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Short Communication

Determination of clozapine and desmethylclozapine in human plasma by high-performance liquid chromatography with ultraviolet detection

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ABSTRACT

A method using reversed-phase high-performance liquid chromatography for the simultaneous determination of clozapine and its desmethyl metabolite in human plasma has been established. Clozapine and N-desmethylclozapine were extracted with *n*-hexane–isoamyl aclohol (98.5:1.5, v/v). Protriptyline served as the internal standard. The limits of detection for clozapine and desmethylclozapine are 2 and 1 ng/ml, respectively. The sensitivity and precision of this method can be utilized for pharmacokinetic studies and therapeutic drug monitoring regimens.

INTRODUCTION

The antipsychotic agent clozapine (Fig. 1) was originally introduced into clinical practice in the early 1970s. Its distribution was severely limited owing to the problem of agranulocytosis; although available in Europe on a limited basis, clozapine was removed from the US market. Eventually, clozapine was reintroduced in the USA in 1990 [1]. Clozapine is referred to as an "atypical" antipsychotic. It differs from standard antipsychotics by its failure to produce various biochemical, behavioural and other dopaminergic effects typically observed with standard antipsychotics in animal models [2,3]. Various dopamine receptors (D-1 to D-5) have been detected in the central nervous system [4–6]. At the suggested therapeutic plasma concentrations, clozapine was

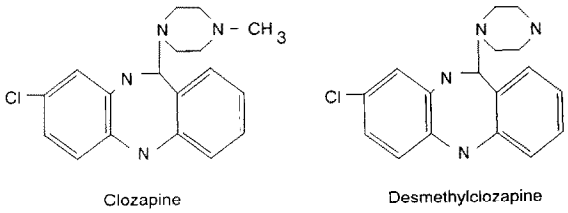

Fig. 1. Structures of clozapine and desmethylclozapine.

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reported to possess the highest binding affinity for the D-4 receptor [5]. The complex interaction between these various dopaminergic receptors with regard to clozapine's clinical efficacy and minimal extrapyramidal side-effects remains to be investigated.

The determination of clozapine plasma concentrations by gas chromatography [7], gas chromatography–mass spectrometry [8], high-performance liquid chromatography (HPLC) [9–11] and radioimmunoassay [12] has been described previously. This paper describes an isocratic reversed-phase HPLC method for the separation and measurement of clozapine and its metabolite desmethylclozapine in human plasma.

EXPERIMENTAL

Chemicals

Clozapine and N-desmethylclozapine were gifts from the Sandoz Research Institute (Basle, Switzerland). Protriptyline was supplied by USPC. Water obtained from a Milli-Q system (Millipore, Milford, MA, USA) and analytical-reagent grade chemicals were used throughout.

Extraction

To 1 ml of plasma in a 12-ml glass-stoppered tube were added 100 μl of the internal standard protriptyline (4 μg/ml) and 200 μl of NaOH (2 mol/l). The plasma mixture was then mixed with a hand vortex mixer and allowed to stand for 5 min. *n*-Hexane–isoamyl alcohol (98.5:1.5, v/v) (5 ml) was added and the solution was shaken for 20 min and centrifuged for 5 min at 3000 *g*. The organic phase was transferred into another 12-ml glass-stoppered tube containing 1 ml of 0.1 mol/l HCl. The solution was shaken for 5 min and centrifuged at 3000 *g* for 2 min. The organic layer was discarded by aspiration through a Pasteur pipette. To the remaining acid layer were added 200 μl of 2.0 mol/l NaOH and the compounds re-extracted with 5 ml of *n*-hexane–isoamyl alcohol solution (98.5:1.5, v/v). These steps were repeated and the resulting organic extracts pooled into a 75 mm × 10 mm I.D. borosilicate glass tube. The extract was evaporated under a gentle stream of dry nitrogen at 45°C. The residue was reconstituted with 100 μl of 0.1 mol/l HCl and 80 μl were injected into the chromatograph.

High-performance liquid chromatography

Analyses were conducted by HPLC with a double-piston pump (Waters Model 6000 A), a 100-μl loop injector (Waters U6K) and a variable-wavelength UV detector (Kratos 773) set at 230 nm with the sensitivity set at 0.001 a.u.f.s. The detector was connected to an autointegrator (Waters 740 data module) to analyse the chromatographic data. Reversed-phase HPLC was performed with a Whatman Partisil 10 ODS-3 (10 μm) column (250 mm × 4.6 mm I.D.) and a 70 mm ODS Whatman column survival kit (CSK) guard column. Both columns were packed with C_{18} particles (10 μm). The mobile phase was acetonitrile–methanol–buffered aqueous solution containing 5.0 g of dibasic phosphate adjusted to pH 4.0 with phosphoric acid (24:12:64, v/v). The flow-rate was set at 2.0 ml/min. For the determination of clozapine and desmethylclozapine, the peak height was used and compared with that of the internal standard protriptyline.

Patients' samples

Blood samples were obtained from six in-patient male schizophrenics treated with 150–800 mg of clozapine per day. Each patient was treated for 7–105 days as shown in Table I. A 10-ml blood sample was taken from the vein of these patients 12 h after oral administration. The pharmacokinetic profile of clozapine and desmethylclozapine in two schizophrenic patients are presented in Fig. 2A and B. Each patient received a single 100-mg oral dose of clozapine. The blood was immediately separated by centrifugation at 3000 *g* for 10 min. The plasma was stored in polypropylene tubes at −50°C until analysis.

RESULTS AND DISCUSSION

The precisions for clozapine and desmethylclozapine are presented in Table II. Both the intra- and inter-assay coefficients of variation (C.V.) were generally less than 10% except with

TABLE I

SUMMARY OF PATIENT DEMOGRAPHICS, CLOZAPINE (CLZ) AND DESMETHYLCLOZAPINE (DCLZ) PLASMA CONCENTRATIONS

Patient No.	Age (years)	Weight (kg)	Duration of treatment (days)	Dose (mg/day)	Concentration (ng/ml)	
					CLZ	DCLZ
1	27	76	88	800	1302	375
2	31	48	105	600	1132	403
3	27	69	56	500	329	99
4	27	68	15	400	268	87
5	27	95	7	150	167	49
6	43	50	7	150	189	43

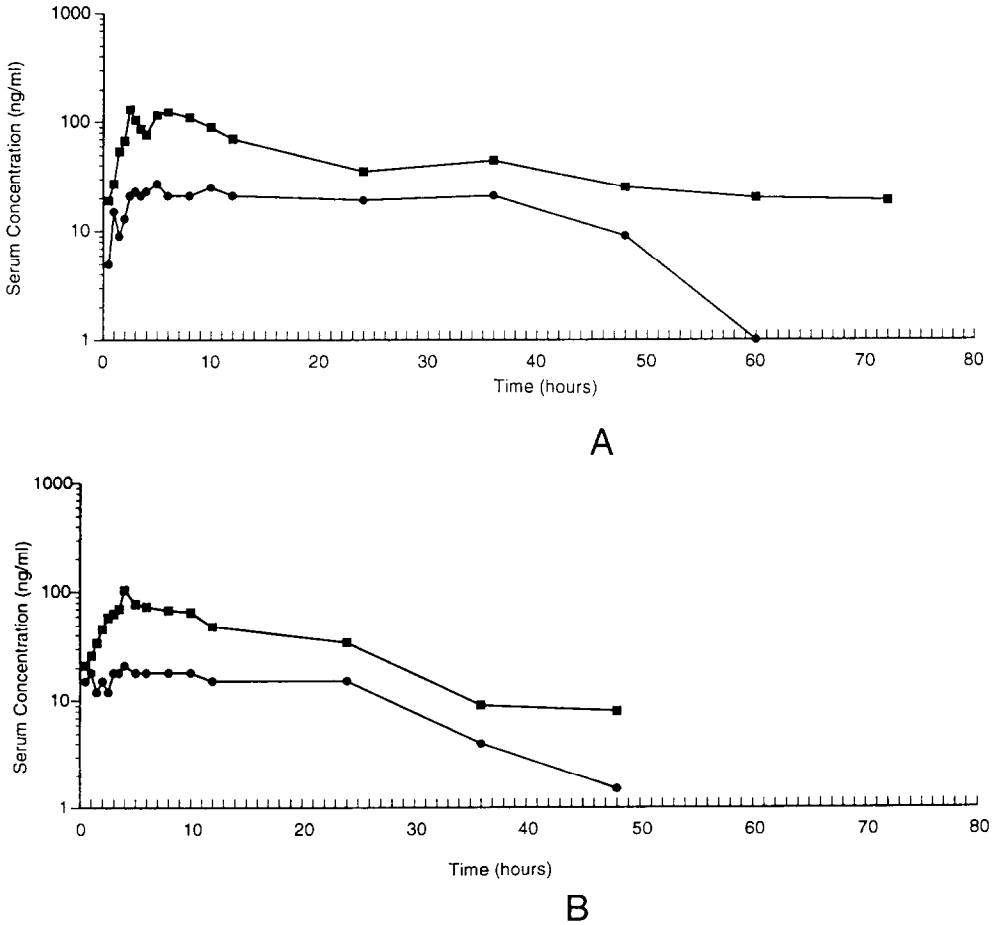

Fig. 2. Plasma level profiles of (■) clozapine and (●) desmethylclozapine in two schizophrenic patients (A and B) receiving a single 100-mg oral dose of clozapine.

TABLE II

PRECISION OF THE ASSAY FOR CLOZAPINE (CLZ) AND DESMETHYLCLOZAPINE (DCLZ)

Concentration (ng/ml)	Intra-assay ($n = 5$)						Inter-assay ($n = 5$)					
	CLZ			DCLZ			CLZ			DCLZ		
	Mean (ng/ml)	S.D. (ng/ml)	C.V. (%)	Mean (ng/ml)	S.D. (ng/ml)	C.V. (%)	Mean (ng/ml)	S.D. (ng/ml)	C.V. (%)	Mean (ng/ml)	S.D. (ng/ml)	C.V. (%)
50	49.6	6.4	12.9	56	6.0	10.7	51.5	7.6	14.7	50.6	4.0	7.9
100	99.3	5.9	5.9	107.1	4.4	4.1	102.0	7.0	6.8	102.8	5.9	5.7
150	169.3	10.2	6.0	172	11.2	6.5	152.2	14.8	9.7	160.7	16.7	10.3
200	198.5	7.7	3.8	201.6	6.6	3.2	197.5	11.4	5.7	196.5	10.3	5.2
300	299.6	14.1	4.8	316.6	42.0	13.2	299.3	15.1	5.1	309.6	28.9	9.3
400	407.0	5.0	1.2	399.6	7.1	1.7	402.1	6.9	1.7	406.5	7.5	1.8

clozapine and desmethylclozapine concentrations of 50 ng/ml. The intra-assay C.V. of 13.2% at a desmethylclozapine concentration of 300 ng/ml is high but comparable to those in previous reports [11]. The intra- and inter-assay C.V.s at 50 ng/ml ranged from 7.9 to 14.7%. These values are also comparable to those for other HPLC assays, with reported overall mean intra- and inter-assay C.V.s of 9.9–13.2% for concentrations between 25 and 400 ng/ml [10,11]. Its importance may not be clinically significant in the routine monitoring of plasma clozapine and desmethylclozapine levels where the typical therapeutic clozapine dose ranges from 300 to 600 mg per day. Plasma concentrations of clozapine and desmethylclozapine will be at sufficient levels to be easily measured as shown in Table I. However, the plasma levels of clozapine and desmethylclozapine can be lower in single-dose pharmacokinetic studies, as shown in Fig. 2, or in clozapine withdrawal studies. A possible reason for the greater variability of clozapine and desmethylclozapine plasma levels at lower concentrations in patients' samples could be the effects of the clozapine N-oxide metabolite. N-Oxide metabolites such as those observed with imipramine and chlorpromazine are converted back to their parent drug [12]. Therefore, any compound which forms an N-oxide metabolite can potentially undergo a reversible metabolic process, thus resulting in a wide variability for clozapine and subsequently desmethylclozapine plasma levels. Unfortunately, the other clozapine metabolite, clozapine N-oxide was not available commercially and was not assayed.

TABLE III

SUMMARY OF THE ABSOLUTE RECOVERIES OF CLOZAPINE AND DESMETHYLCLOZAPINE

Concentration (ng/ml)	Recovery (mean ± S.D., $n = 5$) (%)	
	Clozapine	Desmethylclozapine
50	89.6 ± 2.9	55.8 ± 5.4
150	82.2 ± 6.0	56.0 ± 4.9
300	79.6 ± 10.2	56.6 ± 7.5

The absolute recoveries for clozapine and desmethylclozapine are shown in Table III. The overall recoveries for clozapine and desmethylclozapine were 83.8 and 56.1%, respectively. Retention times determined from the chromatograms included N-desmethylclozapine 7.9 min, clozapine 9.3 min and protriptyline 15.4 min. A sample chromatogram is shown in Fig. 3, where S-100, S-200 and S-400 are parts of the calibration graphs for clozapine and desmethylclozapine at 100, 200 and 400 ng/ml, respectively. The linear regression equations for the calibration graphs were established for clozapine ($y = 0.005 +$

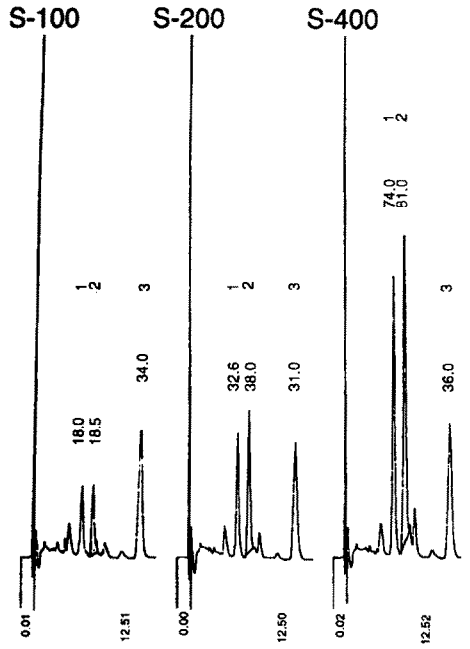

Fig. 3. Chromatograms of clozapine and desmethylclozapine at concentrations of 100, 200 and 400 ng/ml. Peak heights (mm) for (1) desmethylclozapine, (2) clozapine and (3) the 100 ng/ml protriptyline internal standard are reported.

$0.005x$, $r = 0.996$), desmethylclozapine ($y = 0.055 + 0.005x$, $r = 0.999$) and protriptyline ($y = 0.173x - 2.5$, $r = 0.998$) (y = peak-height ratio; x = standard concentration) over the concentration range 50–800 ng/ml ($n = 5$ for each concentration).

Fig. 4 shows a chromatogram of clozapine and desmethylclozapine in plasma from two different schizophrenic patients. In Fig. 4A, the plasma concentrations of clozapine and desmethylclozapine were calculated to be 316 and 217 ng/ml, respectively. Fig. 4B, the peak heights were enlarged four-fold and the calculated clozapine and desmethylclozapine plasma levels were 1.6 and 5.9 ng/ml, respectively. The minimum concentrations that could be determined were 2 ng/ml for clozapine and 1 ng/ml for desmethylclozapine.

Plasma clozapine and desmethylclozapine concentrations from six schizophrenic patients are presented in Table I. The correlation coefficients between dose and plasma concentrations for clozapine and desmethylclozapine were $r = 0.882$ and 0.853, respectively. Desmethylclozapine plasma concentrations are much lower than clozapine plasma levels. Fig. 2A and B show the plasma level profiles for two patients receiving a 100-mg single oral dose of clozapine. Like the plasma clozapine and desmethylclozapine levels observed in Table I, the desmethylclozapine plasma concentrations are much lower than the clozapine plasma levels. The pharmacokinetic profile of clozapine in these patients is similar to those in previously reported kinetic studies [1].

This HPLC method with UV detection offers several advantages over previously published HPLC techniques [9–11]. Haring *et al.* [9] described an HPLC method with UV detection that included a laborious extraction procedure and required 20 ml of blood from the patient. A simplified extraction procedure was later developed that required a smaller sample volume and utilized amperometric detection [10]. However,

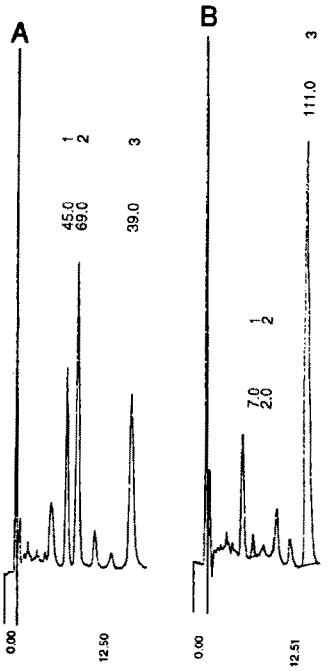

Fig. 4. Chromatograms of (1) desmethylclozapine and (2) clozapine in plasma samples from two schizophrenic patients. 3 = Protriptyline (100 ng/ml). (A) Desmethylclozapine plasma concentration 217 ng/ml and clozapine plasma level 316 ng/ml; (B) enlarged four-fold, showing desmethylclozapine plasma level 5.9 ng/ml and clozapine plasma level 1.6 ng/ml.

neither of those assays could detect the desmethyl metabolite. Lovdahl *et al.* [11] developed an HPLC assay with UV detection that simultaneously measured clozapine and desmethylclozapine. The reported recoveries for clozapine and desmethylclozapine were 84.4 and 28.4%, respectively. The detection limit was reported to be 15 ng/ml for clozapine and 30 ng/ml for desmethylclozapine. Compared with Lovdahl *et al.*'s results [11], the recoveries with the present HPLC assay are similar for clozapine but greater for desmethylclozapine. The detection limit is lower in this study. The inter-assay C.V. for clozapine of 9.7% at 150 ng/ml in this study is comparable to Lovdahl *et al.*'s reported value of 9.9%.

In conclusion, this analysis offers advantages over other HPLC methods utilizing UV detection while determining clozapine and desmethylclozapine simultaneously. This method would allow the conduction of pharmacokinetic studies and therapeutic drug monitoring programmes for clozapine that includes the determination of the desmethyl metabolite.

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REFERENCES

1 M. W. Jann, *Pharmacotherapy*, 11 (1991) 179.
2 H. Y. Meltzer, *Neuropsychopharmacology*, 1 (1988) 193.
3 H. Y. Meltzer, B. Bastani, L. Ramirez and S. Matsubara, *Eur. Arch. Psychiatr. Neurol. Sci.*, 238 (1989) 332.
4 P. Sokoloff, B. Giros, M. P. Martes, M. L. Bouthenet and J. C. Schwartz, *Nature*, 347 (1990) 146.
5 H. H. M. VanTol, J. E. Bunzow, H. C. Guan, R. K. Sunahara, P. Seeman, H. B. Niznik and O. Civelli, *Nature*, 350 (1991) 610.
6 R. K. Sunahara, H. C. Guan, B. F. O'Dowd, P. Seeman, L. G. Laurier, G. Ng, S. R. George, J. Torchia, H. H. M. VanTol and H. B. Niznik, *Nature*, 350 (1991) 615.
7 K. Richter, *J. Chromatogr.*, 434 (1988) 465.
8 U. Bondesson and L. H. Lindstrom, *Psychopharmacology*, 95 (1988) 472.
9 C. Haring, C. Humpel, B. Auer, A. Saria, C. Barnas, W. Fleischhacker and H. Hinterhuber, *J. Chromatogr.*, 428 (1988) 160.
10 C. Humpel, C. Haring and A. Saria, *J. Chromatogr.*, 491 (1989) 235.
11 M. Lovdahl, P. J. Perry and D. D. Miller, *Ther. Drug Monit.*, 13 (1991) 69.
12 M. H. Bickel, *Pharmacol. Rev.*, 21 (1969) 325.