行政院國家科學委員會專題研究計畫期中進度報告

PDE5 inhibitors 的相關交互作用影響研究
Investigation of Potential Drug Interactions of PDE5 Inhibitors

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中文摘要

本計劃主要是研究目前在市面上非常熱門的壯陽藥品第五型磷酸二酯酶抑制劑(sildenafil; vardenafil; tadalafil)的相關交互作用評估。由於國情，本類藥品在國內濫用的情況相當普遍，又因為屬於較新上市的藥物產品，相關的藥物動力學及藥品交互作用的文獻，除了第一個上市的 sildenafil(威而剛)有稍微多一些的資訊外，其餘皆鮮有相關報導。在本類藥品普遍濫用情況下，顯然值得進一步去探討相關的藥物交互作用研究，而能對此類藥品的臨床應用與限制有更進一步的瞭解。

第一年計畫成果針對tadalafil的研究方面，我們已完成蔓越莓汁與tadalafil的交互作用試驗。藉由大鼠體內動力學試驗及體外微粒體代謝試驗，我們發現蔓越莓汁與tadalafil併服時，會經由抑制腸道CYP3A活性而增加tadalafil口服的生體可用率，並使其血中濃度顯著增加1.6倍。此部分成果將於6月份在日本金澤所舉辦的4th World Conference on Drug Absorption, Transport and Delivery (WCDATD)學術研討會中發表。

而針對vardenafil的研究方面，我們已成功開發了第一個以高效液相層析搭配螢光偵測的分析方法，用來定量vardenafil在血漿與膽汁中的濃度，並成功地應用在研究vardenafil在大鼠體內與肝膽排泄之動力學。此部分成果投稿至Journal of Chromatography A期刊後已被接受(doi:10.1016/j.chroma.2007.03.077)，其網路版於今年3月28日刊登。

另外，在原位肝臟灌流實驗以及大鼠體內動力學試驗結果證實 tadalafil 與 vardenafil 的膽汁排泄皆是經由主動運輸步驟；目前正積極研究以釐清特定藥物傳輸子(transporter)在此所扮演之角色。相信此研究的成果，將有助於瞭解此類藥品肝臟排除的機轉，進一步能評估其可能的臨床相關交互作用，及對國內壯陽藥品不正常使用的情況提出有效的注意事項。所收集的數據，並對於生物藥劑分類系統的理論也將能更進一步的瞭解，必能對於現今國家生技重點中草藥製劑開發及健康食品的使用有所助益。

關鍵詞：大鼠、第五型磷酸二酯酶(phosphodiesterase-5, PDE5) 抑制劑、肝膽代謝機轉、藥物交互作用

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Development and validation of a high-performance liquid chromatographic method using fluorescence detection for the determination of vardenafil in small volumes of rat plasma and bile

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Abstract

A new, simple and sensitive high-performance liquid chromatography (HPLC) method with fluorescence detection was developed and validated for the determination of vardenafil in small volumes of rat plasma and bile. The absorbance and fluorescence characteristics of vardenafil were studied and factors that affect the HPLC resolution and fluorescence intensity were examined and optimized. Vardenafil and the internal standard cisapride were extracted using acetonitrile. The separation was achieved on a C18 column at 35 °C using acetonitrile–50 mM ammonium acetate aqueous solution (pH 6.8) (40:60) as mobile phase. At a flow rate of 1 ml/min, the total run time was 18 min. Fluorescence was measured with excitation and emission set at 280 and 470 nm, respectively. The calibration curves were linear from 10 to 1000 ng/ml and 0.2–100 μg/ml for plasma and bile samples, respectively. The intra- and inter-day imprecision did not exceed 10.8%, and the accuracy was within 9.6% deviation of the nominal concentration. The method was used successfully to investigate the disposition and biliary excretion of vardenafil in rats.

Keywords: Vardenafil; Erectile dysfunction; Fluorescence detection; HPLC; Pharmacokinetics

1. Introduction

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 monohydrochloride, trihydrate (Fig. 1), is a new oral selective phosphodiesterase-5 (PDE5) inhibitor marketed for the improvement of erectile dysfunction [1–3]. Following oral administration to healthy subjects, vardenafil is rapidly absorbed and the peak plasma concentration occurs 0.25–3 h postdose with an absolute bioavailability of 15%. [3] The pharmacokinetics of vardenafil are approximately linear over the recommended dose range of 5–20 mg. Vardenafil is highly protein bound in plasma (93–95%) and has a large steady-state volume of distribution of 2.5 l/kg, a high clearance of 56 l/h, and a short elimination half-life of 4–5 h [3].

Vardenafil is predominantly eliminated by hepatic metabolism, mainly by cytochrome P450 3A4 isozyme (CYP3A4). Concomitant use of potent CYP3A4 inhibitors such as erythromycin, ketoconazole, indinavir and ritonavir produced marked increases of plasma concentrations of vardenafil. Vardenafil and its metabolites are excreted predominately via biliary excretion into faeces (∼91–95%) and to a small extent in the urine (∼2–6%) [2,3]. Therefore, investigation on the metabolism-based drug–drug interaction and hepatobiliary transport of vardenafil is of great interest. To investigate these issues, a suitable assay for quantitation of vardenafil is necessary.

The availability of quantification methods for vardenafil in biological fluids is rather limited, and hence its pharmacokinetic characteristics have not been studied extensively. The quantification of vardenafil in tablet formulations has been achieved by capillary electrophoresis [4] and HPLC with UV detection [5]. Liquid chromatography coupled with electrospray ionization mass spectrometry or tandem mass spectrometry methods were used for the simultaneous determination of undeclared

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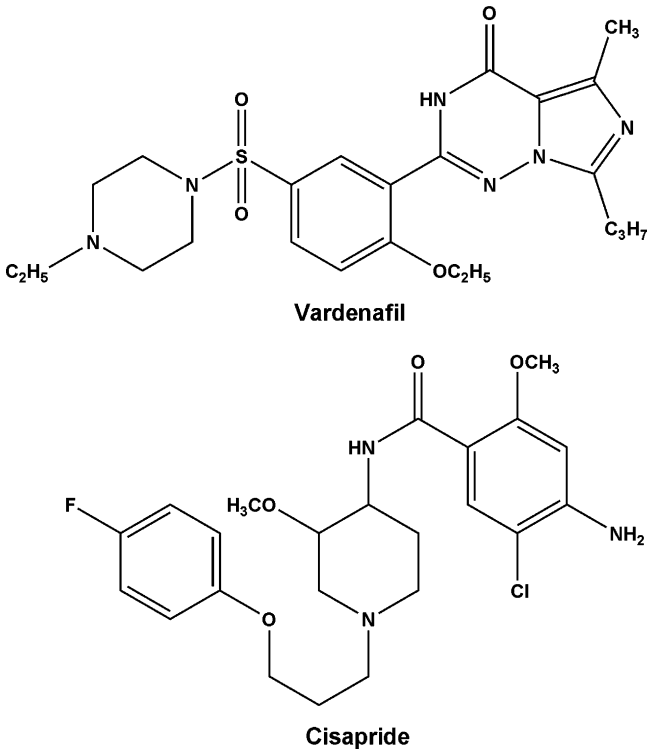

Fig. 1. Structures of vardenafil and internal standard cisapride.

PDE5 inhibitors, sildenafil, vardenafil and tadalafil, in dietary supplements [6–8]. As the matrices of pharmaceutical preparations and dietary supplements are simpler than those of biological samples, these reported methods usually cannot be applied directly to measure vardenafil in biological fluids due to interference by endogenous components. Recently HPLC coupled with electrospray ionization tandem mass spectrometry (LC–MS/MS) method was developed and validated for the determination of vardenafil in 1 ml of human plasma and urine [9]. Another LC–MS/MS method, without given full validation details, was also reported to measure plasma levels of vardenafil and its metabolite in a food effect study [10]. Although these methods offer good sensitivity, the tandem mass spectrometric instrumentation required for these methods is relatively expensive and not readily accessible in most laboratories.

It is interesting to note that vardenafil possesses native fluorescence. However, there is no report for analytical determination of vardenafil using fluorescence detection so far. Therefore, the main objective of this study was to develop and validate a simple and sensitive HPLC method using fluorescence detection to quantify vardenafil. The absorbance and fluorescence characteristics of vardenafil were examined and the effect of the pH value and of the content of acetonitrile on the fluorescence intensity and chromatographic retention of vardenafil were also explored in this work. Based on the high sensitivity and specificity provided by fluorescence detection, we have developed and validated the first fluorimetric HPLC method for the determination of vardenafil in small volumes of rat plasma and bile.

2. Experimental

2.1. Chemicals and reagents

Vardenafil hydrochloride trihydrate (batch 050323, purity 99.92%) was from Polymed Therapeutics (Shenzhen, China). Cisapride monohydrate (CIS/0304006, purity 99.49%) was from Medefarma (Lugano, Switzerland) (Fig. 1). Ammonium acetate was obtained from Merck (Darmstadt, Germany). HPLC grade acetonitrile and methanol were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Milli-Q Reagent Water (Millipore, Bedford, MA, USA) was used in the preparation of buffers and solutions. Rat plasma and bile were obtained from male Sprague-Dawley rats. All other chemicals were of analytical grade.

2.2. UV and fluorescence spectrometry

The UV spectra of vardenafil (10 μg/ml) in aqueous solution with various pH values were recorded with 10 mm quartz cell using a Hitachi U2010 spectrophotometer (Tokyo, Japan). The aqueous solutions used were 0.1M HCl and 50 mM phosphate buffer (pH 4.5, 6.4, 9.4).

Fluorescence was measured on a Hitachi F2500 fluorescence spectrophotometer (Tokyo, Japan) equipped with a xenon lamp. The excitation and emission slit widths were fixed at 10 nm. The fluorescence spectra of vardenafil (10 μg/ml) in mobile phase and in aqueous solution with various pH values were recorded with 10 mm quartz cell.

2.3. Instrumentation and chromatography

The Hitachi HPLC system consisted of a Model L-7100 pump, an L-7200 autosampler, an L-7300 column oven, an L-7485 fluorescence detector, an L-7455 diode array detector and a Hitachi D-7000 Chromatography Data Station (Tokyo, Japan). The analytical column was a Hypersil-100 C18 (5 μm, 25 cm × 4.6 mm I.D., Astmoor, Runcorn, UK) column. A Hypersil guard column (H5 ODS, 10 mm × 3.2 mm) was used. The temperature of the columns was maintained at 35 °C. Chromatographic conditions were identical for plasma and bile samples. Mobile phase consisted of a mixture of acetonitrile and 50 mM ammonium acetate aqueous solution (pH 6.8) (40/60, v/v). The prepared mobile phase was filtered through a 0.45-μm Millipore HVLP membrane filter and degassed ultrasonically before use. Analyses were run at a flow-rate of 1 ml/min. The eluents were detected by fluorescence at an excitation wavelength of 280 nm and an emission wavelength of 470 nm, and the peak area recorded. The bandwidths for excitation and emission were fixed at 15 nm.

2.4. Standards and controls

Master stock solutions of vardenafil (1 mg/ml in methanol) and the internal standard cisapride (1 mg/ml in acetonitrile) were prepared monthly and kept tightly sealed at −20 °C. The stock solution of vardenafil was diluted with drug-free plasma

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to give the working standard solution of 100 μg/ml, which was further diluted to obtain plasma calibration standards at concentrations of 10, 20, 50, 100, 200, 500 and 1000 ng/ml vardenafil. The quality controls (QCs) were prepared independently at concentrations of 10, 100 and 1000 ng/ml prior to the start of sample collection and stored at −20 °C until analysis. Two additional 2000 and 4000 ng/ml QC samples were spiked at concentrations higher that the upper limit of quantitation (ULOQ, 1000 ng/ml) and are known as dilution QCs. They were diluted 10-fold with blank plasma prior to analysis. The bile calibration standards (0.2, 1, 2, 10, 20, 100 μg/ml) and QC (0.2, 5, 100 μg/ml) were prepared by appropriate dilution of the stock solution of vardenafil in blank rat bile. The working solution of cisapride was obtained by diluting the stock solution in acetonitrile to 20 μg/ml. A complete calibration curve was generated with each run.

2.5. Sample preparation

The samples to be analyzed 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 100-μl aliquot of the internal standard working solution. After vortex-mixed for 30 s and upon centrifugation at 15,850 × *g* for 10 min, the supernatant was transferred to an Xpertek vial insert (4 mm × 25 mm) with bottom spring (P. J. Cobert, St. Louis, MO, USA). An aliquot of 100-μl supernatant was injected onto the column for HPLC analysis.

For bile samples, 10 μl were spiked with 190 μl of the internal standard working solution. After vortex-mixed for 30 s and upon centrifugation at 15,850 × *g* for 10 min, an aliquot of 150-μl supernatant was diluted with 150 μl of mobile phase and an aliquot of 100-μl solution was injected onto the column for HPLC analysis.

2.6. Method validation

The model for the calibration curve of vardenafil used the peak area ratio of vardenafil to cisapride (PAR) and the vardenafil concentration (*C*) in plasma and bile, 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, Mountain View, CA, USA), using nominal concentrations and measured PARs from calibration standards with a weighting scheme of $1/C^2$. Vardenafil concentrations of QCs and unknown samples were estimated by back-calculation from the associated calibration curves.

Intra-day precision was evaluated by analysing the spiked plasma and bile 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 six different days. Assay precision (relative standard deviation, RSD) 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 (RSD < 20%) determined. Assay specificity was examined in relation to interference from endogenous substances in six independent batches of drug-free plasma and bile.

Absolute recovery of vardenafil (100 ng/ml) in plasma was determined by assaying the extracted samples and comparing the peak areas of vardenafil to those obtained from un-extracted control samples. The recovery of the internal standard, cisapride, was evaluated at the concentration used in sample analysis (20 μg/ml). Freeze–thaw stability of vardenafil (10 and 1000 ng/ml) in plasma samples was determined for three 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 vardenafil in plasma at ambient temperature (ca. 20 °C) was studied for 4 h. The post-preparative stability of vardenafil in processed samples left at ambient temperature was followed for 16 h. The short-term stability of vardenafil in plasma at 4 °C was examined for 24 h and the long-term stability of vardenafil was assessed for 28 days.

2.7. Application

The assay was applied to a single dose (5 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 jugular vein, 0.2-ml of blood samples for analytical determinations were collected via the right jugular vein at designated time for 8 h. Bile sample were collected at specific time intervals for 8 h. Plasma and bile samples were stored at −20 °C until analysis.

3. Results

3.1. UV and fluorescence spectra

UV absorption spectra of vardenafil at pH 1.2, 4.5, 6.4, 9.4 were shown in Fig. 2. Note that the UV absorption spectra of vardenafil were very similar in the aqueous buffer solution with pH ranging from 4.5 to 9.4, and showed absorption maxima at 214 nm. Nonetheless, at the acidic pH 1.2, changes in the spectra were observed.

Fig. 3 showed the fluorescence spectra of vardenafil in the mobile phase and aqueous buffer solution with pH 1, 4.5, 7 and 9. The excitation maximum was at 250–260 nm and the emission maximum at 460–480 nm. In acidic solution the fluorescence intensity was much lower than in alkaline media, where vardenafil was in the non-ionized form. The presence of organic solvent acetonitrile significantly enhanced the fluorescence intensity.

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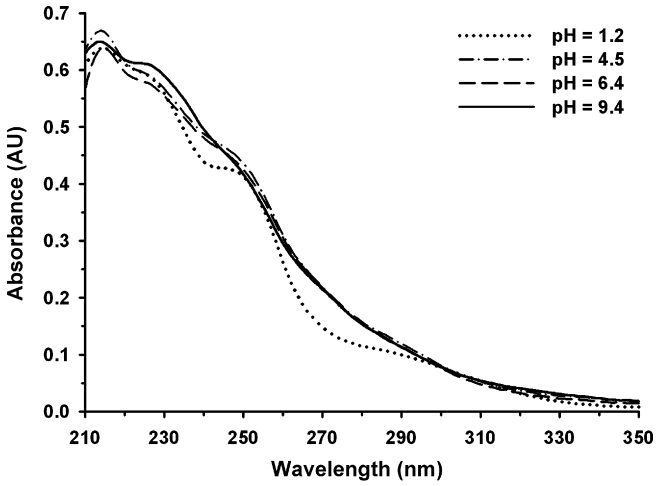

Fig. 2. UV spectra of vardenafil (10 μg/ml) in aqueous solution with various pH values.

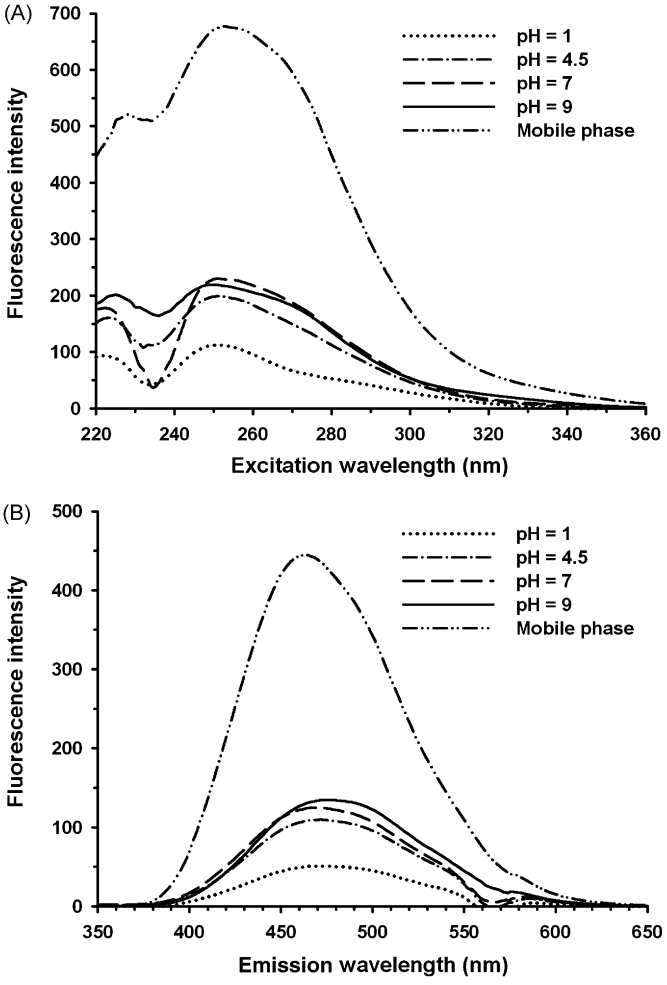

Fig. 3. Fluorescence spectra of vardenafil (10 μg/ml) in mobile phase and aqueous solution with various pH values. (A) Excitation spectra measured with emission at 470 nm, (B) emission spectra measured with excitation at 280 nm.

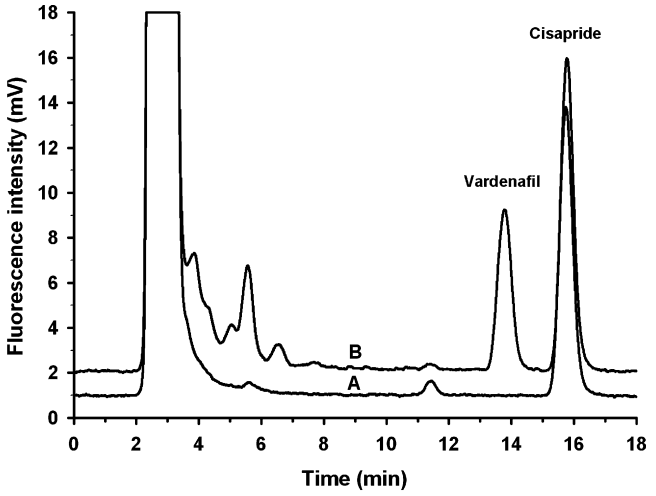

Fig. 4. The HPLC chromatograms of extracts from pre-dosing (A) and 180 min (B) plasma samples from a rat after intravenous bolus of 5 mg/kg vardenafil. Experimental conditions are given in Section 2.3.

3.2. Chromatography

Fig. 4 showed chromatograms of plasma extracts from pre-dose (A) and 180 min (B) after administration of 5 mg/kg vardenafil to a rat. The chromatograms of bile samples collected from pre-dose (A) and 360–480 min (B) were shown in Fig. 5 after administration of 5 mg/kg vardenafil to a rat. Vardenafil and cisapride were eluted after 14 and 16.5 min, respectively. As can be seen in Figs. 4 and 5, a good separation of vardenafil and the internal standard was achieved under the chromatographic conditions specified in Section 2. The simple protein precipitation with acetonitrile was sufficient to isolate vardenafil and cisapride from plasma and bile without any interfering endogenous peaks. The method is specific for vardenafil. No interfering endogenous peaks were observed at the retention of vardenafil when drug-free rat plasma and bile samples were analyzed (Fig. 4A and Fig. 5A).

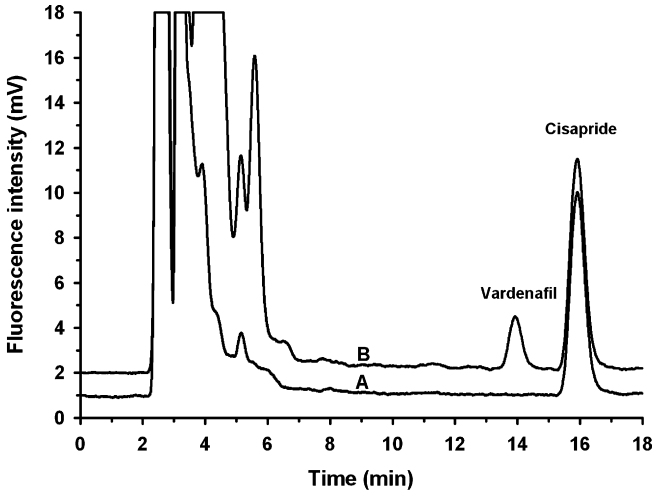

Fig. 5. The HPLC chromatograms of extracts from pre-dosing (A) and 360–480 min (B) bile samples from a rat after intravenous bolus of 5 mg/kg vardenafil. Experimental conditions are given in Section 2.3.

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

	Intra-day			Inter-day		
$C_{nominal}$ (ng/ml)	C_{est} (ng/ml)	RSD (%)	Error (%)	C_{est} (ng/ml)	RSD (%)	Error (%)
10	10.9	8.6	9.3	9.8	9.7	−1.7
100	101	3.1	1.3	103	2.4	3.0
1000	972	3.1	−2.8	998	3.5	−0.2
2000	1910	4.4	−4.5	1939	1.4	−3.0
4000	3617	5.5	−9.6	3678	2.7	−8.0

$C_{nominal}$: nominal concentration; $C_{est.}$: estimated concentration; the dilution QCs (2000 and 4000 ng/ml) were diluted 10-fold with blank plasma prior to analysis.

3.3. Method validation

3.3.1. Plasma samples

The calibration curves for plasma samples were linear from 10 to 1000 ng/ml. The mean (±SD) regression equation for six replicated calibration curves constructed using 50 μl of rat plasma samples on different days was: PAR = (0.0022 ± 0.0001) × C + (0.0002 ± 0.0013), $r^2 = 0.997 \pm 0.002$. We had also prepared the calibration curves ($n = 6$) using blank human plasma, and the corresponding regression equation was: PAR = (0.0022 ± 0.0002) × C + (0.0002 ± 0.003), $r^2 = 0.997 \pm 0.002$.

Precision and accuracy were investigated by replicated analyses of spiked controls (Table 1), and in all cases the intra- and inter-day precision was acceptable at a RSD of 9.7% or less. In addition, accuracy was within 9.6% deviation when compared with nominal concentrations across the range of 10–4000 ng/ml. From this experiment, the LOQ of the method was determined to be 10 ng/ml, with the intra-day imprecision and error of 9.7 and −1.7%, and the inter-day imprecision and error of 8.6 and 9.3% (Table 1).

The efficiencies of extraction of both compounds were comparable. The mean absolute recovery of vardenafil was 86.9 ± 9.5% ($n = 3$), and that of cisapride was 85.3 ± 1.7%. The high recoveries from plasma indicated that the losses of vardenafil and cisapride due to high plasma protein binding or during the protein precipitation process were minimum.

Vardenafil is stable when stored in the refrigerator and freezer. In this study, vardenafil was stable in plasma at 20 °C for up to 4 h, at 4 °C for up to 24 h, and in frozen plasma (−20 °C) for up to 28 days. The mean concentration for the quality control samples (10 and 1000 ng/ml) was within 10% of nominal concentrations for vardenafil following the three freeze–thaw cycle. The present study also shows that vardenafil was stable in processed samples left at ambient temperature for up to 16 h (Table 2).

Table 2
Stability of vardenafil in spiked plasma and post-preparative samples (mean ± SD, $n = 4$)

Conditions	10 ng/ml	1000 ng/ml
	% Remained	% Remained
Bench-top stability (20 °C, 4 h)	97.1 ± 11.5	102.0 ± 0.4
Short-term stability (4 °C, 24 h)	92.3 ± 9.4	99.9 ± 0.6
Long-term stability (−20 °C)		
1 Day	95.2 ± 8.8	98.0 ± 0.6
7 Days	90.1 ± 4.7	93.2 ± 0.8
14 Days	100.7 ± 4.3	100.1 ± 2.2
28 Days	105.7 ± 10.1	92.1 ± 3.5
Freeze–thaw stability (−20 °C/20 °C)		
Cycle 1	96.7 ± 7.9	97.9 ± 0.9
Cycle 2	91.3 ± 4.2	98.8 ± 1.3
Cycle 3	98.0 ± 8.8	98.8 ± 0.9
Post-preparative stability (20 °C)		
6 h	97.6 ± 3.5	101.8 ± 3.2
16 h	97.9 ± 8.8	98.8 ± 0.9

3.3.2. Bile samples

The calibration curves for bile samples were linear from 0.2 to 100 μg/ml. The mean (±SD) regression equation for six replicated calibration curves constructed using 10 μl of rat bile samples on different days was: PAR = (0.2372 ± 0.0015) × C + (0.0008 ± 0.0047), $r^2 = 0.994 \pm 0.007$.

Precision and accuracy were investigated by replicated analyses of spiked controls (Table 3), and in all cases the intra- and inter-day precision was acceptable at a RSD of 10.8% or less. In addition, accuracy was within 6.4% deviation when compared with nominal concentrations across the range of 0.2–100 μg/ml. From this experiment the LOQ of the method was determined to be 0.2 μg/ml, with the intra-day imprecision and error of 10.8 and 6.4%, and the inter-day imprecision and error of 7.0 and −1.0% (Table 3).

Table 3
Intra- and inter-day accuracy and precision for the determination of vardenafil in 10-μl bile aliquots ($n = 6$)

	Intra-day			Inter-day		
$C_{nominal}$ (μg/ml)	C_{est} (μg/ml)	RSD (%)	Error (%)	C_{est} (μg/ml)	RSD (%)	Error (%)
0.2	0.21	10.8	6.4	0.20	7.0	−1.0
5	5.04	5.4	0.8	4.99	5.3	−0.3
100	99.7	7.7	−0.3	104	7.4	4.2

$C_{nominal}$: nominal concentration; $C_{est.}$: estimated concentration.

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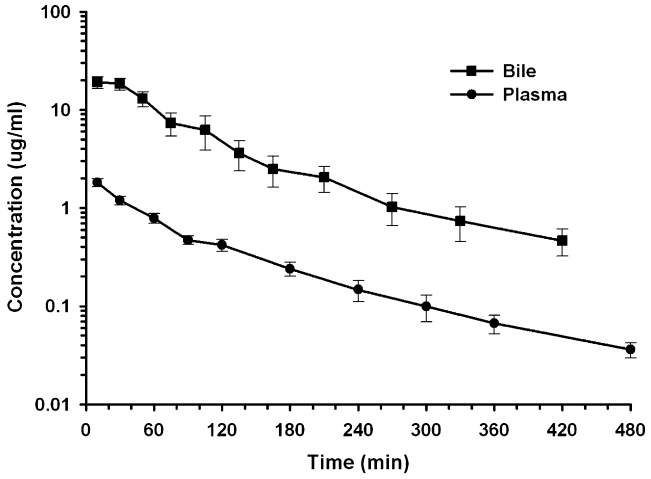

Fig. 6. The mean (±SE) plasma and bile concentration–time profiles of vardenafil after intravenous bolus of 5 mg/kg vardenafil to six male rats.

3.4. Application

The assay was applied to a preliminary pharmacokinetic experiment in rats. A single bolus dose of 5 mg/kg of vardenafil was administered intravenously to male rats. The plasma and bile concentration–time profiles are illustrated in Fig. 6. The results showed that this simple and rapid method is sufficiently sensitive to follow plasma and bile levels of vardenafil. Of interest to note, a peak was observed at the retention time of 5.5 min during the analysis of unknown samples. Since this peak was presented only in the rat plasma and bile samples after vardenafil dosing and possessed similar UV and fluorescence spectra with vardenafil, it probably corresponds to a metabolite of vardenafil.

4. Discussion

4.1. Optimization of HPLC conditions

Separation of vardenafil in HPLC has been accomplished mainly by C18 columns with mobile phases consisting of acetonitrile–acidic aqueous buffers (Table 4) [5–9]. An optimized mobile phase of acetonitrile–10 mM phosphate buffer (pH 3) (30:70) was chosen by Aboul-Enein et al. [5] for the determination of vardenafil in Levitra tablet using a monolithic column with UV detection. They found that the peak shape of vardenafil was poor when the proportion of acetonitrile in the mobile phase exceeded that of buffer and a large negative peak that interfered with vardenafil was observed when buffer concentration was increased from 10 to 100 mM. They also showed that increasing the pH of mobile phase from 2 to 7.5 delayed the elution of vardenafil [5].

For the quantification of vardenafil in plasma and urine by LC–MS/MS, gradient elution with acetonitrile–10 mM ammonium acetate buffer (pH 3) was used [9]. For simultaneous determination of sildenafil, vardenafil and tadalafil, elution with acetonitrile–aqueous solutions containing modifiers were utilized in LC–MS and LC–MS/MS methods [6–8]. These authors

Table 4
Analytical characteristics of reported HPLC methods for the determination of vardenafil

Ref.	Detection	Column	Mobile phase	Flow (ml/min)	Matrix	Sample (ml)	LOQ
This study	Fluorescence (Ex 280 nm, Em 470 nm)	C18	CH_3CN:50 mM NH_4OAc (pH 6.8) = 40:60	1	Plasma	0.05	10 ng/ml
					Bile	0.01	200 ng/ml
[8]	MS/MS (*m/z* 489→312)	C18[a]	CH_3CN:10 mM ammonium formate = 38:62	0.25	Dietary supplement	30 mg	0.07 ng
	DAD	C18		1	(Bulk powder)		7 ng
[7]	MS (*m/z* 489)	C18	CH_3CN:20 mM NH_4OAc and 0.2% formic acid; gradient	1	Dietary supplement (Liquid form)	1	0.011 ng[b]
	UV (292 nm)						4.48 ng
[5]	UV (230 nm)	C18	CH_3CN: phosphate buffer (pH 3) = 20:80	1	Tablet	2 Tablets	310 ng/ml
		C18[c]	CH_3CN:10 mM phosphate buffer (pH 3) = 30:70	2			320 ng/ml
[9]	MS/MS (*m/z* 489→151)	C18	CH_3CN:10 mM NH_4OAc (pH3); gradient	1	Plasma	1	0.1 ng/ml
					Urine	1	0.25 ng/ml
[6]	MS (*m/z* 489)	C18[a]	CH_3CN:0.1% formic acid; gradient	0.4	Dietary supplement	–	1.4 ng[b]

[a] Microbore column.
[b] LOD: limit of detection.
[c] Monolithic column.

reported that the use of methanol in the mobile phase produced considerably more background noise compared to acetonitrile [6], and sildenafil and vardenafil were better separated by the use of acetonitrile and could not be separated using aqueous–methanolic mobile phases with any composition [7,8]. As the PDE5 inhibitors all have amino groups in their structures, the use of ammonium acetate and triethylamine [7] or ammonium formate and diethylamine [8] as modifiers suppressed peak-tailing. Nonetheless, the addition of diethylamine or triethylamine resulted in significant baseline shifting and ionization suppression [7,8].

In this study, the optimized resolution of vardenafil and baseline separation from the internal standard was achieved using a C18 column and an isocratic mobile phase composed of acetonitrile–50 mM ammonium acetate buffer (40:60). Increasing the acetonitrile content in the mobile phase decreased the retention of analytes, which resulted in unsatisfactory resolution between vardenafil and cisapride, and the co-elution of the plausible metabolite of vardenafil with the solvent peaks. The retention time of vardenafil decreased as the pH of buffer decreased, however, it was pH-independent in the pH range of 6.7–8.8. As the fluorescence intensity of vardenafil was maximized under alkaline pH and in the presence of acetonitrile (see Section 4.2), the pH of aqueous phase in the mobile phase was adjusted at 6.8 after considering the fluorescence characteristics and retention behavior of analytes, and the stability of conventional C18 column. It is worth noting that alkaline pH is preferred for fluorimetric detection of vardenafil, while acidic pH is commonly employed to protonate vardenafil for mass spectrometric detection.

4.2. Optimization of fluorescence detection

The structure of vardenafil contains conjugated configuration and exhibits strong UV absorption and native fluorescence. Also noted is that the structure of vardenafil includes several amine groups, and it has three p*K*a values of 3.4, 6.7 and 8.8, which might be ionized under acidic conditions. Because the degree of ionization could potentially affect the UV absorbance and fluorescence intensity of a drug [11–13], it is worthy to evaluate the effect of pH value on the UV and fluorescence spectra of vardenafil.

As displayed in Fig. 2, while there is similarity in the absorption spectra of vardenafil in acidic, neutral and basic medium with high molar absorptivities around 214, 230 and 250 nm, the greatest difference is seen at acidic pH. Vardenafil can be measured by UV detection at 292 and 230 nm with LOQs (S/N = 10) of 4.48 and 7 ng, respectively, for liquid and bulk forms of dietary supplements [7,8]. The LOQ of the present method with UV detection at 230 nm was 6.7 ng. Our initial attempt to detect vardenafil in plasma extracts using 230 nm was unsatisfactory due to significant interferences. Furthermore, the sensitivity offered by UV detection was not adequate for determination of vardenafil in small volume of plasma. Therefore, fluorescence detection was considered to improve both specificity and sensitivity.

As shown in Fig. 3, optimal fluorescence response was obtained with excitation at 250–260 nm and emission at 460–480 nm in aqueous solution. Intensity of the excitation and emission fluorescence profiles varied with the pH of solutions; the maximal fluorescence intensity occurred between pH 7 and 9. The intensity decreases when decreasing the pH of solutions. The addition of acetonitrile greatly improved the fluorescence intensity, probably by creating a hydrophobic environment in which intra-molecular quenching of fluorescence is hindered [14]. Under the current LC conditions, maximal on-column fluorescence response and S/N ratio for vardenafil were obtained with excitation at 260 nm and emission at 460 nm. However, the fluorescence intensity of cisapride was weak with excitation at 250–260 nm. Therefore, in the present method a slightly sub-optimal detection with excitation at 280 nm and emission at 470 nm was employed. The LOQ of the fluorescence detection was 0.33 ng, which was 20 times more sensitive than that of the UV detection.

4.3. Selection of extraction solvent

Because of its lipophilicity, vardenafil was extracted easily into methanol [4,7], acetonitrile [8], or acetonitrile–water (1/1) [6] from the simple matrices of pharmaceutical and nutraceutical preparations. Vardenafil can be efficiently isolated from plasma by solid-phase extraction with SPEC C18 cartridges [9]. In this study, acetonitrile was used as the de-protein agent and extraction solvent as it gave high extraction recoveries for both vardenafil and cisapride, and provided relatively clean chromatograms under fluorescence detection. Furthermore, acetonitrile enhanced considerably the fluorescence intensity of vardenafil as mentioned above.

4.4. Selection of internal standard

Among the reported methods only one LC–MS/MS method had used an internal standard, [$^2H_5$]-vardenafil, for the determination of vardenafil [9]. It is general believed that stable isotopically labeled internal standards yield better assay performance results for quantitative LC/MS methods. However, these compounds are not always available or are very costly and beyond the financial means of most small laboratories.

In the present study, cisapride was chosen as the internal standard because it is commercially available, has native fluorescence, and can be extracted by acetonitrile with high recovery [15]. Cisapride also displayed appropriate chromatographic retention with its peak sufficiently separated from that of vardenafil in the present method. The suitability of cisapride as internal standard is also demonstrated as the relative recovery of vardenafil (101.8 ± 9.3%) determined by the PARs of the processed samples with that of unprocessed control samples was close to 100%. Because of its fatal side effect of torsade de pointe, cisapride was withdrawn from most of the markets in the world; therefore, the possibility of interference encountered from a therapeutic blood concentration of cisapride is low. Like vardenafil, cisapride is lipophilic, highly bound to plasma proteins and is also a CYP3A4 substrate.

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4.5. Calibration range, quantitation limit and sample volume

Vardenafil is effective in treating erectile dysfunction for on-demand application with the recommended starting dose of 10 mg. The maximum dose is 20 mg once daily [1–3]. Following oral administration of a single 20-mg dose to healthy male subjects, the mean peak plasma concentration of vardenafil was 17.1–24.7 ng/ml and the terminal elimination half-life was about 3–4 h [1–3,9,10]. When co-administered with CYP3A inhibitors, such as erythromycin, ketoconazole, indinavir and ritonavir, the systemic exposure of vardenafil increased dramatically [3]. In particular, a tremendous level of interaction was demonstrated when vardenafil was taken with ritonavir. There was a 49-fold increase in area under plasma concentration-time curve (AUC) and a 13-fold increase in maximal plasma concentration of vardenafil, with individual ratios as high as 300-fold. And the terminal half-life was prolonged 10-fold [3].

Detection of vardenafil by mass spectrometry provided excellent sensitivity with on-column detection limit of 11–1400 pg [6–8] and lower LOQs of 0.1 and 0.25 ng/ml have been reported for the determination of vardenafil in human plasma and urine by an LC–MS/MS method (Table 4) [9]. The ULOQs of calibration curves were set at 50–100 ng/ml for human plasma and urine by LC–MS/MS methods to characterize the plasma and urine concentration profile of vardenafil in healthy subjects [9,10]. We have extended and validated the dynamic calibration range over 10–4000 ng/ml for rat plasma, after considering the maximal therapeutic dose, clinical concentrations and potential drug–drug interactions for vardenafil. Up to now, there is no analytical method available for the quantification of vardenafil in rat bile. Based on our preliminary study in rats, vardenafil can be excreted into bile, with concentration of vardenafil in bile about 10-fold higher than that in plasma. Therefore, the calibration range for rat bile was set at 0.2–100 μg/ml.

In the published LC-MS/MS method, a relative large amount (1000 μl) of plasma and urine was utilized for sample preparation [9]. Thus, efforts were made in this study to lower considerably the volume of sample needed, from 1000 μl to 50 μl for plasma and 10 μl for bile, and still providing good sensitivity for vardenafil 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 biological waste.

5. Conclusion

In this paper, the fluorescence characteristics of vardenafil were studied and a sensitive fluorimetric HPLC method for the quantification of vardenafil has been developed and validated for the first time. Vardenafil has native fluorescence with maximal intensity occurs at pH 7–9. The fluorescence intensity is enhanced in the presence of acetonitrile. Using acetonitrile as extraction solvent, vardenafil and the internal standard cisapride can be isolated with high recovery and analyzed efficiently. The optimal separation was achieved on a C18 column using acetonitrile–50 mM ammonium acetate (pH 6.8) (40:60, v/v) as mobile phase. The present method offers good accuracy, precision and sensitivity, and provides wider linear range for monitoring concentration of vardenafil. This method requires only 50 μl of plasma and 10 μl of bile, making it suitable for studying the pharmacokinetics of vardenafil in small animals.

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