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Journal of Pharmaceutical and Biomedical Analysis 45 (2007) 176–184
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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Short communication

Development of LC/MS/MS assay for the determination of 5-ethyl-2-{5-[4-(2-hydroxyethyl)piperazine-1-sulfonyl]-2-propoxyphenyl}-7-propyl-3,5-dihydropyrrolo[3,2-*d*]pyrimidin-4-one (SK3530) in human plasma: application to a clinical pharmacokinetic study

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Received 23 April 2007; received in revised form 23 June 2007; accepted 27 June 2007
Available online 30 June 2007

Abstract

5-Ethyl-2-{5-[4-(2-hydroxyethyl)piperazine-1-sulfonyl]-2-propoxyphenyl}-7-propyl-3,5-dihydropyrrolo[3,2-*d*]pyrimidin-4-one (SK3530) is a new phosphodiesterase type-5 inhibitor currently undergoing a Phase III investigation for the treatment of male erectile dysfunction. This study first describes a rapid and sensitive LC/MS/MS assay method for the quantification of SK3530 and its major metabolite, SK3541, in human plasma. The assay was validated to demonstrate the specificity, linearity, recovery, lower limit of quantification (LLOQ), accuracy, and precision. The multiple reaction monitoring was based on the transition of $m/z = 532.5 \rightarrow 99.1$ for SK3530, $488.6 \rightarrow 295.5$ for SK3541, and $520.3 \rightarrow 99.1$ for SK3304 (internal standard). The assay utilized a single liquid–liquid extraction and isocratic elution, and the LLOQ was 1 ng/ml using 0.2 ml human plasma. The assay was linear over a range from 1 to 1000 ng/ml for both SK3530 and SK3541, with correlation coefficients >0.9999. The mean intra- and inter-day assay accuracy ranged from 94.7 to 101.6% and 96.8 to 101.1% for SK3530 and 92.6–105.7% and 97.4–107.8% for SK3541, respectively. The mean intra- and inter-day precision was between 7.2–12.1% and 5.7–7.4% for SK3530 and 4.6–13.2% and 5.0–14.1% for SK3541, respectively. The developed assay was applied to a clinical pharmacokinetic study after oral administration of SK3530 in healthy male volunteers (dose 100 mg).

Keywords: SK3530; SK3541; PDE-5 inhibitor; LC/MS/MS; Pharmacokinetics

1. Introduction

Male erectile dysfunction (ED) is a common medical problem affecting approximately 18 million men or 18.4% of the US male population aged 20 years or older [1]. The prevalence of ED increases with age from 5.1% in men aged 20–39 years to 14.8, 43.8, and 70.2% in men aged 40–59, 60–69, and 70 years and older, respectively [1]. The occurrence of ED across different ethnicities and countries appears comparable [1–2]. ED is known to be associated with various diseases, including diabetes, lower urinary tract symptoms, depression, hypertension, atherosclerosis, and cardiovascular diseases [3–6]. The most common cause of ED is considered to be related to abnormal vascular supply to penile and erectile tissues. Recently, orally active phosphodiesterase type-5 (PDE-5) inhibitors, sildenafil, vardenafil, and tadalafil, have been widely used for the treatment of ED [7–9]. PDE-5 is a cyclic guanosine monophosphate (cGMP)-specific hydrolyzing enzyme, which is present in high concentrations in the smooth

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doi:10.1016/j.jpba.2007.06.021

muscle of the penile corpus cavernosum. The PDE-5 inhibitors improve erectile function by binding to cGMP and maintaining sufficient cellular levels in the smooth muscles [7,10–11]. The therapeutic outcomes and clinical pharmacokinetics of PDE-5 inhibitors have been reviewed [8–9,12–13]. Common adverse reactions such as headache, flushing, nasal congestion, dyspepsia, and visual changes have been reported in association with the use of PDE-5 inhibitors [8]. 5-Ethyl-2-{5-[4-(2-hydroxyethyl)piperazine-1-sulfonyl]-2-propoxyphenyl}-7-propyl-3,5-dihydropyrrolo[3,2-*d*]pyrimidin-4-one (SK3530) is a new PDE-5 inhibitor designed to alleviate drawbacks of these common adverse reactions. SK3530 appears to be safe and effective in the treatment of male ED and is currently under Phase III clinical investigation. The HPLC assay has been reported for the quantification and purity determination of SK3530 (linear dynamic range 200–300 μg/ml) in pharmaceutical dosage forms [14], but an assay method for the determination of SK3530 in biological fluids has been not been reported. The metabolism, biliary, urinary, and fecal excretion of SK3530 were determined in rats after oral administration of ^{14}C-SK3530 [15]. The biliary excretion accounted for 38.82% of the administered radioactivity, indicating that SK3530 is cleared by the hepatobiliary system. When SK3530 is incubated with human liver microsomes, SK3541 was identified as the major metabolite [15].

The present study was conducted to develop a sensitive liquid chromatographic/electrospray tandem mass spectrometry (LC/MS/MS) assay for the determination of SK3530 and its major metabolite, SK3541, in human plasma (lower limit of quantification 1 ng/ml). The developed assay was applied to a pharmacokinetic study following oral administration of SK3530 (dose 100 mg) to healthy male volunteers.

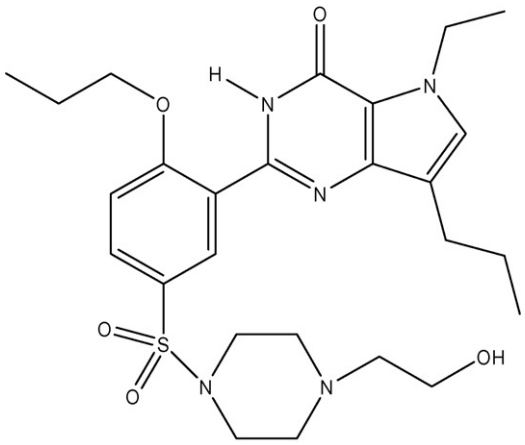
2. Experimental

2.1. Chemicals

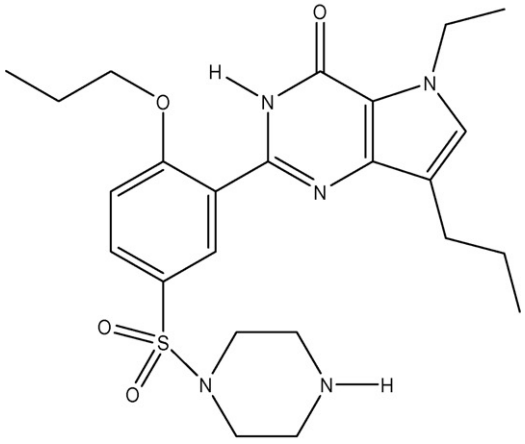
SK3530, SK3541, and SK3304 (internal standard) were supplied by SK Chemicals Co., Ltd. (Suwon, Korea). The structures of these chemicals are shown in Fig. 1. HPLC grade acetonitrile, methanol, *t*-butyl methyl ether were purchased from J.T. Baker (Phillipsburg, NJ, USA). Ammonium acetate was purchased from Sigma (St. Louis, MO, USA). Water was de-ionized prior to use using a Millipore Milli-Q water purification system (Milford, MA, USA).

2.2. Preparation of standard and quality control solutions

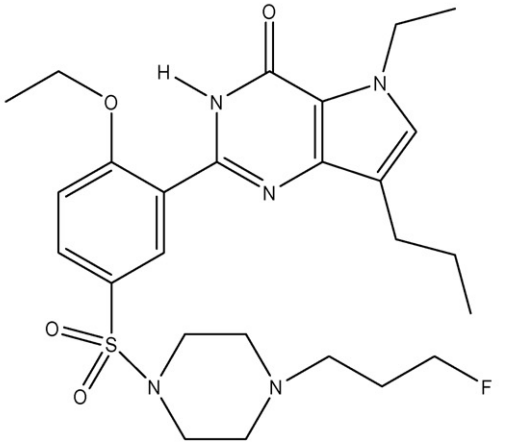
Stock solutions (100 μg/ml) were prepared by separately dissolving 10 mg each of SK3530 and SK3541 in 100 ml of methanol. Calibration samples were prepared by serial dilution with methanol, yielding final concentrations of 1000, 500, 100, 50, 10, 5, and 1 ng/ml. The internal standard stock solution (100 μg/ml) was prepared by dissolving 2.5 mg of SK3304 in 25 ml of methanol and the solution was further diluted to 10 μg/ml. Quality control (QC) samples were prepared by spiking the standard working solutions of SK3530 and



SK3530 ($C_{26}H_{37}N_5O_5S$)



SK3541 ($C_{24}H_{33}N_5O_4S$)



SK3304 ($C_{26}H_{36}FN_5O_4$)

Fig. 1. Chemical structures of SK3530, SK3541, and SK3304.

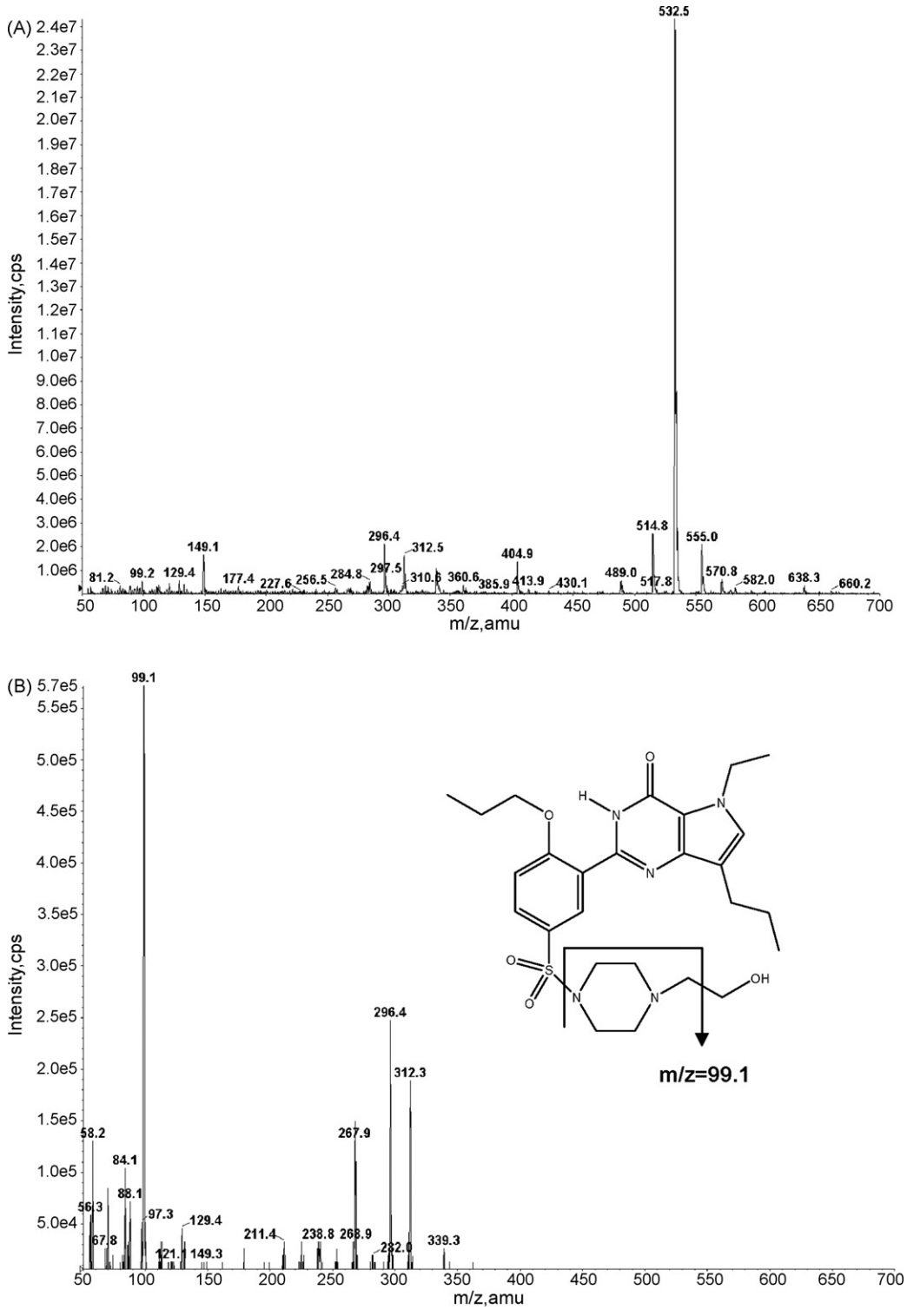


Fig. 2. (A) Positive ion Q1 mass spectrum of SK3530 and (B) product ion mass of protonated SK3530 ($[M+H]^+$, $m/z = 532.5$).

SK3541 to blank human plasma to provide high concentration QC (800 mg/ml), medium concentration QC (200 ng/ml), low concentration QC (4 ng/ml), and lower limit of quantification (LLOQ) QC (1 ng/ml). Each QC sample was prepared once, and aliquots (200 μl) were placed in borosilicate glass tubes. QC samples, stock, and standard working solutions were stored at −20 °C.

2.3. Sample extraction

The internal standard solution (35 μl, SK3304 10 μg/ml in methanol) and methanol (100 μl) were added to human plasma (0.2 ml) in a borosilicate glass tube, and the mixture was basified with 0.5 ml of a saturated ammonium carbonate solution. The plasma samples were extracted with *t*-butyl methyl ether (2 ml)

on a vortex mixer for 7 min followed by centrifugation for 10 min at 2840 × *g*. The upper organic layer was transferred to a clean test tube and evaporated on a heating block (Dry Thermobath MG-2100, Tokyo Rikakikai, Tokyo, Japan) at 40 °C under nitrogen. The residue was dissolved in the mobile phase (200 μl), mixed on a vortex mixer for 5 min, and centrifuged at 2840 × *g* for 1 min. This solution was transferred to a glass flat bottom insert (volume 250 μl), and a portion (15 μl) was injected into LC/MS/MS.

2.4. LC/MS/MS conditions

HPLC was performed with Shimadzu 10Avp system (Kyoto, Japan) consisting of SCL-10Avp system controller, LC-10ADvp pump, SIL-10ADvp auto sampler, CTO-10Avp column oven, and DGU-14A degasser. Compounds were separated on a Atlantis-C_{18} column (150 mm × 2.1 mm i.d., 3 μm, Waters, Milford, MA, USA) with a Security Guard column (4 mm × 2 mm i.d., Phenomenex, Torrance, CA, USA). The isocratic mobile phase consisted of acetonitrile and 10 mM ammonium acetate solution (90:10, v/v). The flow rate of the mobile phase was set at 0.35 ml/min and the column oven temperature was 40 °C.

The HPLC system was coupled to an API 2000 triple-quadrupole mass spectrometer equipped with a turbo ion spray ionization (ESI) source (AB MDS Sciex, Toronto, Canada). The ESI source was operated in a positive mode with the curtain and collision gas (nitrogen) set at 25 and 6 psi, respectively. The turbo-gas temperature was set at 350 °C, and the ion spray needle voltage was adjusted at 4500 V. The mass spectrometer was operated at a unit resolution for both Q1 and Q3 in the multiple reaction monitoring (MRM) mode with a dwell time of 300 ms in each transition. The transition of the precursors to the product ion was monitored at 532.5 → 99.1 for SK3530, 488.6 → 295.5 for SK3541, and 520.3 → 99.1 for SK3304. Data acquisition was preformed with Analyst 1.4 software (AB MSD Sciex, Toronto, Canada).

2.5. Validation

Calibration curves ranging from 1 to 1000 ng/ml were prepared by spiking the blank human plasma (200 μl) with SK3530 and SK3541 at concentrations of 1, 5, 10, 50, 100, 500, and 1000 ng/ml. The calibration curves were constructed by the weighted regression method ($1/x$) of peak area ratios of drug to internal standard versus actual concentration. The linearity of calibration curves was validated with five different calibration curves. The limit of detection (LOD) was the plasma drug concentration that yielded a signal-to-noise (S/N) ratio > 3, and the lower limit of quantification (LLOQ) was defined as the lowest plasma drug concentration that yielded a S/N ratio > 10 with acceptable accuracy and precision (<15%). The precision was expressed as the coefficient of variance of each concentration, and the accuracy was expressed as the percentage of mean calculated versus actual concentrations. Intra- and inter-day assay variability was determined by assaying LLOQ (1 ng/ml), low (4 ng/ml), medium (200 ng/ml), and high QC (800 ng/ml) samples on 5 consecutive days with 5 replicate samples of each day. The extracted QC samples were compared to the neat standard solutions at 4, 200, and 800 ng/ml with the internal standard at 1750 ng/ml, and the blank plasma extract was compared to the neat internal standard solution of 1750 ng/ml. The stability of SK3530 and SK3541 was examined under four different conditions described below using five replicates of low, medium, and high QC samples. To assess the stock solution stability, SK3530 and SK3541 were dissolved in methanol at 4, 200, and 800 ng/ml, and drug concentrations left at room temperature for 12 h were determined. To assess the short-term stability, human plasma was spiked with SK3530 and SK3541 at 4, 200, and 800 ng/ml, and drug concentrations left at room temperature for 12 h were determined. To assess the auto sampler stability, extracted low, medium, and high QC samples of SK3530 and SK3541 dissolved in the mobile phase were left in the auto sampler rack at room temperature for 12 h, and drug concentrations were determined and compared with the immediately work-up concentrations. Lastly, to assess the short-term stability, human plasma spiked with SK3530 and SK3541 at 4, 200, and 800 ng/ml were subject to three freeze-thaw cycles, and remaining drug concentrations were determined. The results were expressed as the percentage of mean deviation over actual concentrations.

2.6. Application to a pharmacokinetic study

Healthy male volunteers ($n = 12$, age 25–37 years, body weight 64.3 ± 6.8 kg) were fasted for 12 h before drug administration. SK3530 was orally administrated to the volunteers (dose 100 mg), and the fasting state was maintained for an additional 4 h. Blood samples were collected before and 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, and 10 h after drug administration. Plasma samples were harvested by centrifugation at 2840 × *g* for 10 min and stored at −70 °C. The plasma samples were analyzed within 2 months of storage. The plasma drug concentration–time data were subjected to a non-compartmental analysis using the non-linear least-squares regression program WinNonlin (Pharsight, Cary, USA).

3. Results and discussion

3.1. Mass spectrometry and chromatography

This study reports the development of a sensitive LC/MS/MS assay for the simultaneous determination of plasma levels of SK3530 and SK3541 following oral administration of SK3530 (100 mg) to healthy male volunteers. SK3541 was previously reported as a major metabolite in rats [15]. The positive Q1 mass spectra and the product ion mass spectra for SK3530, SK3541, and SK3304 (internal standard) are shown in Figs. 2–4, respectively. In the full scan Q1 mass spectrum, the most abundant ions for SK3530, SK3541, and SK3304 were the protonated molecular ions $[M+H]^+$ found at $m/z = 532.5$, 488.6, and 520.3, respectively. The most prominent product ions of the protonated SK3530, SK3541, and SK3304 were shown at m/z of 99.1, 295.5, and 99.1, respectively. Subsequently, the mass transitions were

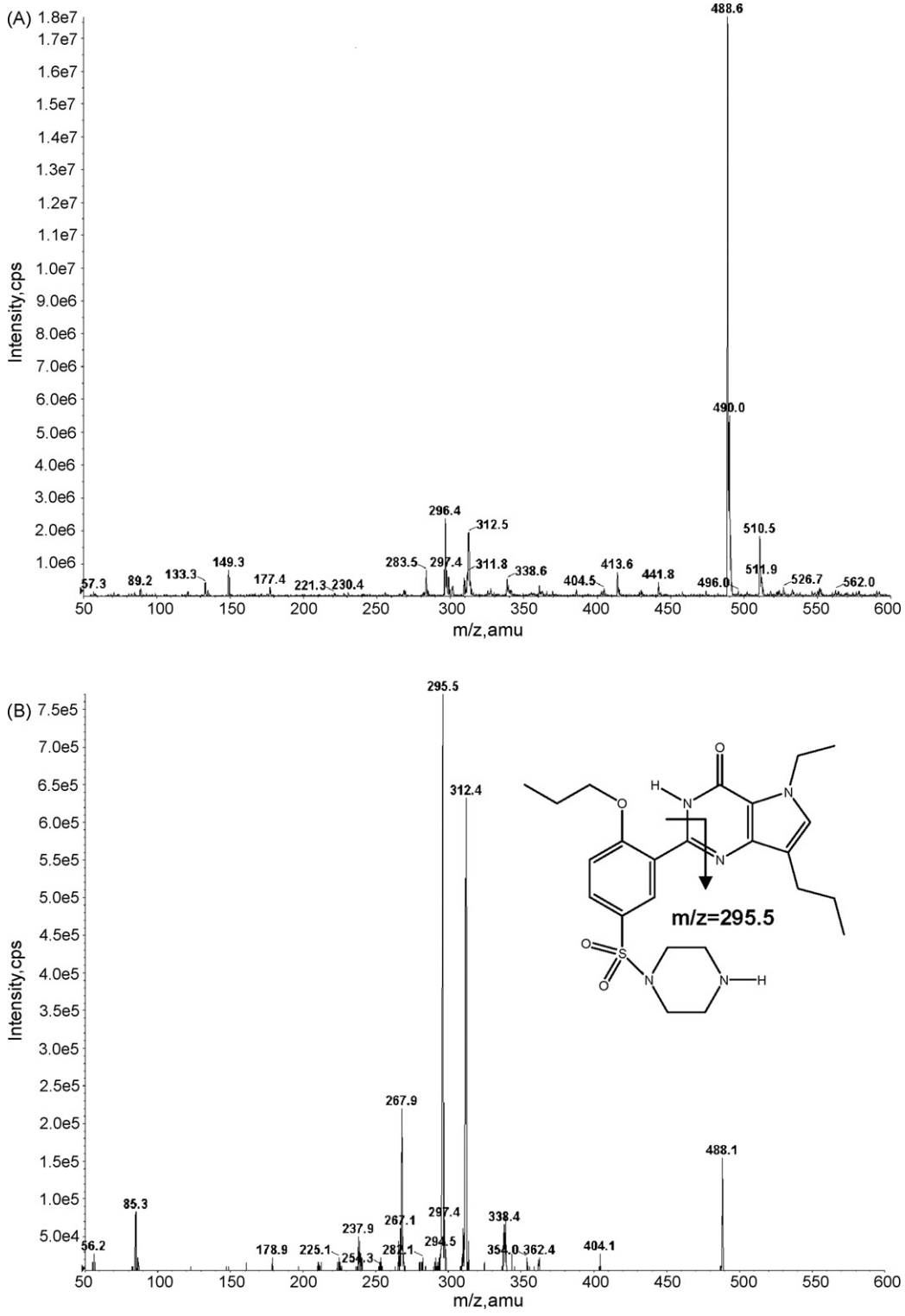


Fig. 3. (A) Positive ion Q1 mass spectrum of SK3541 and (B) product ion mass of protonated SK3541 ($[M+H]^+$, $m/z = 488.6$).

monitored at 532.5 → 99.1, 488.6 → 295.5, and 520.3 → 99.1 for SK3530, SK3541, and SK3304, respectively, and the optimum MS/MS parameters were selected for the assay. Mass chromatograms of SK3530, SK3541, and SK3304 obtained by extraction of blank human plasma, plasma spiked with each compound, and plasma obtained in a human volunteer after administration of SK3530 are shown in Fig. 5. SK3530, SK3541, and SK3304 were eluted at 1.6, 2.6, and 1.7 min, respectively. No endogenous or extraneous peaks interfering with the analytes were observed.

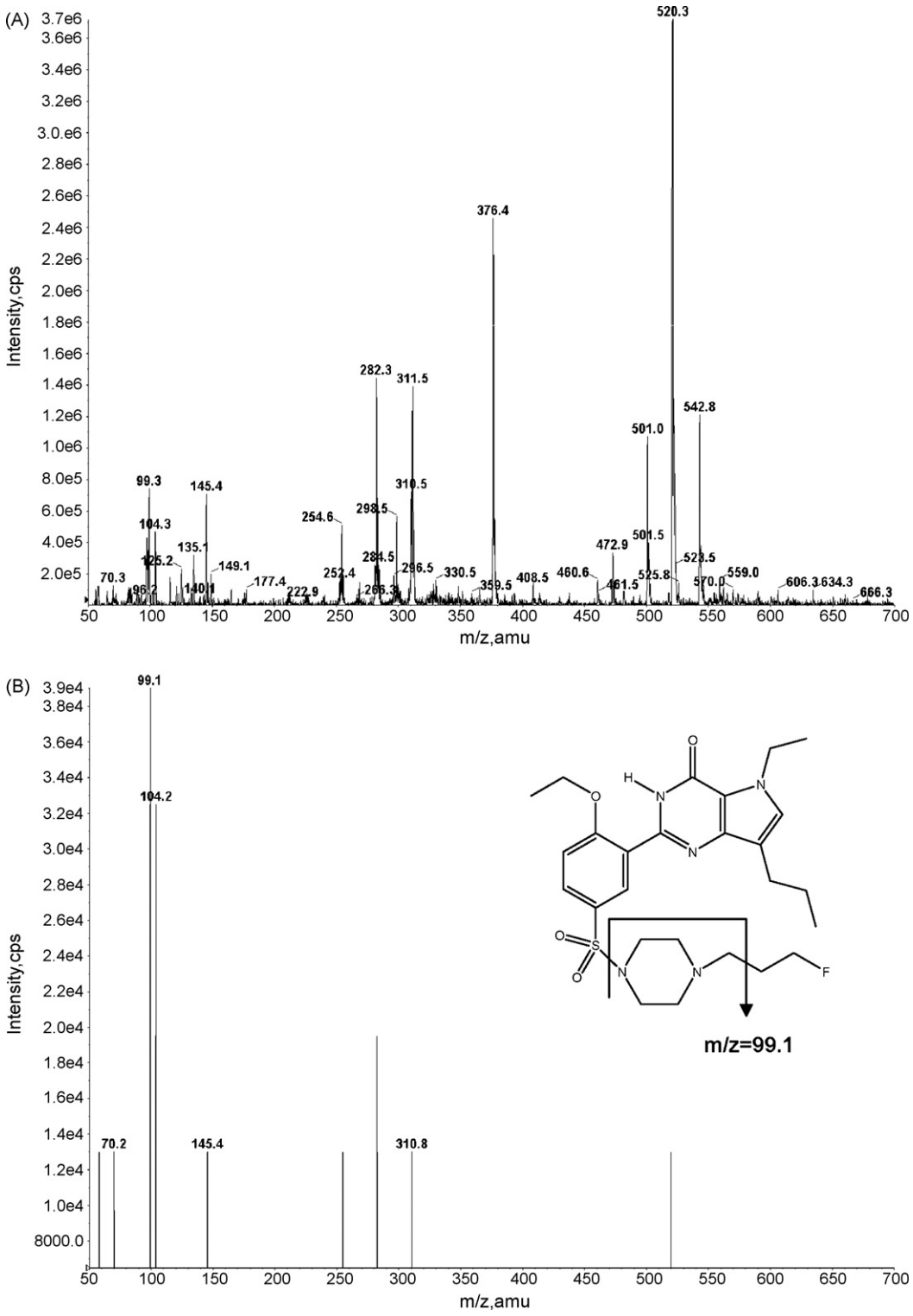


Fig. 4. (A) Positive ion Q1 mass spectrum of SK3304 (internal standard) and (B) product ion mass of protonated SK3304 ($[M+H]^+$, $m/z = 520.3$).

3.2. Assay validation

The extraction recoveries determined at 4, 200, 800 ng/ml were 96.6 ± 9.7, 94.9 ± 7.7, and $100.7 \pm 9.3\%$, respectively, for SK3530, and 111.6 ± 11.2, 106.3 ± 6.7, and $112.3 \pm 3.0\%$, respectively, for SK3541, while that of the internal standard determined at 1750 ng/ml was $99.3 \pm 7.5\%$ (Table 1). A high linearity was achieved over a concentration range from 1 to 1000 ng/ml for both SK3530 and SK3541, with correlation coefficients > 0.999. Values for the intra- and inter-day accuracy and precision are shown in Table 2. The intra- and inter-day accuracy ranged from 94.7 to 101.6% and 96.8 to 101.1%, respec-

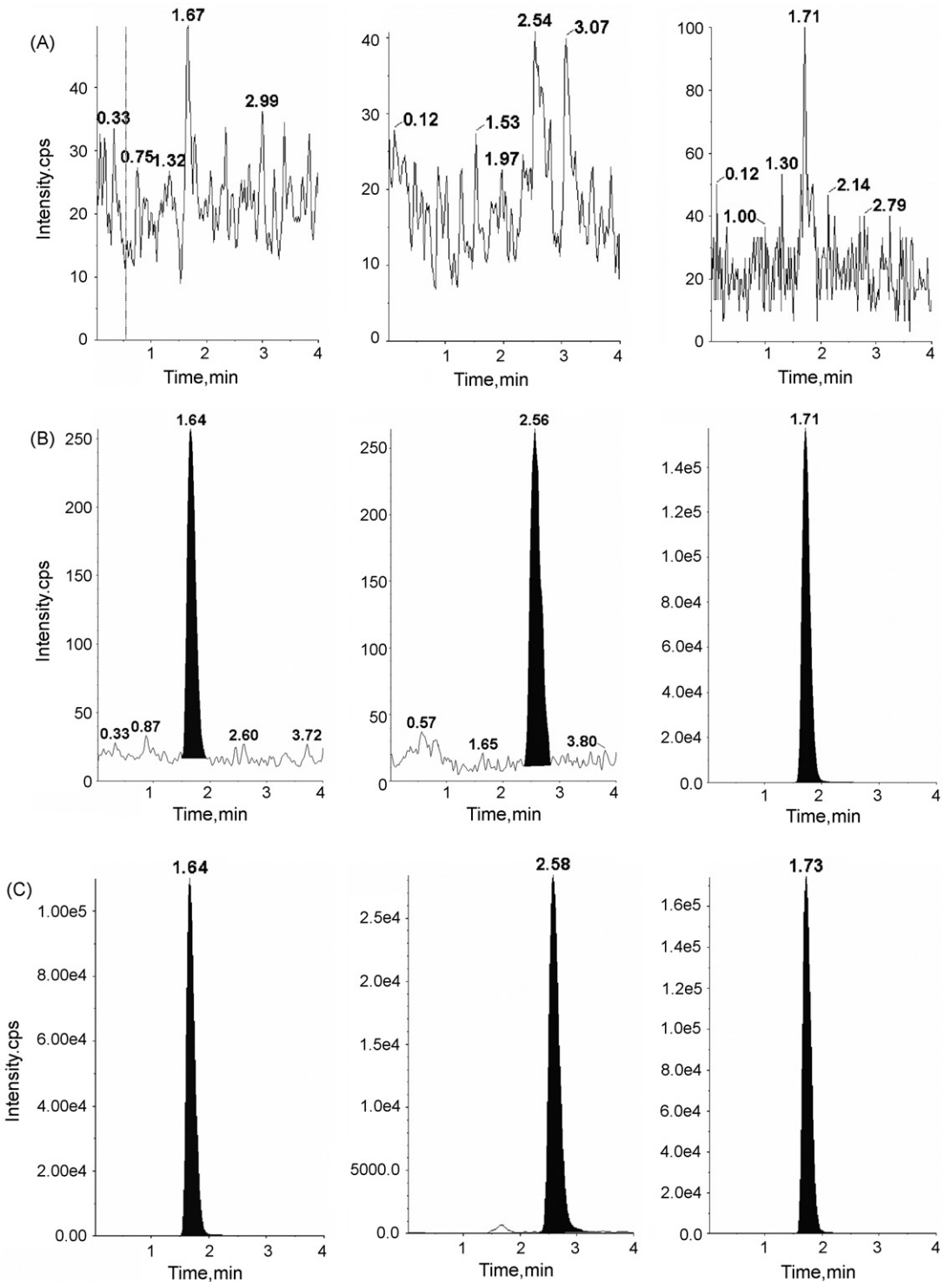


Fig. 5. MRM chromatograms of SK3530 (left) at 532.5 → 99.1, SK3541 (center) at 488.6 → 295.5, and SK3304 (right) at 520.3 → 99.1 obtained by extraction of (A) blank human plasma, (B) plasma spiked with SK3530 (1 ng/ml), SK3541 (1 ng/ml), and SK3304, and (C) plasma obtained 1.5 h after oral administration of SK3540 to a human volunteer (dose 100 mg).

tively, with their respective %CV being <12.1% and <7.4% for SK3530. For SK3541, the intra- and inter-day accuracy ranged from 92.6 to 105.7% and 97.4 to 107.8%, respectively, with their respective %CV being <13.2% and <14.1%. The LLOQ and LOD of this assay were 1 and 0.2 ng/ml, respectively, for both SK3530 and SK3541 when 0.2 ml of human plasma was used. Results of the stock solution, short-term, freeze-thaw, and auto sampler stability are shown in Table 3. The percentages of deviation of calculated versus theoretical concentrations were less than 10.6, 10.8, 6.8, and 12.0% for the stock solution, short-term, freeze-thaw, and auto sampler stability determined at three concentrations of 4, 200, and 800 ng/ml.

Table 1
Average extraction recovery ($n = 5$, mean ± S.D.) of SK3530, SK3541, and SK3304 (internal standard)

Compound	Plasma concentration (ng/ml)	Recovery (%)
SK3530	4	96.6 ± 9.7
	200	94.9 ± 7.7
	800	100.7 ± 9.3
SK3541	4	111.6 ± 11.2
	200	106.3 ± 6.7
	800	112.3 ± 3.0
SK3304	1750	99.3 ± 7.5

3.3. Application of method

The developed assay was applied to a pharmacokinetic study after oral administration of SK3530 to healthy male volunteers at a dose of 100 mg. The LLOQ of the assay was sufficient to characterize the pharmacokinetics of SK3530. The mean concentration–time profiles of SK3530 and its major metabolite, SK3541 are shown in Fig. 6. Pharmacokinetic parameters of SK3530 and SK3541 are summarized in Table 4. Upon oral administration, SK3530 was rapidly absorbed ($T_{max} = 1.0 \pm 0.3$ h) and average $t_{1/2,\lambda n}$, C_{max}, and AUC were 1.6 ± 0.4 h, 354.9 ± 162.9 ng/ml, and 984.4 ± 460.4 ng h/ml, respectively. The T_{max} of SK3541 was also short (1.1 ± 0.3 h) and average $t_{1/2,\lambda n}$, C_{max}, and AUC were 2.7 ± 0.4 h, 109.9 ± 69.4 ng/ml, and 483.5 ± 344.3 ng h/ml, respectively.

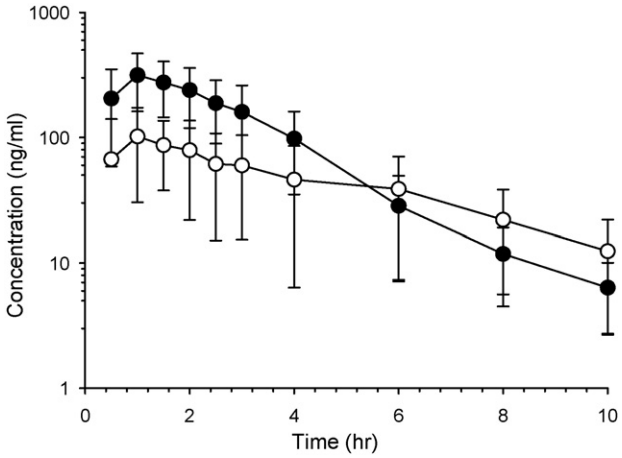


Fig. 6. Average plasma concentration–time profiles (mean ± S.D.) of SK3530 (●) and its metabolite SK3541 (◯) in healthy volunteers ($n = 12$) following oral administration of SK3530 at a dose of 100 mg.

Table 4
Pharmacokinetic parameters (mean ± S.D) of SK3530 and its metabolite, SK3541 in human volunteers ($n = 12$) obtained after oral administration of SK3530 (dose 100 mg)

Parameters	SK3530	SK3541
AUC (ng h/ml)	948.4 ± 460.4	483.5 ± 344.3
C_{max} (ng/ml)	354.9 ± 162.9	109.9 ± 69.4
T_{max} (h)	1.0 ± 0.3	1.1 ± 0.3
$t_{1/2,\lambda n}$ (h)	1.6 ± 0.4	2.7 ± 0.4

Table 2
Average intra- and inter-day accuracy (mean ± S.D.) and precision of SK3530 and SK3541 assay in human plasma

Concentration (ng/ml)	Intra-day ($n = 5$)		Inter-day ($n = 5$)	
	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
SK3530				
1	101.6 ± 12.2	12.1	98.2 ± 6.3	5.7
4	98.2 ± 6.1	7.5	101.1 ± 4.7	7.2
200	94.7 ± 5.2	7.2	96.8 ± 2.0	6.9
800	96.4 ± 5.6	7.5	99.6 ± 1.7	7.4
SK3541				
1	105.7 ± 6.4	13.2	97.4 ± 12.5	14.1
4	92.6 ± 5.8	9.9	101.2 ± 7.4	10.4
200	102.9 ± 5.0	4.9	107.8 ± 10.0	9.7
800	98.3 ± 4.6	4.6	101.9 ± 4.3	6.0

Table 3
Stability of SK3530 and SK3541

Concentration (ng/ml)	Deviation over theoretical conc. (%)			
	Stock solution	Short-term	Freeze-thaw	Auto sampler
SK3530				
4	6.8	6.7	0.2	10.3
200	5.0	−1.0	0.2	7.3
800	6.6	0.6	0.6	12.0
SK3541				
4	0.4	−1.5	5.9	−6.7
200	5.1	10.8	6.8	2.4
800	10.6	2.4	−4.2	−10.0

Given the longer elimination half-life of the metabolite and the relatively high ratios of metabolite-to-drug C_{max} (0.31) and AUC (0.49) warrants further work to determine the contribution of the metabolite to overall therapeutic outcome.

4. Conclusions

A rapid and sensitive LC/MS/MS assay method was developed for the simultaneous determination of SK3530 and its major metabolite, SK3541 in human plasma. The assay showed a wide linear dynamic range of 1–1000 ng/ml for both SK3530 and SK3541, with excellent intra- and inter-day accuracy and precision. The developed assay was successfully applied to a pharmacokinetic study in healthy male volunteers after oral administration of SK3530 (dose 100 mg).

References

[1] E. Selvin, A.L. Burnett, E.A. Platz, Am. J. Med. 120 (2007) 151–157.
[2] A. Nicolosi, E.D. Moreira Jr., M. Shirai, M.I.B.M. Tambi, D.B. Glasser, Urology 61 (2003) 201–206.
[3] C.B. Johannes, A.B. Araujo, H.A. Feldman, C.A. Derby, K.P. Kleiman, J.B. McKinlay, J. Urol. 163 (2000) 460–463.
[4] M.E. Sullivan, S.R. Keoghane, M.A. Miller, BJU Int. 87 (2001) 838–845.
[5] M.M. Fung, R. Bettencourt, E. Barrett-Connor, J. Am. Coll. Cardiol. 43 (2004) 1405–1411.
[6] E.O. Laumann, S. West, D. Glasser, C. Carson, R. Rosen, J.H. Kang, J. Sex. Med. 4 (2007) 57–65.
[7] W.D. Steers, Rev. Urol. 4 (Suppl. 3) (2002) S17–S25.
[8] G.A. Broderick, Rev. Urol. 5 (Suppl. 7) (2003) S9–S20.
[9] C.C. Carson, T.F. Lue, BJU Int. 96 (2005) 257–280.
[10] T.M. Lincoln, Mol. Pharmacol. 66 (2004) 11–13.
[11] A. Aversa, R. Bruzziches, M. Pili, G. Spera, Curr. Pharm. Des. 12 (2006) 3467–3484.
[12] M. Gupta, A. Kovar, B. Meibohm, J. Clin. Pharmacol. 45 (2005) 987–1003.
[13] R. Shabsigh, A.D. Seftel, R.C. Rosen, H. Porst, S. Ahuja, M.C. Deeley, C.S. Garcia, F. Giuliano, Urology 68 (2006) 689–696.
[14] J.G. Oh, W.J. Jang, S.C. Chi, J. Pharm. Biomed. Anal. 43 (2007) 1179–1184.
[15] J. Lee, H.H. Yoo, K.J. Rhim, D.R. Sohn, D.H. Kim, Rapid Commum. Mass Spectrom. 21 (2007) 1139–1149.