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BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF TADALAFIL IN HUMAN PLASMA USING RP-HPLC

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ABSTRACT

Purpose: A simple reverse phase liquid chromatographic analytical method has been developed and validated for estimation of Tadalafil in Human plasma. **Methods:** The separation was carried out on Eclipse XBD-C8 Plasma 5 µm (4.6× 150 mm) as Stationary phase, Mobile Phase: Acetonitrile: Aqueous solution containing 0.012M triethyl amine + 0.020M orthophosphoric acid. (40:60) Flow rate: 0.5 mL/min using uv detector at 225 & 285 nm. **Results**: The described HPLC method was linear over a concentration range of 100-3200 ng/mL. Sildenafil was used as a internal standard. The Tadalafil showed retention time 9.4 min & sildenafil showed retention time 3.7min respectively. The Accuracy and Precision**,** recovery, selectivity for Tadalafil was 100-3200 ng/mL respectively. The stability of thedrug spiked human plasma samples during three freeze thaw cycles were stable in plasma for about one month when stored at frozen state. **Conclusions:** It can be concluded that the developed bioanalytical method is capable of quantifying Tadalafil from spiked human plasma samples. The method meets the requirements of the USFDA Guidelines and can be applied to Bioavailability/ Bioequivalence studies of Tadalafil.

KEYWORDS: Bioanalytical method, development, validation.

INTRODUCTION

Tadalafil is white amorphous powder with melting point 295-300ºC, spluble in DMSO, water, methanol, ethanol and Dichloromethane. Is a an oral treatment for erectile dysfunction, is a selective inhibitor of cyclic guanosine monophosphate (cGMP)-specific phosphodiesterase type-5 (PDE-5). Through the inhibition on PDE-5, Tadalafil increases the

concentration of cyclic guanosine monophosphate (cGMP), producing smooth muscle relaxation and increased blood flow to the corpus cavernosum, thereby enhancing erectile response. Initial dose: 10 mg orally once a day, as needed, prior to sexual activity. Maintenance dose: 5 to 20 mg orally once a day, as needed, prior to sexual activity based on individual efficacy and tolerability (dose refer from drug bank)

Pharmacodynamics: Tadalafil is used to treat male erectile dysfunction (impotence) and pulmonary arterial hypertension (PAH). Part of the physiological process of erection involves the release of nitric oxide (NO) in the corpus cavernosum. This then activates the enzyme guanylate cyclase which results in increased levels of cyclic guanosine monophosphate (cGMP), leading to smooth muscle relaxation in the corpus cavernosum, resulting in increased inflow of blood and an erection. Tadalafil is a potent and selective inhibitor of cGMP specific phosphodiesterase type 5 (PDE5) which is responsible for degradation of cGMP in the corpus cavernosum. This means that, with tadalafil on board, normal sexual stimulation leads to increased levels of cGMP in the corpus cavernosum which leads to better erections. Without sexual stimulation and no activation of the NO/cGMP system, tadalafil should not cause an erection. Literature survey revealed that Tadalafil is estimated by High-performance Liquid Chromatography, liquid chromatographic–ultraviolet estimation, RP-HPLC-PDA, UV Spectrophotometric, Derivative Spectrophotometry**,** GC/MS, Liquid chromatography–tandem mass spectrometry with electrospray ionization, LC-MS/MS. Several methods have been reported for quantification of Tadalafil in plasma as mentioned above.

The present investigation reports A highly selective, sensitive and rapid HPLC method for analysis of Tadalafil in plasma, using sildenafil as internal standard (IS) (shakya et al., Thejomoorthy. Karavadi1 and B. R. Challa).

The Plan of the present study is as follows: Optimization of chromatographic conditions were proposed to be developed and optimized like selection of initial separation conditions, nature of the stationary phase, nature of the mobile phase (pH, peak modifier, solvent strength, ratio and flowrate) and Selection of internal standard. The developed method were also proposed to be validated using the various validation parameters such as, Accuracy, Precision, Selectivity, Recovery, Stability. as per US-FDA guidelines.

MATERIALS AND METHODS

Chemicals, reagents and Instrumental Conditions: Working Standard of Tadalafil was gifted by Glenmark Pharmaceuticals Pvt. Ltd., Taloja, Navi Mumbai and Sildenafil internal standard was gifted by Watson Pharma Pvt Ltd, Ambernath East, Thane Acetonitrile of HPLC grade by Merck, Methanol of HPLC grade by Merck, diethyl ether (Loba Chemie Pvt.Ltd), Triethyl amine (Avantor Performance Materials india Ltd), Orthophosphoric acid (RFCL Ltd.). All other reagents used were of HPLC grade. HPLC Agilent Technologies, system with following configuration was used i.e Analytical columns of Eclipse XBD-C8 Plasma 5 µm (4.6× 150 mm), manual injector, UV-VIS detector, Shimadzu1700 UV-VIS spectrophotometer, Infrared spectrophotometer Shimadzu, Vortex mixer Remi, Centrifuge Remi, Sartorius single pan digital balance Shimadzu, Micropippetes Cyberopippetor, Ultra Sonicator were used for investigation.

Ethical approval: The detail of the study was approved by the Institutional Ethical Committee of R.C.Patel institute of pharmaceutical education & Research, shirpur. The volunteers were also instructed to refrain from consuming alcohol, smoking or other stimulant drinks during investigation period.

Chromatographic Conditions: HPLC conditions stationary phase

[Eclipse XBD-C8 Plasma 5 µm (4.6× 150 mm). Mobile Phase: Acetonitrile: Aqueous solution containing 0.012M triethyl amine + 0.020M orthophosphoric acid. (40:60). Elution mode: Gradient =. (40:60v/v) Flow rate: 0.5 mL/min. Injection volume: 20µl using manual injector Detection: at 285nm for Tadalafil and 284nm for Sildenafil. mobile phase degassed using ultrasonicator. The experiments were carried out at room temperature of about 35-37 ºC

Validation of the method: Validation is a process which involves confirmation or establishment by laboratory studies that a method / procedure / system / analyst can give the required accuracy, precision, sensitivity, ruggedness, etc. In the most basic form, validation of an analytical procedure demonstrates that the procedure developed is suitable for its intended purpose. Validation of the method was carried out after the development of the HPLC method. This section describes the procedure followed for the validation of the methods developed.

Accuracy: The accuracy of the drug was calculated by comparing the concentration obtained from the relative recovery of drug supplemented plasma to the actually added concentration.

To drug supplemented plasma, standard Tadalafil solution (Three levels) and internal standard solution were added. The resulting sample solution was analysed and the response factor was calculated. The absolute recovery of Tadalafil was determined by comparing the response factor of the drug obtained from the plasma with response factor obtained by the direct injection of Tadalafil in mobile phase at three different levels. Recovery studies were carried out for three levels at six times and the % recovery, mean, standard deviation and % CV was calculated.

Precision: The precision of the method was determined by intraday precision and interday precision. The intraday precision was evaluated by analysis of plasma samples containing Tadalafil at three different concentrations containing internal standard using nine replicate determinations for three occasions. The interday precision was similarly evaluated over two week period. Precision studies were carried out for three levels at nine times and three occasions. The mean concentration, standard deviation and % CV were calculated.

Selectivity Method I: The six blank plasma samples obtained from six different volunteers were analysed and the spectrums were recorded. These spectrums were compared with the spectrums obtained from standard solutions. Each spectrum was tested for interference. The combination of the sample preparation procedure and spectrums provided an assay which must be free from significant interfering endogenous plasma components at the retention times of Tadalafil and the internal standard.

Linearity and Range: The different concentrations of standard solutions were prepared to contain 100-3200ng/mL of Tadalafil containing 10.00 µg/ml of internal standard. These solutions were analysed and the peak areas and response factors were calculated. The calibration curve was plotted using response factor Vs concentration of the standard solutions. The calibration curve was constructed on six different days over a two weeks period to determine the variability of the slopes and intercepts.

Stability Studies: The stability studies of plasma samples spiked with Tadalafil were subjected to three Freeze thaw cycles, Short term stability at room temperature for 2,4,6 hrs and Long term stability at -20˚C over four weeks. The stability of triplicate spiked human plasma samples following three freeze thaw cycles was analysed. The mean concentrations of the stability samples were compared to the theoretical concentrations. The stability of triplicate short term samples spiked with Tadalafil was kept at room temperature for 2.00 to

6.00 h before extraction. The plasma samples of the long term stability were stored in the freezer at -20 ºC until the time of analysis. The mean concentrations of the stability samples were compared to the theoretical concentrations. The stability of the internal standard stock solution was also performed by comparing a freshly prepared standard solution containing internal standard.

Preparation of Tadalafil standard stock solution: The standard stock solution of Tadalafil was appropriately diluted with methanol to get six different working standard solutions having concentrations 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2µg/mL. The standard stock solution of internal standard was diluted with methanol to yield appropriate working solution of internal standard.

Preparation of calibration curve (cc) standards and qc samples: Plasma spiked calibration standards and QCs samples of Tadalafil were prepared by spiking 100 µL of working solutions of Tadalafil to 1.0 mL of Tadalafil free plasma, added 100 µL of working solution of internal standard (1000 ng/mL) the samples were mixed by vortexing and extracted using liquid- liquid extraction procedure.

Preparation of plasma samples

At the time of analysis, the samples were removed from the deep freezer and kept in the room temperature and allowed to thaw. 1.0 mL of sample was pipetted into 10 ml centrifuge tube with with this 100 µl of internal standard solution (10.0 µg/ml) and 5mL of LLE solvent (Diethyl Ether) was added. The resulting solution was vortexed for 10 minutes and centrifuged at 5000 r/min for 10 min. 5000 RPM for effective phase separation. organic layer was pipette out into a separate tube and evaporated to dryness. The residue was then reconstituted with 500µL mobile phase and subjected to chromatographic analysis.

Results of the investigation of Tadalafil in plasma by RP-HPLC estimation method

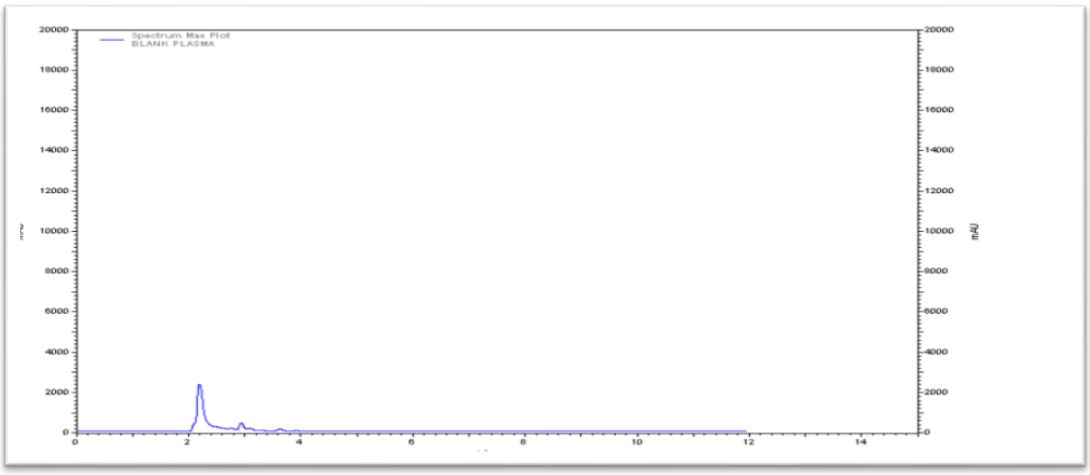

Fig. no. 1: Typical chromatogram of blank plasma.

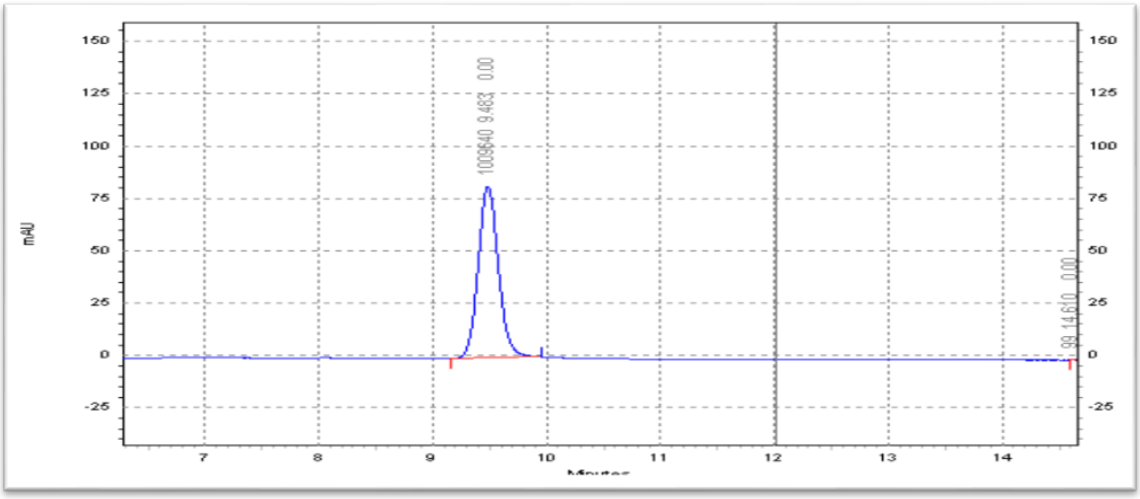

Fig. no. 2: RP-HPLC Chromatogram of Tadalafil.

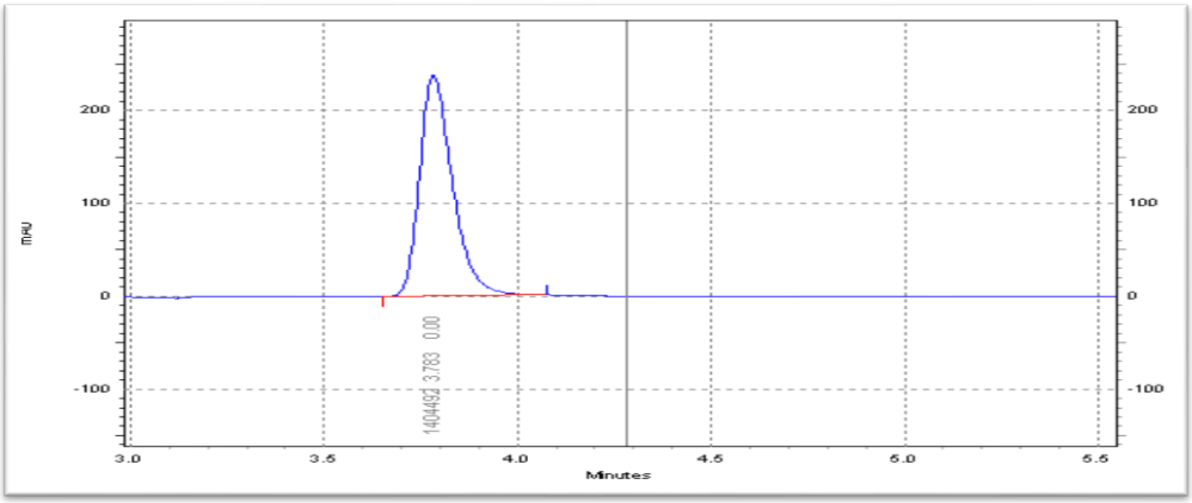

Fig. no. 3: RP-HPLC Chromatogram of internal standard.

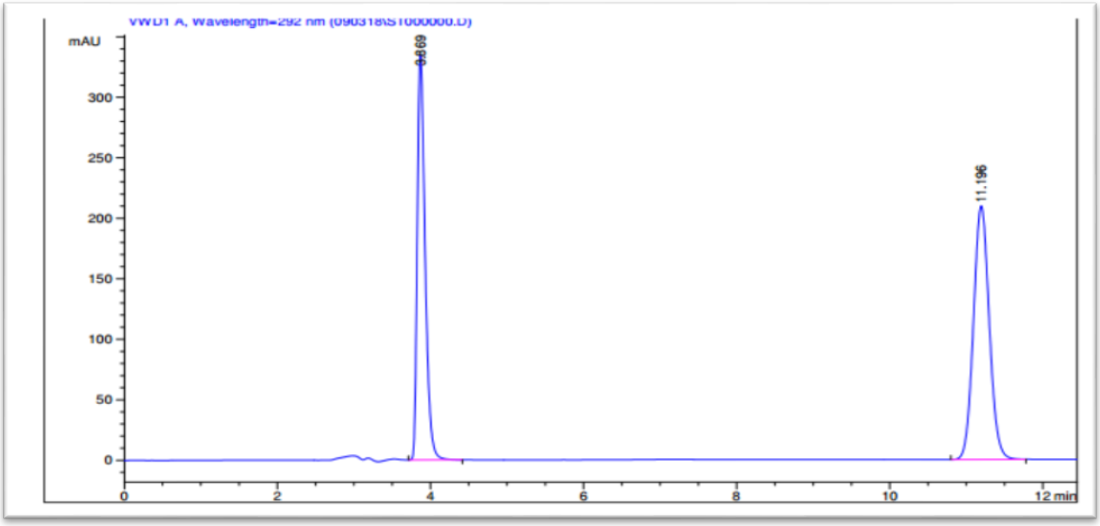

Fig. no. 4: Typical standard chromatogram of Tadalafil and internal standard.

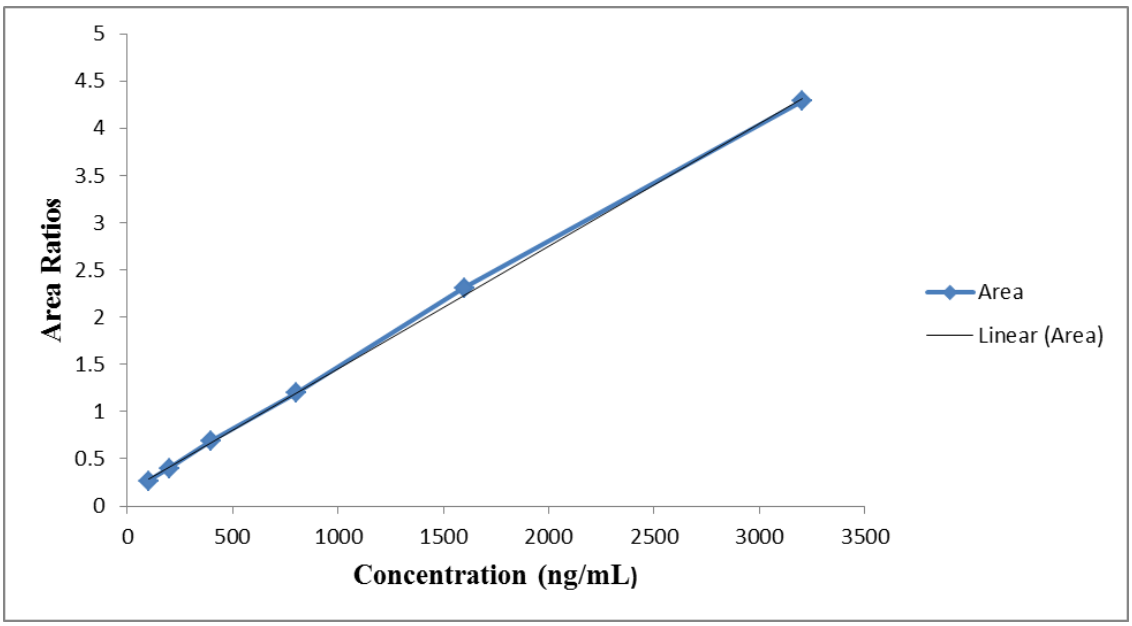

Fig. no. 5: Calibration curve of Tadalafil.

The results observed by RP-HPLC method for recovery studies of Tadalafil are showed in table no. 1. The Selectivity studies were indicated in in table no 2. & fig 6. The Accuracy and Precision studies were indicated in table no.3.and chromatogram of LQC, MQC & HQC show in fig no. 7, 8, 9. Followed by linearity and range results in table no. 4. The table no.5 indicates Stability of Tdalafil in plasma during storage and sample handling followed by RP-HPLC System.

Table 1: Tadalafil recovery studies.

	Unextracted Sample Peak Response	Extracted Sample Peak Response	% Recovery	Mean
LQC	245892.86	132971.59	54.07	56.72
	200466.12	119034.44	59.37	
MQC	519051.78	309860.08	59.69	58.85
	506741.68	294034.79	58.02	
HQC	1057652.01	605861.02	57.28	58.38
	988812.76	588210.31	59.48	
IS	453105.88	286738.34	63.28	

Table 2: Tadalafil selectivity studies.

Sr. No.	Blank response	Peak areas at lloq	% peak area in blank
1	1350.4	99319.75	1.36%
2	1580.5	101429.96	1.55%
3	1431.1	97213.03	1.47%
4	1615.9	88929.96	1.81%
5	1504.5	105928.75	1.42%
6	1657.1	119034.44	1.39%

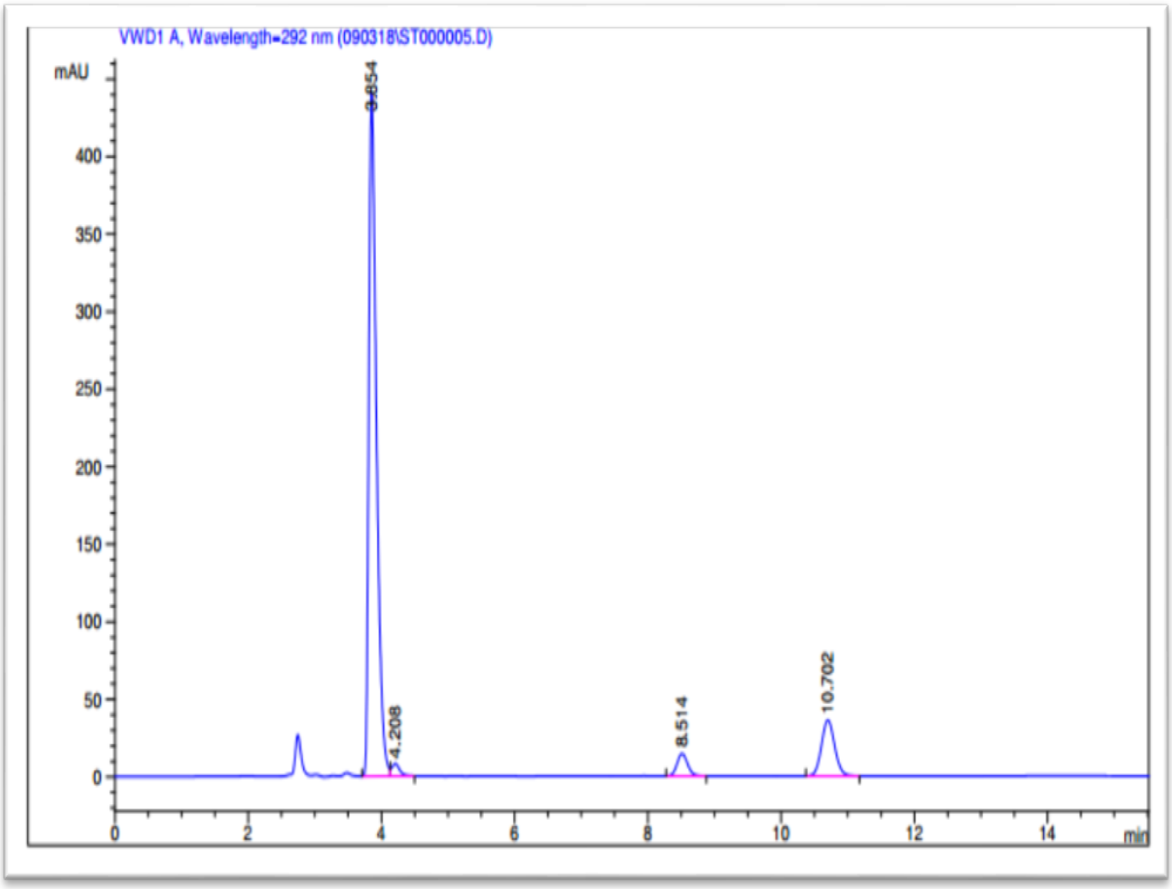

Fig. no. 6: Representative chromatogram of LLOQ.

Table 3: Tadalafil Accuracy and Precision studies.

	LQC (300ng)	MQC (800ng)	HQC (3200ng)
DAY 1	308.593147	790.924637	3205.389608
	305.946117	804.974834	3204.711306
	307.513634	797.124058	3199.669053
	291.172319	785.092861	3196.265289
	295.159683	805.974020	3201.430459
Mean	301.676980	796.818082	3201.493143
SD	7.952	8.9815	3.7483
% RSD	2.635	1.1271	0.1170
% RE	-1.676	3.1819	-1.4931
DAY 2	308.222565	806.921074	3192.131207
	313.038541	799.927467	3198.113470
	297.610809	808.187278	3204.838230
	307.172291	791.122958	3205.401221
	281.970651	812.798756	3202.383885
Mean	301.602971	803.791507	3200.573603
SD	12.3184	8.4508	5.5248
RSD	4.0843	1.0513	0.1726
%RE	-1.6029	-18.9575	-0.5736
DAY 3	308.435264	784.999981	3193.555266
	309.265918	778.356769	3210.537594
	300.194301	801.416438	3206.374411
	302.011705	808.596804	3191.548456
	304.061280	799.873890	3203.999086
Mean	304.793694	794.648776	3201.202963
SD	3.9589	12.5117	8.2673
RSD	1.2988	1.5745	0.2582
%RE	-4.7936	5.3512	-1.2029
DAY 4	301.479970	798.142889	3196.355879
	306.730621	799.319657	3207.266040
	300.207473	789.807762	3191.858739
	297.646130	808.276323	3207.772667
	304.106176	791.924104	3205.122976
Mean	302.034074	797.494147	3201.675261
SD	3.5088	7.2476	7.9608
RSD	1.1617	0.9088	0.2486
%RE	-2.0340	2.5058	-1.6752
DAY 5	295.648918	798.142889	3210.578203
	290.960959	799.319657	3212.783155
	298.477182	789.807762	3203.772429
	286.731734	808.276323	3198.325066
	299.731126	810.527513	3192.686312
Mean	294.309984	801.214829	3203.629033
SD	5.4127	8.3627	8.3663
RSD	1.8391	1.0437	0.2611
%RE	5.6900	-1.2148	-3.6290
Overall mean	300.883540	798.793468	3201.714800

Overall SD	6.629	9.110	6.773
Overall RSD	2.203	1.140	0.211
% RE	-0.883	-1.826	-1.714

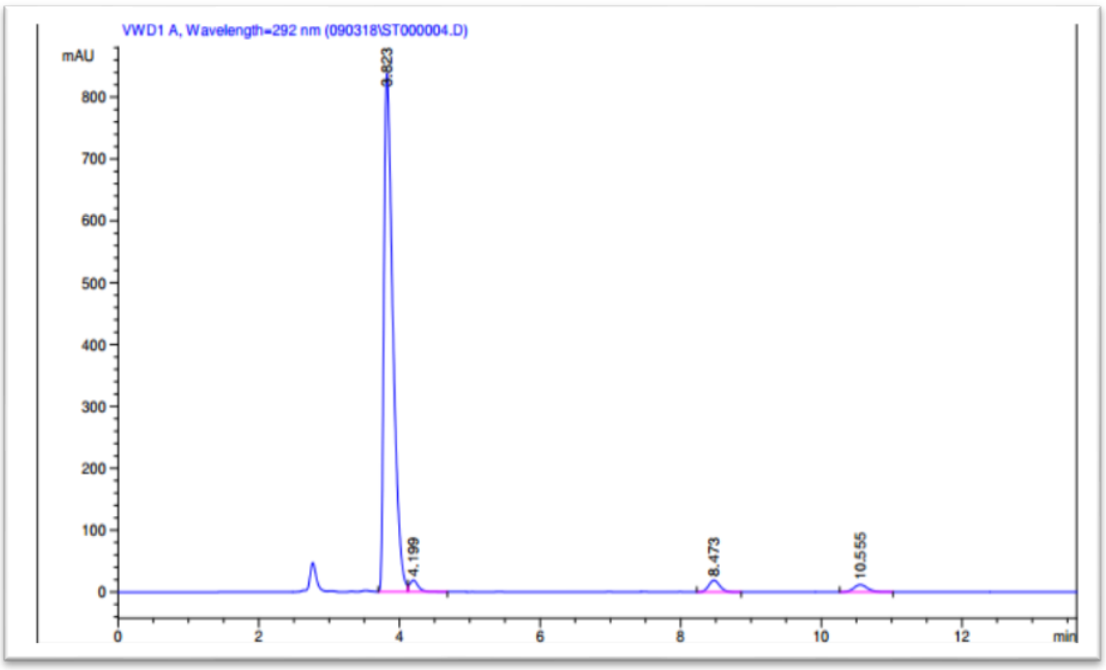

Fig. no. 7: Representative Chromatogram of LQC of Tadalafil.

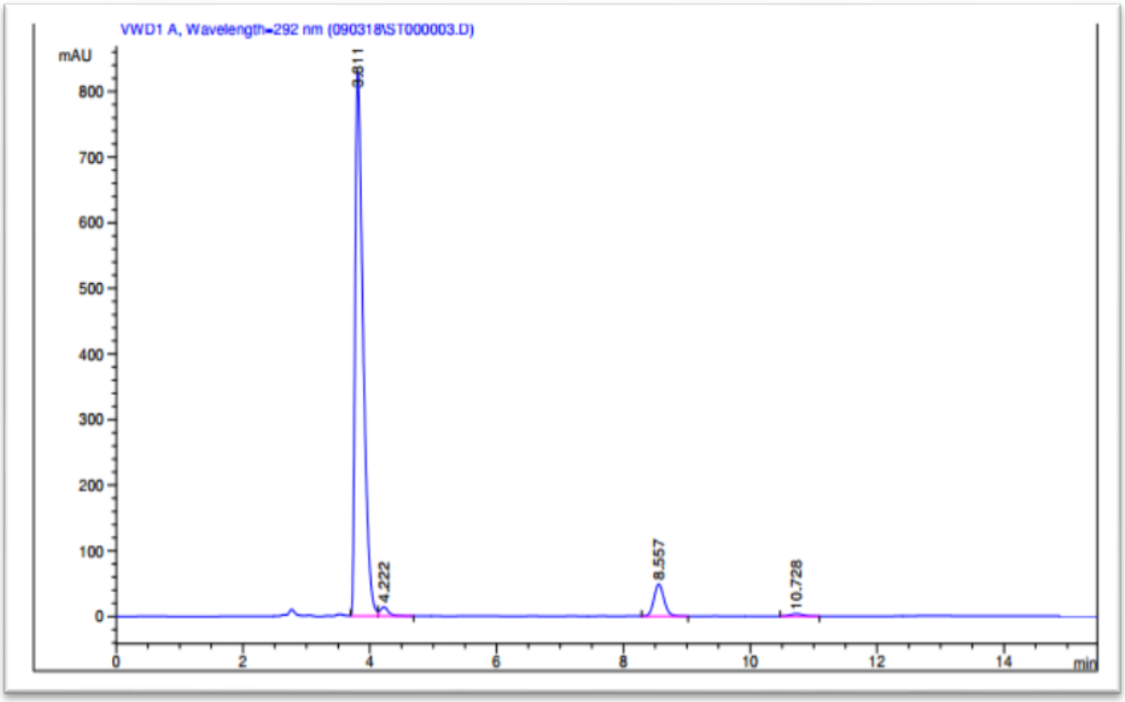

Fig. no. 8: Representative Chromatogram of MQC of Tadalafil.

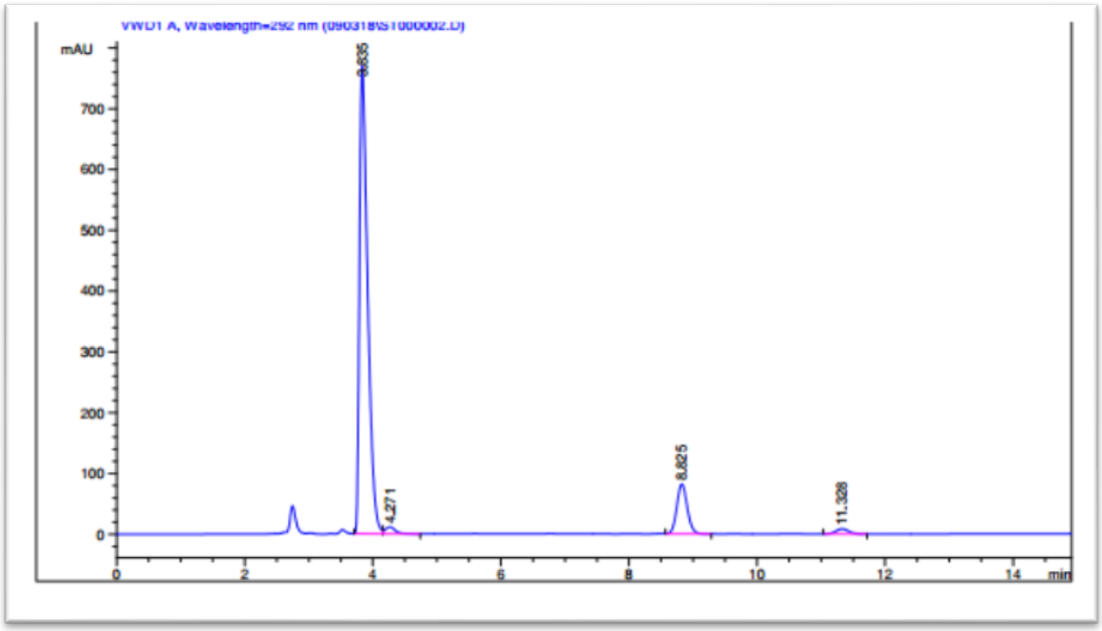

Fig. no. 9: Representative Chromatogram of HQC of Tadalafil.

Table 4: Linearity and Range of Tadalafil.

CC no.	Amount of Drug (ng/mL)	Area ratios							±SD of area ratios
		1	2	3	4	5	6	Mean	
CC-1	100	0.2599	0.2669	0.2698	0.2611	0.2586	0.2688	0.2642	0.0048
CC-2	200	0.3902	0.3883	0.3964	0.4076	0.3972	0.4198	0.3999	0.0118
CC-3	400	0.6933	0.7156	0.6720	0.6474	0.6966	0.7141	0.6898	0.0262
CC-4	800	1.1893	1.1768	1.2299	1.1690	1.1941	1.2114	1.1951	0.0224
CC-5	1600	2.3049	2.4261	2.1518	2.2947	2.2628	2.4027	2.3072	0.0995
CC-6	3200	4.1945	4.5979	4.5304	4.1241	4.2241	4.0491	4.2867	0.2242

Table 5: Tadalafil stability studies.

QC Level	Stability at Room Temperature					
	% Nominal			% RSD		
	2 hr	4 hr	6 hr	2 hr	4 hr	6 hr
LQC	101.02	100.55	99.38	1.493	1.748	2.884
HQC	99.82	100.02	100.54	0.194	0.331	0.478
QC Level	**Stability at -20°C**					
	% Nominal			% RSD		
	10 Days	20 Days	30 Days	10 Days	20 Days	30 Days
LQC	101.33	100.43		99.97	0.73	1.23
HQC	99.99	99.99		100.04	0.05	0.09
QC Level	**Freeze Thaw Stability**					
	% Nominal			% RSD		
	FT1	FT2	FT3	FT1		FT2
LQC	102.49	99.20	101.09	2.94		5.77
HQC	101.58	100.05	99.65	0.35		0.66

DISCUSSION

From the results obtained during method development and subsequent calibration and validation experiments, the following conclusions can be drawn, A mobile phase comprise of Aqueous solution containing 0.012M triethyl amine + 0.020M orthophosphoric acid & acetonitrile (60:40 v/v) is successful in resolving Tadalafil and Sildenafil from each other and from co-extracted plasma components.

Both Tadalafil and Sildenafil are well extracted from spiked plasma using Diethyl ether as an extracting solvent.

The procedure for LLE and chromatographic analysis described provide satisfactory extraction and adequate resolution of Tadalafil and the internal standard Sildenafil. When calibration experiments were performed on CC standards in concentration range of 100-3200 ng/mL, the use of unweighted linear regression as calibration model in Tadalafil and bioanalysis resulted in heteroscedasticity of calibration data and a large total %RE of the interpolated concentrations of CC standards. The heteroscedasticity was minimized with consequent minimization of the total %RE by using weighted least square linear regression with a weighting factor of 1/X2. Hence weighted (1/X2) least square linear regression is the preferred calibration model in the method developed for the estimation of Tadalafil in plasma.

In the evaluation of selectivity of the developed method, it was observed that the detector response for the six blank extracts was less than 20% of the peak area of the LLOQ sample. The method can thus be deemed to be selective at LLOQ of 100ng/mL, as per the requirements of US-FDA guidance. The standard curve generated on the validation days gave back calculated concentrations between 85-115% of the nominal. This is in accordance with the criteria for standard curves given by the US-FDA guidance for Bioanalytical method validation. The accuracy and precision of the method were demonstrated statistically using %RSD and %RE as shown in TABLE 3. %RE at all three levels tested i.e. LQC, MQC and HQC was between ±15 while %RSD was below 15%, this meets the USFDA criteria for accuracy and precision.

The results of the stability evaluation indicate that the concentrations of Tadalafil found in the stability samples were between 85-115% of the nominal concentration with %RSD less than 15. Since these results were within the acceptance limit of the assay variability, the deviation of experimental values from nominal could not be attributed instability. It can be concluded that Tdalafil is stable in plasma after three freeze thaw cycles, on keeping the QC samples at room temperature for 6 hrs, and upon storage of QC samples at -20˚C for 1 month. It can be concluded that the developed bioanalytical method is capable of quantifying Tadalafil from spiked human plasma samples. The method meets the requirements of the USFDA Guidelines and can be applied to Bioavailability/ Bioequivalence studies of Tadalafil.

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