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Short communication

Simplified method for determination of clarithromycin in human plasma using protein precipitation in a 96-well format and liquid chromatography–tandem mass spectrometry

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ABSTRACT

A simplified method to determine clarithromycin concentrations in human plasma using protein precipitation in a 96-well plate and liquid chromatography–tandem mass spectrometry was developed and validated. Plasma proteins were precipitated with acetonitrile and roxithromycin was used as the internal standard. After vortex mixing and centrifugation, the supernatants were directly injected onto a Phenomenex Luna Phenyl–Hexyl column (50 mm × 2.0 mm ID, 3 μm). The mobile phase consisted of water and methanol (30:70, v/v) containing 0.1% formic acid and 5 mM ammonium acetate. The flow rate was 0.22 mL/min and the total run time (injection to injection) was less than 3 min. Detection of the analytes was achieved using positive ion electrospray tandem mass spectrometry in selected reaction monitoring (SRM) mode. The linear standard curve ranged from 100 to 5000 ng/mL and the precision and accuracy (inter- and intra-run) were within 7.9% and 4.9%, respectively. The method was successfully used to determine clarithromycin concentrations in human plasma samples obtained from healthy subjects who were given clarithromycin 500 mg for 3 days. The method is rapid, simple, precise and directly applicable to clarithromycin pharmacokinetic studies.

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1. Introduction

Clarithromycin is a macrolide antibiotic that is commonly used to treat infections such as pneumonia, bronchitis, sinusitis, and otitis media; it is also used in combination drug regimens to treat *Helicobacter pylori* infections [1,2]. Clarithromycin is mainly metabolized by cytochrome P450 (CYP) 3A enzymes (CYP3A4 and CYP3A5) to an active 14-hydroxy metabolite [3]. Clarithromycin is a strong mechanism-based CYP3A inhibitor [4] and has been used as an inhibitor in drug–drug interaction studies [5–7].

Clarithromycin concentrations in human plasma have been determined by high-performance liquid chromatography (HPLC) with electrochemical [8–17], fluorescent (after derivatization) [18], ultraviolet [19,20], and mass spectrometric detection [21–24]. Most of the non-MS-based methods suffer from long run times (>7 min) and require large sample volumes (≥0.5 mL). In addition, most of the methods involve liquid–liquid extraction, which is often laborious and time-consuming; none of the methods use sample preparation in a convenient 96-well plate format.

Here we report a simplified liquid chromatography–tandem mass spectrometry assay using protein precipitation in a 96-well plate, which allows fast and reliable determination of clarithromycin plasma concentrations using a very small volume of sample (25 μL).

2. Experimental

2.1. Chemicals and reagents

Clarithromycin reference standard (97.7% purity) was obtained from the United States Pharmacopoeia (Rockville, MD, USA). Roxithromycin (>90% purity) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Ammonium acetate, formic acid (88%), and acetonitrile were obtained from VWR (West Chester, PA, USA). Methanol was purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA). Blank plasma was obtained from UF & Shands Hospital (Gainesville, FL, USA). HPLC grade deionized water was obtained from a Barnstead Nanopure Diamond UV Ultrapure Water System (Dubuque, IA, USA).

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2.2. Preparations of standards and quality control (QC) samples

A stock solution of clarithromycin was prepared at a concentration of 1 mg/mL in acetonitrile and stored in a glass vial at 4 °C. Dilutions prepared in acetonitrile at concentrations of 25, 100 and 500 μg/mL were used to prepare calibration standards and quality control (QC) samples. Blank plasma was spiked with appropriate stock solutions. The total volume of acetonitrile in the standards and QC samples was less than 2%. Six calibration standards were prepared at concentrations of 100, 250, 500, 1000, 2500 and 5000 ng/mL. QC samples were prepared at concentrations of 200 ng/mL (low QC), 2000 ng/mL (medium QC) and 4000 ng/mL (high QC). A dilution QC was prepared at a concentration of 10,000 ng/mL. Standards and QC samples were prepared at the beginning of the validation, aliquoted into 1.5 mL microcentrifuge tubes (Thermo Fisher Scientific, Waltham, MA, USA) and stored at −20 °C until analysis. A stock solution (1 mg/mL) of the internal standard (ISTD) roxithromycin was prepared in acetonitrile and was further diluted with acetonitrile to 1 μg/mL and used for all analyses. The ISTD stock solutions were stored in glass vials at 4 °C.

2.3. Sample preparation

Frozen samples were thawed at room temperature. An aliquot (25 μL) of plasma was transferred to a well of a 96-well polypropylene 2.0 mL deepwell plate with conical bottom (Thermo Fisher Scientific, Waltham, MA, USA). The plasma was combined with acetonitrile (200 μL) and ISTD (10 μL or 10 ng) (except for the double blank without ISTD) and the plate was sealed and vortex mixed briefly. Then, deionized water (500 μL) was added to each well and the plate was sealed, vortex mixed briefly, and centrifuged for 10 min at 2630 × *g* in an Eppendorf 5810R centrifuge equipped with a four-place microplate swing-bucket rotor (Eppendorf North America, Westbury, NY, USA). The plate was put in the autosampler and an aliquot (5 μL) was injected onto the analytical column. The autosampler needle depth was adjusted so that the needle was in the upper portion of the supernatant and the precipitated proteins were not disturbed.

2.4. LC–MS/MS conditions

The LC–MS/MS system consisted of a Surveyor HPLC autosampler, Surveyor MS quaternary pump and a TSQ Quantum Discovery triple quadrupole mass spectrometer (Thermo Scientific, San Jose, CA, USA). The TSQ Quantum mass spectrometer was equipped with an electrospray (ESI) ion source and operated in the positive mode. The ESI source spray was set orthogonal to the ion transfer tube. The mass spectrometer was calibrated with a solution of polytyrosine-1,3,6 according to the manufacturer. The MS/MS conditions were optimized by infusing clarithromycin in the mobile phase. The ESI source parameters were tuned for maximum abundance of $[M+H]^+$ ions of clarithromycin and roxithromycin at the LC flow rate of 0.22 mL/min. For quantification, the TSQ Quantum was operated in high-resolution selected reaction monitoring (SRM) mode. The acquisition parameters were: spray voltage 5.0 kV, source CID 5 V, and ion transfer tube temperature at 350 °C. Nitrogen was used as the sheath and auxiliary gas and set to 35 and 10 (arbitrary units), respectively. The argon collision gas pressure was set to 1.5 mTorr. The collision energy was 34 eV for clarithromycin and 25 eV for roxithromycin. The SRM scheme followed transitions of the $[M+H]^+$ precursor to selected product ions with the following values: m/z 748.5 → 157.9 for clarithromycin and m/z 837.5 → 679.4 for roxithromycin. The instrument was operated in enhanced (high) resolution mode with the peak width (full width at half maximum, FWHM) set to 0.2 m/z at Q1 and to 0.7 m/z at Q3. The scan time was 250 ms for each transition. Data were acquired and processed using ThermoFinnigan XCalibur® software version 1.4, service release 1 (Thermo Scientific, San Jose, CA, USA).

2.5. Chromatographic conditions

Chromatographic separations were performed using a Phenomenex (Torrance, CA, USA) Luna Phenyl–Hexyl, 50 mm × 2.0 mm ID, 3 μm analytical column at ambient temperature protected by an inline stainless steel precolumn filter (0.5 μm). The mobile phase used for the analysis was 0.1% formic acid, 5 mM ammonium acetate in deionized water and methanol (30:70, v/v) delivered at a flow rate of 0.22 mL/min. The mobile phase was degassed and filtered through a 0.22-μm Nylon 66 membrane before use. The autosampler sample tray was maintained at 10 °C.

2.6. Standard curve

Duplicate calibration curves were analyzed each run with the lowest standard concentration (100 ng/mL) prepared in triplicate. For each standard calibration curve, the clarithromycin peak area to ISTD peak area ratio was calculated and plotted against nominal clarithromycin concentrations. Weighted ($1/\text{concentration}^2$) linear regression analysis was used to construct calibration curves from the standards. The regression equations were used to calculate the concentrations in QC and clinical samples.

2.7. Method validation

The current LC–MS/MS method was validated for precision, accuracy, linearity, dilution integrity, selectivity, carry-over, matrix effect, recovery, and stability. Precision and accuracy were determined by the analysis of clarithromycin QC samples spiked at concentrations of 200, 2000 and 4000 ng/mL. Six of each QC level was analyzed for two runs and twelve of each QC level was analyzed for one run. The difference in the calculated mean concentration relative to the spiked concentration was used to express accuracy as the relative error (RE, %). Means, standard deviations and relative standard deviations (RSD, %) were calculated from the QC values and used to estimate the intra- and inter-run precision. A dilution QC was prepared at a concentration of 10,000 ng/mL and six replicates were processed after a 10-fold dilution. The mean accuracy was expressed as the RE%.

Selectivity was evaluated by processing and analyzing blank plasma obtained from six different sources. Carry-over was evaluated by placing vials of blank mobile phase at several locations in the analysis set including after high standards. The potential for a matrix effect (suppression or enhancement of ionization) was evaluated by continuous infusion of clarithromycin post-column and injection of processed plasma samples from six different sources [25]. In addition, the matrix effect was evaluated by comparing the clarithromycin peak areas of blank samples spiked post-processing with corresponding peak areas of standards prepared in the injection solution. Recovery was determined by comparing the response obtained in (A) QC samples processed normally and (B) the supernatant from processed blank plasma samples, which was spiked to contain clarithromycin and roxithromycin at concentration values corresponding to the QC concentrations (reference samples). Responses obtained from the reference samples were defined as 100%.

Processing and analysis of QC samples tested the stability of the analytes (post-processing). The stability of the extracts in the autosampler was tested after the extracts were left in the autosam-

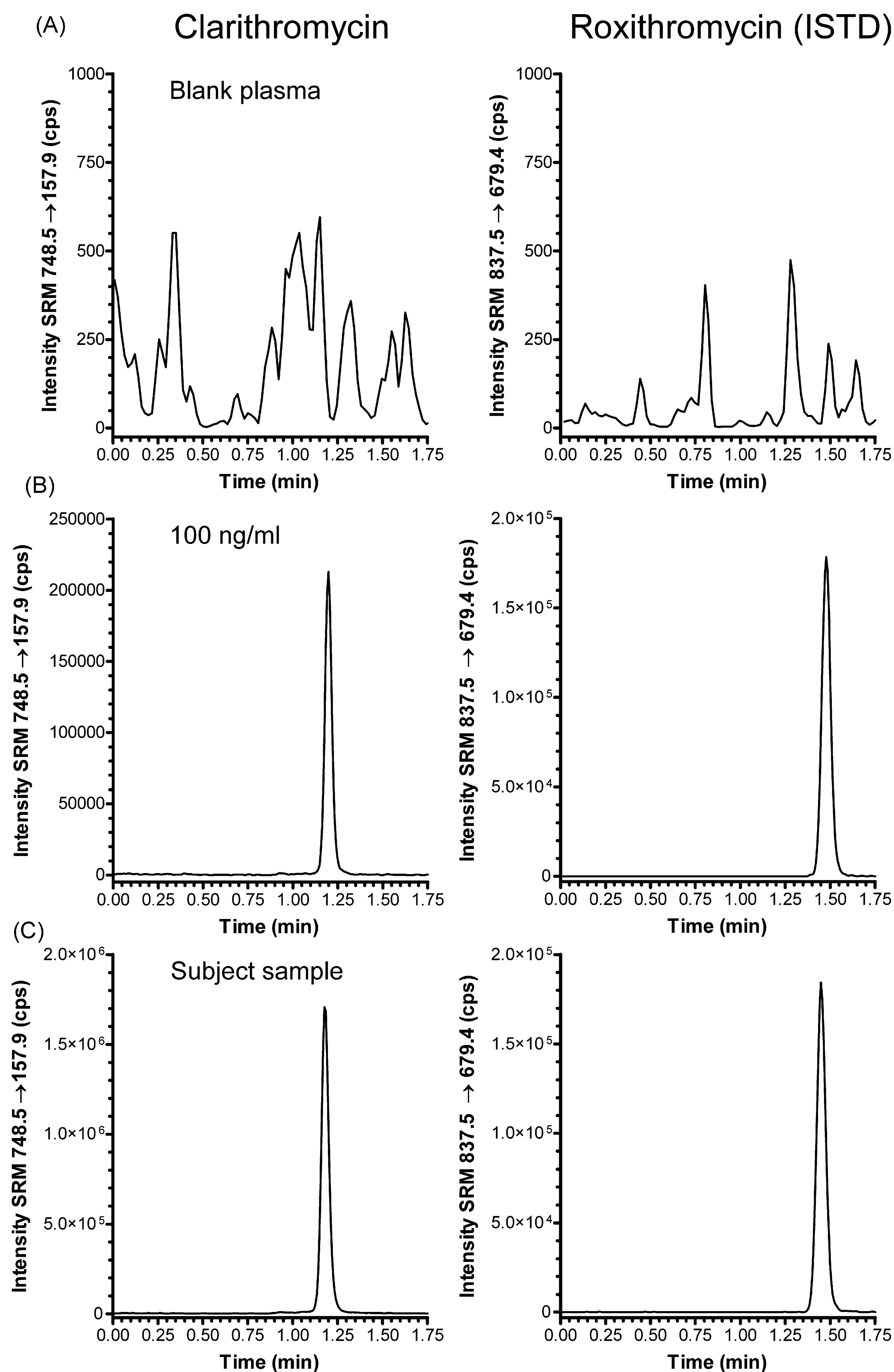

Fig. 1. The extracted LC–MS/MS chromatograms of: (A) double blank plasma (no clarithromycin or ISTD); (B) clarithromycin lowest standard (100 ng/mL); (C) plasma sample from a healthy volunteer obtained 3.0 h after oral administration of clarithromycin 500 mg (concentration = 2407 ng/mL). Clarithromycin is shown in the left column and the internal standard roxithromycin is shown in the right column.

pler at 10 °C for at least 24 h. The effect of freeze and thaw on the stability of the samples was tested using low and high concentration QC samples. Three aliquoted tubes of each QC were stored at 4 °C as a reference and the other tubes were subjected to three freeze–thaw cycles prior to processing and analysis. The thawed samples were processed and analyzed as described above. Effects of freeze and thaw were measured by concentrations of each QC sample relative to the reference.

2.8. Application to plasma sampling

The method was used for the analysis of plasma samples obtained from healthy volunteers who were given multiple doses of clarithromycin 500 mg. The protocol was approved by the University of Florida Institutional Review Board and all study subjects provided written informed consent. Clarithromycin 500 mg (Roxane Laboratories Inc., Columbus, OH, USA) was given orally at the General Clinical Research Center at the University of Florida after a 3-day pretreatment with clarithromycin 500 mg twice daily. Blood samples were collected in tubes containing sodium heparin at multiple time points within the dosing interval; plasma harvested by centrifugation was stored at −20 °C until analyzed. Plasma concentrations of clarithromycin were determined as described above. A linear one compartment model with first order absorption was used to describe clarithromycin pharmacokinetics [26]. The maximum clarithromycin concentration (C_{max}), time at which C_{max} occurred (T_{max}), apparent oral clearance (CL/*F*, where *F* is bioavailability), and half-life are reported. The pharmacokinetic parameter estimates were calculated using WinNonlin software (version 2.1, Pharsight Corporation, Mountain View, CA, USA).

3. Results and discussion

3.1. Chromatographic method

Representative LC–MS/MS chromatograms of plasma samples are shown in Fig. 1. Fig. 1A shows a double blank plasma sample (no clarithromycin or ISTD), Fig. 1B is a plasma sample spiked with clarithromycin 100 ng/mL (limit of quantitation, LOQ) and roxithromycin (ISTD; 10 ng/mL), and Fig. 1C depicts a plasma sample obtained 3 h after the seventh dose of clarithromycin 500 mg. Retention times were approximately 1.2 and 1.5 min for clarithromycin and roxithromycin, respectively. The peaks of interest were well separated and free from interference with endogenous substances.

3.2. Method validation

3.2.1. Linearity, precision, accuracy, and dilution integrity

Linear calibration curves ($n = 6$) with a mean correlation coefficient of 0.995 were obtained for clarithromycin over the concentration range of 100–5000 ng/mL. The LOQ was set at 100 ng/mL for which the signal-to-noise ratio was greater than 2000:1. A lower LOQ was not necessary for our application but may be desirable for single-dose pharmacokinetic studies. During pre-validation a standard of 25 ng/mL (signal-to-noise ratio greater than 500:1) was successfully used but was not included later because of the concentration values expected in our study.

The accuracy and precision data from QC samples demonstrate suitability of the method (Table 1). Intra- and inter-run precision (RSD, %) was ≤7.9% and accuracy (RE, %) was within ±4.9% (Table 1). The dilution QC (10,000 ng/mL) was processed after a 10-fold dilution with blank plasma to determine dilution integrity. The mean concentration found for the dilution QC was 10,426 ng/mL. The mean precision was 4.4% and the accuracy was 4.3%, which were within the acceptance criteria (<15%).

Table 1
Intra- and inter-run precision (%RSD) and accuracy (%RE) for clarithromycin quality control samples in human plasma

Concentration (ng/mL)		Precision RSD (%)	Accuracy RE (%)
Nominal	Observed (mean ± SD)		
Intra-run ($N = 12$)			
200.0	193.7 ± 14.71	7.6	−3.1
2000	1903 ± 121.8	6.4	−4.9
4000	4144 ± 291.9	7.0	3.6
Inter-run ($N = 24$)			
200.0	191.6 ± 14.20	7.4	−4.2
2000	1906 ± 149.7	7.9	−4.7
4000	4004 ± 281.1	7.0	0.1

3.2.2. Selectivity, matrix effect, recovery, and stability

No endogenous interference with clarithromycin or roxithromycin was detected in six different sources of blank plasma and there was no evidence of sample carry-over. The post-column infusion experiments used to assess a matrix effect showed no evidence of a change in signal in the regions where clarithromycin and roxithromycin eluted. Further, the matrix effect assessed by spiking samples post-processing showed <10% difference from spiked injection solvent. The mean extraction recoveries for clarithromycin were 78.3 ± 1.4% and 90.5 ± 6.4% at concentrations of 200 and 4000 ng/mL, respectively. The extraction recovery for the roxithromycin was 98.1 ± 5.4%.

Clarithromycin and roxithromycin were both stable in processed samples held in the autosampler at 10 °C for at least 24 h with mean recoveries within 10% of the nominal concentration. No degradation of clarithromycin was observed in the samples subjected to three freeze–thaw cycles with concentration values being 93.6–102.9% of the nominal concentration.

3.3. Method application

The mean clarithromycin concentration–time profile obtained from six healthy volunteers after an oral dose of clarithromycin is shown in Fig. 2. The observed C_{max} concentration of

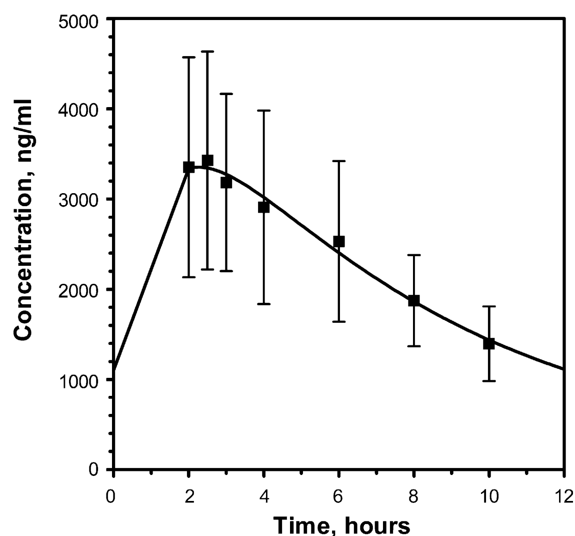

Fig. 2. Mean (±SD) concentration–time profile of clarithromycin after multiple dose clarithromycin 500 mg in six healthy volunteers. The solid line represents the best fit to the data.

3470 ± 1160 ng/mL occurred at 2.4 ± 0.4 h. The clearance of clarithromycin after oral drug administration (CL/*F*) was 19.5 ± 5.5 L/h and the estimated half-life was 5.5 ± 1.3 h. The concentration of clarithromycin in all of the samples was greater than the LOQ, which was set at 100 ng/mL. The results demonstrate that the assay is suitable for pharmacokinetic studies of clarithromycin in human subjects.

4. Conclusions

We have developed and validated a simplified LC–MS/MS method for clarithromycin using protein precipitation in a 96-well format plate. Sample preparation in the 96-well format and the short cycle time made the analysis of three to four batches of 96 samples per day feasible. The method requires only a small amount of plasma (25 μL) and can be applied to quantitate concentrations of clarithromycin in human plasma samples. The method was shown to be rapid, sensitive, selective, and reproducible.

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References

[1] M.H. Gotfried, Chest 125 (2004) 52S.
[2] W. Malaty, S. Stigleman, L.B. Hansen, J. Fam. Pract. 52 (2003) 799.
[3] A.D. Rodrigues, E.M. Roberts, D.J. Mulford, Y. Yao, D. Ouellet, Drug Metab. Dispos. 25 (1997) 623.
[4] S.F. Zhou, C.C. Xue, X.Q. Yu, C. Li, G. Wang, Ther. Drug Monit. 29 (2007) 687.
[5] B.J. Gurley, A. Swain, M.A. Hubbard, F. Hartsfield, J. Thaden, D.K. Williams, W.B. Gentry, Y. Tong, Clin. Pharmacol. Ther. 83 (2008) 61.
[6] A.G. Pinto, Y.H. Wang, N. Chalasani, T. Skaar, D. Kolwankar, J.C. Gorski, S. Liangpunsakul, M.A. Hamman, M. Arefayene, S.D. Hall, Clin. Pharmacol. Ther. 77 (2005) 178.
[7] S.K. Quinney, B.D. Haehner, M.B. Rhoades, Z. Lin, J.C. Gorski, S.D. Hall, Br. J. Clin. Pharmacol. 65 (2008) 98.
[8] A. Pappa-Louisi, A. Papageorgiou, A. Zitrou, S. Sotiropoulos, E. Georgarakis, F. Zougrou, J. Chromatogr. B 755 (2001) 57.
[9] Y.G. Kim, H.J. Kim, J.W. Kwon, W.B. Kim, M.G. Lee, Int. J. Clin. Pharmacol. Ther. 39 (2001) 356.
[10] I. Niopas, A.C. Daftsios, Biomed. Chromatogr. 15 (2001) 507.
[11] M. Lohitnavy, O. Lohitnavy, S. Wittaya-areekul, K. Sareekan, S. Polnok, W. Chaiyaput, Drug Dev. Ind. Pharm. 29 (2003) 653.
[12] S.Y. Chu, L.T. Sennello, R.C. Sonders, J. Chromatogr. 571 (1991) 199.
[13] M. Hedenmo, B.M. Eriksson, J. Chromatogr. A 692 (1995) 161.
[14] I. Kanfer, M.F. Skinner, R.B. Walker, J. Chromatogr. A 812 (1998) 255.
[15] F. Kees, S. Spangler, M. Wellenhofer, J. Chromatogr. A 812 (1998) 287.
[16] T.D. Rotsch, M. Spanton, P. Cugier, A.C. Plasz, Pharm. Res. 8 (1991) 989.
[17] J.I. Wibawa, P.N. Shaw, D.A. Barrett, J. Chromatogr. B 783 (2003) 359.
[18] G. Bahrami, B. Mohammadi, J. Chromatogr. B 850 (2007) 417.
[19] H. Amini, A. Ahmadiani, J. Chromatogr. B 817 (2005) 193.
[20] P.O. Erah, D.A. Barrett, P.N. Shaw, J. Chromatogr. B 682 (1996) 73.
[21] Y. Jiang, J. Wang, H. Li, Y. Wang, J. Gu, J. Pharm. Biomed. Anal. 43 (2007) 1460.
[22] W. Li, J. Rettig, X. Jiang, D.T. Francisco, W. Naidong, Biomed. Chromatogr. 20 (2006) 1242.
[23] G.F. van Rooyen, M.J. Smit, A.D. De Jager, H.K. Hundt, K.J. Swart, A.F. Hundt, J. Chromatogr. B 768 (2002) 223.
[24] P. Benninger, A. Cooper, R. Moisan, P. Patel, A. Elvin, J.J. Thiessen, Int. J. Clin. Pharmacol. Ther. 42 (2004) 342.
[25] R. King, R. Bonfiglio, C. Fernandez-Metzler, C. Miller-Stein, T. Olah, J. Am. Soc. Mass Spectrom. 11 (2000) 942.
[26] J.L. Ferrero, B.A. Bopp, K.C. Marsh, S.C. Quigley, M.J. Johnson, D.J. Anderson, J.E. Lamm, K.G. Tolman, S.W. Sanders, J.H. Cavanaugh, et al., Drug Metab. Dispos. 18 (1990) 441.