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Simultaneous assay of sildenafil and desmethylsildenafil in human plasma using liquid chromatography–tandem mass spectrometry on silica column with aqueous–organic mobile phase

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Abstract

A liquid chromatography–tandem mass spectrometry method was developed for the analysis of sildenafil (SIL) and its metabolite desmethylsildenafil (DMS) in human plasma. Samples were accurately transferred to 96-well plates using a liquid handler (Multiprobe II). Solid-phase extraction was carried out on a 96-channel programmable liquid handling workstation (Quadra 96) using a C_8 and cation-exchange mixed-mode sorbent. The extract was injected onto a silica column with an aqueous–organic mobile phase, a combination that was novel for improving the method sensitivity. The low limit of quantitation was 1.0 ng/ml for both SIL and DMS. The method was validated to meet the criteria of current industrial guidance for quantitative bioanalytical methods.

Keywords: Sildenafil; Desmethylsildenafil

1. Introduction

Sildenafil (SIL) has been widely used by men with erectile dysfunction to achieve and maintain an erection. SIL is readily absorbed and metabolized to desmethylsildenafil (DMS) by cytochrome P450 3A4 [1]. Analysis of SIL and/or DMS in dosage formulations [2,3] and biological fluids [4–7] by high-performance liquid chromatography (HPLC) and ultraviolet (UV) detection have appeared in the literature. Identification of SIL and metabolites by liquid chromatography–mass spectrometry (LC–MS) in forensic toxicology was also described [8] but quantitative LC–MS–MS methods suitable for the routine analysis of SIL and DMS have not been reported.

In this paper, we present an LC–MS–MS method for the simultaneous assay of SIL and DMS in human plasma. As a direct result of the short analysis times offered by LC–MS–MS, sample preparation has become the rate-limiting step [9]. Much effort has been devoted to automate the sample preparation step by using the 96-well plate format [10–13]. Therefore, the method described here also utilized automated sample transferring and solid-phase extraction (SPE) in the 96-well plate format.

2. Experimental

2.1. Chemicals and reagents

SIL (purity 99.9%), DMS hydrochloride salt (purity

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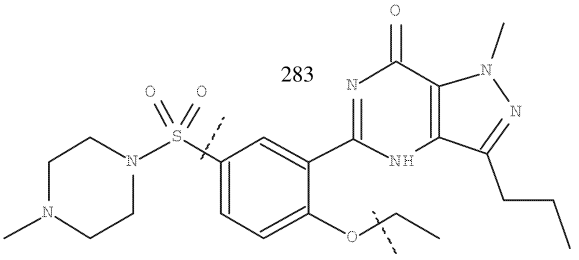
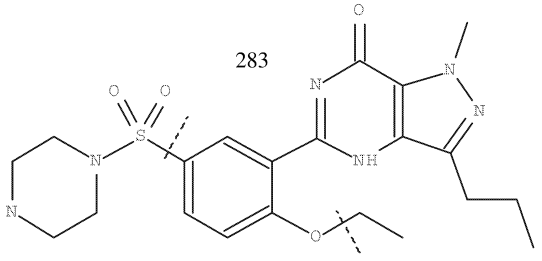
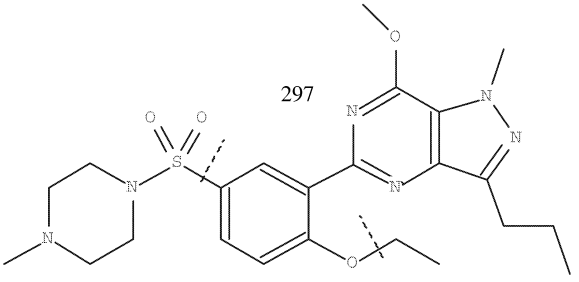
97.6%) and internal standard (I.S.; purity 97.3%) were purchased from Custom Synthesis Services (Madison, WI, USA). The chemical structures of SIL, DMS, and I.S., a derivative of SIL, are shown in Fig. 1. Acetonitrile, methanol, and water were of HPLC grade and were from Fisher Scientific (St. Louis, MO, USA). Trifluoroacetic acid (TFA) was from Sigma (St. Louis, MO, USA). Control human sodium edetate (EDTA) plasma was from Biochemed (Winchester, VA, USA) and was stored in a freezer at −20 °C.



Sildenafil



Desmethylsildenafil



Internal Standard

Fig. 1. Chemical structures of sildenafil (SIL), its metabolite desmethylsildenafil (DMS) and internal standard (I.S.). Fragmentation patterns in tandem mass spectrometry are also shown here.

2.2. Calibration standards and quality control samples (QCs)

Standards and QCs were made from two separate stock solutions (in methanol–water, 1:1, v/v) of SIL and DMS. Calibration standards at concentrations of 1.00, 2.00, 5.00, 20.0, 75.0, 200, 400 and 500 ng/ml for both SIL and DMS were prepared in blank plasma. QCs at levels of 3.00, 30.0, and 350 ng/ml for both SIL and DMS were prepared for the determination of inter-day accuracy and precision. QCs exceeding the upper limit of the calibration curve were prepared at 700 ng/ml and low limit of quantitation (LLOQ) QCs were prepared at 1.00 ng/ml. All standards and QCs were aliquoted and stored frozen at −20 °C.

2.3. LC–MS–MS

The LC–MS–MS system consisted of a Shimadzu HPLC system (Kyoto, Japan) and a Sciex API 3000 tandem mass spectrometer (Ontario, Canada) with electrospray ionization in the positive ion mode [(+)-ESI]. The analytical column, Betasil silica of 5 μm, 50 mm×3.0 mm I.D., was from Keystone Scientific (Bellefonte, PA, USA). The injection volume was 10 μl. The run time was 2.5 min and the flow-rate was 0.4 ml/min. Autosampler carry-over was determined by injecting the highest calibration standard followed by an extracted blank sample. No carry-over was observed. Without any column regeneration, one column could be used for at least 500 injections of the extracted samples.

Sensitivity of the multiple reaction monitoring (MRM) mode was optimized by infusing 0.1 μg/ml SIL and DMS in a mixture of acetonitrile–water (1:1, v/v). The ionspray needle was maintained at 5 kV. The turbo gas temperature was 400 °C and the auxiliary gas flow was 8.0 l/min. Nebulizing gas, curtain gas, and collision gas flows were at instrument settings of 12, 8, and 4, respectively. The declustering potential (DP) and focusing potential (FP) were at 46 and 200 V, respectively. The mass spectrometer was operated under MRM mode with a collision energy (CE) of 77 V for both SIL and

DMS. The transitions (precursor to product) monitored were m/z 475→283 for SIL, m/z 461→283 for DMS and m/z 489→297 for I.S.. The fragmentation patterns are shown in Fig. 1. The dwell time was 200 ms. Both quadrupoles were maintained at unit resolution.

Chromatograms were integrated using the Analyst version 1.1 software. A weighted $1/\text{concentration}^2$ linear regression was used to generate calibration curves from standards and calculate the concentrations of quality control samples.

2.4. Sample preparation

Samples were briefly vortex-mixed and centrifuged at 1140 *g* for 5 min on a Beckman J6-MC centrifuge (Fullerton, CA, USA). A 350-μl volume was then transferred from vials into 1-ml 96-well deep well plates from Porvair Sciences (Shepperton, UK) by the Packard Multiprobe II robotic liquid handler (Meriden, CT, USA) controlled by WinPrep software. Between two consecutive pipettings, the Multiprobe needles were washed with water, 0.5% TFA in acetonitrile–water (1:1, v/v), and water. Carry-over from the Multiprobe II needles was not observed. A 20-μl volume of I.S. working solution (500 ng/ml in water–methanol, 1:1) was then added to all samples except blanks. The sample plate was brought to the Tomtec Quadra 96-320 robot (Hamden, CT, USA) and automated SPE was carried out. The 25 mg Certify mixed-mode SPE Versaplate cartridge plate (Varian Sample Preparations, Walnut Creek, CA, USA) was first conditioned by 0.5 ml of methanol followed by 0.5 ml of 5% acetic acid in water. For method ruggedness test, a 25 mg Isolute mixed-mode SPE cartridge plate (Jones Chromatography, Lakewood, CO, USA) was used. Samples were mixed with 0.35 ml of 5% acetic acid in water and the mixture was loaded onto the cartridge plate. The plate was washed by 0.5 ml of 5% acetic acid in water, followed by 0.5 ml of methanol. After drying for ~3 min, samples were eluted using two portions of 0.35 ml of 2% (v/v) ammonium hydroxide in acetonitrile into another deep well collection plate. The collection plate was dried using a TurboVap 96 concentrator (Zymark, Hopkinton, MA, USA) and residue was reconstituted with 200 μl of 0.05% (v/v) TFA in acetonitrile using the Tomtec Quadra 96-320 liquid handling workstation. The advantage of using a reconstitution solution with elution strength weaker than the mobile phase has been discussed [14]. The plate was then heat-sealed with a Uniseal film (Whatman, Clifton, NJ, USA) for injection into the LC–MS–MS system.

3. Results and discussion

3.1. Method development

The selection of silica column and aqueous–organic mobile phase for quantitative analysis of SIL and DMS was based on our previous experiences [15–19]. A silica column combined with aqueous–organic mobile phase is a viable means of analyzing polar compounds in biological fluid. Use of a silica stationary phase and aqueous–organic mobile phases significantly enhance LC–MS–MS method sensitivity. Because of the basic functional groups both SIL and DMS possess, the selection of mixed-mode (reversed-phase and cation-exchange) SPE for the extraction was used. Several assays for basic compounds and metabolites have been developed in the author's group using this mixed-mode sorbent, with little or no changes to the extraction procedure described here.

The Packard Multiprobe II was programmed to aliquot samples from individual tubes to 96-well deep well plates. The plate was then brought to the Tomtec Quadra 96 for the SPE sample cleanup. This separation of the two steps not only maximized the ability of Multiprobe II's ability to accurately and flexibly transfer samples and I.S., but also fully utilized the Tomtec Quadra 96's parallel processing capability.

3.2. Validation results

The method was validated to meet the acceptance criteria of industrial guidance for bioanalytical method [20]. Recoveries were determined by comparing the peak areas of extracted standard samples with peak areas of post-extraction plasma blanks spiked at corresponding concentrations. The recoveries of SIL, DMS, and I.S. were 70, 67, and 69%, respectively. Six lots of blank control plasma were tested for

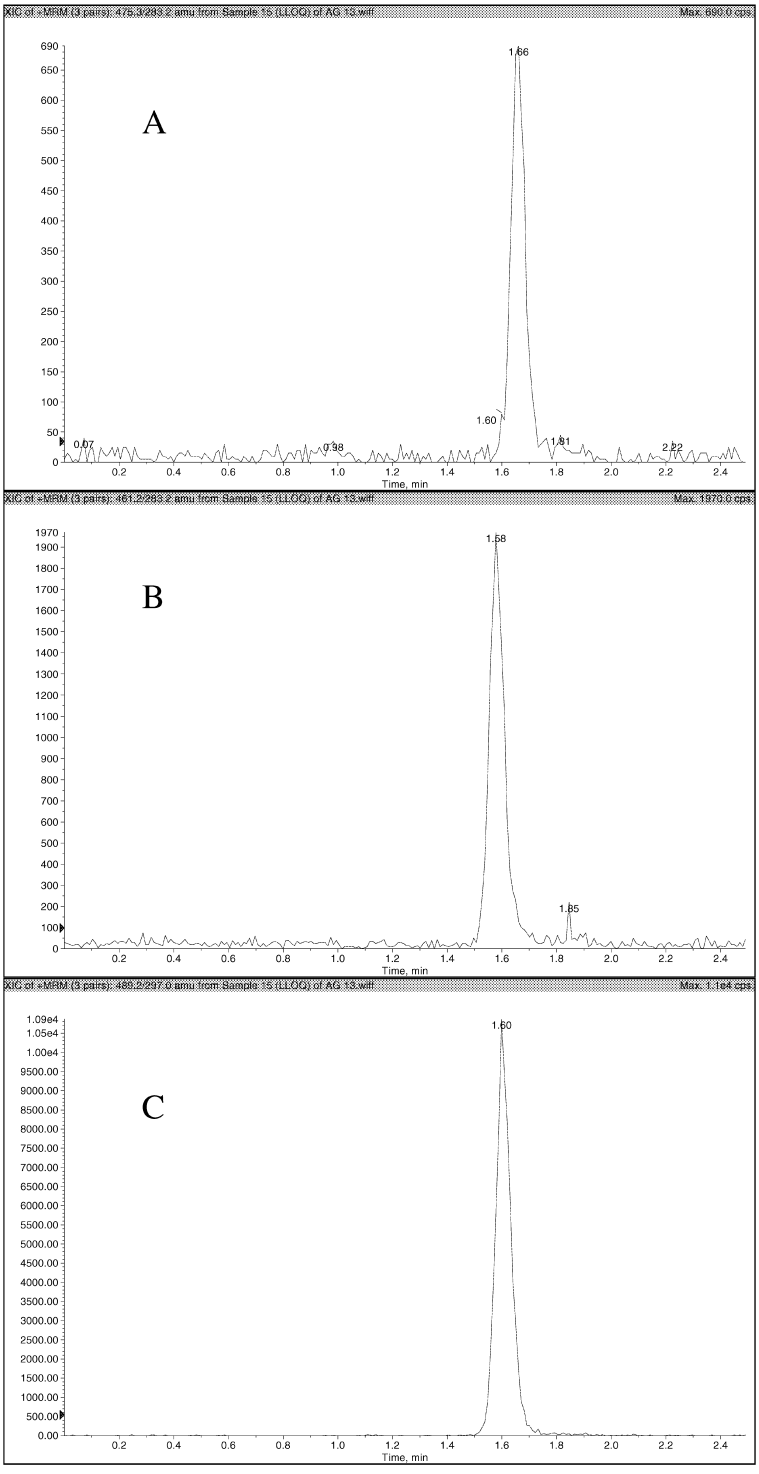


Fig. 2. Chromatogram of an extracted human plasma sample spiked with SIL/DMS at LLOQ (1.0/1.0 ng/ml) and internal standard. (A) SIL channel, 475→283; (B) DMS channel, 461→283; (C) I.S. channel, 489→297.

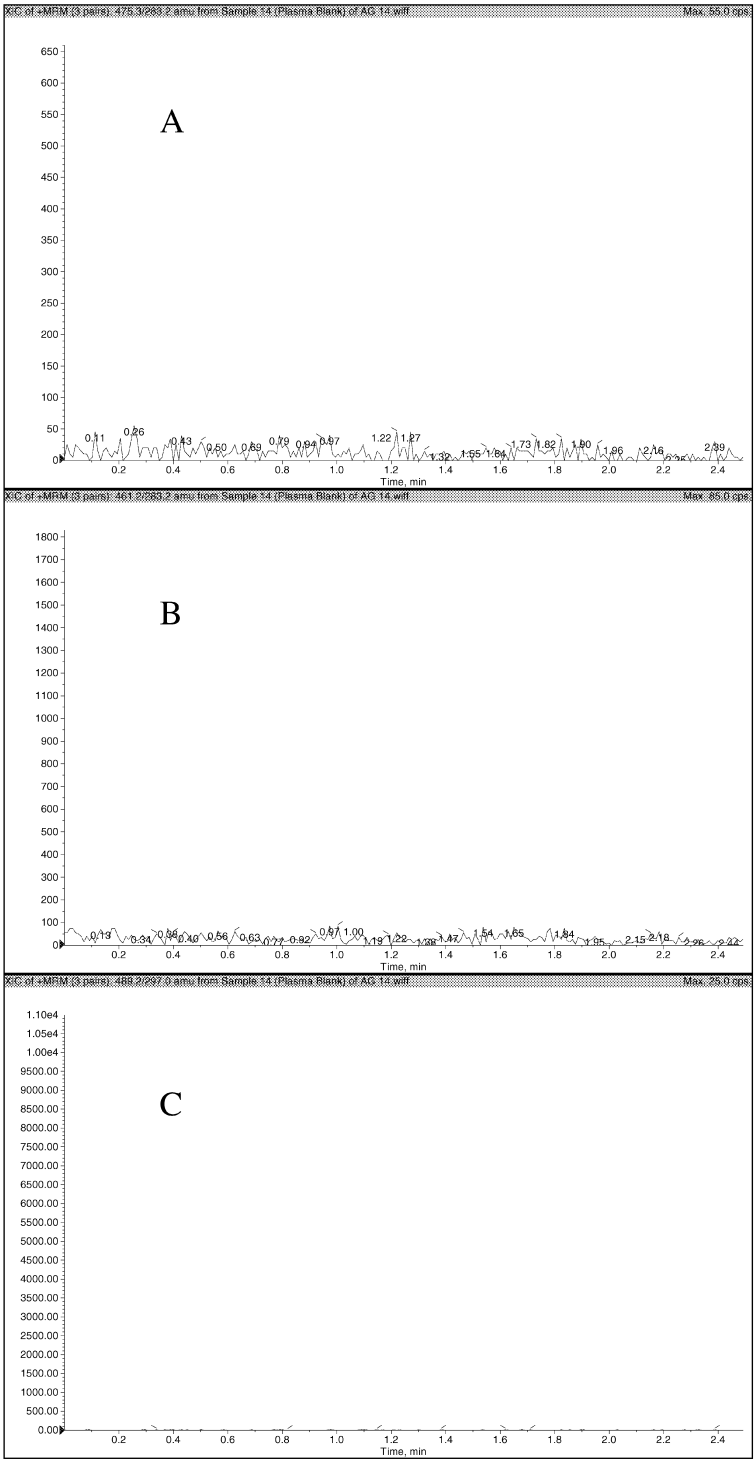


Fig. 3. Chromatogram of a blank human plasma sample. (A) SIL channel, 475→283; (B) DMS channel, 461→283; (C) I.S. channel, 489→297.

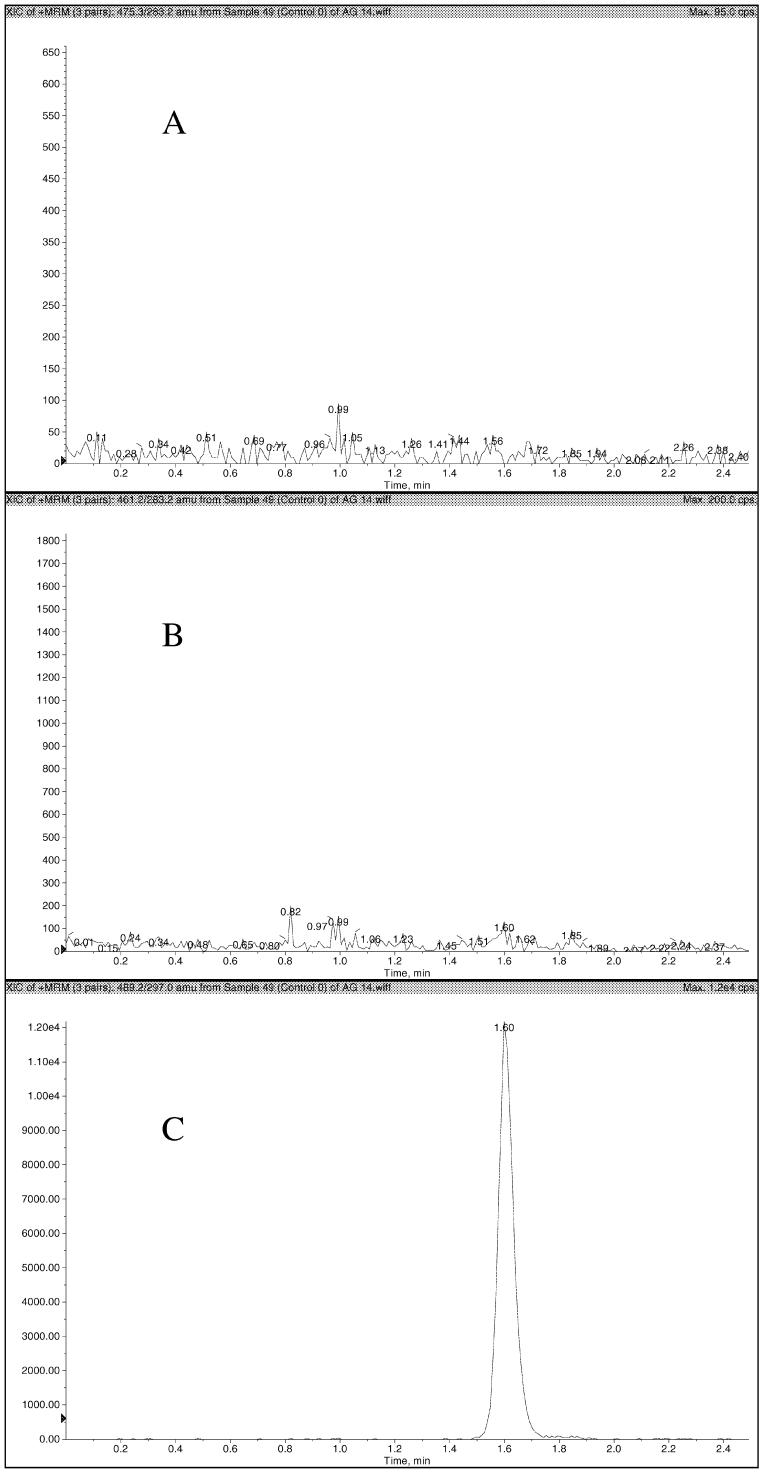


Fig. 4. Chromatogram of a blank human plasma sample spiked with only internal standard. (A) SIL channel, 475→283; (B) DMS channel, 461→283; (C) I.S. channel, 489→297.

Table 1
Precision and accuracy of calibration standards ($n=3$)

	SIL (ng/ml)								Slope	r
	1.00	2.00	5.00	20.0	75.0	200	400	500		
Mean	0.984	2.04	5.10	19.7	74.7	200	397	501	0.061	0.9988
RSD (%)	4.1	8.4	2.4	7.7	1.7	3.1	1.0	2.3	5.7	
RE (%)	−1.6	+2.0	+2.0	−1.5	−0.4	0.0	−0.8	+0.2		
	DMS (ng/ml)									
	1.00	2.00	5.00	20.0	75.0	200	400	500		
Mean	0.980	2.10	4.97	18.3	73.0	207	408	516	0.175	0.9976
RSD (%)	4.0	6.2	4.6	3.3	7.8	5.8	1.8	4.0	3.7	
RE (%)	−2.0	+5.0	−0.6	−8.5	−2.7	+3.5	+2.0	+3.2		

matrix interference. No interference peaks were observed. For QCs with the analytes at 3.00 ng/ml for both SIL and DMS, the relative standard deviations (RSDs) and relative errors (REs) were 3.9 and 9.6% and +2.0 and +0.3% for SIL and DMS, respectively. For QCs with the analytes at 350 ng/ml for both SIL and DMS, the RSDs and REs were 4.1 and 3.0% and −6.3 and −0.6%, respectively. These tight RSD and RE values indicate no significant lot-to-lot variation in matrix effects.

Representative chromatograms of an LLOQ, a blank matrix, and a control zero (blank plasma spiked with I.S. only) are shown in Figs. 2–4. The signal-to-noise ratio for SIL/DMS is about 20 at 1.0 ng/ml. When injected separately, SIL, DMS and I.S. showed no interference in the other two channels.

Calibration curve results are shown in Table 1. The low RSD values for the slope of each analyte indicated reproducible LC–MS–MS instrument conditions. The standards show a linear range of 1–500 ng/ml for both SIL and DMS. The precision and accuracy data for QCs are summarized in Table 2. The data show that this method is consistent and reliable with low RSD and RE values. At the LLOQ, the RSDs ($n=6$) of the measured concentration were 4.4 and 4.6% for SIL and DMS, respectively. The REs of the mean of the measured concentrations were +2.0 and +4.0% for SIL and DMS, respectively.

Stabilities of sample processing (freeze–thaw, bench-top and storage), and chromatography (extracts) were tested and established (Table 3). Three freeze–thaw cycles and ambient temperature storage of the QCs for up to 24 h prior to analysis, appeared to have little effect on the quantitation. QCs stored in a freezer at −20 °C remained stable for at least 3 months. Extracted calibration standards and QCs were allowed to stand at ambient temperature for 48 h prior to injection. No effect on quantitation of the calibration standards or QCs was observed. When

Table 2
Precision and accuracy of quality control samples

	Intra-day ($n=6$)						Inter-day ($n=18$)		
SIL (ng/ml)	1.00	3.00	35.0	350	350*	700*	3.00	30.0	350
Mean	1.02	3.06	29.9	338	367	752	3.14	30.2	345
RSD (%)	4.4	3.4	3.6	2.8	4.9	3.3	7.0	5.1	4.3
RE (%)	+2.0	+2.0	−0.3	−3.4	+4.9	+7.4	+4.7	+0.7	−1.4
DMS (ng/ml)	1.00	3.00	35.0	350	350*	700*	3.00	3.00	350
Mean	1.04	3.15	30.1	337	372	764	3.10	28.3	343
RSD (%)	4.6	4.5	3.5	3.2	6.8	4.0	8.0	8.2	5.8
RE (%)	+4.0	+5.0	+0.3	−3.7	+6.3	+9.1	+3.3	−5.7	−2.0

*Samples were diluted fivefold with blank plasma prior to analysis

Table 3
Stability of the samples

	Concentration (ng/ml)			
	SIL		DMS	
	3.00	350	3.00	350
Three freeze–thaw cycles				
Mean (n=6)	3.14	338	3.16	344
RSD (%)	4.1	4.8	4.8	5.7
RE (%)	+4.7	−3.4	+5.3	−1.7
24 h bench-top				
Mean (n=6)	3.25	340	3.10	351
RSD (%)	4.0	2.3	5.6	4.4
RE (%)	+8.3	−2.9	+3.3	+0.3
3 months at −20 °C				
Mean (n=3)	2.96	365	2.92	382
RSD (%)	11	6.1	9.6	6.2
RE (%)	−1.3	+4.3	−2.7	+9.1
48 h extract				
Mean (n=6)	3.06	350	3.14	351
RSD (%)	4.0	4.0	6.4	4.5
RE (%)	+2.1	0	+4.6	−1.1

stock standard solutions in a mixture of methanol–water (1:1, v/v) were stored at 2–8 °C, the analytes were stable for at least 3 months.

The method robustness was demonstrated by using multiple analytical columns and multiple LC–MS–MS instruments. Mixed-mode SPE cartridges from another vendor (Isolute) were also evaluated by using the same method and acceptable results (RSD<5.2% and RE<3.3%) and were obtained. This method has been successfully used to analyze samples from clinical trials of drug–drug interaction studies. The selectivity of the method towards the test materials was established by fortifying the blank control plasma and SIL/DMS QCs with the test materials. No interference peak or quantitation bias was observed with the addition of test materials.

4. Conclusion

A sensitive and reliable LC–MS–MS method for the measurement of SIL and its metabolite DMS in human sodium EDTA plasma has been successfully developed and validated. SPE in the 96-well format was used to extract SIL and DMS from the plasma. A silica column and an aqueous–organic mobile phase were used to improve the sensitivity. The LLOQ is 1.00 ng/ml for both SIL and DMS. The automated nature of the method significantly improved the sample analysis throughput and method reliability.

References

[1] Physicians' Desk Reference, 54th ed., Medical Economics Company, Montvale, NJ, 2000, p. 2382.
[2] A.I. Segall, M.F. Vitale, V.L. Perez, M.L. Palacios, M.T. Pizzorno, J. Liq. Chromatogr. 23 (2000) 1377.
[3] N. Daraghmeh, M. Al-Omari, A.A. Badwan, A.M.Y. Jaber, J. Pharm. Biomed. Anal. 25 (2001) 483.
[4] H.S. Lee, M.-S. Jang, C.K. Chang, W.B. Kim, H.-Y. Lee, J. Chromatogr. B 752 (2001) 141.
[5] N. Daraghmeh, M. Al-Omari, A.A. Badwan, A.M.Y. Jaber, J. Pharm. Biomed. Anal. 25 (2001) 483.
[6] I.C. Bhoir, S.I. Bhoir, V.R. Bari, A.M. Bhagwat, M. Sundaresan, Indian Drugs 37 (2000) 319.
[7] J.D. Cooper, D.C. Muirhead, J.E. Taylor, P.R. Baker, J. Chromatogr. B 701 (1997) 87.
[8] W. Weinmann, M. Bohnert, A. Wiedemann, M. Renz, N. Lehmann, S. Pollak, Int. J. Legal Med. 114 (2001) 252.
[9] J. Janiszewski, R.P. Schneider, K. Hoffmaster, M. Swyden, D. Wells, H. Fonda, Rapid Commun. Mass Spectrom. 11 (1997) 1033.
[10] J.P. Allanson, R.A. Biddlecombe, A.E. Jones, S. Pleasance, Rapid Commun. Mass Spectrom. 10 (1996) 811.
[11] H. Simpson, A. Berthemy, D. Buhrman, R. Burton, J. Newton, M. Kealy, D. Wells, D. Wu, Rapid Commun. Mass Spectrom. 12 (1998) 75.
[12] D.T. Rossi, LC·GC 17 (1999) S4.
[13] N.H. Huang, J.R. Kagel, D.T. Rossi, J. Pharm. Biomed. Anal. 19 (1999) 613.
[14] W. Naidong, Y.-L. Chen, W.Z. Shou, X. Jiang, J. Pharm. Biomed. Anal. 26 (2001) 753.
[15] W. Naidong, J.W. Lee, X. Jiang, M. Wehling, J.D. Hulse, P.P. Lin, J. Chromatogr. B 735 (1999) 255.
[16] W. Naidong, X. Jiang, K. Newland, R. Coe, P.P. Lin, J.W. Lee, J. Pharm. Biomed. Anal. 23 (2000) 697.
[17] W. Naidong, W.Z. Shou, Y.-L. Chen, X. Jiang, J. Chromatogr. B 754 (2001) 387.
[18] W.Z. Shou, M. Pelzer, T. Addison, X. Jiang, W. Naidong, J. Pharm. Biomed. Anal. 27 (2002) 143–152.
[19] W.Z. Shou, X. Jiang, B.D. Beato, W. Naidong, Rapid Commun. Mass Spectrom. 15 (2001) 466.
[20] Guidance for Industry—Bioanalytical Method Validation, US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER) and Center for Veterinary Medicine (CVM), May 2001, http://www.fda.gov/cder/guidance/index.htm.