ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
16 (1997) 311–318

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Assay for quantitation of clozapine and its metabolite *N*-desmethylclozapine in human plasma by high-performance liquid chromatography with ultraviolet detection

L. Edno [a,*], I. Combourieu [a], M. Cazenave [b], J. Tignol [b]

[a] *Laboratoire de Biologie, Centre Hospitalier Charles Perrens, 121 rue de la Béchade, 33076 Bordeaux Cedex, France*
[b] *Laboratoire de Psychiatrie, Université Bordeaux II, 121 rue de la Béchade, 33076 Bordeaux Cedex, France*

Received 7 February 1997

Abstract

A high-performance liquid chromatographic method with UV detection has been developed for the analysis of clozapine and its active *N*-desmethylated metabolite (*N*-desmethylclozapine = DMC) in human plasma. A liquid/liquid procedure was used to extract clozapine and DMC from human plasma. The analysis was performed on a C8 Nucleosil column and the mobile phase comprised acetonitrile–water–Pic B5 diethylamine (63:37:25:0.04, v/v/v/v). The detection wavelength was 245 nm. The intra-assay and inter-assay precision was satisfactory within the concentration range 10–900 ng ml^{-1}. The lower detection limit for clozapine and for DMC was 5 ng ml^{-1}. The recovery and reproducibility values of this method were better or similar to those found by other authors. This method, which is simple, selective and avoids an evaporation step, can be used routinely for therapeutic drug monitoring. © 1997 Elsevier Science B.V.

Keywords: Clozapine; *N*-desmethylclozapine; HPLC; Drug monitoring; Human plasma

1. Introduction

Clozapine (Clozaril;8-chloro-11-(4-methyl-1-piperazinyl)-5H-dibenzo[b,e]-[1,4]-diazepine) (Fig. 1), first synthesised in 1960, is a member of a class of antipsychotic drugs known as dibenzodiazepines. The drug has a pharmacological profile unlike standard antipsychotics, and it is known as an 'atypical antipsychotic' in the clinical literature; although it may produce a great improvement in both positive and negative symptoms in schizophrenics, it does not produce extrapyramidal side-effects. Unfortunately, the absence of these adverse effects is offset by agranulocytosis, which occurs in 1–2% of patients. Several methods have been developed for the determination of plasma levels of clozapine: gas chromatography [1–5], HPLC [6–10] and radioimmunoassay [11].

In this work, the authors have tried to develop a sensitive, specific, rapid, easy and inexpensive HPLC method for the determination of clozapine

* Corresponding author. Tel.: + 33 56 563512; fax: + 33 56 563570.

PII S0731-7085(97)00048-4

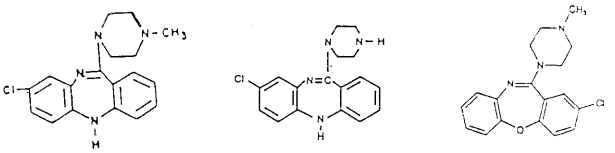

Fig. 1. Chemical structure of clozapine, *N*-desmethylclozapine and loxapine.

and its active *N*-desmethylated metabolite in human plasma [13]. This assay needs only simple extraction without an evaporation step and offers good recovery. Its easy adaptation allows its use for drug routine monitoring.

2. Experimental

2.1. Materials

Clozapine and its metabolite, *N*-desmethylclozapine (DMC), were kindly provided by Sandoz laboratories (Paris, France). The internal standard (loxapine) was supplied by Lederle laboratories (St Quentin Fallavier, France). Stock solutions of clozapine, DMC and loxapine (1 mg ml^{-1}) were prepared by dissolving the appropriate amount in 20 ml of methanol in volumetric flasks. Acetonitrile, hexane (ICS, Toulouse, France), Pic B5 (Waters, USA) and methanol (Merck, Germany) were of HPLC grade. Isoamyl alcohol and diethylamine were both analytical grade and were purchased from Prolabo (France). NaOH and HCl (0.1 M) were provided by Merck (France). Purified water was prepared with a Milli-Q system (Millipore, Milford, MA, USA). Pooled plasma samples from healthy volunteers, provided by a blood bank (CTS, Bordeaux, France), were used for the validation of the method.

2.2. Instrumentation

For development and evaluation of this method an ICS instrument with a pump M220 and an automatic sampler model 738 (ICS Toulouse, France) were used. The 125 × 4.6 mm i.d. analytical column, packed with 5-μm Ecotube Nucleosil, C8, (Bischoff, Leonberg, Germany), was supplied by ICS (Toulouse, France); a 20 × 4.6 mm i.d. precolumn (C8, Bischoff, Germany) was placed just before the inlet of the analytical column. The detector was a variable wavelength UV detector (Model SPD-1OA, Shimadzu Instruments, Touzart Matignon, France). The wavelength was set at 245 nm. A Shimadzu CR5-A Chromatopac data workstation was used to collect and analyse chromatographic data.

2.3. Chromatographic conditions

The mobile phase comprised purified water–acetonitrile–Pic B5 (water–methyl alcohol 1-pentane sulfonic acid–acetic acid)–diethylamine 63:37:2.5:0.04, v/v/v/v); diethylamine was used to block the residual OH group of the stationary phase and also to avoid streaking of the peaks). Pic B5 was used as a counter-ion to allow formation of a neutral-ion pair and therefore to enhance retention of the compounds. The mobile phase was adjusted to pH 6.35 (pH meter Schott CG825, Bioblock Scientific, Strasbourg, France) with 2 M sodium hydroxyde, filtered through a membrane filter (0.45 μm; Millipore, Molsheim, France) and degassed ultrasonically before use. The column was maintained at 56°C with an Eurosas oven (ICS, France). The flow-rate was 1.7 ml min^{-1}. The pressure was about 180 bar.

2.4. Extraction procedure

Sample preparation consisted of a dual liquid–liquid extraction. To 1 ml of plasma in a 12 ml

borosilicate silanised glass tube (100 × 17 mm) were added 40 μl of the internal standard loxapine (10 μg ml^{-1}) and 200 μl of 0.33 M NaOH. The plasma was mixed with a hand vortex mixer (Rotamixer, CCFH, France). Then the preparation was extracted with 6 ml of hexane (95%)–isoamylid alcohol (99%) (985:15, v/v) for 30 min and shaken with an Agitest 86212 (Bioblock Scientific, Strasbourg, France). After shaking, the tubes were centrifuged in a table-top centrifuge at 4000 rpm for 5 min. The organic layer was collected in a 12 ml conical bottomed silanised tube (115 × 19 mm). 150 μl of 0.1 M HCL was added to this phase. This tube was mixed in a vortex mixer for 1 min. Most of the organic layer was withdrawn and discarded by aspiration through a Pasteur pipette; the acidic aqueous phase containing clozapine and DMC was collected into a poly-propylene microtube (ICS, Toulouse, France). 50 μl of this phase was injected into the chromatograph.

2.5. Instrument calibration

Stock solutions were prepared by dissolving the drug or internal standard as supplied (free base) in methanol (1 mg ml^{-1}) and were stored in the freezer at −20°C. Working stock solutions (10 μg ml^{-1}) were prepared weekly.

Calibration standards for control plasma were prepared at concentrations of 10, 25, 50, 100, 20O, 400, 600 and 900 ng ml^{-1} in human plasma. The standard samples were prepared from clozapine and DMC working stock solutions and were treated like the samples from patients.

2.6. Data analysis

For plasma, the ratio of the peak area of clozapine and DMC to that of internal standard was used as the assay parameter. Peak-area ratios were plotted against theoretical concentrations. Standard calibration lines were obtained from unweighted least-squares linear regression analysis of the data. The linearity of the method was statistically tested by comparison of the intercept with zero and calculation of correlation coefficients.

2.7. Stability study

The stability of clozapine and DMC was assessed during all the storage steps and during all steps of the analytical method. During the first days of the study, quality control samples (in plasma) were spiked with standard solutions of clozapine and DMC to provide concentrations of 50, 400 and 800 ng ml^{-1}. Then aliquots of the quality control samples were stored in a freezer at −20°C and randomly removed at various times in each analytical sequence during one month.

3. Results

3.1. Retention times

Under the specified conditions the retention times were: 4.16 min for DMC; 8.70 min for clozapine; and 12.70 min for loxapine. This allowed clear separation. There were no interfering peaks in the control plasma at the retention times of the respective analyses in the HPLC chromatograms of plasma samples (Fig. 2).

3.2. Linearity

Standard curves for clozapine and DMC were linear in each case over a range of 10–900 ng ml^{-1}. The mean correlation coefficients r for calibration curves were 0.999 ± 0.000641 for clozapine and 0.997 ± 0.00441 for DMC. For each point of the calibration standards, the concentrations were recalculated from the equation of the linear regression curves (experimental concentrations). The values of the precision for clozapine and DMC are presented in Table 1. The small percentage differences between nominal and found concentrations of the standards in the standard curves for both intra- and inter-day data confirmed that the assay was linear over the concentration range investigated. Both the intra- and inter-assay RSD values were generally less than 11% except for clozapine and DMC concentrations of 10 and 25 ng ml^{-1}. The intra-assay RSD of 15.02% at a clozapine concentration of 50 ng ml^{-1} is high but comparable to those in previous reports [16].

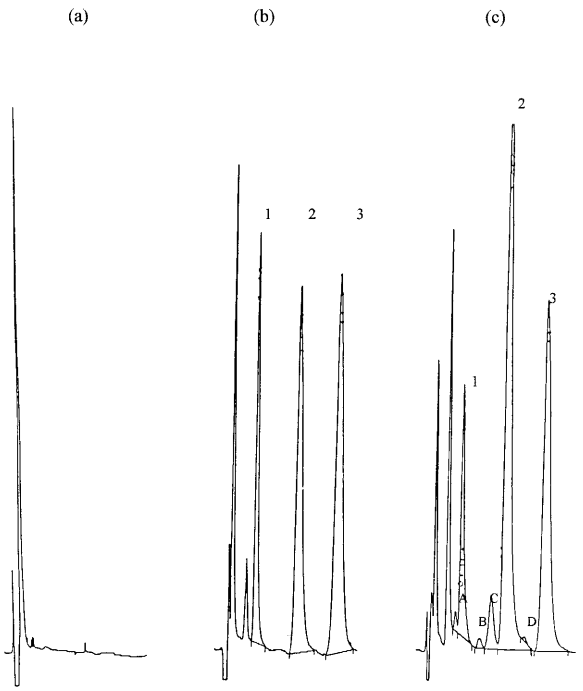

Fig. 2. Representative chromatograms of: (a) blank plasma; (b) human plasma spiked with clozapine, desmethylclozapine and loxapine, the internal standard, at a concentration of 200 ng ml^{-1}; and (c) plasma sample from a patient receiving clozapine. Peaks: (1) desmethylclozapine, (2) clozapine, (3) loxapine. A, B, C, D: Unidentified metabolites. For comments and chromatographic conditions, see text.

3.3. Precision and accuracy

Between- and within-run accuracy and precision in human plasma were assessed by performing replicate analysis of spiked samples (50, 400 and 800 ng ml^{-1}) against calibration standards. The precision and accuracy of the method were calculated as percentage deviation of the observed concentration from the theoretical concentration and absolute error, respectively. Results are presented in Table 2.

3.4. Recovery

The extraction efficiency (recover) was determined by comparing peak-height ratios of extracted standards (prepared from drug-free plasma spiked with known amounts of clozapine and DMC at concentrations of 100, 400 and 900 ng ml^{-1}) with peak-height ratios of unextracted standards (compounds added at the same concentrations to methanol just before HPLC injection). The mean recoveries for clozapine and DMC were $88.06 \pm 6.87\%$ ($n = 4$) and $84.96 \pm 9.08\%$ ($n = 4$), respectively.

3.5. Determination of the limit of quantification (LOQ)

The LOQ was based on the lowest calibration sample (10 ng ml^{-1}); was determined from the peak and the standard deviation of the noise level; it was 10 ng ml^{-1} for clozapine and DMC. At these levels, the analytical error was $< 25\%$.

3.6. Determination of the limit of detection (LOD)

The LOD, defined as three times the signal-to-noise ratio, was 5 ng ml^{-1} for clozapine and

Table 1
Precision of the assay for clozapine (CLZ) and desmethylclozapine (DMC)

Concentration (ng ml^{-1})	Intra-assay ($n = 6$)						Inter-assay ($n = 6$)					
	CLZ			DMC			CLZ			DMC		
	Mean (ng ml^{-1})	SD (ng ml^{-1})	RSD (%)	Mean (ng ml^{-1})	SD (ng ml^{-1})	RSD (%)	Mean (ng ml^{-1})	SD (ng ml^{-1})	RSD (%)	Mean (ng ml^{-1})	SD (ng ml^{-1})	RSD (%)
10	13.23	1.82	13.76	10.44	2.36	22.61	12.32	1.27	10.28	11.87	1.35	11.41
25				22.32	1.23	5.51	29.10	2.02	6.95	26.38	3.49	13.23
50	47.84	7.19	15.02	48.25	5.28	10.94	47.50	4.12	8.68	53.50	5.72	10.70
100	92.41	3.67	3.98	97.92	5.26	5.37	94.78	8.13	8.57	95.28	10.38	10.89
200	198.50	12.26	6.18	209.80	10.98	5.23	198.36	14.22	7.17	191.77	14.58	7.61
400	417.21	12.77	3.06	408.90	26.45	6.47	396.14	23.64	5.97	401.37	15.57	3.88
600	578.88	11.97	2.04	586.10	36.05	6.16	600.75	24.29	4.04	608.78	34.13	5.61
900	900.52	10.15	1.13	892.20	54.94	6.16	893.43	17.12	1.92	909.76	14.90	1.64

Table 2
Accuracy of the HPLC method

Theoretical concentration (ng ml^{-1})	n	Experimental concentration (mean ± SD), (ng ml^{-1})	RSD (%)	Deviation from theoretical value (%)
Clozapine				
Within-day				
50	4	47.30 ± 4.28	9.05	5.39
400	6	409.38 ± 22.54	5.51	−2.34
800	5	818.61 ± 20.19	2.47	−2.33
Between-day				
50	7	49.31 ± 2.37	4.81	1.39
400	9	387.04 ± 18.47	4.77	3.24
800	8	814.08 ± 43.23	5.31	−1.76
N-Desmethylclozapine				
Within-day				
50	4	53.88 ± 4.39	8.13	−7.76
400	6	362.98 ± 9.92	2.74	9.26
800	5	717.89 ± 38.76	5.39	10.26
Between-day				
50	5	53.96 ± 4.59	8.51	−7.92
400	6	395.94 ± 63.89	16.14	1.02
800	5	810.78 ± 71.46	8.81	−1.35

DMC, demonstrating the high sensitivity of this method.

3.7. Stability

The stability of clozapine and DMC on the autosampler were determined for each point of the calibration standards in the mobile phase. The concentrations were expressed as a percentage of the concentrations at time zero. For all concentrations, no significant difference appeared between $t = 0$, $t = 8$ h and $t = 20$ h. The stability of stock solutions was also assessed after 30 days of cold storage (+4°C and frozen at −20°C) and of room temperature storage, in the dark. No decomposition of stock solutions was noted during one month.

3.8. Specificity

When testing standard solutions containing various other psychotropic drugs that may be used in combination with clozapine, minimal interference with clozapine and DMC was found for the drugs and metabolites tested (Table 3). Two columns were used for the study.

3.9. Application to plasma of patients

Blood samples were collected about 18 h after the last dose of clozapine. The samples were drawn into heparinized tubes and centrifuged at 2100 g for 10 min within 2 h after collection. Plasma was separated and stored at −20°C until use.

In eleven patients who had been treated with doses of clozapine of 150–600 mg per day, the plasma concentrations ranged between 79 and 623 ng ml^{-1} for clozapine and between 52 and 295 ng ml^{-1} for the active metabolite *N*-desmethylclozapine (Table 4). The plasma from patients shows peaks in addition to those seen in the blank and spiked plasma (Fig. 2). These unknown peaks may be due to metabolites or other drugs administered to the patients. The mean ratios of the concentration of DMC to the concentration of clozapine, as indices of *N*-desmethylation, were 0.590 ± 0.180 (mean ± SD) at the steady state. Patient 4, who had received 500 mg of clozapine, behaved differently; the concentrations of the metabolite and of clozapine were equal.

Table 3
Retention times of psychotropic drugs and some related metabolites analysed for interferences

Drug	Retention time (min)
Clozapine (Leponex®)	8.73
N-desmethylclozapine	4.16
Loxapine	12.70
Paroxetine (Deroxat®)	5.05
Clorazepate (Tranxene®)	3.10
Triazolam (Halcion®)	3.65
Lorazepam (Temesta®)	2.50
Diazepam (Valium®)	4.48
Nitrazepam (Mogadon®)	2.13
Alprazolam (Xanax®)	3.19
Temazepam (Normison®)	2.65
Chlordiepoxide (Librium®)	2.79
Flunitrazepam (Rohypnol®)	2.76
Prazepam (Lysanxia®)	7.18
Oxazepam (Seresta®)	1.99
Bromazepam (Lexomil®)	1.83
Haloperidol (Haldol®)	4.13

4. Discussion and conclusions

The present HPLC method involves a rapid and automated assay for the determination of clozapine and its metabolite in plasma. The object of this method was to establish an HPLC method suitable for the simultaneous determination of clozapine and its major active metabolite (DMC) in the plasma of patients undergoing clozapine therapy.

Recoveries (88–85%) and day-to-day variations of 1–20% were better or in the range of those found by others [6,7,12,13,17–19]. Compared with some authors' results [2,10,15–17,20], the recoveries with the present HPLC assay are similar for clozapine but better for DMC.

The sensitivity of the assay was satisfactory, since plasma levels of clozapine and its *N*-desmethylated metabolite were far above their detection limits of 5 ng ml^{-1}. The detection limit of the method may be considered acceptable for routine determination.

Detection, at two different wavelengths (254 and 245 nm), was considered because many authors present their results for 254 nm. A wavelength of 245 nm was chosen since most of the observed drugs have a higher absorption at 245 mm. That allows the ratio of the signals to be calculated for better estimation of the amounts. The plasma levels determined in a number of selected patients (with low and high doses of clozapine) were estimated to be between 50 and 900 ng ml^{-1}. These values indicate that this method is sensitive enough for pharmacokinetics studies and would allow therapeutic drug monitoring programmes for clozapine to be conducted to include the determination of DMC.

Table 4
Concentration of clozapine and DMC in the serum of schizophrenic patients

Patient	Final dose (mg of clozapine per day)	Mean plasma concentrations (ng ml^{-1})		
		Clozapine	DMC	Ratio DMC/Clozapine
ALB-1	600	530	284	0.536
BIC-2	400	136	67	0.493
BAY-3	500	272	145	0.533
MOR-4	500	292	295	1.010
RUF-5	300	362	243	0.671
SOU-6	300	383	215	0.561
CAL-7	400	107	74	0.692
DES-8	200	79	52	0.658
DUB-9	400	346	205	0.592
DUC-10	475	623	176	0.282
TRA-11	150	355	165	0.465
	Mean ± SD	316.82 ± 168.47	174.64 ± 84.50	0.590 ± 0.180

In summary, this HPLC method with UV detection offers several advantages over previously published HPLC techniques. The present study allows the quantitative determination of clozapine and DMC in a low volume of blood, after a single extraction step without an evaporation step. The recoveries of DMC were better than those found by others. Another advantage was the specificity of this method. The retention times obtained for each psychotropic drug indicate that this method is specific.

The limited data obtained with these patients can be used to determine whether a correlation exists between clozapine concentration and therapeutic response by administering a fixed dose of clozapine over a long period to obtain a steady-state. The range and the high inter-individual variations in the serum levels of clozapine found were in agreement with data reported in the literature [14,17]; in contrast, the intra-individual variations in this same group were low. These results must be confirmed by a wide study including a larger group of patients.

References

[1] G.M. Simpson, T.A. Cooper, Am. J. Psychiatry 135 (1978) 99–100.
[2] R. Heipertz, H. Pilz, W. Beckers, Arch. Toxicol. 37 (1977) 313–318.
[3] B. Stock, G. Spiteller, R. Heipertz, Arzneimittel-Forsch. 27 (1977) 982–990.
[4] M. Ackenheil, Psychopharmacology 99 (1989) 532–537.
[5] K. Richter, J. Chromatogr. 434 (1988) 465–468.
[6] M.G. Choc, R.G. Lehr, F. Hsuan, G. Honigfeld, H.G.H.G. Smith, R. Borison, J. Volavka, Pharm-Res. 4 (1987) 402–405.
[7] C. Haring, C. Humpel, B. Auer, A. Saria, C. Barnas, W. Fleischhacker, H. Hinterhuber, J. Chromatogr. 428 (1988) 160–166.
[8] C. Humpel, C. Haring, A. Sana, J. Chromatogr. 491 (1989) 235–239.
[9] U. Bondesson, L.H. Lindstrom, Psychopharmacology 95 (1988) 472–475.
[10] G. Dollo, H. Bun, M. Bonnefont-Cornet, J. Pharm. Clin. 13 (1994) 17–22.
[11] M.H. Bickel, Pharmacol. Rev. 21 (1969) 325–355.
[12] H. Weigmann, C. Hiemke, J. Chromatogr. 583 (1992) 209–216.
[13] M.J. Lovdahl, P. Perry, D.D. Miller, Ther. Drug Monit. 13 (1991) 69–72.
[14] P.J. Perry, D.D. Miller, S.V. Arndt, R.J. Cadoret, Am. J. Psychiatry 148 (1991) 231–235.
[15] B. Jeanny, J.L. Vailleau, P. d'Athis, V. Lahet, Analysis 13 (1994) 7–11.
[16] M.C. Chung, S.K. Lin, W.H. Chang, J. Chromatogr. 613 (1993) 168–173.
[17] D. Wilhem, A. Kemper, J. Chromatogr. 525 (1990) 218–224.
[18] U. Breyer, K. Villemsen, Eur. J. Clin. Pharmacol. 9 (1976) 457–465.
[19] W. Zeren, L. Minglian, X. Peipei, Z. Yanlin, Biomed. Chromatogr. 1 (1986) 53–57.
[20] C. Haring, J. Chromatogr. 428 (1988) 160–166.

.