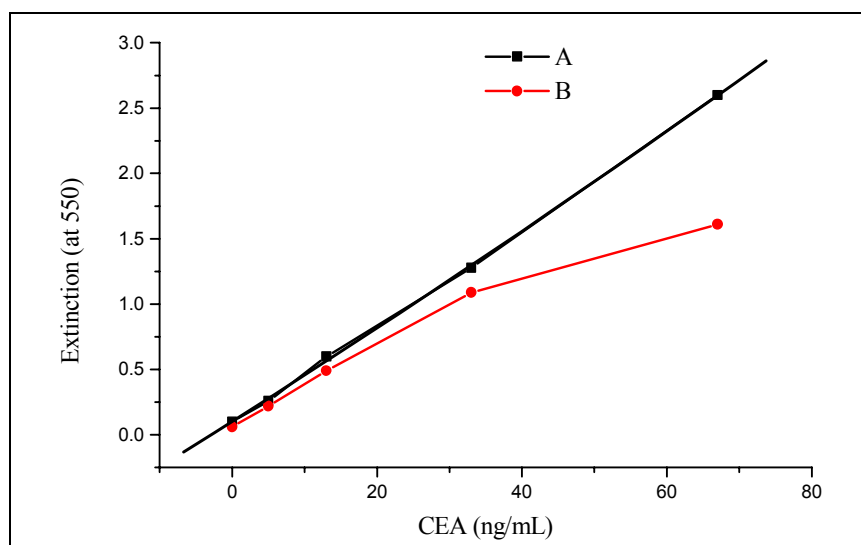


FIG. 6. Immunoreactivity of ^{188}Re -antiCEA (expresses by 550 nm extinction) vs. CEA concentration: A - standard curve; B - experimental curve.

The values of absorbance are with approximation unchangeable in comparison with the standard curves values in 0–33 ng/mL range. The same aspect has the radioactive curve.

3.5. Biodistribution of ^{188}Re -lanreotide

We used for biodistribution studies 256 Walker tumour bearing rats. ^{188}Re -Lanreotide was injected i.v (10 μg Lanreotide/rat; 50 μCi). The animals were sacrificed at 4, 24 and 48 h post injection and various organs were dissected, weighed and counted. The results are shown in Table II. Before sacrifice the animals were imaged at 4, 24 and 48h. The maximum accumulation of ^{188}Re -Lanreotide in tumour is 24 and 48 h post injection as we see in Fig. 8.

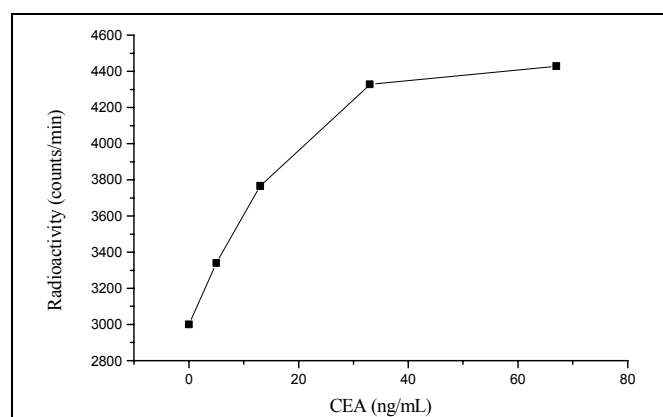


FIG. 7. Binding curve of ¹⁸⁸Re-AntiCEA to CEA.

TABLE III. BIODISTRIBUTION OF ¹⁸⁸RE-LANREOTIDE (3 ANIMALS/GROUP) EXPRESSED IN% DOSE/ORGAN

Organ	Time (h) postinjection		
	4	24	48
Tumour	5.6	10.3	9.8
Bone	0.9	1.2	1.1
Blood	5.4	3.7	1.8
Small intestine	2.1	1.3	0.4
Large intestine	2.3	1.2	0.1
Kidney	10.1	6.2	2.3
Spleen	1.1	1.6	3.7
Pituitary gland	2.8	4.5	4.3
Liver	13.1	2.5	2.0



FIG. 8. Images of tumour bearing rats at 4, 24 and 48 h (left to right) after ¹⁸⁸Re-lanreotide administration.

3.6. Biodistribution of ¹⁸⁸Re-antiCEA-Mab

Wistar London rats with 256 Walker induced breast (mammalian gland) tumour were used for the biodistribution studies. 10 µg of ¹⁸⁸Re-antiCEA-Mab with 26 MBq (0.7 mCi) radioactivity were i.v. administrated to tumour bearing rats. The animals were sacrificed at 4, 24 and 48 h post injection and various organs were dissected, weight and counted. The results are shown in Table IV.

TABLE IV. BIODISTRIBUTION OF ^{188}Re -ANTICEA (3 ANIMALS/GROUPS) EXPRESSED IN% DOSE/GRAM ORGAN

Organ	Time (h) postinjection		
	4	24	48
Tumour	10.5	14.6	13.2
Bone	0.5	0.7	0.2
Blood	8.2	3.3	3.6
Small intestine	1.6	0.9	0.3
Large intestine	2.6	4.1	0.9
Kidney	12.3	7.1	3.4
Liver	14.1	3.7	2.9
Lung	5.2	2.1	0.7

The high tumour accumulation of ^{188}Re -anti CEA still starts from the first hours as it is seen in Fig. 9.

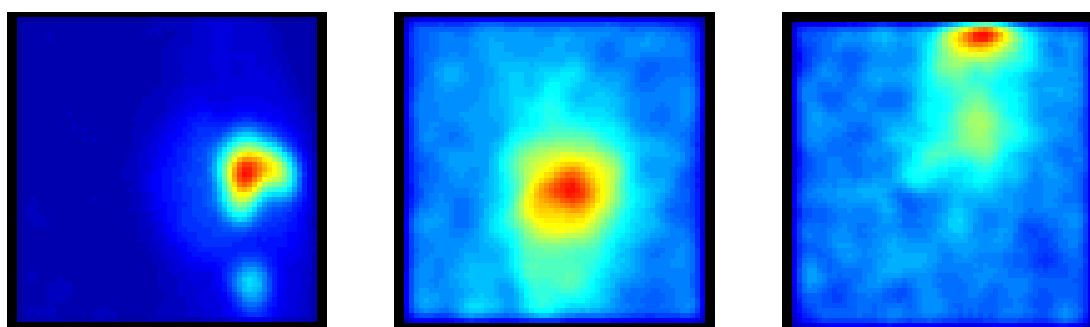


FIG. 9. Images of tumour bearing rats at 4, 24 and 48 h (left to right) after ^{188}Re -antiCEA administration.

4. DISCUSSION AND CONCLUSIONS

The results obtained in this study on the ^{188}Re -radiolabelling of lanreotide and antiCEA-Mab by direct and indirect methods show that the reactions of labelling process are controlled by stannous ion concentration (as reducing agent of perrhenate). A supplemental stannous ion amount is essential and is asked by a low redox potential of perrhenate [5]. When we used 0.6-1.3 mg stannous ions in acid pH, long reaction time and incubation temperature up to 90°C high efficiency of ^{188}Re radiolabelling was obtained.

In vitro studies show that only lanreotide 8mer has the conformational structure acknowledged by somatostatin receptors in 10^{-11} - 10^{-8} mol/L range, after labelling. For this biomolecules, tumour uptake remains up to 48 h after injection with an over-regulation of somatostatin receptors in the pituitary gland. The binding of ^{188}Re -DOTA-lanreotide to somatostatin receptors has a major alteration as a function of ^{188}Re attachment.

^{188}Re -radiolabelling process of antiCEA-Mab is sustainable by the *in vitro* and *in vivo* results. The immunoreactivity of ^{188}Re -antiCEA-Mab is unaltered in relation to the binding to CEA for 5-33 ng/mL concentrations. The imaging and biodistribution results show us that

^{188}Re -antiCEA consistently remains in tumour up to 48 h after injection. At this time the tumour/blood and tumour /liver ratios were approximately 3.6 and 4.5, respectively.

In summary, ^{188}Re -lanreotide and ^{188}Re -antiCEA-Mab may be prepared in kit form. It follows the pre-clinical and clinical studies, which will define the properties of this labelled biomolecules as radiotherapeutic agents.

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RADIOLABELLING OF MONOCLONAL ANTIBODIES FOR RADIOTHERAPY

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Abstract

Preparation of beta emitting radioisotope and development of labelling techniques with antibodies, peptides and their conjugates was investigated. Samarium-153 was labelled to DTPA – antibody conjugate giving low yields due to instability of labelled products and low specific activity of ^{153}Sm produced. Rhenium-188 was labelled to antibodies and lanreotide peptides by direct method and indirect method via MAG_3 -conjugate giving maximum yields of 86.1% and 79.5% labelling for ^{188}Re -labelled antibody and 99.3% and 85.0% labelling for ^{188}Re -labelled lanreotide, respectively. Synthesis of DOTA and DOTA-lanreotide was performed and the products were characterized by instrumental analysis and compared with standard sample. Yttrium-90 was produced by $^{90}\text{Sr}/^{90}\text{Y}$ generator system and was labelled to DOTA-lanreotide with more than 97% labelling yields. All radiolabelled products were determined for radiochemical purity by ITLC and HPLC, *in vitro* and serum stability, immunoreactivity or receptor binding affinity and some of them were evaluated for *in vivo* biodistribution in model animals.

1. INTRODUCTION

Targeted radiotherapy is a new form of radiotherapy that is very useful in the treatment of many tumour types. This technique involves the use of radiolabelled tumour-seeking biomolecules to deliver a cytotoxic dose of radiation to tumour cell. A number of anti tumour-associated antigen monoclonal antibodies, some specific proteins or somatostatin analogue peptides are the examples of biomolecules usually used. After labelling with beta emitting radionuclide, which have suitable chemical and physical properties, the labelled products are formulated as radiopharmaceuticals and administered to the patients. Samarium-153 (^{153}Sm), Rhenium-188 (^{188}Re) and Yttrium-90 (^{90}Y) are the most attractive radionuclides usually used in this application and their physical characteristics were summarized in Table I.

TABLE I. PHYSICAL CHARACTERISTICS OF ^{153}SM , ^{188}RE AND ^{90}Y

Radionuclide	Physical half life	Decay mode (MeV)	Particle maximum range in soft tissue
^{153}Sm	1.95 days	β (0.8), γ (0.103)	3.0 mm
^{188}Re	16.95 h	β (2.13), γ (0.155)	11.0 mm
^{90}Y	2.67 days	β (2.27)	12.0 mm

Radiolabelling of these radiometals with biomolecules had been developed for many years. The labelling techniques were mostly based on two methods: the first is the direct attachment of radiometal to the biomolecules [1, 2, 3]; the second is labelling via suitable bifunctional chelating agent or ligand, with one site that attaches to tumour specific biomolecules and the other site that binds to radiometal [4, 5]. The selected ligand should have to bind very tightly to radiometal to yield stable radiolabelled products both *in vitro* and *in vivo* under physiological conditions. Besides the chemical stability of labelled products,

their biological activity has to be preserved as much as possible during the processes to ensure their ability to bind to the target.

The objective of this project is to develop laboratory procedures to label monoclonal antibodies, peptides and other proteins with beta emitting radionuclides in order to prepare radiopharmaceuticals for therapeutic purpose. Preparation of ^{153}Sm and ^{90}Y radioisotope, synthesis and radiolabelling of ^{153}Sm -DTPA antibody, ^{188}Re antibody, ^{188}Re -MAG₃ antibody, ^{188}Re -lanreotide peptide, ^{188}Re -MAG₃ lanreotide, ^{90}Y -DOTA lanreotide (Fig. 1), radiochemical quality control and evaluation of specific biological characteristics will be described.

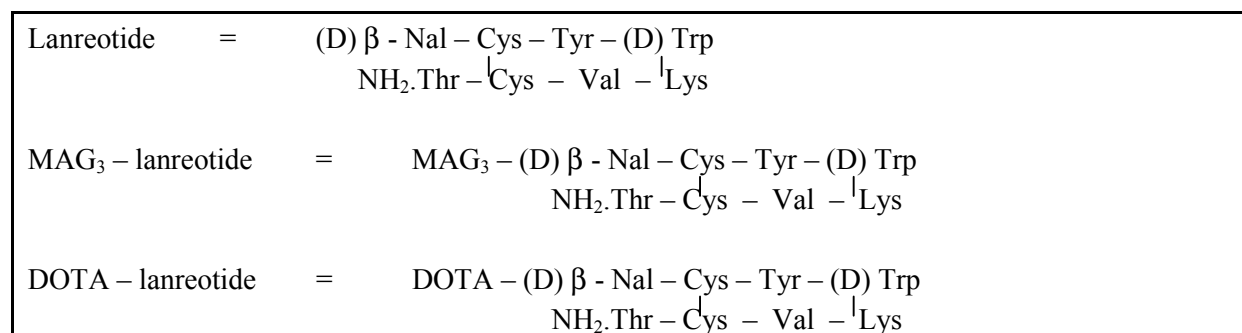


FIG. 1. Structure formulas of lanreotide peptide, MAG₃ — lanreotide, and dota — lanreotide.

2. MATERIALS AND METHODS

2.1. Antibody, protein, peptide and chemical

Goat IgG (Sigma), goat anti human IgG (Sigma), anti human prostate antigen MoAb. (Fitzgerald, USA.), PSA antigen (Fitzgerald, USA.), lanreotide and DOTA-lanreotide (Pi Chem, Austria) were used as model antibodies and model peptides.

All other reagents and chemicals were obtained from commercial sources except benzoyl-MAG₃ and DOTA, which were locally synthesized. Standard sample of DOTA was provided by Dr. M. Chinol.

2.2. Radionuclide and radioactivity calibration

Iodine-125 was a product of Amercham. $^{188}\text{W}/^{188}\text{Re}$ generators were provided by the IAEA (product of MAP Medical Technology Oy, Finland). Rhenium-188 radioactivity was measured in Victoreen Isotope Calibrator II (Model 34-056) dial setting 025.4. Samarium-153 was produced by neutron irradiation in the TRIGA III Research Reactor (OAEP) using a thermal neutron flux of $2 \times 10^3 \text{ n. cm}^{-2} \cdot \text{sec}^{-1}$ and was formulated in 0.9% NaCl or acetate buffer solution depending on experimental condition.

Y-90: The Yttrium-90 was produced by $^{90}\text{Sr}/^{90}\text{Y}$ generator system (Fig. 2). The generators were prepared by ion exchange method described previously by W.J. Skraba, et al. [6] and M. Chinol, et al. [7]. Strontium-90 was purchased from CIAE China. The nuclear characteristics of the decay system for this pair are shown below.

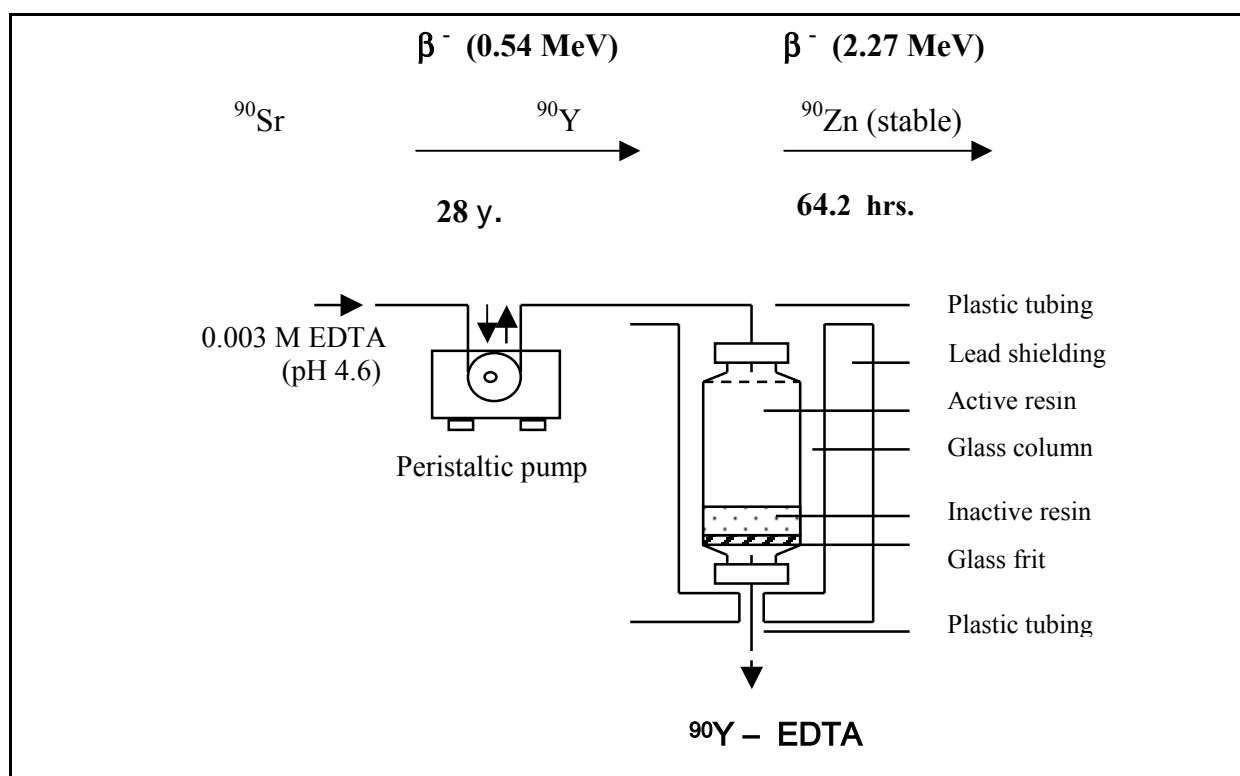


FIG. 2. Schematic diagram of ^{90}Sr - ^{90}Y generator system.

The ^{90}Y radioactivity was measured in Capintec Dose Calibrator (Pittsburgh, PA) dial setting 048 multiplied by 10 [8]. The generator yield of ^{90}Y was determined by measuring the count rate of the product by Liquid Scintillation Counter (LSC, ALOKA 5000 series, Japan) in the energy range above 0.54 MeV, comparing the count rate with that of a standard ^{90}Sr - ^{90}Y sample at equilibrium.

The Yttrium-90 radionuclidic purity and ^{90}Sr breakthrough in the ^{90}Y -product were estimated by paper chromatographic technique on Whatman No. 1 or ITLC-SG with 0.9% NaCl as eluant, ^{90}Sr migrates with solvent front with $R_f \sim 0.8 - 1.0$ while ^{90}Y as acetate remains at origin at $R_f \sim 0.0 - 0.1$. The results were confirmed by counting the remaining trace of ^{90}Sr in GM counter after allowing ^{90}Y to decay for more than 30 days or determined by Doering's method [9].

2.3. Synthesis of bifunctional chelating agents

2.3.1. *Bz-MAG₃*; The *s*-benzoyl *MAG₃* was synthesized by the method described previously by Fritzburg, A. R. et al. [10] and the chemical purity was confirmed by mp., IR and ^1H -NMR spectrometry.

2.3.2. *DOTA*; 1, 4, 7, 10 - Tetraazacyclododecane 1, 4, 7, 10 - Tetraacetic acid was synthesized by two methods.

Method I: The synthetic route were shown in the following chemical reactions [11] (Scheme 1).

NaHCO₃ pH 8.2 at room temperature for 1 h. After incubation, the DTPA-antibody conjugate was separated from free DTPA by Sephadex G-50 gel column chromatography or by dialysis against 1 l of 0.9% NaCl.

2.4.2. MAG₃ antibody

Conjugation of MAG₃ to antibody was done after ¹⁸⁸Re-MAG₃ labelling took place and the details were described in the labelling processes.

2.4.3. MAG₃ lanreotide

The conjugation was performed using the method described previously by P. Smith Jone [14] and by Decristoforo and Mather, S.J. [15]. It was divided into three steps: *step 1* – protection with Boc; *step 2* – MAG₃ conjugation; *step 3* – deprotection as shown in Scheme 3.

The correct structures of Boc lanreotide and MAG₃ lanreotide were confirmed by means of ESI-MS and their purity by RPC₁₈-HPLC.

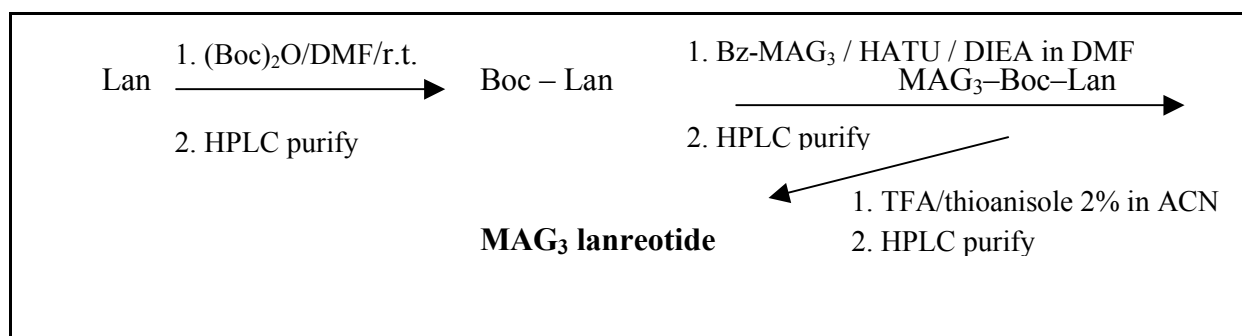


FIG. 5. Scheme 3: synthesis of Bz-Mag₃-lanreotide.

2.4.4. DOTA lanreotide. The conjugation of DOTA to Boc-lanreotide was carried out as the method described previously by Peter Smith-Jones [15] (Scheme 4).

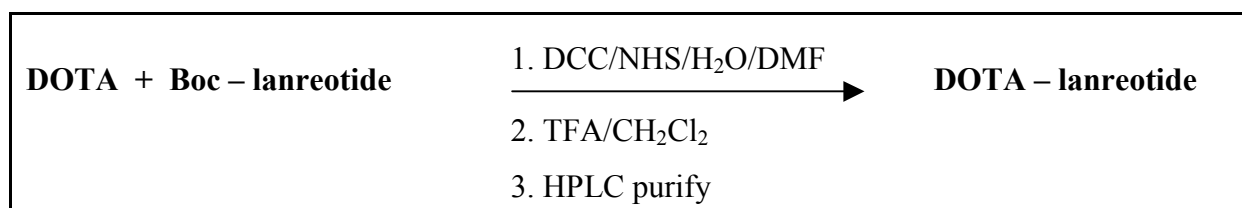


FIG. 6. Scheme 4: synthesis of DOTA-lanreotide.

HPLC conditions: Column — Sphericlone ODS 5 μ 4.6 mm \times 250 mm. (Phenomenex). Mobile phase — **A** 1% TFA/H₂O **B** 1% TFA/acetonitrile gradient — 0 – 3 mins. 100% A, 3 – 13 mins. 0 – 50% B, 13 – 23 mins. 50% B, 23 – 26 mins. 50 – 70% B, 26 – 30 mins. 70% B, 30 – 32 mins. 70 – 0% B, 33 – 40 mins. 0% B. flow rate ; 1 ml/min, detector UV 280 nm.

The product concentration was determined by UV spectrometric assay. The chemical purity was analysed by HPLC in comparison with standard sample.

2.5. Radiolabelling and quality control

2.5.1. ^{153}Sm -DTPA antibody

Purified DTPA antibody conjugate (500 μg) was used for Sm-153 labelling in three different buffer solutions in order to find optimum condition. The buffers used were 0.1 M acetate buffer pH 4.6 – 5.0, 0.1 M citrate buffer pH 5.6 and 0.1 M Hepes buffer pH 7.0 plus excess EDTA. The mole ratio of Sm: DTPA was adjusted to be 5: 1 and the reaction mixtures were incubated at room temperature for 1 h (Scheme 5). After the buffer was optimized, the mole ratios of Sm: DTPA were increased to 50: 1 and 100:1.

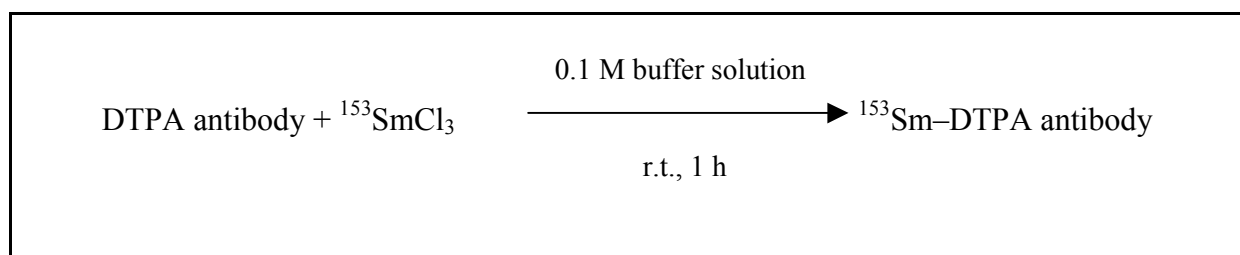


FIG. 7. Scheme 5: radiolabelling of ^{153}Sm -DTPA antibody.

The labelling yields were determined by two ITLC systems.

System 1: ITLC-SG / 0.9% NaCl, ^{153}Sm DTPA antibody and ^{153}Sm colloid remained at origin ($R_f \sim 0.0$).

System 2: ITLC-SG presoaking with 0.5% BSA/saline develop in solvent EtOH: NH_3 : H_2O 1: 2: 10 ^{153}Sm colloid remained at origin ($R_f \sim 0.0$) while ^{153}Sm -DTPA antibody and ^{153}Sm -DTPA moved with solvent front ($R_f \sim 0.9 - 1.0$).

The labelled product, ^{153}Sm -DTPA antibody, was purified by Sephadex G-50 gel column and its radiochemical purity confirmed by ITLC, paper electrophoresis on cellulose acetate paper and size exclusion HPLC; column SEC-3000 (Phenomenex) mobile phase; 0.2 M Tris buffer pH 7.0, flow rate ; 1 ml/min detector ; UV 280 nm and gamma (NaI) detector.

2.5.2. ^{188}Re antibody

Antibodies were labelled with ^{188}Re by both direct and indirect method. For direct labelling, the antibodies were reduced with 3500-fold excess of ascorbic acid (AA) at pH 5.0 for 1 h [16] or modified by 2-iminothiolane (2IT) at molar ratio 2IT: Ab = 100: 1 for 30 min [17] and then labelled with ^{188}Re by transchelation with pre-form $^{188}\text{Re(V)}$ gluconate or $^{188}\text{Re(V)}$ citrate at room temperature for 1 h (Scheme 6). By indirect labelling, MAG_3 ligand was labelled with ^{188}Re in the first step by solid phase synthesis of $^{188}\text{Re-MAG}_3$ [5], which was converted to $^{188}\text{Re-MAG}_3\text{-TFP}$ ester before being conjugated to antibody (Scheme 7). The ^{188}Re labelled antibodies were purified by Sephadex G-50 using 0.9 NaCl as eluent or by

dialysis against 1 litre of 0.9% NaCl for 2–6 h with 2–3 changes. Quality controls were determined by ITLC and HPLC.

2.5.3. ^{188}Re lanreotide

Lanreotide peptide was labelled directly with ^{188}Re by exchange reaction with ^{188}Re gluconate using SnCl_2 as reducing agent (Scheme 8).

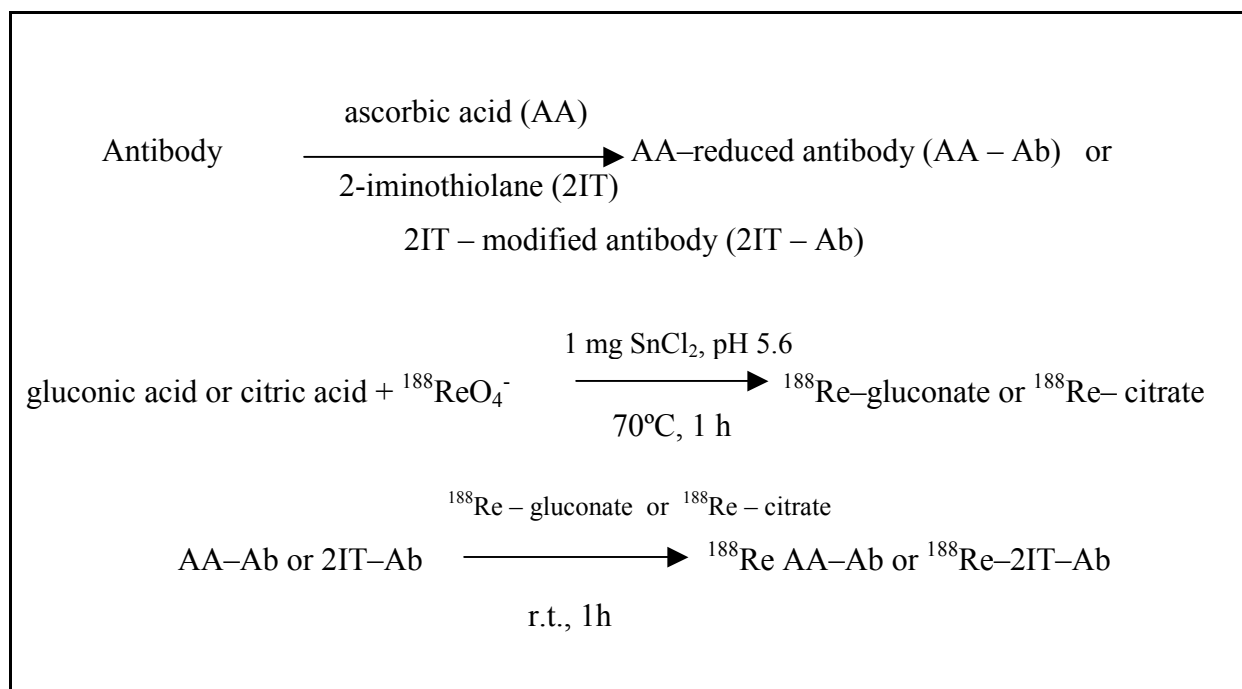


FIG. 8. Scheme 6: ^{188}Re direct labelling of antibody.

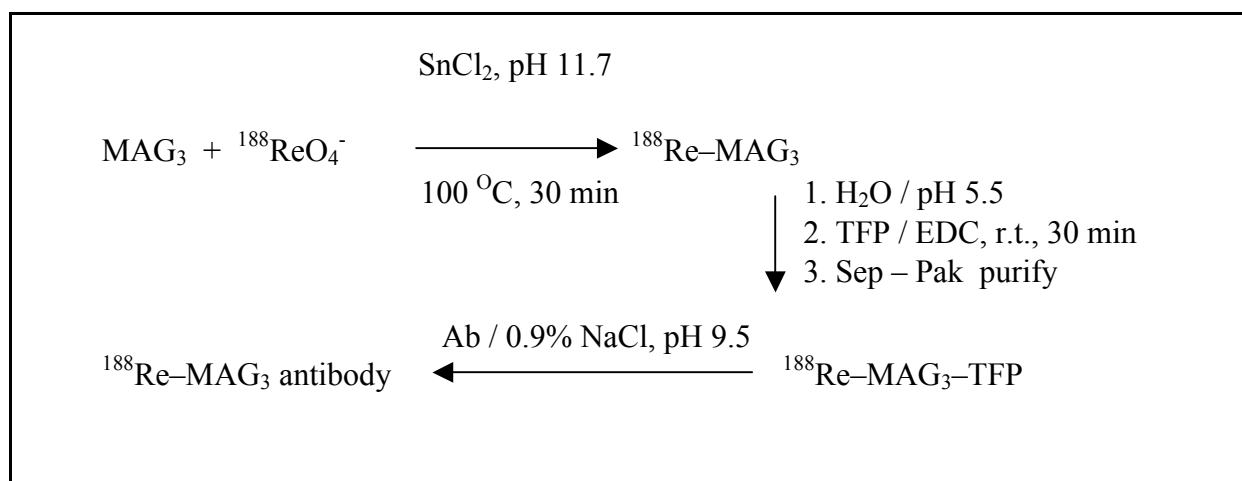


FIG. 9. Scheme 7: ^{188}Re indirect labelling of antibody.

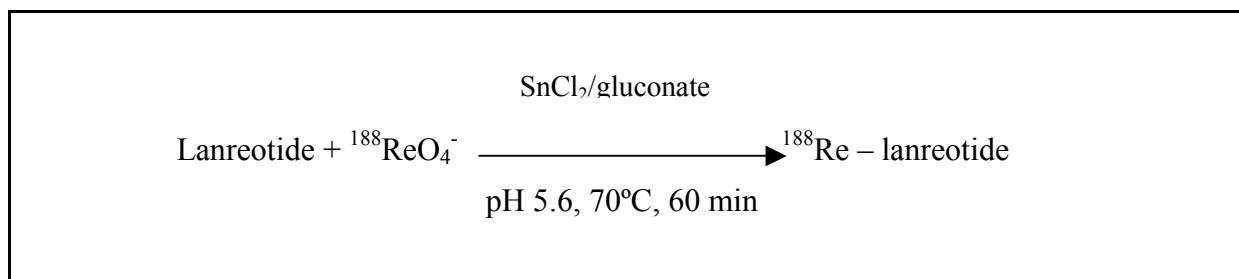


FIG. 10. Scheme 8: ^{188}Re direct labelling of lanreotide.

To a reaction vial containing 250 μl of SnCl_2 gluconate solution (0.5-2 mg SnCl_2 , 7.5-30 mg gluconate) pH 5.6, was added 10 μg lanreotide and 3-5 mCi $^{188}\text{ReO}_4^-$ (0.2-1 ml), N_2 -filled and closed with rubber stopper and aluminium cap. The vial was heat at 70 $^\circ\text{C}$ for 1 h in water bath. After cooling, the labelling yield was determined by ITLC – SG / 0.9% NaCl.

The ^{188}Re lanreotide product was purified by Sep-Pak, free $^{188}\text{ReO}_4^-$ and ^{188}Re -gluconate were washed out with 5 ml dilute (0.001 N) HCl and eluted ^{188}Re lanreotide with acidified (10% 0.01 N acetic acid) EtOH. The radiochemical purity was checked by ITLC–SG/0.9% NaCl and by HPLC employing RPC_{18} column with water/acetonitrile/ 0.1% TFA solvent system.

2.5.4. ^{188}Re -MAG₃ lanreotide

^{188}Re -MAG₃ lanreotide was labelled by exchange reaction with pre-formed ^{188}Re (V)-gluconate at 90 $^\circ\text{C}$ for 10 min and cooled down for 5 min (Scheme 9).

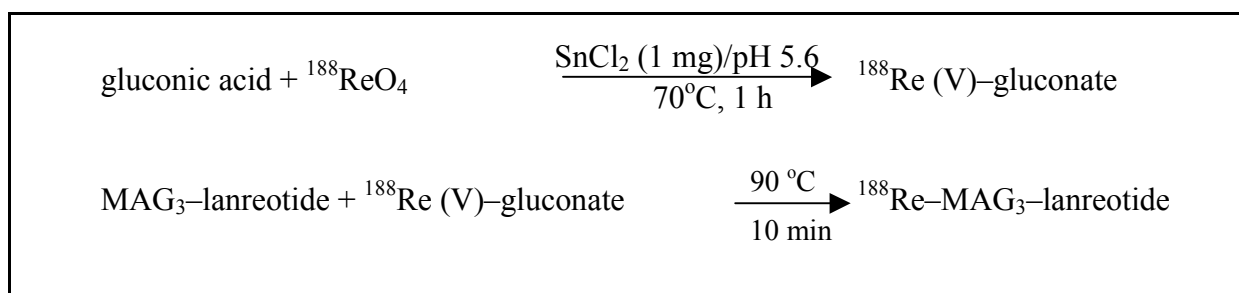


FIG. 11. Scheme 9: ^{188}Re indirect labelling of MAG₃ lanreotide conjugate.

The product ^{188}Re -MAG₃ lanreotide was purified by Sep-Pak with 80% ACN or 80% ethanol as eluant. The labelling yield and radiochemical purity were determined by ITLC/ 0.9% NaCl and RPC_{18} -HPLC with UV and gamma detector (NaI).

2.5.6. ^{90}Y -DOTA lanreotide

Labelling conditions of ^{90}Y -DOTA lanreotide; pH, ligand concentration, reaction temperature and incubation time, were optimized under limited amount of ^{90}Y activity (Scheme 10).

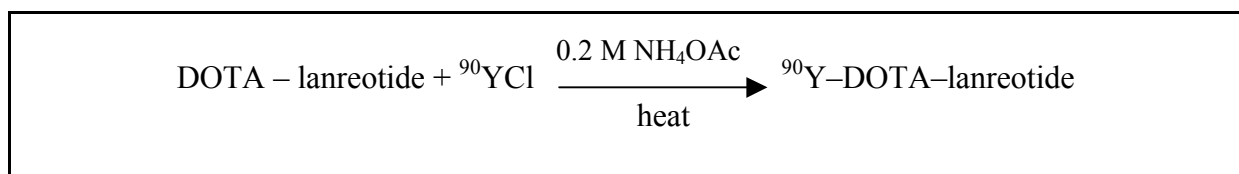


FIG. 12. Scheme 10: ^{90}Y labelling of DOTA lanreotide.

One to forty micrograms (0.60–26.5 nmole) of DOTA lanreotide in 150 μ l 0.2 M NH_4OAc buffer pH ranging from pH 4 to pH 8, was reacted with 20–30 μCi ($4\text{--}6\times 10^{-4}$ nmole) ^{90}YCl in acid washed glass vial. The solutions were allowed to react at 100°C in water bath for 30 min. The labelling yields were determined by ITLC-SG using 0.004 M EDTA pH 4.0 as solvent, the ^{90}Y -DOTA lanreotide remained at origin with $R_f \sim 0.0 - 0.1$ while unbounded ^{90}Y moved with the solvent front as EDTA complex with $R_f \sim 1.0$. The reaction time and incubation temperature were also optimized and the labelling yield was determined in the same manner.

Low labelling yield products were purified by silica gel column; Sep-Pak, using 80% acetonitrile as eluant, and the radiochemical purity were confirmed by ITLC/0.004 M EDTA pH 4.0 and/or HPLC RPC_{18} column Spherisorb ODS 4.5×150 nm. (5 μ); UV detection $\lambda = 280$; flow rate 1 ml/min; mobile phase A: 0.1% TFA in H_2O ; mobile phase B: 0.1% TFA in $\text{H}_2\text{O}/\text{CH}_3\text{CN}$ (20/80); linear gradient 0% to 100% B over 20 min.

2.6. *In vitro* stability studies

2.6.1. *In vitro* and serum stability studies of Re-labelled antibodies

In vitro stability of Re-labelled antibodies was checked by cysteine challenge (mole ratio cysteine: Ab 0–500: 1 in 0.4 M phosphate buffer pH 7.0) at 37°C for 1 h and by incubation in fresh human serum (~ 10 μg Ab/ml) at 37°C for 24 h using PBS as control.

2.6.2. *In vitro* and serum stability studies of ^{188}Re lanreotide, ^{188}Re -MAG₃ lanreotide and ^{90}Y -DOTA lanreotide

Purified ^{188}Re and ^{90}Y labelled lanreotides were formulated in 0.075 M NaCl, 0.05 M NH_4OAc and 0.2 M ascorbic acid for *in vitro* stability test at 4°C for 24 h. For serum stability, small volume of this solution was added to 500 μ l of fresh human serum to final concentration of 0.3–1 μg peptide/ml and incubated at 37°C , 5% CO_2 for 24 h. The stability was determined at each time interval by ITLC/0.9% NaCl for free $^{188}\text{ReO}_4$ and / or ^{188}Re -MAG₃ dissociated or by ITLC-SG/0.004 M EDTA pH 4.0 for free Y-90.

2.7. Immunoreactivity test of ^{188}Re labelled antibodies

Immunoreactivity of labelled antibodies was determined as the method described by T. Lindmo [18]. The assay was performed with model antibody (human IgG as antigen and goat anti-hIgG as antibody) labelled with ^{188}Re by both direct and indirect methods and comparing to its immunoreactivity after being labelled with I-125 (chloramines-T technique).

2.8. Receptor binding of ^{188}Re and ^{90}Y labelled peptide

The binding affinity of ^{188}Re lanreotide, ^{188}Re -MAG₃ lanreotide and ^{90}Y -DOTA lanreotide was tested in saturation binding and competition binding assay as methods described previously by J.C. Reubi (1985) using rat brain cortex membrane as a source of somatostatin receptors. The membrane was prepared using a modified method of Raynor and Reisine (1989). In case of ^{188}Re lanreotide and ^{188}Re -MAG₃ lanreotide, the final pellet was counted for radioactivity by HPGe detector equipped with multichannel \square nalysed. For ^{90}Y –

DOTA – Lnareotide, the membrane was solubilized in concentrated hydrochloric acid (~1 ml.) for one night and the solution was heated to evaporate the excess acid out before mixing with Scintillation cocktail (Optiphase Hisafe², Wallac, UK) and measured the radioactivity in LSC (Wallac wind spectra 1441, UK). The data were analysed using the method of Scatchard (1949) and specific binding is total binding minus binding persisting in the presence of 1 μ M sandostatin (non-specific).

2.9. Biodistribution studies

2.9.1. Biodistribution of $^{188}\text{Re-MoAb}$ and $^{188}\text{Re-MAG}_3\text{-MoAb}$ in normal animals (mice)

Biodistribution of ^{188}Re labelled antibody were first evaluated in normal animal with model antibody, anti Prostate Specific Antigen monoclonal antibody (anti. PSA. MoAb), labelled by both direct labelling ($^{188}\text{Re-AA-anti PSA. MoAb}$) and indirect labelling ($^{188}\text{Re-MAG}_3\text{ anti PSA MoAb}$). Two group of mice (weight 24-26 g) were injected with each Re-labelled MoAb. After 2 and 24 h post injection, the animals were killed and the organs dissected. Samples were weighed and counted in gamma counter and the results were expressed as percentage of injected dose per gram of tissue.

2.9.2. Biodistribution of $^{188}\text{Re lanreotide}$ and $^{188}\text{Re-MAG}_3\text{ lanreotide}$ in normal animals (rats)

In order to study receptor uptake, the rats were divided into two groups, the control group was injected 150–160 μg unlabelled peptide to block the receptor. Thirty minutes later all rats were injected with $^{188}\text{Re lanreotide}$ or $^{188}\text{Re-MAG}_3\text{ lanreotide}$ (50–70 μCi). Two hours after injection, the animals were killed, dissected the organs and the sample of blood, muscle and bone are taken. Samples were weighed and counted in gamma counter and the results were expressed as percent of injected dose per gram of tissue.

3. RESULTS

3.1. Sm-153 and Y-90 production

Samarium–153 yields were 205–269 mCi for 1 day (12 h) irradiation with specific activity 85.3–99.0 mCi/mg and 10,11 mCi for 3 days (36 h, 12 h/day) irradiation with specific activity 152 mCi/mg. The generator yields (Fig. 3) of Y-90 for every seven day elution were 297–453 μCi for 1 mCi ^{90}Sr -loaded generators (59.8–76.6%) and 1,360–1,485 μCi for 5 mCi ^{90}Sr -loaded generator (48.5 – 54.7%). The ^{90}Y -radionuclidic purities determined by ITLC (Fig. 4) were greater than 99% and ^{90}Sr -breakthrough were less than 0.1% ($1.2\text{--}3.0 \times 10^{-2}\%$).

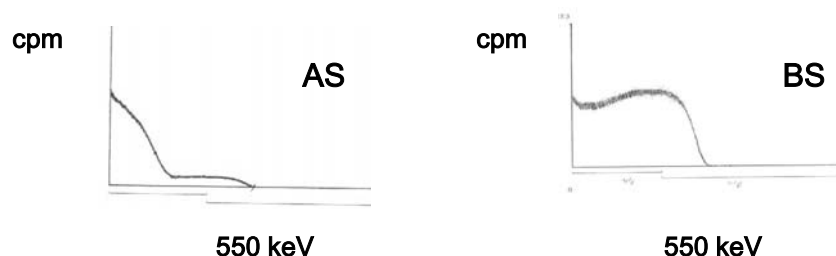


FIG. 13. Beta ray spectra of ^{90}Sr - ^{90}Y at equilibrium (a) and pure ^{90}Y (b) by LSC.

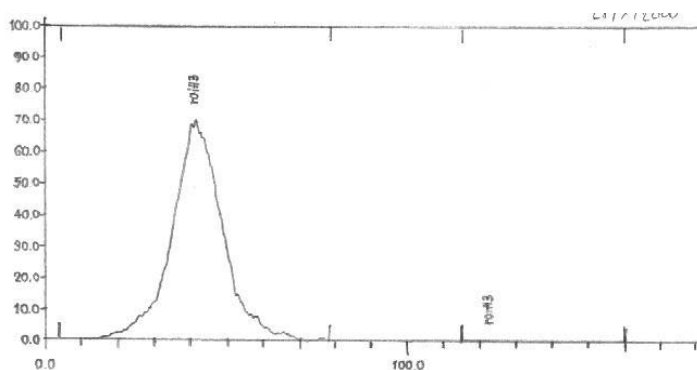


FIG. 14. Radiochromatogram of ^{90}Y (acetate) by ITLC – SG / 0.9% NaCl.

3.2. Ligand synthesis and peptide conjugation

The yields for s-Bz-MAG₃ synthesis were 1.44–2.28 g/batch mp. 194–196 °C; IR and $^1\text{H-NMR}$ were corresponded to the previous work [10].

Synthesis of DOTA by Method I gave low yield, 90 mg. or 5.57%, from 4.71 mmole cyclene and 27 mmole ethyl bromoacetate while good yield was obtained by method II, 400 mg. (96.2%) of pure DOTA was recovered (using 0.99 mmole cyclene). Instrumental analysis by IR (cm^{-1}): 3407.2 (sharp, broad, -COOH), 2,847.3 (sharp, C-O) and by $^1\text{H-NMR}$ (ppm.): 3.6 (s, 8H, CH₂COO), 3.27 (s, 16H, CH₂N).

DTPA antibody: modified method of Hnatowich [13] was applied to estimate DTPA incorporated into the antibody molecule and found to be 0.883 mole DTPA/mole antibody for conjugation ratio cDTPAa: antibody 10: 1.

MAG₃ lanreotide; Boc lanreotide was synthesized (Fig. 5A) with maximum yield of 54.2% (914 μg) and this product (183–400 μg) was conjugated to MAG₃ chelator (Fig. 5B) giving recovery yields of MAG₃-lanreotide, after deprotection and HPLC purified process (Fig. 5C), ranging between 30 μg (16.49%) and 64.5 μg (26.7%).

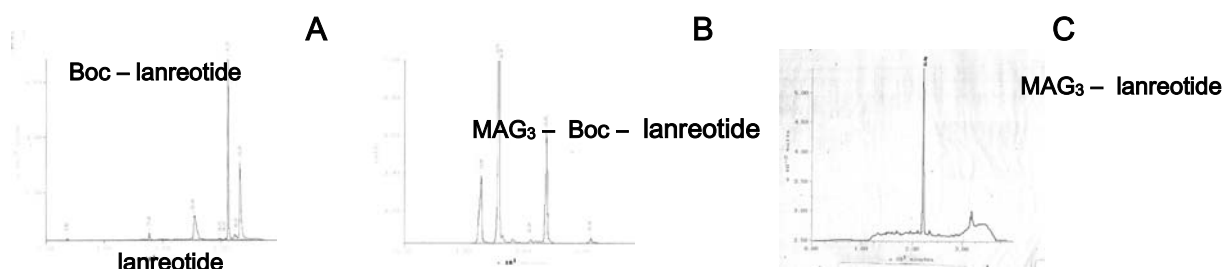


FIG. 15. HPLC (UV – detector) of Boc – LAN (A), MAG₃-Boc-LAN (B) and MAG₃-LAN (C).

DOTA lanreotide: Mole ratio and sequence of reaction had been optimized. The mole ratio was found to be Boc-lan: DOTA: DCC: NHS 1: 5: 10 : 5 and the sequences of addition were as follow: DOTA was mixed with NHS for 5 mins. before addition of DCC and mixed

well, and then added this solution dropwise to the vial of Boc–lan, mixed and stirred at room temperature for 16 h as show in scheme 4. DOTA lanreotide yield, after deprotection and HPLC purified (Fig. 6), was 90.4 µg (29.2%) from 0.2089 mmol Boc–lan comparing to 60% by peak integration. Chemical purity was identified by HPLC using standard sample of DOTA–lanreotide as reference.

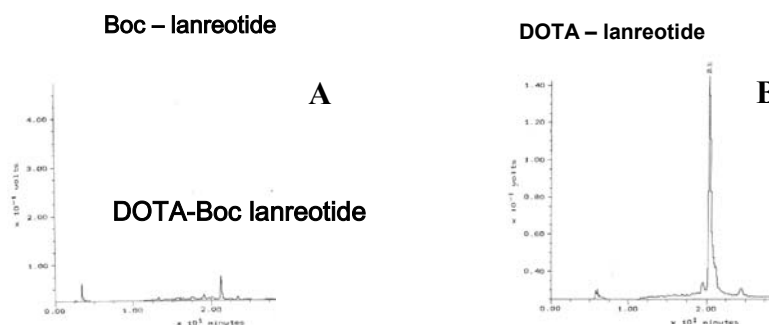


FIG. 16. HPLC (UV detector) of DOTA–Bo–LAN conjugation (A) and pure DOTA–LAN (B).

3.3. Labelling yields

3.3.1. ¹⁵³Sm–DTPA antibody

Labelling yields of ¹⁵³Sm–DTPA antibody in 0.1 M acetate buffer, 0.1 M citrate buffer and 0.1 M Hepes buffer and mole ratio Sm: DTPA = 5: 1 were 41.3, 19.4 and 24.6 µCi Sm–153/mg. antibody or their chemical yields were 49.5, 23.3 and 29.6%, respectively. When the mole ratio Sm: DTPA was increased to 500: 1, the recovery yield after purification was only 58.1 µCi/mg. Radiochemical purity analysis by ITLC and PE were 94% and 93.8%, respectively, and by HPLC, it gave a peak at T_r 5.28 min.

3.3.2. ¹⁸⁸Re–labelled antibody

¹⁸⁸Re antibody by direct labelling: Ascorbic acid (AA) and 2–iminothiolane (2IT) reduced antibodies were labelled with ¹⁸⁸Re by exchanged with ¹⁸⁸Re–gluconate or ¹⁸⁸Re–citrate. ¹⁸⁸Re–Antibody had T_r 7.5 min y HPLC and R_f 0.0–0.1 by ITLC/0.9% NaCl.

¹⁸⁸Re–MAG₃–Antibody by indirect labelling: MAG₃ was first labelled with ¹⁸⁸Re under optimized condition; 10 µg MAG₃, 1 mg. SnCl₂, pH 11.0 and 100 °C reaction temperature, giving maximum labelling yield of 79.5% determined by ITLC. The formation of ¹⁸⁸Re–MAG₃–TFP ester was optimized at pH 5.5–5.7 with 61.85% yield. ¹⁸⁸Re–MAG₃–Antibody was purified efficiency by dialysis. ITLC analysis gave R_f 0.75–0.9 for ¹⁸⁸Re–MAG₃ in ITLC–SG/MeOH, R_f 0.1–0.2 for ¹⁸⁸Re–MaG₃–TFP and R_f 0.75–0.9 for ¹⁸⁸Re–MAG₃ in ITLC–SG/20% NaCl, R_f 0.0–0.1 for ¹⁸⁸Re – MAG₃–Antibody in ITLC–SG/0.9% NaCl. HPLC determination of ¹⁸⁸Re–MAG₃–antibody gave T_r 7.61 min.

Labelling yield and radiochemical purity analysis of ¹⁸⁸Re–Antibody (direct labelling) and ¹⁸⁸Re–MAG₃–Antibody (indirect labelling) are summarized in Table II.

3.3.3. ¹⁸⁸Re labelled peptide

¹⁸⁸Re–lanreotide and ¹⁸⁸Re–MAG₃–lanreotide had R_f 0.0 in ITLC–SG/0.9% NaCl analysis and by HPLC gave T_r 21 min and 17.2 min respectively. Labelling yields and radiochemical analysis of both were summarized in Table III.

TABLE II. LABELLING YIELDS AND RADIOCHEMICAL PURITY OF ^{188}Re -LABELLED ANTIBODIES BY ITLC AND HPLC

	Labelling yield (ITLC)	Radiochemical purity ITLC	Radiochemical purity HPLC	Specific activity $\mu\text{Ci} / \mu\text{g Ab.}$
<i>Direct labelling</i>				
$^{188}\text{Re-AA-Ab}$	79.6 – 84.2%	80.5 – 99.0%	94.15%	5.8 – 7.1
$^{188}\text{Re-2IT-Ab}$	27.3 – 86.1%	90.05 – 97.4%	94.01%	5.3 – 6.5
<i>Indirect labelling</i>				
$^{188}\text{Re-MAG}_3\text{-Ab}$	10.0 – 79.5%	87.0 – 98.6%	95.25%	2.1 – 5.7

TABLE III. LABELLING YIELDS AND RADIOCHEMICAL PURITY OF ^{188}Re LANREOTIDE AND $^{188}\text{Re-MAG}_3$ LANREOTIDE

	% labelling (ITLC)	%RCP (ITLC)	Sp. Act. ($\mu\text{Ci} / \mu\text{g}$)
$^{188}\text{Re-lan}$	74.94 – 99.34	85.00 – 98.90	0.018 – 0.365
$^{188}\text{Re-MAG}_3\text{-lan}$	53.60 – 82.40	89.03 – 95.31	0.016 – 0.093

3.3.4. ^{90}Y -DOTA lanreotide

Labelling yields of ^{90}Y -DOTA lanreotide depended mainly on reaction temperature, pH and incubation time. At optimum condition, 10 μg DOTA-Lanreotide, pH 5–5.5 0.2 M NH_4OAc buffer, 100 °C reaction temperature and 25–30 min, the labelling efficiencies were greater than 97% (98.00 ± 0.4 , $n=10$) even though at reaction temperature 90 °C or at low ligand concentration to 22.4 μm . Radiochemical purity analysis by ITLC, ^{90}Y -DOTA-Lanreotide had R_f 0.0–0.2, by HPLC had T_r 18.5–19.5 mins. with 97.83% purity (Fig. 7).

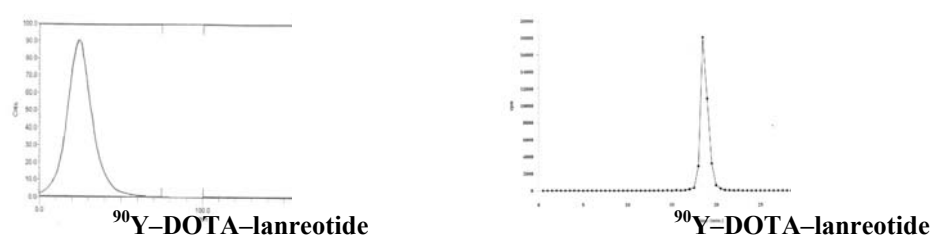


FIG. 17. Radiochromatogram of ^{90}Y -DOTA-Lanreotide by ITLC (A) and by HPLC (B).

3.4. In vitro stability

3.4.1. ^{188}Re labelled antibody

^{188}Re antibody and $^{188}\text{Re-MAG}_3$ antibody had good stability under cysteine challenging test, less than 10% of ^{188}Re activity was dissociated. In fresh human serum incubation at 37°C for 24 h, 26–36% of ^{188}Re activity dissociated comparing to 32–44% lost in PBS.

3.4.1. ^{188}Re labelled peptides

Both ^{188}Re lanreotide and ^{188}Re -MAG₃ lanreotide showed good *in vitro* stability at 4°C upto 4 h, that is less than 3% of ^{188}Re activity dissociated. In fresh human serum at 37°C, 5% CO₂, for 24 h, the dissociations of Re-activity were 38.4% and 39.3%, respectively.

3.4.2. ^{90}Y -DOTA lanreotide

^{90}Y -DOTA lanreotide had very high chemical stability both *in vitro* at 4°C for 72 h incubation or in fresh human serum at 37°C for 24 h, in which less than 0.3% of free ^{90}Y activity dissociated.

3.5. Immunoreactivity and receptor binding

Immunoreactivity test with model antibody (anti h. IgG) gave the immunoreactive reaction (r) for ^{188}Re -AA antibody (direct), ^{188}Re -2IT antibody (direct) and ^{188}Re -MAG₃ antibody (indirect) 0.51, 0.57 and 0.28, respectively, or 71.8%, 80.2% and 39.4% when comparing to ^{125}I antibody (r = 0.71).

Receptor binding: Receptor binding assay of ^{188}Re -lanreotide, ^{188}Re -MAG₃ lanreotide and ^{90}Y -DOTA lanreotide are shown in Figs 18 to 20.

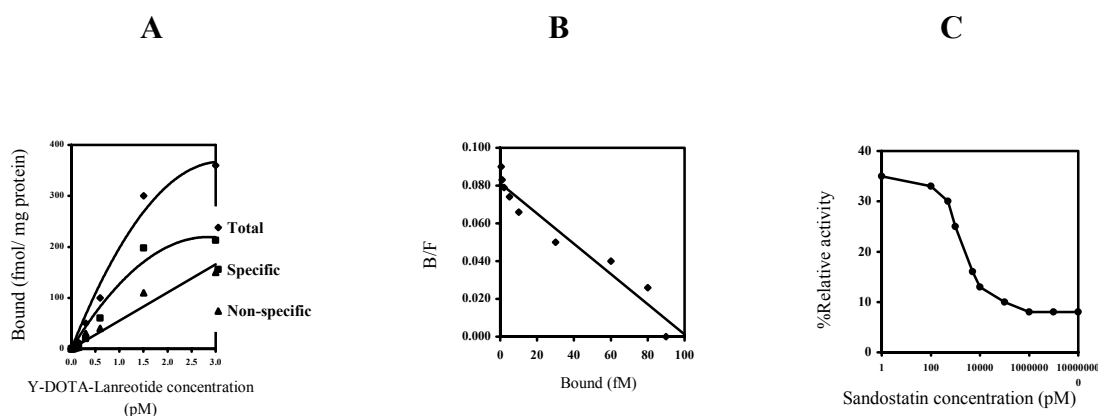


FIG. 18. Saturation binding (A), scatchard plot (B) and competitive binding (C) ^{90}Y -DOTA lanreotide.

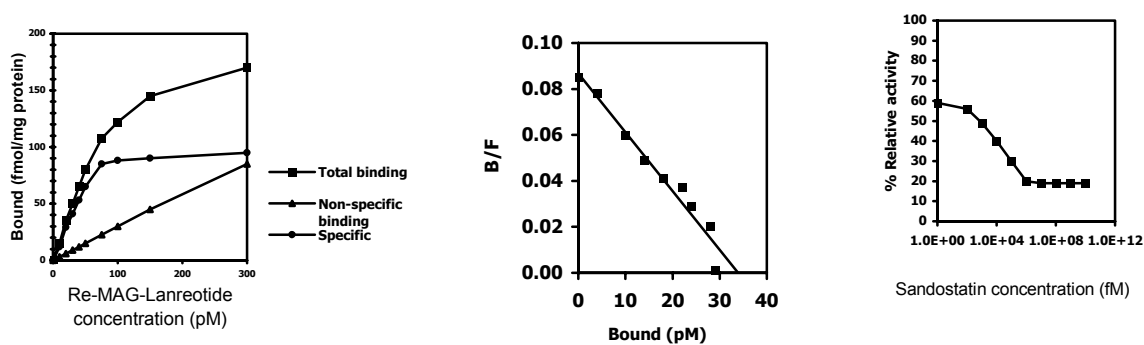


FIG. 19. Saturation binding (A), scatchard plot (B) and competitive binding (C) ^{188}Re -MAG₃ lanreotide.

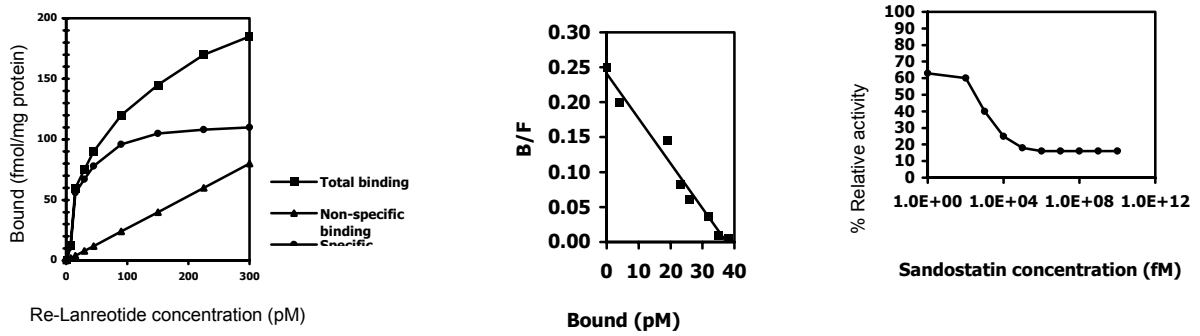


FIG. 20. Saturation binding (A), scatchard plot (B) and competitive binding (C) ^{188}Re lanreotide.

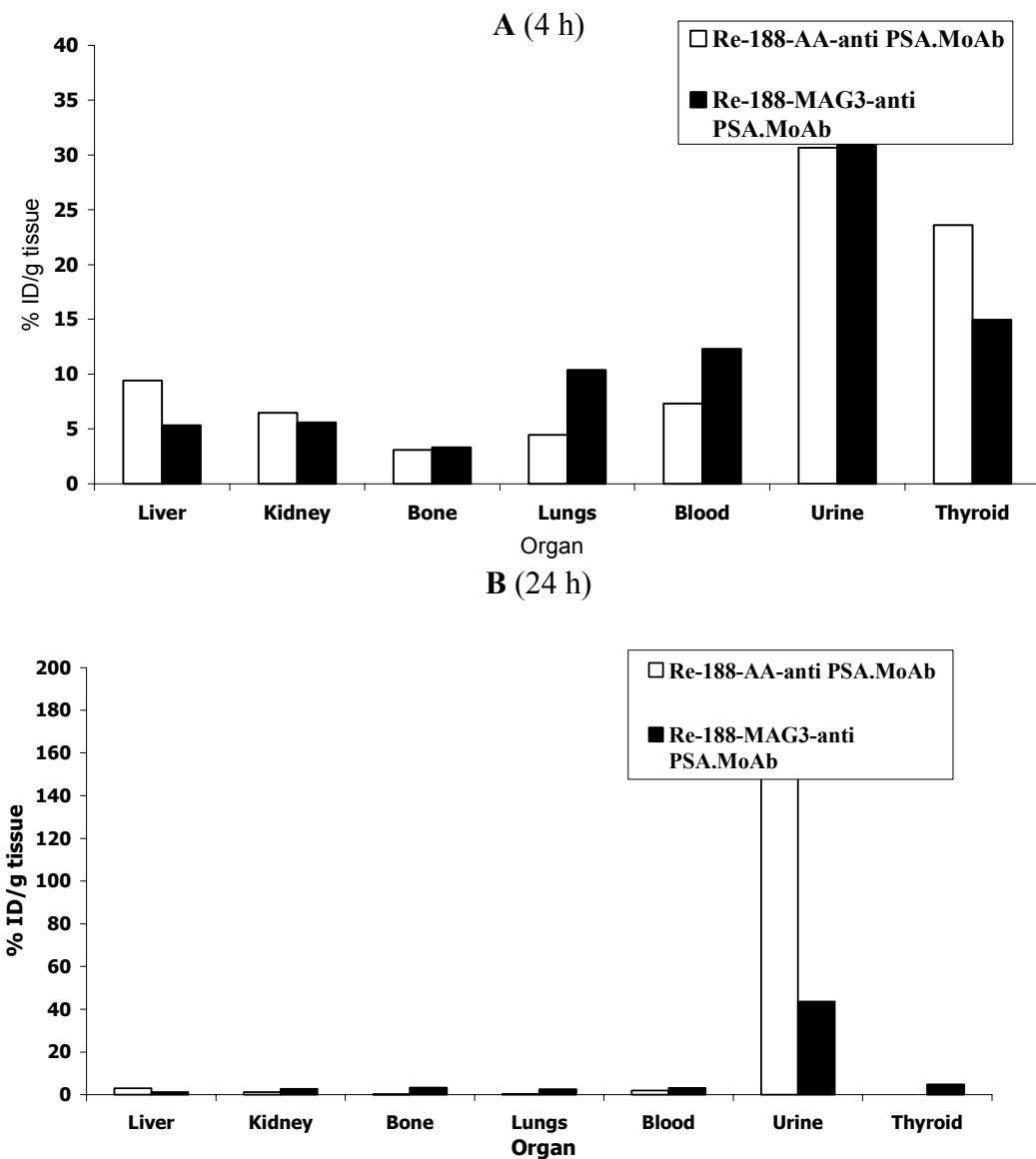


FIG. 21. Biodistribution in normal mice of ^{188}Re – labelled anti PSA. MoAb by direct and indirect labelling at 4 h (a) and 24 h (b) p.i.

3.6. Animal biodistribution

3.6.1. ¹⁸⁸Re labelled MoAb

Biodistribution of ¹⁸⁸Re-labelled MoAb by direct labelling (¹⁸⁸Re-AA anti PSA MoAb) and by indirect labelling (¹⁸⁸Re-MAG₃ anti PSA MoAb) in normal mice at 4 and 24 h P.I. are shown in Fig. 11 (A) and (B). At 24 h, both ¹⁸⁸Re labelled MoAb's had low uptake in most organs accepted in bone (3.3% ID / g.) for ¹⁸⁸Re-MAG₃ anti PSA MoAb, and the activity was excreted mainly by kidney and liver.

3.6.2. ¹⁸⁸R-labelled peptide

Normal animal biodistribution of ¹⁸⁸Re lanreotide and ¹⁸⁸Re-MAG₃ lanreotide had been evaluated in normal rats to demonstrate its ability to bind to somatostatin receptor-positive organ *in vivo*. The results are shown in Table IV.

TABLE IV. TISSUE DISTRIBUTION (% ID/g TISSUE, MEAN±SD, N = 3) OF ¹⁸⁸RE LANREOTIDE AND ¹⁸⁸RE-MAG₃ LANREOTIDE IN NORMAL RATS AT 2 H P.I

Tissue	¹⁸⁸ Re lanreotide		¹⁸⁸ Re-MAG ₃ lanreotide	
	Blocked	Unblocked	Blocked	Unblocked
Liver	1.24±0.10	2.28±0.32	1.07±0.08	1.53±0.02
Spleen	0.57±0.09	2.10±0.51	0.28±0.14	0.74±0.07
Kidney	0.66±0.08	1.60±0.16	0.81±0.11	1.26±0.24
Muscle	0.00	0.00	0.02±0.02	0.21±0.05
Bone	0.00	0.00	0.12±0.01	0.26±0.10
Heart	0.25±0.08	0.96±0.09	0.15±0.06	0.41±0.08
Blood	0.13±0.02	0.33±0.50	0.14±0.04	0.17±0.01
Urine	19.37±10.11	18.43±4.70	22.82±15.21	46.50±13.30
Brain	0.00	0.011±0.009	0.03±0.02	0.06±0.02
Pancreas	0.789±0.20	3.60±0.40	1.17±0.30	1.85±0.10
Adrenal	0.00	0.81±0.57	0.78±0.98	2.73±0.54

4. DISCUSSION AND CONCLUSION

Samarium-153 antibody labelling has been investigated via DTPA antibody conjugate at conjugation ratio cDTPAa: Antibody 10: 1 and was labelled with ¹⁵³Sm acetate. The maximum recovery yields of ¹⁵³Sm-DTPA antibody after Sephadex G-50 column purification were in the range of 41.3–58.1 µCi/mg (antibody) with 94% radiochemical purity. The labelling yields were low because of low specific activity of Sm-153 produced (<160 mCi/mg. Sm) and instability of product.

Rhenium-188 antibody labelling was performed with three different labelling techniques, two by direct labelling and one by indirect, with their quality controls determined. The maximum labelling yields for direct and indirect labelling were 86.1% and 79.5% and maximum radiochemical purities were 99.0% and 98.6%, respectively. Rhenium-188 labelled antibodies had good *in vitro* stability under cysteine challenging test, less than 10% of Re-188 activity was dissociated. In fresh human serum incubation at 37°C for 24 h, the dissociation of Re-188 activity was 26–36%. Labelling by direct method was more efficient and simpler than indirect method. Labelling by the addition of ¹⁸⁸Re-MAG₃ chelate to form ¹⁸⁸Re-MAG₃ antibody conjugate led to loss of immunoreactivity and required several steps of formulation more than the labelling by direct approach.

¹⁸⁸Re lanreotide and ¹⁸⁸Re-MAG₃ lanreotide were synthesized and labelled with Re-188 by ligand exchange through ¹⁸⁸Re (V) gluconate. The labelling yields of ¹⁸⁸Re lanreotide were ranging between 74.9–99.34% and the radiochemical purity, 85.0–98.9%. For MAG₃ lanreotide conjugation, the overall yields of the final product were low (16.4–26.7%) due to multisteps of synthesis and purification. The labelling yield of ¹⁸⁸Re-MAG₃ lanreotide depended directly on the yield of ¹⁸⁸Re (V) gluconate that formed earlier before exchange to MAG₃ lanreotide. In most cases, the exchange was higher than 90% (data not shown) but the overall yields were in the range of 53.6–82.4% and the radiochemical purities were 89.0–95.3%.

Construction of ⁹⁰Sr/⁹⁰Y generators succeeded. Three generators were prepared and all of them were working well. The ⁹⁰Y elution efficiencies were about 50–70% yield with more than 99% radionuclidic purity and less than 0.1% ⁹⁰Sr breakthrough. Yttrium-90 was labelled to DOTA lanreotide giving ⁹⁰Y DOTA lanreotide radioligand of maximum labelling yields higher than 97%. Radiochemical purity analysis of ⁹⁰Y DOTA lanreotide was 99.76% by ITLC and 97.83% by HPLC.

¹⁸⁸Re lanreotide and ¹⁸⁸Re-MAG₃ lanreotide had good *in vitro* stability for 4 h at 4°C but had moderate stability in fresh human serum after incubation at 37°C for 24 h, ⁹⁰Y DOTA lanreotide had very high chemical stability both *in vitro* at 4°C and in fresh human serum incubation.

Receptor binding studies of ¹⁸⁸Re lanreotide, ¹⁸⁸Re-MAG₃ lanreotide and ⁹⁰Y DOTA lanreotide showed binding affinity for somatostatin receptor in rat cortex membrane under saturation binding and competition binding tests. Scatchard analysis gave the dissociation constant (k_d) of 0.15 nM, 0.38 nM and 1.25 pM and maximum binding (B_{max}) of 112, 100 and 101.5 fmole/mg. protein, respectively. ¹⁸⁸Re lanreotide and ¹⁸⁸Re-MAG₃ lanreotide had been tested for *in vivo* study in normal rat and showed specific uptake in somatostatin-positive organs (pancreas, adrenal and brain).

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The authors wish to thank Dr. M. Chinol for the helpful discussions, Dr. T. Vilaiwan. of the Faculty of Science, Chulalongkorn University for the mass-spectrometry analysis, Mr. P. Soonthornmondha for the LSC counting, and the Isotope Production Division Staff of OAEP for their help in all the experiments. This work was supported in part by the IAEA.

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LABELLING AND CONTROL OF BIOMOLECULES WITH ^{188}Re AND ^{153}Sm

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Abstract

The aim of this work was to develop and/or standardize the labelling of different biomolecules with beta emitters as well as their radiochemical and biological quality control methods. Lanreotide was labelled with ^{188}Re with yields >90% with good stability using SnF_2 in the presence of ascorbic acid and HEDP (molar ratio HEDP/lanreotide=260 and SnF_2 /lanreotide=40) at pH 1-2. In order to conduct indirect labelling, the synthesis of ^{188}Re -MAG3-TFP ester was done. Lanreotide was also labelled with ^{125}I by chloramine-T method and Bolton-Hunter method with yield >95% and RCP >98% after purification. Somatostatin receptors from rat brain cortex were prepared, their control with ^{125}I -somatostatin gave maximum binding capacity near to 100%. ^{188}Re -lanreotide binding was low and could be inhibited with unlabelled lanreotide but not with somatostatin. ^{153}Sm -EB1 was obtained with RCP >90%, without affecting the binding site for avidin-biotin complex using molar ratio EB1- ^{153}Sm 20:1, at 80°C, 10 min. Conjugation of 83D4 and IgG bovine with cDTPA was done and labelled with ^{153}Sm (yield <20%). Radioiodination (chloramine-T method) of native and biotinylated 83D4, scFv and VH with BMCC-biotin in 1:50, 1:25 and 1:25 molar ratios respectively, were achieved with yields >50%. Their binding capacity with respect to Tn structure and avidin by immunoradiochemical analysis and by formation of triple complex [AcB]-[avidin]-[^{111}In -DTPA-biotin] was higher for 83D4 than for the recombinant fragments: %B/T=36 for native and %B/T=38 for 83D4-biotin. The highest affinity constant, determined by surface plasmon resonance, was obtained for 83D4-biotin, $K_A=1 \times 10^{10} \text{ M}^{-1}$, while the value for the 83D4 was $7 \times 10^9 \text{ M}^{-1}$. In order to gain experience and then switch to therapeutic applications by replacing $^{99}\text{Tc}^m$ with ^{188}Re , the labelling conditions of $^{99}\text{Tc}^m$ -N₄-Lys-Biotin were investigated. The best results were obtained using 50 µg of the ligand (61 µmol), 50 µg of SnCl_2 (224 µmol) at pH 12, incubating at RT. A diagnostic study in a patient with colon carcinoma by using a pretargeting system showed the location of the tumour at 2 h and a predominant renal excretion of the labelled molecule. Bifunctional chelating agents cDTPA and TETA were synthesized.

1. INTRODUCTION

In view of the potential of targeted radiotherapy to treat a wide range of malignant conditions, biomolecules such as monoclonal antibodies, peptides and other receptor avid molecules as carriers have been strongly investigated during the last decade. The over-expression of somatostatin receptors on most of tumours has made somatostatin analogues favourable target molecules, i.e. lanreotide. Also, alterations in carbohydrate profile has been demonstrated in a wide range of cancer cells. One of them, the Tn structure (N-acetylgalactosamina-Ser/Thr) is aberrantly expressed in breast, ovarian and other cancers. So, the use of molecules directed towards Tn, could have potential application in this field.

On the other hand, the three step pre-targeting approach based on the avidin-biotin system, makes it important to develop new biotin conjugates suitable for labelling with beta emitters.

In addition to the therapeutic nuclides of the first generation, many other beta emitting radionuclides with relatively short half lives such as ^{153}Sm , ^{188}Re have also been made available for therapy and used with good results.

So, the aim of the project is centred in the search of chemical and radiochemical approaches for labelling selected biomolecules with ^{188}Re and ^{153}Sm , while preserving the *in vivo* capability of recognising their specific receptors or antigens, as well as to develop and/or standardize quality control methods. ^{125}I , ^{111}In and $^{99}\text{Tc}^m$ were used for molecule characterization and/or to gain experience looking their replacement/analogy with therapeutic radionuclides.

2. MATERIALS AND EQUIPMENT

2.1. Radioisotopes

$\text{Na}^{188}\text{ReO}_4$ was obtained from a $^{188}\text{W}/^{188}\text{Re}$ generator system (MAP Medical Technologies Oy, Finland; OAK Ridge)

$^{153}\text{SmCl}_3$ was received from IPEN, Perú, under the scope of the IAEA ARCAL Programme

Na^{125}I high specific activity, highly concentrated, was obtained from Amersham and CIS biointernational

$^{111}\text{InCl}_3$ was obtained from CIS biointernational, France.

2.2. Biomolecules

- Lanreotide (8 mer, Cys²⁻⁷, cyclo) D-β-Nal-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr.NH₂, were provided by piChem Research Development, Austria through the IAEA
- 3-[¹²⁵I] iodotyrosyl¹¹Somatostatin-14 (¹²⁵I-SMT), specific activity approx. 74 TBq/mmol (2000 Ci/mmol) was from Amersham, England
- Somatostatine (SMT) and IgG bovine was from Sigma Chemical Co.
- EDTA-Biotin (EB1) was a gift from Silvia Castiglia, CNEA, Argentina.
- Anti Tn proteins: IgM Mab (83D4), scFv and VH fragments were provided by LOBBM, Faculty of Medicine, Uruguay
- Anti-CEA monoclonal antibody, avidin and biotinylated-HSA were provided by Dr. Chinol, IEO, Milan, Italy.

2.3. Chemicals

- Reagents were purchased from Aldrich Sigma Chemical Co. except where otherwise stated and used as they were received.

2.4. Equipment

- A solvent module (Varian Associates, model 5000) with UV detector
- coupled to and on-line (NaI(Tl) detector, was used for HPLC analysis.
- A dose calibrator (Capintec CRC-7, USA), a solid scintillation counter (ORTEC, USA) with plane (3 x 3'') NaI(Tl) detector, an automatic well-type gamma counter (Compac-120, Picker, USA) and a multichannel analyser coupled with a NaI(Tl) 3'' detector were used for radioactive measures.

3. METHODS

3.1. Labelling and quality control of biomolecules with ^{188}Re

$^{188}\text{W}/^{188}\text{Re}$ generator was evaluated until six months after calibration, analysing the elution yield and profile, pH, radiochemical purity (RCP) by paper chromatography, levels of some radionuclidic impurities using a multichannel analyser (with an efficiency of 1,76% for photons of 290 keV and 8.8% for photons of 155 keV), and presence of Al^{13} as chemical impurity (CP).

3.1.1. Lanreotide

Labelling by direct method using SnF_2 , $\text{SnCl}_2 \cdot \text{H}_2\text{O}$ or potassium borohydride as reducing agents, and HEDP, MDP or pyrophosphate as transchelating agents were

investigated. Different molar ratios and pH were studied, incubating at 100°C during 30 min. Sep-pak (C18) purification and chromatography systems were standardized. HPLC controls for the labelled molecule before and after purification were done using MCH-5-n-capp (C18) column with radiometric and UV detection. The solvent programme was linear gradient from 90% H₂O (TFA 0,1%):10% ACN (TFA 0,1%) up to 10% H₂O (TFA 0,1%):90%ACN (TFA 0.1%) in 30 min with flow 1mL/min.

In order to conduct binding studies, rat brain membrane somatostatin receptors were prepared according a modification of the IAEA protocol: 25 mM Tris-Mg buffer was used without adding antibiotics. Tissues were dissected in cold environment, homogenization was done with a Polytron mixer and centrifugation at 500 g during 10 min was used to avoid all big tissue debris. Tissues were centrifuged 4 times at 2500 g during 30 min each and keeping precipitate for further homogenization. Supernatants were discarded. Precipitates were resuspended in Hepes buffer. Protein concentration was determined by Lowry method. These tissue preparations were controlled by challenging with ¹²⁵I-SMT. The assay procedure was also modified: an overnight incubation at room temperature was preferred instead of 2 h at 37°C. Separation of bound activity to receptor was done by addition of polyethylene glycol solution at 6% concentration, with incubation at 4°C during 30 min. After this step, centrifugation at 4°C was done at 2500 g during 50 min.

Inhibition of binding of labelled biomolecules was done by addition of SMT concentrations from 3pM to 8µM to a receptor preparation of 800 µg/mL in HEPES buffer.

Determination of maximum binding capacity (MBC) was performed by challenging a fixed amount of ¹²⁵I-SMT (aprox. 20000cpm) to various dilutions of receptor preparation (from 100µg/mL to 800µg/mL) in a reaction volume of 250 µL, and measurement of the bound activity (B), as extrapolation at y-axis of inverse corrected bound activity versus inverse receptor volume. Non-specific binding was estimated by evaluation of the bound activity in the presence of 8µM cold somatostatin. MBC was also estimated for ¹²⁵I-SMT locally prepared; for ¹²⁵I lanreotide labelled by chloramine-T method and Bolton-Hunter method, as well as for ¹⁸⁸Re lanreotide using cold somatostatin or cold lanreotide.

3.1.2. IgG bovine

Forty µg/mL of reduced immunoglobulin with 2-mercaptoethanol (equal volume of 10% solution; 30 min.; RT) purified by PD-10 column (Sephadex G-25) was added to 1 mL of Na¹⁸⁸ ReO₄ in the presence of SnCl₂.2H₂O (17 µmoles), pH 3,5. Labelling yield and stability of the final product was tested by ITLC-SG chromatography control systems at different incubation conditions.

3.1.3. MAG₃ and synthesis of ¹⁸⁸ Re-MAG₃-TFP ester, coupled to amines (preconjugate approach)

Six mg of SnCl₂.H₂O was dissolved in 1 mL of 0.1 M citrate buffer, pH 5,5 (sol. A). 1,5 mg of benzoil-MAG₃ was dissolved in CNCH₃:H₂O (500 µL, 9:1 v/v) (sol. B).

250 µL of solution A (1,5 mg SnCl₂.H₂O) and 150 µL of solution B was added to 26,4 mCi of Na¹⁸⁸ReO₄ fresh solution previously evaporated through dryness under nitrogen atmosphere. The reaction mixture was heated for 60 min at 90°C and chromatographic quality control in ITLC-SG using saline solution and octanol as a solvents were done.

To the ^{188}Re -MAG₃ solution, 50 μL (30 μmol) of 2,3,5,6-tetrafluorophenol (TFP) (100 $\mu\text{g}/\text{mL}$ CNCH₃:H₂O; 9:1) and 20 mg (106 μmol) of 1 ethyl-3-(3-dimethylaminopropyl)-carboiimide (EDC) were added. Incubation for 40 min at RT was done with gently agitation. The molar ratios used were 70:1 (TFP:MAG₃) and 1:3,5 (TFP:EDC).

HPLC analysis was done using MCH-5-n-capp (C18) column with radiometric and UV detection. The solvent programme was: 0-3 min 100% H₂O (TFA 0,1%); linear gradient from 3 to 13 min up to 50%ACN:50% H₂O (TFA 0,1%); isocratic mode up to 18 min following by linear gradient 18- 20 min up to 70% ACN: 30% H₂O (TFA 0,1%) at flow 1 mL/min.

3.2. Labelling and quality control of biomolecules with ^{153}Sm

3.2.1. EDTA-biotin

Based on the strategy of administration of biotin conjugated Mabs followed by avidin and final addition of biotin labelled with beta emitter, labelling of EB1 at different reagent ratios and incubation conditions was investigated. Different thin layer chromatography systems were evaluated. HPLC in TSK-Gel column Type G3000SW_{XL} 7,8mm \times 300mm with isocratic programme (flow 1mL/min) with phosphate buffer 0.1M, pH 6,9 sodium azide 0.01% with and without EDTA 0.002M were done.

3.2.2. 83D4 (IgM) and IgG bovine

For that purpose, the synthesis of cDTPA was performed using as starting material DTPA according to the Hnatowich procedure (J. Nucl. Med. (1985) 26:503).

Conjugation to immunoglobulins was done, as follows: 5 mg of cDTPA were dissolved in 10 mL chloroform. Different volumes of this solution were added to a solution of 83D4 1,1mg/mL (1,2 μM) to achieve a molar ratio of cDTPA to 83D4 20:1, 100:1 and 2500:1.

For the preparation of IgG conjugate, a solution of cDTPA in CHCl₃ was added to 1 mg of IgG bovine in 100 μL (69 mM). In both cases pH reaction was adjusted to 8,3 by adding NaHCO₃ 0.1M and incubated 30 min at RT. Purification was done by gel filtration on PD10 column (Sephadex G-25) eluting with acetate buffer 0.2M, pH 5,5 measuring protein concentration by Lowry method.

Labelling was attempted by mixing equal volumes of ^{153}Sm -acetate and protein conjugates. Labelling yield was estimated by ITLC-SG chromatography using saline solution, MeOH 85% and trichloroacetic acid (TCA) 10% as solvents and gel filtration in PD10 column.

3.3. Biotinylation and control of biomolecules ¹

Sulphydril groups were introduced into proteins by reaction with 2-Iminothiolane (2-IT) in the following molar ratios 83D4 to 2-IT: 1:100, 1:500 and 1:1000; scFv and VH to 2-IT: 1:10.

¹ Work performed by H. Balter during a stage in Unité INSERM 463, Nantes, France, in collaboration with A. Faivre-Chauvert, J.Le Boterff and P. Vusio.

Conjugation with BMCC-biotin in 1:50 molar ratio for 83D4 and 1:25 for scFv and VH. Purification in both steps was done by gel permeation in PD-10 columns (Sephadex G-25) and concentration was done by ultracentrifugation in C-10 or C-30 cartridges depending on the molecular weight. Binding capacity of native and biotinylated products respect to Tn structure, was done by immunoradiochemical analysis. Affinity constant was determined by surface plasmon resonance (Biacore) system immobilizing asialo bovine submaxilar mucin (aBSM) in the sensorchip.

3.4. Labelling and quality control of biomolecules with ¹²⁵I

3.4.1. 83D4, scFv and VH

The 83D4 (15 µg; 1.7×10^{-11} mol) was labelled with 227 to 540 µCi of ¹²⁵I (100 mCi/mL; 17400 Ci/g without reducing agents) by chloramine-T limiting method and purification was done by gel filtration in PD 10 column. Binding studies with coated tubes with aOSM (5µg/mL), a Tn rich mucin, were conducted in order to verify its capability to recognize them after labelling. Biodistribution studies were performed in normal rats and with breast tumours induced with N-nitroso methyl urea.

Radiodination of native and biotinylated 83D4, scFv and VH was done by means of chloramine-T limiting method [Robles, et al: Nucl. Med. Biol. (2002)]. Specific binding to aBSM or avidin immobilized to solid phase was determined. Avidin was immobilized to poliestirene tubes (Nunc maxisorp) by incubating 0,2mL of 3.175×10^{-3} U/mL (1U binds 1,0 µg biotin) solutions in carbonate buffer 0,1M, pH 9.6 during 14-18h and washing two times with PBS 0.05M with Tween 20, 0.05%. Radiolabelled biomolecules (30000-100000cpm, 0.2mL) were incubated 14-18hs at RT under continuous gentle agitation. Unbound activity was removed by decantation and tubes were washed twice with 1mL PBS 0.05M, Tween 20, 0.05%. Bound activity was measured in a well gamma counter.

3.5. Labelling and quality control of biomolecules with ¹¹¹In

DTPA-biotin was labelled with ¹¹¹In and purified by Sep-pak C18. RCP was determined by chromatography on CCM silice using MeOH-NH₄Ac. Specific binding was determined using avidin immobilized to solid phase.

Formation of triple complex “in vitro”: [Ab-B]-[Avidin²]-[B-DTPA-¹¹¹In] was done by secuencial incubation and determination of binding to Tn-FS.

Also, with the expertise of Dr. Chinol of CNEA, Argentina, participation of our staff allowed gained some experience in the biotin labelling with ¹¹¹In and the radiochemical and biological controls.

3.6. Preparation of ⁹⁹Tc^m -N₄-Lys-biotin

The labelling of this biotin derivative provided by Dr. Chinol, was carried out in aqueous media (pH: 10-12) at low ligand amounts (50 µg) and using Sn (30-50µg as tin chloride or fluoride) in presence of citrate for the reduction of ⁹⁹Tc^mO₄⁻ (20-40 mCi in 0.2-0.4 mL). Also, a commercial kit manufactured by a local radiopharmaceutical company containing tin and tartrate was tested. The labelling was conducted either at room temperature

² And/or streptavidin

or at 60°C. After that, an aliquot was mixed with 20 µL of avidin solution and the labelling yields were checked by ITLC-SG (saline eluant) and HPLC (size exclusion column). Gel filtration on Sephadex G-25 was also performed.

Patient study: During the mission of Dr. Chinol, a patient with a CEA positive colon carcinoma was injected on Day 1 with 2.0 mg of biotinylated anti-CEA monoclonal antibody. On the Day 2, the patient received 2.0 mg of avidin in bolus “chase” followed by other 10,0 mg of avidin as slow infusion. On Day 3, the patient was injected with 2.6 mg of biotinylated-HS (2° chase) followed 15 min later by 31,8 mCi of ⁹⁹Tc^m-N₄-Lys-Biotin. Planar and SPECT images were acquired at 2 h and 4 h post-injection.

3.7. Synthesis of TETA

1,4,8,11-tetraazacyclotetradecane-N,N',N'',N'''-tetraaceticacid (TETA) synthesis and control was performed according technique for DOTA synthesis reported by Desreux (Inorg. Chem., 1980, 19:1319-1324) and the advise of Dr. Varvarighou, Greece. Briefly, 1 g of 1,4,8,11-tetraazacyclo-tetradecane (cyclam) was suspended in cold water. In a three mouth balon, a solution of 2,2 g of chloroacetic acetic was prepared (10% excess respect to cyclam) and neutralized with NaOH 10M in an ice bath. Cyclam is added to the balon and then heated at 80°C with stirring during 24 h maintaining pH range between 9–10. After this time, mixture is cooled and acidified with HCl 12N until pH 2,5. A white precipitate is formed. Purification was carried out by cationic exchange (Dowex). The obtained precipitate is dissolved in NaOH and incubated for 1 h with stirring in a beaker with ten-fold resin excess. Solution is then filtered and evaporated in a rotavapor. After this the product is dried in phosphorus pentoxid desicator and analysed by melting point, IR and NMR spectra.

4. RESULTS

¹⁸⁸W/¹⁸⁸Re generator (MAP) quality control indicate that it is according to specifications until six month after calibration in view of pH, levels of Al³⁺ (<5ppm), levels of radionuclidic impurities (⁶⁰Co: 2,3x10⁻⁴ µCi/mL of eluate; 1,6x10⁻⁵%; ¹³⁴Cs: 2,5x10⁻⁵ to 1,4x10⁻⁴ µCi/mL of eluate; 4,3x10⁻⁶ to 5,9x10⁻⁶% and presence of ¹⁸⁸W ranged from 5,2 to 9,6x10⁻³%). The elution yield was 71±4% with the maximum of activity in the first three mililiters (88% of total activity).

The best results for direct labelling of Lanreotide with ¹⁸⁸Re were obtained with the use of SnF₂ as reducing agent in the presence of ascorbic acid, HEDP as transchelating agent at pH 1-2 and molar ratios HEDP/lanreotide and SnF₂/lanreotide of 260 and 40 respectively. The chromatography quality control systems selected are shown in Table I. The labelling yield were more than 90%. Sep-pak (C18) purification allow the separation of radiochemical impurities with good yield. Figure 1 shows a representative elution profile obtained. Figure 2 shows the HPLC profiles for the labelled lanreotide with an without purification (radiometric detection). Stability of the purified peptide at RT revealed that after 24 h the RCP is in the order of 90%. Specific activity of labelled lanreotide was about 1,74x10¹⁴ Bq/mol.

The in-house somatostatin receptor tissue membranes tested with a reference material is presented in Fig. 3. The locally prepared iodinated tracers in all cases did not show any biological activity. The receptors protein concentration by Lowry for the two tissue batches were 3.8 mg/mL and 3.06 mg/mL. Fig. 4 shows the inhibition test. For 8µM mass of cold SMT, inhibition of tracer binding was 66%, considering bound counts at zero mass added as 100% binding. Representation of MBC shows a steady straight line with positive slope with

aproximately 100% of binding activity for ^{125}I -SMT Amersham and cold somatostatin (Sigma). Assays using cold lanreotide as mass show a decrease of the MBC to 16%. The other iodinated tracers prepared at laboratory gave no specific binding. About ^{125}I -lanreotide, tagging of high amounts of tracer was observed but no specific inhibition with SMT. Nevertheless, when MBC assays of ^{188}Re lanreotide were done, there was a very low binding capacity which was inhibited by cold lanreotide but not by cold somatostatine.

TABLE I. RF VALUES OF LABELLED PRODUCTS IN THE SPECIFIED SYSTEMS

	MEK Whatman 1	NaCl 0.9% Whatman 3MM	EtOH-HCl0.01N 90:10 ITLC-SG
^{188}Re -Lanreotide	0	0	0.9-1.0
^{188}Re -HEDP	0	0.9-1.0	0
$^{188}\text{ReO}_4^-$	0.9-1.0	0.9-1.0	0.9-1.0
^{188}Re red-hidr.	0	0	0

Labelling of IgG bovine with ^{188}Re was achieved with very low yield (less than 20%) and very poor stability of the labelled compound.

The procedure conducted for labelling MAG_3 with ^{188}Re was succesfully achieved with quantitative yields. Fig. 5 shows a typical HPLC chromatogram of the ^{188}Re - MAG_3 . Synthesis of ^{188}Re - MAG_3 -TFP ester was achieved with low yield due probably for hydrolysis of the EDC.

Fig. 6 shows the labelling yield obtained for ^{153}Sm -EB1 at different EB1: ^{153}Sm molar ratios where the influence of temperature for 20:1 molar ratio is presented in Fig. 7.

ITLC-SG chromatography in the different solvent systems assay: a) EtOH: NH_3 : H_2O (3:1:5), b) MeOH: NH_3 : H_2O (10:1:20) and c) saline allowed to determine the labelling yield of ^{153}Sm -EB1. HPLC in TSK-Gel column, Type G3000SW_{XL} 7,8 mm×300 mm with isocratic program, flow 1 mL/min with phosphate buffer 0,1M, pH 6.9, sodium azide 0,01% with and without EDTA 0,002M allowed to resolve the species ^{153}Sm -acetate from ^{153}Sm -EB1 and complex ^{153}Sm -EB1-avidine. Considering all the conditions analysed, the best yield for the labelling of ^{153}Sm -EB1 was obtained at 80°C during 10 min with a molar ratio of EB1- ^{153}Sm 20:1. Up to 5 mCi of ^{153}Sm were used in the labellings. The incorporation of ^{153}Sm did not affect the binding site for avidin-biotin complex.

Labelling of $^{83}\text{D4}$ with ^{153}Sm was achieved with very low yields (less than 10%) in all cases. For the labelling with ^{125}I , the yield was higher than 50%, obtaining a product with RCP>90% after purification by gel filtration (PD-10, sephadex G25). Binding studies with coated tubes with aOSM (5µg/mL), a rich Tn mucin, gave 28% B/T. Table II shows the biodistribution results obtained in rats with breast tumours induced with N-nitroso methyl urea as well as in normal rats. Although the tumour/muscle ratio is promising, blood clearance must be improved in order to evaluate the potential application of this antibody in 3-step pretargetting; further studies are in course.

Related to biotinization of biomolecules, the Table III depict the results obtained for the diverse biomolecules studied in function of the molar ratio used in each case. Immunoreactivity results are expresed considering the direct binding to aBSM and avidin for

the respective radioiodinated molecules and, in the case of formation of triple complex the activity due to [Ab-B]-[Avidin³]-[B-DTPA-¹¹¹In].

TABLE II. BIODISTRIBUTION OF ¹²⁵I-83D4 IN RATS

	Rat bearing tumour 2 h	Rat bearing tumour 24 h	Normal rat 2 h
% Activity in tumour	5.65	1.19	---
% Act/g in tumour	4.50	1.08	---
% Act/g in muscle	0.60	0.26	0.30
% Act/g in blood	4.64	1.24	4.80
% Act/g in thyroid	1.90	0.45	1.22
Tumour/Muscle ratio	7.5	4.2	

TABLE III. IMMUNOREACTIVITY OF NATIVE AND BIOTINYLATED MOLECULES

Protein	Molar ratio		Immunoreactivity (%B/T)			K _A (Biacore) (M ⁻¹)
	P:2-IT	P/BMCC-B	ABSM	aBSM	Avidin	
			¹²⁵ I-P	¹¹¹ In-B	¹²⁵ I-P	
VH native			6			2x10 ⁸
VH-biotin	1:10	1:25	3	8	57	ND
ScFv D native			5			2x10 ⁷
ScFv D-biotin	1:10	1:25	3	2	51	ND
83D4 native			36			7x10 ⁹
83D4-biotin	1:100	1:50	38	3	7	1x10 ¹⁰
83D4-biotin	1:500	1:50	34	1	7	4x10 ⁹
83D4-biotin	1:1000	1:50	18	6	18	2x10 ⁹

P: protein; B: biotin; ND: non detectable

The best labelling yield in the ⁹⁹Tc^m-N₄-Lys-Biotin preparation was obtained using 224 μmol of stannous chloride with 61 μmol of N₄-Lys-Biotin, adjusting to pH 12 and incubating 1 h at RT. Fig. 8 shows a representative gel filtration profile for the labelled molecule incubated with avidin. The patient study revealed that although, the location of the tumour was in close proximity of the bladder, the 2h images showed already a mass separated by the bladder (Fig. 9). The radiopharmaceutical showed a predominant renal excretion although the presence of some free pertechnetate disturbed the images in the bowel area.

³ And/or streptavidin

The synthesis of TETA was performed with 95% yield obtaining a product with melting point of 211-214°C. IR spectra showed an intense band in 1735cm⁻¹ and 3413cm⁻¹ corresponding to the functional groups C=O and O-H, respectively. NMR peaks correspond to the structure proposed with four carboxylic groups.

5. CONCLUSIONS

¹⁸⁸Re lanreotide, ¹²⁵I lanreotide, ¹⁵³Sm-EB1, ⁹⁹Tc^m-N₄-Lys-biotin were obtained with high radiochemical purity, high *in vitro* stability and their biological characteristics were determined. On the other hand, the indirect labelling of lanreotide with ¹⁸⁸Re must be improved for what availability of chemicals represent a problem for our laboratory work.

A technique for efficient conjugation of biotine to antibodies and fragments by means of introduction of SH groups was set up. This technique could be adapted to other bifunctional agents capable to be attached to sulphhydryl groups. The binding capacity to Tn was higher for the integer antibody (83D4) than for the recombinant fragments produced by genetic engineering and further studies would indicate its potential as radiotherapeutic agent in three steps RIT. Research should be continued in order to achieve fragments with immunoreactivity compatible for RIT.

It was possible to prepare by a modified method rat brain cortex membrane somatostatine receptors which not require an ultra centrifuge although is more time consuming, and whose bioactivity was confirmed by commercial reference material. Locally produced tracers did not exhibit significant specific binding and in the case of lanreotide (cold and labelled) a high non specific tagging was observed. So, more work is needed to conclude our findings.

In summary, significant progress have been made acquiring and applying different techniques including direct labelling of biomolecules with ¹⁸⁸Re, biotin and bifunctional chelate conjugation, purification of conjugates and their labelling with ¹⁵³Sm as well as with ¹²⁵I and ¹¹¹In, which can be easily replaced by others radionuclides for therapeutic applications. Valuable experience was also gained on the application of analytical techniques for the separation and detection of different components. Methods for preparing rat brain membrane receptors and for measuring radioligand binding assays were also acquired. Progress in bifunctional chelating agents synthesis was achieved.

The possibility of local preparation of ⁹⁹Tc^m-N₄-Lys-Biotin and its clinical use by 3-steps pretargeting approach beside the promotion activities carried out during the expertise of Dr. Chinol, has increased the scientists and clinicians interest in the development of new radiolabelled biomolecules as well as in their clinical application.

National interdisciplinary collaboration, regional co-operation and expertise from other participants of the CRP, which have enabled the transfer of materials and information, made possible for us to gain insight into many aspects of the research that was done in our laboratory.

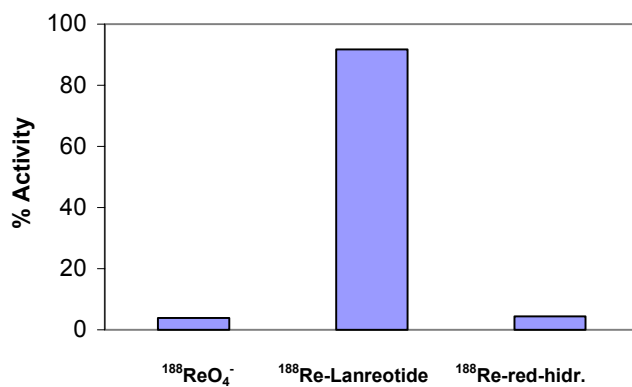


FIG. 1. Sep-pak purification pattern of ^{188}Re lanreotide.

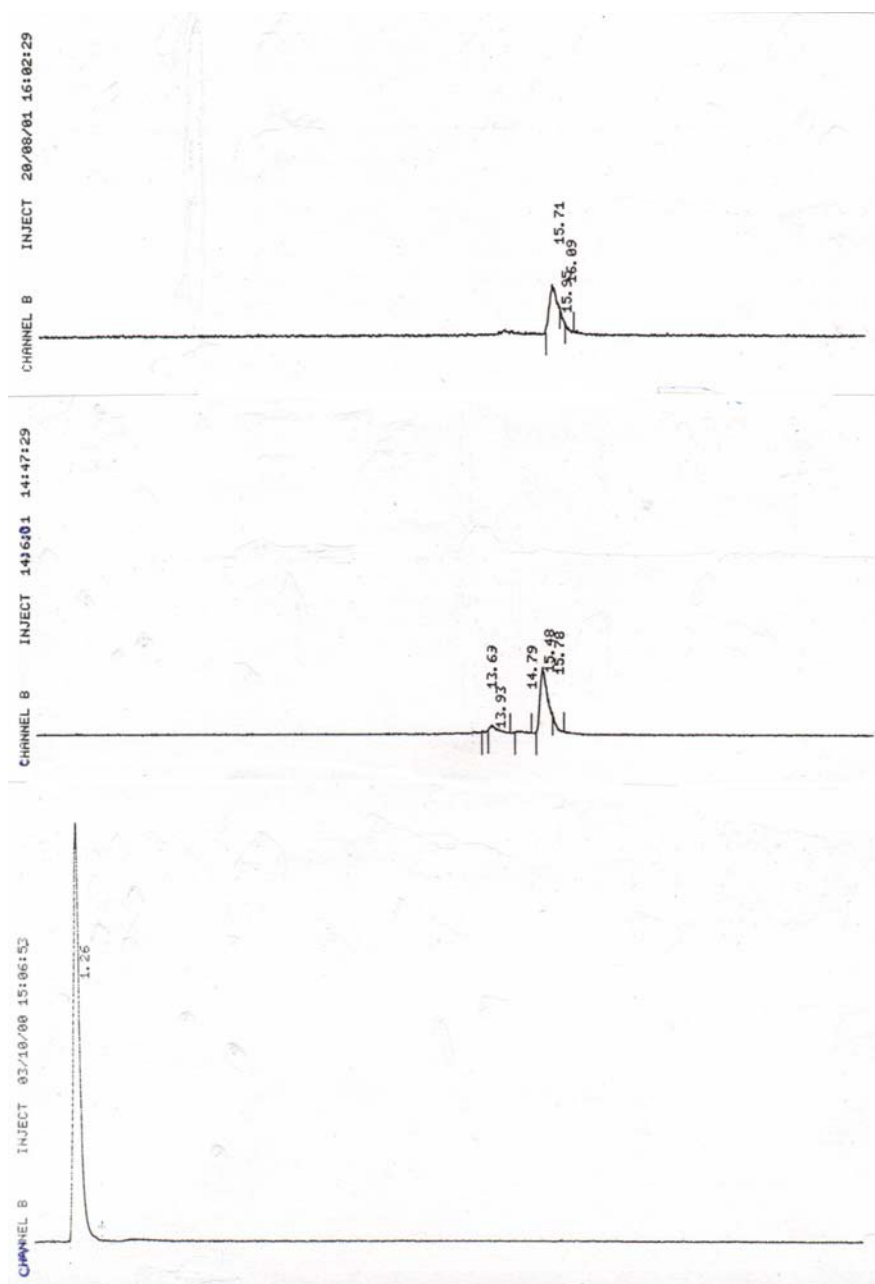


FIG. 2. HPLC profiles of ^{188}Re -lanreotide purified, not purified and $^{188}\text{ReO}_4^-$.

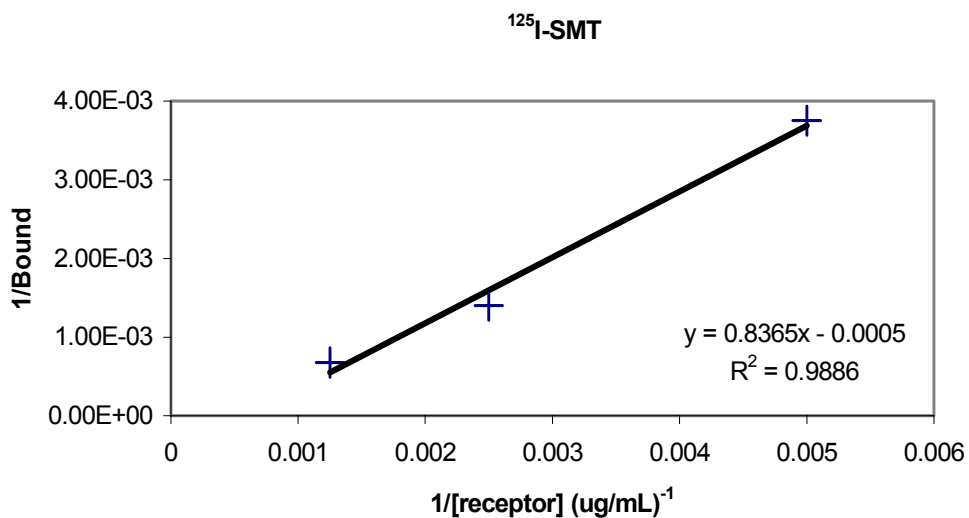


FIG. 3. Maximum binding capacity for rat brain cortex membrane receptors.

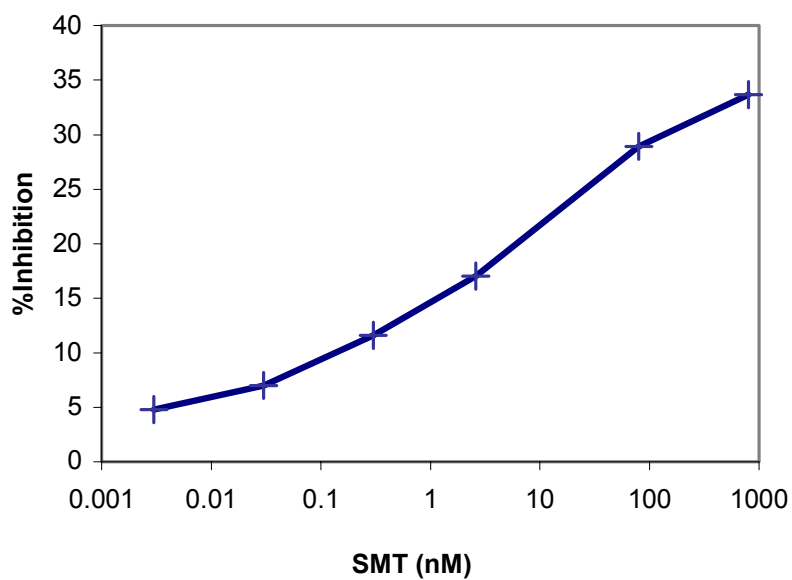


FIG. 4. Inhibition test for rat brain cortex membrane receptors.

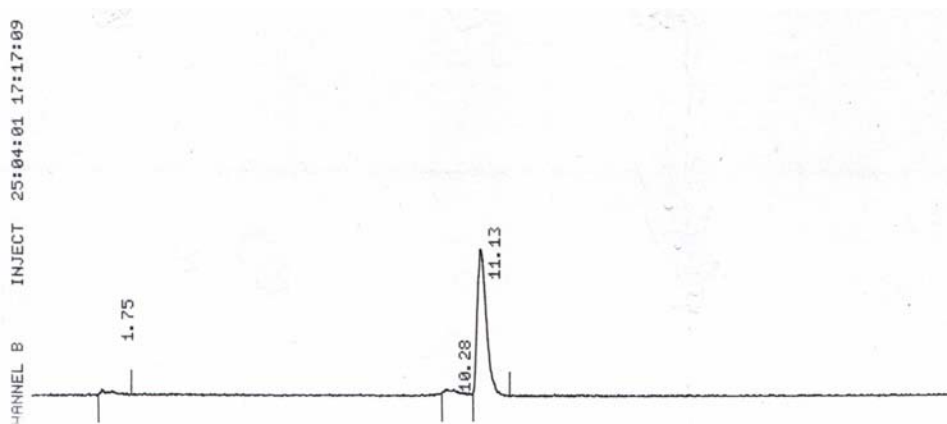


FIG. 5. HPLC profile of OF ¹⁸⁸Re-MAG₃.

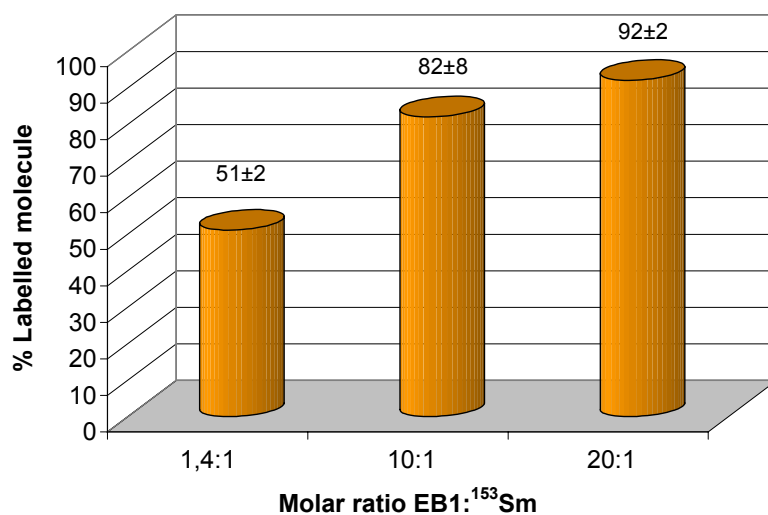


FIG. 6. ¹⁵³Sm-EB1 labelling yields at different molar ratios, incubated at 80° C, 10 min.

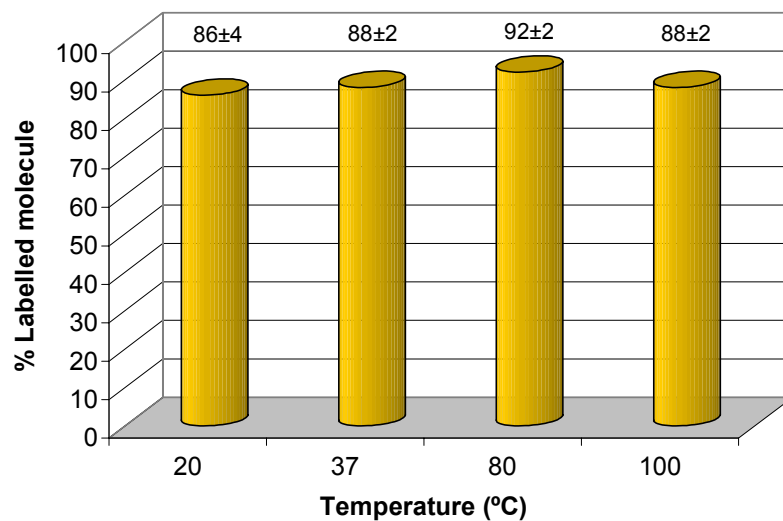


FIG. 7. ¹⁵³Sm-EB1 labelling yields at different incubation temperatures, 10 min, molar ratio EB1/¹⁵³Sm 20:1.

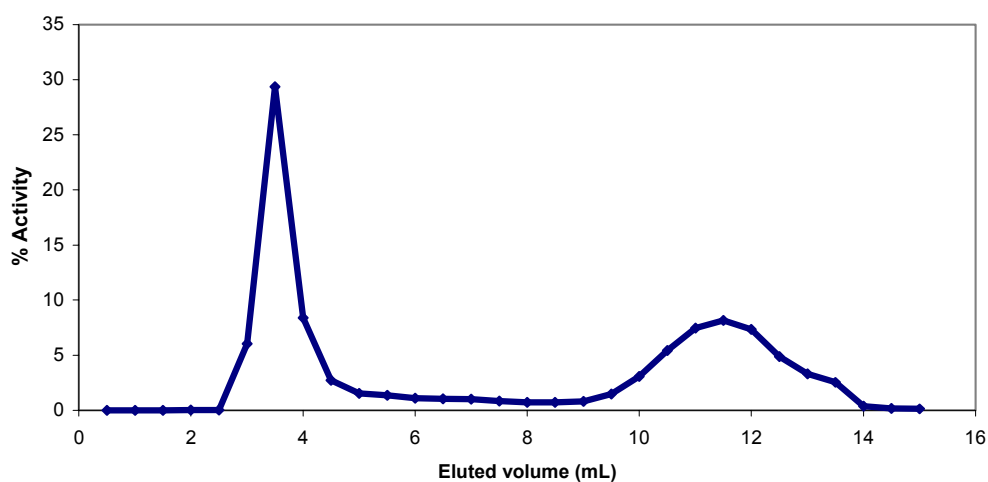


FIG. 8. Gel filtration profile of ^{99m}Tc-N₄-Lys-biotin incubated with avidin.

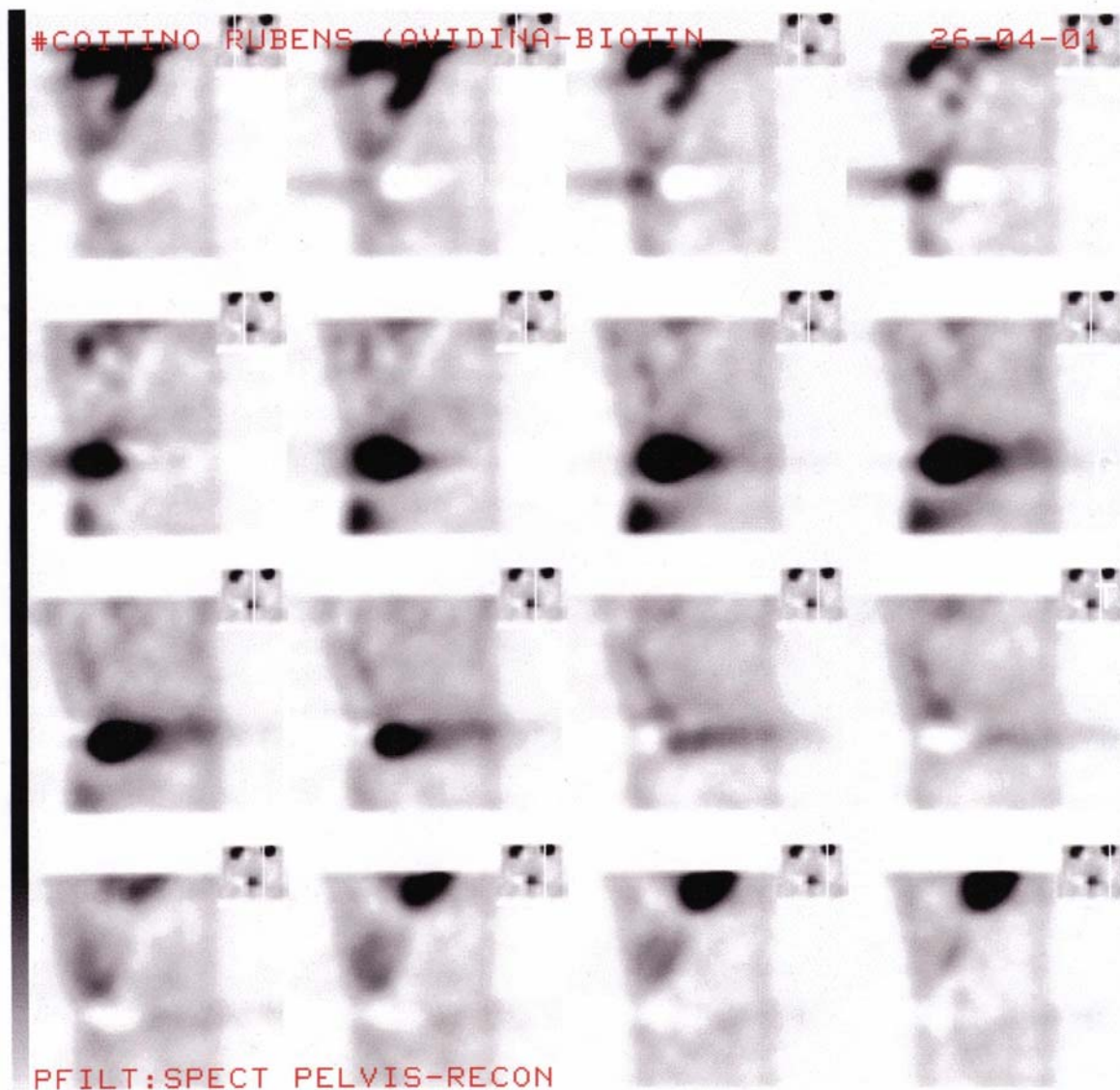


FIG. 9. SPECT images obtained 2 h after injection of $^{99}\text{Tc}^m\text{-N}_4\text{-biotin}$ in a patient with colorectal carcinoma. Although the lesion was located in proximity of the bladder was clearly visible in the tomographic images.

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