ELSEVIER

Journal of Chromatography A, 916 (2001) 289–296

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Rapid capillary electrophoretic method for the determination of clozapine and desmethylclozapine in human plasma

Maria Augusta Raggi[a,*], Francesca Bugamelli[a], Roberto Mandrioli[a], Cesare Sabbioni[a], Vittorio Volterra[b], Salvatore Fanali[c]

[a]*Dipartimento di Scienze Farmaceutiche, Università di Bologna, Via Belmeloro 6, 40126 Bologna, Italy*
[b]*Clinica Psichiatrica, Università di Bologna, Viale Pepoli 5, 40123 Bologna, Italy*
[c]*CNR, Istituto di Cromatografia, Area della Ricerca di Roma, P.O. Box 10, 00016 Monterotondo Scalo (Roma), Italy*

Abstract

A rapid and sensitive high-performance capillary electrophoretic method for the determination of clozapine and its main metabolite desmethylclozapine in human plasma was developed. The separation of the two analytes was carried out in an untreated fused-silica capillary [33 cm (8.5 cm effective length)×50 μm I.D.] filled with a background electrolyte at pH 2.5 containing β-cyclodextrin. Baseline separation of clozapine and desmethylclozapine was recorded in less than 3 min. An accurate sample pretreatment by means of solid-phase extraction and subsequent concentration allows for reliable quantitation of clozapine in the plasma of schizophrenic patients under treatment with the drug. The method showed good precision (mean RSD=4.0%) as well as satisfactory extraction yields (~ 88%) and a good sensitivity (limit of quantitation= 0.075 μg ml^{-1}, limit of detection=0.025 μg ml^{-1}).

Keywords: Clozapine; Desmethylclozapine

1. Introduction

Clozapine {8-chloro-11-(4-methyl-1-piperizinyl)-5H-dibenzo[*b*,*e*] [1,4]diazepine, Fig. 1} is a widely used antipsychotic drug first synthesised in the 1960s and introduced into clinical practice between 1972 and 1975 [1]. Its use, however, was discontinued for several years due to some cases of fatal agranulocytosis associated with its administration [2]. Clozapine has recently been reintroduced on the market because it showed good efficacy especially in patients who were non-responders to traditional antipsychotics (i.e. chlorpromazine, haloperidol) [3,4]. Furthermore, clozapine does not cause extrapyramidal motor side effects [5].

In order to avoid the onset of agranulocytosis, patients treated with clozapine must undergo constant monitoring of hematic parameters. Most side effects of clozapine (e.g. seizures, constipation and agranulocytosis) have been associated with high plasma concentrations of the drug [6–8] and the mechanism of bone marrow toxicity has been correlated with the presence of the main active metabolite of clozapine, namely desmethylclozapine [9,10] (Fig. 1). For these reasons, reliable therapeutic monitoring of the plasma levels of the two clozapines is very important for the safety of the patients.

Several papers on the analysis of clozapine and desmethylclozapine in human plasma are reported in the literature. A few papers report the use of gas-

*Corresponding author. Tel.: +39-051-209-9700; fax: +39-051-209-9734.

E-mail address: raggima@alma.unibo.it (M.A. Raggi).

0021-9673/01/$ – see front matter
PII: S0021-9673(01)00520-9

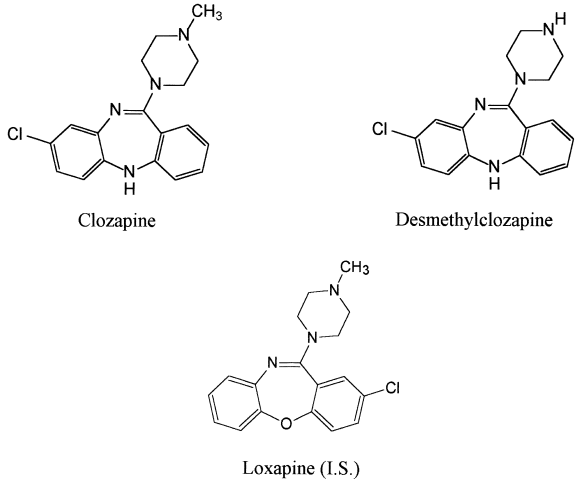

Fig. 1. Structural formulae of the analytes and the internal standard.

chromatography methods [11] sometimes coupled with mass spectrometry [12]. More recently, a number of papers have proposed the employment of high-performance liquid chromatography (HPLC) with UV [13–19] or electrochemical detection [20,21].

Recently, we have developed some HPLC methods with amperometric detection for the determination of clozapine [22] and its metabolites [23,24] in the plasma of schizophrenic patients.

The proposed HPLC methods allowed good resolution and sensitivity even though they exhibited some drawbacks such as a long analysis time and the consumption of very high volumes of mobile phase containing toxic and polluting solvents (e.g., methanol or acetonitrile).

High-performance capillary electrophoresis (HPCE), a recent analytical technique, is a powerful tool which offers very high resolution capability and high efficiency in a short time. Furthermore the use of very low volumes of buffer (μL) and samples (nL) make this new technique very interesting for rapid and practical analysis also in the biomedical field [25–28].

The aim of this study is the development of a rapid HPCE method suitable for the determination of clozapine and its metabolite desmethylclozapine in human plasma. Several experimental parameters were investigated, e.g., buffer pH, complexing agent in order to find the optimum conditions for the baseline separation of the two analytes to be determined in samples of biological interest.

2. Experimental

2.1. Apparatus

A 3D capillary electrophoresis apparatus from Agilent Technologies (Palo Alto, CA, USA), equipped with a diode array detector set at 240 nm was used for the electrophoretic experiments.

The analysis was carried out in an untreated fused-silica capillary, 33 cm (effective length 8.5 cm)×50 μm I.D. The background electrolyte (BGE) was composed of a pH 2.5, 50 m*M* phosphate buffer containing 7 m*M* β-cyclodextrin.

The sample was injected by pressure (50 mbar for 15 s) at the anodic end of the capillary and the separation achieved by applying a potential of 20 kV. The capillary was thermostatted at 25°C.

2.2. Chemicals and reagents

Clozapine (CLZ) and desmethylclozapine (des-CLZ) were kindly supplied by Novartis (Basel, Switzerland), while Loxapine used as the internal standard (I.S.) was from Lederle Labs. (Gosport, UK). For the chemical structures of the analytes and the I.S., see Fig. 1.

Methanol, 85% phosphoric acid, 1 *M* and 0.1 *M* sodium hydroxide were analytical grade form Carlo Erba (Milan, Italy). β-Cyclodextrin (β-CD) was purchased from Sigma (St. Louis, MO, USA). Ultrapure water (18.2 MΩ cm) was obtained by means of a Millipore (Bedford, MA, USA) Milli-Q apparatus.

The stock solutions of clozapine, desmethylclozapine and loxapine were 1 mg ml^{-1} in methanol. All subsequent dilutions were made in a mixture composed of methanol–5 m*M* phosphate buffer pH 2.5 (50:50, v/v). The stock solutions were stored at −20°C and were stable for at least 2 months, while the standard solutions were prepared every day.

2.3. Human plasma sampling

The study was performed on schizophrenic pa-

tients subjected to treatment with Leponex tablets at a constant daily dose for at least 8 weeks. Blood samples were obtained immediately before a first daily dose of CLZ, and 12 h from the last dose.

Blood samples were drawn into test tubes containing EDTA and centrifuged at 3000 rpm for 20 min. The supernatant plasma was transferred into test tubes and frozen at −20°C until analysis, usually within 1 week. This procedure was also used to obtain and store the plasma of healthy volunteers (''blank'' plasma).

2.4. Sample analysis

Waters (Milford, MA, USA) Oasis HLB (30 mg, 1 ml) cartridges were used in a Varian (Harbor City, CA, USA) VacElut apparatus for the solid-phase extraction (SPE) procedure.

The cartridges were activated and conditioned twice with 1 ml of methanol and then 1 ml of water. An amount of 500 μl of blank plasma spiked with the analytes or 500 μl of patient plasma were diluted with 500 μl of water and loaded onto the cartridge.

After loading, the cartridge was washed twice with water and dried under vacuum (−30 kPa) for 10 s. The analytes were eluted with 500 μl of methanol and the cartridge dried under vacuum again for 10 s, in order to obtain a quantitative elution. The eluate was then brought to dryness and redissolved with 200 μl of a mixture composed of methanol–5 m*M* phosphate buffer pH 2.5 (50:50, v/v).

2.5. Capillary conditioning

Accurate washing of the capillary is essential in order to obtain reproducible results and to avoid interference due to the matrix and the analytes sticking to the internal wall of the capillary.

At the beginning of every working day the capillary was rinsed with: water (5 min), 0.1 *M* NaOH (3 min), water (6 min) and BGE for 20 min.

After each electrophoretic run the capillary was flushed with water (1 min), 0.1 *M* HCl (1 min), water (2 min) and with the BGE (2 min).

At the end of the day the capillary was washed for 2 min with water, 3 min with 1 *M* HCl, 3 min with water, 5 min with methanol, 5 min with water. It was then air-dried for 3 min.

3. Results and discussion

3.1. Method optimization

In order to optimize the separation of clozapine and its main metabolite (desmethylclozapine) by HPCE, we studied the effect of several experimental parameters such as background electrolyte type, pH, and concentration. A pH 2.5 buffer was chosen, because a recent voltammetric investigation [29] on clozapine tablets showed that at higher pH values clozapine is more easily oxidised and thus less stable. Among the buffers investigated 50 m*M* phosphate buffer at pH 2.5 allowed us to achieve the best separation of the two analytes, albeit complete resolution was not obtained (results not shown). At the operating experimental conditions, the two analytes (basic compounds) were fully protonated and migrated towards the cathode. In order to find the I.S. to be used for the assay of the drugs investigated, several attempts were done analyzing several compounds (amoxapine, dibenzepine, imipramine and loxapine) mixed with clozapine and desmethylclozapine. Among the I.S. considered we selected loxapine due to its chemical structure which is very similar to that of the studied analytes. For this reason loxapine has a migration time similar to those of the clozapines and it should be extracted from plasma together with clozapine.

In order to obtain a baseline separation of the studied compounds and the I.S., β-cyclodextrin was added to the BGE. It has been reported that cyclodextrins, added to the BGE, can be helpful in order to improve the selectivity of the separation due to the formation of inclusion complexes with the analytes modifying their effective mobility. The β-CD concentration was varied in the 0.5–10 m*M* range, and the best resolution was obtained at a concentration of 7 m*M*.

Fig. 2 shows the electrophoretic separation of clozapine, desmethylclozapine and loxapine (I.S.) using the optimum experimental conditions. As can be observed in this figure the separation of the three compounds was achieved in a relatively short time, less than 3.0 min; the peak with migration time (t_m)=2.4 min corresponded to clozapine, while that of desmethylclozapine was at t_m=2.1 min and the peak at t_m=2.9 min corresponded to the I.S.

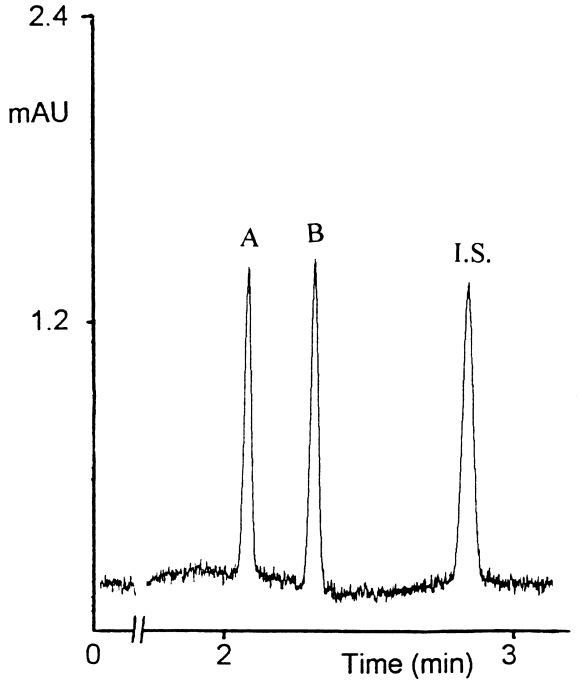

Fig. 2. Electropherogram of a standard solution containing 400 ng ml^{-1} of the clozapines and 800 ng ml^{-1} of the internal standard. BGE: 50 m*M*, pH 2.5 phosphate buffer containing 7 m*M* β-cyclodextrin. Peaks: A = desmethylclozapine; B = clozapine; I.S. = internal standard (loxapine).

In order to improve the sensitivity of the CE method some preliminary assays were carried out at a detection wavelegth of 205 nm, however the baseline was much noisier than at 240 nm. The latter wavelength was thus chosen for all subsequent assays. Calibration curves were set up in the 0.25–5.00 μg ml^{-1} concentration range, by plotting the analyte peak height against the respective analyte concentrations. Good linearity was obtained (r_c > 0.999).

3.2. New plasma pretreatment procedure

Due to the very narrow bore of the electrophoretic capillary, the optical path of the UV detector is limited and thus is the limited sensitivity which can be reached with the given instrumentation. Since the plasma clozapine levels found in patients usually range from 100 to 1500 ng ml^{-1}, an accurate sample pretreatment step is necessary which should concentrate the analytes as much as possible, thus obtaining a final analyte concentration suitable for the detector sensitivity.

Different procedures were tested in order to eliminate the matrix interference and to obtain the highest possible extraction yield of the analytes.

A simple precipitation with methanol (1 ml of methanol added to 200 μl of plasma), followed by drying of the supernatant and redissolving gave acceptable recovery however interference was heavy. A protein precipitation (again with methanol) followed by purification with SPE was tried. Oasis cartridges, loaded with 0.5 ml of solution and eluted with 0.5 ml of methanol were used for the SPE. The eluate was then brought to dryness and redissolved in pH 2.5 phosphate buffer. In this case a good deal of interference was eliminated, however the analyte recovery was not satisfactory. Better results were obtained by eliminating the first precipitation step, however the final drying and redissolving step had a negative effect on the extraction yield, probably because the dissolution of the analytes in 5 m*M* phosphate buffer was difficult and thus not complete. We tried to redissolve the residue with different buffer–methanol mixtures, ranging from a 70:30 (v/v) to a 20:80 (v/v) ratio. Best results were obtained using 200 μl of a methanol–buffer (50:50, v/v) mixture.

The final leading conditions were as follows: 500 μl of plasma diluted with water were loaded onto the conditioned cartridge. After suitable washings the analytes were eluted with 500 μl of methanol. The eluate was brought to dryness, redissolved with 200 μl of a mixture composed of methanol and pH 2.5 phosphate buffer (50:50) and injected into the electrophoretic apparatus.

The electropherogram of a blank plasma sample spiked with 800 ng ml^{-1} of the I.S is reported in Fig. 3a, while the electropherogram of the same plasma sample spiked with 800 ng ml^{-1} of both clozapines and 800 ng ml^{-1} of the I.S. is reported in Fig. 3b. The separation was still good and no interference was present.

The extraction yield or absolute recovery was evaluated on blank plasma samples spiked with different concentrations of the clozapines (0.250, 0.800 and 2.000 μg ml^{-1}) and resulted to be very satisfactory, as reported in Table 1. The mean

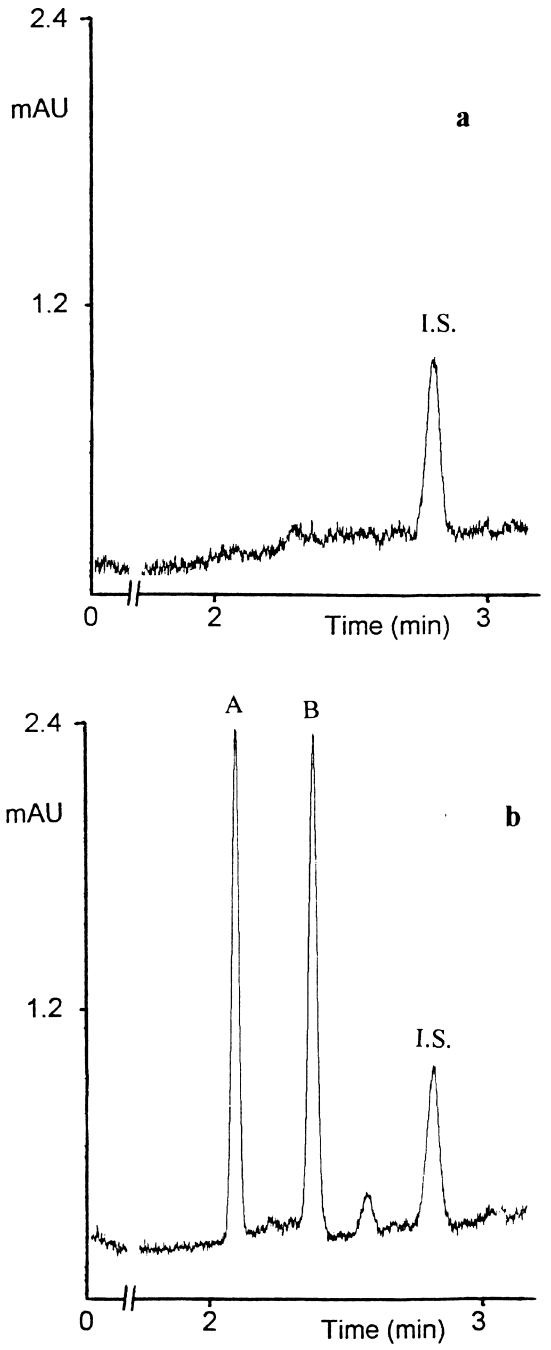

Fig. 3. Electropherograms of a blank plasma sample spiked with 800 ng ml^{-1} of the I.S. (a) and the same plasma sample spiked with 800 ng ml^{-1} of the clozapines and 800 ng ml^{-1} of the internal standard (b). Peaks: A = desmethylclozapine; B = clozapine; I.S. = internal standard (loxapine).

recoveries were 89.7% for clozapine and 87.0% per desmethylclozapine; that of loxapine was 65.5%. For both analytes a calibration curve was set up in the 0.250–5.000 μg ml^{-1} concentration range (which becomes 0.100–2.000 μg ml^{-1} on the original plasma if one takes into account the concentration which takes place during the sample pretreatment). Good linearity was obtained for the dependence of peak height of clozapine and desmethylclozapine respectively on concentration expressed in μg ml^{-1}. The linear regression equations (obtained on 10 experimental points) were $y = 1.729x + 0.015$ (R_c = 0.9999) for clozapine and $y = 1.503x - 0.025$ (R_c = 0.9997) for desmethylclozapine. The limits of detection (LOD) and quantification (LOQ) were calculated according to USP XXIV guidelines [30] and are reported in Table 1.

The results of the precision assays were also very satisfactory with regard to both peak heights and migration times. The mean RSDs of peak heights were less than 3.8% for desmethylclozapine and 4.2% for clozapine for concentrations higher than 250 ng ml^{-1}. The mean RSDs of migration times were less than 2.1% for desmethylclozapine and 2.9% for clozapine.

3.3. Application to patient plasma

Having thus validated the method, it was applied to the determination of clozapines in the plasma of psychotic patients treated with Leponex tablets. All patients were in a steady state (i.e. they had been taking clozapine for more than 8 weeks). As an example, the electropherogram of a plasma sample from a patient treated with 600 mg day^{-1} of clozapine is shown in Fig. 4. As one can see, clozapine is detected at 2.4 min, desmethylclozapine at 2.1 min and the I.S. at 2.9 min. The concentrations of clozapine and desmethylclozapine, obtained by interpolation on the corresponding calibration curve, were 850 and 350 ng ml^{-1}, respectively. In order to evaluate the accuracy of the method, known amounts of the standard analyte solutions were added to known amounts of plasma samples from patients treated with Leponex. The recovery was obtained by subtracting the original clozapine concentrations from the total amounts found after the addition and

Table 1
Method characteristics

Compound	Linearity range ($\mu g\ ml^{-1}$)	Amount added to plasma ($\mu g\ ml^{-1}$)	Absolute recovery (%)[a]	LOQ ($\mu g\ ml^{-1}$)	LOD ($\mu g\ ml^{-1}$)
Clozapine	0.100–2.000	0.250	88±7	0.075	0.025
		0.800	91±6		
		2.000	90±4		
Desmethylclozapine	0.100–2.000	0.250	86±6	0.075	0.025
		0.800	88±5		
		2.000	87±3		
Loxapine	0.200–2.000	0.800	65±3	0.200	0.100
		1.600	66±2		

[a] Each value is the mean of 3 independent assays±SD.

comparing this difference with the theoretical concentration added. These recovery assays were repeated for three added concentrations (0.250, 0.800 and 1.600 $\mu g\ ml^{-1}$) and three times for each concentration. The accuracy data are satisfactory, being the recovery values from 86 to 90% for clozapine and from 80 to 86% for desmethylclozapine.

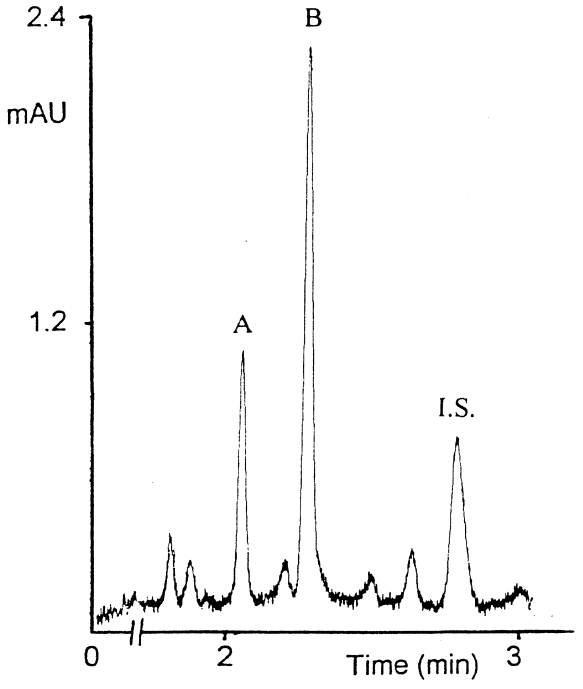

Fig. 4. Electropherogram of a plasma sample from a patient treated with Leponex® (clozapine 600 mg day^{-1}). Peaks: A = desmethylclozapine; B = clozapine; I.S. = internal standard (loxapine).

3.4. Comparison with an HPLC method

The results obtained using the method described herein were compared to those obtained by means of an HPLC method with amperometric detection previously implemented by us [24]. The results are reported in Table 2. As can be seen, the plasma levels of clozapines obtained by means of the two methods are in good agreement.

It should be noted that the HPLC method with amperometric detection, thanks to its superior sensitivity, allows for the determination of the minor clozapine metabolite, namely clozapine *N*-oxide, which is usually present in the plasma at very low levels (a few ng ml^{-1}).

The CZE method described herein manages to separate clozapine *N*-oxide from the other analytes, however the clozapine *N*-oxide concentrations in plasma samples are too low to be quantified with this method, due to its lower sensitivity.

We intend to develop, in the near future, a new method for the determination of all three clozapines by means of an apparatus which couples capillary electrophoresis with electrochemical detection.

Table 2
Concentrations of clozapine and desmethylclozapine in the plasma of schizophrenic patients treated with Leponex® tablets

Patient No.	CLZ dosage (mg day^{-1})	CZE		HPLC	
		CLZ (ng ml^{-1})	des-CLZ (ng ml^{-1})	CLZ (ng ml^{-1})	des-CLZ (ng ml^{-1})
1	300	320	140	305	128
2	350	337	165	329	156
3	400	450	230	412	214
4	500	547	236	527	245
5	600	850	350	810	367

4. Conclusion

The HPCE method allows for a very rapid separation and quantifications of clozapine and desmethylclozapine. Good sensitivity is achieved by using a new sample pretreatment procedure allowing determination levels of clozapine as low as 100 ng ml^{-1}. Furthermore the method shows satisfactory precision and accuracy and thus can be useful for the clinical monitoring of patients under treatment with clozapine. Further assays are in progress in order to fully implement this application, e.g., using electrochemical detection in order to improve the sensitivity.

Acknowledgements

This research was supported by a grant (40%) from MURST (Ministero dell'Università e della Ricerca Scientifica e Tecnologica), Italy. Thanks are due to Novartis Pharma AG for providing pure drug compounds enabling the development of this assay, and to Mr. Ettore Marziali and Miss Alessandra Maurizio for their technical assistance.

References

[1] J. Angst, D. Bente, P. Berner, H. Helmann, H. Helmchen, H. Hippius, Pharmakopsychiatr./Neuro-Psychopharmakol. 4 (1971) 201.
[2] J. Indänpään-Heikkilä, E. Alhava, M. Olkinura, Eur. J. Clin. Pharmacol. 11 (1977) 193.
[3] R.J. Baldessarini, F.R. Frankenburg, New Engl. J. Med. 324 (1991) 746.
[4] A.J. Wagstaff, H.M. Brison, CNS Drugs 4 (1995) 370.
[5] Martindale, The Extra Pharmacopoeia, 31st ed, Royal Pharmaceutical Society, London, 1996, p. 697.
[6] M.H. Kronig, R.A. Munne, S. Szymansky, A.Z. Safferman, S. Pollack, T. Cooper, J.M. Kane, J.A. Lieberman, Am. J. Psychiat. 152 (1995) 179.
[7] O.V. Olesen, K. Thomsen, P.N. Jensen, C.H. Wulff, N.A. Rasmussen, C. Refshammer, J. Soresen, M. Bysted, J. Christensen, R. Rosenberg, Psycopharmacology 117 (1995) 371.
[8] G.M. Simpsons, T.A. Cooper, Am. J. Psychiat. 135 (1978) 99.
[9] S.L. Gerson, H.Y. Meltzer, Drug Safety 7 (1992) 17.
[10] S.L. Gerson, C. Arce, H.Y. Meltzer, Brit. J. Haematol. 85 (1994) 555.
[11] K. Richter, J. Chromatogr. 434 (1988) 465.
[12] V. Bondesson, L.H. Lindstrom, Psycopharmacology 95 (1988) 472.
[13] M.C. Chung, S.K. Lin, W.H. Chang, J. Chromatogr. 613 (1993) 168.
[14] S.A. Volpicelli, F. Centorrino, P.R. Puopolo, J. Knado, F.R. Frankenburg, R.J. Baldessarini, J.G. Flood, Clin. Chem. 39 (1993) 1656.
[15] E.O. Fadiran, J. Leslie, M. Fossler, D. Young, J. Pharm. Biomed. Anal. 13 (1995) 185.
[16] R.N. Gupta, J. Chromatogr. B 673 (1995) 311.
[17] U. Hariharan, M. Hariharan, J.S. Naickar, R. Tandon, J. Liq. Chromatogr. R. T. 19 (1996) 2409.
[18] L. Edno, I. Combourieu, M. Cazenave, J. Tignol, J. Pharm. Biomed. Anal. 16 (1997) 311.
[19] C. Guitton, J.M. Kinowski, R. Aznar, F. Bressolle, J. Chromatogr. B 690 (1997) 211.
[20] C. Humpel, C. Haring, A. Saria, J. Chromatogr. 491 (1989) 235.
[21] E. Schulz, C. Fleischhacker, H. Remschmidt, Pharmacopsychiatry 28 (1995) 20.
[22] M.A. Raggi, F. Bugamelli, R. Mandrioli, D. De Ronchi, V. Volterra, Chromatographia 47 (1998) 8.
[23] M.A. Raggi, F. Bugamelli, R. Mandrioli, D. De Ronchi, V. Volterra, Chromatographia 49 (1999) 75.
[24] M.A. Raggi, F. Bugamelli, C. Sabbioni, D. De Ronchi, S. Pinzauti, V. Volterra, Chromatographia 51 (2000) 147.

[25] C. Desiderio, S. Fanali, A. Kupfer, W. Thormann, Electrophoresis 15 (1994) 87.
[26] C. Desiderio, S. Rudaz, M.A. Raggi, S. Fanali, Electrophoresis 20 (1999) 3432.
[27] D.B. Strickmann, B. Chankvetadze, G. Blaschke, C. Desiderio, S. Fanali, J. Chromatogr. A 887 (2000) 393.
[28] A. Ramseier, J. Caslavska, W. Thormann, Electrophoresis 20 (1999) 2726.
[29] M.A. Raggi, V. Pucci, F. Bugamelli, V. Volterra, J. AOAC Int., in press.
[30] United States Pharmacopeia, United Stated Pharmacopeial Convention, Rockville, MD, XXIV ed., 2000, pp. 2150–2151.