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Journal of Chromatography B, 752 (2001) 141–147

JOURNAL OF CHROMATOGRAPHY B

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Narrowbore high-performance liquid chromatography for the simultaneous determination of sildenafil and its metabolite UK-103,320 in human plasma using column switching

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Received 27 April 2000; received in revised form 28 September 2000; accepted 28 September 2000

Abstract

A fully automated narrowbore high-performance liquid chromatography method with column switching was developed for the simultaneous determination of sildenafil and its active metabolite UK-103,320 in human plasma samples without pre-purification. Diluted plasma sample (100 μl) was directly introduced onto a Capcell Pak MF Ph-1 column (20×4 mm I.D.) where primary separation occurred to remove proteins and concentrate target substances using 15% acetonitrile in 20 m*M* phosphate solution (pH 7). The drug molecules eluted from the MF Ph-1 column were focused in an intermediate column (35×2 mm I.D.) by a valve switching step. The substances enriched in the intermediate column were eluted and separated on a phenyl-hexyl column (100×2 mm I.D.) using 36% acetonitrile in 10 m*M* phosphate solution (pH 4.5) when the valve status was switched back. The method showed excellent sensitivity (detection limit of 10 ng/ml), good precision (RSD≤2.3%) and accuracy (bias: ±2.0%) and speed (total analysis time 17 min). The response was linear (r^2≥0.999) over the concentration range 10–1000 ng/ml. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Sildenafil; UK-103,320

1. Introduction

Sildenafil, 1-[4-ethoxy-3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo[4,3-d]pyrimidin-5-yl)-phenylsulphonyl]-4-methyl piperazine (Fig. 1) is a novel drug used in the treatment of erectile dysfunction [1–3]. Five major pathways of the sildenafil metabolism were identified in rat, rabbit, dog and man, i.e., piperazine *N*-demethylation, pyrazole *N*-demethylation, *N*,*N*′-deethylation, oxidation of piperazine ring and aliphatic hydroxylation [4]. The piperazine *N*-desmethyl metabolite, UK-103,320 (Fig. 1) was identified as a major metabolite having a similar potency to sildenafil in dog, mouse, rat and man [4–7]. The simultaneous determination of sildenafil and its active metabolite UK-103,320 was necessary for the evaluation of sildenafil pharmacokinetics.

High-performance liquid chromatographic (HPLC) methods have been reported for the simultaneous determination of sildenafil and UK-103,320 in biological samples [4–7]. These methods involve

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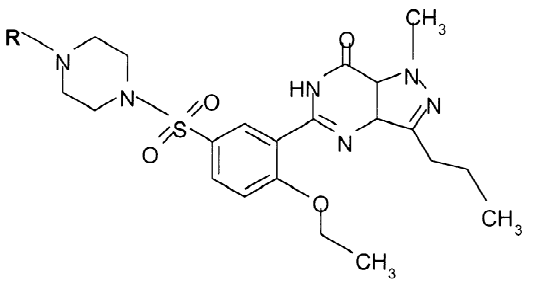
PII: S0378-4347(00)00536-3



Sildenafil : R = CH_3
UK-103,320 : R = H

Fig. 1. Structures of sildenafil and UK-103,320.

liquid–liquid extraction (LLE) [7], solid-phase extraction (SPE) [4] or automated sequential trace enrichment of dialysate (ASTED) [4,7] as sample preparation procedures. LLE and SPE were simple but had some disadvantages such as time constraints, the large sample volume (≥500 μl) and the possibility of contamination during handling. The ASTED system [7,8] requires the dialysis system for on-line dialysis as well as a switching valve for trace enrichment, and therefore, the present method using column switching offers practical advantages over the ASTED system with respect to the equipment and sample volume (640 μl).

Column switching [9–13] is an on-line trace enrichment technique that can directly analyze biological samples in the hundreds of microliters for semi-microbore liquid chromatography (LC) [11–13] as well as conventional LC [9,10]. In microbore LC, a triple column switching system using an intermediate column has advantages such as shorter analysis time and the protection of the main column from the high pressure over dual column switching [11–13].

The purpose of the present study was to describe an automated narrowbore HPLC method using triple column switching for the simultaneous determination of sildenafil and its active metabolite UK-103,320 from a small amount of human plasma samples without coupling on-line dialysis. This method was based on two separation processes: (1) the primary separation on a polymer-coated mixed function phase (MF Ph-1) for deproteinization and concentration of analytes and (2) the main separation using a narrowbore phenyl-hexyl column. The applicability of the method was proven in the study of the pharmacokinetics of sildenafil in humans following a single oral administration of sildenafil.

2. Experimental

2.1. Materials and reagents

Sildenafil and UK-103,320 were synthesized in Dong-A (Yongin, South Korea). HPLC-grade acetonitrile and methanol were purchased from Burdick & Jackson (Muskegon, MI, USA). Stock solutions of sildenafil and UK-103,320 were prepared by dissolving in methanol (1 mg/ml) and aliquots were spiked to drug-free human blank plasma to obtain the calibration plasma standards at five concentrations of 10, 50, 250, 500 and 1000 ng/ml.

2.2. Triple column switching system

A column switching system using three columns consisted of the Nanospace SI-1 series (Shiseido, Tokyo, Japan), i.e., two 2001 pumps, a 2002 UV–Vis detector, a 2003 autosampler, a 2004 column oven, a 2012 high-pressure switching valve, and a 2009 degassing unit; the configuration of which is shown in Fig. 2. The system was operated by Syscon (Shiseido) and the signals were processed by S-MicroChrom (Shiseido).

2.3. Chromatographic conditions

The pre-column for the primary separation of sildenafil and UK-103,320 from plasma samples was a Capcell Pak MF Ph-1 cartridge (20×4 mm I.D.; Shiseido). The intermediate column was a Capcell Pak C_{18} UG 120 (35×2 mm I.D.) to enrich the drug molecules separated on the MF Ph-1 pre-column and the main separation was performed on a Luna 2 phenyl-hexyl column (3 μm, 100×2 mm I.D.; Phenomenex, Torrance, CA, USA) using 36% acetonitrile in 10 m*M* phosphate solution (pH 4.5) at a

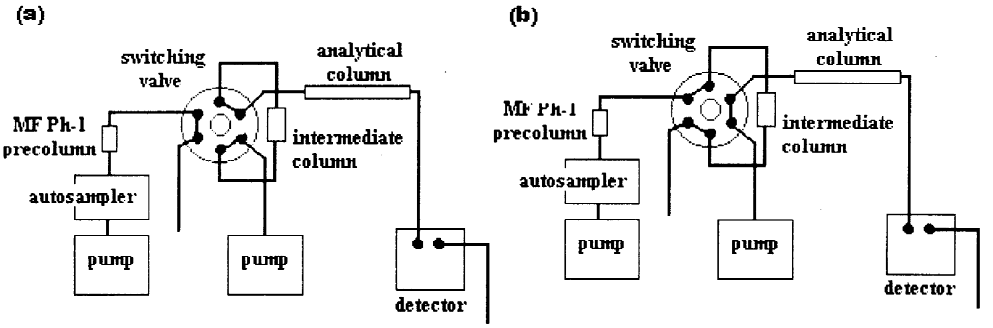


Fig. 2. Triple column system for the column switching in semi-micro LC. (a) Position A, (b) position B.

flow-rate of 0.2 ml/min. The column temperature was 30°C and the effluent was monitored at 230 nm.

2.4. Analytical procedure

Plasma samples (70 μl) were diluted with the same volume of 50 m*M* phosphate solution (pH 7) and passed through a low-protein-binding membrane syringe filter (0.22 μm, PVDF; Millipore, Bedford, MA, USA).

Step 1 (0–1.8 min, valve position A): filtered plasma sample (100 μl) was injected onto the Capcell Pak MF Ph-1 pre-column where plasma proteins, sildenafil and UK-103,320 were separated using 15% acetonitrile in 20 m*M* phosphate solution (pH 7) at a flow-rate of 0.5 ml/min. The intermediate column and analytical column were equilibrated using the mobile phase.

Step 2 (1.8–5.4 min, valve position B): when the valve status was changed to B, the drug-containing fractions separated in the Capcell Pak MF Ph-1 pre-column were focused onto an intermediate column using 15% acetonitrile in 20 m*M* phosphate solution (pH 7) at a flow-rate of 0.5 ml/min. The analytical column was equilibrated using the mobile phase.

Step 3 (5.4–17 min, valve position A): the valve was switched back to the A position, and then, the analytes trapped in the intermediate column were transferred in back-flush mode to a narrowbore phenyl-hexyl column and separated with 0.2 ml/min of 36% acetonitrile in 10 m*M* phosphate solution (pH 4.5). In the meantime, the MF Ph-1 column was equilibrated with a 15% acetonitrile in 50 m*M* phosphate solution (pH 7).

2.5. Method validation

The limits of quantitation (LOQs) for sildenafil and UK-103,320 were determined as the concentration of drug giving a signal-to-noise ratio greater than 5:1. Five sildenafil and UK-103,320-spiked plasma standard samples over the concentration range 10–1000 ng/ml were quantified to evaluate the recovery, linearity, precision [the relative standard deviation (RSD) of replicate analysis] and accuracy (the bias between theoretical and actual concentration).

2.6. Pharmacokinetics of sildenafil and UK-103,320 in volunteers

Four healthy male volunteers (40–45 years old) received a single oral dose of sildenafil (50 mg). Blood samples (1 ml) were withdrawn from the forearm vein at 0.5, 0.75, 1.0, 1.5, 2, 4, and 6 h post dosing, transferred to Vacutainer tubes and centrifuged. Following centrifugation (3000 rpm, 15 min, 4°C), plasma samples were transferred to Eppendorf tubes and stored at −70°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}) of sildenafil and its active metabolite UK-103,320 were determined by visual inspection from each volunteer's plasma concentration–time plots for sildenafil and UK-103,320, respectively. Area under the plas-

ma concentration–time curves (AUC) was calculated by the linear trapezoidal method from 0 to 6 h. Plasma elimination half-life ($t_{1/2}$) of sildenafil and UK-103,320 was determined from the descending slope of the concentration–time profiles after logarithmic transformation of the concentration data.

3. Results and discussion

3.1. Chromatography

The effect of different narrowbore bonded-phase columns such as octadecyl, octyl and phenyl-hexyl on the simultaneous determination of sildenafil and UK-103,320 was examined. In the octadecyl and octyl columns, the retention of sildenafil increased severely but the retention of UK-103,320 was not affected when compared to the phenyl-hexyl column. The phenyl-hexyl column was chosen for the simultaneous determination of sildenafil and UK-103,320 from human plasma because of excellent resolution, short analysis time and good sensitivity (Fig. 3). The use of a narrowbore column resulted in advantages such as small plasma sample volume (50 μl), high column efficiency, and lower solvent consumption over conventional HPLC. Since sildenafil and UK-103,320 are basic drugs (pK_a=8.7), the more acidic the pH of mobile phase becomes, the shorter analysis time is. However, more acidic mobile phase (<pH 4.0) gave more interference peaks in column switching, and therefore the pH of mobile phase was appropriate at pH 4.5. The mixture of acetonitrile–10 m*M* phosphate solution (pH 4.5) (36:64, v/v) was used as the mobile phase.

3.2. Column switching procedure

To establish the triple column switching system for the simultaneous determination of sildenafil and its active metabolite UK-103,320 from plasma, the choice of pre-column, washing solvent and valve-switching time must be considered.

Capcell Pak MF Ph-1 was widely used as pre-column packing in microbore column switching [11,12]. MF Ph-1 phase consists of hydrophilic

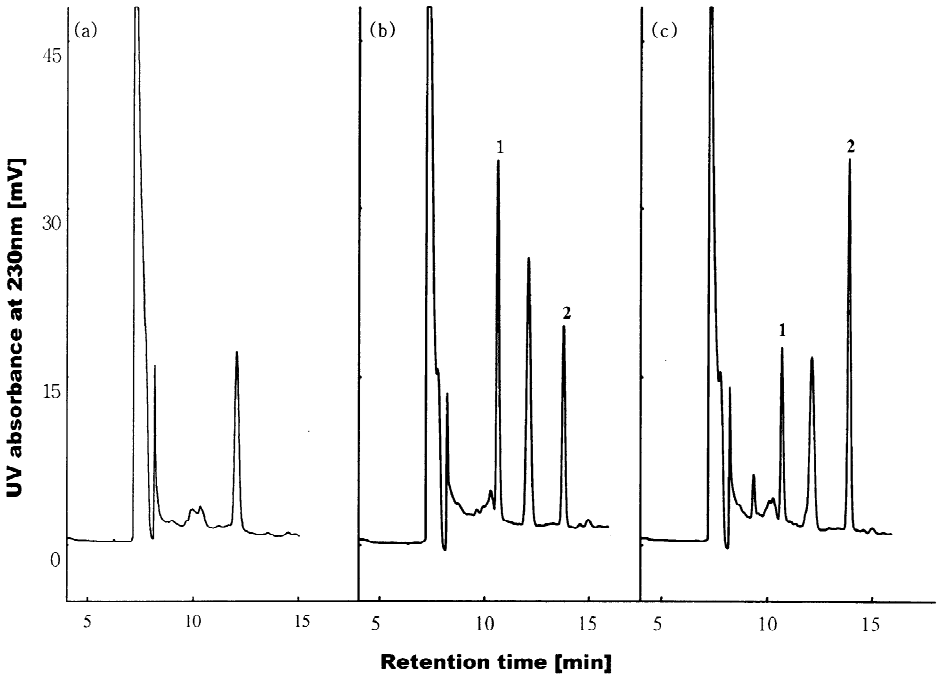


Fig. 3. Chromatograms of (a) blank plasma, (b) blank plasma spiked with sildenafil and UK-103,320 (250 ng/ml), and (c) plasma sample at 1 h after single oral dose of 50 mg sildenafil to a volunteer. Peaks: 1=UK-103,320, 2=sildenafil.

polyoxyethylene groups and hydrophobic phenyl groups bonded on the surface of 80 Å silica and proved to be appropriate to remove proteins and concentrate sildenafil and UK-103,320 from plasma. The pH of the mobile phase on the MF-Ph 1 pre-column was appropriate at pH 7.0 for little protein denaturation of plasma sample. To obtain good recovery and clean chromatogram within a relatively short time, the mixture of pH 7.0 phosphate solution and acetonitrile was appropriate for the deproteinization and fractionation of sildenafil and UK-103,320 from plasma on the MF Ph-1 cartridge. To determine the appropriate time for column switching, the separation profile of sildenafil and UK-103,320 in plasma on the MF Ph-1 column (20×4 mm I.D.) was evaluated using 15% acetonitrile in 50 m*M* phosphate solution (pH 7.0) (Fig. 4).

The analytes (equivalent to 1.8 ml volume) fractionated from MF Ph-1 by the valve switching step were focused in the top of intermediate C_{18} column (35×2 mm I.D.) to obtain sharp peaks in the final separation. The use of an intermediate column resulted in the protection of ME Ph-1 column from high pressure as well as the saving of analysis time, that is, the focusing time was reduced from 9 min at 0.2 ml/min to 3.6 min at 0.5 ml/min.

The MF Ph-1 pre-column was exchanged after injection of 80 plasma samples (equivalent to 4.0 ml plasma). The intermediate and main columns showed no decrease in efficiency after more than 250 injections of plasma sample.

3.3. Method validation

The mean recoveries of sildenafil and UK-103,320 from plasma samples were 93.5±2.4% and 93.2±2.1%, respectively. The calibration curves of peak areas versus the concentrations of sildenafil and UK-103,320 in plasma were linear giving a correlation coefficient of 0.999 over the range of 10–1000 ng/ml. The LOQ of sildenafil and UK-103,320 was 10 ng/ml using 50 μl plasma.

The intra- and inter-day precision and accuracy of the assay are shown in Table 1. Actual concentrations deviated from −2.0 to 2.0% of the theoretical concentrations in the spiked plasma samples and the assay was precise because the RSD was less than 2.3%.

3.4. Pharmacokinetics of sildenafil in humans

The suitability of this method was proved in the pharmacokinetic study of sildenafil after a single oral administration of sildenafil (50 mg) to four healthy

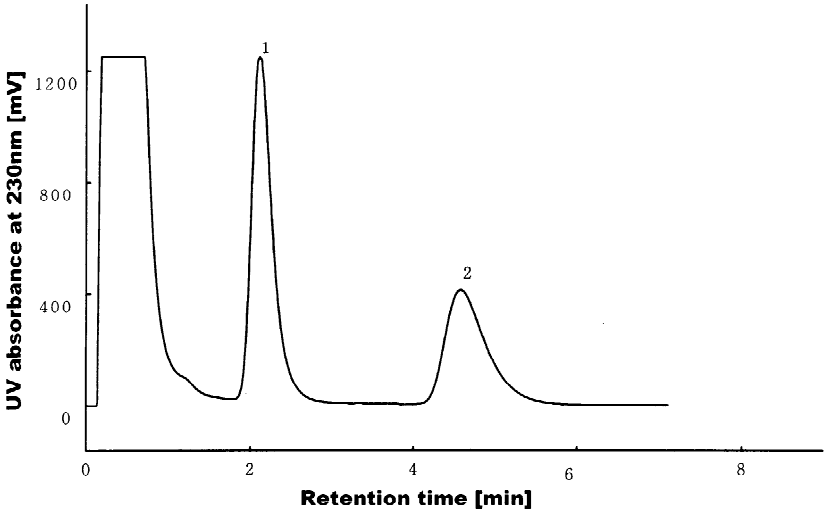


Fig. 4. Separation of sildenafil and UK-103,320-spiked plasma on the MF Ph-1 column (20×4 mm I.D.). Conditions: mobile phase: acetonitrile–50 m*M* phosphate solution (pH 7.0) (15:85, v/v), flow-rate: 0.5 ml/min, injection volume: 50 μl plasma. Peaks: 1=UK-103,320, 2=sildenafil.

Table 1
Reproducibility of sildenafil and UK-103,320 in human plasma samples (n=6)

Concentration added (ng/ml)	Concentration found (ng/ml)		RSD (%)	
	Sildenafil	UK-103,320	Sildenafil	UK-103,320
Intra-day				
10.0	10.2	9.9	2.1	1.9
50.0	50.1	49.3	2.1	2.2
250	251	248	2.2	1.9
500	498	500	1.6	2.2
1000	999	1010	1.5	1.8
Inter-day				
10.0	10.1	10.0	2.1	1.9
50.0	49.8	49.9	1.8	1.9
250	252	251	1.9	2.1
500	500	499	2.3	2.0
1000	1004	1002	1.9	1.8

male volunteers. The plasma chromatogram of a volunteer administered sildenafil is shown in Fig. 3c. Fig. 5 shows the mean (±SD) plasma concentration–time curves of sildenafil and its active metabolite UK-103,320. The pharmacokinetic parameters of sildenafil and UK-103,320 are shown in Table 2. C_{max} of sildenafil (419±150 ng/ml, 4.9) and ratio C_{max} sildenafil/UK-103,320 were higher than those (212±59 ng/ml, 2.1) of Walker et al. [4] but T_{max} was similar (1.0 h vs. 1.2±0.3 h).

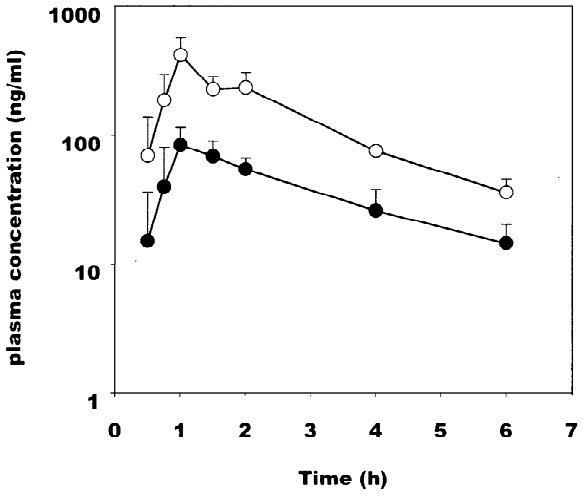


Fig. 5. Plasma concentration–time profiles of sildenafil (○) and UK-103,320 (●) following a single oral administration of sildenafil (50 mg).

4. Conclusion

An automated narrowbore HPLC method using column switching has been developed for the simultaneous determination of sildenafil and its active metabolite UK-103,320 from human plasma samples. The method shows excellent sensitivity (10 ng/ml using 50 μl plasma), reproducibility, specificity, and speed (total analysis time 17 min). The suitability of the method was confirmed in the pharmacokinetic study of sildenafil in human volunteers and dogs.

Acknowledgements

This study was supported by a grant of the Korea Health R&D Project, Ministry of Health & Welfare, Republic of Korea (HMP-99-D-07-0003).

Table 2
Pharmacokinetic parameters of sildenafil and UK-103,320 in human after single oral administration of sildenafil (50 mg) (n=4, mean±SD)

Parameter	Sildenafil	UK-103,320
C_{max} (ng/ml)	419±150	86±27
T_{max} (h)	1.0	1.13±0.25
$t_{1/2}$(h)	1.07±0.21	1.26±0.35
AUC (ng h/ml)	870±160	217±74

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