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Validated liquid chromatographic–ultraviolet method for the quantitation of tadalafil in human plasma using liquid–liquid extraction

Ashok K. Shakya [a,*], Ahmed N.A. Abu-awwad [b], Tawfiq A. Arafat [b], Munther Melhim [b]

[a] Department of Medicinal and Pharmaceutical Chemistry, Faculty of Pharmacy and Medical Sciences, Amman University, P.O. Box 263, Amman 19328, Jordan
[b] Jordan Center for Pharmaceutical Research, P.O. Box 950435, Amman 11195, Jordan

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Abstract

A highly selective, sensitive and rapid HPLC method has been developed and validated to quantify tadalafil in human plasma. The tadalafil and internal standard (loratadine, I.S.) were extracted by liquid–liquid extraction technique followed by an aqueous back-extraction allowing injection of an aqueous solvent in the HPLC system. The chromatographic separation was performed on a reverse phase BDS Hypersil C-18 column (250 mm × 4.6 mm, 5 μm, Thermo Separation Co., USA) with a mobile phase of acetonitrile and aqueous solution containing 0.012 M triethylamine + 0.020 M orthophosphoric acid (50/50, v/v). The analytes were detected at 225 nm. The assay exhibited a linear range of 5–600 ng/mL for tadalafil in human plasma. The lower limit of quantitation (LLOQ) was 5 ng/mL. The within- and between batch precision (expressed as coefficient of variation, C.V.) did not exceed 10.3% and the accuracy was within −7.6% deviation of the nominal concentration. The recovery of tadalafil from plasma was greater than 66.1%. Stability of tadalafil in plasma was excellent with no evidence of degradation during sample processing (auto-sampler) and 30 days storage in a freezer. This validated method is applied for the clinical study of the tadalafil in human volunteers.
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Keywords: Tadalafil; Liquid–liquid extraction; HPLC–UV; Plasma; Quantitation

1. Introduction

Tadalafil (Cialis®) (Fig. 1), (6R, 12aR)-2,3,6,7,12,12a-hexahydro-2-methyl-6-(3,4-methylene dioxyphenyl) pyrazino(1′, 2′:1,6) pyrido-(3,4-b)indole-1,4-dione, 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 following appropriate sexual stimulation. This drug has been launched in more than 40 countries worldwide [1].

Tadalafil is rapidly absorbed after oral administration. After single oral dose administration, tadalafil achieves the maximum observed plasma concentration (C_{max}) between 0.5 and 6 h (median time of 2 h). Absolute bioavailability of tadalafil following oral dosing has not been determined. The rate and extent of absorbance of Tadalafil are not influenced by food; thus it may be taken with or without food. Over a dose range of 2.5–20 mg, tadalafil exposure (AUC) increases proportionally with dose in healthy subjects. Based on the reported literature the peak plasma concentration following administration of 20 mg dose of tadalafil was 378 μg/L [2–4]. Tadalafil easily gets distributed (V_d/F) into tissues to the extent of 63 L. At therapeutic concentration, 94% of tadalafil in plasma is bound to proteins. Protein binding is not affected by impaired renal function. Samples collected from healthy human subjects approximately 5 h after dosing indicated that <0.0005% of the total dose of tadalafil is distributed to semen. Tadalafil gets metabolized by the cytochrome *p* 450 (CYP) 3A4 to a catechol metabolite. The major circulating metabolite is the methylcatechol glucuronide, which is at least 13,000-fold less potent than tadalafil for PDE5. Consequently, it is not expected to be clinically active at observed metabolite concentrations. Tadalafil gets eliminated

* Corresponding author. Tel.: +962 79 5783428.
E-mail addresses: ashokshakya@hotmail.com (A.K. Shakya), tawfiqarafat@yahoo.com (T.A. Arafat).

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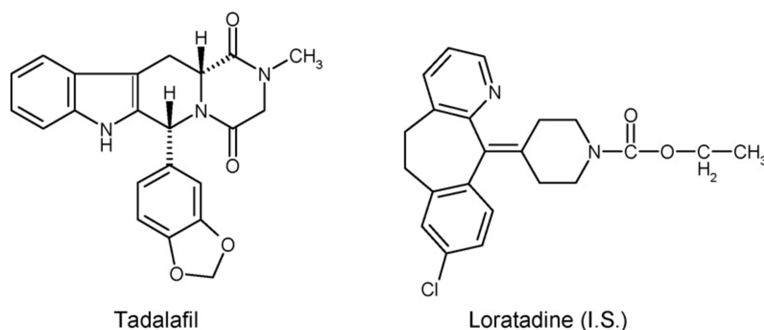

Fig. 1. . Chemical structure of tadalafil and internal standard (loratadine).

from the body mainly in the form of faeces (61%) and urine (36%). The mean oral clearance for tadalafil is 2.5 L/h and the mean half-life is 17.5 h in healthy subjects [1,5].

A few analytical methods have been reported for the estimation of tadalafil in formulation using HPLC [6], and capillary electrophoresis with UV detection [7,8]. A high through-put validated analytical method for the quantitation of tadalafil in human plasma using LC–MS/MS has been published [4]. It was validated over a calibration range of 10–1000 ng/mL using 250 μL plasma. However the LC–MS/MS machine is quite expensive and not readily available in the most clinical, bioanalytical, educational research laboratories. To date, no simple, sensitive and rapid high performance liquid chromatographic method with commonly used ultraviolet absorbance detection has been reported for the tadalafil quantitation at therapeutic concentration in human plasma or serum. HPLC–UV method for determination of tadalafil in rat plasma has been reported [9]. The calibration range was 10–2000 ng/mL and the run time was 18 min. The same method cannot be used for the detection of tadalafil in human plasma due to interference by endogenous components. For pharmacokinetic studies, sensitive and precise method that can detect low concentration of tadalafil (at least 2.5% of $C_{\max}$) is required. We tried to develop LC–MS method for the estimation of tadalafil using ion trap mass analyzer, but could not succeed due to poor detectability of drug. Tadalafil contains conjugated system and exhibit intensive UV absorption around 220, 280 and 290 nm [9]. So, we developed a simple, rapid, sensitive and selective HPLC–UV method for the quantitation of tadalafil in human plasma using liquid–liquid extraction. This extraction method selectively extracts the tadalafil and internal standard from the plasma, which are easily detected at 225 nm without any interference from endogenous components of plasma. The lower limit of quantitation of this validated method is 5 ng/mL using 500 μL of human plasma. The total run time was ∼6 min. This method has been successfully used for the clinical studies of tadalafil at JCPR, Amman.

2. Experimental

2.1. Chemicals

Tadalafil (purity 99.25%) drug substance and loratadine (99.12%) (I.S.) were obtained from Beir-Ziet Pharmaceutical Manufacturing Company (Palestine). Chemical structures are presented in Fig. 1. HPLC-grade Lichrosolv acetonitrile, water, diethylether, dichloromethane and isopropanol were purchased from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

2.2. Standard Solution

Stock solution of tadalafil (0.5 mg/mL) and loratadine (I.S., 0.5 mg/mL) were prepared in acetonitrile, separately and stored at 4 °C. Working solutions were prepared by appropriate dilution in acetonitrile:water (50/50, v/v) as required.

2.3. High performance liquid chromatography

For chromatographic analysis, Dionex® HPLC (Dionex-Softron GmbH, Munchen, Germany) system equipped with a quaternary pump (model P-580), an automated sample injector (Model ASI-100) holding 100 μL loop, a column oven (Model STH-585), a photodiode array detector (UVD-340S) and a data system (Chromoleon Version 6.2) was used. The separation of the compounds was made on a BDS Hypersil C-18 column (250 mm × 4.6 mm, 5 μm, Thermo Separation Co.) at temperature 20 ± 1 °C. Detector was set at a wavelength of 225 nm. The mobile phase was prepared by mixing an aqueous solution of triethylamine (0.012 M, 3.35 mL/2 L of water) + orthophosphoric acid (0.020 M, 2.65 mL/2 L of water) with acetonitrile (50/50, v/v). The mobile phase was filtered through 0.45 μm nylon membrane filters before use. Elution was performed at a flow rate of 1.5 mL/min.

2.4. Sample processing

A 0.5 mL volume of plasma was transferred to a 15 mL polypropylene tube, and then 25 μL of loratadine working solution (25 μg/mL) was added. After addition of 50 μL of 1 M sodium carbonate buffer solution, a 6 mL aliquot of extraction solvent (diethyl ether/dichloromethane, 7/3, v/v) was added using Dispensette Organic (Brand GmbH, Postfach, Germany). The sample was vortexed for 2 min using a Vibrax Type VX-Z, VXR Basic Vortexer (IKA-Werke GmBH & Co. Staufen, Germany) and centrifuged using Multitude 3S-R (Sorvall-Heraeus, Germany) for 5 min at 800 × *g*. The organic layer was transferred by freeze-decant (−70 °C, Platinum 500 V, Angelantoni Industrie S.p.a, Italy) to a 8 mL glass test tube and evaporated to

dryness at 40 °C under a stream of nitrogen. The dried extract was dissolved in 1 mL hexane and the solvent (hexane) was transferred to a tapered glass test tube containing 200 μL of 0.1 M sulfuric acid–isopropanol (85/15, v/v) solution. The analytes were back extracted into the aqueous layer by 1 min vortex mixing and centrifugation at 800 × *g* for 2 min. The aqueous layer was collected using Pasteur pipette and 100 μL of this sample was injected on to analytical column.

2.5. Bioanalytical method validation

2.5.1. Calibration and control samples

Working solutions for calibration (0.1, 0.2, 0.4, 1, 2, 4, 8, 12 μg/mL) and controls (0.1, 0.3, 6 and 9.6 μg/mL) were prepared from the stock solution by an adequate dilution using acetonitrile:water (50/50, v/v) separately. The loratadine (IS) working solution 25 μg/mL was prepared by diluting stock solution with diluent. Fifty microliters of working solutions of tadalafil were added to 950 μL of drug-free plasma to obtain tadalafil concentrations of 5, 10, 20, 50, 100, 200, 400 and 600 ng/mL. The quality control samples were prepared in pool, at concentrations of 5 ng/mL (LLOQ), 15 ng/mL (low), 300 ng/mL (medium) and 480 ng/mL (high), as a single batch at each concentration, and then divided in aliquots that were stored in the freezer at −70 °C until analysis. A calibration curve was constructed from a blank sample (a plasma sample processed without an I.S.), a zero sample (a plasma processed with I.S.) and eight non-zero samples covering the total range (5–600 ng/mL), including lower limit of quantification (LLOQ).

Each validation run consisted of system suitability sample, blank samples, a zero sample (a plasma processed with IS) calibration curve consisting of eight non-zero samples covering the total range (5–600 ng/mL) and quality control samples at three concentrations ($n = 6$, at each concentration). Such validation samples were generated on six consecutive days. Calibration samples were analyzed from low to high concentration at the beginning of each validation run and the other samples were distributed randomly through the run. Linearity was assessed by a weighted ($1/x^2$) least squares regression analysis. The calibration curve had to have a correlation coefficient (r) of 0.998 or better. The acceptance criterion for each back-calculated standard concentration was 15% deviation from the nominal value except LLOQ, which was set at 20%. At least 67% of non-zero standard should meet the above criteria including acceptable LLOQ and upper limit of quantitation.

2.5.2. Specificity

To evaluate the specificity of the method, drug free plasma samples were carried through the assay procedure and the retention times of the endogenous compounds in the plasma were compared with those of tadalafil (5 ng/mL) or internal standard. Specificity of the method was assessed to test the matrix influence between different plasma samples.

2.5.3. Recovery

Recovery of tadalafil was evaluated by comparing the mean peak areas of six extracted low, medium and high quality control samples to mean peak areas of six neat reference solutions (unprocessed). Recovery of Loratadine (I.S.) was evaluated by comparing the mean peak areas of ten extracted quality control samples to mean peak areas of ten neat reference solutions (unprocessed) of the same concentration.

2.5.4. Accuracy and precision

Within-batch accuracy and precision evaluations were performed by repeated analysis of tadalafil in human plasma. The run consisted of a calibration curve plus six replicates of each LLOQ, low, medium and high quality control samples. Between-batch accuracy and precision were assessed by analysis of samples consisting of a calibration curve and six replicates of LLOQ, low, medium and high quality control samples for tadalafil on three separate days. The overall precision of the method expressed as relative standard deviation and accuracy of the method expressed in terms of bias (percentage deviation from true value).

2.5.5. Stability

The bench top stability was examined by keeping replicates of the low and high plasma quality control samples at room temperature for approximately 12 h. Freeze–thaw stability of the samples was obtained over three freeze–thaw cycles, by thawing at room temperature for 2–3 h, refrozen for 12–24 h. Auto-sampler stability of tadalafil was tested by analysis of processed and reconstituted low and high plasma QC samples, which are stored in the auto-sampler tray for 24 h at 5 ± 1 °C. Stability of tadalafil in human plasma was tested after storage at approximately −70 °C for 30 days. For each concentration and each storage condition, six replicates were analyzed in one analytical batch. The concentration of tadalafil after each storage period was related to the initial concentration as determined for the samples.

2.5.6. Robustness

Variation of organic strength by ±1% did not have any significant effect on chromatographic resolution in the HPLC method. Robustness of the method was also evaluated by using different machines and/or the analyst.

2.5.7. Stock solution stability

The working aqueous solution (600 ng/mL) of tadalafil was repeatedly injected into the chromatograph immediately after preparation (time 0) and at 3, 6 and 9 h after bench top storage at room temperature and at 4 °C. This injection protocol was repeated after 1, 3, 6, 8, 15, 30, 45 and 60 days storage of this solution at 4 °C.

3. Result and discussion

3.1. Separation

Fig. 2 shows the representative chromatograms of blank plasma (A), blank plasma with internal standard (B), plasma spiked with tadalafil at LLOQ (5 ng/mL) with IS (C), with

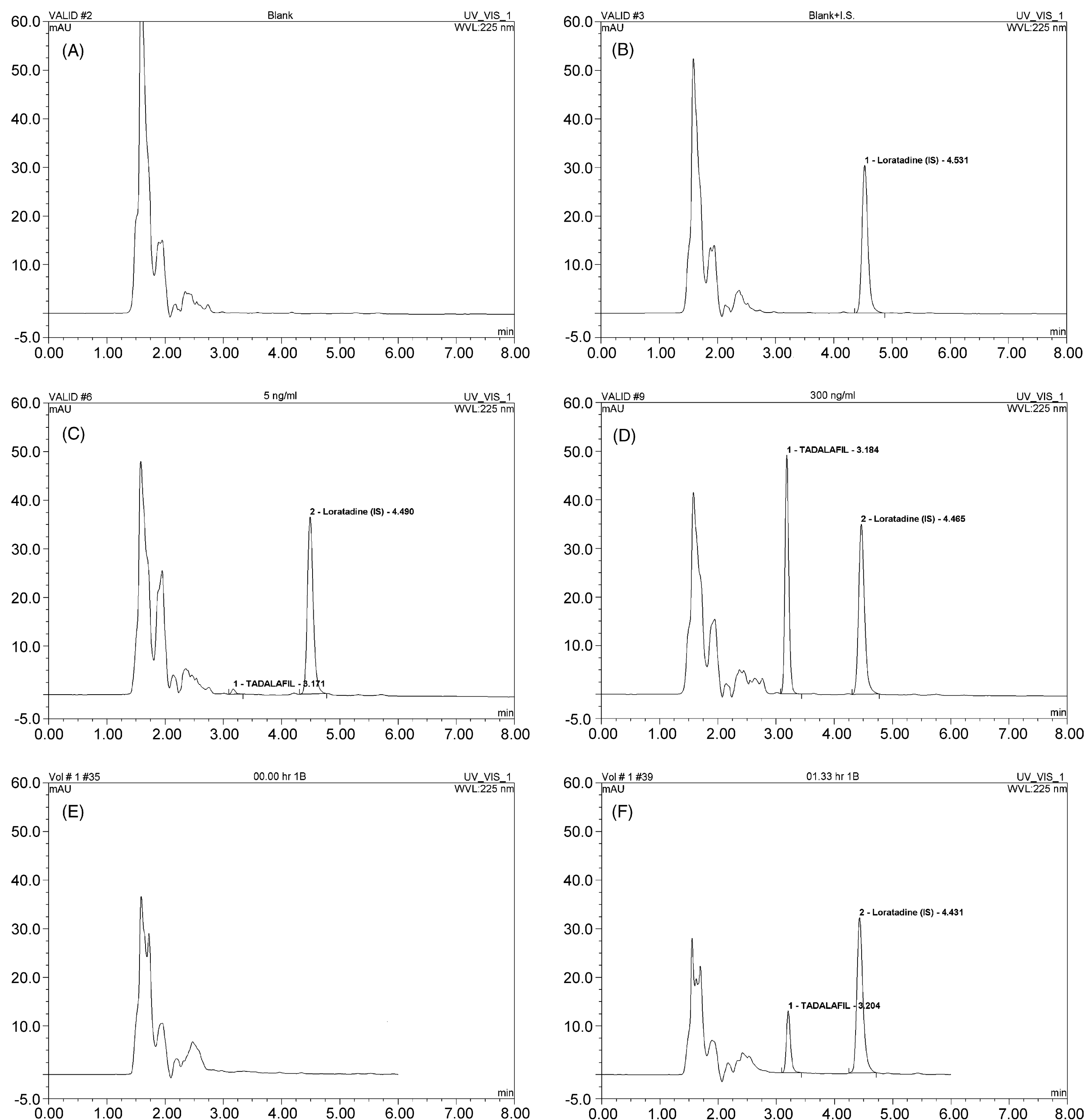

Fig. 2. Chromatograms resulting from (A) the analysis of blank human plasma, (B) human plasma spiked with 1.25 μg/mL of IS, (C) human plasma spiked with 5ng/mL tadalafil and IS, (D) human plasma spiked with 300 ng/mL tadalafil and I.S. (E) Volunteer sample obtained at zero hour and (F) volunteer sample obtained 1.33 h after oral administration of 20 mg tadalafil tablet.

300 ng/mL and IS (D), plasma obtained from a healthy subject at 0 h (E), and after 1.33 h following oral 20 mg dose of tadalafil, Cialis® (F). The analytes were well separated from co-extracted material under the present chromatographic conditions at retention time of ∼3.1 and ∼4.5 min for tadalafil and loratadine, respectively. The total run time was ∼6 min. The peaks were of good shape, completely resolved one from another at therapeutic concentration of tadalafil. The two-step extraction processes were sufficient to isolate the tadalafil and loratadine from plasma without any interfering endogenous peaks at 225 nm. The method is specific and more sensitive than the other reported methods [4,9]. No interference with constituent from the plasma matrix was observed.

3.2. Linearity and sensitivity of the method

The peak area ratio of tadalafil to IS in human plasma was linear with respect to the analyte concentration over

Table 1
Standard curves for HPLC assay of tadalafil in plasma[a]

	Slope	y intercept	r
Day 1	0.002119	−0.003112	0.999935
Day 2	0.001656	−0.00702	0.999611
Day 3	0.002548	−0.00099	0.998793
Day 4	0.001919	0.00555	0.999391
Day 5	0.001797	−0.002577	0.999892
Day 6	0.001722	−0.014017	0.999366
Mean	0.001960	−0.003694	0.999498
SD	0.000331	0.006511	0.000421

[a] Eight calibration standards were included in each calibration curve.

the range 5–600 ng/mL. The mean linear regression equation ($y = mx + c$) of calibration curve for the analyte was $y = 0.001960(\pm 0.000331)x - 0.003694$ (± 0.00651), where y was the peak area ratio of the analyte to the IS and x was the concentration of the analyte. The correlation coefficient (r) for the tadalafil was above 0.998 over the concentration range used. Table 1 summarizes the calibration curve results for the analyte.

The lower limit of quantitation, the lowest concentration of the standard curve which can be measured with acceptable accuracy and precision for the analyte from normal human plasma was 5 ng/mL. The mean response for the analyte peak at the assay sensitivity limit (5 ng/mL) was seven times greater than the mean response for the peak in six blank human plasma samples at the retention time of the analyte. Table 2 summarizes the precision and accuracy data of back-calculated concentration of calibration samples for tadalafil in human plasma. The precision for the analyte covering the concentration of 5–600 ng/mL ranged from 1.2 to 12.5% and the relative error (bias, %) was between −4.3 and 1.8%.

Table 2
Statistical evaluation of the analysis results for tadalafil in standard curves during six days

Concentration added (ng/mL)	Concentration found (mean ± SD, $n = 6$) (ng/mL)	Precision (%)	Bias (%)
5	5.09 ± 0.63	12.5	1.8
10	10.10 ± 0.66	6.5	1.0
20	19.13 ± 0.31	1.6	−4.3
50	48.36 ± 3.66	7.6	−3.3
100	98.11 ± 9.82	10.0	−1.9
200	199.91 ± 14.26	7.1	−0.0
400	399.21 ± 6.93	1.7	−0.2
600	599.20 ± 6.93	1.2	−0.1

Table 4
Accuracy and precision of the HPLC method for determining tadalafil concentrations in plasma samples

Concentration added (ng/mL)	Concentration found (mean ± SD) (ng/mL)	Precision (%)	Bias (%)
Within-batch precision ($n = 6$)			
5 (LLOQ)	5.04 ± 0.43	8.6	0.8
15 (Low)	14.16 ± 0.37	2.6	−5.6
300 (Med)	310.85 ± 16.22	5.2	3.6
480 (High)	443.42 ± 9.45	2.1	−7.6
Between-batch precision ($n = 3$)			
5 (LLOQ)	5.06 ± 0.52	10.3	1.2
15 (Low)	14.60 ± 0.53	3.6	−2.7
300 (Med)	293.79 ± 4.57	1.6	−2.1
480 (High)	460.50 ± 7.43	1.6	−4.1

The published LC–MS/MS (4) and HPLC–UV (9) methods are validated over a calibration range of 10–1000 ng/mL (in human plasma) and 10–2000 ng/mL (rat plasma), respectively. On considering the single dose and clinical concentration of tadalafil formulation, we have developed the analytical method which is more sensitive than the above mentioned methods. This method is fully validated as per FDA guideline [10] over the calibration range of 5–600 ng/mL. The samples (*if any*) which are having concentration above the higher calibration curve can be analyzed after dilution with blank plasma.

3.3. Extraction recovery

The recovery of tadalafil was 66.1 ± 2.8% on an average, and the recovery of internal standard, loratadine was 78.9 ± 1.9% at the concentration used in the assay (Table 3).

3.4. Specificity

There were no interfering peaks present in six different randomly selected samples of drug free human plasma used for analysis at the retention times of either analyte or internal standard.

3.5. Precision and accuracy

The precision and accuracy at the LLOQ and at low, medium and high concentration of tadalafil in plasma were within acceptable limits (Table 4). Within- and between days relative standard deviation (precision, % CV) were less than 8.6 and 10.3%. Within- and between-day relative errors (bias, %) were less than −7.6 and −4.1%, respectively. Accuracy was expected as

Table 3
Extraction recovery of tadalafil and loratadine from plasma

Analyte	Concentration (ng/mL)	Concentration found (mean ± SD) (ng/mL)	% Recovery (mean ± SD)	Mean recovery
Tadalafil ($n = 6$)	15	9.98 ± 0.36	66.5 ± 2.4	
	300	196.35 ± 7.69	65.5 ± 2.6	
	480	318.81 ± 8.80	66.4 ± 1.8	66.1 ± 2.8
Loratadine ($n = 10$)	1250	985.80 ± 24.1	–	78.9 ± 1.9

Table 5
Stability of the samples

Sample concentration (ng/mL)	Concentration found (ng/mL)	Precision (%)	Bias (%)
Short-term stability for 12 h ($n = 6$) in plasma			
15	15.50	9.1	3.3
480	443.30	3.2	−7.6
Three freeze and thaw cycles ($n = 6$)			
15	14.40	8.1	−4.0
480	439.90	7.6	−8.4
Auto-sampler stability for 24 h ($n = 6$), (*after extraction and reconstitution*)			
15	14.63	5.0	−2.5
480	455.74	5.2	−5.1
Thirty days stability at −70 °C ($n = 6$)			
15	15.02	8.4	0.1
480	482.75	5.6	0.6

percent error (relative error) [(measured concentration − spiked concentration)/spiked concentration] × 100%, while precision was quantitated by calculating within and between day % CV values.

3.6. Dilution integrity

The dilution integrity was also conducted to assess whether the upper concentration limit (600 ng/mL) can be extended or not. Quality control samples (in six replicate) at concentration 1000 ng/mL were diluted by two times with blank plasma, and the assay, precision, and accuracy were determined as described in Section 2.5.4. For, tadalafil, the concentration found was 1035 ± 35 ng/mL and bias was 3.6%. The result indicated that samples whose concentrations were greater than the upper limit of the standard curve could be re-analyzed by appropriate dilution.

3.7. Stability

Stock solution of the tadalafil and loratadine were prepared in acetonitrile. The solutions were stable for at least 2 months when stored at 4 °C. Stability of plasma samples were performed as described earlier in the text. Three freeze-thaw cycle and 12 h room temperature storage for low and high quality control samples indicated that tadalafil was stable in the human plasma under the experimental condition. QC sample were stable for at least 30 days if stored frozen at approximately −70 °C in deep freezer (Table 5). Auto-injector stability of the samples is also mentioned in the Table 5. Results indicate that the samples were stable when kept in the auto-injector for up to 24 h at 5 ± 1 °C.

3.8. Application

The validated method has been successfully used to quantitate the tadalafil concentration in the human plasma samples after oral administration of single dose of 20 mg tadalafil tablet (JCPR, data on file). The analyses were accomplished in accordance with the FDA bio-analytical method validation guidance.

4. Conclusion

The developed HPLC/UV method employing simple liquid–liquid extraction for sample preparation is economical, very simple and convenient for the quantitation of tadalafil in human plasma. The previously reported methods for analysis of tadalafil in biological fluid were less sensitive. The method described here is simple, sensitive, specific and fully validated as per FDA guideline. The method has shown acceptable precision, accuracy and adequate sensitivity for use in the clinical studies. The method has been applied for the analysis of tadalafil in healthy volunteers. The validated method allows quantification of tadalafil in the 5–600 ng/mL range. In addition, this method has a short turnover time (less than 6.5 min) and is suitable for clinical pharmacokinetic studies. In conclusion, this paper describes a very simple, rapid and sensitive HPLC method for the quantitation of tadalafil in human plasma with dilution integrity.

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