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Proper retention factor for Avanafil and Dapoxetine Hydrochloride Separation and Validation with RP-HPLC in Bulk, Dosage Form and Rat Plasma; Validation, Degradation, and Pharmacokinetic Application

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Abstract

Environmental changes and fluctuated storage conditions are the main markers for API degradation and drug-drug interactions. Avanafil (Ava), phosphodiesterase inhibitor and dapoxetine (Dap), selective serotonin reuptake inhibitor helps to treat erectile dysfunction and increase ejaculation retention. The aim of the current study is to investigate the best chromatographic condition for the determination of Ava, Dap and their degradation products at different stress conditions and develop a sensitive, rapid, and reproducible method for their simultaneous estimation either in their combined dosage form or in rat plasma. The chromatographic conditions involve separation with RP-Eclipse-XDP C18 column, (25 cm ×4.6 mm, 5µ) column at 239 nm detection wavelength and mobile phase consisting of buffer (phosphate, pH 3) to acetonitrile with ratio 55:45 (v/v) and the injection volume was 20 µl. The method was subsequently used to evaluate the mixture of Ava and Dap pharmacokinetics in rat plasma. The pharmacokinetic parameters Cmax, Tmax, t1/2, AUC0- 24, AUCtotal, Ke, Vd, and CL of the cited drugs after oral administration was determined. The obtained data demonstrate that the method was applied successfully for separating each component and their degradation products with all accepted validation parameters and the method can be applied for preclinical pharmacokinetic investigation.Keywords: Avanafil, dapoxetine, degradation, HPLC, UV detector, and pharmacokinetics.

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1. Introduction

Erectile dysfunction (impotence) is the inability to get and/or maintain erection enough for satisfactory sexual performance. Many drugs are prescribed for impotency However, it should be noted that some of these treatments interfere with other medications. In addition, drug-drug interaction may lead to serious symptoms also at bad storage conditions inevitable degradation occurs [1]

Avanafil (Ava) is a phosphodiesterase type 5 inhibitor (PDE5) acts after desire stimulation resulting in smooth muscle relaxation of the corpus cavernosum, to increase the inflow of blood [2]. The mediator of this response is nitric oxide. Nitric oxide activates guanylyl cyclase, which form cyclic guanosine monophosphate (GMP) from guanosine triphosphate produced smooth muscle relaxation through a reduction in the intracellular (Ca^{2+}) concentration. PDE-5 enzyme is responsible for the degradation of GMP in the corpus cavernosum. The duration of action of cyclic nucleotide is controlled by the action of phosphodiesterase (PDE) [3].

Dapoxetine (Dap) is from serotonin reuptake inhibitor (SSRI) family and works by decreasing serotonin's activity at the postsynaptic cleft, inhibiting the serotonin transporter, and thereby promoting ejaculatory latency.

The presence of degradation, even in small amounts for each mixed API, may affect the efficacy and safety of pharmaceuticals [4]. Degradation product is

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an impurity that results from chemical changes that occur during storage condition due to the effects of temperature, light, humidity, and pH, reaction with excipients, or contact with primary packaging [5].

Several studies have stressed stability indication methods for Ava. One of them, the analytical method used by Can. et al was focused on Stability-Indicating LC-MS/MS and LC-DAD methods for determination of Ava. In addition, LCMS-IT-TOF was used to identify a new degradation product from medicinal formulations. [6]. Liew and Peh. reported that Dap slightly degrades under alkalin and acidic conditions but was stable underneath UV light and heat [7]. But no attempt has been carried out for identification of degradation products.

In addition, a detailed literature survey reveals that Dap can be estimated by chiral recognition of Dap enantiomers with methylated gamma-cyclodextrin [8]. One more study presented the Dap has been analyzed either alone or in presence of related substances by HPLC [9,10]. An UV spectroscopic method was reported for simultaneous estimation of sildenafil and Dap [11]. Also, UPLC-MS/UV [12] and HPLC and HPTLC [13] method for determination of Dap in combination with sildenafil or fluoxetine were reported.

A thorough survey of the literature revealed that there are only few analytical methods reported for the analysis of Dap in mixture with Ava either by UV spectrophotometric method [14] or spectrofluorimetric method [15] or HPLC-fluorometric detection [16] or LC- MS/MS in human plasma [17]. Based on the aforementioned concerns, there is no data for simultaneous determination paired with degradation products separation and identification for any of them.

The metabolic pathway of avanafil involves hepatic biotransformation primarily by cytochrome P450 enzymes, leading to the formation of several metabolites, with M4, M16, and M17 being the most prominent ones. Understanding the metabolic pathway of avanafil is important for assessing its pharmacokinetics, drug interactions, and potential for adverse effects in clinical practice [18].

The metabolic pathway of dapoxetine involves Phase I and Phase II biotransformation reactions in the liver, primarily mediated by cytochrome P450 enzymes and conjugation enzymes such as UGTs and sulfotransferases. These metabolic processes lead to the formation of multiple metabolites, which are eventually eliminated from the body via urine and feces. Understanding the metabolic fate of dapoxetine is crucial for optimizing its therapeutic use and minimizing the risk of adverse effects and drug interactions [19].

In order to simultaneously determine Ava and Dap HCL in combined pharmaceutical dosage forms and their degradation products under various stress conditions, a sensitive, accurate, economical, and stability-indicating RP-HPLC method was developed in this study.

The pharmacokinetic investigation in rats had effectively used the technique with low detection limits and with high selectivity from any plasma components. On the other hand, the official method required expensive and complicated mass spectrometry equipment.

2. Experimental

2.1. Instruments

The Chromatographic system (Agilent 1260 from Santa Clara, California, U.S) was equipped with UV detector G1314A, auto sampler ALS G1313A, 1260 Degasser G1322A, and Quaternary pump G1311A. Separation was made on Agilent- Eclipse XDB- C18 column (250 mm, 4.6 mm, and 5 µm). A sonicator (ultrasonic cleaner UCS-05 jeio tech Co. from DongCheng, Dongguan, Guangdong, 523000, China) was used. A digital pH meter (Hanna PH211, Romania) was employed for the pH adjustment. A balance (Sartorious, 37070 Goettingen, Germany) was used. (0.45µm Millipore membrane filters, Sartorius Germany) were used for mobile phase filtration; a thermostatic water bath (Memmert, Germany) and a Rotavapor (Buchi, Switzerland) were used.

2.2. Materials and Reagents

Ava (100% potency was kindly provided from EVA pharma), Dap HCL (100% potency was kindly provided from Penta pharma). Super Avana® tablets (batch no. 541415) were purchased on-line and the box contains 4 tablets (100mg Ava and 60 mg Dap HCL/Tablet). Acetonitrile and methanol were purchased from Sigma-Aldrich, Darmstadt, Germany (HPLC grade). Potassium dihydrogen phosphate was obtained from LOBA CHEMIE Mumbai, India and used for preparation of the buffer; HCL 37% (CAS Number: 7647-01-0) phosphoric acid 85% (CAS No.:7664-38-2) (Fig. 1).

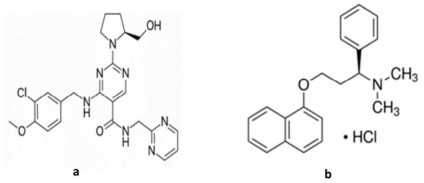

Figure 1: Chemical structures of (a) Ava ($C_{23}H_{26}ClN_7O_3$), (b) Dap ($C_{21}H_{23}NO$)

2.3. Stock preparation

A stock solution of Ava (1000 µg/mL) was created by dissolving an exact weight of Ava (20 mg) in 20 mL of methanol. To create a Dap HCL stock solution

(1000 µg/mL), a precise weight of Dap HCL (20 mg) was dissolved in methanol in a second 20 mL volumetric flask. In order to create stock solutions for Ava degradation products and Dap HCL degradation products, 5 mg of each degradation product (Previously prepared) were dissolved in methanol in two different 50 mL volumetric flasks to reach a concentration of (100 µg/mL).

2.4. Sample preparation:

Four tablets were weighed, then ground into a fine powder. In a 100 mL volumetric flask, an amount of the powder equal to 100 mg Ava and 60 Dap HCL was transferred, 50 mL methanol was added, and the flask was sonicated for 10 min. With the same solvent, the volume was finished to the mark to create a sample stock solution (1000 g/mL Ava and 600 g/mL Dap HCL). Whatman filter paper was used to filter the resulting sample stock solutions, with the first few milliliters being discarded. In order to determine the concentration of both medications, aliquots from the stock solution were transferred to two sets of 10 mL volumetric flasks, and the volume was then finished with mobile phase.

2.5. Chromatographic Conditions

A RP column (Agilent- Eclipse XDB- C18) with dimensions (250 mm, 4.6 mm, and 5 m) was used for the chromatographic separation and quantification. Acetonitrile: 20 mM potassium dihydrogen phosphate, pH 3 adjusted with o-phosphoric acid (45:55, v/v), and a flow rate of 1 mL/min made up the mobile phase and the injection volume was 20 µl. Before injecting all of the solutions, after 30 minutes of saturation with the mobile phase, the system was brought to equilibrium. At 25O C, all experiments were conducted. Based on UV detection at 239 nm, the quantification was conducted.

2.6. Preparation of Avanafil Acid Degradation Product

Fifteen mL of 5N HCL were added after 20 mg of Ava had been dissolved in 5 mL of methanol. The degradation was monitored by LC using the specified conditions while the solution was refluxed at 100 °C for around 45 hours, or until the Ava peak disappeared. The solution was adjusted in volume after being neutralized with 5 N NaOH. The solution was next extracted three times with chloroform (10 mL each time), after which the chloroform extract was filtered on anhydrous sodium sulfate and dried using an oven.

2.7. Preparation of Dap HCL Acid Degradation Product

Fifteen mL of 5N HCL were added after 15 mg of Dap HCL (20 mg) had been dissolved in 5 mL of methanol. The solution was heated in a water bath at 65 °C, and the degree of degradation was examined by LC under the specified chromatographic conditions for approximately 72 hours. The solution was adjusted in volume after being neutralized with 5 N NaOH. After that, three extractions of the solution were made with 10 mL of chloroform, which was then filtered on anhydrous sodium sulfate and dried with an oven.

2.8. Method Validation:

According to ICH recommendations [20], the suggested method's validation for the following factors was carried out: accuracy, specificity, precision, limit of detection, limit of quantification, range, linearity, robustness, and ruggedness.

2.8.1. Linearity

Linearity was determined by plotting a graph of concentration versus peak area of avanafil and dapoxtine HCL. The solutions were prepared at eight concentration levels rang ng from limit of quantification (0.1- 200) µg/mL and (0.06-120) µg/mL respectively. the target concentration (about 100 µg/ml for avanafil and 60 µg/ml for Dapoxtine HCL) and Injected into the HPLC system. The correlation coefficient value, slope, y-intercept, and bias at 100%level were all calculated.

2.8.2. Accuracy

By including standards in the test preparation for the tablets containing Ava and Dap HCL, an accuracy study for those substances was undertaken. At varied concentrations (80%, 100%, 120%, and 160% of the desired concentration), samples were generated in triplicate.

2.8.3. Precision

Repetition and intermediate precision served as proof of the test method's accuracy. By evaluating three samples of Ava and Dap HCL for concentrations of (100 µg/mL, 80 µg/mL, 120 µg/mL) and (60 µg/mL, 48 µg/mL, 72 µg/mL) for Ava and Dap HCL, respectively, and calculating the % RSD for content of each peak, the repeatability of the test technique was assessed. The study was run on various three distinct days to show intermediate precision.

2.8.4. Robustness

To test the resilience of the suggested method, experiments were carried out by changing the conditions. The primary criterion for this study's evaluation was system appropriateness. In this investigation, the variables were the change in the temperature of column from 20 °C to 30 °C, the change in the flow rate from 0.9 mL/min to 1.1 mL/min (10%), and the change in wavelength from 239 nm to 234 nm (2%).

2.8.5. Limit of detection (LOD) and limit of quantitation (LOQ)

Based on the signal-to-noise ratio approach, the limits of detection and quantification for Ava and Dap HCL were established. The concentration where the signal to noise ratio was close to 3 was used to calculate the limit of detection. The concentration at which the Ava and Dap HCL peak signal-to-noise ratio was

found to be close to 10 served as the limit of quantitation. Ava and Dap HCL precision testing was done at or near the limit of quantification. Six test preparations of Ava and Dap HCL tablets were made and administered into the system at the limit of quantification levels (0.1 g/mL and g/mL). Six replicate preparations' percent RSD was calculated.

2.9. System suitability

To assess the system performance, system suitability characteristics were examined [21]. A standard preparation containing Ava at a concentration level of 100 g/mL and Dap at 60 g/mL were injected three times to determine the system's precision. The tailing factor was less than 2.0 for the Ava peak and this met the acceptable criteria of the United States Pharmacopeia (USP), and the acceptability criteria were less than 1% relative standard deviation (RSD) for the Ava and Dap peak areas.

2.10. Forced degradation study.

HCL forced degradation studies were carried out to comprehend the degradation behavior of Ava and Dap. Separate stress tests were performed on Ava and Dap HCL. Acid hydrolysis (5 N HCl, 100 °C, 45 h), alkaline hydrolysis (5 N NaOH, 65 °C, 24 h), water hydrolysis (65 °C, 24 h), thermal (105 °C, 6 h), oxidation (5% H2O2, 25 °C, 5 h), and photolytic (200 Wh/m2, 16 h) conditions were among the stress conditions. The suggested method was then used to analyze the strained solutions.

2.11. Application of the suggested method to an in-vivo pharmacokinetic study design

Ava and Dap were given a single crossover dose at random. In the EDA animal house, thirty male albino rats (weighing approximately 250±20 g) were raised. Wistar rats (Rattus norvegicus) from animal house and kept in regular settings with 12-hour darkness at night and unrestricted access to food and water throughout the experiment. The test subject's animal kept up a seven-day acclimation period. Two groups of rats were created randomly from the rats. The first group served as a negative control and received 2 ml/kg BWt of distilled water orally, while the second received 9 and 5.4 mg/kg BWt, respectively, of the study medication, which contained Ava and Dap. The present study involving rats was conducted in strict accordance with the guidelines provided by the NODCAR Committee (Pys-D9-2022) which equivalent for the Institutional Animal Care and Use Committee and relevant to national and international regulations. Paget and Barnes' [22] worldwide criteria for the handling and management of laboratory animals were followed throughout all experimental methods.

2.11.1. Sampling

A heparinized tube containing whole blood was left at 25ºC for one hour. The samples were then centrifuged for 10 minutes at 5000 rpm. For sample preparation with the medication and/or tadalafil as internal standard (IS) for chromatographic analysis, a 500µL aliquot of plasma was obtained. The blood was sampled at intervals of 0.25, 0.5, 1, 2, 4, 6, 8, 10, and 12 hr over a 10-day period. To prevent unexpected death, each group was split into 5 subgroups of 3 rats. Until the test, the plasma was obtained and kept at -20 ºC. Using the previously mentioned HPLC chromatographic conditions, plasma samples were examined for Ava and Dap.

2.11.2. Plasma Sample preparation

Protein precipitation was used to remove and prepare the plasma samples using acetonitrile. After reconstitution, 500 µL of plasma samples were combined with 50 µL of IS (Tadalafil, 100 µg/mL) and 1.5 mL of acetonitrile to produce a final concentration of 5 µg/mL. Samples were vortexed for 30 s and centrifuged for 10 min at 5000 rpm. After the supernatant was filtered, a portion of it was reconstituted under vacuum and the volume was then adjusted to 1 ml with mobile phase.

2.11.3. Validation of the bioanalytical method

The method's linearity, intraday and inter-day precision, and accuracy have all been confirmed in accordance with FDA requirements [23]. Ava and Dap reference solutions (20, 10 µg/mL) were serially diluted to create solutions with drug concentrations ranging from 0.1 to 10.0 µg/mL and from 0.06 to 5 µg/mL, respectively. These solutions were tested for linearity and range. The same HPLC described procedure was used to treat and evaluate these solutions after they had been generated in triplicate. The internal standard, as well as known doses of Ava and Dap, were spiked into blank plasma to provide standard samples with final concentrations that were within the ranges of 100 to 10000 ng/mL and 60 to 5000 ng/mL, respectively.

2.11.4. Calibration curves

Ava and Dap concentrations in each reference sample and the resultant peak area ratios were used to generate calibration curves. The calibration curves were used to estimate the amounts of in the unknown samples.

2.11.5. Within-run precision studies

Six injections of processed Ava and Dap mixture samples incorporating lower limit of quantitation (LLOQ) (0.1 and 0.06 µg/mL for Ava and Dap, respectively) and low, medium, and high-quality control samples (QCs) (1, 2, and 5 µg/mL) were used for within-run precision studies. All samples were analyzed on the same day. Three injections of the various QCs, prepared and administered over the course of two days, were used to evaluate between-run precision. The coefficient of variation CV% of each computed analyte concentration was used to

express precision. The percentage of the two medicines recovered from the three QC samples was used to measure accuracy. The same previously manufactured solution of three different concentrations (1, 2, and 5 µg/mL) were used to prepare the unfortified processed sample solution of Ava and Dap. For the fortified solutions, 1500 mL of acetonitrile with concentrations of 0.66, 1.33, and 3.33 g/ml was added to 500 ml of plasma, which was then thoroughly mixed. After 10 minutes of protein precipitation at 5000 rpm, the supernatant was reconstituted and diluted to one ml with the mobile phase. For Ava and Dap, the fortified drug concentration was 1, 2, and 5 g/mL, respectively, as unfortified. The accuracy was calculated as the percent recovery of the fortified compared with the unfortified drug concentrations, and the drug concentration in each fortified and unfortified solution was determined (n = 3).

Bench-top stability testing was done on Ava and Dap in rat plasma at concentrations of QC samples (1, 2, and 5 g/mL) for 4 hours at 25°C. The stability of freeze/thaw was evaluated by analyzing QC samples instantly and then again after 18 hours of storage in the autosampler setting, 10°C was assessed. All of the QC samples were quantitated using freshly processed standard samples. All stability QC samples underwent triplicate analysis.

2.11.6. Pharmacokinetic and statistical calculations

WinNonlin® software (v 6.1; Pharsight Corporation, Mountain View, CA, USA) was used to analyze the non-compartmental plasma concentration-time profiles of Ava, and Dap. The observed data were used to calculate the pharmacokinetic values for the peak concentration (Cmax) and time to Cmax (Tmax). Individual concentration versus time profiles were shown, and the log linear regression of at least three data points deemed to be in the terminal phase was used to calculate the terminal elimination rate constant (ke). The trapezoidal rule was used to calculate the area under the curve (AUC) from time zero to the last recorded concentration of AUC last plus AUC last/k. Using dose/AUC, the total body clearance (CL) was calculated; Volume of distribution (Vd) was calculated as dose/C0, where C0 is the concentration measured just after the administration. All results were expressed as arithmetic mean ± standard deviation (SD).

3. Results and discussion

3.1. Optimization of the chromatographic conditions:

The method was aimed to separate degradation products formed under various stress conditions from Ava and Dap HCL as there is no report till the date for this combination. The Optimization retention K prime (retention factor) was adjusted to obtained the best retained by the stationary phase relative to its time spent in the mobile phase. Accordingly, the composition of the mobile phase, pH, organic modifiers, flow rate, temperature of the column and wavelength were optimized. Various mobile phases were tried to achieve well resolved detection of the drugs from their degradation products like different ratios of methanol: water or acetonitrile: water but no well resolved peaks were obtained. Then different buffers were tried, and the combination of phosphate buffer with acetonitrile gave sharp peaks but similar retention times for the cited drugs, it was decided to use this combination further in various ratios with adjustment of pH of the buffer to (3). This has resulted in improved shape for both drugs and well resolved peaks from their degradation products. The optimized method, utilizing HPLC with a UV detector and degradation technique, was compared to a single method utilizing HPLC with a UV detector without degradation technique, as described by Patel et al. (2016) [24]. The optimized HPLC conditions involve a Phenomenex Gemini C18 column with an acetonitrile–buffer mobile phase (ammonium acetate buffer at pH 3.7 with acetic acid) at a flow rate of 0.7 mL/min. Detection is carried out at 239 nm with a temperature of 20°C. The developed methods are validated and effectively applied for the simultaneous determination of Ava and Dap in the dosage form. LOQ of obtained data VS. Patel was lower for obtained results with 20 and 30-fold for Ava and Dap respectively.

3.2. Method Validation

For Ava and Dap HCL, respectively, the calibration curve was linear in the range of (0.1-200) µg/mL and (0.06-120) µg/mL, as shown in (Table 1). LOD and LOQ values for Ava and Dap HCL were calculated as 0.033/0.099 µg/mL and 0.019/0.059 µg/mL, respectively. These results demonstrate the method's ability to examine diluted solutions. For Ava and Dap HCL, the correlation coefficient was found to be 0.9997 and 0.9989, respectively. The y-intercept values were discovered to be minimum. Furthermore, the residuals were dispersed at random around the mean and do not exhibit any pattern. The technique was discovered to be precise and effective at extracting and quantifying the analytes. Repeatability and intermediate precision were taken into consideration as accuracy parameters.

The peak area's %RSD was discovered to be less than 2, showing that the suggested strategy works. According to the recovery studies shown in (Table 1), the proposed method's accuracy has been demonstrated. The technique was effectively used to quantify both medications from approved tablet formulations without excipient interference or degradation products.

Table 1: validation parameter and result of HPLC method for Ava and Dap in their drug substances and products

Parameter	Ava	Dap
Range (µg/mL)	0.1-200µg	0.06-120µg
Regression Data		
Slope	90.367	67.997
SD of slope	0.31	0.64
Intercept	55.825	43.052
SD of intercept	0.24	0.11
Correlation coefficient (r)	0.9997	0.9989
SD of (r)	0.0001	0.0006
LOD/LOQ (µg/ mL)	0.033/ 0.099	0.019/ 0.059
Accuracy		
Drug substance	100.72±0.72	100.2±0.98
Drug product	99.87±0.54	100.54±0.36
Added standard	100.39±1.09	100.84±1.39
Precision (RSD %)		
Intra-day a	0.077%	0.099%
Inter-day b	0.066%	0.086%

As part of the optimization of the LC technique settings, system suitability testing is carried out to ensure that the repeatability and resolution are suitable for the analysis being carried out. A 20 µg/mL as% RSD solution was injected six times, and the test criteria were column efficiency, resolution, theoretical plates, capacity factor, chromatographic peak tailing, selectivity factor, and repeatability of peak area. Examples of the test results that were recorded for the recently developed approach are shown in (Table 2). Furthermore, the resolution of the Active Pharmaceutical Ingredient (API) and its degradation products were identified as 3.47, 8.98, and 17.32 for Ava compared to Ava degradation, and Dap compared to Ava, followed by Dap degradation compared to Dap. These values represent the separation achieved between the API and its degradation products in the analytical method employed. The resolution is a critical parameter in chromatographic analysis as it determines the degree of separation between adjacent peaks, indicating the method's ability to distinguish between closely eluting compounds.

Table 2: system suitability parameters for HPLC method for determination of Ava and Dap in presence of their degradation products.

Parameter	Ava	Dap	Recommended value
Rt	2.8	4.7	The small value indicates rapid resolution
k	1.89	3.73	1-10 acceptable
α	1.77	1.98	>1
R	3.47	8.98	>2
T	0.68	0.54	≤ 2 T=1 for a typical symmetric peak
N	6034	5260	>2000 Increases with efficiency of the separation
HETP	0.0012	0.0021	The smaller the value, the higher the column efficiency
RSD%	0.097	0.106	RSD≤1, n =6

Retention time (RT); Capacity factor (k); Selectivity factor (α); Resolution (R); Tailing factor (T); Theoretical plates (N).

Forced degradation study:

Studies on the forced degradation of intact drugs can help with the discovery of key degradants, the establishment of the degradation route, the validation of the method's ability to indicate stability, and the assessment of the drug's stability. Under alkaline hydrolysis (5 N NaOH, 65 °C, 24 h), water hydrolysis (65 °C, 24 h), and photolytic stress (200 Wh/m2, 16 h), Dap HCL and Ava were both determined to be stable. Complete degradation was seen mostly under conditions of acid stress (5 N HCl, 100 °C, 45 h) for Ava and (5 N HCL, 65 °C, 72 h) for Dap, as well as under conditions of oxidation stress (5% H_2O_2, 25 °C, 5 h) and thermal stress (105 °C, 6 h). Under acid stress, acidic impurity was one of the main degradants seen.

At retention times of approximately 2 minutes for Ava degradation and approximately 9.1 minutes for Dap degradation, an unidentified contaminant was found. Ava and Dap HCL cannot completely degrade under the specified conditions of oxidative stress and heat stress. By infusing the standards of the full degradation solutions of both Ava and Dap HCL separately, the retention times of the known impurity in the stressed samples were verified. Dap is heated in a water bath for 72 hours at 65 degrees Celsius and Ava is refluxed for 45 hours at 100 degrees, both of which cause hydrolysis and result in a consistent drop in drug concentration with increasing time intervals. However, the medicines completely disintegrate under extreme acid hydrolysis conditions. According to (Fig. 2), the retention durations for Ava, Dap, and Dap degradation are 2.1, 2.8, 4.8, and 9.2 minutes, respectively.

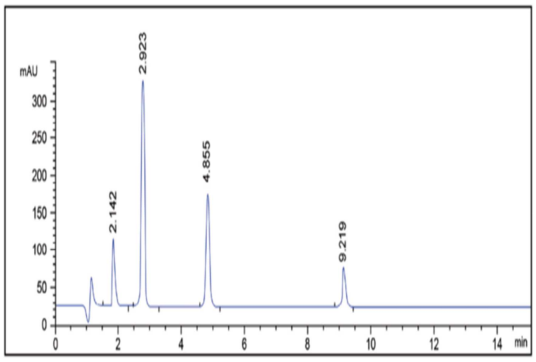

Figure 2: Chromatographic chart for mixture of Ava and Dap spiked with degradation products

Ava and Dap's degradation products were also identified by IR followed by ^{1}H-NMR, as shown in Table 3 and Figs. 3 and 4 as well as (Fig. S1 to S8 in the supplemental materials).

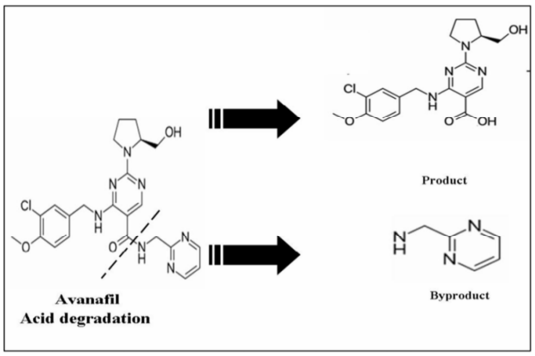

Figure 3: Predicted Structure of Ava degradation **[**4-((3-chloro-4-methoxybenzyl)amino)-2-(2-(hydroxymethyl)pyrrolidin-1-yl)pyrimidine-5-carboxylic acid**]**

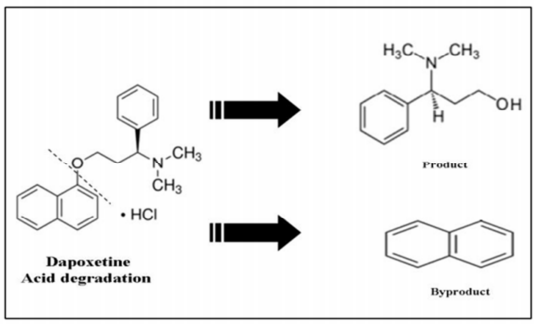

Figure 4: Predicted Structure of Dap degradation

Table 3: Characterization of function groups of Ava and Dap degradation via FTIR followed by proton NMR

	IR		**NMR**	
	Wavelength nm	**Function group**	**Wavelength nm**	**Function group**
Ava	3441	-NH	1.8	-CH-
	3217	-OH	1.9	-C=C-CH-
	3035	0	3.1	Cl- CH
	1670	-C=O	3.2	C-OH
	1660	Aromatic ring	3.5	-CH-O
	1636	C=C-	5.5	C=CH
	1404	Aromatic ring	7	Ar-H
	1068	C-O		
	1029	C-N		
	676	C-Cl		
DAP	3583	N-H	1.2	0
	3062	CH_3	1.9	C=C-CH-
	1660	Aromatic ring	2.4-2.7	Ar-CH3
	1636	C=C-	3.5	-CH-O
	1068	C-O	4.5	C=CH
	1026	C-N	7	Ar-H

3.3. Application of the proposed method to *in vivo* pharmacokinetic parameters

The method was validated according to FDA guidelines for linearity, within-run and between- run precision, and accuracy [16].

3.3.1. Linearity and lower limit of quantitation of bioanalytical method

Constructing calibration curves for Ava and Dap required graphing the peak area ratio of the drug to the IS (Tadalafil) vs. the drug concentration. The mean regression coefficients were calculated using intercepts and slopes weighted linear regression with (1/x) as the weighing factor. Over the linearity range of Ava and Dap, the developed calibration curves were determined to be linearity (100 – 10000 and 60 to 5000 ng/mL) respectively. The LLOQ for Ava and dap was (100, and 60 ng/mL) and showed good outcomes in terms of precision and accuracy. Additionally, the regression equations were calculated, and for Ava and Dap, the correlation coefficients were found to be 0.9949 and 0.9998, respectively.

3.3.2. Selectivity

The LC chart of blank plasma from six different rats showed no interfering peaks. The suggested chromatographic technique has great specificity because of the suitable mobile phase and specific wavelength. The symmetrical resolution of the peaks and absence of considerable chromatographic interference around the retention times of the analyte and IS in specimens devoid of analytes demonstrate the analysis's selectivity. (Fig.5) is a representative chromatogram of blank rat plasma, plasma spiked with IS, Ava and Dap, besides rat plasma sample collected at 60 min after p.o. administration.

3.3.3. Within-run and between-run precision and accuracy

As indicated in (Table 4), satisfactory results were obtained for each of the listed drugs in terms of CV% and recoveries for both within run and between run precision. According to the acceptance criteria for the assessment of precision, the variation of each concentration level from the nominal concentration for QC samples was within 15%, except for LLOQ 20%. Similar to this, except for LLOQ 20%, the mean accuracy for QC samples did not vary by more than 15% of the nominal concentration [24].

By analyzing four QC samples in quintuplicate at LLOQ-the low QC, medium QC, and high QC-it was possible to determine the accuracy and precision of the assay for Ava and Dap in plasma, as well as its accuracy and precision between and within days. The entire analytical method was carried out over the

course of two days and the results from the QC samples were scrutinized for between-day precision and accuracy. For QC samples, the between-run and between-run variability (precision) was within the acceptable range of 15%. The mean accuracy of Ava and Dap concentration, varied from 102.36% to 105.45% and 104.76% to 105.90% respectively.

Table 4: Within-run and between-run precision and accuracy results of Avanafil and dapoxitine in rat plasma by the proposed HPLC method

	Parameters	Precision (CV %)		Accuracy	
		Intraday	Interday	Unfortified (µg/ml)	Fortified (Recovery %)
Ava	LLOQ	0.98	1.26	0.1	102.36%
	LQC	0.81	1.04	1	102.91%
	MQC	0.96	1.09	2	104.70%
	HQC	1.20	1.28	5	105.45%
	Parameters	Precision CV %		Accuracy	
		Intraday	Interday	Unfortified (µg/ml)	Fortified (Recovery %)
Dap	LLOQ	1.04	1.30	0.06	105.90%
	LQC	1.20	0.86	1	104.76%
	MQC	1.09	1.32	2	105.21%
	HQC	0.45	1.27	5	105.59%

Lower Limit of Quantification (LLOQ), Low Quality Control (LQC), Medium Quality Control (MQC), High Quality Control (HQC).

3.3.4. Recovery from rat plasma matrix

The recommended method's extraction effectiveness was evaluated using the mean recovery of the specified pharmaceuticals from rat plasma. Within the bounds of variability, good recovery data were obtained and reported in (Table 4), demonstrating a good extraction efficiency of the suggested approach. Furthermore, it was discovered that the Tadalafil as IS mean recovery from rat plasma was 104.8 4.8%. The extent of an analyte's recovery does not have to be 100%, but it must be consistent, exact, and repeatable (FDA), according to the acceptance requirements. Additionally, the impact of the matrix was evaluated by contrasting the direct injection of comparable standard solutions with pretreatment blank samples spiked at a concentration of QC samples.

3.3.5. Bioanalytical stability parameters

Stability parameters of bioanalytical method were illustrated at autosampler at low, medium and high QCs at 10ºC, Bench top (Room temperature), Freeze/Thaw (-80 °C), at three cycle, and long term -80 °C, for 28 days. Obtained data were stable for each parameter and as shown at (Table 5).

Table 5: Assessment of stability for Ava and Dap in rat plasma

Cond.	NC. µg/mL	Ava		Dap	
		Calc. Conc. µg/mL	% RE	Calc. Conc. µg/mL	% RE
AS 10ºC	1	1.014 ± 0.007	1.4	1.034 ± 0.012	3.37
	2	1.985 ± 0.007	-0.75	1.96 ± 0.021	-2
	5	4.999 ± 0.041	-0.02	4.875 ± 0.075	-2.5
BT	1	1.028 ± 0.019	2.8	0.951 ± 0.012	-4.87
	2	2.053 ± 0.017	2.65	1.947 ± 0.057	-2.63
	5	4.92 ± 0.182	-1.6	4.823 ± 0.178	-3.53
Cycle 1	1	0.971 ± 0.003	-2.9	0.933 ± 0.024	-6.67
	2	1.945 ± 0.043	-2.75	1.907 ± 0.043	-4.63
	5	4.893 ± 0.097	-2.14	4.917 ± 0.103	-1.67
Cycle 2	1	0.946 ± 0.038	-5.4	0.884 ± 0.008	-11.6
	2	1.931 ± 0.071	-3.45	1.799 ± 0.06	-10.07
	5	4.763 ± 0.185	-4.74	4.53 ± 0.137	-9.4
Cycle 3	1	0.952 ± 0.044	-4.8	0.9 ± 0.017	-10.03
	2	1.926 ± 0.047	-3.7	1.783 ± 0.073	-10.87
	5	4.79 ± 0.188	-4.2	4.382 ± 0.083	-12.37
LT	1	0.915 ± 0.072	-8.5	0.872 ± 0.015	-12.8
	2	1.81 ± 0.075	-9.5	1.686 ± 0.03	-15.7
	5	4.675 ± 0.322	-6.5	4.327 ± 0.089	-13.47

Conditions: (Cond); Nominal Concentration (NC); Relative estimated (RE); Bench top room temperature (BT); Long term (LT).

3.3.6. Pharmacokinetics of Ava and Dap after oral administration to rats

The vehicle and treated rats were given calculated dose according to [20] Paget and Barnes, by oral administration. Fig (5) represents the

chromatographic separation of mixed Ava and Dap with high resolution (5.2, 6.3) for Dap: Ava and IS: Dap, respectively.

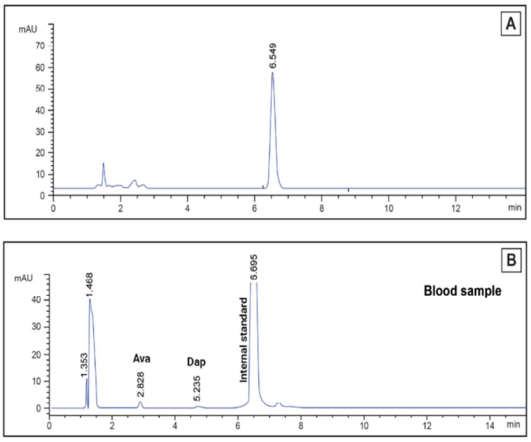

Figure 5: Representative chromatograms of (A) blank rat plasma with IS, (B) plasma spiked with Ava and Dap, plasma sample obtained from 1 h after oral administration at the dose of 9, 5.4 mg/kg of Ava and Dap respectively

On the other hand, Ava separates from the plasma clustered component which indeed the low proper interference. Fig. (6) showed the average plasma concentration-time profiles of the cited drugs. After a single oral administration dose of 9, 5.4 mg/ kg BWt of Ava and Dap, respectively, was easily absorbed and was detectable within the first 6 min in plasma, the pharmacokinetic parameters are shown in Table (6).

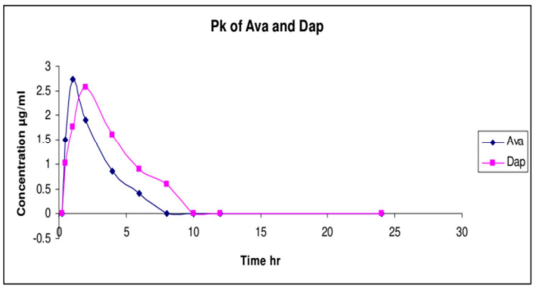

Figure 6: Plasma concentration-time curves of Ava and Dap after a single oral administration at the dose of 9 mg/kg. Each point represents an average of six determinations, standard deviation of the mean (n = 6).

At 1, 2 hours, the maximal plasma concentration (C_{max}) was discovered to be 2.743, 2.573 g/mL. According to calculations, the plasma elimination $t_{1/2}$ value is 1.832, 2.752 h. According to calculations, the apparent volume of distribution was only 0.6, 0.3 mL/kg, and the apparent clearance was 0.225, 0.076 mL/min/kg. Additionally, for Ava and Dap, the AUC to 24 hours was 7.617, 11.118, and the sum was 8.71, 13.372.

Table 6: Pharmacokinetic Parameters of Ava, Dap after oral Administration at the Dose of 9, 5.4 mg/kg in Rats, respectively (Mean ± SD, n = 6)

PK parameters	**Ava**	**Dap**
Cmax (µg/mL)	2.743 ± 0.232	2.573 ± 0.181
Tmax hr	1 ± 0.084	2 ± 0.177
$T_{1/2}$ hr	1.832 ± 0.146	2.752 ± 0.221
AUC $_{0\text{-}24}$ (µg/h/L)	7.617 ± 0.675	11.118 ± 1
AUC $_{total}$ (µg/h/L)	8.71 ± 0.727	13.372 ± 0.963
Ke (h^{-1})	0.375 ± 0.03	0.252 ± 0.02
Vd (mL/kg)	0.6 ± 0.043	0.3 ± 0.025
CL (mL/hr.kg)	0.225 ± 0.016	0.076 ± 0.005

4. Conclusion

A simple rapid and reproducible stability indicating LC method was successfully developed to simultaneously quantify Ava and Dap in pharmaceutical dosage form and in spiked rat plasma. In addition, the analysis of mixture was obtained in the presence of acid degradation products. The method is found to be specific as there is no interference of excipient and the degradation products or rat plasma matrix. Obtained data concluded that the method was successfully applied for separation of each component and each degrading product with all accepted validation parameters and applied for preclinical pharmacokinetic investigation.

Compliance with Ethical Standards:

Conflict of Interest: The authors declare that they have no conflict of interest.

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References

[1] A.W. Pastuszak, Current diagnosis and management of erectile dysfunction. Curr Sex Health Rep 2014; 6 (3): 164–76, (n.d.).

[2] S.A. Huang, J.D. Lie, Phosphodiesterase-5 (PDE5) inhibitors in the management of erectile dysfunction, Pharm. Ther. 38 (2013) 407.

[3] M. Lorigo, N. Oliveira, E. Cairrao, PDE-mediated cyclic nucleotide compartmentation in vascular smooth muscle cells: From basic to a clinical perspective, J. Cardiovasc.Dev. Dis. 9 (2022) 4.

[4] M.D. Waldinger, Drug treatment options for premature ejaculation, Expert Opin. Pharmacother. 19 (2018) 1077–1085.

[5] International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use Guideline, Impurities in new drug substances Q3A (R2), in:

Proc. Int. Conf. Harmon. Tech. Requir. Regist. Pharm. Hum. Use, Geneva, Switz., 2006.
[6] N. CAN, Development of Validated and Stability-Indicating and LC-DAD LC-MS/MS Methods for Determination of Avanafil in Pharmaceutical Preparations and Identification of a Novel Degradation Product by LCMS-IT-TOF, Molecules. 23 (2018) 1771. https://doi.org/10.3390/molecules23071771.
[7] K. Bin Liew, K.K. Peh, Stability indicating HPLC-UV Method for determination of dapoxetine hcl in pharmaceutical product, Acta Pol. Pharm. - Drug Res. 71 (2014) 393–400.
[8] G. Neumajer, T. Sohajda, A. Darcsi, G. Tóth, L. Szente, B. Noszál, S. Béni, Chiral recognition of dapoxetine enantiomers with methylated-gamma-cyclodextrin: A validated capillary electrophoresis method, J. Pharm. Biomed. Anal. 62 (2012) 42-47.
[9] P. Mehta, U. Sahoo, A.K. Seth, Development and validation of a RP-HPLC method for the determination of dapoxetine hydrochloride in pharmaceutical formulation using an experimental design, Int. J. Pharm. Sci. Rev. Res. 6 (2011) 76–82.
[10] T. Rohith, S. Ananda, S. Ananda, A validated chiral liquid chromatographic method for the enantiomeric separation of dapoxetine hydrochloride, Int. J. Adv. Res. Pharm. Bio Sci. 2 (2012) 311–319.
[11] A.P. Chabukswar, B.S. Kuchekar, S.L. Patil, S.A. Moon, S.G. Chate, B.D. Pagare, Spectrophotometric simultaneous determination of dapoxetine and sildenafil in combined tablet dosage form by absorbance corrected method, Der Pharma Chem. 4 (2012) 1404–1407.
[12] V.S. Tambe, M.N. Deodhar, V. Prakya, Stability-indicating UPLC-MS/UV method for simultaneous determination of sildenafil citrate and dapoxetine hydrochloride from bulk and formulation, Indian J. Pharm. Sci. 78 (2017) 663–672.
[13] S.M. Soliman, H.M.Y. El-Agizy, A.E.A. El Bayoumi, Validated Stability-Indicating UPLC Method for Determination of Dapoxetine and Fluoxetine: Characterization of Their Hydrolytic Degradation Products, Kinetic Study and Application in Pharmaceutical Dosage Forms, Pharm Anal Acta. 8 (2017) 571.
[14] M.N. Patel, C.S. Kothari, Multivariate Approaches for Simultaneous Determination of Avanafil and Dapoxetine by UV Chemometrics and HPLC-QbD in Binary Mixtures and Pharmaceutical Product, J. AOAC Int. 99 (2016) 649–663. https://doi.org/10.5740/jaoacint.15-0259.
[15] T.A. Mohamed, S.A. Atty, Native and synchronous fluorescence spectroscopy for determination of avanafil in presence of its co-formulated drug (dapoxetine hydrochloride): Application to pharmaceutical product, biological fluid and content uniformity, Spectrochim. Acta Part A Mol. Biomol. Spectrosc. 229 (2020) 117898.https://doi.org/10.1016/j.saa.2019.117898.
[16] M. Hegazy, A. Kessiba, M. Abdelkawy, A.E. El-Gindy, RP-HPLC with Time Programmed Fluorescence Detection for Quantitation of Avanafil and Dapoxetine Hydrochloride: Application to Pharmaceutical Dosage Form and Biological Fluid, J. Liq. Chromatogr. Relat. Technol. 38 (2015) 1660–1665. https://doi.org/10.1080/10826076.2015.1087860.
[17] M.N. Abou-Omar, A.M. Annadi, N.M. El Zahar, A.O. Youssef, M.A. Amin, M.S. Attia, E.H. Mohamed, Simultaneous determination of Avanafil and Dapoxetine in human plasma using liquid chromatography/tandem mass spectrometry (LC-MS/MS) based on a protein precipitation technique, RSC Adv. 11 (2021) 29797–29806. https://doi.org/10.1039/D1RA05492A.
[18] Almazroo, O. A., Miah, M. K., & Venkataramanan, R. (2017). Drug Metabolism in the Liver. Clinics in liver disease, 21(1), 1–20. https://doi.org/10.1016/j.cld.2016.08.001
[19] Skibiński, R., Trawiński, J., & Gawlik, M. (2021). Characterization of Phase I Hepatic Metabolites of Anti-Premature Ejaculation Drug Dapoxetine by UHPLC-ESI-Q-TOF. Molecules (Basel, Switzerland), 26(13), 3794. https://doi.org/10.3390/molecules26133794.
[20] P. Borman, D. Elder, Q2(R1) Validation of Analytical Procedures, ICH Qual. Guidel. (2017) 127–166. https://doi.org/10.1002/9781118971147.CH5.
[21] The United States Pharmacopeia (USP 30), National Formulary (NF 25), 2007, Rockville, MD., n.d.
[22] G.E. Paget, J.M. Barnes, Toxicity tests, Eval. Drug Act. Pharmacometrics. 1 (1964) 135–165.
[23] Fda, Cder, Bioanalytical Method Validation Guidance for Industry Biopharmaceutics Bioanalytical Method Validation Guidance for Industry Biopharmaceutics Contains Nonbinding Recommendations, 2018.
[24] Darwish, M. K., Soliman, M. M., & Abdel-Razeq, S. A. (2019). Validated Stability Indicating HPTLC and UV-Spectrophotometric Techniques for the Determination of Avanafil. Asian Journal of Chemical Sciences, 6(4), 1–16.
[25] Mital N Patel, Charmy S Kothari, Multivariate Approaches for Simultaneous Determination of Avanafil and Dapoxetine by UV Chemometrics and HPLC-QbD in Binary Mixtures and Pharmaceutical Product, Journal of AOAC INTERNATIONAL, Volume 99, Issue 3, 1 May 2016, Pages 649–663, https://doi.org/10.5740/jaoacint.15-0259.
[26] Ahmed, Tarek A et al. “Two-Step Optimization to Develop a Transdermal Film Loaded with Dapoxetine Nanoparticles: A Promising Technique to Improve Drug Skin Permeation.” Dose-response: a publication of International Hormesis Society vol. 18,2 1559325820923859. 7 May. 2020, doi:10.1177/1559325820923859