Journal of Chromatography B, 915–916 (2013) 28–32

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Determination of phenylephrine in human plasma using ultra-performance liquid chromatography–tandem mass spectrometry

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ARTICLE INFO

Article history:
Received 15 September 2012
Accepted 16 December 2012
Available online 26 December 2012

Keywords:
Determination
Phenylephrine
UPLC–MS/MS
Human plasma

ABSTRACT

This paper described a sensitive and rapid method based on ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC–MS/MS) for the determination of phenylephrine in human plasma. Plasma samples were pre-purified by solid-phase extraction (SPE). The chromatographic separation was achieved with BEH HILIC column using a mixture of 10 mM pH 3.5 ammonium formate and acetonitrile (10:90, v/v) under isocratic conditions at a flow rate of 0.4 mL/min. The mass spectrometry was carried out using positive electrospray ionization (ESI) and data acquisition was carried out in the multiple reaction monitoring (MRM) mode. The method was fully validated over the concentration range of 10.0–5000 pg/mL. The lower limit of quantification (LLOQ) was 10.0 pg/mL. Inter- and intra-batch precision was less than 15% and the accuracy was within 85–115%. Extraction recovery was 78.5%. Selectivity, matrix effects and stability were also validated. The method was applied to the pharmacokinetic study of phenylephrine hydrochloride in Chinese subjects.

1. Introduction

Phenylephrine hydrochloride is a potent vasoconstrictor, possessing both direct and indirect sympathomimetic effect. It is indicated for use by adults and children for the temporary relief of nasal congestion inducing by the common cold, hay fever, or other upper respiratory allergies [1,2].

Up to date, a few methods have been reported for the determination of phenylephrine in human plasma or serum. High performance liquid chromatography (HPLC) with coulometric detection [3] has been reported for the determination of phenylephrine in human serum, but a volume of 1 mL sample was used with a lowest limit of quantification (LLOQ) of 0.35 ng/mL. And the run time for the HPLC was 25.0 min. The LLOQ of the liquid chromatography with electrochemical detection [4] method was only 2.0 ng/mL and a volume of 1 mL sample was also needed. Although, the more sensitive LC–MS/MS method has been reported [5], LLOQ was only 51.0 pg/mL, the assay range was 51.0–5500 pg/mL and the run time was 8.0 min. Therefore, these assay methods have some defaults including narrow linearity range, low sensitivity, time consuming and requiring large volume sample for sample preparation, which limit their widespread use in clinical application. In contrast, UPLC–MS/MS offers improved resolution, speed, and sensitivity for analytical determinations [6], allowing rapid analysis of analytes. This analytical platform has been used widely for the assessment of analytes such as pharmaceuticals, long chain fatty acids, underivatized amino acids and opiates in various matrices [7–10].

Based on previous pharmacokinetic study results [11], detection of phenylephrine in human plasma required a LLOQ of 10.0 pg/mL.

Here we present a more sensitive and rapid method for the determination of phenylephrine using only 250 μL plasma for each sample preparation. The run time was only 2.0 min using UPLC. And the use of a stable isotope of the analyte as the internal standard was thought to be able to yield better assay performance results [12].

The objective of this study was to establish and validate an UPLC–MS/MS method with high sensitivity and specificity for the determination of phenylephrine in human plasma and to support pharmacokinetic studies of phenylephrine.

2. Experimental

2.1. Chemicals and solvents

Phenylephrine (purity 99.9%) and its internal standard [$^2H_3$] phenylephrine were provided by U.S. Pharmacopeia and BDG Synthesis Limited, respectively. Acetonitrile was of chromatographic grade and obtained from Burdick&Jackson Lab. Ammonium formate and formic acid were of analytical grade and purchased from Sigma–Aldrich Co. LLC. and Sinopharm Chemical Reagent Co., Ltd., respectively. Drug-free human plasma (anticoagulant: EDTA K_3)

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http://dx.doi.org/10.1016/j.jchromb.2012.12.019

was obtained from six different healthy subjects who were drug-free for at least two weeks. Distilled water was prepared with a Milli-Q water purifying system.

2.2. Calibration standard (CS) and quality control (QC) samples in human plasma

Stock solutions of phenylephrine for CS and QC were prepared separately in acetonitrile–water (50:50, v/v) after correction for purity. The concentrations of stock solutions of phenylephrine for CS and QC were 500 and 400 μg/mL, respectively. A stock solution of internal standard was also prepared in acetonitrile–water (50:50, v/v). These stock solutions were further diluted to yield working solutions at several concentration levels.

Calibration standards and QC samples in plasma was prepared by diluting corresponding working solutions with drug-free human plasma, respectively. The final concentrations of calibration standards were 10.0, 20.0, 100, 200, 500, 1000, 2000, 5000 pg/mL. The final concentrations of QC were 25.0, 200 and 4000 pg/mL. Internal standard working solution (1.00 ng/mL) was prepared with acetonitrile–water (50:50, v/v). Plasma samples were stored at −70 °C.

2.3. Sample preparation

Biological samples were extracted by a solid phase extraction (SPE) method using a Discovery DSC-18 SPE 96-well Plate (100 mg, Supelco). Plasma, 250 μL, was mixed with 50 μL of internal standard (1.00 ng/mL). The SPE plate was conditioned with 3 mL 1% formic acid in acetonitrile and 1 mL water. The mixture, 300 μL, was then loaded onto the plate. SPE columns were washed with 1 mL water and 1 mL acetonitrile–water (50:50, v/v). The elution of these compounds was carried out with 1 mL 1% formic acid in acetonitrile. The eluates were added 50 μL formic acid and evaporated to dryness under nitrogen gas at room temperature. 1% formic acid in acetonitrile, 100 μL, was finally added to the residues and well mixed before injection. The operation of mixing the biological matrices and internal standard was done in an ice bath.

2.4. Liquid chromatography–tandem mass spectrometry

Plasma samples were analyzed using Acquity UPLC Core system (Waters Co., MA, USA) coupled with API 5500 Qtrap tandem MS equipped with an electrospray ionization (ESI) source (AB SCIEX, CA, USA). Chromatography separation for plasma was carried out on an ACQUITY UPLC BEH HILIC (2.1 mm × 50 mm, 1.7 μm) at 35 °C. The autosampler temperature was 10 °C. The mobile phase was 10 mM pH 3.5 ammonium formate–acetonitrile (10:90, v/v). The flow rate was 0.4 mL/min and the run time was 2.0 min with an injection volume of 7.5 μL.

Analysis was performed with an ionizing voltage of 4500 V. The ion source temperature was set at 550 °C with ultrahigh-purity nitrogen as curtain gas (30 psi), nebulizer gas (60 psi) and auxiliary gas (60 psi). Multiple reaction monitoring (MRM) was carried out using nitrogen as collision gas (6 psi), and with a dwell time of 100 ms for each transition. The analytes were detected by monitoring the transitions m/z 168.1 → 150.1 and 171.2 → 153.1 with the same collision energy 15 eV. The product ion mass spectra of the analyte and internal standard are depicted in Fig. 1.

2.5. Method validation

The method was validated for selectivity, sensitivity, linearity, recovery, matrix effects, precision, accuracy and stability according to the US Food and Drug Administration (FDA) [13] and Chinese State Food and Drug Administration (SFDA) guidelines [14] for the validation of bioanalytical methods.

The specificity of this method was evaluated by comparison of UPLC–MS/MS chromatograms of phenylephrine at the LLOQ to those of six individual human blank plasma samples.

Calibration standards in human plasma were prepared and analyzed in three independent runs. The calibration curves were constructed by weighted ($1/x^2$) least-square linear regression analysis of the peak area ratio of analyte to its internal standard against nominal analyte concentration. The LLOQ is defined as the lowest concentration on the calibration curve. The deviations of back-calculated concentrations of calibration standards from their nominal values should be within ±20% for LLOQ and ±15% for all other calibration levels.

The extraction recovery of phenylephrine was calculated at three levels (low, 25.0 pg/mL; medium, 200 pg/mL; and high, 4000 pg/mL) by comparing two groups of control samples: (A) drug spiked to plasma and prepared normally (pre-extraction); (B) drug spiked after extraction of blank plasma (post-extraction). The ratio ($A/B \times 100$) is defined as the extraction recovery. The reproducibility of the extraction procedure was determined as RSD%.

The matrix effects were evaluated at three levels (low, 25.0 pg/mL; medium, 200 pg/mL; and high, 400 pg/mL) too. Two groups of samples were prepared: group 1 was prepared in plasma originating from six different subjects and submitted to the sample preparation process and spiked with phenylephrine after processing (B); group 2 was prepared to evaluate the MS/MS response for a pure standard of phenylephrine dissolved in the mobile phase (C). The ratio ($B/C \times 100$) is defined as the absolute matrix effects. The assessment of the relative matrix effects, which was expressed as RSD%, was made by a direct comparison of B values between six different lots of plasma. The inter-subject variability of matrix effects at every concentration level should be less than 15% [15–17].

Intra- and inter-batch precision and accuracy were determined by measuring the concentrations of analyte in plasma in five replicates of QC samples at three different concentrations for three separate batches.

The stability of phenylephrine in biological matrices and in working solution at different storage conditions were evaluated as follows: The short-term stability of analyte in biological matrices and in working solution was assessed after 3 h of storage in an ice bath and at room temperature, respectively. The long-term stability of analyte in human plasma was assessed after 249 days of storage in a freezer at −70 °C. The stability of analyte was assessed after two freeze–thaw cycles (−70 °C to room temperature). The stability of analyte in extracts was also tested after 6 h at 10 °C.

3. Result and discussion

3.1. Extraction procedure optimization

Owing to the high polarity, recovery of phenylephrine by the liquid–liquid extraction (LLE) method using diethyl ether, ethyl acetate and dichloromethane is low. The matrix effects are significant for the precipitating protein method. We therefore chose the SPE method in which recovery is higher than LLE, and inter-subject variability is lower.

Based on the pKa and polarity of phenylephrine, different SPE columns were tried, such as Waters Oasis HLB and MCX, Phenomenex Strata-X CW. Thus, Supelco Discovery DSC-18 column exhibited the highest recovery. However, when the Discovery DSC-18 96-well plates were used, there was interference in the double blank sample though the plates were new, which indicated there

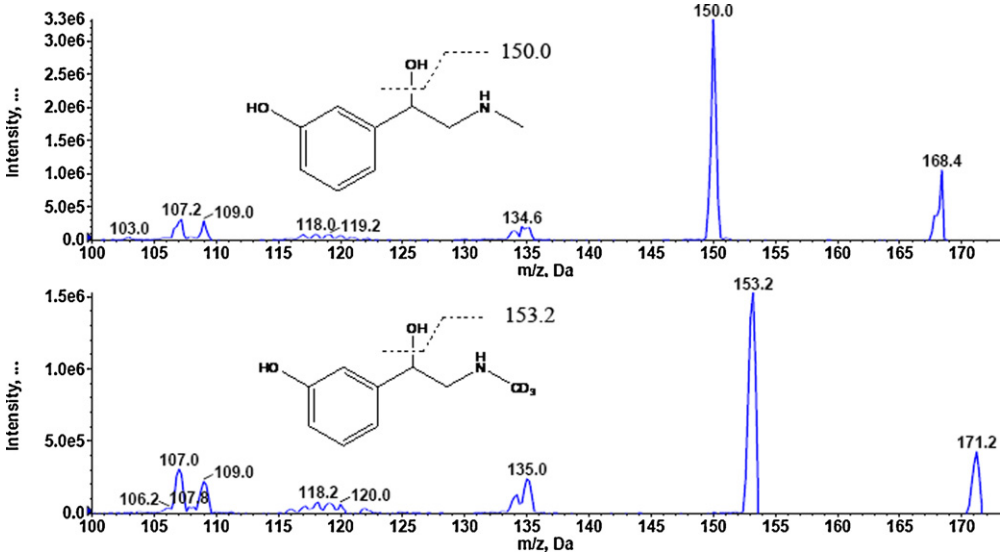

Fig. 1. Product ion spectrum and chemical structures of phenylephrine (upper) and internal standard (lower).

was interference in the new plates. Therefore, before using, the new plates were washed with 3 mL 1% formic acid in acetonitrile to make sure there was no interference left in the plates.

The composition of the elution solvent was also optimized. It was found that if the samples were washed with water and acetonitrile–water (50:50, v/v) and eluted using 1% formic acid in acetonitrile during the SPE procedure, the highest and most robust extraction efficiency was obtained.

Because phenylephrine is unstable in human plasma at room temperature, mixing the biological matrices and internal standard was done in an ice bath. Phenylephrine was stable during this sample preparation procedure for 3 h.

3.2. UPLC–MS/MS optimization

Because of the polarity, phenylephrine has no retention on simple reverse-phase columns. Though it has retention on amide columns, the sensitivity is low. We therefore chose the HILIC column which is suitable for the analysis of polar analytes. The peak shape and sensitivity for phenylephrine and the internal standard are both good.

Since phenylephrine is unstable in basic solution, a series of aqueous formic acid with different pH values and buffers were investigated. When 10 mM pH 3.5 ammonium formate is used as the aqueous phase, a good chromatographic profile and sensitivity was achieved.

The analyte was introduced into the mass spectrometer using an electrospray interface, and the parameters such as ionizing voltage (IS), declustering potential (DP) and entrance potential (EP) were optimized to obtain protonated molecular ions $[M+H]^+$ based on the structures. In order to achieve high specificity and sensitivity, the MRM scan mode was selected to assay the analyte at the most suitable collision energy (CE). Negative ionization was also applied, but compared to the positive mode, the sensitivity was much lower.

3.3. Validation procedure

3.3.1. Selectivity, linearity and LLOQ

Chromatograms of a blank plasma, a calibration standard and a subject's plasma sample are shown in Fig. 2. No peaks eluting at the retention times of the phenylephrine or internal standard were detected in samples from 6 lots of human plasma.

A calibration curve was established ranging from 10.0 to 5000 pg/mL for plasma. Calibration curve was regressed using linear equation with a weighting factor of $1/x^2$. Coefficients of correlation of all calibration curves were more than 0.99. The LLOQ was 10.0 pg/mL in plasma.

3.3.2. Recovery and matrix effects

The observed recovery for the extraction method from plasma (mean value and RSD%, $n = 6$) is shown in Table 1. The mean recovery at different concentrations of phenylephrine was 78.5% in plasma with little inter-subject variability.

Matrix effects and inter-subject variability data from plasma of individual subjects who had not received phenylephrine are summarized in Table 1. The inter-subject variabilities were up to 9.6%. This indicated little or no difference in ionization efficiency of phenylephrine from different plasma lots.

3.3.3. Accuracy and precision

Precision and accuracy values were determined on three different batches by measuring five replicates of QC samples at three concentration levels. The results are listed in Table 2. Intra-batch accuracy ranged from 98.5% to 101.0%, while precisions were less than 9.5%. Inter-batch accuracy ranged from 98.0% to 107.6%, while precisions were less than 10.1%.

3.3.4. Stability

The stability tests of the analyte were designed to cover expected conditions of handling of clinical samples. The stability of the analyte in human plasma was investigated under a variety of

Table 1
Results of extraction recovery and matrix effects of phenylephrine in human plasma ($n = 6$).

Nominal concentration (pg/mL)	Recovery% (RSD%)	Matrix effects% (Inter-subject variability%[a])
25.0	77.9 (9.5)	60.4 (4.9)
200	79.1 (8.1)	46.5 (9.6)
4000	78.4 (7.4)	47.1 (3.8)

[a] Expressed as RSD%.

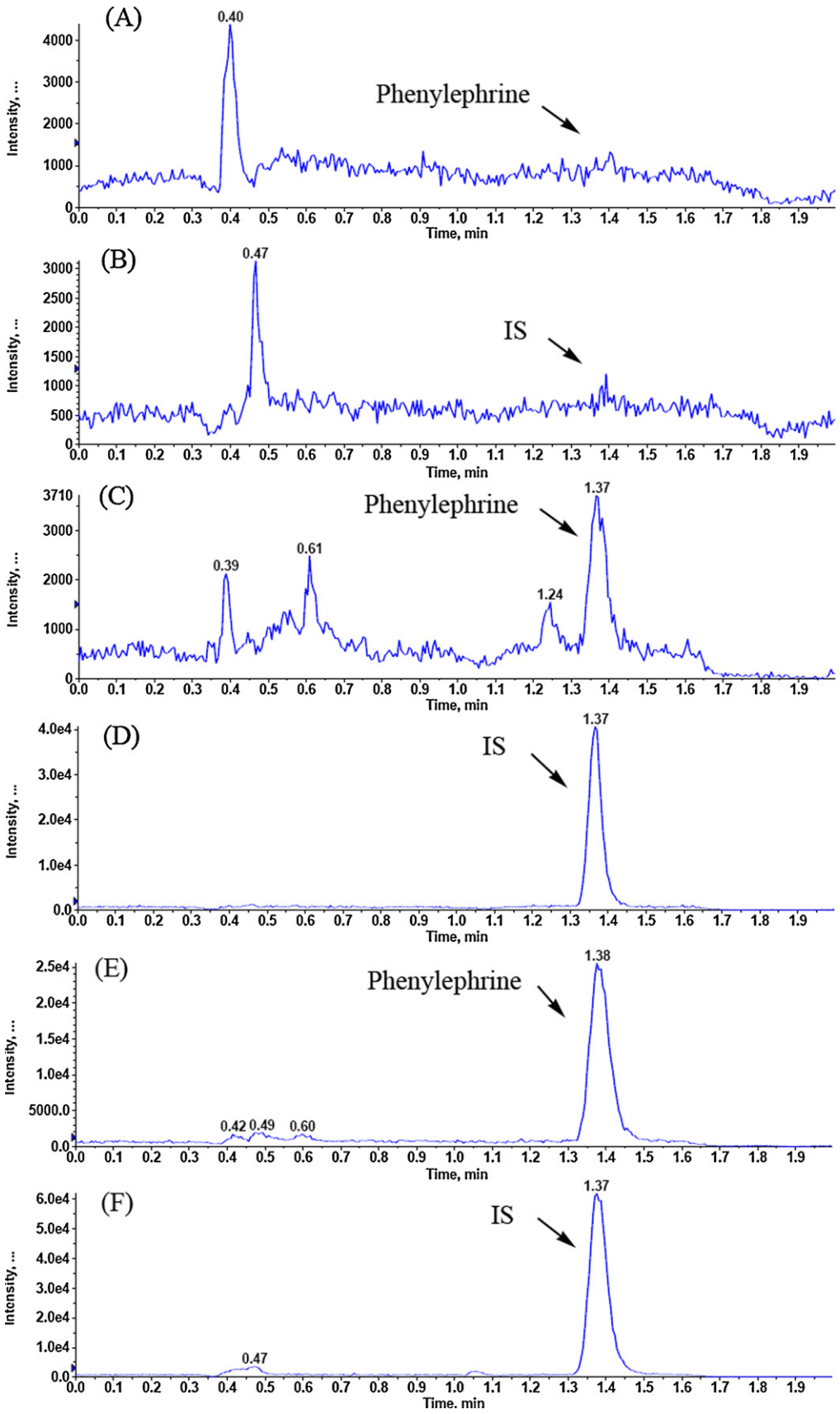

Fig. 2. MRM chromatograms of phenylephrine and internal standard in blank plasma (A and B), LLOQ (C and D) and plasma from a subject 2.5 h post-dose of phenylephrine hydrochloride (E and F).

Table 2
Accuracy and precision of intra- and inter batch for the detection of phenylephrine in plasma.

	Low QC	Medium QC	High QC
Nominal concentration (pg/mL)	25.0	200	4000
Intra-batch			
Mean found concentration (pg/mL) (n = 5)	25.1	197	4040
Precision (RSD%)	9.5	3.7	2.8
Accuracy (%)	100.4	98.5	101.0
Inter-batch			
Mean found concentration (pg/mL) (n = 15)	26.9	196	4060
Precision (RSD%)	10.1	8.2	8.9
Accuracy (%)	107.6	98.0	101.5

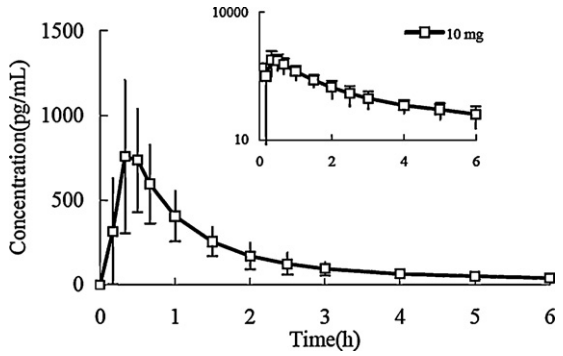

Fig. 3. The mean (±SD) concentration–time curve of phenylephrine in plasma from 20 Chinese subjects after oral administration of 10 mg phenylephrine hydrochloride.

storage and processing conditions. Briefly, two freeze/thaw cycles and ice bath storage of the QC samples up to 3 h appeared to have no effect on results of quantification of phenylephrine in plasma. QC samples stored at −70 °C remained stable for at least 249 days in plasma. Processed samples were allowed to stand at 10 °C in extracts for 6 h prior to analysis, with no observed effect on results of quantification. When working solutions of phenylephrine were stored at room temperature for 6 h, the analyte was found to be stable.

3.4. Application of the method in pharmacokinetic studies

In the present study, a specific, sensitive and rapid method based on UPLC–MS/MS was developed and fully validated to quantify phenylephrine in human plasma. This method was successfully applied to determine the pharmacokineticprofile of phenylephrine after orally administration of phenylephrine hydrochloride to Chinese subjects. The study was approved by the Ethics Committee of Peking Union Medical College Hospital, and all subjects signed the Informed Consent Form before the study. The mean plasma concentration–time curve for the 10-mg phenylephrine dose (n = 20) is shown in Fig. 3.

4. Conclusion

A fast, sensitive and specific UPLC–MS/MS method based on SPE extraction has been developed and validated for the determination of phenylephrine concentrations in plasma. The extraction procedure and UPLC–MS/MS conditions were optimized in order to improve the sensitivity and robustness of the method. The procedure was fully validated. This method was successfully applied to determine phenylephrine in human plasma and characterize the pharmacokinetic profile of phenylephrine in Chinese subjects.

Acknowledgments

This study was financially supported by Shanghai Johnson&Johnson Pharmaceuticals, Ltd. This work was also supported by a grant from the National Program on Key Research Project of New Drug Innovation (No. 2008ZX09312-016).

References

[1] R.C. Hatton, A.G. Winterstein, R.P. McKelvey, J. Shuster, L. Hendeles, Ann. Pharmacother. 41 (2007) 381.
[2] P.J. Desjardins, R.G. Berlin, Br. J. Clin. Pharmacol. 64 (2007) 555.
[3] V. Vuma, I. Kanfer, J. Chromatogr. B 678 (1996) 245.
[4] K. Gumbhir, W.D. Mason, J. Pharm. Biomed. Anal. 14 (1996) 623.
[5] P. Ptacek, J. Klima, J. Macek, J. Chromatogr. B 858 (2007) 263.
[6] M.I. Churchwell, N.C. Twaddle, L.R. Meeker, D.R. Doerge, J. Chromatogr. B 825 (2005) 134.
[7] A.L. Batt, M.S. Kostich, J.M. Lazorchak, Anal. Chem. 80 (2008) 5021.
[8] J. Mensch, M. Noppe, J. Adriaensen, A. Melis, C. Mackie, P. Augustijns, M.E. Brewster, J. Chromatogr. B 847 (2007) 182.
[9] W.A. Waterval, J.L.J.M. Scheijen, M.M.J.C. Ortmans-Ploemen, C.D. Habets-van der Poel, J. Bierau, Clin. Chim. Acta 407 (2009) 36.
[10] T. Berg, E. Lundanes, A.S. Christophersen, D.H. Strand, J. Chromatogr. B 877 (2009) 421.
[11] Understanding Phenylephrine Metabolism, 2007, Pharmacokinetics, Bioavailability and Activity. http://www.fda.gov/ohrms/dockets/ac/07/briefing/2007-4335b1-01-Schering-Plough.pdf
[12] E. Stokvis, H. Rosing, J.H. Beijnen, Rapid Commun. Mass Spectrom. 19 (2005) 401.
[13] Guidance for Industry Bioanalytical Method Validation, 2001. http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070107.pdf
[14] State Food and Drug Administration, 2005. http://www.sda.gov.cn/gsz05106/07.pdf
[15] P.J. Taylor, Clin. Biochem. 38 (2005) 328.
[16] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 30.
[17] B.K. Matuszewski, J. Chromatogr. B 830 (2006) 293.