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Determination of clozapine and its major metabolites in human plasma and red blood cells by high-performance liquid chromatography with ultraviolet absorbance detection

Claire Guitton[a], Jean-Marie Kinowski[a], Regis Aznar[a], Françoise Bressolle[a,b,*]

[a]*Laboratoire de Pharmacocinétique, Hôpital Carémeau, 30900 Nîmes, France*
[b]*Laboratoire de Pharmacocinétique, Faculté de Pharmacie, 34060 Montpellier Cedex 01, France*

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Abstract

An isocratic high-performance liquid chromatographic (HPLC) method with UV absorbance detection is described for the quantification of clozapine (8-chloro-11-(4′-methyl)piperazino-5*H*-dibenzo[*b*,*e*]-1,4-diazepine) and its two major metabolites in plasma and red blood cells (RBCs). The method involves sample clean-up by liquid–liquid extraction with ethyl acetate. The organic phase was back-extracted with 0.1 *M* hydrochloric acid. Loxapine served as the internal standard. The analytes were separated by HPLC on a Kromasil Ultrabase C_{18} analytical column (5 μm particle size; 250×4.6 mm I.D.) using acetonitrile–phosphate buffer pH 7.0 (48:52, v/v) as eluent and were measured by UV absorbance detection at 254 nm. The limits of quantitation were 20 ng/ml for clozapine and N-desmethylclozapine and 30 ng/ml for clozapine N-oxide. Recovery from plasma or RBCs proved to be higher than 62%. Precision, expressed as % C.V., was in the range 0.6–15%. Accuracy ranged from 96 to 105%. The method's ability to quantify clozapine and two major metabolites simultaneously with precision, accuracy and sensitivity makes it useful in therapeutic drug monitoring.

Keywords: Clozapine; N-Desmethylclozapine; Clozapine N-oxide

1. Introduction

Clozapine, 8-chloro-11-(4-methyl-1-piperazinyl)-5*H*-dibenzo[*b*,*e*]diazepine, is a high-dose neuroleptic drug. This drug does not induce the extrapyramidal side-effects [1,2] that occur frequently with standard neuroleptics [3]. The most common side effects of clozapine are sedation, orthostatic hypotension and hypersalivation [1,4], but convulsions have also been reported in patients with serum concentrations above 1000 ng/ml [5]. Clozapine is effective in many patients with resistant schizophrenia who do not respond to classical neuroleptics such as chlorpromazine or haloperidol [2,6]. However, in spite of its high efficacy, the use of clozapine is limited by a high incidence of drug-induced agranulocytosis [7]. Recent reports describe the use of clozapine in treatment of psychotic mood disorders [8,9]. To minimize risk of potentially fatal but reversible agranulocytosis, therapeutic drug monitoring is necessary. Clozapine is mainly metabolized to N-desmethylclozapine (active metabolite) and clozapine N-oxide [10].

Several assays to quantify clozapine and some of

*Corresponding author.

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its metabolites in plasma have been reported. They include gas chromatography with nitrogen-specific [11,12] or mass-spectrometric [13] detection. Although these methods provided good results, they involved expensive equipment and time-consuming pretreatment of samples and are not easily available for routine drug monitoring. High-performance liquid chromatography (HPLC) with either ultraviolet [14–24] or electrochemical [25] detection have been also developed. These methods involved liquid–liquid [14,15,20,22,25] or solid-phase extraction of the samples [16,17,19,23,24]; an on-line automated method has been also described [21]. Few methods involved both the quantitation of clozapine and its two main metabolites [16,19,22,24]. The limit of detection ranged from 2 [20] to 40 ng/ml [16] for clozapine, from 1 [20] to 40 ng/ml [16] for N-desmethylclozapine, and from 3 [22] to 40 ng/ml [16] for clozapine N-oxide. Limits of quantitation of 0.45 [17] when volume losses during sample work-up and injection were taken into account and 15–20 ng/ml [22,25] for clozapine and of 15 ng/ml [24] for its two metabolites have been reported.

The present paper describes an isocratic reversed-phase HPLC method for the separation and measurement of clozapine and its two metabolites, the N-desmethylclozapine and the clozapine N-oxide, in human plasma and RBCs. This method was validated with respect to accuracy, precision, selectivity, and limits of quantitation and of detection according to good laboratory practice guidelines [26,27]. Moreover, stability tests under various conditions have been performed.

2. Experimental

2.1. Materials and reagents

Clozapine, N-desmethylclozapine and clozapine N-oxide were obtained from Sigma (St. Louis, MO, USA). Loxapine was a gift from Wyeth Léderlé laboratories (Paris, France). Ethanol and ethyl acetate were Chromasol grade (SDS, Peypin, France) and used without further purification. Acetonitrile was of high purity solvent (HPLC grade) manufactured by Scharlau (Barcelona, Spain). Hexadecyltrimethylammonium bromide (Cetrimide, Merck, Darmstadt, Germany), 85% phosphoric acid and disodium hydrogen phosphate (Prolabo, Paris, France), 33% hydrochloric acid (SDS) were all analytical grade. 0.1 *M* hydrochloric acid was then prepared by dilution in purified water (Laboratoires Fandre, Ludres, France). The buffer (pH 7.0) consisted of di-sodium hydrogen phosphate (0.716 g) and cetrimide (2 g) in purified water (1 l) adjusted to pH 7.0 with phosphoric acid.

The structures of clozapine, its two main metabolites and the internal standard are shown in Fig. 1. Stock solutions of clozapine and its desmethyl and N-oxide metabolites (0.1 mg/ml) were prepared in absolute ethanol. Working solutions were obtained by dilution of the stock solutions with ethanol in a 1:10 ratio. These solutions were stored at −30°C. Specific volumes of the stock or working solution were used to spike the plasma or RBC samples prior to extraction. A stock solution of loxapine, internal standard, was prepared extemporaneously at 50 μg/ml in purified water.

An unextracted working aqueous solution containing the three drugs to be analysed at the concentration of 300 ng/ml and the internal standard at the concentration of 600 ng/ml was prepared daily to check the resolution of the chromatographic system.

For the validation of the method, human plasma and RBCs were obtained from pooled blood samples collected from healthy volunteers. Coagulation was prevented by adding EDTA-sodium salt then, the blood was centrifuged at 2000 *g* for 10 min. The

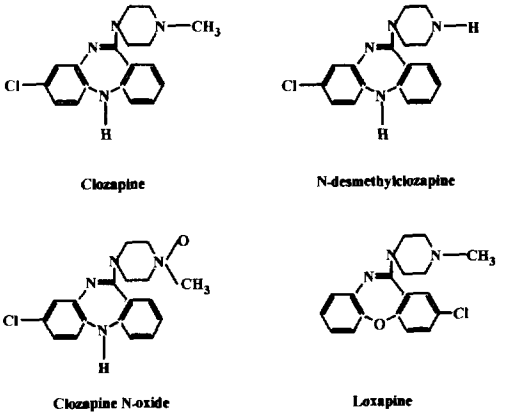

Fig. 1. Structural formulae of clozapine, N-desmethylclozapine, clozapine N-oxide and loxapine.

obtained drug-free plasma was stored at −30°C before use. RBCs were washed twice with an equal volume of 0.9% sodium chloride. Before storage at −30°C, RBCs were haemolysed with an equal volume of purified water.

2.2. Instrumentation

Analysis by HPLC was performed using a Gilson instrument with a Model 805 manometric module, a Model 305 pump, an automatic sample injection system (Model 231; Gilson Medical Electronic, Villiers le Bel, France) with a Rheodyne loading valve (Model 7010) fitted with a 100-μl sample loop and an oven (Du Pont de Nemours, Les Ulis, France). Reversed-phase HPLC was performed with a stainless-steel column (250×4.6 mm I.D., Hypersil, Cheshire, UK) packed with Kromasil Ultrabase C_{18} (particle size, 5 μm). A guard column (20×4.6 mm I.D.; SFCC, Neuilly Plaisance, France) packed with Hypersil ODS (5 μm) was placed just before the inlet of the analytical column. Effluent was monitored at 254 nm with a variable-wavelength UV detector (model SPD-6AV; Shimadzu Instruments, Touzart Matignon, France). Chromatograms were recorded and peaks integrated with a Shimadzu C-R3A integration system.

2.3. Chromatographic conditions

Acetonitrile and the phosphate buffer (pH 7.0) were filtered through a membrane filter (0.45 μm; Millipore, Molsheim, France). The HPLC eluent was acetonitrile–phosphate buffer (48:52, v/v). Prior to use, it was first degassed by vacuum and then with a stream of helium during use. The isocratic separation was performed at 1.5 ml/min flow-rate at 50°C, which corresponds to a pressure of about 11.1 MPa.

2.4. Sample processing

Sample preparation consisted of a dual liquid–liquid extraction. Plasma (0.5 ml) or RBCs (0.5 ml diluted with 0.5 ml of purified water) was pipetted into a 15-ml glass centrifuge tube, fitted with a PTFE-lined screw cap. To this, 300 ng of internal standard was added and the sample was briefly mixed by manual agitation. Then 6 ml of ethyl acetate was added to the tube. The mixture was vortex-mixed for 2 min, after which the tubes were placed in a table-top centrifuge at 2000 *g* for 5 min. The organic phase was drawn off and put into another 15-ml glass tube, to which 250 μl 0.1 *M* hydrochloric acid was added. This tube was vortex-mixed for 2 min and then centrifuged at 2000 *g* for 5 min. A 200-μl volume of the acid layer was transferred into a 5-ml glass tube then evaporated to dryness at 40°C. The residue was dissolved in 200 μl of mobile phase and 100 μl was injected into the chromatograph.

2.5. Instrument calibration

Calibration standards in plasma were prepared using concentrations of 50, 100, 200, 300, 500, 1000 and 2000 ng/ml for clozapine and concentrations of 50, 80, 100, 200, 300, 400 and 500 ng/ml for its desmethyl and N-oxide metabolites. In RBCs, the concentrations of 50, 80, 100, 200, 300, 400 and 500 ng/ml for clozapine and its metabolites were prepared. The volume added was always smaller than or equal to 2% of total volume of the samples, so that the integrity of the plasma was maintained.

2.6. Data analysis

From recorded peak areas the ratios of the drug to internal standard were calculated. Unweighted least squares linear regression of the peak area as a function of the theoretical concentration was applied to each standard curve. The resulting slopes and intercepts were used to obtain concentration values for that day's quality control samples and unknown samples.

The linearity of the method was confirmed by comparing the slopes and the intercepts of linear calibration curves with zero, and the correlation coefficients with 1. Moreover, the Kolmogorov–Smirnov test was used to compare the distribution of the residuals (difference between nominal and back-calculated concentrations) to the expected one (N(0,1)).

2.7. Specificity

To evaluate the specificity of the method, several pre-dose human plasma and RBC samples from different healthy subjects and patients were tested for the absence of interfering compounds. The retention times of endogenous compounds in the matrix were compared with that of clozapine, N-desmethylclozapine and clozapine N-oxide.

Plasma from patients receiving antipsychotic drugs commonly used in combination with clozapine was analysed for interference with clozapine, N-desmethylclozapine or clozapine N-oxide determination. The following drugs were checked: amitriptyline, clomipramine, droperidol, clonazepam, clorazepate, diazepam and hydroxyzine.

2.8. Precision and accuracy

Inter-day and intra-day reproducibilities of the assay were assessed by performing replicate analyses of quality control samples in plasma and RBCs, containing all the three compounds (80, 800 and 1400 ng/ml for clozapine in plasma; 80, 200 and 400 ng/ml for clozapine in RBCs, 80, 200 and 400 ng/ml for N-desmethylclozapine and clozapine N-oxide in plasma and RBCs) against a calibration curve. The procedure was repeated on different days on the same spiked standards to determine inter-day repeatability. Intra-day repeatability was determined by treating spiked samples in replicate the same day.

The accuracy was evaluated as percent error [(mean of measured − mean of added)/mean of added] × 100, while the precision was given by the inter-day and intra-day coefficients of variation.

2.9. Determination of the limits of quantitation (LOQ) and detection (LOD)

The LOQ was defined as the lowest drug concentration which can be determined with an accuracy and precision ≤20% on a day-to-day basis [27]. Its corresponds to sample concentrations of clozapine, N-desmethylclozapine and clozapine N-oxide resulting in a peak area of 10 times the noise level (SN). To determine the analytical error in the LOQ, spiked samples were used.

The LOD was defined as the sample concentration resulting in a peak area of three times the signal-to-noise ratio.

2.10. Recovery

The extraction efficiency (recovery) was determined by comparing peak areas from drug-free plasma and RBCs spiked with known amounts of drugs (80, 800 and 1400 ng/ml for clozapine in plasma; 80, 200 and 400 ng/ml for clozapine in RBCs, 80, 200 and 400 ng/ml for N-desmethylclozapine and clozapine N-oxide in plasma and RBCs), assayed accordingly, vs. peak areas of the same concentrations prepared in purified water injected directly onto the analytical column. Each sample was determined in triplicate.

In order to study the effect of co-extracted biological material, recoveries were also computed by comparison of extracts from spiked samples with blank extracts spiked after the extraction.

2.11. Stability study

The stability at −30°C of stock and working solutions of clozapine and of its two metabolites was assessed.

For stability studies in biological matrix, control human plasma samples were spiked with 80, 800 and 1400 ng/ml of clozapine and 80, 200 and 400 ng/ml of the two metabolites. Samples spiked with 80, 200 and 400 ng/ml of clozapine and of its metabolites were prepared to study stability in RBCs. The short-term stability was assessed after 1, 2, 4 and 6 h of storage at both ordinary laboratory conditions (20°C at daylight exposure) and 4°C. The stability of the drugs in frozen human plasma and RBCs (−30°C) was determined by periodic analysis over a span of 1 month; prior to their analyses, samples were brought to room temperature and vortex-mixed well. Spiked samples were analysed immediately after preparation (reference values) and after storage. Each determination was performed in triplicate.

The freeze–thaw stability was also determined. Spiked plasma and RBC samples were analysed

immediately after preparation and on a daily basis after repeated freezing–thawing cycles at −30°C on three consecutive days.

The stability of clozapine, desmethylclozapine and clozapine N-oxide in extract was inspected during 20 h at 20°C.

3. Results

3.1. Retention times and specificity

Observed retention times were 2.8, 4.9, 11 and 20 min for clozapine N-oxide, N-desmethylclozapine, clozapine and loxapine, respectively. In drug-free plasma and RBC samples, no peak interfered at the retention times of the respective analytes (Fig. 2a and Fig. 3a). Representative chromatograms are shown in Figs. 2 and 3. No interference was found with all drugs tested that could be co-administered (Fig. 4).

3.2. Linearity

Peak-area ratios of clozapine and its two metabolites over the internal standard varied linearly with concentration over the range used. The correlation coefficients (r) for calibration curves were equal to or greater than 0.992. Intra-assay reproducibility was determined for calibration curves prepared the same day in replicate ($n=6$) using the same stock solutions. Inter-assay reproducibility was determined for calibration curves prepared on different days ($n=9$). Results are given in Table 1 and Table 2. For each point of calibration standards, the concentrations were back-calculated from the equation of the linear regression curves (experimental concentrations) and the coefficients of variation (C.V.) were computed. Inter-day and intra-day variabilities at concentration of calibration standards are presented in Table 3. A linear regression of the back-calculated concentrations vs. the nominal ones provided a unit slope and an intercept equal to zero (Student's t-test).

For each calibration curve, the slope was statisti-

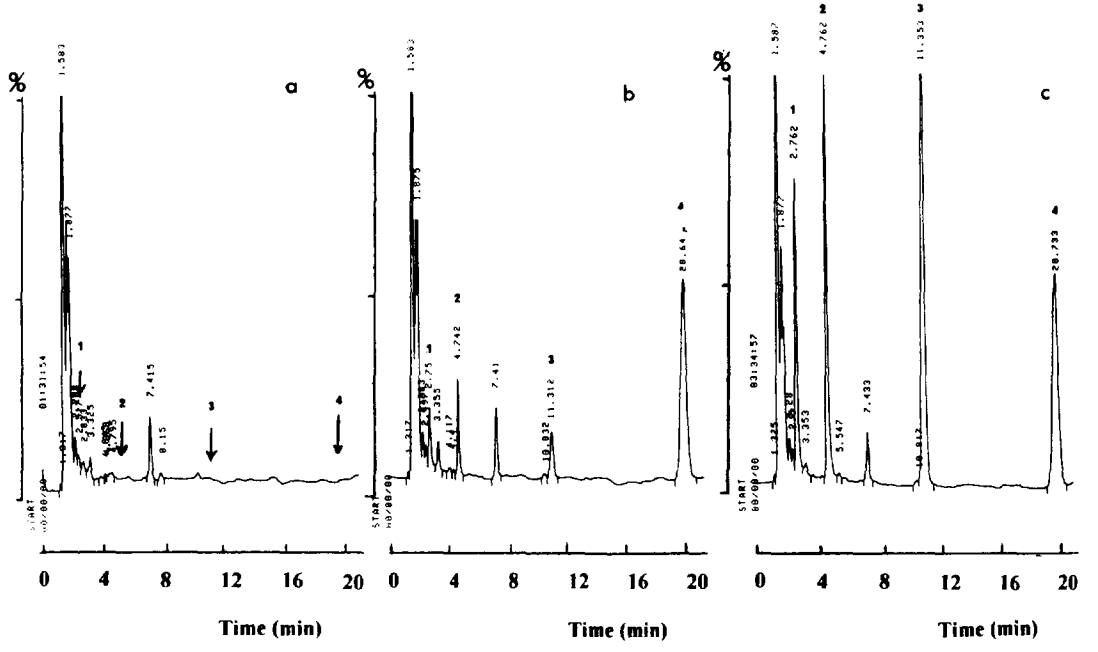

Fig. 2. Chromatograms of (a) blank plasma, (b) plasma spiked with 50 ng/ml clozapine and its N-oxide and desmethylated metabolites, and (c) plasma spiked with 500 ng/ml clozapine and 300 ng/ml N-desmethylclozapine and clozapine N-oxide. Peaks: 1=clozapine N-oxide; 2=N-desmethylclozapine, 3=clozapine; 4=the internal standard. For chromatographic conditions see Section 2.

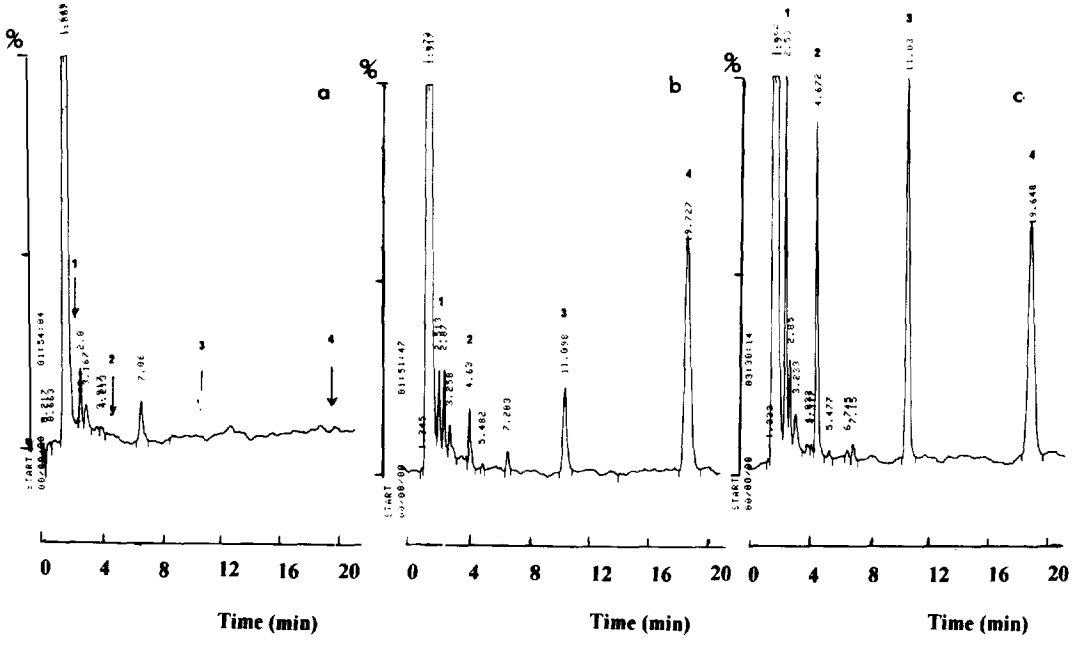

Fig. 3. Chromatograms of (a) blank RBCs, (b) RBCs spiked with 50 ng/ml clozapine and its N-oxide and desmethylated metabolites, and (c) of RBCs spiked with 300 ng/ml of the three analytes. Peaks: 1=clozapine N-oxide, 2=N-desmethyl clozapine, 3=clozapine, and 4=internal standard. For chromatographic conditions see Section 2.

cally different from zero, and the intercept was not statistically different from zero. Moreover, the residuals (difference between nominal and back-calculated concentrations) were normally distributed and centred around zero (Kolmogorov–Smirnov test).

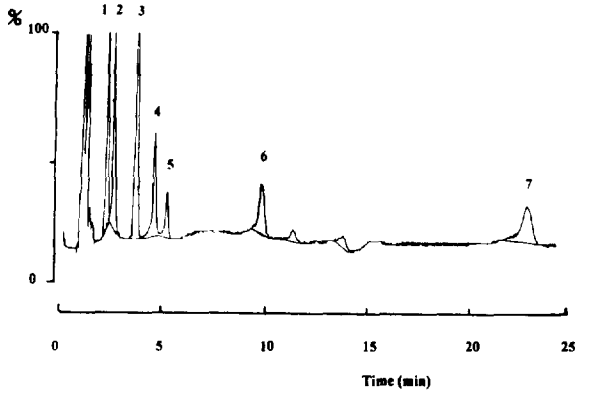

Fig. 4. Chromatogram obtained from a spiked plasma with droperidol (peak 1, 1 μg/ml); clozapine N-oxide (peak 2, 500 ng/ml), clonazepam (peak 3, 1 μg/ml), N-desmethylclozapine (peak 4, 500 ng/ml), clorazepate (peak 5, 1 μg/ml), clozapine (peak 6, 500 ng/ml) and amitriptyline (peak 7, 1 μg/ml). Clomipramine, diazepam, and hydroxyzine are not detected. For chromatographic conditions see Section 2.

3.3. Precision and accuracy

For concentrations of calibration standards, the precision around the mean value did not exceed 15% (Table 3). The results for accuracy, intra-day and inter-day precision are presented in Table 4.

3.4. Recovery

In plasma, the mean recovery (n=9) averaged 72.7±5.4% for clozapine, it was 80.2±6.8% for N-desmethylclozapine and 67.8±5.3% for clozapine N-oxide. In RBCs, recoveries were 71.9±5.3, 62.6±3.2 and 64.5±5.0% for the three analytes (n=9), respectively. The extraction efficiency is not statistically different over the range of concentrations studied. For the internal standard, the recovery averaged 73.0±3.5% (n=6) in plasma and 68.0±4.3% (n=6) in RBCs. No effect of the co-extracted biological material was detected

Table 1
Assay linearity for clozapine

	Correlation coefficient of the linear regression analysis[a] (r) (mean±S.D.)	Slope (b) (mean±S.D.)	Intercept (a) (mean±S.D.)
Human plasma			
Intra-day reproducibility ($n=6$)	$0.9992\pm5.97\ 10^{-4}$ (C.V.=0.06%)	$0.00172\pm4.93\ 10^{-5}$ (C.V.=2.87%)	0.0163±0.0172
Inter-day reproducibility ($n=9$)	$0.9991\pm6.89\ 10^{-4}$ (C.V.=0.069%)	$0.00191\pm1.52\ 10^{-4}$ (C.V.=7.96%)	$6.96\ 10^{-3}\pm0.026$
Red blood cells			
Intra-day reproducibility ($n=6$)	$0.999\pm5.27\ 10^{-4}$ (C.V.=0.053%)	$0.0020\pm1.45\ 10^{-4}$ (C.V.=7.25%)	$0.00107\pm1.02\ 10^{-2}$
Inter-day reproducibility ($n=12$)	$0.997\pm3.27\ 10^{-3}$ (C.V.=0.33%)	$0.00194\pm7.55\ 10^{-5}$ (C.V.=3.89%)	$0.01\pm2.66\ 10^{-2}$

[a] Linear unweighted regression, formula: $y=a+bx$.

3.5. Limit of quantitation and limit of detection

The limit of quantitation was 20 ng/ml for clozapine and N-desmethyl clozapine, it was 30 ng/ml for clozapine N-oxide. At these levels the analytical error averaged 11±6.4, 9.6±5.92, 13.3±3.18% in plasma, and 11.6±6.0, 8.67±6.9, 12.8±3.0 in RBCs, respectively ($n=3$). The limit of detection was 10 ng/ml for clozapine and N-desmethyl clozapine, and 20 ng/ml for clozapine N-oxide.

3.6. Stability

Stock and working solutions of clozapine and its desmethyl and N-oxide metabolites were stable for at least 2 months and 7 days, respectively without measurable decomposition.

Stability results at 20 and at 4°C are given in Table 5. After bench-top storage at 20°C, losses averaging 13.7% were observed for N-desmethyl clozapine at the concentration of 80 ng/ml in plasma. In the other cases no degradation occurred.

Frozen at −30°C clozapine and its metabolites were stable during one month; compared to the reference values, there was no statistical difference. Recoveries were 96±4.5% for clozapine, 99.3±3.1% for N-desmethyl clozapine and 103.3±3.1% for clozapine N-oxide in plasma. These percentages were 97.4±5.9, 96.1±3.8 and 95.4±3.6% in RBCs, respectively.

Run-time stability at room temperature of processed samples after extraction was determined for each point of calibration standard. After 20 h no significant losses occurred.

At least three freeze–thaw cycles can be tolerated without losses higher than 10%. Indeed, after the third cycle, losses ≤4% were observed.

4. Discussion and conclusions

The present HPLC method requiring a small sample volume (0.5 ml) enables the determination of both clozapine and its two major metabolites in the plasma or RBCs of patients undergoing clozapine therapy within a single run. The method had to be rapid, simple, selective, accurate and robust.

Assay performance was assessed both on the basis of the statistical characteristics of individual calibration lines and from the results of quality control samples. This method has been validated for concentrations ranging from 50 to 2000 and 50 to 500 ng/ml for clozapine in plasma and RBCs, respective-

Table 2
Assay linearity for desmethylclozapine and clozapine N-oxide

	N-desmethylclozapine			Clozapine N-oxide		
	Correlation coefficient of the linear regression analysis[a] (r) (mean±S.D.)	Slope (b) (mean±S.D.)	Intercept (a) (mean±S.D.)	Correlation coefficient of the linear regression analysis[a] (r) (mean±S.D.)	Slope (b) (mean±S.D.)	Intercept (a) (mean±S.D.)
Human plasma						
Intra-day reproducibility ($n=6$)	0.993±4 10^{-3} (C.V.=0.4%)	0.00176±9.77 10^{-5} (C.V.=5.5%)	0.012±5 10^{-3}	0.990±6.5 10^{-3} (C.V.=0.66%)	0.00386±3.4 10^{-4} (C.V.=8.8%)	0.0854±0.032
Inter-day reproducibility ($n=9$)	0.994±3.22 10^{-3} (C.V.=0.32%)	0.00205±1.94 10^{-4} (C.V.=9.5%)	0.012±0.012	0.993±3.16 10^{-3} (C.V.=0.32%)	0.00438±3.98 10^{-4} (C.V.=9.1%)	0.0521±0.0425
Red blood cells						
Intra-day reproducibility ($n=6$)	0.996±6.98 10^{-3} (C.V.=0.701%)	0.00195±3.67 10^{-5} (C.V.=1.88%)	$-3.69\ 10^{-3}\pm4.15\ 10^{-3}$	0.994±5.97 10^{-3} (C.V.=0.601%)	0.00514±2.55 10^{-4} (C.V.=4.96%)	0.00340±0.0255
Inter-day reproducibility ($n=12$)	0.997±2.3 10^{-3} (C.V.=0.231%)	0.00207±1.81 10^{-4} (C.V.=8.76%)	$-4.28\ 10^{-3}\pm8.1\ 10^{-3}$	0.994±3.68 10^{-3} (C.V.=0.370%)	0.00472±4.18 10^{-4} (C.V. =8.86%)	0.0437±0.0414

Table 3
Intra- and inter-assay reproducibilities of the HPLC analysis for clozapine, desmethylclozapine and clozapine N-oxide

Theoretical concentration (ng/ml)	Clozapine				N-Desmethylclozapine				Clozapine N-oxide			
	Intra-assay reproducibility ($n=6$)		Inter-assay reproducibility ($n=9$)		Intra-assay reproducibility ($n=6$)		Inter-assay reproducibility ($n=9$)		Intra-assay reproducibility ($n=6$)		Inter-assay reproducibility ($n=9$)	
	Experimental concentration (ng/ml) (mean±S.D.)	C.V. (%)	Experimental concentration (ng/ml) (mean±S.D.)	C.V. (%)	Experimental concentration (ng/ml) (mean±S.D.)	C.V. (%)	Experimental concentration (ng/ml) (mean±S.D.)	C.V. (%)	Experimental concentration (ng/ml) (mean±S.D.)	C.V. (%)	Experimental concentration (ng/ml) (mean±S.D.)	C.V. (%)
Human plasma												
50	48.3±4.4	9.1	51.2±5.7	11.1	47.7±4.6	9.6	49.8±3.8	7.6	51.3±5.1	9.9	52.1±2.7	5.2
80					81.7±1.7	2.1	79.2±5.0	6.3	82.0±3.8	4.6	80.0±5.4	6.7
100	108±8.6	8.0	96.3±8.2	8.5	104±4.5	4.3	105±6.9	6.6	103±3.4	3.3	101±2.2	2.2
200	202±8.4	4.2	202±8.9	4.4	189±14.7	7.8	204±8.7	4.3	198±17.9	9.0	200±10.9	5.4
300	295±10.4	3.5	297±8.8	3.0	291±12.4	4.3	288±15.3	5.3	290±16.0	5.5	286±16.3	5.7
400					408±25.4	6.2	417±27.3	6.5	393±24.4	6.2	409±16.2	4.0
500	491±19.7	4.0	486±14.3	2.9	500±18.4	3.7	496±19.1	3.8	506±15.7	3.1	501±16.7	3.3
1000	1016±33.0	3.2	1022±35.3	3.5								
2000	1994±19.0	0.95	1991±18.6	0.93								
Red blood cells												
50	53.1±3.0	5.6	50.9±4.4	8.6	50.6±2.1	4.2	51.6±3.2	6.2	49.8±4.5	9.0	50.9±7.6	14.9
80	81.3±1.2	1.5	80.4±5.2	6.5	82.1±3.1	3.8	81.2±3.8	4.7	84.2±3.3	3.9	88.6±10.9	12.3
100	105±4.4	4.2	100±6.7	6.7	102±5.1	5.0	101±7.0	6.9	104±5.9	5.7	109.4±2.5	2.3
200	193±5.2	2.7	199±6.9	3.5	193±8.4	4.4	191±11.6	6.1	200±3.5	1.8	208±10.6	5.1
300	292±7.7	2.6	289±15.9	5.5	292±7.8	2.7	289±13.6	4.7	281±14.3	5.1	288±13.5	4.7
400	401±8.9	2.2	395±10.6	2.7	400±9.3	2.3	405±10.3	2.5	408±17.3	4.2	398±20.4	5.1
500	505±8.7	1.7	510±13.8	2.7	502±6.9	1.4	505±3.0	0.59	506±9.3	1.8	503±9.83	2.0

Table 4
Intra-day and inter-day precision and accuracy of the HPLC method for clozapine, N-desmethylclozapine and clozapine N-oxide

Theoretical concentration (ng/ml)	n	Clozapine				N-Desmethylclozapine				Clozapine N-oxide			
		Experimental concentration (ng/ml) (mean±S.D.)	C.V. (%)	Mean recovery (%)	Relative error (%)	Experimental concentration (ng/ml) (mean±S.D.)	C.V. (%)	Mean recovery (%)	Relative error (%)	Experimental concentration (ng/ml) (mean±S.D.)	C.V. (%)	Mean recovery (%)	Relative error (%)
Human plasma													
Intra-day precision													
80	6	83.8±2.6	3.2	105.0	5.0	83.6±6.3	7.5	104.5	4.5	82.9±4.1	5.0	103.6	3.6
200						198±12.9	6.5	99.0	1.0	198±11.5	5.8	99.0	1.0
400						391±22.0	5.6	97.8	2.2	395±33.4	8.5	98.8	1.2
800	6	785±30.7	3.9	98.1	1.9								
1400	6	1417±55.2	3.9	101.0	1.2								
Inter-day precision													
80	7	79.9±6.7	8.4	99.9	0.1	80.5±6.09	7.6	100.6	0.6	79.7±6.7	8.4	99.6	0.4
200						202±10.6	5.2	101.0	1.0	204±17.6	8.6	102.0	2.0
400						402±21.5	5.3	100.5	0.5	404±31.7	7.9	101.0	1.0
800	7	791±34.4	4.4	98.9	1.1								
1400	7	1441±95.8	6.6	102.9	2.9								
Red blood cells													
Intra-day precision													
80	6	83.8±3.5	4.2	104.7	4.7	78.8±2.6	3.3	98.5	1.5	82.9±3.90	4.7	103.6	3.6
200	6	206±17.9	8.7	103.1	3.1	210±11.0	5.2	105.0	5.0	209±18.5	8.9	104.5	4.5
400	6	405±23.3	5.8	101.2	1.2	420±27.3	6.5	105.0	5.0	390±19.4	5.0	97.5	2.5
Inter-day precision													
80	6	80.3±5.9	7.3	100.4	0.4	80.3±5.4	6.7	100.4	0.4	81.4±1.8	2.2	101.7	1.7
200	6	199±17.4	8.7	99.5	0.5	195±15.8	8.1	97.5	2.5	199±11.4	5.7	99.5	0.5
400	6	393±31.9	8.1	98.3	1.7	413±15.3	3.7	103.3	3.3	387±25.2	6.5	96.8	3.2

Table 5
Mean percent recoveries after 6 h of storage at 20 and 4°C ($n=4$)

Concentration added (ng/ml)	Recovery (mean±S.D.)(%)		Concentration added (ng/ml)	Recovery (mean±S.D.)(%)			
	Clozapine			N-Desmethylclozapine		Clozapine N-oxide	
	20°C	4°C		20°C	4°C	20°C	4°C
Human plasma							
80	93.0±1.3	101.9±6.9	80	86.3±2.4	102.6±5.3	100.5±1.9	100.7±1.3
800	103.3±2.0	104.6±2.1	200	103.7±5.0	101.0±4.8	99.4±5.5	99.7±2.3
1400	100.0±2.2	99.7±4.2	400	99.8±2.9	98.0±4.2	103.0±6.0	100.7±5.9
Red blood cells							
80	105.0±4.1	99.1±6.0	80	101.2±1.1	98.2±5.2	98.9±6.3	97.2±5.3
200	101.9±3.9	96.7±1.6	200	100.7±2.7	100.6±1.8	101.4±6.2	95.8±4.9
400	101.9±4.1	95.8±2.1	400	101.8±2.5	95.8±2.1	102.9±7.0	98.4±3.3

ly, and from 50 to 500 ng/ml for its N-oxide and desmethylated metabolites in plasma and RBCs that spans what is currently though to be the clinically relevant range of these compounds in body fluids. The limits of quantitation of the present assay (20 ng/ml for clozapine and N-desmethylclozapine) were satisfactory, since plasma levels of clozapine and its N-desmethylated metabolite were far above these limits. Indeed, the therapeutic range of clozapine in plasma determined in a number of selected patients (with low and high doses) was estimated to be between 70 and 1000 ng/ml, it was between 60 and 500 ng/ml for the N-desmethylclozapine. For the clozapine N-oxide, higher limit of quantitation was found (30 ng/ml); since this metabolite seems to be the least pharmacologically effective of the three substances [28], this limit may be considered acceptable for routine determination. Recoveries ranged from 63 to 80% according to the analyte and day-to-day variations were always lower than 15%. Loxapine was regarded as an acceptable internal standard because it exhibits similar extraction properties.

The limit of detection reported in the present study was similar to that published in the literature [15,18,19,21,23,25], however, it was higher than that reported by Chung et al. [20] (2 ng/ml for clozapine, 1 ng/ml for the desmethylated metabolite). These authors described an HPLC method that included a laborious extraction procedure and required 1 ml of plasma.

The proposed method allows therapeutic drug monitoring of clozapine that include the determination of the N-desmethyl and N-oxide metabolites. This method has been used for more than three months in the routine therapeutic drug monitoring in our hospital. Our aim is to relate the results of the clozapine and its two metabolites plasma concentrations to the antipsychotic effect and side effects of the drug. In some patients, the ratios (RBC concentrations/plasma concentrations) have been determined. After multiple oral-doses (400 to 800 mg/day), these ratios averaged 0.29±0.14 for clozapine, 1.63±0.45 for N-desmethylclozapine and 0.73±0.55 for clozapine N-oxide ($n=10$). These results show an important accumulation of the active metabolite in RBCs. These findings are of great interest in the

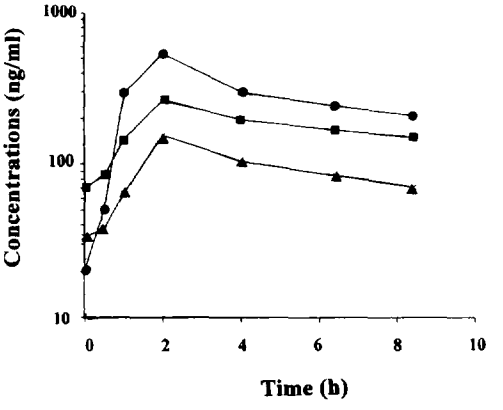

Fig. 5. Plasma concentration–time curves for clozapine, and its desmethylated and N-oxide metabolites following oral administration of clozapine in a schizophrenic patient. The dose (300 mg) was administered 36 h after the previous dose. (●) Clozapine, (■) N-desmethylclozapine, (▲) clozapine N-oxide.

choice of the pharmacokinetic model used to determine the population pharmacokinetic parameters of clozapine in order to optimize individual dosage regimens. Fig. 5 shows plasma level profiles of clozapine, N-desmethylclozapine and clozapine N-oxide in a schizophrenic patient.

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