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Quantification of cyclizine and norcyclizine in human plasma by liquid chromatography–tandem mass spectrometry (LC–MS/MS)

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ABSTRACT

A rapid and simple liquid chromatography-tandem mass spectrometry (LC–MS/MS) assay was developed and validated for quantification of cyclizine and its main metabolite norcyclizine in human plasma. Samples were prepared by protein precipitation with acetonitrile and cinnarizine was used as internal standard (recovery >87%). The analytes were eluted from a C8 50 mm × 2.0 mm analytical column using a linear gradient of methanol and 0.05% formic acid with a total analysis time of 4 min. Analytes were detected by MS/MS using electrospray ionisation in the positive mode with multiple reactions monitoring (MRM) of the precursor ion/product ion transitions 267.2/167.2 for cyclizine and 253.2/167.2 for norcyclizine. Matrix effects were negligible. Standard curves for cyclizine and norcyclizine were linear ($r^2 \geq 0.996$) over the range 2–200 ng/mL, with 2 ng/mL representing the lower limit of quantification. Relative standard deviations were <14% for intra- and inter-day precision and the accuracy was within ±8%. The assay was successfully applied to a clinical study.

1. Introduction

Cyclizine (Fig. 1), is a piperazine histamine H_1-receptor antagonist. It is widely used clinically in the management of nausea and vomiting and is administered as cyclizine hydrochloride tablets or cyclizine lactate solution for injection [1]. Despite its widespread use, the pharmacokinetics of cyclizine in humans have not been well studied, which in part has been related to analytical difficulties due to low therapeutic plasma concentrations [2]. The complete metabolic pathways of cyclizine in humans have yet to be elucidated. Cyclizine is metabolised in the liver mainly by *N*-demethylation to norcyclizine, which is largely inactive [2,3]. Other routes include *N*-glucuronidation resulting in the formation of quaternary N^+-glucuronides, which are polar and non-volatile metabolites that are not easy to detect [4]. In greyhounds several urinary metabolites have been identified including conjugated hydroxy-metabolites [5,6].

For a clinical study on cyclizine pharmacokinetics a sensitive and specific analytical assay was required to determine concentrations of cyclizine and norcyclizine in plasma. A literature review revealed only two published assays for concomitant analysis of cyclizine and norcyclizine in plasma at therapeutic concentrations. These assays, both by Walker and Kanfer, employed HPLC with coulumetric [7] or UV [8] detection and had run times of 15–20 min. To achieve adequate sensitivity, the sample preparation consisted of solid-phase extraction of 1 mL of plasma followed by a phase separation step [7,8]. As our clinical study involved several hundred samples, a faster and simpler assay was desired.

By using liquid chromatography-tandem mass spectrometry (LC–MS/MS) a higher degree of selectivity and sensitivity can generally be achieved permitting less elaborate sample preparation and a smaller sample volume to be used. An added advantage is that LC–MS/MS allows for the use of shorter analytical columns and thus shorter run times can be achieved [9]. We were unable to locate any LC–MS/MS assays in the literature involving both cyclizine and norcyclizine. Cyclizine, but not norcyclizine, has been determined in human plasma by LC–MS/MS as reported by the Walker and Kanfer group [10], and an LC–MS/MS antihistamine screening assay, which includes cyclizine, has been described [11]. As no existing methods were suitable for our purposes, the aim of the current work was to develop and validate a rapid and simple LC–MS/MS assay for simultaneous determination of cyclizine and norcyclizine concentrations in human plasma to be used for a clinical study.

2. Experimental

2.1. Materials

Cyclizine (1-benzhydryl-4-methyl-piperazine), norcyclizine (1-benzhydryl-piperazine) and the internal standard (IS) cinnarizine

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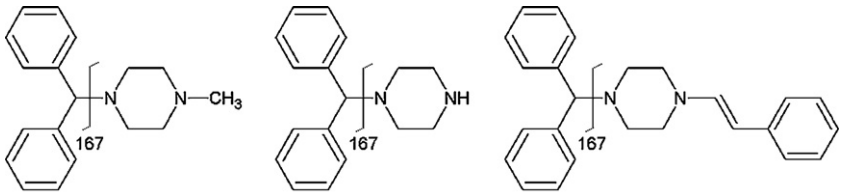

Fig. 1. Chemical structures of cyclizine (M_w 266.4), norcyclizine (M_w 252.4) and the internal standard (IS) cinnarizine (M_w 366.4). Proposed site for the most intense MS fragment (m/z 167) is indicated.

(1-benzhydryl-4-cinnamyl-piperazine) (Fig. 1) were purchased from Sigma–Aldrich (St. Louis, MO, USA). LC-grade methanol, acetonitrile and formic acid were purchased from Merck (Darmstadt, Germany). Distilled, deionised water was produced by a locally installed Milli-Q Reagent Water System (Millipore, MA, USA). Human plasma used for assay blanks and preparation of calibration standards was expired plasma from voluntary donations to New Zealand Blood Services (Christchurch, New Zealand).

2.2. Calibration standards and quality control samples

Stock solutions of cyclizine, norcyclizine and IS were prepared in methanol at 1 mg/mL. Standard solutions were prepared in water and spiked into plasma to produce calibration standards of 0, 2, 5, 10, 20, 50, 100 and 200 ng/mL of cyclizine and norcyclizine. Quality control (QC) samples were prepared from separately weighed stock solutions and spiked into plasma to concentrations of 5 ng/mL (LQC), 20 ng/mL (MQC) and 200 ng/mL (HQC) of both cyclizine and norcyclizine. An IS solution of 100 ng/mL cinnarizine was prepared in acetonitrile. All solutions and spiked samples were stored at −30 °C.

2.3. Sample preparation

Protein precipitation was performed by adding 100 μL of IS 100 ng/mL solution in acetonitrile to 50 μL of plasma samples followed by vortexing and centrifuging at 16,000 × *g* for 5 min. A 75 μL aliquot of clear supernatant was mixed with 50 μL formic acid 0.05% in the wells of a 96-well plate. A volume of 10 μL was injected into the LC–MS/MS system.

2.4. Instrumentation and chromatographic conditions

The LC–MS/MS system consisted of a Shimadzu LC-20AD HPLC system (Shimadzu Corporation, Kyoto, Japan) interfaced with a 3200 Q TRAP® mass spectrometer (Applied Biosystems, Foster City, Canada) equipped with a TurboIonSpray® source. Analyst™ software ver. 1.5 was used for instrument control and data acquisition. Positive mode electrospray ionisation was used, the ionspray voltage was +4500 V and the source temperature was 500 °C. Curtain gas, nebuliser gas and auxiliary gas were set to flow rates of 20, 60 and 50 psi. Data acquisition was performed via multiple reactions monitoring (MRM) with a dwell time of 150 ms. MRM transistions, collision energy (CE) and declustering potential (DP) were optimised for the individual analytes; cyclizine 267.2/167.2 (CE 19 V, DP 21 V), norcyclizine 253.2/167.2 (CE 19 V, DP 21 V), cinnarizine (IS) 369.2/167.2 (CE 25 V, DP 36 V). Calibration curves were constructed by plotting analyte/IS peak area ratios versus analyte concentrations using the Analyst™ quantification software. Analysis of linearity was performed by 1/*x*-weighted linear regression.

A Luna C8 (2) 50 mm × 2.0 mm i.d., 3 μm column was used with a C8 guard column 4 mm × 2.0 mm (Phenomenex, Torrance, CA, USA). The column was heated to 40 °C whilst the auto-sampler was cooled to 15 °C. Mobile phase A consisted of 0.05% formic acid in water and mobile phase B was methanol. The flow rate was 0.3 mL/min and a fast gradient was applied as follows: the run was started at 30% B for 0.1 min followed by a linear gradient to 90% B over 0.4 min, held at 90% B for 0.1 min, returned to 30% B over 0.1 min and allowed to equilibrate at 30% B for 2.3 min. The run time was 3 min and the first 1.5 min of the run was sent to waste. Between each run the injection needle was washed before and after injection with methanol, allowing the column another 1 min to equilibrate, resulting in a total analysis time of 4 min.

2.5. Assay validation

A set of spiked calibration standards (n = 6 for 2 ng/mL; and n = 2 for 5, 10, 20, 50, 100 and 200 ng/mL) and QC samples (n = 6 for 5, 20 and 200 ng/mL) were prepared and analysed on three different days to evaluate linearity, precision and accuracy according to FDA guidelines [12]. This included the lowest concentration calibration standard (2 ng/mL), which represented the lower limit of quantification (LLOQ) of the assay.

Absolute extraction recoveries of cyclizine, norcyclizine and IS were determined by comparing the obtained peak areas of the MQC samples (n = 4) to the peak areas of blank plasma extracts spiked with cyclizine, norcyclizine and IS at concentrations corresponding to 100% recovery. Matrix effects from the plasma extracts were assessed by comparing the peak areas of the spiked blank plasma extracts (n = 4) to the peak areas from cyclizine, norcyclizine and IS spiked in mobile phase A, which represented 100% i.e. no matrix effect [13].

Stock solution stability was determined by comparing peak areas of cyclizine and norcyclizine stock solutions stored at −30 °C for 6 months with those of freshly prepared ones. Long term stability in plasma was determined by comparing concentrations determined from MQC samples stored at −30 °C for 6 months (n = 3) with those of freshly prepared MQC samples. For a combination of short term and freeze-thaw stability, QC samples (n = 4 at 3 levels) were subjected to three freeze-thaw cycles (thaw at room temperature, freeze at −30 °C for 12–24 h) followed by bench top storage at room temperature for 4 h. The results were then compared with those of freshly made QC samples. Processed sample stability was evaluated by determining the concentrations of a set of QC samples (n = 4 at 3 levels) and storing the samples in the 96-well plate at 15 °C in the autosampler for 56 h. The samples were reanalysed and the results compared with original values.

3. Results and discussion

3.1. Assay development

Since the assay was to be used for several hundred clinical samples, protein precipitation was considered preferable for sample preparation as it is a fast and simple technique compared to the more complex procedures such as solid phase or liquid–liquid

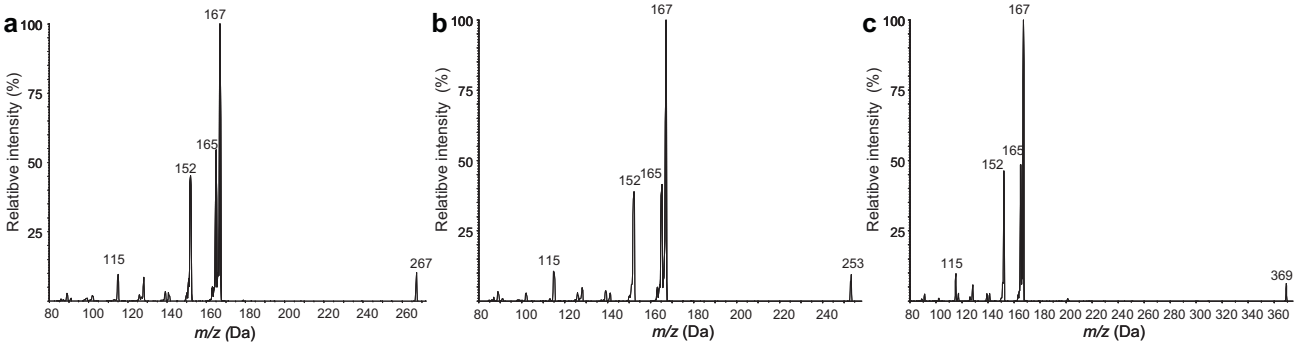

Fig. 2. Positive mode product ion spectra (MS/MS) of (a) cyclizine (m/z 267→spectrum), (b) norcyclizine (m/z 253→spectrum) and (c) cinnarizine (IS) (m/z 369→spectrum).

extraction [9]. As protein precipitation produces relatively 'dirty' extracts a potential issue is co-elution with matrix components that can lead to changes in the ionisation processes in the electrospray source of the mass spectrometer (matrix effects) [13]. The chromatography was therefore developed with the aim of obtaining separation of the analytes from potentially interfering endogenous compounds as well as a fast run time. This was obtained using a relatively short (50 mm) analytical column and a fast methanol gradient with a total analysis time of 4 min.

The MS/MS parameters were optimised to produce the most abundant product ions for cyclizine, norcyclizine and IS using electrospray ionisation. The highest signal to noise ratios were achieved in the positive mode and product ion spectra are shown in Fig. 2. For all three compounds the m/z 167 fragment was dominant and was therefore used for quantification. This fragment is likely to be a diphenylmethyl cation [5] as indicated in Fig. 1. Product ions of m/z 165, 152 and 115 were also observed for all compounds but were of lower intensity (Fig. 2). Positive mode ionisation and the MRM transition 267/167 for cyclizine have also been used in the two previously reported LC–MS/MS assays for cyclizine [10,11].

Cinnarizine was chosen as internal standard as isotopically labelled cyclizine was not available. It was found to be suitable as an internal standard as it has relatively similar chromatographic properties to the analytes and the same ionisation properties (Figs. 1 and 2), and recovery was similar (see below). Representative chromatograms of spiked plasma samples, a patient sample and a blank sample are shown in Fig. 3.

3.2. Assay validation

A set of spiked standard and quality control samples were prepared and analysed on three different days. The standard curves for cyclizine and norcyclizine were linear over the range 2–200 ng/mL in plasma ($r^2 \geq 0.996$) using $1/x$-weighted linear regression anal-

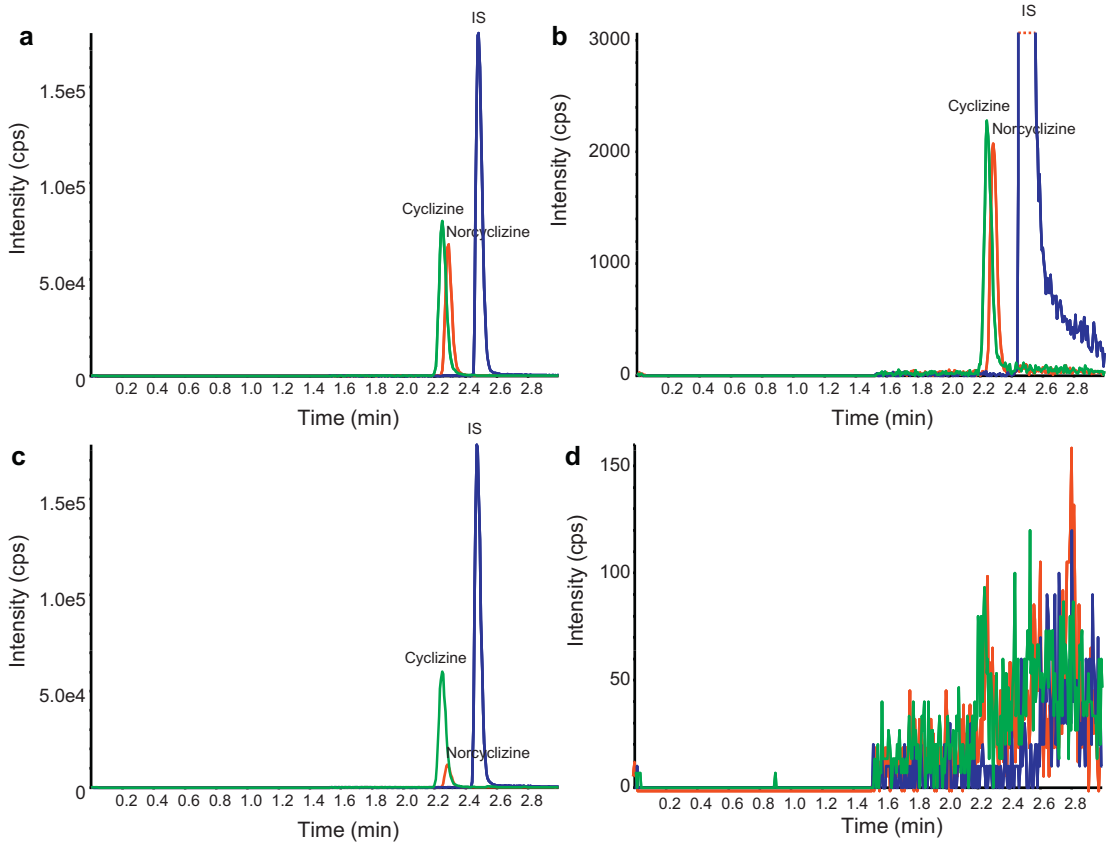

Fig. 3. LC–MS/MS example chromatograms of (a) plasma sample spiked with cyclizine and norcyclizine at 100 ng/mL, (b) plasma sample spiked cyclizine and norcyclizine at 2 ng/mL (LLOQ), (c) patient sample and (d) blank plasma. The internal standard (IS) was spiked at concentrations corresponding to 200 ng/mL in plasma.

Table 1
Intra- and inter-day precision (relative standard deviation, RSD) and accuracy (relative error, RE) for cyclizine and norcyclizine in human plasma ($n = 6$ at each level on 3 separate days).

	Intraday				Interday		
Sample	Nominal conc. (ng/mL)	Mean conc. ± SD (ng/mL)	RSD (% CV)	RE (%)	Mean conc. ± SD (ng/mL)	RSD (% CV)	RE (%)
Cyclizine							
LLOQ	2	2.00 ± 0.26	12.8	−0.1	2.02 ± 0.27	13.5	0.8
LQC	5	4.73 ± 0.29	6.2	−5.4	4.77 ± 0.44	9.2	−4.7
MQC	20	18.3 ± 1.8	10.0	−8.4	20.5 ± 2.2	10.5	2.6
HQC	200	193 ± 12	6.5	−3.4	200 ± 16	8.1	0.1
Norcyclizine							
LLOQ	2	2.01 ± 0.28	13.9	0.6	2.01 ± 0.20	10.0	0.3
LQC	5	4.89 ± 0.47	9.5	−2.2	4.80 ± 0.42	8.8	−3.9
MQC	20	18.9 ± 1.8	9.4	−5.7	21.6 ± 2.4	11.0	8.0
HQC	200	186 ± 14	7.6	−6.8	199 ± 21	10.4	−0.4

ysis. The limit of quantification was 2 ng/mL and a representative chromatogram at this concentration is shown in Fig. 3b.

Results for precision and accuracy of the assay over the range 2–200 ng/mL cyclizine and norcyclizine in human plasma are summarised in Table 1. Both precision and accuracy were within acceptable ranges for bioanalytical purposes according to FDA guidelines [8]. Relative standard deviations (RSD) were <14% for intra- and inter-day precision and the accuracy (RE) was within ±8%. Blank plasma samples from 6 different sources were tested and found to have no interference with the assay. A representative blank plasma sample is shown in Fig. 3d.

Absolute extraction recoveries following plasma protein precipitation (mean ± SD, $n = 4$) were 98% ± 4% for cyclizine, 95% ± 4% for norcyclizine and 87% ± 4% for IS. To investigate whether the matrix (protein precipitated plasma) influenced the ionisation, the response of protein precipitated plasma spiked with either cyclizine, norcyclizine or IS was compared to areas of the compounds spiked in mobile phase A (representing 100%, i.e. no matrix effect). The matrix was found to have negligible impact on the ionisation, as the matrix effects (mean ± SD, $n = 4$) were 93% ± 11% for cyclizine, 96% ± 12% for norcyclizine and 110% ± 13% for IS as compared to mobile phase.

Cyclizine and norcyclizine stock solutions in methanol were found to be stable during freezing at −30 °C for 6 months, as the concentrations of stored solutions deviated <9% for cyclizine and <3% for norcyclizine from freshly prepared stock solutions. Cyclizine and norcyclizine were found to be stable in plasma during freezing at −30 °C for 6 months, as stored samples deviated <10% for cyclizine and <2% for norcyclizine from freshly prepared samples. Plasma samples subject to three freeze-thaw cycles and storage at room temperature for 4 h, deviated <7% from freshly prepared samples for both analytes. Processed samples stored in the autosampler at 15 °C for 56 h deviated less than 7% from freshly prepared samples. In the literature, cyclizine has been reported to be stable in serum for up to 4 weeks at −10 °C and 4 °C [8,10] and able to tolerate three freeze-thaw cycles [10]. No data regarding norcyclizine stability in plasma could be found, but norcyclizine was reported to be stable in urine for 4 weeks at −10 °C and for 2 weeks at 4 °C [8]. This paper thus extends the published stability data for cyclizine and norcyclizine.

Compared to previous assays the developed assay is superior in that it allows simultaneous quantification of cyclizine and its main metabolite norcyclizine in a fast and simple manner with a total analysis time of 4 min, using only 50 μL of plasma and with 89–95% recovery of analytes. Previously published assays employed solid-phase extraction of 1 mL of plasma followed by a phase separation step (overall recovery 65–83%) and run times of 15–20 min [7,8]. The only other published LC–MS/MS assay for cyclizine (but not norcyclizine) used liquid–liquid extraction (80% recovery) and had a 14 min run time [10]. The LC–MS/MS antihistamine screening assay had a short run time of 4 min, but the sample preparation consisted of liquid–liquid extraction with a reported cyclizine recovery of 137% [11], which might be acceptable for screening but not quantification. All assays had limits of quantification of 1–5 ng/mL of cyclizine, which is similar to that obtained with the developed assay and clinically appropriate as therapeutic concentrations have been reported to be around 50–100 ng/mL [10].

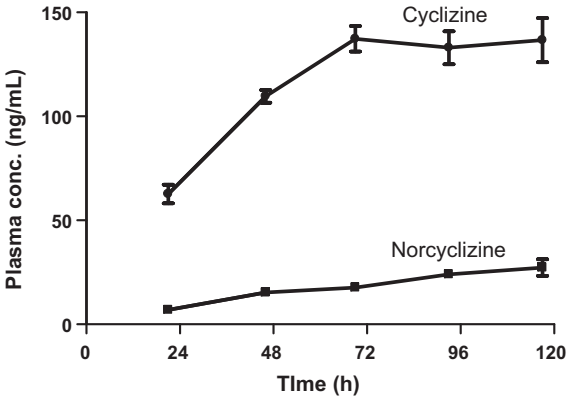

Fig. 4. Plasma concentration–time curves (mean ± SD of triplicate samples) for cyclizine (●) and its main metabolite norcyclizine (■) following initiation of subcutaneous infusion of 150 mg cyclizine/24 h to a patient.

3.3. Assay application

This LC–MS/MS assay was used in a clinical study of cyclizine pharmacokinetics involving triplicate analysis of ∼100 plasma samples and it was found to be robust and reliable. The average precision (RSD) of the triplicate determinations was 6% for both cyclizine and for norcyclizine, and the triplicate determinations were within 80–120% of the mean value in 99% of the samples. An example of plasma concentration–time curves of cyclizine and norcyclizine following initiation of subcutaneous infusion of cyclizine at 150 mg/24 h is shown in Fig. 4. The clinical study was approved by the Upper South A Regional Ethics Committee, New Zealand. The outcomes of the clinical study will be reported elsewhere.

4. Conclusion

A rapid and simple LC–MS/MS assay for determining cyclizine and its main metabolite norcyclizine in human plasma at concentrations of 2–200 ng/mL was developed and validated. The assay was found to be reproducible, accurate and precise and has been applied successfully to a clinical study.

Conflict of interest statement

The authors do not have a commercial or other association that might pose a conflict of interest.

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References

[1] S.C. Sweetman (Ed.), Martindale: The Complete Drug Reference, Pharmaceutical Press, London, 2009.
[2] R.B. Walker, I. Kanfer, Eur. J. Pharm. Sci. 4 (1996) 301.
[3] R. Kuntzman, A. Klutch, I. Tsai, J.J. Burns, J. Pharmacol. Exp. Ther. 149 (1965) 29.
[4] H. Luo, E.M. Hawes, G. McKay, E.D. Korchinski, K.K. Midha, Xenobiotica 21 (1991) 1281.
[5] M.C. Dumasia, L. Grainger, E. Houghton, Xenobiotica 32 (2002) 795.
[6] M.C. Dumasia, Xenobiotica 32 (2002) 809.
[7] R.B. Walker, I. Kanfer, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 672 (1995) 172.
[8] R. Walker, I. Kanfer, Chromatographia 24 (1987) 287.
[9] B. Law, I.D. Wilson (Eds.), Bioanalytical Separations, Elsevier, Amsterdam The Netherlands, 2003.
[10] A. Mohammadi, I. Kanfer, V. Sewram, R.B. Walker, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 824 (2005) 148.
[11] M. Gergov, J.N. Robson, I. Ojanpera, O.P. Heinonen, E. Vuori, Forensic Sci. Int. 121 (2001) 108.
[12] Guidance for Industry, Bioanalytical Method Validation, US Department of Health and Human Services, US FDA Center for Drug Development and Research, USA, (2001).
[13] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019.