Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 809 (2004) 111–116
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Field-amplified sample stacking in capillary electrophoresis for the determination of clozapine, clozapine N-oxide, and desmethylclozapine in schizophrenics' plasma

Yu-Hsiang Ho [a], Wei-Kung Ko [c], Hwang-Shang Kou [a], Hsin-Lung Wu [a], Shou-Mei Wu [a,b,*]

[a] *Faculty of Pharmacy, College of Pharmacy, Kaohsiung Medical University, Kaohsiung 807, Taiwan*
[b] *Faculty of Fragrance and Cosmetics, College of Pharmacy, Kaohsiung Medical University, Kaohsiung 807, Taiwan*
[c] *Jing-Ho Mental Hospital, Kaohsiung 807, Taiwan*

Received 4 March 2004; received in revised form 27 May 2004; accepted 8 June 2004

Available online 13 July 2004

Abstract

A method of field-amplified sample stacking in capillary electrophoresis is described for the simultaneous determination of clozapine (CZP) and its metabolites, clozapine N-oxide (CNO), and desmethylclozapine (DMC), in human plasma. Plasma (0.2 mL) was extracted with organic solvents (ethyl acetate/*n*-hexane/isopropyl alcohol, 8/1/1 by volume) and centrifuged. An aliquot of supernatant was evaporated and suitably reconstituted with water for CE analysis. An untreated fused-silica capillary was used (31.2 cm; effective length, 20 cm; 50 μm i.d.) for the analysis. The background buffer was phosphate buffer (400 mM, pH 3.0) containing 50% ethylene glycol. The separation voltage was 25 kV with a detection wavelength of 214 nm. In the method validation, the calibration curves were linear ($r \geqq 0.98$) over a range of 50–800 ng/mL for CZP, 30–180 ng/mL for CNO, and 25–600 ng/mL for DMC. The relative standard deviation (R.S.D.) and relative error (R.E.) were all less than 11% for the intra- and inter-day assays. The limits of detection ($S/N = 3$, electric-driven injection, 99.9 s) of CZP, DMC, and CNO were 5, 5, and 10 ng/mL, respectively. After continuing treatment with the CZP tablets, a blood sample from one male schizophrenic patient (41-year-old, 62 kg) who had been receiving ongoing treatment with the CZP tablets was prepared and analyzed. The levels of CZP, DMC, and CNO were determined and the feasibility of the method's application in clinical treatment was proven.

Keywords: Field-amplified sample stacking; Clozapine; Clozapine N-oxide; Desmethylclozapine

1. Introduction

Field-amplified sample stacking in capillary electrophoresis (FASS-CE) is a simple technique to improve the accuracy of measurements of clozapine (CZP) and its metabolites, clozapine N-oxide (CNO), and desmethylclozapine (DMC), in human plasma. This technique overcomes the problem of decreased sensitivity caused by small injection volumes and narrow optical path length. The technique described herein requires no additional equipment and is based on the idea that ions electrophoretically migrating through a low-conductivity solution into a high-conductivity solution slow down dramatically at the boundary of the two buffers and stack into a narrow zone [1,2]. This method could result in up to a 100-fold sensitivity enhancement [3], and can be applied to biological materials [4].

CZP (Fig. 1) is the first atypical antipsychotic (AP) that has been found to be a potent serotonin–dopamine antagonist [5]. CZP has shown superiority over other APs in randomized clinical trials involving treatment-resistant schizophrenia [6]. It can improve both positive and negative symptoms and has fewer side effects than other drugs. In the mid-1970s, reports of agranulocytosis [7] resulted in the withdrawal of CZP from the market in the USA and other countries, including Taiwan. But in the early 1990 s, it was re-approved for use in the USA and Taiwan. CZP is extensively metabolized in the liver by cytochrome P450 isoenzymes yielding several derivatives, mainly desmethylclozapine (DMC, Fig. 1) and clozapine N-oxide (CNO, Fig. 1). DMC is pharmacologically active. Some authors have suggested that the

* Corresponding author. Tel.: +886 7 3121101x2164; fax: +886 7 3159597.
E-mail address: shmewu@kmu.edu.tw (S.-M. Wu).

1570-0232/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2004.06.011

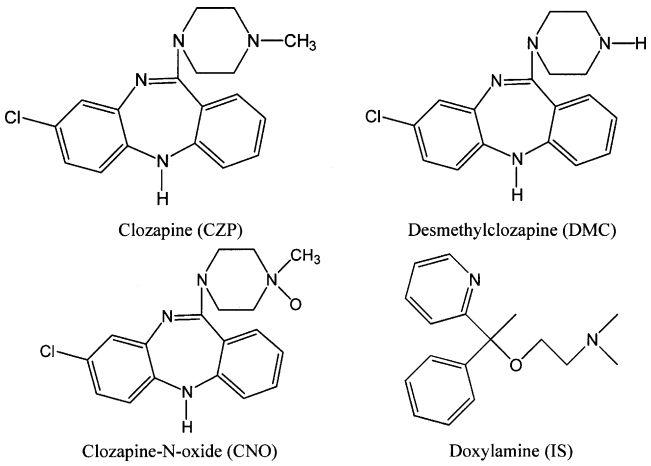

Fig. 1. Structures of clozapine (CZP), clozapine N-oxide (CNO), desmethylclozapine (DMC), and doxylamine (IS).

main cause of agranulocytosis is DMC rather than the parent drug [8]. The other metabolite, CNO, can be converted back to the parent drug through a reduction process [5]. Therefore, CNO could influence the pharmacokinetics of the parent compound. Due to the large inter-individual variability in bioavailability, elimination, the pharmacokinetic interactions with other drugs, and the avoiding the onset of agranulocytosis, therapeutic drug monitoring is suggested in the clinical management of patients treated with CZP.

We have established one HPLC method [9], and were interested in developing an alternative CE analysis. In a survey of recent CE methods, Raggi and co-workers established some CZE methods [10–14], which were applied using a phosphate buffer, or coupled with β-cyclodextrin or polyvinylpyrrolidone. Jin et al. employed the CZE following end-column amperometric detection for the analysis of CZP, but did not include metabolites [15]. They discussed the separation parameters for determining the optimum conditions for separation and applications in the quality control of pharmaceuticals [10,11] or patient's plasma [12]. None of the above-mentioned experiments showed, without additional facilities, that sample stacking in CE enhanced sensitivity in the detection of CZP and its metabolites.

In this study, we established a FASS-CE to determine the plasma levels of CZP and its metabolites. The parameters, including water plug, sampling voltage, concentration and pH of buffer, and percentage of ethylene glycol, were applied to plasma samples and successfully optimized and validated. This method was proven effective in monitoring the therapeutic drug levels of schizophrenic patients treated with CZP.

2. Experimental

2.1. Reagents, reference, and plasma sample standards

CZP, CNO, DMC, doxylamine (internal standard, IS) (Sigma, St. Louis, MO, USA), Clozaril® (CZP tablet, Novartis Pharmaceutica, England), methanol, isopropanol, *n*-hexane, ethyl acetate (Tedia, USA), sodium dihydrogen phosphate, phosphoric acid (85%), and ethylene glycol (Merck, Darmstadt, Germany) were used without further treatment. Water treated by a Milli-Q apparatus (Millipore, MA, USA) was used for the preparation of buffer and related aqueous solutions. Plasma was obtained by centrifuging at 1800 × *g* the whole blood of healthy subjects for 10 min.

Stock solutions of the analytes at 100 μg/mL were prepared in methanol and suitably diluted by water as reference standards.

Plasma sample standards were prepared by spiking various levels of reference standards into healthy plasma.

2.2. CE conditions

A Beckman CE (Model P/ACE MDQ, Fullerton, CA, USA) equipped with a filter UV detector and a liquid-cooling device was used. CE was performed in an uncoated fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 31.2 cm (effective length, 20 cm) × 50 μm i.d. The detector was set at 214 nm. The run buffer was composed of 50% (v/v) ethylene glycol in phosphate buffer (400 mM; pH 3.0). Capillary conditioning between runs was carried out by applying positive pressure at the injection end to rinse the capillary with 0.1N NaOH (5 min) and run buffer (10 min). The capillary tip was dipped for 3 s into a vial containing water. Additionally, a water plug (application of 0.5 psi for 5 s) was introduced into the capillary. The sample was electrokinetically injected using a voltage of 6 kV for 99.9 s. The separation voltage was 25 kV. Due to the low conductivity of the sample zone, the current gradually increased from about 0 to 80 μA within the first 10 s of power application. All of the operations and electropherograms were computer-controlled using Beckman P/ACE MDQ software.

2.3. Extraction procedure

A 0.2 mL aliquot of plasma was extracted with 0.5 mL of ethyl acetate:*n*-hexane:isopropanol (8:1:1 (v/v)) containing IS (500 ng/mL), vortexed for 1 min, and centrifuged at 10,000 × *g* for 5 min. A 350 μL aliquot of supernatant was evaporated in centrifugal vaporizer (EYELA CVE-200D, Japan) and suitably reconstituted with water (0.2 mL) for CE analysis.

2.4. Method validation parameters

Known levels of plasma sample standards were prepared for the calibration curves. Following Section 2.3 in Section 2, the calibration graphs were established with the peak area ratio of each analyte to IS as ordinate (*Y*) versus the concentration of respective analyte in ng/mL as abscissa (*X*).

The absolute recoveries of these analytes from the plasma matrix were determined by comparing the calculated concentrations with those of the standards.

Intra-day precision and accuracy were tested by analyzing five identically spiked plasma samples for three concentrations of the analytes: 100, 400, 600 ng/mL for CZP, 40, 90, 150 ng/mL for CNO, and 50, 200, 400 ng/mL for DMC. Inter-day precision and accuracy were calculated from repeated analysis of identically spiked plasma samples on five successive days for these three concentrations of five analytes.

The limits of detection were determined by spiking plasma with decreasing concentrations of each analyte until the ratio of signal to noise equaled 3 ($S/N = 3$; injection, 99.9 s).

2.5. Application in patients' plasma

One schizophrenic patient (male, 41-year-old; 62 kg) received continual medicinal therapy (CZP, 150 mg per day) for more than 1 year. Blood samples were drawn daily at 18 h after drug intake. All blood samples were centrifuged immediately, and plasma was separated and stored at −60 °C until analysis.

3. Results and discussion

3.1. Water plug for sensitivity and voltage of electrokinetic sampling

Enhanced stacking and sample loading were processed by injection of a water plug into the capillary immediately prior to the sample injection. The electric field strength will be higher than the buffer's over the low-conductivity water zone. Therefore, the velocity of the analytes will be high in the zone until it reaches the buffer interface. The injection time of the water plug (1–10 s) at 0.5 psi was optimized for the highest absorbance of reasonable peak width. According to the results (data not shown), 5 s of injection time provided the highest absorbance. By comparing the difference between injecting and not injecting the water plug, we are able to gain higher reproducibility when using water plug.

Electrokinetic injection was used with analytes dissolved in water; the injection voltages (1, 3, 6, 8, and 10 kV) were tested when the injection time was set at 99.9 s. There were no differences between these voltages (data not shown), so the voltage was arbitrarily set at 6 kV.

3.2. Concentration and pH of phosphate buffer

CE of the drugs using 50% (v/v) ethylene glycol and phosphate buffer (pH 3.0) in different concentrations (100–600 mM) was studied. The results shown in Fig. 2 indicate that the optimal concentration was set at 400 mM to reach the rapid and better baseline resolution.

Based on the basic properties of the analytes, a preliminary study was conducted in buffers with pH < 6. The results showed a better resolution in lower pH due to the smaller EOF. The 50% (v/v) ethylene glycol and phosphate buffer (400 mM) in different pHs (2.6–3.4) were studied. The results indicated that a baseline resolution could be reached during these pHs, the best separation and shortest time was obtained at pH 3.0.

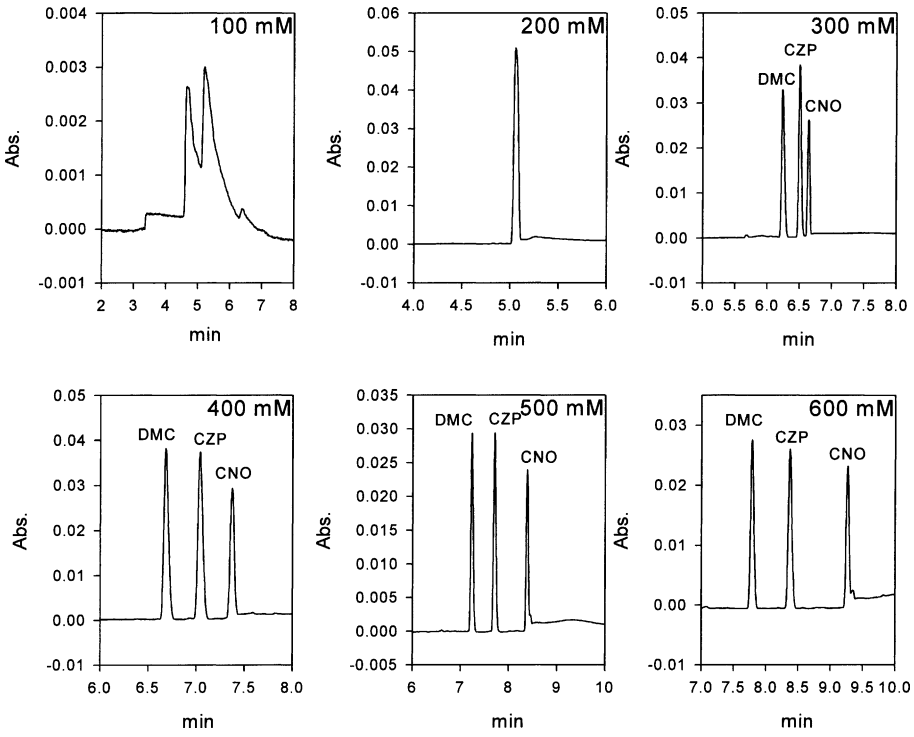

Fig. 2. Electropherograms of phosphate concentrations in the migration of the analytes.

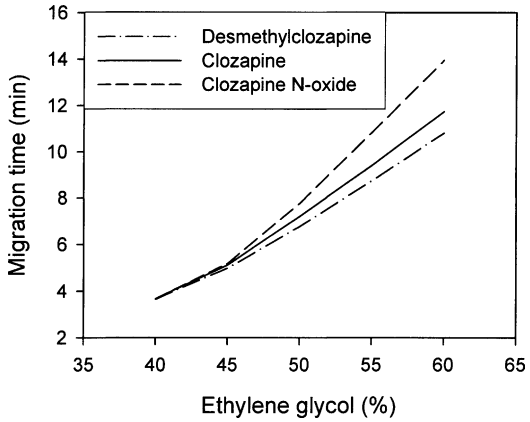

Fig. 3. Effects of concentrations of ethylene glycol on the migration of the analytes.

3.3. Concentration of ethylene glycol

Injection of a highly viscous buffer, which contained ethylene glycol, prior to the preinjection plug of water, acted as a trap to slow the velocity of the analytes. The effects of ethylene glycol, at a concentration range of 40–60% (v/v) in a phosphate buffer (400 mM, pH 3.0) on analyte separation were discussed. The results shown in Fig. 3 indicate that electrophoresis of the drugs with a BGE containing less than 40% (v/v) of ethylene glycol resulted in no baseline resolution. The more viscous buffer needed more separation time. The optimal ethylene glycol concentration was set at 50%, which effectively retained and separated the drugs.

This FASS-CE proceeded as following: the capillary was dipped into water before the insertion of a 5 s water plug from a different vial. The electrokinetic injection (6 kV, 99.9 s) of extracted plasma followed. The capillary was 31.2 cm long, 50 μm i.d., and was operated at a separation voltage of 25 kV. The background buffer was 50% (v/v) ethylene glycol and phosphate buffer (400 mM, pH 3.0). Baseline separation was achieved within 10 min as shown in Fig. 4, and there was no interference from the plasma blank.

3.4. Validation of the analytes spiked in plasma

The linear regression equations are listed in Table 1. The mean correlation coefficients for the calibration curves were obtained from three separate experiments which ranged from 0.98 to 1.00 during a 5-day-analysis. The data indicated good linearity of this method for intra- and inter-day assays. The relative recoveries of extracted analytes from plasma were all greater than 90%. The absolute recoveries of them compared with standards were 75% for CZP, 60% for DMC, and 25% for CNO.

The precision (relative standard deviation, R.S.D.) and accuracy (relative error, R.E.) of the proposed method for spiked samples were studied. The results (Table 2) showed that the intra- and inter-day R.S.D. and R.E. at these concentrations were all below 11%. When comparing the peak

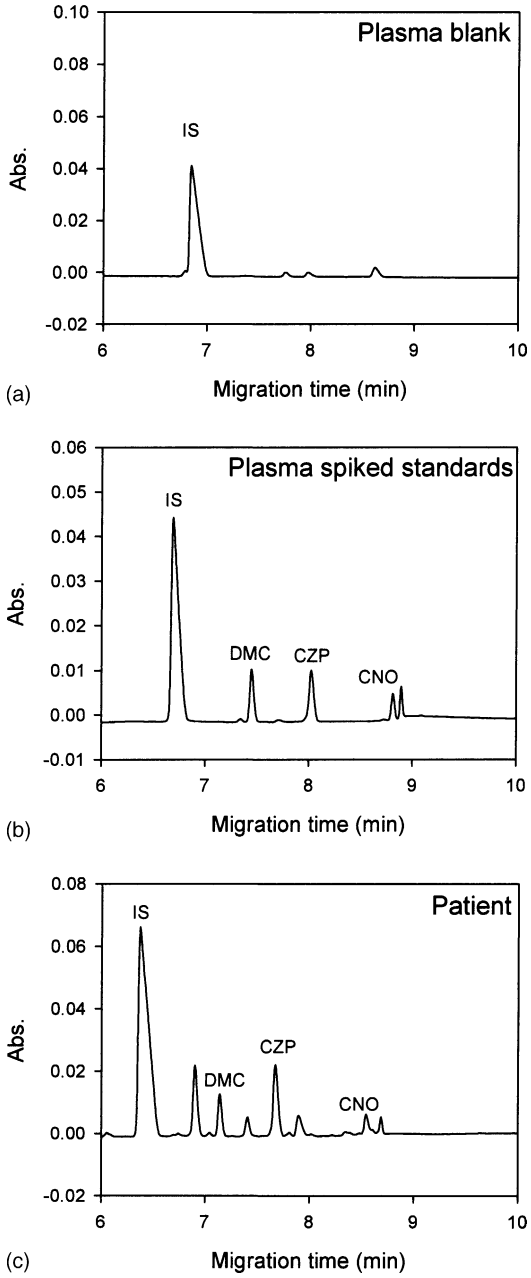

Fig. 4. Electropherograms of plasma blank, plasma spiked standards, and plasma from one schizophrenia patient (41-year-old, 62 kg) treated with CZP. CE conditions: buffer, 400 mM phosphate buffer (pH 3.0) containing 50% ethylene glycol; applied voltage, 25 kV; uncoated fused-silica capillary, 20 cm (effective length) × 50 μm i.d.; sample injection, 99.9 s by 6 kV; detection wavelength, 214 nm.

area ratio of the analytes without extraction, the absolute recoveries were about 75% for CZP, 60% for DMC, and 25% for CNO. The detection limits ($S/N = 3$; electrokinetic injection, 6 kV, 99.9 s) were found to be 5 ng/mL for CZP and DMC, and 10 ng/mL for CNO. In our lab, the sensitivity of this stacking CE method could improve 200-folds for analyzing CZP when comparing to that of conventional CE. Surveying the detection limits of Raggi et al.'s method used for human plasma [12], we took smaller volume of plasma

Table 1
Regression analysis for the determination of CZP, DMC, and CNO in plasma

Concentration range (ng/mL)[a]	Regression equation	Correlation coefficient (r)
Intra-day		
CZP (50–800)	$Y = (0.0018 \pm 0.0018)X - (0.0116 \pm 0.0090)$	1.00
DMC (25–600)	$Y = (0.0016 \pm 0.0001)X + (0.0180 \pm 0.0106)$	1.00
CNO (30–180)	$Y = (0.0006 \pm 0.0006)X - (0.0027 \pm 0.0022)$	0.98
Inter-day		
CZP (50–800)	$Y = (0.0025 \pm 0.0005)X + (0.0189 \pm 0.0758)$	0.99
DMC (25–600)	$Y = (0.0024 \pm 0.0004)X + (0.0415 \pm 0.0355)$	0.99
CNO (30–180)	$Y = (0.0010 \pm 0.0001)X - (0.0032 \pm 0.0163)$	0.98

[a] The regression equations for intra- and inter-day analyses, respectively, from the assay values of prepared standards on a single day ($n = 5$) and on five consecutive days.

and got higher sensitivity. This FASS-CE can enhance more than one order of sensitivity, and provided the reliable clinical data even in lower concentrations.

3.5. Plasma levels from patients being administered CZP

Compliance problems are frequent in patients taking CZP due to a delayed response (more than 10–24 weeks). Together with the variability of responsiveness and side effects to this drug, great attention has been given to its therapeutic monitoring to optimize dosing strategies. Trough concentrations are often inconvenient because the CZP is normally dosed at bedtime. The steady-state plasma levels (C_{ss}) of CZP were guaranteed by maintaining subjects at the CZP dosage for at least 7 days (CZP half-life, 12–26 h) [16]. A 12-h post-dose CZP serum concentration of at least 250 ng/mL is recommended if the patient is receiving divided CZP doses, or 350 ng/mL if the patient is being dosed once daily [6]. In the present study, we developed a sensitive and selective FASS-CE method for the determination of CZP and its metabolites after administration. We tried this method of analysis at the delayed sampling time to check whether it provided greater sensitivity in detection. The electropherograms resulting from the analysis of the real plasma samples at 18 h intervals is shown in Fig. 4. There were other undefined drugs coexisting in the real plasma samples. The levels of our plasma samples were 120.0 ng/mL for CZP, 75.2 ng/mL for CNO, and 82.72 ng/mL for DMC. This method could detect lower levels of analytes, and provide some information for dosage adjustment in clinical use. Further investigation of the relationship between the C_{ss} of the parent drug and its metabolites and the clinical outcome has been urged by the psychiatrists involved in this study. We hope to assist the clinician in making therapeutic decisions regarding the prescription of drugs to patients with refractory schizophrenia.

Table 2
Precision and accuracy of the determination of CZP, DMC, and CNO in plasma

Concentration known (ng/mL)		Concentration found (ng/mL)	R.S.D. (%)	R.E. (%)
Intraday ($n = 5$)				
CZP	100	103.02 ± 7.43	7.22	3.02
	400	400.37 ± 26.21	6.55	0.99
	600	612.17 ± 43.05	7.03	2.03
DMC	50	47.63 ± 4.33	9.10	−4.74
	200	217.92 ± 8.93	4.10	8.96
	400	406.38 ± 28.86	7.10	1.60
CNO	40	38.77 ± 2.89	7.45	−3.08
	90	86.08 ± 6.43	7.47	−4.35
	150	152.81 ± 16.28	10.65	1.87
Interday ($n = 5$)				
CZP	100	90.86 ± 5.41	5.95	−9.14
	400	367.70 ± 5.02	1.37	−8.08
	600	604.93 ± 19.92	3.29	0.82
DMC	50	49.18 ± 3.38	6.88	−1.65
	200	187.07 ± 8.24	4.41	−6.47
	400	390.21 ± 33.39	8.56	−2.45
CNO	40	39.91 ± 2.97	2.43	−6.21
	90	85.45 ± 5.41	6.49	−5.25
	150	140.04 ± 8.76	6.26	−6.64

Acknowledgements

We gratefully acknowledge the financial support of the National Science Council of Taiwan (NSC92-2113-M-037-031) in funding this work.

References

[1] L. Gross, E.S. Yeung, J. Chromatogr. 480 (1989) 169.
[2] C.X. Zhang, W. Thormann, Anal. Chem. 68 (1996) 2523.
[3] A.B. Wey, C.X. Zhang, W. Thormann, J. Chromatogr. A 853 (1999) 95.
[4] S.M. Wu, Y.H. Ho, H.L. Wu, S.H. Chen, H.S. Ko, Electrophoresis 22 (2001) 2717.
[5] M.W. Jann, S.R. Grimsley, E.C. Gray, W.H. Chang, Clin. Pharmacokinet. 24 (1993) 161.
[6] J.T. Dipiro, R.L. Talbert, G.C.Yee, G.R. Matzke, B.G. Wells, L.M. Posey, Pharmacotherapy—A Pathophysiologic Approach, fifth ed., McGraw-Hill Co., NY, USA, 2002, pp.1227, 1229.
[7] J.M. Kane, G. Honigfeld, J. Singer, H.Y. Meltzer, Arch. Gen. Psychiatry 45 (1988) 789.

[8] S.L. Gerson, H.Y. Meltzer, Drug Saf. 7 (1992) 17.
[9] Y.L. Shen, H.L. Wu, W.K. Ko, S.M. Wu, Anal. Chim. Acta 460 (2002) 201.
[10] V. Pucci, M.A. Raggi, E. Kenndler, J. Liq. Chromatogr. Rel. Technol. 23 (2000) 25.
[11] M.A. Raggi, V. Pucci, F. Bugamelli, V. Volterra, J. AOAC Int. 84 (2001) 361.
[12] M.A. Raggi, F. Bugamelli, R. Mandrioli, C. Sabbioni, V. Volterra, S. Fanali, J. Chromatogr. A 916 (2001) 289.
[13] V. Pucci, M. Raggi, E. Kenndler, J. Chromatogr. B 728 (1999) 263.
[14] V. Pucci, M. Raggi, E. Kenndler, J. Chromatogr. A 853 (1999) 461.
[15] W. Jin, Q. Xu, W. Li, Electrophoresis 21 (2000) 1415.
[16] L. Ereshefsky, M.D. Watanabe, T.K. Tran-Johnson, Clin. Pharmacol. 8 (1989) 691.