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Quantitation of tadalafil in human plasma by liquid chromatography–tandem mass spectrometry with electrospray ionization

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Abstract

A simple, rapid, sensitive and specific liquid chromatography–tandem mass spectrometry method was developed and validated for quantitation of tadalafil (I) in human plasma, a new selective, reversible phosphodiesterase 5 inhibitor. The analyte and internal standard (sildenafil, II) were extracted by liquid–liquid extraction with diethyl ether/dichloromethane (70/30, v/v) using a Glas-Col Multi-Pulse Vortexer. The chromatographic separation was performed on reverse phase Xterra MS C18 column with a mobile phase of 10 mM ammonium formate/acetonitrile (10/90, v/v, pH adjusted to 3.0 with formic acid). The protonate of analyte was quantitated in positive ionization by multiple reaction monitoring with a mass spectrometer. The mass transitions m/z 390.4 → 268.0 and m/z 475.5 → 58.3 were used to measure I and II, respectively. The assay exhibited a linear dynamic range of 10–1000 ng/mL for tadalafil in human plasma. The lower limit of quantitation was 10 ng/mL with a relative standard deviation of less than 15%. Acceptable precision and accuracy were obtained for concentrations over the standard curve ranges. Run time of 1.2 min for each sample made it possible to analyze a throughput of more than 400 human plasma samples per day. The validated method has been successfully used to analyze human plasma samples for application in pharmacokinetic, bioavailability or bioequivalence studies.
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Keywords: Tadalafil

1. Introduction

Tadalafil (Fig. 1) (6R, 12aR)-2,3,6,7,12,12a-hexahydro-2-methyl-6-(3,4-methylenedioxyphenyl)pyrazino(1′,2′:1,6)-pyrido(3,4-b)indole-1,4-dione, is a potent, reversible, and selective phosphodiesterase 5 (PDE5) inhibitor for the treatment of erectile dysfunction (ED) [1,2]. On 21 November 2003 US FDA approved tadalafil and it has been available and in widespread use in Europe and Latin America for several months [3].

Compared with sildenafil and vardenafil, tadalafil has a unique and potentially advantageous pharmacologic profile. Tadalafil exhibits a prolonged plasma residence and window of therapeutic response. The terminal plasma elimination half-life ($t_{1/2}$) of tadalafil is 17.5 h and the t_{max} is approximately 2.0 h (range, 0.5–12.0 h; normalized for a 20 mg dose) in healthy volunteers [4]. Tadalafil was rapidly absorbed from the gastrointestinal tract after oral dosing, reaching a maximum plasma concentration (C_{max}) of 378 μg/L; distributed in tissues, with a mean apparent volume of distribution (V_d/F) of 62.6 L; and predominantly eliminated by the liver [1,5]. Neither intrinsic factors (e.g., age, diabetes status) nor extrinsic factors (i.e., food or alcohol intake, time of dosing) had any clinically significant influence on tadalafil plasma concentrations or systemic exposure, as indicated by the area under the plasma concentration–time curve (AUC) [6]. The foregoing advantages, particularly the reduced need to plan sexual activity around the time of either tadalafil dosing or meal/alcohol consumption, may translate in clinical practice into enhanced convenience

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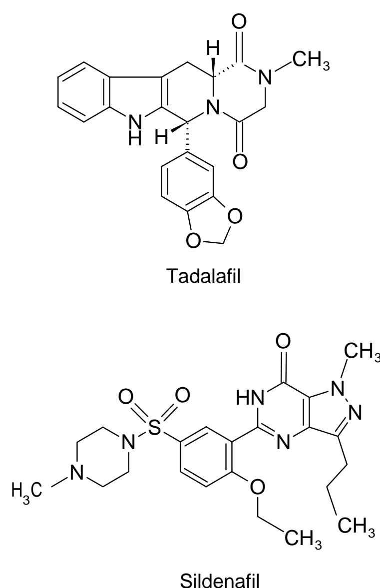

Fig. 1. Chemical structures of tadalafil (I) and internal standard (sildenafil, II).

and acceptability of tadalafil to the ED patient and/or his partner.

To date, no simple chromatographic method has been reported for tadalafil quantitation in plasma. In order to quantify plasma concentrations of tadalafil in clinical trials, it was necessary to develop and validate an assay with appropriate sensitivity, selectivity, accuracy and precision. The most attractive approach for an assay, which would be robust, sensitive, selective and would allow high throughput, was a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method [7]. We now report for the first time an LC–MS/MS method developed and validated for the quantitative determination of tadalafil in human plasma. It was essential to establish an assay capable of quantifying tadalafil at concentration down to 10 ng/mL. At the same time, it was expected that this method would be efficient in analyzing large number of plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies after therapeutic doses of tadalafil.

In recent years, a number of laboratories have reported the use of high-throughput bioanalytical procedures using LC–MS/MS [7–11]. Our method is simple, rapid, robust, specific, sensitive and novel that makes it an attractive procedure in high-throughput bioanalysis.

2. Experimental

2.1. Chemicals

The pure substances of Tadalafil (99.57%) and Sildenafil (99.62%) (internal standard, I.S.) were from Vimta Labs Ltd. (Hyderabad, India). Chemical structures are presented in Fig. 1. Stock solutions of tadalafil (1 mg/mL) and I.S. (0.5 mg/mL) were separately prepared in 10-mL volumetric flasks with methanol. HPLC-grade LiChrosolv methanol, LiChrosolv acetonitrile, diethyl ether and dichloromethane were from Merck (Darmstadt, Germany). Ammonium formate GR and formic acid GR were from Merck (Worli, Mumbai, India). HPLC Type I water from Milli-Q system (Millipore, Bedford, MA, USA) was used. All other chemicals were of analytical grade.

Table 1
Tandem mass-spectrometer main working parameters

Parameter	Value
Source temperature (°C)	150
Dwell time per transition (ms)	200
Ion source gas (Gas 1) (psi)	18
Ion source gas (Gas 2) (psi)	10
Curtain gas (psi)	15
Collision gas (psi)	12
Ion spray voltage (V)	5400
Entrance potential (V)	10
Declustering potential (DP) (V)	80 (analyte) and 110 (I.S.)
Collision energy (V)	16 (analyte) and 80 (I.S.)
Collision cell exit potential (V)	20 (analyte) and 8 (I.S.)
Mode of analysis	Positive
Ion transition for tadalafil (*m/z*)	390.4/268.0
Ion transition for sildenafil (*m/z*)	475.5/58.3

2.2. LC–MS/MS apparatus and conditions

The HPLC Agilent 1100 Series (Agilent Technologies, Waldbronn, Germany) is equipped with G1312A binary pump, G1379A degasser, G1367A autosampler equipped with a G1330B thermostat, G1316A thermostatted column compartment and G1323B control module. The chromatography was on Waters Xterra MS C18 column (3.5 μm, 100 mm × 3 mm i.d.) at 30 °C temperature. The mobile phase composition was a mixture of 10mM ammonium formate buffer/acetonitrile (10/90, v/v, pH adjusted to 3.0 with formic acid), which was pumped at a flow-rate of 0.8 mL/min.

Mass spectrometric detection was performed on an API 4000 triple quadrupole instrument (ABI-SCIEX, Toronto, Canada) using multiple reaction monitoring (MRM). A turbo electrospray interface in positive ionization mode was used. The main working parameters of the mass spectrometer are summarized in Table 1. Data processing was performed on Analyst 1.3 software package (SCIEX).

2.3. Sample processing

A 250-μL volume of plasma sample was transferred to a 15-mL glass test tube and then 20 μL of I.S. working solution (5 μg/mL) was spiked. After vortexing for 30 s, 3-mL aliquot of extraction solvent, diethyl ether/dichloromethane (70/30) was added using Dispensette Organic (Brand GmbH, Postfach, Germany). The sample was vortex-mixed for 3 min using Multi-Pulse Vortexer (Glas-Col, Terre Haute, USA). The organic layer (2.5 mL) was quantitatively transferred to a 5-

mL glass tube and evaporated to dryness using TurboVap LV Evaporator (Zymark, Hopkinton, MA, USA) at 40 °C under a stream of nitrogen. Then the dried extract was reconstituted in 250 μL of diluent (water–acetonitrile, 50:50, v/v) and a 5-μL aliquot was injected into chromatographic system.

2.4. Bioanalytical method validation

2.4.1. Calibration and control samples

Working solutions for calibration and controls were prepared from the stock solution by an adequate dilution using water/methanol (1/1). The I.S. working solution (5 μg/mL) was prepared by diluting its stock solution with water/methanol (1/1). Working solutions were added to drug-free plasma to obtain the tadalafil concentration levels of 10, 20, 50, 100, 200, 500, 800 and 1000 ng/mL. Seeded quality control (SQC) samples were prepared as a bulk, at concentrations of 10 ng/mL (lower limit of quantitation, LLOQ), 50 ng/mL (low), 400 ng/mL (medium) and 800 ng/mL (high).

2.4.2. Calibration curve

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 (10–1000 ng/mL), including lower limit of quantitation. Eight samples of each concentration were measured. Linearity was assessed by a weighted ($1/x^2$) least squares regression analysis. The calibration curve had to have a correlation coefficient (r^2) of 0.99 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%.

2.4.3. Precision and accuracy

The within-batch precision and accuracy was determined by analyzing six sets of quality control samples in a batch. The between-batch precision and accuracy was determined by analyzing six sets of seeded quality control samples on four different batches. The quality control samples were randomized daily, processed and analyzed in position either (a) immediately following the standard curve, (b) in the middle of the batch, or (c) at the end of the batch. The acceptance criteria of within- and between-batch precision were 20% or better for LLOQ and 15% or better for the rest of concentrations and the accuracy was 100 ± 20% or better for LLOQ and 100 ± 15% or better for the rest of concentrations.

2.4.4. 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 reference solutions spiked in extracted drug-free plasma samples. Recovery of I.S. was evaluated by comparing the mean peak areas of ten extracted quality control samples to mean peak areas of ten reference solutions spiked in extracted plasma samples of the same concentration.

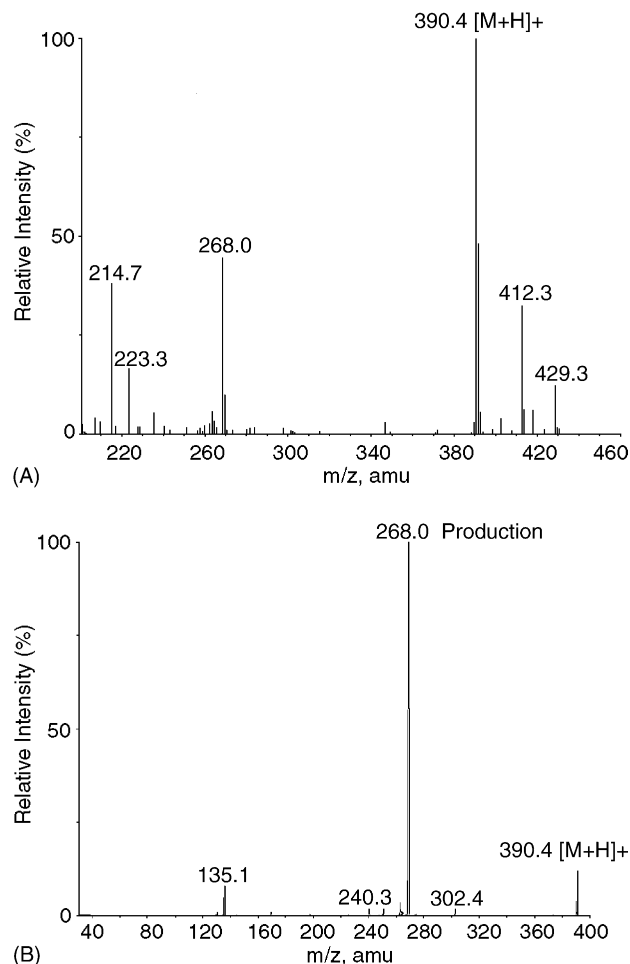

Fig. 2. Full scan positive ion TurboIonspray (A) Q1 mass spectra and (B) product ion mass spectra of tadalafil.

3. Results and discussion

Electrospray MS–MS was used to analyze tadalafil as it is beneficial in developing a selective and sensitive method. The positive ion TurboIonspray Q1 mass spectrum and product ion mass spectrum of tadalafil and the I.S. are shown in Figs. 2 and 3, respectively. $[M + H]^+$ was the predominant ion in the Q1 spectrum and was used as the precursor ion to obtain product ion spectra. The most sensitive mass transition was from m/z 390.4 to 268.0 for Tadalafil and m/z 475.5 to 58.3 for the I.S. LC–MRM is a very powerful technique for pharmacokinetic studies since it provides sensitivity, selectivity and specificity requirements for analytical methods. Thus, the MRM technique was chosen for the assay development. The MRM state file parameters were optimized to maximize the response for the analyte. The parameters presented in Table 1 are the result of this optimization.

3.1. Method development

Different mobile phases consisting of water–methanol or water–acetonitrile were evaluated to improve HPLC sep-

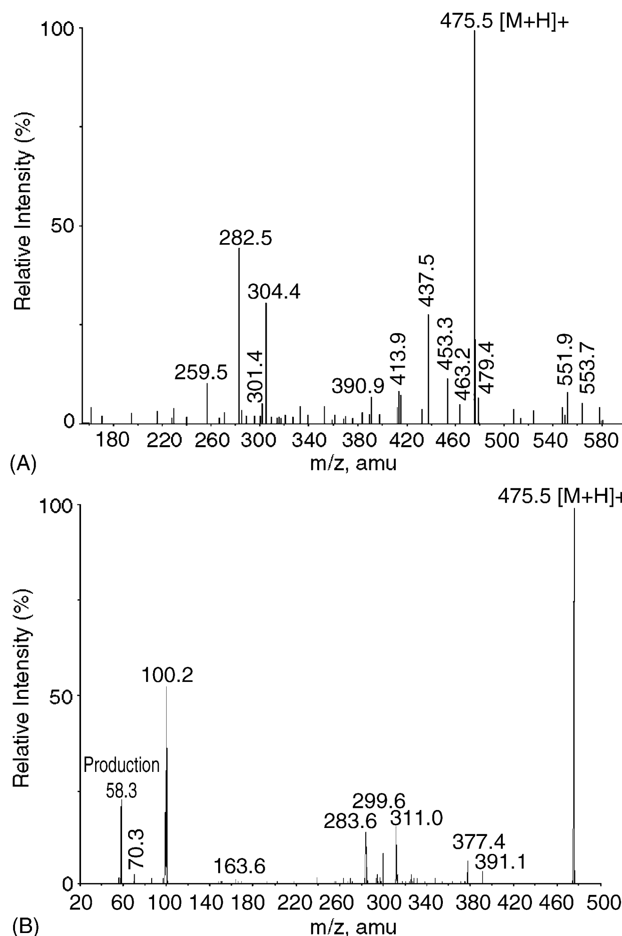

Fig. 3. Full scan positive ion TurboIonspray (A) Q1 mass spectra and (B) product ion mass spectra of internal standard (sildenafil).

aration and enhance sensitivity in MS. Modifiers such as formic acid and ammonium formate alone or in combination in different concentrations were added. The best signal was achieved using 10 mM ammonium formate buffer/acetonitrile (10/90, v/v), with pH adjusted to 3.0 with formic acid. The formic acid was found to be necessary in order to lower the pH to protonate the tadalafil and thus deliver good peak shape. The percentage of formic acid was optimized to maintain this peak shape whilst being consistent with good ionization and fragmentation in the mass spectrometer.

The tandem mass spectrometer allows the selective detection of substances with varying masses or fragments without chromatographic separation. The development of the chromatographic system was focused on short retention times and coelution of Tadalafil and I.S., paying attention to matrix effects as well as good peak shapes. A high proportion of organic solvent [10 mM ammonium formate/acetonitrile (10/90, v/v; pH 3.0)] was used to coelute both substances at retention time of 0.65 min. Flow rate of 0.8 mL/min produced a good peak shape and brought the runtime to 1.2 min. Ideally, elution of the analyte with a capacity factor of 1.5–2.0 is recommended. However, in the current reported method

Table 2
Precision and accuracy data of back-calculated concentrations of calibration samples for tadalafil in human plasma

Concentration added (ng/mL)	Concentration found (mean ± S.D., $n = 8$) (ng/mL)	Precision (%)	Accuracy (%)
10	10.24 ± 0.86	8.36	102.38
20	19.92 ± 1.15	5.76	99.60
50	50.25 ± 5.05	10.05	100.49
100	95.13 ± 5.02	5.28	95.13
200	202.01 ± 13.69	6.78	101.01
500	501.35 ± 37.79	7.54	100.27
800	795.26 ± 27.94	3.51	99.41
1000	971.98 ± 65.84	6.77	97.20

a high throughput LC–MS/MS analysis was attempted. The method was subjected to specificity and matrix effect to establish the ability of mass spectrometer to separate and quantify the analyte in the presence of endogenous species and co-administered medications. Finally, the validated method was used in the analysis of 1184 samples from a clinical pharmacokinetic study.

3.2. Internal standard

The best way to cope with sample matrix effects is to use a stable isotope labeled analyte as internal standard. Since such internal standard is not commercially available, an alternative approach has been used. Internal standard substance should match the chromatographic retention, recovery and ionization properties with the matrix of tadalafil. Sildenafil (Fig. 1) was found to fulfill these criteria sufficiently. The matrix effects were similar to the matrix effects of tadalafil. Hence sildenafil has been chosen as internal standard in the quantitative assay for tadalafil from plasma. A high proportion of organic solvent was used to achieve the coelution of both substances.

3.3. Calibration curves

Calibration curve was linear over the concentration range of 10–1000 ng/mL for the analyte. The eight-point calibration curve gave acceptable results for the analyte and was used for all the calculations. The mean correlation coefficient of the weighted calibration curve generated during the validation was 0.998 for the analyte. Table 2 summarizes the calibration curve results for the analyte. The precision and accuracy for the analyte covering the concentration of 10–1000 ng/mL ranged from 3.51 to 10.05% and 95.13 to 102.38%, respectively. The calibration curve obtained as described above was suitable for generation of acceptable data for the concentrations of the analyte in the samples during the validations.

3.4. Specificity

LC–MS/MS analysis of the blank human plasma samples showed no interference with the quantitation of tadalafil

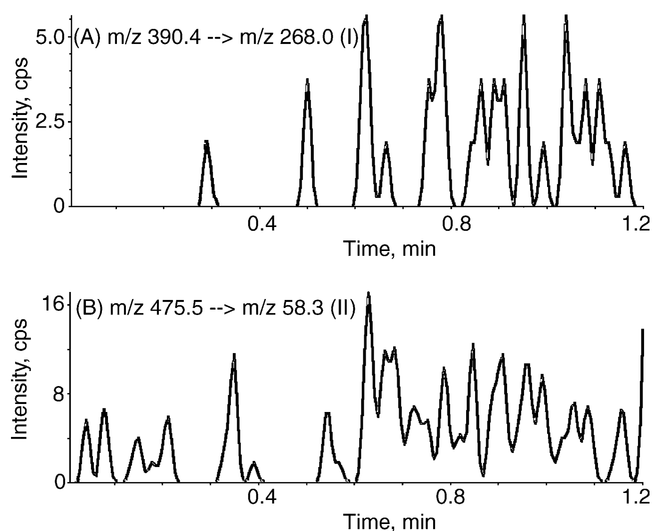

Fig. 4. MRM ion-chromatograms resulting from the analysis of blank (drug and internal standard free) human plasma for tadalafil and internal standard.

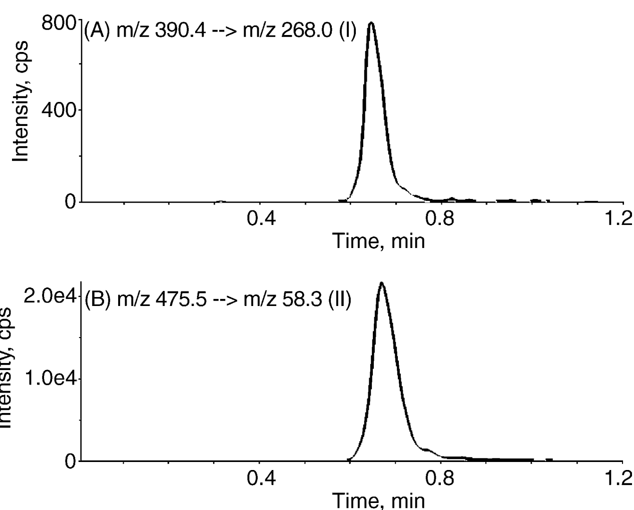

Fig. 6. Representative MRM ion-chromatograms resulting from the analysis of 10 ng/mL (LLOQ) of tadalafil spiked with the internal standard.

and the I.S. The specificity of the method was established with pooled and individual plasma samples from six different sources. Representative chromatograms of extracted blank plasma (Fig. 4), blank plasma fortified with I.S. (Fig. 5) and tadalafil (Fig. 6), demonstrating the specificity and selectivity of the method. As shown in Fig. 4, no significant interference in the blank plasma traces was seen from endogenous substances in drug-free human plasma at the retention time of the analyte. Fig. 5 shows the absence of interference from the I.S. to the MRM channels of the analyte. Fig. 6 depicts a representative ion-chromatogram for the LLOQ (10 ng/mL) of the calibration curve. Excellent sensitivity was observed for 5-μL injection volume corresponding to ca. 50 fg on-column.

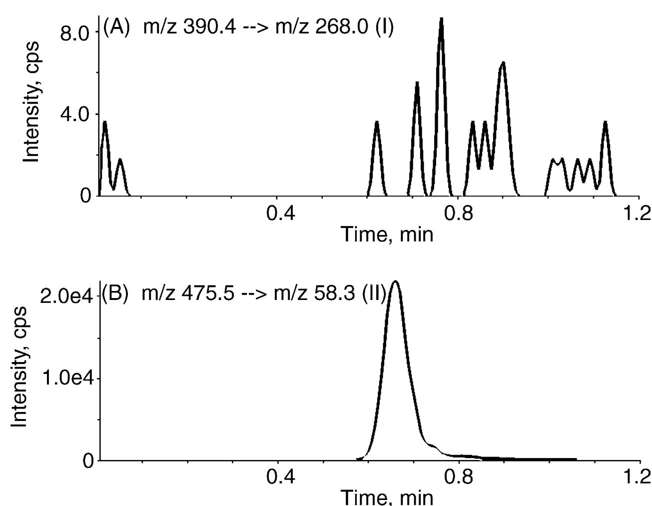

Fig. 5. MRM ion-chromatograms resulting from the analysis of blank (drug-free spiked with internal standard) human plasma for tadalafil and internal standard.

3.5. Matrix effect

The matrix effects in the LC–MS/MS method were evaluated by spiking blank plasma extracts with low and high QC samples. Six independent plasma lots were used with six samples from each lot. Percent nominal concentrations estimated were well within the acceptable limits. Hence the effect of matrix on estimation of drug is negligible.

3.6. Extraction recovery

The extraction recovery of tadalafil was 65.16 ± 1.81% on average, and the recovery ranges from 63.43 to 67.04%. The recovery of the I.S. was 63.54 ± 2.74% at the concentration used in the assay (5 μg/mL). With the moderate extraction recovery of tadalafil and I.S., the assay has proved to be robust in high throughput bioanalysis.

3.7. Lowest concentration

The lower limit of quantitation of tadalafil in human plasma assay was 10 ng/mL. The between-batch precision at the LLOQ was 8.90%. The between-batch accuracy was 103.95% (Table 3). The within-batch precision was 6.49% and the accuracy was 114.12% for tadalafil.

3.8. Middle and upper concentrations

The middle and upper quantitation levels of tadalafil ranged from 50 to 800 ng/mL in human plasma. For the between-batch experiment, the precision ranged from 3.42 to 6.35% and the accuracy ranged from 96.48 to 101.38% (Table 3). For the within-batch experiment, the precision and accuracy for the analyte met the acceptance criteria (<±15%) and precision was below 8% at all concentrations tested.

Table 3
Precision and accuracy of the LC–MS/MS method for determining tadalafil concentrations in plasma samples

Concentration added (ng/mL)	Within-batch precision (n = 6)			Between-batch precision (n = 3)		
	Concentration found (mean ± S.D.) (ng/mL)	Precision (%)	Accuracy (%)	Concentration found (mean ± S.D.) (ng/mL)	Precision (%)	Accuracy (%)
10	11.41 ± 0.74	6.49	114.12	10.39 ± 0.88	8.90	103.95
50	51.46 ± 2.04	3.96	102.93	48.50 ± 2.90	6.35	97.00
400	403.58 ± 32.05	7.94	100.90	385.92 ± 16.68	4.50	96.48
800	841.37 ± 18.85	2.24	105.17	811.07 ± 26.99	3.42	101.38
1600[a]	1592.02 ± 41.23	2.59	99.50	1594.56 ± 60.27	3.78	99.66

[a] The sample was processed with four-fold dilution.

3.9. Dilution

The upper concentration limits can be extended with acceptable precision and accuracy to 1600 ng/mL by a four-fold dilution with control human plasma. For the between-batch experiment, the precision and accuracy at 1600 ng/mL were 3.78 and 99.66%, respectively (Table 3). For the within-batch experiment, the precision was 2.59% and accuracy was 99.50%, respectively. These data suggested that samples whose concentrations are greater than the upper limit of the standard curves could be reanalyzed by dilution to obtain acceptable data.

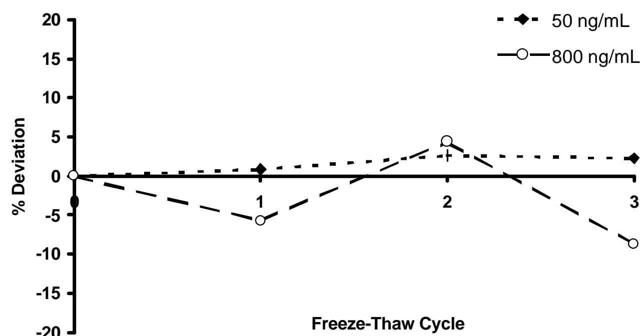

Fig. 7. Freeze–thaw cycle stability of tadalafil at two concentration levels (50 and 800 ng/mL). Deviations are less than 10% even after third freeze–thaw cycle.

3.10. Stability

As per in-house clinical blood sampling SOP, blood samples were collected at room temperatures (24 ± 1 °C) and the samples were centrifuged using refrigerated centrifuge at 4 °C within 30 min from collection.

To examine the batch size that could be analyzed, two batches were prepared containing 144 samples and a system suitability sample (145 in total) and analyzed consecutively. The run was then examined for changes in retention time and sensitivity throughout. The retention time for tadalafil ranged from 0.64 to 0.66 min. There was no noticeable change in sensitivity for tadalafil or the internal standard. With the run time of 1.2 min and a batch size of approximately 150 samples, it was evident that the desired high throughput for the assay would be achievable.

The freeze–thaw stability of the analyte was determined by measuring the assay precision and accuracy for the samples which underwent three freeze−thaw cycles. The stability data were used to support repeat analysis. In each freeze–thaw cycle, the frozen plasma samples were thawed at room temperature for 2–3 h and refrozen for 12–24 h. After completion of each cycle the samples were analyzed and results were compared with that of zero cycle. The results showed that the analyte was stable in human plasma through three freeze−thaw cycles (Fig. 7). The results demonstrated that human plasma samples could be thawed and refrozen without compromising the integrity of the samples.

The storage time in long-term stability evaluation brackets the time between the first sample collection and the last sample analysis. The sample long-term storage stability at −72 °C was evaluated to establish acceptable storage conditions for subject samples. Aliquots of human plasma samples spiked with analyte at concentrations of 50 and 800 ng/mL were analyzed on day 1. Then the samples from the same pools were analyzed against calibration curves from freshly prepared standards after storage at −72 °C for 15 days. The precision and accuracy for the analyte on day 15 ranged from 2.20 to 6.64% and 94.24 to 95.74%, respectively (Table 4).

Stability of the tadalafil and its internal standard after processing in the autosampler provides advantage to determine a large number of plasma samples. Twelve sets of quality control samples were prepared as described in Section 2.3, and placed into the autosampler to +10 °C. Six sets were analyzed at once (controls) and six sets 24 h later. The results indicated that the analyte and I.S. were stable for at least 24 h. It took less than 10 h to run 400 samples with a sample turnover rate of 1.2 min/sample. This rapid assay method facilitates to analyze several hundred samples in one working day.

Differences in responses of spiked standards at time 0 h and after 24 h for Tadalafil were less than 10%. As per in-house SOP system significance was defined if the percent change in response between 0 and 24 h is more than 10%. Therefore, the differences in responses were not significant, indicating the stability of analyte at room temperature up to 24 h. Further, the analyte was found to be stable after reconstitution in diluent for at least 12 h at 4 °C. The re-injection reproducibility was established to determine if an analytical batch could be reanalyzed in case of an unexpected delay in analyses. The same set of QC samples were repeated after

Table 4
Stability of human plasma samples of tadalafil

Sample concentration (ng/mL) (n = 6)	Concentration found (mean ± S.D.) (ng/mL)	Precision (%)	Accuracy (%)
Short-term stability (24 h)			
50	45.43 ± 4.89	10.76	96.74
800	717.83 ± 21.57	3.01	88.86
Long-term stability (15 days at −72 °C)			
50	44.97 ± 2.99	6.64	95.74
800	761.33 ± 16.74	2.20	94.24
Autosampler stability (24 h)			
50	47.79 ± 4.82	10.08	104.66
800	762.75 ± 68.63	8.99	96.60

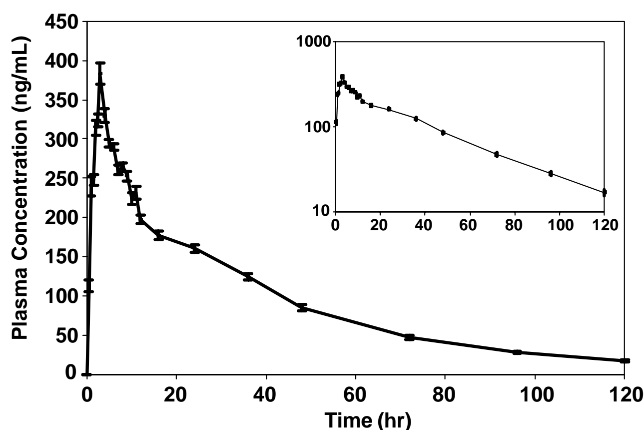

Fig. 8. Representative data showing mean plasma concentration–time profiles of 24 healthy subjects after the administration of an oral single dose of 20 mg tablet of tadalafil. The error bars represents ± standard deviation.

the analysis with a 3 h gap between, during which the samples were stored at 4 °C, and in all cases the deviations were less than 15%. On similar lines, stability of the extracted dry residue was also established to be over 24 h (deviations observed <10%). In addition, the stock solutions of tadalafil and I.S. were also found to be stable for at least 3 months at 4 °C.

3.11. Application

The validated method has been successfully used to quantitate the tadalafil concentration in the human plasma samples after the oral administration of a single dose of 20 mg tadalafil tablet. The analyses were accomplished in accordance with the FDA bioanalytical method validation guidance [12]. The mean plasma concentration–time profile of 24 healthy subjects is represented in Fig. 8.

4. Conclusions

In summary, this is the first method described here for the quantitation of tadalafil from human plasma by LC–MS/MS in positive ionization mode using multiple reaction monitoring. The current method has shown acceptable precision and adequate sensitivity for the quantitation of tadalafil in human plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies. Furthermore, it was utilized for the analysis of hundreds of subject samples. The method described is simple, rapid, sensitive, specific and fully validated as per FDA guidelines. The cost-effectiveness, simplicity and speed of liquid/liquid extraction and sample turnover rate of 1.2 min/sample make it an attractive procedure in high-throughput bioanalysis of tadalafil. The validated method allows quantitation of tadalafil in the 10–1000 ng/mL range.

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References

[1] M.P. Curran, G.M. Keating, Drugs 63 (2003) 2203.
[2] U. Gresser, C.H. Gleiter, Eur. J. Med. Res. 7 (2002) 435.
[3] K.K. Gaines, Urol. Nurs. 24 (2004) 46.
[4] G. Brock, Eur. Urol. 1 (Suppl.) (2002) 12.
[5] H. Porst, Eur. Urol. 1 (Suppl.) (2002) 19.
[6] B. Patterson, A. Bedding, H. Jewell, C. Payne, M. Mitchell, Int. J. Impot. Res. 13 (Suppl. 4) (2001) 43.
[7] M.S. Lee, E.H. Kerns, Mass Spectrom. Rev. 18 (1999) 187.
[8] M. Jemal, Biomed. Chromatogr. 14 (2000) 422.
[9] N.V.S. Ramakrishna, K.N. Vishwottam, S. Puran, S. Manoj, M. Santosh, S. Wishu, M. Koteshwara, J. Chidambara, B. Gopinadh, B. Sumatha, J. Chromatogr. B 805 (2004) 13.
[10] N.V.S. Ramakrishna, K.N. Vishwottam, S. Puran, S. Manoj, M. Santosh, M. Koteshwara, J. Mass Spectrom. (2004) (in press).
[11] N.V.S. Ramakrishna, K.N. Vishwottam, S. Puran, M. Koteshwara, S. Manoj, M. Santosh, J. Chromatogr. B (2004) (in press).
[12] Guidance for Industry: Bioanalytical Method Validation, US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, Rockville, MD, 2001.