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Determination of tadalafil in small volumes of plasma by high-performance liquid chromatography with UV detection

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Abstract

Tadalafil is a potent reversible phosphodiesterase-5 inhibitor used for the treatment of erectile dysfunction. This study describes a simple and sensitive high-performance liquid chromatographic (HPLC) method for the determination of tadalafil in 50 μl of rat plasma. Tadalafil and the internal standard lamotrigine were extracted with 0.5 ml of *tert*-butyl methyl ether, after the samples alkalinized with 20 μl of sodium hydroxide solution (1N). Chromatographic separation was achieved on a C18 column with the mobile phase of acetonitrile–water containing 20 mM phosphate buffer (pH 7) (35/65, v/v), at a flow rate of 1 ml/min. The eluant was detected at 290 nm. The retention time was about 4.5 min for lamotrigine and 15 min for tadalafil. No endogenous substances were found to interfere. Calibration curves were linear from 10 to 2000 ng/ml. The recovery of tadalafil from plasma was greater than 77%. The limit of quantitation was 10 ng/ml. The intra- and inter-day imprecision (expressed as coefficient of variation, C.V.) did not exceed 10.7%, and the accuracy was within 5.9% deviation of the nominal concentration. The method is suitable in pharmacokinetic investigation and monitoring tadalafil concentration.

Keywords: Tadalafil; Erectile dysfunction; HPLC; Pharmacokinetics

1. Introduction

Tadalafil (Cialis®), (6*R*,12a*R*)-2,3,6,7,12,12a-hexahydro-2-methyl-6-[3,4-(methylenedioxy) phenyl]pyrazino[1′,2′:1,6]pyrido[3,4-b]indole-1,4-dione (Fig. 1) [1], is a newly approved oral selective phosphodiesterase-5 (PDE5) inhibitor indicated for the treatment of erectile dysfunction [1–4]. The mechanism of action of tadalafil is similar to the other PDE5 inhibitors, sildenafil (Viagra®; 1-[4-ethoxy-3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo-[4,3-d] pyrimidin-5-yl)-phenylsulphonyl]-4-methylpiperazine) and vardenafil (Levitra®; 2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3H-imidazo[5,1-f]-[1,2,4]triazin-4-one [2–4]. Through the inhibition on PDE5, tadalafil increases the concentrations 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 [2–4].

The chemical structure and the pharmacokinetic profile of tadalafil differ significantly with those of sildenafil and vardenafil, albeit they are classifed in the same pharmacological category. Following oral administration of a 20-mg dose to healthy subjects, tadalafil is rapidly absorbed with the peak plasma concentration of 378 ng/ml occurs 2 h postdose [2,5]. Tadalafil has a relative large apparent volume of distribution (Vd/F) of 62.6 L, and a low apparent oral clearance (CL/F) of 2.48 L/h. As a result, the mean elimination half-life of tadalafil is about 17.5 h, which is substantially longer than that of sildenafil or vardenafil. The long residence of tadalafil in the body results in a favorable long duration of action of 36 h, and hence greater sexual spontaneity, over the other PDE5 inhibitors [2–5].

Being newly launched, the pharmacokinetic information of tadalafil is limited as compared to the first of this class

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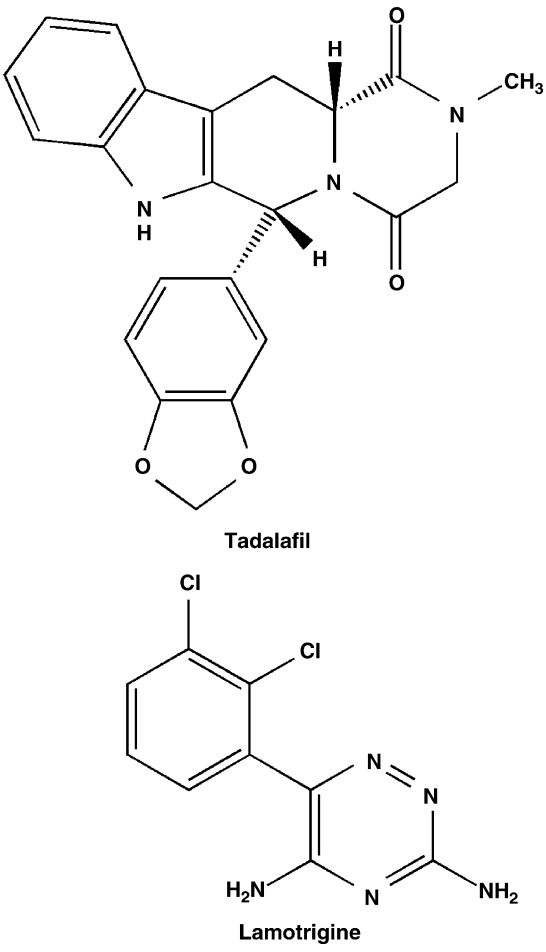

Fig. 1. Structures of tadalafil and internal standard lamotrigine.

sildenafil. Tadalafil is predominantly metabolized by hepatic cytochrome P450 3A4 isozyme (CYP3A4) [2,5]. In vitro and in vivo studies demonstrated that at therapeutic concentrations tadalafil had no significant effect on the metabolism of drugs that are substrates of CYP3A [4]. On the other hand, concomitant with drugs that are inhibitors (e.g. ketoconazole) or inducers (rifampicine) of CYP3A4 can dramatically increase or decrease the systemic exposure of tadalafil, respectively [2]. Therefore, investigation on the metabolism-related drug–drug interaction of tadalafil is of great importance. To address these issues, a suitable assay for quantitation of tadalafil is necessary.

The determination of tadalafil in tablet formulations has been achieved by high-performance liquid chromatography (HPLC) [6] and capillary electrophoresis with UV detection [7,8]. Two liquid chromatography–electrospray ionization mass spectrometry methods were used for the simultaneous determination of undeclared PDE5 inhibitors, sildenafil, vardenafil and tadalafil, in dietary supplements [1,9]. As the concentrations of tadalafil prepared for analysis from pharmaceutical preparations and dietary supplements are much higher than those in plasma, these reported methods in general can not be used directly to measure plasma levels of tadalafil, due to inadequate sensitivity or interference by endogenous components in plasma. Recently, an HPLC coupled with electrospray ionization tandem mass spectrometry (LC–MS/MS) method was developed and validated for the determination of tadalafil in 250 μl of plasma [10]. Another LC–MS/MS method, without given full validation details, was also reported to measure plasma levels of tadalafil in a drug–drug interaction study [5]. Although these methods offer high-throughput analysis, they are not readily accessible because of the use of relative expensive tandem mass spectrometry.

For pharmacokinetic studies, a suitable sensitive HPLC method that allows an accurate measurement of low concentration of tadalafil in biological matrices is needed. For routine drug monitoring and experimental research in small animals such as rats, assays that require small sample volumes are very useful. In this report, we present a simple, sensitive and specific HPLC method with UV detection to determine tadalafil concentration in plasma. The limit of quantitation (LOQ) of this validated method was 10 ng/ml using 50 μl of rat plasma. The applicability of this assay was demonstrated in animal pharmacokinetic studies.

2. Experimental

2.1. Chemicals and reagents

Tadalafil (Batch 021212, purity 99.78%) was from Xiamen Fine Chemical Import & Export Co. Ltd. (Xiamen, China). Lamotrigine (purity 99.75% by HPLC) was kindly provided by U-Chu Pharmaceutical Co. Ltd. (Taoyuan, Taiwan) (Fig. 1) [11]. All chemicals were of analytical grade and used as received without further purification. HPLC grade acetonitrile, methanol, and phosphoric acid were obtained from Fisher Scientific (Fair Lawn, NJ, USA). *tert*-Butyl methyl ether was from Merck (Darmstadt, Germany). Milli-Q Reagent Water (Millipore, Bedford, MA, USA) was used in the preparation of mobile phase. Rat plasma used for calibration and validation of the assay was obtained from male Sprague–Dawley rats.

2.2. Instrumentation and chromatography

The HPLC system consisted of a Hitachi L-7100 pump, a Hitachi L-7200 autosampler, a Hitachi L-7400 UV detector, and a Hitachi D-7000 Chromatography Data Station (Tokyo, Japan). The analytical column was a Hypersil ODS (5 μm, 25 cm × 4.6 mm i.d., Runcorn, Cheshire, UK) column. The mobile phase comprised acetonitrile and 20 mM phosphate buffer (pH7) (35/65, v/v). The prepared mobile phase was filtered through a 0.45-μm Millipore filter and degassed ultrasonically before use. Analyses were run at a flow-rate of 1 ml/min at ambient temperature. The detector wavelength was set at 290 nm, and peak areas were measured.

2.3. UV spectrometry

The UV spectra of tadalafil (5 μg/ml) in aqueous solutions with various pH values were recorded with 10 mm quartz cell using a Hitachi U2010 spectrophotometer (Tokyo, Japan). The aqueous solutions used were 0.1N HCl (pH 1.2) and 50 mM phosphate buffers (pH 4.5, 6.8 and 9.2).

2.4. Standards and controls

Master stock solutions of tadalafil and the internal standard lamotrigine (both 100 μg/ml in methanol) were prepared monthly and kept tightly sealed at −20 °C. The stock solution of tadalafil was diluted with drug-free rat plasma to give the calibration standards at concentrations of 10, 20, 50, 100, 200, 500, 1000 and 2000 ng/ml tadalafil. The quality controls were prepared independently at concentrations of 10, 20, 200 and 2000 ng/ml prior to the start of sample collection and stored at −20 °C until used. The working solution of lamotrigine was obtained by diluting the stock solution in methanol to 1 μg/ml. A complete calibration curve was generated with each analytical run.

2.5. Sample preparation

The samples to be analysed were removed from the freezer and thawed. Calibration standards, controls, and unknown samples were pipetted into 1.5-ml microcentrifuge tubes and processed as a batch. To 50-μl aliquots of plasma samples were added a 20-μl aliquot of the internal standard working solution, 10 μl of 1N sodium hydroxide solution, and 0.5 ml of *tert*-butyl methyl ether. After vortex-mixed for 30 s and upon centrifugation at 15,850 × *g* for 10 min, the upper organic layer was transferred by freeze-decant (−70 °C) to a 1.5-ml microcentrifuge tubes and evaporated to dryness under a stream of nitrogen at room temperature. The residue was reconstituted with 200 μl of acetonitrile and a 100 μl of aliquot was injected onto the column for HPLC analysis.

2.6. Assay validation

The model for the calibration curve of tadalafil used the peak area ratio of tadalafil to lamotrigine (PAR) and the tadalafil concentration (*C*), as given in the following equation: PAR = slope × *C* + (*y* intercept). The slope and y intercept were determined by a nonlinear least-squares program (*WinNonlin*, Professional Version 2.1, Pharsight Inc., Mountain View, CA, USA), using nominal concentrations and measured PARs from calibration standards with a weighting scheme of 1/*C*. Tadalafil concentrations were estimated from PARs using the formula: *C* = (PAR − (*y* intercept))/slope.

Intra-day precision was evaluated by analysing the spiked controls six times over 1 day in random order, while inter-day precision was evaluated from the analysis of each control once on each of 6 different days. Assay precision (coefficient of variation, C.V.) was assessed by expressing the standard deviation of the measurements as a percentage of the mean value. The accuracy was estimated for each spiked control by comparing the nominal concentration with the assayed concentration. The lower LOQ was the lowest non-zero concentration level, which could be accurately (relative error < 20%) and reproducibly (C.V. < 20%) determined.

Assay specificity was examined in relation to interference from endogenous substances in six independent batches of drug-free rat plasma. Absolute recoveries of 10, 20, 200 and 2000 ng/ml concentrations of tadalafil in plasma were determined by assaying the samples as described above and comparing the peak areas of both tadalafil and lamotrigine with those obtained from direct injection of the standard solutions.

Freeze-thaw stability of tadalafil (10, 20 and 2000 ng/ml) in rat plasma samples was determined for two freeze-thaw cycles. The samples were thawed at the room temperature without any assistance, and then kept in the freezer (−20 °C) for 4 h before taking out for the next thawing. The bench-top stability of tadalafil in plasma at ambient temperature (ca. 20 °C) was studied for 4 h. The post-preparative stability of tadalafil in processed samples left at ambient temperature was followed for 24 h.

2.7. Pharmacokinetic application

The assay was applied to a single dose (1 mg/kg) pharmacokinetic study in rats. Male Sprague–Dawley rats were obtained from the Animal Breeding Center of National Cheng Kung University. The study protocol complied with the Institutional Guidelines on Animal Experimentation of National Cheng Kung University. After intravenous bolus administration via the left femoral vein, 0.2 ml of blood samples for analytical determinations were collected via the right femoral vein at specific time intervals for 12 h. Plasma samples were stored at −20 °C until analysis.

3. Results

3.1. UV absorption spectra

As shown in Fig. 2, the UV absorption spectra of tadalafil were similar in the aqueous buffer solutions with pH ranging from 1.2 to 9.2, and showed absorption maxima at about 220, 280 and 290 nm.

3.2. Chromatography

Fig. 3 shows chromatograms of extracts from pre-dose (A), 4 h (B), and 12 h (C), respectively, after administration of 1 mg/kg tadalafil to a rat. The concentration of tadalafil was 42.1 and 15.5 ng/ml at 4 and 12 h post-dose, respectively. Lamotrigine and tadalafil were eluted after 4.5 and 15 min, respectively. As can be seen in Fig. 3, a good separation of tadalafil and the internal standard was

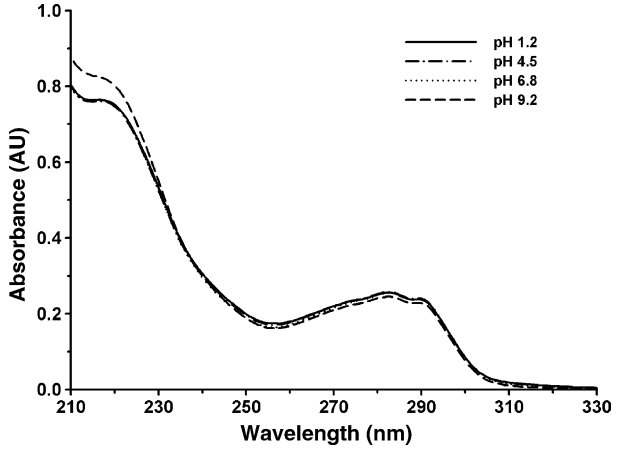

Fig. 2. The UV spectra of tadalafil (5 μg/ml) in aqueous solutions with various pH values.

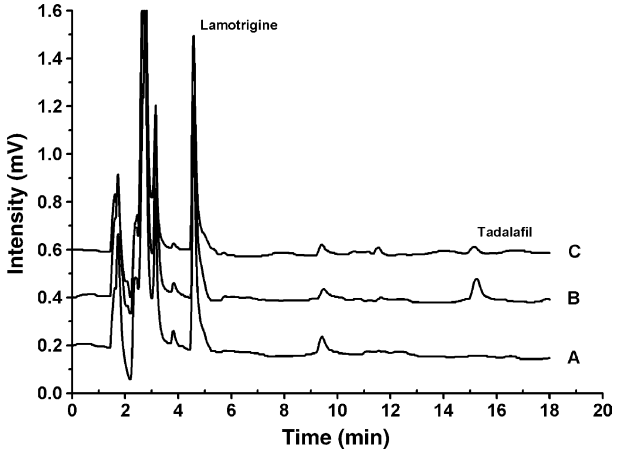

Fig. 3. The HPLC chromatograms of extracts from pre-dosing (A), 4 h (B) and 12 h (C) plasma samples from a rat after intravenous bolus of 1 mg/kg tadalafil. The concentration of tadalafil was 42.1 and 15.5 ng/ml at 4 and 12 h post-dose, respectively.

achieved under the chromatographic conditions specified in Section 2. The one-step liquid–liquid extraction by *tert*-butyl methyl ether was sufficient to isolate tadalafil and lamotrigine from plasma without any interfering endogenous peaks. The method is specific for tadalafil. No interfering endogenous peaks were observed at the retention of tadalafil when drug-free plasma and blank pre-dose rat plasma samples were analyzed (Fig. 3A).

Table 2
Recovery of tadalafil in spiked rat plasma in the presence of internal standard (mean ± S.D., $n = 3$)

Concentration (μg/ml)	Absolute recovery (%)		Relative recovery (%)
	Tadalafil	Lamotrigine	Tadalafil/lamotrigine
10	76.6 ± 12.1	87.7 ± 6.3	87.2 ± 10.8
20	79.5 ± 8.2	86.8 ± 5.8	90.1 ± 10.1
200	78.7 ± 1.8	88.2 ± 3.0	93.5 ± 1.6
2000	81.4 ± 4.1	88.0 ± 4.7	92.1 ± 1.3

3.3. Assay validation

The calibration curves were linear from 10 to 2000 ng/ml. The mean (± S.D.) regression equation for 10 replicated calibration curves constructed using 50 μl of rat plasma samples on different days was: $PAR = (4.1 \pm 0.2) \times 10^{-3} \times C + (-0.009 \pm 0.009)$, $r^2 = 0.998 \pm 0.001$.

Precision and accuracy (10–2000 ng/ml) were investigated by replicated analyses of spiked controls (Table 1), and in all cases the intra- and inter-day precision was acceptable at a C.V. of 10.7% or less. In addition, accuracy was within 6% deviation when compared with nominal concentrations across this range. From this experiment, the LOQ of the method was determined to be 10 ng/ml, with the intra-day imprecision and error of 4.8 and 1.2%, and the inter-day imprecision and error of 9.8 and −4.5% (Table 1).

Satisfactory assay sensitivity was assisted by adequate and reproducible recoveries for tadalafil and lamotrigine as shown in Table 2. The mean absolute recovery of tadalafil from rat plasma in the conditions of the assay between 10 and 2000 ng/ml was 79.0 ± 6.8% ($n = 12$). The mean absolute recovery of the internal standard was 87.7 ± 4.4% ($n = 12$).

Tadalafil is stable when stored in the refrigerator and freezer. It is stable in methanol at a concentration of 1 mg/ml for at least 3 months at 4 °C. Short-term (24 h) and long-term (−72 °C, 15 days) stability of tadalafil in human plasma have been investigated previously [10]. In this study, tadalafil was stable in rat plasma at 20 °C for up to 4 h, and in frozen rat plasma (−20 °C) for up to 30 days. The mean concentration for the quality control samples (10, 20 and 2000 ng/ml) was within ±5.7% of nominals for tadalafil following the second freeze-thaw cycle (Table 3). The present study also showed that tadalafil was stable in processed samples left at ambient temperature for up to 24 h (Table 3).

Table 1
Intra- and inter-day accuracy and precision for the determination of tadalafil in 50-μl rat plasma aliquots ($n = 6$)

$C_{nominal}$ (ng/ml)	Intra-day			Inter-day		
	C_{est} (ng/ml)	C.V. (%)	Error (%)	C_{est} (ng/ml)	C.V. (%)	Error (%)
10	10.1	4.8	1.2	9.6	9.8	−4.5
20	19.1	3.4	−4.5	18.8	10.7	−5.9
200	202	2.2	1.0	202	7.2	1.1
2000	1969	2.6	−1.5	1928	1.6	−3.6

$C_{nominal}$: nominal concentration; $C_{est.}$: estimated concentration. Error: deviation from the nominal concentration.

Table 3
Stability of tadalafil in spiked rat plasma and post-preparative samples (mean ± S.D., $n = 3$)

Conditions	%Remained (ng/ml)		
	10	20	2000
Bench-top stability in plasma (20 °C, 4 h)	98.2 ± 4.8	91.0 ± 2.7	99.6 ± 0.9
Long-term stability in plasma (−20 °C)			
15 days	100.7 ± 2.3	105.4 ± 4.2	97.4 ± 5.2
30 days	98.4 ± 5.6	97.8 ± 4.6	101.7 ± 4.0
Freeze–thaw stability (−20 °C/20 °C)			
Cycle 1	101.1 ± 6.5	101.5 ± 6.1	99.3 ± 1.3
Cycle 2	102.5 ± 3.5	94.3 ± 8.1	98.0 ± 6.1
Post-preparative stability (20 °C, 24 h)	97.5 ± 3.6	93.2 ± 2.6	94.7 ± 2.5

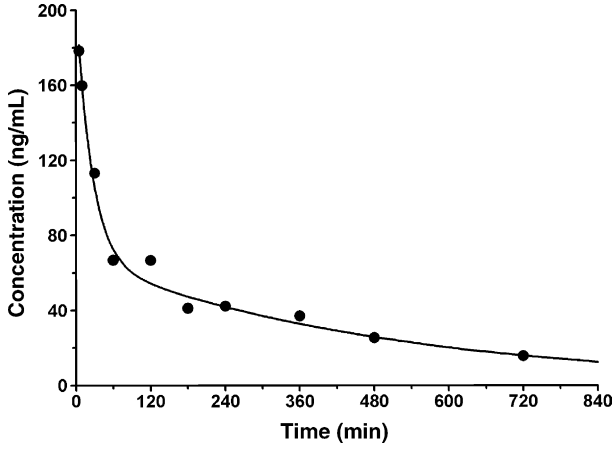

Fig. 4. The plasma concentration–time profile of tadalafil after intravenous bolus of 1 mg/kg tadalafil to a male rat. The solid line represents fitted plasma concentrations according to a two-compartment model using WinNonlin Pro 2.1, Pharsight, USA.

3.4. Pharmacokinetic application

The assay was applied to a preliminary pharmacokinetic experiment in rats. A single bolus dose of 1 mg/kg of tadalafil was administered intravenously to a male rat. The plasma concentration–time profile is illustrated in Fig. 4. The pharmacokinetic parameter estimates of clearance, volume of distribution and terminal half-life were 29 ml/min/kg, 13.1 L/kg and 341 min, respectively. The results showed that this simple and rapid method is sufficiently sensitive to follow blood levels of tadalafil.

4. Discussion

4.1. Selection of column and mobile phase

Separation of tadalafil in HPLC has been achieved by reverse phase C18 columns with mobile phases consist of acetonitrile-aqueous buffers [1,6,9,10]. An optimized mobile phase of acetonitrile–0.1 M phosphate buffer (pH 3) (2/8, v/v) was selected for the determination of tadalafil and sildenafil by a monolithic C18 column with UV detection; and it was found that the detection was poor at low concentration of acetonitrile [6]. For simultaneous determination of the three PDE5 inhibitors, sildenafil, vardenafil and tadalafil, gradient elution with acetonitrile-aqueous solutions containing modifiers (formic acid and ammonium acetate) were utilized in LC–MS methods using conventional C18 columns [1,9]. These authors reported that the presence of methanol in the mobile phase created considerably more background noise compared to acetonitrile [9], and sildenafil and vardenafil could not be separated using aqueous–methanolic mobile phases with any composition [1]. For the quantification of tadalafil in plasma by LC–MS/MS, isocratic elution of a C18 column with acetonitrile–10 mM ammonium fumorate buffer (pH 3) (9/1, v/v) was used [10]. Interestingly, a phenyl–hexyl column was employed recently in an LC–MS/MS method with a mobile phase of methanol–water (9/1) [5]. On considering the reported chromatographic conditions, we have used a C18 column with a simple acetonitrile–phosphate buffer system. The retention time of tadalafil was not affected by the pH values of the systems; therefore the pH of phosphate buffer was adjusted to 7 in this work.

4.2. Selection of UV wavelength

The structure of tadalafil contains conjugated configuration and exhibit intensive UV absorption, thus it can be measured by UV detection with quite a low LOQ [1]. Also noted is that the structure of tadalafil possesses three amine groups, which might be ionized under acidic conditions. Based on the migration behavior in capillary zone electrophoresis (CZE), Ali and Aboul-Enein reported that tadalafil existed as a cation at pH 3 and migrated towards cathode [7]. On the contrary, Flores et al. concluded that tadalafil remained neutral in the running buffer with pH 2.2–13, as tadalafil migrated with the electro-osmotic flow under these pH conditions [8]. Because the degree of ionization could potentially affect the UV absorbance of a compound, it is worthy to evaluate the effect of pH value on the UV spectrum of tadalafil.

As displayed in Fig. 2, tadalafil has similar absorption spectrum in acidic, neutral and basic medium, with high molar absorbance around 220, 280 and 290 nm. Because higher sensitivity can be attained at low UV wavelength, irrespective to the pH of the mobile phase or running buffer, the reported HPLC and CE methods applied UV detection at the region of 220–254 nm to determine tadalafil in pharmaceutical and nutraceutical preparations [6–8]. However, the use of shorter wavelength is less specific and more prone to interferences from endogenous substances of biological matrix. Our initial attempt to detect tadalafil in plasma extracts using 220 nm was unsuccessful due to significant interferences, therefore, UV detection was performed at 290 nm in the present study.

4.3. Selection of extraction solvent and internal standard

Because of its lipophilicity, tadalafil was extracted easily into methanol or acetonitrile–water (1/1) from the simple matrices of pharmaceutical and nutraceutical preparations [6–9]. Tadalafil can be efficiently isolated from plasma by liquid–liquid extraction using a mixture of diethyl ether–dichloromethane (7/3) with an average recovery of 65.2% [10], or by solid-phase extraction with C2 disk cartridges [5]. In this study, *tert*-butyl methyl ether was used as the extraction solvent as it gave high extraction recoveries for both tadalafil (79.0%) and lamotrigine (87.7%), and provided relative clean chromatograms as detected under UV 290 nm.

In the published LC–MS/MS methods, [$^{13}C^{2}H_{3}$]-tadalafil and sildenafil were used as the internal standards [5,10]. In the present study, lamotrigine was chosen as the internal standard because it is commercially available, has good UV absorbance at 290 nm in pH 1.2–10, and can be extracted by ethers with high recoveries [11]. Lamotrigine also displayed appropriate chromatographic retention with its peak sufficiently separated from that of tadalafil in the present method.

4.4. Calibration range, quantitation limit and sample volume

Tadalafil is effective in treating erectile dysfunction for on-demand or once-daily applications. For on-demand dosing, the recommend dose is 5–20 mg. Once-daily dose of up to 50 mg have been used in non-diabetic patients with mild or moderate erectile dysfunction with longest treatment duration of 3 weeks [3]. Following oral administration of a single 20-mg dose to healthy subjects, the mean peak plasma concentration of tadalafil was 378 ng/ml [2,5,10] and the concentration at 36 h postdose (the duration of action) was above 100 ng/ml [10]. Steady state was reached within 5 days of once-daily dosing with an accumulation factor of approximately 1.6. And the steady-state peak plasma concentration of tadalafil was greater than 500 ng/ml after once-daily multiple dosing of 20-mg tadalafil [5]. When coadministered with the CYP3A inhibitor ketoconazole, the systemic exposure of tadalafil was increased by 107% [1].

Detection of tadalafil by mass spectrometry provided excellent sensitivity with on-column detection limit of 40–300 pg [1,9,10] and a lower LOQ of 0.5 ng/ml has been reported for the quantitation of tadalafil in plasma by LC–MS/MS method [5]. Nevertheless, a LC–MS/MS method with a validated calibration range of 10–1000 ng/ml was adequate enough to characterize the plasma concentration profile of tadalafil for up to 5 days (i.e. more than six times elmination half-life) after a signle oral dose of 20-mg tadalafil tablet [10]. On considering the maximal therapeutic dose, clinical concentrations and potential drug–drug interactions for tadalafil, we have extended and validated the dynamic calibration range over 10–2000 ng/ml.

In the published LC–MS/MS method, a relative large amount (250 μl) of human plasma was utilized for sample preparation [10]. Thus, efforts were made in this study to lower considerably the volume of plasma sample needed, from 250 to 50 μl, and still providing good sensitivity for tadalafil quantitation. This is very useful in reducing the blood collection, offering the possibility to make sufficient numbers of blood samples for pharmacokinetic study, and minimizing the amount of blood-derived biological waste.

In conclusion, a simple and sensitive HPLC–UV method for the determination of tadalafil in rat plasma has been developed. The method was validated over the concentration range of clinical interest and it offers good accuracy, precision and sensitivity for monitoring therapeutic concentration of tadalafil. This method is flexible and required only 50 μl plasma, making it suitable for studying the pharmacokinetics of tadalafil in small animals. The application of this method was demonstrated in a pharmacokinetic study in rats.

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