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Short communication

Liquid chromatographic-tandem mass spectrometric method for the quantitation of sildenafil in human plasma

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Abstract

A method to determine sildenafil in human plasma involving liquid chromatography–tandem mass spectrometry (LC–MS/MS) has been developed. Sildenafil and the internal standard (I.S.), diazepam, are extracted from human plasma with ether–dichloromethane (3:2, v/v) at basic pH and analyzed by reversed-phase high-performance liquid chromatography (HPLC) using methanol–10 mM ammonium acetate pH 7.0 (85:15, v/v) as the mobile phase. Detection by electrospray positive ionization mass spectrometry in the multiple-reaction monitoring mode was linear over the concentration range 0.125–40.0 ng/ml. Intra- and inter-day precision of the assay at four concentrations within this range were 2.5–8.0%. The method was used to evaluate plasma concentration–time profiles in healthy volunteers given an oral dose of 20 mg sildenafil as a combination tablet also containing apomorphine.

Keywords: Sildenafil

1. Introduction

Sildenafil is a potent and selective phosphodiesterase type 5 (PDE-5) inhibitor used to treat male erectile dysfunction by enhancing relaxation of the penile corpus cavernosum [1]. It has been widely used by men to improve penile tumescence and facilitate satisfactory sexual performance. The usual dose is 25 mg and peak blood levels are of the order of 100 ng/ml.

Various analytical methods have been developed to determine sildenafil in biological samples including micellar electrokinetic capillary chromatography, gas chromatography–mass spectrometry [2,3] and high-performance liquid chromatography (HPLC) with ultraviolet or mass spectrometric detection [4–13]. These methods suffer from a number of disadvantages including low sensitivity, the need for extensive sample preparation and occasionally a column-switching system [4]. The sensitivity of these methods is generally inadequate for studies involving low oral doses or for the topical formulations currently under development [14].

This paper describes the development and validation of an improved method for the assay of sildenafil in human plasma using HPLC with tandem mass spectrometric detection (LC–MS/MS). Diazepam is used as the internal standard (see Fig. 1 for structures). The assay has been applied to a clinical pharmacokinetic study of sildenafil given as a multicomponent tablet containing 20 mg sildenafil.

2. Experimental

2.1. Materials

Sildenafil citrate and diazepam (purity > 99.5% in each case) were kindly supplied by Jilin Aotai Scientific Research Center, Changchun, China. Acetonitrile and methanol were HPLC-grade. All other chemicals were of analytical grade and used without further purification. Distilled water, prepared from demineralized water, was used throughout the study.

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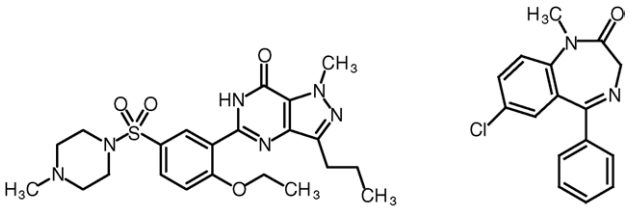

Fig. 1. Structures of sildenafil and diazepam (internal standard).

2.2. Standard and QC solutions

All concentrations of sildenafil refer to the free base. Stock solutions (100 μg/ml) of sildenafil citrate and diazepam were prepared in methanol. A series of sildenafil standard solutions with concentrations of 0.125, 0.250, 0.400, 1.25, 2.50, 4.00, 12.5, 25.0 and 40.0 ng/ml were prepared by dilutions of aliquots of the stock solution with 30% methanol. A working internal standard (I.S.) solution (diazepam, 50 ng/ml) was also prepared in 30% methanol. QC solutions (0.125, 0.250, 2.50 and 25.0 ng/ml, respectively) were prepared in a similar way, stored at 4 °C and used within 1 month of preparation.

2.3. Sample preparation

To 100 μl human plasma were added 100 μl I.S. solution, 100 μl 30% methanol or a standard or QC solution of sildenafil, and 100 μl 1 M NaOH. The mixture was extracted with 3 ml ether–dichloromethane (3:2, v/v) by shaking for 15 min. After centrifugation at 3000 × *g* for 5 min, the organic phase was transferred to another tube and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was reconstituted in 100 μl mobile phase and 20 μl injected into the LC–MS/MS system. The samples with concentrations greater than the maximum standard in the calibration curve were determined by dilution of these samples with blank plasma.

2.4. Chromatography

The LC–MS/MS system consisted of an Agilent 1100 series (Agilent Technologies, Palo Alto, CA, USA) binary pump and autosampler connected to a Nucleosil C_{18} column (5 μm, 50 mm × 4.6 mm I.D. from Dalian Johnsson Separation Science and Technology Corp., Dalian, China) and Applied Biosystems Sciex API 4000 mass spectrometer (Applied Biosystems Sciex, Ont., Canada) using electrospray ionization (ESI). The mobile phase consisted of methanol:10 mM ammonium acetate (adjusted to pH 7.0 with ammonia before mixing) 85:15 (v/v) delivered at 0.7 ml/min at ambient temperature.

ESI was performed in the positive ion mode with nitrogen as the nebulizer, heater and curtain gas. High-flow gas flow parameters were optimized by making successive flow injections while introducing mobile phase into the ionization source at 0.7 ml/min. Optimum values for nebulizer, heater and curtain gas flow rates were 65, 40 and 30 units, respectively. The ionspray needle voltage and heater gas temperature were set at 5100 V and 500 °C, respectively. Instrument response was optimized by syringe pump infusion (5 μl/min) of a solution in mobile phase containing sildenafil citrate and diazepam into the stream of mobile phase eluting from the column. The pause time was set at 5 ms and the dwell time at 400 ms.

The detector was operated at unit resolution in the multiple-reaction monitoring (MRM) mode using the transitions of the protonated molecular ions of sildenafil at m/z 475.4 → 100.2 and diazepam at m/z 285.0 → 193.0. The collision gas (N_2) was set at 3 units and collision energies of 42 and 45 eV were used for sildenafil and diazepam, respectively. The instrument was interfaced to a computer running Applied Biosystems Analyst version 1.3 software.

2.5. Assay validation

Linearity was assessed by preparation of three independent calibration curves each based on nine spiked plasma samples with concentrations in the range 0.125–40.0 ng/ml. Calibration curves were analyzed by weighted linear regression ($1/x^2$) of drug-internal standard peak area ratios. Intra- and inter-day precision (as relative standard deviation (R.S.D.)) were determined by assay of six replicates of QC samples at 0.125, 0.250, 2.50 and 25.0 ng/ml on three different days. Accuracy (as relative error (R.E.) i.e. percentage deviation of the mean from the true value) was determined on the basis of the total data set ($n = 24$). The limit of quantitation (LOQ) was the concentration below which signal-to-noise ratio <5.0, R.E. > 20% and CV > 20%. The limit of detection (LOD) was the concentration with signal-to-noise ratio of 3. Recoveries of sildenafil and I.S. were determined by comparing peak areas of extracted standard samples with peak areas of post-extraction plasma blanks spiked at corresponding concentrations.

Matrix effects for sildenafil were evaluated by comparing the peak areas of analyte in extracted samples of blank plasma from six different drug free volunteers spiked with high and low concentrations with the corresponding areas obtained by direct injection of standard solutions. Matrix effects for the I.S. were also investigated. Stability was evaluated using six replicates of QC samples at 2.50 and 25.0 ng/ml. But the preparation is a little different from QC samples it was prepared by adding small volume standard solution into 5 ml blank plasma in order to mimic the real sample condition. Stability tests included three freeze–thaw cycles, storage for 1 month at −20 °C and at room temperature for 24 h. Stability in mobile phase on storage in plastic autosampler vials under autosampler conditions for 12 h was also assessed.

2.6. Pharmacokinetic study

After a 12 h fast, 10 healthy male volunteers received a single multicomponent tablet containing sildenafil (20 mg) and apomorphine (3 mg) with 200 ml of water. Blood samples (1 ml) were collected by venepuncture into heparinized tubes prior to dosage and at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 10, 12 and 16 h thereafter. Following centrifugation (3000 × *g* for 10 min),

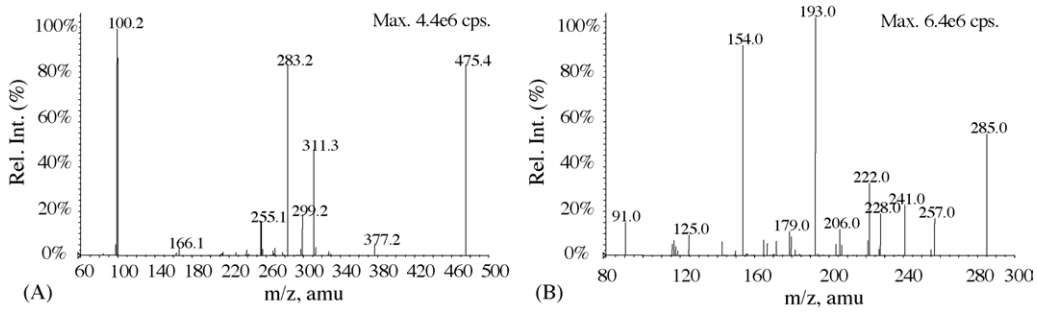

Fig. 2. Full-scan product ion spectra of $[M + H]^+$ for (A) sildenafil and (B) diazepam.

plasma samples were stored in polypropylene tubes at −20 °C and analyzed within 1 month. Each analytical run included a plasma blank, a zero-level standard (blank plasma plus I.S.), a set of calibration standards and QC samples in duplicate. Pharmacokinetic parameters were calculated using Topfit 2.0.

3. Results and discussion

3.1. Mass spectrometry

Analyte and internal standard responded best to positive ionization and protonated molecular ions $[M + H]^+$ were present as major peaks for both compounds. Small amounts of $[M + Na]^+$ were also detected. Product ion spectra of $[M + H]^+$ showed fragment ions at *m*/*z* 311, 283, 100 and 99 for sildenafil and at *m*/*z* 222, 193 and 154 for diazepam (Fig. 2). The fragment ions at *m*/*z* 100 and 193 were present in highest abundance and were chosen for MRM acquisition of sildenafil and diazepam, respectively. The most suitable collision energy was determined by observing the maximum response obtained for the two product ions.

3.2. Chromatography

In order to minimize the run time of the assay, a short C_{18} column was used. Of the commercial columns evaluated (Nucleosil, Nova-Pak, Hypersil and Zorbax), Nucleosil was found to give the best chromatography with minimal matrix effects at pH 7.0. Under the optimum conditions, analyte and internal stan-

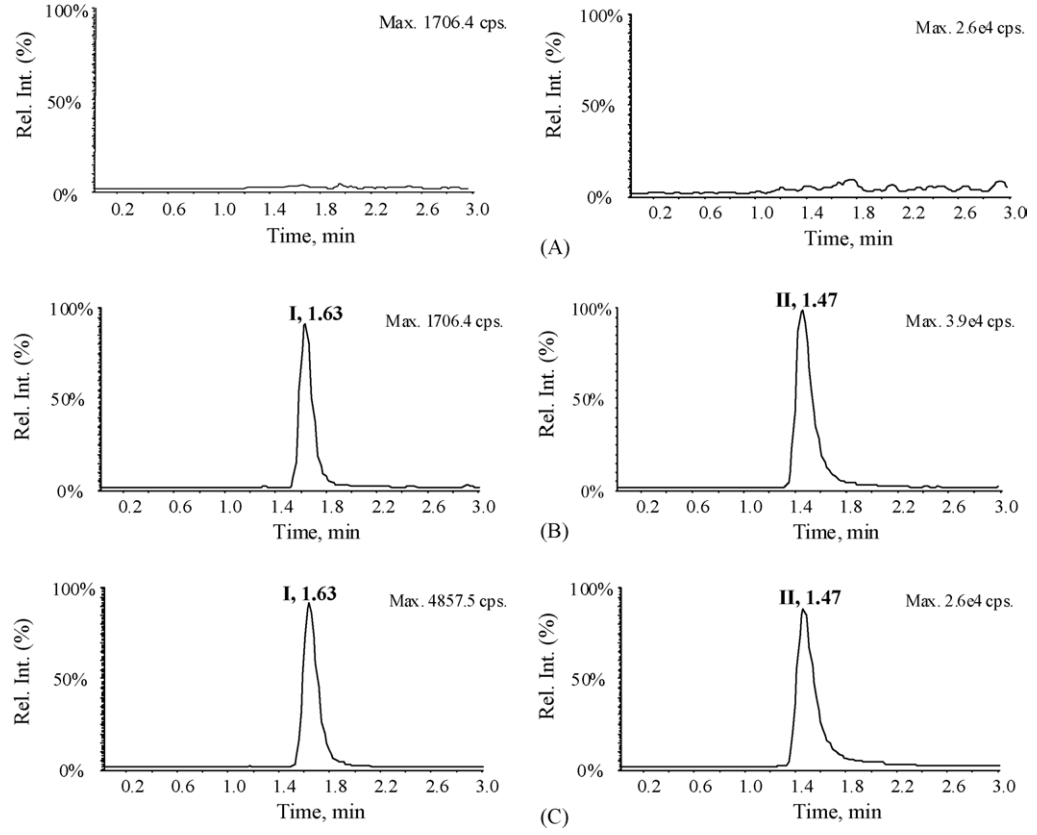

Fig. 3. Representative MRM chromatograms of: (A) blank plasma; (B) plasma spiked with sildenafil at the limit of quantitation (0.125 ng/ml) and (C) a plasma sample 16 h after administration of a single multicomponent tablet containing sildenafil (20 mg) and apomorphine (3 mg) to a healthy male volunteer. Peak I, sildenafil; Peak II, diazepam.

Table 1
Precision and accuracy for the determination of sildenafil in human plasma (precision values are based on analysis of QC samples ($n = 6$) on three different days; accuracy values are based on all data ($n = 24$))

Added concentration (ng/ml)	Found concentration (ng/ml)	Intra-day R.S.D. (%)	Inter-day R.S.D. (%)	Relative error (%)
0.125	0.128	3.84	4.62	2.09
0.250	0.253	2.45	6.21	1.09
2.50	2.51	7.93	3.77	0.22
25.0	25.6	3.82	4.52	2.51

dard were free of interference from endogenous substances and gave retention times of 1.63 and 1.47 min, respectively. In order to avoid shifts in retention time due to variations in mobile phase pH, a volume of mobile phase sufficient for the anticipated number of samples to be analyzed was prepared.

3.3. Assay validation

Representative chromatograms of blank plasma, blank plasma spiked with sildenafil at the limit of quantitation (0.125 ng/ml) and a study sample containing a low concentration of sildenafil are shown in Fig. 3. Matrix effects for sildfenafil and I.S. were minimal based on concentrations being 94.2–103.1% of nominal concentrations.

The assay was found to be linear in the concentration range 0.125–40.0 ng/ml ($r > 0.995$) with the LOD equal to 50 pg/ml. Precision and accuracy were satisfactory at the four concentrations studied (Table 1). Accuracy and the inter-day precision of dilution of 100 ng/ml QC sample was also acceptable (R.E. < 10% and R.S.D. < 5% $n = 6$).

Absolute recoveries of sildenafil at concentrations of 0.250, 2.50 and 25.0 ng/ml were 78 ± 4.6, 80 ± 2.5 and 76 ± 5.1%, respectively. Sildenafil was stable under all the storage conditions evaluated with mean recoveries of 95.6–105.3% of the nominal concentrations.

3.4. Pharmacokinetic study

The mean concentration–time profile after administration of a multicomponent tablet containing sildenafil (20 mg) to healthy volunteers ($n = 10$) is shown in Fig. 4. The C_{max} was 50.3 ± 19.4 ng/ml occurring at 1.50 ± 0.41 h. The plasma elimination half-life was 2.90 ± 0.50 h and the area under the plasma concentration–time curve 172 ± 73.1 ng h/ml. The C_{max} is lower than expected on the basis of previous results obtained with single component sildenafil 25 mg tablets. Nevertheless it is clear that the sensitivity of the assay far exceeds what is required to define almost the entire concentration–time curve at this dose. Thus the assay is likely to be useful for pharmacokinetic studies of formulations containing lower doses of sildenafil and in particular of systemic levels after application of topical formulations.

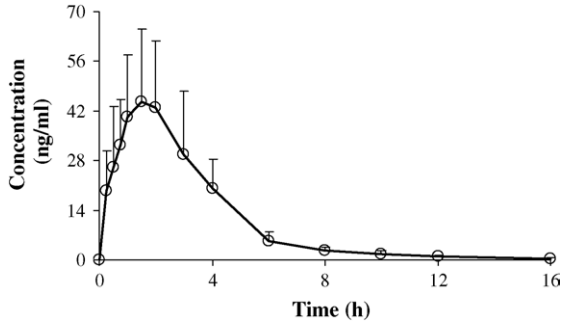

Fig. 4. Mean plasma concentration–time profile of sildenafil after administration of a single multicomponent tablet containing sildenafil (20 mg) and apomorphine (3 mg) to healthy male volunteers. Data are mean ± S.D. ($n = 10$).

4. Conclusion

A rapid and sensitive LC–MS/MS method is reported for the determination of sildenafil in human plasma. The assay was successfully applied to determine concentration–time profiles of the drug in a single oral dose study of a sildenafil tablet psprt. The method allows high sample throughput due to the short run time and relatively simple sample preparation procedure.

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