Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 809 (2004) 351–356
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

New method for the chiral evaluation of mirtazapine in human plasma by liquid chromatography

Fernando José Malagueño de Santana, Evandro José Cesarino, Pierina Sueli Bonato*

Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Av. Café SN, CEP 14040-903, Ribeirão Preto, SP, Brazil

Received 6 May 2004; received in revised form 15 July 2004; accepted 16 July 2004

Available online 3 August 2004

Abstract

A simple, rapid and sensitive high-performance liquid chromatography (HPLC) method was developed for the enantioselective analysis of the new antidepressant drug mirtazapine in human plasma. The procedure involved liquid–liquid extraction using toluene, followed by liquid chromatography coupled to UV detection at 292 nm. The chromatographic separation of the (+)-(*S*)- and (−)-(*R*)-enantiomers of mirtazapine was achieved on a Chiralpak AD column (250 mm × 4.6 mm, 10 μm particle size) protected with a CN guard column, using hexane–ethanol (98:2, v/v) plus 0.1% diethylamine as the isocratic mobile phase, at a flow rate of 1.2 ml/min. The total analysis time was less than 12 min per sample. The recoveries of (+)-(*S*)- and (−)-(*R*)-mirtazapine were in the 88–111% range with a linear response over the 6.25–625 ng/ml concentration range for both enantiomers. The quantification limit (LOQ) was 5 ng/ml. Within-day and between-day assay precision and accuracy were studied at three concentration levels (10, 50 and 250 ng/ml). For both mirtazapine enantiomers, the coefficients of variation (CV) and deviation from the theoretical value were lower than 15% at all concentration levels. The method proved to be suitable for pharmacokinetic studies.

Keywords: Enantiomer separation; Mirtazapine; Antidepressant; Chiral stationary phase

1. Introduction

Mirtazapine is a chiral drug (1,2,3,4,10,14*b*-hexahydro-2-methylpyrazino-[2,1-*a*]-pyrido[2,3-*c*][2]benzazepine), commercialized as a 50:50 mixture of (+)-(*S*)- and (−)-(*R*)-enantiomers, and used as an antidepressant in the treatment of moderately and severely depressed hospitalized and out-patients [1]. Mirtazapine acts as an antagonist of α_2-adrenergic auto and heteroreceptors, resulting in increased release of norepinephrine and serotonin. It is also an antagonist of postsynaptic serotonin type 2 (5-HT_2) and type 3 (5-HT_3) [1,2].

After oral administration, mirtazapine is rapidly and completely absorbed, and then extensively biotransformed in the liver [3]. The biotransformation of mirtazapine includes 8-hydroxylation, *N*(2)-demethylation, *N*(2)-oxidation, as well as direct conjugation of the drug with glucuronic acid and conjugation of its metabolites with glucuronic or sulphuric acid [3,4].

The pharmacokinetics and pharmacodynamics of mirtazapine appear to be enantioselective, as shown by the differences in the kinetic parameters and effects of its enantiomers [3,5]. The enantiomers of mirtazapine show different receptor binding affinity. The α_2-autoreceptor and 5-HT_2 blocking effects of mirtazapine are present primarily in the (+)-(*S*)-enantiomer of mirtazapine, whereas the α_2-heteroreceptor and 5-HT_3 type receptor antagonistic activities reside predominantly in the (−)-(*R*)-enantiomer [6]. After single oral dose administration, the (−)-(*R*)-enantiomer appears in plasma at up to three times the concentration and has a longer plasma half-life than the other enantiomer [3,5]. A study of the metabolism of mirtazapine enantiomers by human cytochrome P450 enzymes demonstrated that (+)-

* Corresponding author. Tel.: +55 16 6024261; fax: +55 16 6332960.
E-mail address: psbonato@fcfrp.usp.br (P.S. Bonato).

1570-0232/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2004.07.012

(*S*)-mirtazapine was extensively metabolised by CYP2D6 [7].

The separation and quantification of the enantiomers may be important to allow therapeutic drug monitoring and the determination of the clinical response and of the pharmacokinetic properties of the drug. Several non-chiral analytical methods have been described for the determination of racemic mirtazapine (*rac*-mirtazapine) in human plasma and serum, including high-performance liquid chromatography (HPLC) with fluorescence [8–11] and ultraviolet detection [12] and gas chromatography (GC) [13,14]. Some methods to quantify *rac*-mirtazapine in pharmaceutical forms were described using capillary electrophoresis (CE) [15,16] and UV–vis spectrophotometry [17].

To our knowledge, only one chromatographic chiral method has been described in the literature for the determination of mirtazapine enantiomers in biological fluid by high-performance liquid chromatography with UV detection [18].

This study describes a novel, rapid and sensitive analytical chiral method for the quantification of mirtazapine enantiomers in plasma samples using high-performance liquid chromatography under normal-phase conditions. Validation parameters of the method were calculated in terms of recovery, linearity, precision, quantification limit and accuracy.

2. Experimental

2.1. Chemicals and reagents

Rac-mirtazapine (laboratory code Org 3770, purity ≥98%) and *rac*-demethylmirtazapine were kindly supplied by Analytical Control Labs., N.V. Organon (Oss, The Netherlands). Hexane, methanol, ethanol and toluene were purchased from Merck (Darmstadt, Germany) and were of chromatography grade. Diethylamine was supplied by Fluka (Buchs, Switzerland) and sodium hydroxide was obtained from Nuclear (São Paulo, Brazil), both of analytical grade.

2.2. Apparatus and chromatographic conditions

Analyses were conducted using a Shimadzu (Kyoto, Japan) liquid chromatograph, with an LC-AT VP solvent pump unit and an SPD-10A UV–vis detector operating at 292 nm. Injections were performed manually through a 50 μl loop with a Rheodyne model 7125 injector (Rheodyne, Cotati, USA). Data were collected using a Chromatopak CR6A integrator (Shimadzu, Kyoto, Japan). To establish the elution order, a Jasco CD-1595 circular dichroism detector (Jasco, Tokyo, Japan) was used. The resolution of the mirtazapine enantiomers was evaluated at 23 (±2) °C on several chiral columns, i.e. Chiralpak AD (250 mm × 4.6 mm, 10 μm particle size), Chiralpak AD-RH (150 mm × 4.6 mm, 5 μm particle size), Chiralcel OG (250 mm × 4.6 mm, 10 μm particle size), Chiralcel OJ (250 mm × 4.6 mm, 10 μm particle size) (all purchased from Chiral Technologies, Exton, PA, USA), Ultron ES-OVM (150 mm × 4.6 mm, Rockland Technologies, Newport, DE, USA) and a Chiral AGP column (150 mm × 4.0 mm, 5 μm particle size, ChromTech AB, Hägersten, Sweden). A CN column (4 mm × 4 mm, 5 μm particle size, Merck, Darmstadt, Germany) was used as guard column. The best resolution was achieved on the Chiralpak AD column using hexane–ethanol (98:2, v/v) plus 0.1% diethylamine as the mobile phase, at a flow-rate of 1.2 ml/min.

2.3. Calibration and internal quality control solutions

Human plasma samples from healthy volunteers were supplied by The Blood Center of the University Hospital, Faculty of Medicine of Ribeirão Preto (University of São Paulo, São Paulo, Brazil). Individual plasma samples were evaluated for endogenous interference before use.

Stock standard and working solutions were prepared in methanol in the concentration range of 6.25–625 μg/ml. They were stored frozen at −20 °C and protected from direct light, remaining stable for at least 3 month.

Measurements were performed on 1 ml drug-free fresh frozen plasma spiked with 25 μl of standard solutions of (+)-(*S*)-mirtazapine and (−)-(*R*)-mirtazapine. No internal standard was used in this method. Plasma quality controls (QC) spiked with 10, 50 and 250 ng/ml of both enantiomers were prepared to measure the accuracy and precision of the method.

2.4. Sample preparation procedure

A 100 μl aliquot of 10 mmol sodium hydroxide and 4 ml of the extracting solvent toluene were added to 1 ml of unknown plasma samples, spiked plasma samples or quality control samples. The mixture was submitted to mechanical shaking at 200 rpm for 30 min and centrifuged at 1800 × *g* for 10 min. Appropriate volumes (3 ml) of the upper organic phases were transferred to conical tubes and the contents were evaporated to dryness under a gentle stream of compressed air at room temperature. The residues were dissolved in 100 μl of the mobile phase and 50 μl was injected into the chromatographic system.

2.5. Validation of the method

The recovery of each mirtazapine enantiomer was determined at plasma concentrations of 6.25, 12.5, 62.5, 125 and 625 ng/ml (n = 3 for each concentration). Drug-free plasma samples (1 ml) were spiked with known amounts of mirtazapine to obtain the concentrations previously specified. These samples were submitted to the extraction procedure and their concentrations were determined on the basis of a calibration curve obtained by the direct injection of mirtazapine enantiomers in the mobile phase.

Linearity of the analytical method was evaluated by analysing spiked plasma samples for each concentration (n = 2) over the concentration range of 6.25–625 ng/ml for each enantiomer of mirtazapine. The results were plotted on a graph of peak height (Y) versus plasma concentration (X) and the best relationship was obtained by linear least-squares regression analysis.

The sensitivity of the method was evaluated by determining the quantification limit (LOQ). The LOQ was defined as the lowest mirtazapine enantiomers concentration that could be determined with an accuracy and a precision below 20% over five analytical runs with 1.0 ml of plasma and was obtained using plasma samples (n = 5) spiked with 5 ng/ml of each mirtazapine enantiomer.

The precision and accuracy of the method were determined over 4 days. Each day, one calibration curve and 15 determinations of five quality controls in three concentrations, 10, 50 and 250 ng/ml of each mirtazapine enantiomers were performed. The within-day (n = 5) and between-day (n = 4) results were expressed as relative standard deviations (coefficient of variation, CV) and deviation from the theoretical value, respectively.

To assess the applicability of the validated method, mirtazapine enantiomers were determined in plasma samples collected from a healthy volunteer after a single oral administration of 30 mg of *rac*-mirtazapine (Remeron®, N.V. Organon). Venous blood was collected into heparinized tubes at 0, 1, 2, 3, 5 and 12 h after drug administration and the tubes were centrifuged for 10 min at 1800 × g. The plasma samples obtained were stored at −20 °C until analysis. We also analyzed a plasma sample collected at steady state (immediately before drug administration) from a patient under depression treatment. The volunteers gave written informed consent to participate in the investigation, which was approved by the Ethics Committee of the Faculty of Pharmaceutical Sciences of Ribeirão Preto–University of São Paulo (process number 19-CEP/FCFRP).

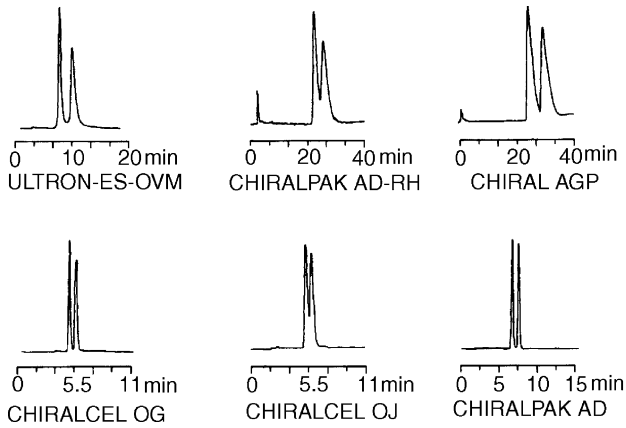

Fig. 1. Chromatograms referring to the separation of mirtazapine enantiomers on different columns and under different chromatographic conditions (as specified in Table 1).

3. Results and discussion

3.1. Chromatographic and extraction conditions

The chiral resolution of mirtazapine enantiomers was evaluated in several cellulose and amylose derivatives and protein-based chiral stationary phases: Chiralcel OG, Chiralcel OJ, Chiralpak AD, Chiralpak AD-RH, Ultron ES-OVM and Chiral AGP column (Fig. 1). The first three columns were evaluated under normal phase conditions using hexane–isopropanol or hexane–ethanol as the mobile phase. Diethylamine was added to these mobile phases in order to reduce the interaction of the basic drug with the silanol groups of the silica support. The other three columns were evaluated under reversed phase conditions using buffer–acetonitrile or buffer–methanol mixtures. Table 1 describes the optimized chromatographic conditions as well as the retention and separation parameters.

Table 1
Optimized chromatographic conditions for the chiral separation of mirtazapine in plasma

Mobile phase	N	Rs	F	k_1	α	As
Chiralpak AD						
Hexane:ethanol (98:2, v/v) + diethylamine (0.1%, v/v)	4010	1.82	1.5	3.41	1.16	1.1
Chiralcel OJ						
Hexane:ethanol (98:2, v/v) + diethylamine (0.1%, v/v)	1600	0.86	1.5	1.96	1.15	1.2
Chiralcel OG						
Hexane:ethanol (98:2, v/v) + diethylamine (0.1%, v/v)	2153	1.78	1.5	1.84	1.15	1.1
Chiral AGP						
Ammonium acetate 10 mmol/l, pH 5.5:acetonitrile (95:5, v/v)	762	1.23	1.0	27.06	1.20	2.0
ULTRON-ES-OVM						
Sodium phosphate 70 mmol/l, pH 4.8:acetonitrile (95:5, v/v)	563	1.75	1.0	4.36	1.40	1.4
CHIRALPAK AD-RH						
Sodium borate 20 mmol/l, pH 9.2:acetonitrile (70:30, v/v)	416	0.9	1.0	12.23	1.15	1.5

N, theoretical plates; Rs, resolution; F, flow-rate (ml/min); k_1, retention factor for the first eluted enantiomer (t_m was defined as the first significant baseline disturbance, corresponding to the retention time of a non retained solute); α, separation factor; As, asymmetry factor for the first eluted enantiomer.

Under the chromatographic conditions described on Table 1, the Chiralpak AD column was chosen once it provided better resolution and efficiency as well as relatively short retention times (Table 1 and Fig. 2). The use of a CN guard column to protect the column evaluated did not disturb significantly the chromatographic behavior.

Dodd et al. [18] reported the separation of mirtazapine enantiomers in the Chiralpak AD column using hexane–ethanol–isopropanol (98:1:1, v/v) as the mobile phase, imipramine as the internal standard and solid-phase extraction (SPE) to prepare the samples. The detection was carried out at 290 nm. The method described in this paper uses hexane–ethanol (98:2, v/v) as the mobile phase plus 0.1% diethylamine. The addition of diethylamine to the mobile phase resulted in narrower peaks than the above method, improving the resolution. Furthermore, we used a simpler procedure to prepare the sample (liquid–liquid extraction), which resulted in excellent recovery of drug enantiomers with suitable precision and accuracy.

The elution order was defined by submitting *rac*-mirtazapine to circular dichroism (CD) detection using the same chromatographic conditions as described above and comparing the CD spectra of pure enantiomers with those presented by Moody et al. (Fig. 3) [19].

The elution order was also evaluated by analysing the pure enantiomers (obtained by semipreparative analysis under the conditions established in the present paper) using the procedure described by Dodd et al. [18]. The use of a different mobile phase did not change the elution order of mirtazapine enantiomers.

The *N*-demethyl metabolite of mirtazapine is not measured by this method, but under the chromatographic condition established, the enantiomers of the metabolite elute at retention time of 58 min (not resolved). In the analysis of real samples the injection time should be controlled in order to avoid the co-elution of this metabolite from a previously injected sample. The chromatograms were free from interfering peaks e.g. no significant co-elution with endogenous compounds was found. Representative chromatograms of a drug-free plasma sample, a spiked plasma sample and a patient plasma sample are illustrated in Fig. 2.

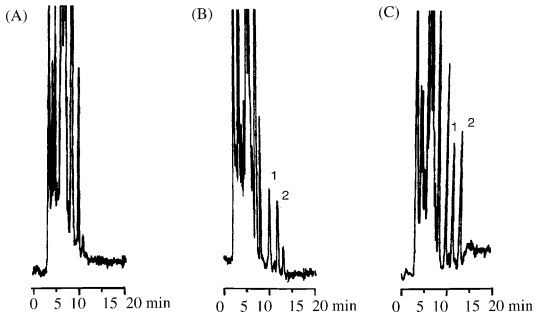

Fig. 2. Chromatograms referring to drug-free plasma (A), plasma spiked with 12.5 ng/ml of (+)-(*S*)-mirtazapine (1) and (−)-(*R*)-mirtazapine enantiomers (2) (B) and plasma sample collected from a patient under mirtazapine treatment (C). The analysis was performed on a Chiralpak AD column using hexane:ethanol (98:2, v/v) plus 0.1% diethylamine at a flow rate of 1.2 ml/min, λ = 292 nm.

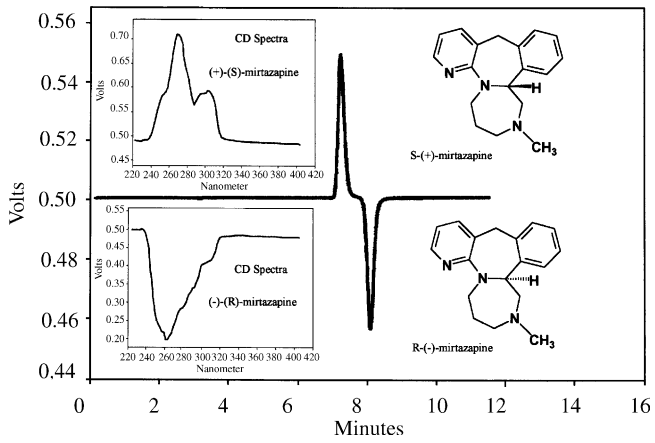

Fig. 3. CD Chromatogram (detection at 250 nm) of a *rac*-mirtazapine and CD spectra of (+)-*S*-mirtazapine and (−)-*R*-mirtazapine. Chromatographic conditions were as specified on Fig. 2.

3.2. Method validation

The liquid–liquid extraction recoveries obtained were between 88 and 111%, with CV values lower than 5.5% for both enantiomers (Table 2) and show that the method is suitable for the analysis of both enantiomers in biological fluids.

The method proved to be linear over the concentration range of 6.25–625 ng/ml, with typical calibration curve equations determined as $Y = -582.44 + 797.91X$ and $Y = -571.05 + 673.24X$ for the (+)-(*S*)- and (−)-(*R*)-enantiomers of mirtazapine, respectively, and a determination coefficient (r^2) ≥ 0.991.

The excellent accuracy and precision of the assay are summarized in Table 3. The within-day assay coefficients of variation (CVs) for all compounds were lower than 3.8% and all between-day assay CVs were below 10.3%. The within-day and between assay accuracies for all compounds were found to be within 1.3 and −7.2% for 10 ng/ml, 3.9 and −4.7% for 50 ng/ml and 5.5 and −4.3% for 250 ng/ml. The lowest concentration quantified by the validated method (LOQs) was 5 ng/ml (Table 3), a lower value than that reported in the literature (10 ng/ml) [18].

Table 2
Mean recovery of mirtazapine enantiomers in plasma

Plasma concentration (ng/ml, *n* = 3)	(+)-*R*-mirtazapine		(−)-*S*-mirtazapine	
	Recovery (%)	CV (%)	Recovery (%)	CV (%)
6.25	111.1	3.8	107.1	4.2
12.5	97.5	5.5	97.5	4.8
62.5	94.3	3.4	95.8	3.8
125	88.6	5.3	90.8	5.4
625	99.9	2.2	92.8	3.0
Range (6.25–625)	96.5	9.2	96.8	7.1

n, Number of samples; CV, coefficient of variation.

Table 3
Precision, accuracy and limit of quantification for the analysis of mirtazapine enantiomers in plasma

Nominal standard concentration (ng/ml)	Analysed concentration (ng/ml)		Accuracy[a]		Precision[b]	
	(−)-(*S*)-	(+)-(*R*)-	(−)-(*S*)-	(+)-(*R*)-	(−)-(*S*)-	(+)-(*R*)-
Within-day (n = 5)[c]						
5[e]	5.00	4.47	0.0	−10.7	10.7	9.0
10	9.94	10.13	−0.6	1.3	3.0	3.1
50	50.18	51.93	0.4	3.9	2.1	3.0
250	252.49	263.62	1.0	5.4	2.2	3.8
Between-day (n = 4)[d]						
10	9.28	9.56	−7.2	−4.4	5.0	10.3
50	47.63	48.33	−4.7	−3.3	4.6	6.1
250	239.15	242.88	−4.3	−2.8	4.0	4.7

[a] Expressed as deviation from the theorical values.
[b] Expressed as coefficient of variation.
[c] Number of samples.
[d] Number of days.
[e] Quantification limit.

The method developed here proved to be highly selective since the retention times for the drugs analyzed under the same chromatographic conditions of mirtazapine analysis were not similar to those obtained for mirtazapine enantiomers (Table 4).

The developed and validated method was used in the analysis of some samples collected from a patient under mirtazapine treatment and from a healthy volunteer after a single oral administration of *rac*-mirtazapine. The chromatogram in Fig. 2C obtained from the patient shows higher concentrations of (−)-(*R*)-mirtazapine ((−)-(*R*)-/(+)-(*S*)-mirtazapine = 1.14), in agreement with literature data [3,5,18].

In contrast, the analysis of the samples collected from the healthy volunteer showed a higher concentration of the (+)-(*S*)-enantiomer (Fig. 4). Since (+)-(*S*)-mirtazapine is preferentially cleaved via 8-hydroxylation catalysed by the CYP2D6 isoenzyme, this result is in accordance with the hypothesis of the subject been a poor metaboliser [3].

Table 4
Evaluation of the interference of some drugs in the analysis of mirtazapine enantiomers

Drug	t_R	Drug	t_R
Alprazolam	ND	Mexiletine	ND
Atenolol	ND	Mirtazapine	9.5/11
Bromazepam	ND	*N*-demethyldiazepam	ND
Caffeine	ND	*N*-demethylmirtazapine	58
Clobazam	ND	Pindolol	ND
Disopyramide	19.2/27.0	Procainamide	ND
Phenobarbital	ND	Propranolol	15.0/33.6
Fluoxetine	ND	Salbutamol	ND
Flunitrazepam	ND	Triazolam	ND
Imipramine	17.1/19.2	Trimetropim	ND
Lidocaine	17.5/19.6	Verapamil	17.5/19.6
Metoprolol	ND		

t_R: Retention time in minutes; ND, not detected by the chromatographic method up to 60 min run time.

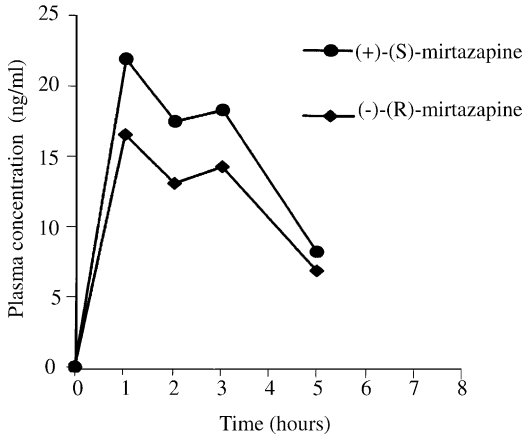

Fig. 4. Time-concentration profile of mirtazapine enantiomers after oral administration of the racemic drug.

4. Conclusion

A simple, rapid, sensitive and reproducible HPLC method using a common UV detector and liquid–liquid extraction was developed for the measurement of the two enantiomers of mirtazapine in human plasma. The Chiralpak AD column proved to be the most suitable column for the resolution of mirtazapine enantiomers under the chromatographic conditions used. The validated method allows the determination of mirtazapine in the 6.25–625 ng/ml range with a quantification limit of 5 ng/ml for both enantiomers. The values of validation presented in this paper demonstrate that the method is superior to the other method described in the literature for the analysis of mirtazapine enantiomers.

Acknowledgments

The authors are grateful to Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP) and CNPq

(Conselho Nacional de Desenvolvimento Científico e Tecnológico) for financial support and for granting a research fellowship.

References

[1] T. Puzantian, Am. J. Health-Sys. Pharma. 55 (1998) 44.
[2] J.M. Gorman, J. Clin. Psychiatry 60 (1999) 9.
[3] C.J. Timmer, J.M. Ad Sitsen, L.P. Delbressine, Clin. Pharmacokinet. 38 (2000) 461.
[4] J. Fawcett, R.L. Barkin, J. Affect. Disorders 51 (1998) 267.
[5] L.P.C. Delbressine, M.E.G. Moonen, F.M. Kaspersen, G.N. Wagenaars, P.L. Jacobs, C.J. Timmer, J.E. Paanakker, H.J.M. van Hal, G. Voortman, Clin. Drug Invest. 15 (1998) 45.
[6] C. McGrath, G.D. Burrows, T.R. Norman, Eur. J. Pharmacol. 356 (1998) 121.
[7] S. Dodd, D.W. Boulton, G.D. Burrows, C.L. DeVane, T.R. Norman, Hum. Psychopharmacol. Clin. Exp. 16 (2001) 541.
[8] F.A. Maris, E. Dingler, S. Niehues, J. Chromatogr. B 721 (1999) 309.
[9] P.E. Morgan, J. Tapper, E.P. Spencer, J. Chromatogr. B 798 (2003) 211.
[10] P. Ptacek, J. Klima, J. Macek, J. Chromatogr. B 794 (2003) 323.
[11] M. Shams, C. Hiemke, S. Härtter, Ther. Drug Monit. 26 (2004) 78.
[12] T. Romiguieres, F. Pehourcq, M. Matoga, B. Begaud, C. Jarry, J. Chromatogr. B 775 (2002) 163.
[13] J.E. Paanakker, H.J.M. van Hal, J. Chromatogr. 417 (1987) 203.
[14] G. Voortman, J.E. Paanakker, Hum. Psychopharmcol. Clin. Exp. 10 (1995) 83.
[15] G.S. Wynia, G. Windhorst, P.C. Post, F.A. Maris, J. Chromatogr. A 773 (1997) 339.
[16] L. Labat, P. Dallet, E. Kummer, J.P. Dubost, J. Pharm. Biomed. Anal. 28 (2002) 365.
[17] N. Karasen, S. Altinöz, J. Pharm. Biomed. Anal. 24 (2000) 11.
[18] S. Dodd, G.D. Burrows, T.R. Norman, J. Chromatogr. B 748 (2000) 439.
[19] J.D. Moody, J.P. Freeman, P.P. Fu, C.E. Cerniglia, Drug Metab. Dispos. 30 (2002) 1274.