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Simultaneous determination of sildenafil and its active metabolite UK-103,320 in human plasma using liquid chromatography–tandem mass spectrometry

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Abstract

A liquid chromatography–tandem mass spectrometric method for the simultaneous determination of sildenafil and its active *N*-demethylated metabolite, UK-103,320 in human plasma was developed. Sildenafil, UK-103,320 and the internal standard (DA-8159) were extracted from human plasma with dichloromethane at basic pH. A reverse-phase LC separation was performed on Luna phenylhexyl column with the mixture of acetonitrile–ammonium formate (10 mM, pH 6.0) (60:40, v/v) as mobile phase. The detection of analytes was performed using an electrospray ionization tandem mass spectrometry in the multiple reaction-monitoring mode. The lower limits of quantification for sildenafil and UK-103,320 were 2.0 ng/ml. The method showed a satisfactory sensitivity, precision, accuracy, recovery and selectivity.

Keywords: Sildenafil; UK-103,320; LC/MS/MS

1. Introduction

Sildenafil is the effective oral therapy for the treatment of erectile dysfunction as a potent and selective inhibitor of cGMP-specific phosphodiesterase type 5 enzyme (PDE5) (Fig. 1). The major circulating metabolite of sildenafil, UK-103,320 (Fig. 1), results from piperazine *N*-demethylation and has a 2.5-fold lower in vitro potency for PDE5 [1,2]. The simultaneous determination of sildenafil and the active metabolite UK-103,320 is necessary in the pharmacokinetic study of sildenafil. High-performance liquid chromatography (HPLC) using a UV detector [2–5], gas chromatography–mass spectrometry (GC/MS) [6], micellar electrokinetic chromatography [7], liquid

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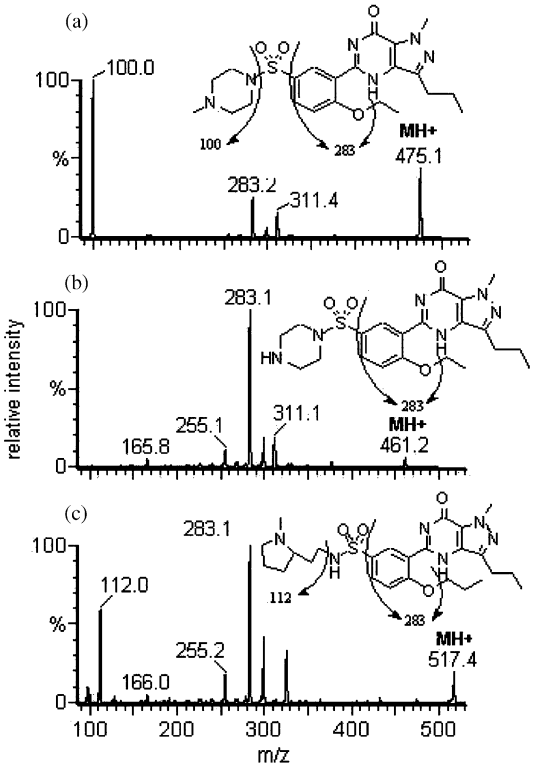

Fig. 1. Product ion mass spectra of (a) sildenafil, (b) UK-103,320 and (c) DA-8159.

chromatography–mass spectrometry (LC/MS) [8,9] and liquid chromatography–tandem mass spectrometry (LC/MS/MS) [9,10] methods have been reported for the analysis of sildenafil and/or UK-103,320 in biological samples. Sample preparation is based on solid-phase extraction [1,2,6–10], on-line trace enrichment [3,4] or protein precipitation using acetonitrile [5]. These sample preparation methods had some disadvantages such as time constraints, large sample volumes or complex equipment for automation.

The objective of this study was to develop and validate an LC/MS/MS method for the rapid analysis of sildenafil and UK-103,320 in human plasma using a one-step liquid–liquid extraction. This method was proved to be successful for the evaluation of sildenafil pharmacokinetics in human.

2. Experimental

2.1. Materials

Sildenafil, UK-103,320 and internal standard (DA-8159) were synthesized by Dong-A Pharm. Co. (Yongin, Korea). Acetonitrile and dichloromethane (HPLC grade) were obtained from Burdick & Jackson, Inc. (Muskegon, MI) and other chemicals were of HPLC grade or the highest quality available. Drug-free human plasma containing heparin as the anticoagulant was obtained from healthy volunteers.

2.2. Preparation of calibration standards and quality control samples

Primary stock solutions of sildenafil, UK-103,320 and DA-8159 (1 mg/ml) were prepared in methanol. Working standard solutions of sildenafil and UK-103,320 were prepared by diluting each primary solution with 50% methanol. The working solution for DA-8159 (1 μg/ml) was prepared by diluting an aliquot of stock solution with 50% methanol.

Human plasma calibration standards of sildenafil and UK-103,320 (2, 10, 40, 100, 250, 500 and 1000 ng/ml) were prepared by spiking appropriate amount of the working standard solutions into drug-free human plasma. Quality control (QC) samples at 5, 50 and 800 ng/ml were prepared in bulk by adding the appropriate working standard solution to drug-free human plasma. The QC samples were aliquoted (100 μl) into polypropylene tubes and stored at −20 °C until analysis.

2.3. Sample preparation

To 100 μl blank plasma, calibration standards and QC samples, 10 μl of DA-8159 working solution and 100 μl of 50 mM sodium hydroxide were added to 2 ml Eppendorf tubes. The samples were extracted with 700 μl of dichloromethane by vortex mixing for 5 min and centrifuged at 5000 × *g* for 5 min. The organic layer was separated and evaporated to dryness under nitrogen at 35 °C. The residues were dissolved in 50 μl of 50%

acetonitrile and 10 μl was injected onto LC/MS/MS system.

2.4. LC/MS/MS analysis

For LC/MS/MS analysis, the chromatographic system consisted of a Nanospace SI-2 pump, an SI-2 autosampler and an S-MC system controller (Shiseido, Tokyo, Japan). The separation was performed on a Luna phenylhexyl column (3 μm, 2 mm i.d. × 100 mm; Phenomenex, Torrance, CA) using a mixture of acetonitrile–ammonium formate (10 mM, pH 6.0) (60:40, v/v) at a flow rate of 0.2 ml/min. The column temperature was 30 °C. The eluent was introduced directly by the tandem quadrupole mass spectrometer (Quattro LC, Micromass UK Ltd., UK) through the positive ionization electrospray interface. The ion source and the desolvation temperature were held at 120 and 250 °C, respectively. The nebulizing and collision gases were nitrogen and argon, respectively. The cone voltage was 35 V and the optimum collision energies for sildenafil, UK-103,320 and DA-8159 were 30, 30 and 25 eV, respectively. Multiple reaction monitoring (MRM) mode using specific precursor/product ion transitions was employed for the quantification. Detection of the ions was performed by monitoring the transitions of m/z 475 → 100 for sildenafil, m/z 461 → 283 for UK-103,320 and m/z 517 → 283 for DA-8159. Peak areas for all components were automatically integrated using MassLynx software.

2.5. Method validation

Batches, consisting of three calibration standards at each concentration, were analyzed on three different days to complete the method validation. In each batch, QC samples at 5, 50 and 800 ng/ml were assayed in sets of five replicates to evaluate the intra- and inter-day precision and accuracy. Five replicates of QC samples at each of the three concentrations processed and stored under autosampler conditions (4 °C) for 24 h were assayed to evaluate processed sample/autosampler stability.

2.6. Application

Four healthy male volunteers, fasted for 12 h, received a single oral dose of sildenafil tablet (100 mg, Viagra® tablets) with 20 ml of water. Blood samples (1 ml) were withdrawn from the forearm vein at 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8 and 12 h post-dosing were transferred to Vacutainer tubes and centrifuged. Following centrifugation (3000 × g, 15 min, 4 °C), plasma samples were transferred to Eppendorf tubes and stored at −20 °C prior to analysis. Drug concentrations were determined as the mean of duplicate samples. The peak concentration (C_{max}) and the time to peak concentration (T_{max}) were determined by visual inspection from each volunteer's plasma concentration–time plots for sildenafil and UK-103,320. Area under the plasma concentration–time curve (AUC) was calculated by the linear trapezoidal method from 0 to 12 h. Plasma elimination half-life ($t_{1/2}$) was determined from the descending slope of the concentration–time profile after logarithmic transformation of the concentration data.

3. Results and discussion

3.1. LC/MS/MS

Luna phenylhexyl column with the mobile phase of acetonitrile and 10 mM ammonium formate (pH 6.0) (60:40, v/v) resulted in short chromatographic run time (3.5 min) with satisfactory separation of sildenafil, UK-103,320 and DA-8159. The retention times for sildenafil, UK-103,320 and DA-8159 were 2.6, 2.1 and 2.5 min, respectively.

The electrospray ionization method gave the optimum sensitivity for sildenafil and UK-103,320 in positive ion mode. The quantification was performed by MRM detection mode using the specific precursor/product ion transitions. The mass spectra of sildenafil, UK-103,320 and DA-8159 indicated the protonated molecule ($[MH^+]$) at m/z 475, 461 and 517, respectively, and no $[M+2H]^{2+}$ ion and other fragment ions were observed. Therefore, $[MH^+]$ ion of each compound was selected as the precursor ion. The collision-induced

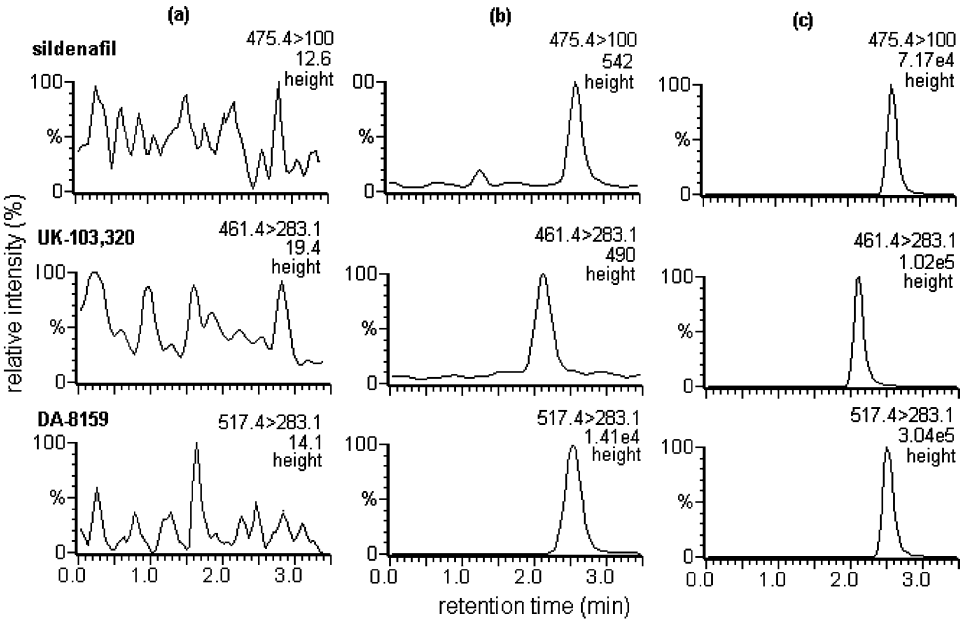

Fig. 2. Representative LC/MS/MS chromatograms of (A) a blank human plasma, and human plasma samples spiked with (B) 2 ng/ml and (C) 500 ng/ml of sildenafil and UK-103,320.

dissociation product ion mass spectra for sildenafil, UK-103,320 and DA-8159 yielded several predominant product ions (Fig. 1). The product ions at *m/z* 100, 283 and 283 were selected based on the specificity and sensitivity for sildenafil, UK-103,320 and DA-8159, respectively. The precursor/product ion transitions were *m/z* 475 → 100 for sildenafil, *m/z* 461 → 283 for UK-103,320 and *m/z* 517 → 283 for DA-8159.

Fig. 2 shows the representative chromatograms of the extracts obtained with blank human plasma and human plasma spiked with sildenafil and UK-103,320 (2.0 and 500 ng/ml). There were no peaks of interfering with sildenafil, UK-103,320 and DA-8159 at their retention times in the blank human plasma (Fig. 2a).

3.2. Linearity

The calibration curves for sildenafil and UK-103,320 in human plasma were linear over the concentration range 2.0–1000 ng/ml and reproducible with mean ± standard deviation for the constants in the regression equation of $y = (0.0159 \pm 0.0013)x - (0.0019 \pm 0.0010)$ and $y = (0.0307 \pm 0.0025)x - (0.0009 \pm 0.0008)$, respectively. The mean correlation coefficients (r^2) were 0.997 and 0.995 for sildenafil and UK-103,320, respectively. Acceptable accuracy and precision were obtained over the concentration range examined for sildenafil and UK-103,320 (Table 1).

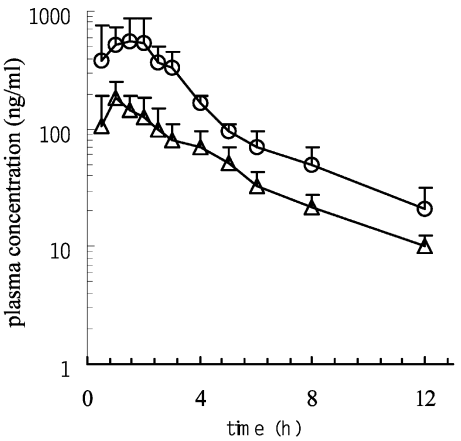

Fig. 3. Mean plasma concentration–time plots of sildenafil (○) and UK-103,320 (△) after a single oral dose of sildenafil (100 mg) to four male volunteers. Each point represents the mean ± S.D.

Table 1
Calculated concentrations of sildenafil and UK-103,320 in calibration standards prepared in human plasma ($n = 9$)

Analytes	Statistical variables	Theoretical concentration						
		2 ng/ml	10 ng/ml	40 ng/ml	100 ng/ml	250 ng/ml	500 ng/ml	1000 ng/ml
Sildenafil	Mean (ng/ml)	1.9	9.7	37.1	95.4	241.2	484.6	1022.2
	CV (%)	5.3	7.2	6.7	6.1	6.8	4.3	3.3
	Accuracy (%)	95.0	97.0	92.7	95.4	96.5	96.9	102.2
UK-103,320	Mean (ng/ml)	2.1	9.9	38.1	99.2	242.7	480.1	997.6
	CV (%)	4.8	9.1	5.2	4.3	6.5	5.1	4.2
	Accuracy (%)	105.0	99.0	92.7	99.2	97.1	96.0	99.8

Table 2
Absolute recoveries of sildenafil, UK-103,320 and DA-8159 from spiked human plasma

Concentration (ng/ml)	Recovery (%, mean ± S.D., $n = 5$)		
	Sildenafil	UK-103,320	DA-8159
5	59.0 ± 4.2	78.8 ± 4.9	–
25	61.4 ± 3.6	79.7 ± 3.3	–
400	59.5 ± 1.9	74.6 ± 4.2	–
1000	65.3 ± 4.1	77.4 ± 3.9	–
20	–	–	55.3 ± 4.1

–, not assayed.

3.3. Recovery

The extraction recoveries of sildenafil and UK-103,320 from spiked human plasma were determined at the concentrations of 5, 25, 400 and 1000 ng/ml in five replicates. The recoveries of sildenafil ranged from 59 to 65.3%. The recoveries of UK-103,320 ranged from 74.6 to 79.7% while that of DA-8159 being 55.3 ± 4.1% (Table 2). The one-step liquid–liquid extraction with dichloromethane has been successfully applied to the extraction of sildenafil and UK-103,320 from human plasma.

3.4. Sensitivity

The lower limit of quantitation (LLOQ) was set at 2.0 ng/ml for sildenafil and UK-103,320 using 100 μl of human plasma. As shown in Fig. 2b, the peak heights for two analytes are at least five times the background noise. The mean percent accuracy values were 95.0% for sildenafil and 105.0% for UK-103,320 with coefficients of variation (CV) 5.3 and 4.8%, respectively, at the LLOQ (Table 1).

3.5. Intra- and inter-batch accuracy and precision

QC samples containing sildenafil and UK-103,320 were prepared and analyzed in three assay batches. Table 3 shows a summary of intra- and inter-batch precision and accuracy. The intra-

Table 3
Precision and accuracy of sildenafil and UK-103,320 in human plasma quality control samples

Analytes	Statistical variable	Intra-batch ($n = 6$)			Inter-batch ($n = 18$)		
		5 ng/ml	50 ng/ml	800 ng/ml	5 ng/ml	50 ng/ml	800 ng/ml
Sildenafil	Mean (ng/ml)	4.9	49.5	771.1	5.0	50.6	794.9
	CV (%)	7.6	6.2	5.5	8.1	6.9	8.1
	Accuracy (%)	98.6	99.0	96.4	99.6	101.2	99.4
UK-103,320	Mean (ng/ml)	4.9	48.8	796.2	4.9	48.2	783.1
	CV (%)	6.9	5.3	3.4	6.4	5.7	4.1
	Accuracy (%)	98.0	97.6	99.5	97.2	96.4	97.9

Table 4
Processed sample stability of sildenafil and UK-103,320 in human plasma for 24 h at 4 °C ($n = 5$)

Statistical variable	Sildenafil			UK-103,320		
	5 ng/ml	50 ng/ml	800 ng/ml	5 ng/ml	50 ng/ml	800 ng/ml
Mean (ng/ml)	5.0	48.4	782.9	4.9	49.6	800.3
CV (%)	7.2	5.4	6.3	7.6	6.3	4.3
Accuracy (%)	100.0	96.8	97.9	98.0	99.2	100.0

batch accuracy for sildenafil and UK-103,320 ranged from 96.4 to 99.5% at three different concentrations with the precision (CV) between 3.4 and 7.6%. The inter-batch accuracy for sildenafil and UK-103,320 ranged from 96.4 to 101.2% at three different concentrations with the precision (CV) between 4.1 and 8.1%. These results indicated that the present method has a satisfactory accuracy, precision and reproducibility.

3.6. Stability

Sildenafil, UK-103,320 and DA-8159 were stable throughout the extraction procedure and in the extracts. The analysis of the reconstituted extracts stored for 24 h at 4 °C showed the acceptable precision and accuracy for QC samples (Table 4).

3.7. Application

The suitability of this method was proved in the pharmacokinetic study of sildenafil and UK-103,320 after a single oral dose of sildenafil (100 mg) to four healthy male volunteers. Fig. 3 shows mean plasma concentration profiles of sildenafil and UK-103,320 in four healthy male volunteers. C_{max}, T_{max}, AUC and $t_{1/2}$ of sildenafil were 735 ± 354 ng/ml, 0.9 ± 0.7 h, 1951 ± 569 ng·h/ml and 3.43 ± 0.93 h, respectively. C_{max}, T_{max}, AUC and $t_{1/2}$ of UK-103,320 were 200 ± 59 ng/ml, 0.97 ± 0.39 h, 633 ± 199 ng h/ml and 3.57 ± 1.06 h, respectively.

In conclusion, a rapid, sensitive and selective LC/MS/MS method using a simple liquid–liquid extraction was developed for simultaneous determination of sildenafil and UK-103,320 in human plasma. The analytes and the internal standard were extracted from plasma with dichloromethane and separated on a phenylhexyl column. The method was proved to be suitable for the clinical pharmacokinetic study of sildenafil.

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