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Short communication

Determination of the unstable drug otilonium bromide in human plasma by LC–ESI-MS and its application to a pharmacokinetic study

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ABSTRACT

Otilonium bromide (OB) degrades rapidly in plasma and readily undergoes hydrolysis by the plasma esterase. In this paper, an LC–ESI-MS method has been developed for the determination of OB in human plasma. The rapid degradation of OB in plasma was well prevented by immediate addition of potassium fluoride (KF, an inhibitor of plasma esterase) to the freshly collected plasma before prompt treatment with acetonitrile. The method was validated over the concentration range of 0.1–20 ng/ml. The data of intra-run and inter-run precision and accuracy were within ±15%. The mean extraction recoveries for OB and the internal standard were higher than 93.0% and the matrix effects were negligible. The method has been successfully used in a pharmacokinetic study.

1. Introduction

Otilonium bromide, diethyl-methyl-[2-[4-[(2-octoxybenzoyl)-amino]benzoyl]oxyethyl] azanium bromide (Fig. 1A), is a potent spasmolytic which acts as muscarinic receptor antagonist and calcium channel blocker [1,2]. It is mainly used for the treatment of irritable bowel syndrome and has shown significant therapeutic effect [3,4]. Analytical techniques such as HPLC [5], derivative spectrophotometry [6–8] and capillary zone electrophoresis [9] have been used for the determination of OB in pharmaceutical formulations. However, the information on the pharmacokinetic disposition of OB is limited. The stability test in our study indicated that OB degrades rapidly in plasma and readily undergoes hydrolysis to yield two degradation products named DP-1 (Fig. 1C) and DP-2 (Fig. 1D). Shin et al. [10] established an LC–MS/MS method to determine OB in rat plasma, in which protein precipitation with acetonitrile was used to prepare the plasma samples. However, they did not mention the stability of OB in plasma. Kim et al. [11] developed an LC–MS/MS method for the determination of OB in human plasma with an LLOQ of 0.2 ng/ml. In their method, the stability of OB was improved by adding hydrochloric acid to human plasma at 2.5% plasma volume. According to our investigation, the addition of hydrochloric acid could improve the stability of OB indeed; however, it could not solve the problem of instability completely. Besides, treatment with hydrochloric acid also resulted in massive amounts of contaminant and interference. This often brings matrix effect and leads to heavy peak tailing of analytes. Moreover, Kim et al. used methanol to prepare the standard solutions of OB. We found that OB was unstable in methanol and may convert into the trans-esterification product DP-3 (Fig. 1E). So, using methanol as the solvent to prepare the stock solution should be avoided.

In the present study, a more sensitive method with an LLOQ of 0.1 ng/ml was established and validated for the determination of OB in human plasma. To solve the problem of instability of OB in plasma, a novel sample preparation method was developed. As OB was unstable in methanol, acetonitrile was used to prepare the stock and standard solutions. After validation, this assay was successfully applied to characterize the pharmacokinetics of OB in healthy volunteers.

2. Materials and methods

2.1. Materials, reagents and instrumentation

The OB reference substance (>99.3% purity) was provided by Venturepharm Laboratories Limited (Beijing, China). The OB tablets (40 mg/Tab) were bought from Berlin-Chemie AG (Menarini Group). Chlorobenzylidine, the internal standard (IS, Fig. 1B), was obtained from Medicinal Chemistry Research Center of China Pharmaceutical University. Acetonitrile (HPLC grade) was purchased

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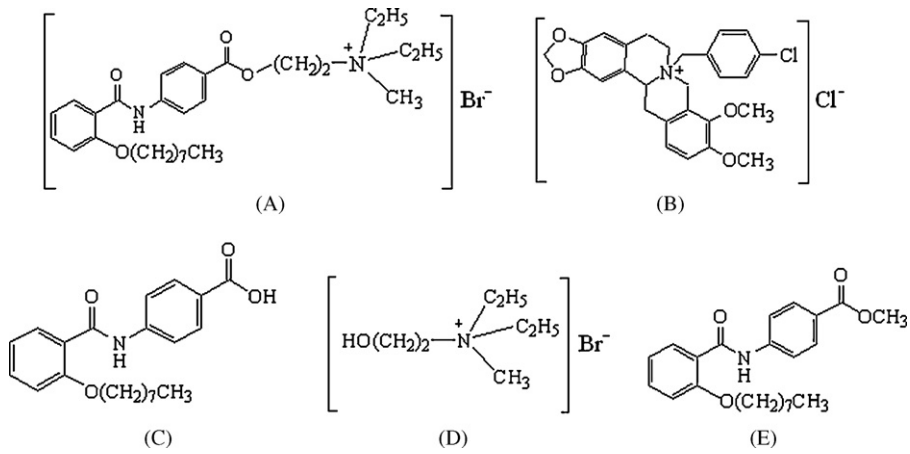

Fig. 1. Structures of OB (A), chlorobenzylidine (B), and the proposed structures of DP-1 (C), DP-2 (D) and DP-3 (E).

from Merck KGaA (Darmstadt, German). Ammonium acetate and formic acid (analytical grade) were purchased from Nanjing Chemical Reagent Co. Ltd. (Nanjing, China). Potassium fluoride dihydrate was purchased from Shanghai Chemical Reagent Co. Ltd. (Shanghai, China).

HPLC–ESI-MS analyses were performed by using an Agilent Technologies Series 1100 LC/MSD SL system (Agilent Technologies, Palo Alto, CA). And the HPLC–ESI-MS was controlled by a computer employing the Agilent 1100 ChemStation (version 10.02) software.

2.2. HPLC–ESI-MS conditions

The chromatographic separation was achieved on an Agilent Zorbax Eclipse Plus C18 column (3.5 μm, 150 mm × 2.1 mm i.d.) with a mobile phase of acetonitrile–20 mM ammonium acetate buffer solution containing 0.3% formic acid (58:42, v/v) at a flow rate of 0.35 ml/min. The injection volume was 3 μl and the column temperature was 30 °C. A single quadrupole mass spectrometer equipped with an ESI source was set with a drying gas (N_2) flow of 10 l/min, nebulizer pressure of 40 psig, drying gas temperature of 350 °C, capillary voltage of 4.0 kV, and the fragmentor voltage of 160 V in positive ion mode. A selected-ion monitoring (SIM) mode was used to detect OB at $[M]^+$ m/z 483.3 and the IS at $[M]^+$ m/z 464.1.

2.3. Preparation of standard solutions

Stock solutions of OB (1.0 mg/ml) and the IS (0.5 mg/ml) were prepared in acetonitrile and stored at −20 °C. Standard solutions of OB at concentrations of 100, 10 and 1 ng/ml were prepared by serial dilution of OB stock solution with acetonitrile. A solution containing 200 ng/ml of the IS was also obtained by further dilution of the IS stock solution with acetonitrile.

2.4. Sample preparation

The blood samples were centrifuged immediately to separate the plasma samples after being drawn from the volunteers. An aliquot of 0.2 ml plasma sample was placed in a tube containing 10 μl of 6.8 mol/l KF. After vortex-mixed for 10 s, 15 μl of the IS solution (200 ng/ml) was added before prompt treatment with 0.8 ml of acetonitrile. The supernatant was collected after centrifugation at 17,800 × g for 5 min and stored at −20 °C until analysis.

2.5. Preparation of calibration curves and quality control samples

Blank human plasma samples were purchased from the local blood center. An appropriate amount of the working solutions was added into 2-ml eppendorf tubes. After being evaporated to dryness under a stream of nitrogen, the residues were dissolved in 0.2 ml blank plasma and 10 μl of 6.8 mol/l KF to prepare the calibration standards of OB at the concentration levels of 0.1, 0.3, 1, 3, 6, 12 and 20 ng/ml. In addition, blank plasma samples were run to discard the presence of interferences. The quality control (QC) samples were designed at three OB concentration levels of 0.25, 2 and 18 ng/ml in the plasma.

3. Results

3.1. LC–MS/MS characteristics

OB and the IS were fully separated chromatographically (Fig. 2). The retention time for OB was 5.2 min and for the IS was 2.0 min, while the full run time was 6 min. No interfering peaks were observed at the retention time of OB and the IS. The matrix effects for OB at concentration levels of 0.25, 2 and 18 ng/ml were 99.9 ± 7.6%, 96.7 ± 2.1% and 97.3 ± 2.4%, respectively. The matrix effects for the IS (200 ng/ml) were 99.4 ± 3.9%. The extraction recoveries of OB were 94.6 ± 6.3%, 93.0 ± 6.2% and 97.3 ± 1.9% (n = 5) at concentration levels of 0.25, 2 and 18 ng/ml, respectively. The extraction recovery of the IS was 98.7 ± 2.1%.

3.2. Linearity, lower limit of quantification, precision and accuracy

The calibration curve was linear over the range of 0.1–20 ng/ml in human plasma with coefficient of correlation (r^2) >0.998. The typical regression equation is as follow: $f = 0.01749(\pm 0.00895) + 0.09655(\pm 0.00927) \times C$, where f represents the peak-area ratio of OB to the IS, and C represents the plasma concentration of OB. At the LLOQ (0.1 ng/ml), the *R.S.D.* (%) was 6.5% (n = 5), and the *R.E.* (%) ranged from −11.7% to 6.1%.

The accuracy, intra- and inter-run precisions of the method were determined by analyzing five replicates at each concentration levels of low, medium and high QCs in each of the three runs. The intra- and inter-run precisions were measured to be less than 4.4% and 12.7%, respectively. The accuracy of the method presented as *R.E.* (%) was from −2.1% to 7.3%.

3.3. Stability

No significant degradation of OB occurred in the acetonitrile extracts at the room temperature for 10 h and at −20 °C for 1 month. The post-preparative samples in autosampler were stable for at

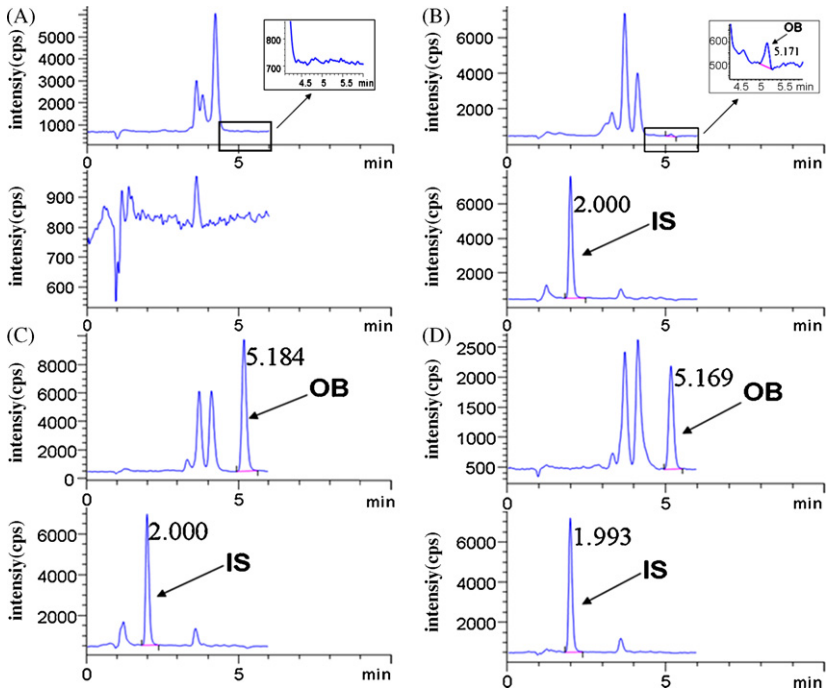

Fig. 2. Typical SIM chromatograms of blank plasma (A), LLOQ for OB in plasma (0.1 ng/ml) and the IS (B), ULOQ for OB in plasma (20 ng/ml) and the IS (C), the plasma sample obtained from a volunteer at 5 h after oral administration of 80 mg OB (D).

least 10 h. The stock solutions of OB and the IS were stable for at least 7 weeks at −20 °C and 6 h at the room temperature.

3.4. Carry-over effect

An acetonitrile/water (1:1, v/v) solution was used to wash the needle before each injection. The carry-over was tested by injecting blank plasma samples and the highest calibration standard containing 20 ng/ml OB and 200 ng/ml IS. No residual was observed at the retention time of OB and the IS in the chromatograms of blank plasma samples following the high concentration samples.

3.5. Application

The method was successfully applied to characterize the pharmacokinetics of OB after a single oral administration of dose 80 mg tablets to 22 healthy male volunteers. The blood samples were collected pre-dose and at 10 min, 20 min, 40 min, 1 h, 2 h, 3 h, 4 h, 5 h, 7 h, 9 h, 12 h, 15 h and 24 h post-dose. The mean plasma concentration–time profile of OB is shown in Fig. 3. The main pharmacokinetic parameters for the 22 volunteers are as follows respectively: the mean maximum OB plasma concentrations (C_{max}) is 4.37 ± 2.41 ng/ml, the elimination half-life ($t_{1/2}$) is 8.5 ± 4.9 h, the time to C_{max} (T_{max}) is 1.5 ± 1.1 h, the mean AUC_{0-24} is 20.4 ± 10.7 ng h/ml.

4. Discussion

4.1. Stability and sample preparation

According to our investigation, OB degraded rapidly in plasma at room temperature and readily underwent hydrolysis to yield two degradation products DP-1 and DP-2. For analysis of unstable drugs, the main challenge is how to keep the drug stable during sample preparation. By comparing the degradation characteristics of OB in plasma with that of in water, it was observed that OB degraded much faster in plasma than in water (Fig. 4A), which implied that the plasma esterase was the main factor leading to the instability of OB. KF was suggested to be an effective plasma esterase inhibitor [12,13], which had been successfully used to solve the problem of instability of drugs in plasma [14–16]. Also, the method of immediately adding acetonitrile to freshly collected plasma could prevent the hydrolysis of drugs by esterase [17]. In this study, OB in plasma was well stabilized by immediate addition of KF before prompt treatment of the plasma samples with acetonitrile, and the samples were stored in the form of acetonitrile extracts. Both KF and acetonitrile are essential factors for preventing the degradation of OB in plasma. Similar research [18] has been reported recently, in which the degradation of the drug was prevented by pre-treatment with enzyme inhibitor before prompt treatment with ice-cold ethanol. And the plasma samples were also stored in the form of extracts until analysis.

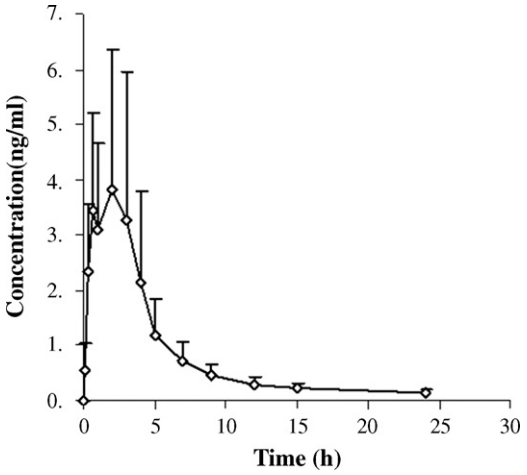

Fig. 3. Mean plasma concentration–time profile of OB determined by LC–ESI-MS method after oral administration of 80 mg OB tablets to healthy male volunteers. Each point represents the mean ± S.D. (n = 22).

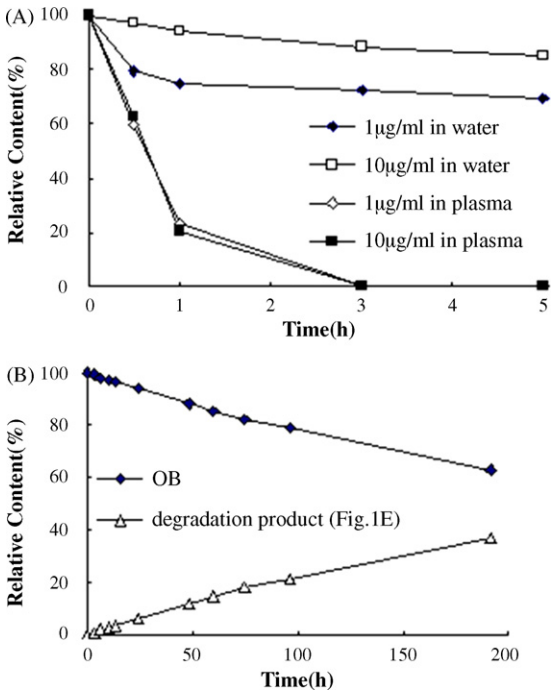

Fig. 4. The degradation characteristics of OB in (A) plasma and in water (1 and 10 μg/ml by HPLC) and (B) methanol (50 μg/ml by HPLC).

Kim et al. [11] found OB in plasma stable at −80 °C for at least 6 weeks and three freeze–thaw cycles. However, OB degrades rapidly in plasma at room temperature, and it would degrade partially even during the process of thawing. Thus, it is essential to prevent the rapid degradation of OB in plasma by using the sample preparation procedures established in this study. First, the addition of KF inhibits plasma esterase. Second, acetonitrile was used for protein precipitation which also makes the plasma esterase inactive. These two factors together make OB totally stable for at least 10 h at room temperature. Such reliable stability can support the accurate evaluation of the OB pharmacokinetics. This method is more important when preparing batch of samples in pharmacokinetic studies. The stability of OB in methanol was examined (Fig. 4B). OB degraded by 10% within 2 days in methanol, while the degradation product DP-3 increased correspondingly. Thus, it should be avoided to use methanol as the solvent to prepare the stock solutions of OB.

The degradation products (*DP-1*, *DP-2* and *DP-3*) of OB were investigated in the present study. The proposed structures of DP-1, DP-2 and DP-3 were identified by LC–ESI-MS. Fig. 5A shows the LC–MS total ion chromatogram of a degradation sample of OB in methanol. The LC peak at 1.46 min refers to the degradation product of DP-2. The ESI mass spectrum of DP-2 (Fig. 5A-1) showed the ion at *m*/*z* 132.2 was assigned to $[M]^+$ of DP-2. The LC peak at 2.99 min refers to the parent drug OB. The LC peak at 15.31 min refers to the degradation product of DP-3. The ESI mass spectrum of DP-3 (Fig. 5A-2) obtained in positive ion mode showed that the ions at *m*/*z* 384.1, 406.1, 422.1 and 789.2 were assigned to $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$ and $[2M+Na]^+$ of DP-3, respectively. The ESI mass spectrum of DP-3 (Fig. 5A-3) obtained in negative ion mode showed the deprotonated molecular ion at *m*/*z* 382.3 $[M-H]^-$ of DP-3.

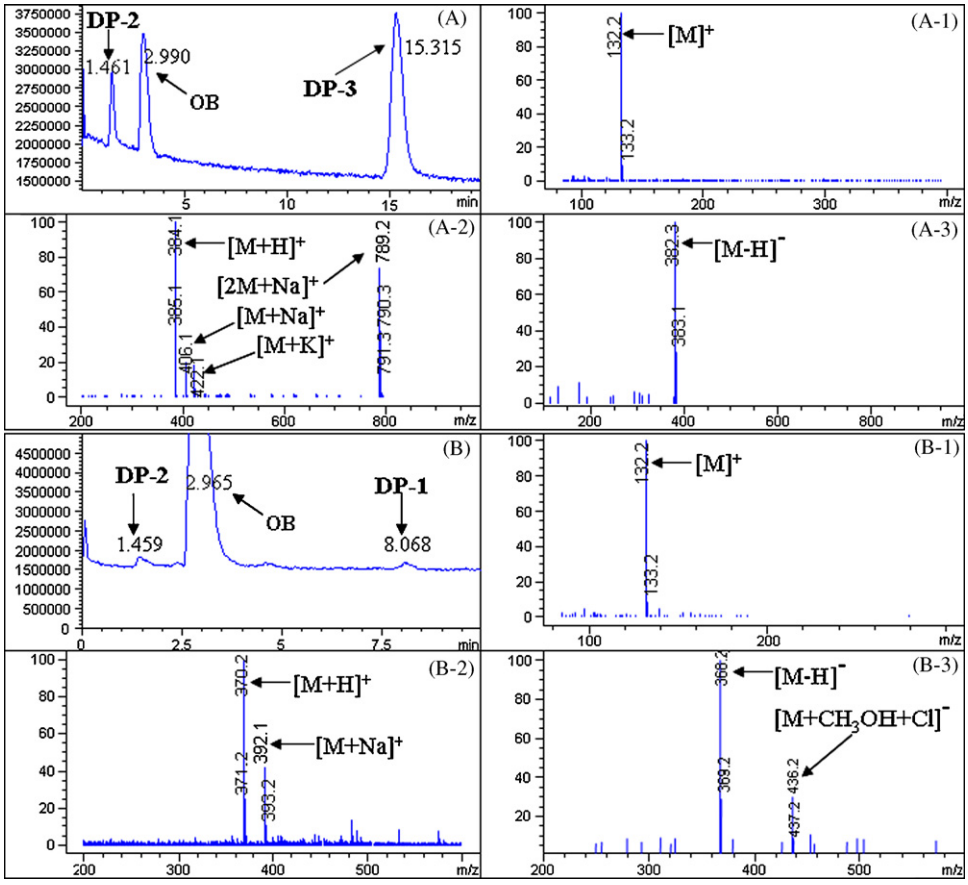

Fig. 5. The LC–MS total ion chromatogram of a degradation sample of OB in methanol (A), the ESI mass spectrum of DP-2 obtained in positive ion mode (A-1), the ESI mass spectrum of DP-3 obtained in positive (A-2) and negative (A-3) ion mode. The LC–MS total ion chromatogram of a degradation sample of OB in water (B), the ESI mass spectrum of DP-2 obtained in positive ion mode (B-1), the ESI mass spectrum of DP-1 obtained in positive (B-2) and negative (B-3) ion mode.

Fig. 5B shows the LC–MS total ion chromatogram of a degradation sample of OB in water. The LC peak at 1.46 min refers to the degradation product of DP-2. The base peak was observed as the $[M]^+$ of DP-2 at m/z 132.2 (Fig. 5B-1). The LC peak at 2.97 min refers to the parent drug OB. The LC peak at 8.07 min refers to the degradation product of DP-1. The ESI mass spectrum of DP-1 (Fig. 5B-2) obtained in positive ion mode showed that ions at m/z 370.2 and 392.1 were assigned to $[M+H]^+$ and $[M+Na]^+$ of DP-1, respectively. The ESI mass spectrum of DP-1 (Fig. 5B-3) obtained in negative ion mode showed that the ions at m/z 368.2 and 436.2 were assigned to $[M-H]^-$ and $[M+CH_3OH+Cl]^-$ of DP-1.

4.2. Application

The dosage of OB received by the volunteers in the present study is 80 mg, and different from that (120 mg) reported by Kim et al. [11]. After dosage adjustment, the pharmacokinetic parameters C_{max} and AUC_{0-24} obtained in the present study are consistent with those reported by Kim et al. [11]. However, the difference on the other two pharmacokinetic parameters T_{max} and $t_{1/2}$ are significant. The difference on T_{max} usually belongs to the different formulations designed by different manufacture. The difference on the elimination half-life maybe resulted by the different dosing amount administered between the two studies.

5. Conclusion

The method achieved good sensitivity for the determination of OB in human plasma. No significant interferences and matrix effect caused by endogenous compounds were observed. The rapid degradation of OB in plasma was well prevented by immediate addition of KF before prompt treatment with acetonitrile. The method is suitable for the pharmacokinetic study and bioavailability evaluation of OB formulations.

References

[1] P. Santicioli, V. Zagorodnyuk, A.R. Renzetti, C.A. Maggi, Naunyn-Schmiedeberg's Arch. Pharmacol. 359 (1999) 420.
[2] S. Evangelista, A. Giachetti, B. Chapelain, G. Neliat, C.A. Maggi, Pharm. Res. 38 (1998) 111.
[3] S. Evangelista, J. Int. Med. Res. 27 (1999) 207.
[4] S. Evangelista, Curr. Pharm. Des. 10 (2004) 3561.
[5] C. Mannucci, J. Bertini, A. Coccini, A. Perico, F. Salvagnini, A. Triolo, J. Pharm. Sci. 82 (1993) 367.
[6] C. Mannucci, J. Bertini, A. Cocchini, A. Perico, F. Salvagnini, A. Triolo, J. Pharm. Sci. 81 (1992) 1175.
[7] B. Morelli, Fresen. J. Anal. Chem. 357 (1997) 1179.
[8] N.M. El-Kousy, L.I. Bebawy, J. AOAC Int. 82 (1999) 599.
[9] S. Furlanetto, S. Orlandini, G. Massolini, M.T. Faucci, E. La Porta, S. Pinzauti, Analyst 126 (2001) 1700.
[10] B.S. Shin, J.J. Kim, J. Kim, S.K. Hu, H.K. Kim, H.S. Lee, S.D. Yoo, Arch. Pharm. Res. 31 (2008) 117.
[11] K. Kim, H. Jung, H.Y. Yun, S.O. Moon, Y.R. Yoon, K.I. Kwon, H. Kim, S. Shon, W. Kang, Chromatographia 66 (2007) 257.
[12] G. Cimasoni, Biochem. J. 99 (1966) 133.
[13] R.M. Krupka, Mol. Pharmacol. 2 (1966) 558.
[14] J.J. Zou, L. Dai, L. Ding, D.W. Xiao, Z.Y. Bin, H.W. Fan, L. Liu, G.J. Wang, J. Chromatogr. B 873 (2008) 159.
[15] M.C. Petersen, R.L. Nation, J.J. Ashley, J. Chromatogr. 183 (1980) 131.
[16] K. Tsujikawa, K. Kuwayama, H. Miyaguchi, T. Kanamori, Y.T. Iwata, H. Inoue, Xenobiotica 39 (2009) 391.
[17] D. Liang, J. Ma, B. Wei, I.O. Poon, E.C. Bell, T.R. Bates, J. Chromatogr. B 863 (2008) 172.
[18] M. Suno, T. Kunisawa, A. Yamagishi, T. Ono, J. Yamamoto, T. Yamada, Y. Tasaki, K. Shimizu, H. Iwasaki, K. Matsubara, J. Chromatogr. B 877 (2009) 1705.