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Original Article

BIO-ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF FINASTERIDE, TADALAFIL, AND ITS APPLICATION TO PHARMACOKINETIC STUDIES IN RAT PLASMA BY USING LC-MS/MS

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ABSTRACT

Objective: An easy, quick, precise, active and reproducible LC-MS/MS (Liquid Chromatography Tandem Mass Spectrometry) technique was developed for the bio-analytical method of Finasteride and Tadalafil using Avanafil as internal standard (IS).

Methods: This article summarizes the recent progress on bioanalytical liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) methods using waters Symmetry C_{18} column (150x4.6 mm, 3.5µ) column and mobile phase of 0.1% Perchloric acid and Acetonitrile (ACN) in 60:40.

Results: The calibration curve was linear in the range of 12.5-100 ng/ml for Finasteride and 12.5-100 ng/ml Tadalafil. The recovery results of Accuracy and Precision of Finasteride and Tadalafil were 95.10, 96.85, 98.76, 98.81% and 95.77, 97.46, 97.99, 97.01% at different QC (Quality Control) concentration levels. Matrix effect results were within the acceptable limit. An electro-spray ionization source was used to study of Finasteride and Tadalafil at m/z 373.5497→142.0085, m/z 390.4047→128.1138 for Finasteride and Tadalafil, m/z 484.9516→104.5326 for Avanafil were ion pairs of mass analysis.

Conclusion: The application denotes all the parameters of system suitability, specificity, linearity and accuracy are in good agreement with USFDA (United States of Food and Drug Administration) guidelines and applied effectively for the investigation of pharmacokinetic studies in rat.

Keywords: Finasteride, Tadalafil, LC-MS/MS, USFDA guidelines, Rat plasma

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INTRODUCTION

Finasteride, sold under the brand names Proscar and Propecia among others, is a medication used to treat pattern hair loss and Benign Prostatic Hyperplasia (BPH) [1-4] in men. It can also be used to treat excessive hair growth in women. [5] It is usually taken orally but there are topical formulations for patients with hair loss [6, 7], designed to minimize systemic exposure by acting specifically on hair follicles [8, 9]. Finasteride is a 5α-reductase inhibitor and, therefore, an antiandrogen [10]. It works by decreasing the production of DHT (Di Hydro Testosterone) by about 70%. In addition to DHT, finasteride also inhibits the production of several anticonvulsant neurosteroids [11, 12], including allopregnanolone, [13] androstanediol, and THDOC (Tetra Hydro De Oxy Corticosterone). Adverse effects from finasteride are rare; however, some men experience sexual dysfunction, depression, and breast enlargement. In some men, sexual dysfunction may persist after stopping the medication. It may also hide the early symptoms of certain forms of prostate cancer.

Tadalafil [14], sold under the brand name Cialis among others, is a medication [15] used to treat erectile dysfunction, benign prostatic hyperplasia, and pulmonary arterial hypertension [16-18]. It is taken by mouth. Onset is typically within half an hour and the duration is up to 36 h. Common side effects include headache, muscle pain, flushed skin, and nausea. Caution is advised in those with cardiovascular disease [19, 20]. Rare but serious side effects include a prolonged erection that can lead to damage to the penis, vision problems, and hearing loss. Tadalafil is not recommended in people taking nitrovasodilators such as nitroglycerin, as this may result in a serious drop in blood pressure [21, 22]. Tadalafil is a PDE5 (Phospho Di Esterase type 5) inhibitor which increases blood flow to the penis. It also dilates blood vessels in the lungs, which lowers the pulmonary artery pressure.

There are two spectrophotometric methods [23, 24] and one HPLC/MS (High-Pressure liquid Chromatography-Mass Spectrometry) [25] method were reported in the literature, but these methods are developed only for routine analysis of the selected drugs in bulk and formulation studies. The developed lCMS (Liquid Chromatography Mass Spectrometry) method was utilized for the estimation of the combined drugs by *in vitro* method.

MATERIALS AND METHODS

Chemicals and reagents

Acetonitrile and Perchloric acid water (HPLC grade) were purchased from Merck (India) ltd, Worli, Mumbai, India. All APIs of Finasteride, Tadalafil and Avanafil as reference standards were procured from Glenmark Pharmaceuticals Pvt ltd, Mumbai. (HPLC-High Pressure liquid Chromatography).

Instrument and conditions

The HPLC system (Waters Alliance model) and the mass spectrometer QTRAP 5500 triple quadrupole instrument (SCIEX) [26, 27] were used to construct the bio-analytical assay. Chromatographic separation was performed at room temperature using an isocratic mode and Symmetry C_{18} (150x4.6 mm x 3.5 µm) column. The mobile phase consisted of acetonitrile and perchloric acid at a ratio of 40:60 (by volume) at a flow rate of 1.0 ml/min. Ten micro litres of liquid were injected, and the entire cycle lasted five minutes. For this study, we employed a QTRAP 5500 triple quadrupole mass spectrometer equipped with a positive ion electrospray ionisation interface. Mass ion pair monitoring using MRM mode: m/z 373.5497→142.0085, m/z 390.4047→128.1138 for Finasteride and Tadalafil, m/z 484.9516→104.5326 for Avanafil (Internal standard). Ion spray voltage 5500V; source temperature 550 °C; drying gas temperature 120-250 °C; collision gas nitrogen; pressure 55psi; drying gas flow rate 5 ml/min; declustering potential 40V; entrance potential 45V; exit potential 15V; capillary voltage 5500V; dwell time 1Sec. Table 1 lays forth all the necessary information on Instrumentation.

Pharmacokinetic study

Selection of animals

In vivo pharmacokinetic studies, 6 healthy rats (app. 250 g) [28, 29] were obtained from Biological E limited, Hyderabad, India. The

protocol of animal study was approved by institute of animal ethics committee (Reg. No: 1074/PO/Re/S/21/CPCSEA). The animals are housed in similar laboratory conditions with access to endive, carrots, fresh corn (few amount only); the animal feed should be kept at temperature of 21-24°C and humidity was 50-55%. Before experimentation, all animals was fasted overnight and had water adlibitum [30, 31].

Chromatographic conditions

Chromatographic separation, using symmetry C_{18} (150 x 4.6 mm, 3.5 micron) column, was administered in isocratic mode at room temperature. As a mobile phase, a mix of 0.1 percent perchloric acid and acetonitrile at 60:40 v/v with a flow of 1.0 ml/min was used. 10µl was the injection rate and the run time was 5 min.

Preparation of standard and internal control samples

Preparation of standard stock solution

Accurately weigh 5 mg of Finasteride, 5 mg of Tadalafil working standards and taken into a 100 ml volumetric flask and 70 ml of diluents and sonicate for 10 min to dissolve the contents completely and make up to the mark with diluent. Further dilution by taking 0.4 ml into 10 ml volumetric flask. From the above solution, 1 ml of the solution is taken into the 10 ml volumetric flask and make up to the mark with the diluent.

Preparation of internal standard stock solution

Accurately weigh 6 mg internal standard (Avanafil) into a 100 ml volumetric flask and make up to the mark with diluent and sonicate for ten minutes to dissolve the contents completely. From this solution take 0.1 ml of solution into 10 ml volumetric flask. From the above solution 1 ml is taken into the 10 ml volumetric flask and make up to the mark with the diluent.

Preparation of standard solution

For standard preparation 200µl of plasma was taken and 300µl of ACN into a 2 ml centrifuge tube and 500µl of standard stock solutions and 500µl of IS stock and 500 µl of diluents were added and vortexed for 10 min. These samples further subjected for centrifuge at 5000rpm for 20 min. Collect the solution and filter through 0.45µ nylon syringe filter and the clear solution was transferred into vial and injected into a system.

Bio-analytical method validation

The method was validated [32-36] in selective, sensitive, linearity, accuracy and precise, matrix condition, recovery study, re-injection reproducibility and stability.

Selectivity

The retention times of Finasteride, Tadalafil, and IS were measured, and interference from untested samples was tested by analysing rat plasma samples from six distinct rats to assess selectivity [37, 38].

Matrix effect

By comparing the peak zone fraction in the post-extract plasma sample of six separate plasma samples devoid of medicine and slick recovery samples, we were able to assess the Effect matrix [39, 40] for Finasteride and Tadalafil. Six different lots of plasma were tested at MQC (Middle-Quality Control) levels in duplicate, with satisfactory accuracy (% Coefficient of variation (CV) 15%).

Recovery

The recovery [41, 42] was calculated by comparing the peak areas of standards that had not been extracted with those of the extracted Finasteride and Tadalafil (6 replicates per QC (Quality Control) concentration).

Dilution integrity

A matrix with an analyte concentration over the ULOQC (Upper limit of Quality Control) must be injected, and the test must be diluted using a blank matrix to prove that the dilution was done correctly [43, 44].

Carryover

The retention of an analyte in the chromatographic system after the injection of a sample is referred to as carry-over [45, 46], and can be identified in subsequent blank or unknown samples.

Precision and accuracy

Quality control replication study was performed on a total of six samples to determine the results at four different quality control levels: low, medium, and high [47-50]. Except for the LLOQ (Lower limit of Quality Control), which should be less than 20%, the %CV level should be less than 15%.

Stability

Comparing the area response of the analyte in the stability samples [51, 52] with the region response of the sample obtained from the fresh stock solution allowed us to draw conclusions about the stock solution's stability. The effects of LQC (Lower Quality Control) and HQC (Higher Quality Control) concentrations on plasma stability were tested using six dose replicates. The US Food and Drug Administration (USFDA) defines stability as a coefficient of variation (CV) of less than 15% for an analyte. Injected rat plasma samples were tested for 24 h of shelf life (bench top stability) after being kept at room temperature. The auto-sampler stability of increased rat plasma was measured over a period of 24 h at 2-8 °C. Extract plasma samples were injected immediately or stored in the autosampler at 2-8 °C for 24 h to assess the stability of the auto sampler. Freeze-thaw stability was evaluated by contrasting newly infused quality control samples with those that had been frozen at-30 °C and thawed three times. Six aliquots were utilized to test the freeze-thaw stability of both the low-and high-quality control concentrations. To evaluate the long-term stability, the 24-hour concentration was compared to the starting concentration.

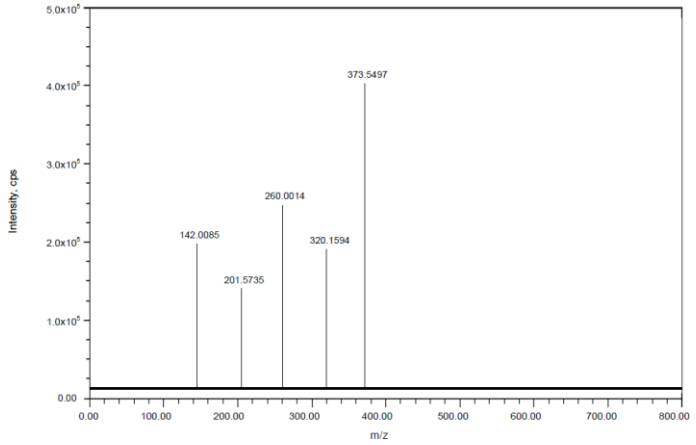
Pharmacokinetic study

Before experimentation, all animals are starved overnight and had water ad-libitum. Topical anesthetic procedure was used. Pharmacokinetic evaluation was performed for Finasteride and Tadalafil formulations. The samples were administered to each rat under fasting conditions. After oral administration of Finasteride and Tadalafil, blood samples were collected from rat marginal ear vein using a 25-guage, 5/8 inch needle by clipping the marginal ear vein with a paper clip with volume of 0.5 ml to 1.0 ml at 0.5, 1, 2, 4, 8, 12, 16 and 20 h. The blood was collected in Eppendorf containing 10% EDTA (Ethylene Diamine Tetra Acetic acid) solution. Blood was centrifuged at 5000 rpm for 30 min at 2-8 °C temperature. The clear supernatant plasma were collected and stored at-30 °C till its analysis. The plasma samples were treated for liquid-liquid phase extraction and analyzed for drug content with developed analytical method. After the study, the animals were returned to animal house for rehabilitation.

The pharmacokinetic parameters [53, 54] for Finasteride and Tadalafil oral administration were determined from plasma concentration data. Pharmacokinetic parameters like AUC (Area under the curve), C_{max} (Maximum Concentration), T_{max} (Time to reach peak concentration) the time at which C_{max} occurred. Data was measured by the trapezoidal rule method from time zero to infinity of concentration-time curve. C_{max} and T_{max} were obtained from the graph. All values are expressed in mean±SD. (SD – Standard Deviation).

RESULTS AND DISCUSSION

The maximum response on air pressure chemical ionization mode selected in this method is by having the electrospray ionization. The mobile phase flow of 1 ml/min Finasteride and Tadalafil are highly responsive in the positive ion mode to offer sensitivity and signal stability with continuous flow to electro spray ion.



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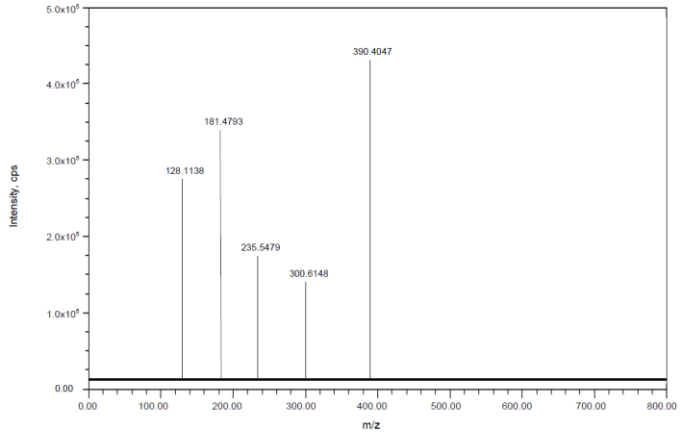

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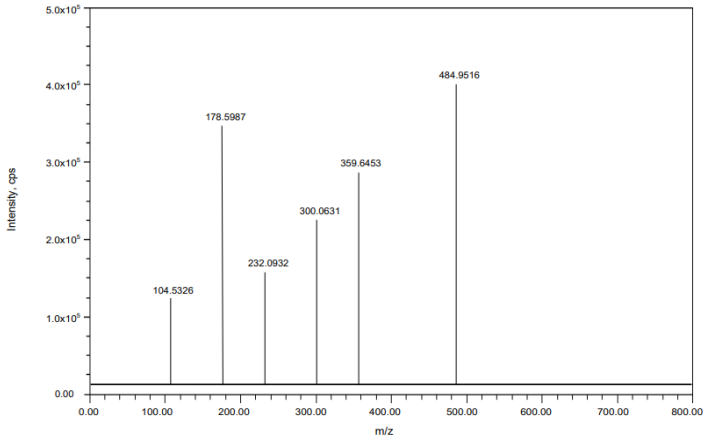

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Fig. 1: MS Spectras of (A) Finasteride, (B) Tadalafil and (C) Avanafil

Specificity

The specificity of the method to research Finasteride and tadalafil simultaneously is proved. The chromatograms of blank and standard as shown in fig. 2, 3. The chromatograms of blank rat plasma and standard having no interference peaks were observed [55, 56].

Matrix effect

Percent RSD (Relative Standard Deviation) for within the signal, ion suppression/enhancement was observed as 1.0 percent for Finasteride and Tadalafil in LC-MS/MS, suggesting that under these circumstances, the matrix effect on analyte ionization is within an acceptable range of ionization [57, 58]. In matrix effect, LQC (Low Quality Control) and HQC (High Quality Control) of Finasteride were 96.1 and 97.9 and tadalafil were 97.6, 97.6%. %CV of both drugs at LQC level were 0.73, 1.28 and HQC level is 0.22, 1.26 respectively. It indicates that the matrix effect on the ionization of the analyte is within the suitable limit.

Linearity

The peak area ratio of calibration standards was proportional to the concentration. The concentration range of Finasteride is 12.5-100 ng/ml and Tadalafil is 12.5-100 ng/ml. linearity results of

Finasteride and Tadalafil were shown in following table 1 and their calibration plots were shown in fig. 4. The calibration curves were appeared linear and the coefficient of correlation was found to be 0.999 for Finasteride and Tadalafil [59, 60].

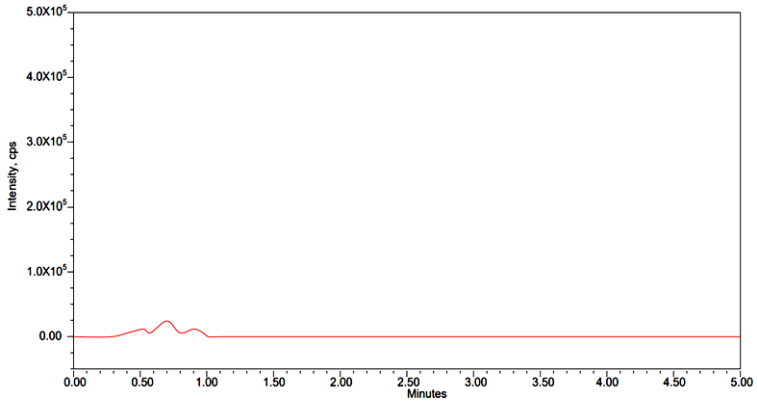


Fig. 2: Chromatogram of blank

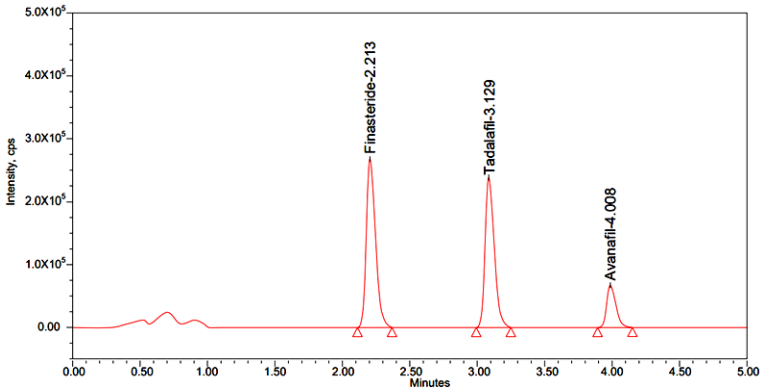
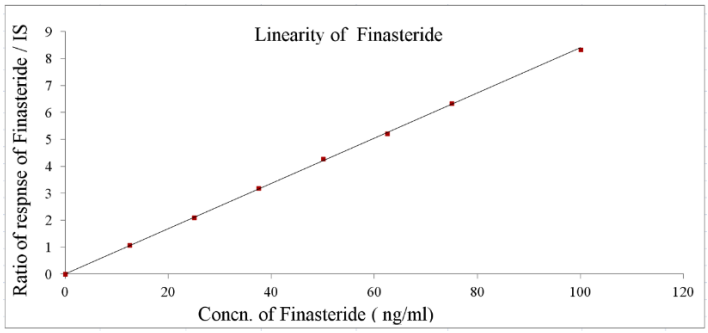


Fig. 3: Chromatogram of standard



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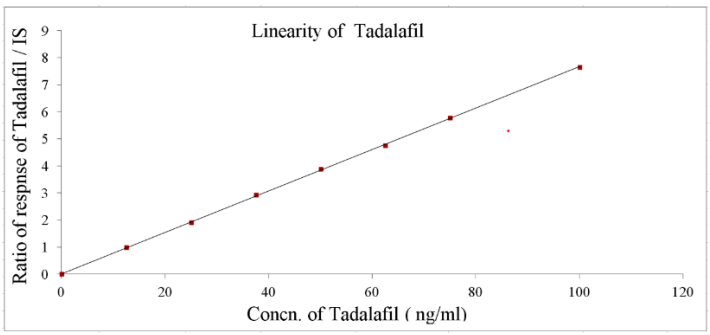

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Fig. 4: Calibration plots of (A) Finasteride and (B) Tadalafil

Table 1: Results of linearity

Linearity	Finasteride		Tadalafil	
	Conc.(ng/ml)	Area response ratio	Conc. (ng/ml)	Area response ratio
1	12.50	1.080	12.50	0.988
2	25.00	2.098	25.00	1.913
3	37.50	3.203	37.50	2.927
4	50.00	4.282	50.00	3.884
5	62.50	5.222	62.50	4.761
6	75.00	6.351	75.00	5.770
7	100.00	8.334	100.00	7.661
Slope		0.0838	Slope	0.0764
Intercept		0.02995	Intercept	0.02969
CC		0.99967	CC	0.99984

Precision and accuracy

By pooling all individual assay results of different internal control samples, the accuracy and precision were calculated. It was obvious, based on the data provided, that the strategy was precise and effective. The precision results of Finasteride and Tadalafil was shown in table 2, 3. Finasteride accuracy results in quality control samples 95.1-98.8 and Tadalafil accuracy results in quality control samples 95.1-99.8. Half of Finasteride and Tadalafil CV (Coefficient Variance) is<5% of total internal control samples [61, 62].

Table 2: Precision and accuracy of finasteride

QC name	LLQC	LQC	MQC	HQC
Conc.(ng/ml)	5	25	50	75
QC sample-1	0.254	1.335	2.713	4.052
QC sample-2	0.267	1.326	2.709	4.063
QC sample-3	0.261	1.318	2.689	4.042
QC sample-4	0.254	1.325	2.702	4.051
QC sample-5	0.271	1.319	2.705	4.065
QC sample-6	0.253	1.321	2.684	4.041
Mean	0.260	1.324	2.700	4.052
SD	0.00764	0.00626	0.01145	0.01011
%CV	2.94	0.47	0.42	0.25
Accuracy	95.10	96.85	98.76	98.81

n=6

Table 3: Precision and accuracy of tadalafil

Qc name	LLQC	LQC	MQC	HQC
Conc.(ng/ml)	5	25	50	75
QC sample-1	0.232	1.202	2.435	3.623
QC sample-2	0.228	1.211	2.428	3.613
QC sample-3	0.241	1.207	2.447	3.622
QC sample-4	0.238	1.224	2.422	3.605
QC sample-5	0.251	1.216	2.444	3.613
QC sample-6	0.235	1.203	2.431	3.618
mean	0.238	1.211	2.435	3.616
Stddev	0.00802	0.00841	0.00957	0.00674
%CV	3.38	0.69	0.39	0.19
Accuracy %	95.77	97.46	97.99	97.01

n=6

Recovery

The recoveries for Finasteride and Tadalafil at LQC, MQC (Medium Quality Control) and HQC levels the results demonstrated that the bioanalytical method had good extraction efficiency. This also showed that the recovery wasn't hooked into concentration. The recoveries for Finasteride (97.25%-98.74%) and Tadalafil (96.86%-98.23%) at LQC, MQC and HQC levels and % CV ranged from 0.35-0.84 for Finasteride and 0.97-1.26 for Tadalafil. the results demonstrated that the bioanalytical method had good extraction efficiency [63-65].

Ruggedness

The percent recoveries and percent CV of Finasteride and Tadalafil determined with two different analysts and on two different columns were within acceptable criteria in HQC, LQC, MQC and LLQC samples [66, 67]. The results proved method is ruggedness. The percent recoveries ranged from 95.49 – 98.73% for Finasteride and 95.11%-98.74% for Tadalafil. The %CV values ranged from 0.19-0.86 for Finasteride and 0.55-1.34 for Tadalafil. The results proved method is ruggedness.

Autosampler carryover

Peak area response of Finasteride and Tadalafil, wasn't observed within the blank rat plasma samples after successive injections of LLQC and ULQC at the retention times of Finasteride and Tadalafil. In auto sampler carryover this method doesn't exhibit auto sampler carryover.

Stability

Finasteride and Tadalafil solutions were prepared with diluents for solution stability analysis and placed in a refrigerator at 2-8 °C [68, 69]. Fresh stock solutions were associated with stock solutions that

were prepared 24 h earlier. The plasma stability of the bench top and autosampler was stable for twenty-four hours and 24 h at 20 °C in the autosampler. It became apparent from future stability that Finasteride and Tadalafil were stable at a storage temperature of-30 °C for up to 24 h [70, 71]. The overall stability results of Finasteride and Tadalafil have been stated in the below table 4, 5.

Table 4: Stability results of finasteride

Stability experiment spiked plasma		Spiked plasma conc. (n=6,ng/ml)	Mean conc. measured (n=6, ng/ml)	Std dev	%CV
Benchtop stability	LQC	25	24.135	3.025	1.16
	MQC	50	49.257	2.541	0.88
	HQC	75	74.458	5.527	0.96
Autosampler stability	LQC	25	24.897	1.658	0.95
	MQC	50	49.589	2.748	0.86
	HQC	75	74.124	3.625	0.77
Long term(Day28)	LQC	25	24.368	0.984	0.95
stability	MQC	50	49.354	5.428	0.85
	HQC	75	74.126	7.486	0.74
Wet extract stability	LQC	25	24.328	3.741	0.79
	MQC	50	49.856	2.487	0.52
	HQC	75	74.175	6.859	0.45
Dry extract stability	LQC	25	24.689	5.201	0.96
	MQC	50	49.657	7.629	0.74
	HQC	75	74.821	3.458	0.94
Freeze thaw stability	LQC	25	24.628	2.246	0.85
	MQC	50	49.145	3.485	0.84
	HQC	75	74.286	2.749	0.74
Short term stability	LQC	25	24.369	1.623	0.41
	MQC	50	49.486	2.485	1.56
	HQC	75	74.289	8.795	1.05

Table 5: Stability results of tadalafil

Stability experiment spiked plasma		Spiked plasma conc. (n=6,ng/ml)	Mean conc. measured (n=6, ng/ml)	Std dev	%CV
Benchtop stability	LQC	25	24.534	1.658	1.42
	MQC	50	49.12	3.457	0.86
	HQC	75	74.548	6.592	0.74
Autosampler stability	LQC	25	24.525	3.642	0.91
	MQC	50	49.321	4.857	0.87
	HQC	75	74.584	9.568	0.94
Long term	LQC	25	24.587	2.013	0.84
(Day 28)stability	MQC	50	49.874	4.563	0.68
	HQC	75	74.582	6.452	0.74
Wet extract stability	LQC	25	24.574	1.423	0.61
	MQC	50	49.369	3.650	0.82
	HQC	75	74.514	7.856	0.95
Dry extract stability	LQC	25	24.542	2.239	1.14
	MQC	50	49.841	4.524	1.25
	HQC	75	74.586	8.562	0.64
Freeze thaw stability	LQC	25	24.564	1.036	0.95
	MQC	50	49.684	5.569	1.26
	HQC	75	74.521	7.741	1.03
Short term stability	LQC	25	24.574	1.115	0.84
	MQC	50	49.231	5.236	0.97
	HQC	75	74.541	8.623	1.45

Table 6: Pharmacokinetic parameters of finasteride and tadalafil

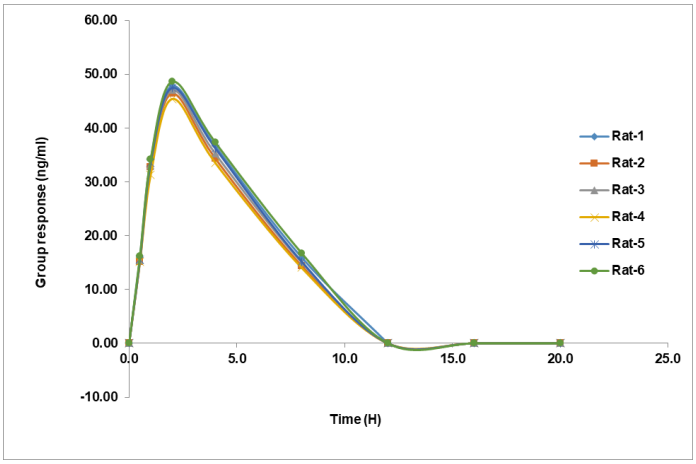
Pharmacokinetic parameters	Finasteride	Tadalafil
AUC_{0-t}	728 ng-h/ml	822 ng-h/ml
C_{max}	47.1 ng/ml	47.5 ng/ml
$AUC_{0-\infty}$	728 ng-h/ml	822 ng-h/ml
T_{max}	2 h	2 h
$T_{1/2}$	8 h	16 h

$AUC_{0-\infty}$: Area under the curve extrapolated to infinity, AUC_{0-t}: Area under the curve up to the last sampling time, C_{max}: The maximum plasma concentration, T_{max}: The time to reach peak concentration, $T_{1/2}$: Time the drug concentration.

In vivo pharmacokinetic evaluation

The plasma concentration-time profiles of Finasteride and Tadalafil in rat are shown in fig. 4. The graph indicated bell shaped curve in both the cases of experimental formulation. Finasteride and Tadalafil could be traced to be present in the blood for 8 h and 16 h after oral administration, which indicates the effectiveness of drug release from the formulation.

The pharmacokinetic parameters C_{max}, T_{max}, T1/2, K_{el}, K_a, AUC_{0-t}, $AUC_{0-\infty}$ were calculated and the data is shown in table 6 [72, 73]. The C_{max} for Finasteride and Tadalafil were found to be 47.147ng/ml and 47.502 ng/ml respectively. The T_{max} for Finasteride and Tadalafil were found to be 2h and 2h respectively. The $t_{½}$ values were 8h and 16h respectively for Finasteride and Tadalafil. The pharmacokinetic parameters of Finasteride and Tadalafil were shown in table 6.



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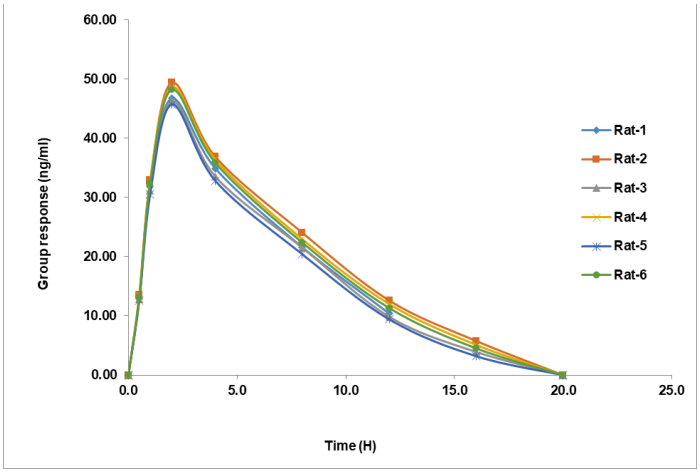

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Fig. 4: Recovery plot (A) Finasteride and (B) Tadalafil

CONCLUSION

For the primary time higher sensitive HPLC-ESI-LCMS/MS method was developed and validated for the determination of Finasteride and Tadalafil in rat plasma. Here the described method is rugged, fast, reproducible bio-analytical method. This method was validated according to USFDA guidelines. Simple and efficient method was developed and may be utilized in pharmacokinetic studies and to see the investigated analyte in body fluids.

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None

AUTHORS CONTRIBUTIONS

Yesupadamu has collected the literature and information about the drug and carried out the research samples and prepared the manuscript. David Raju check the data and reviewed the article.

CONFLICTS OF INTERESTS

Author declares that there have been no conflicts of interest.

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