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Determination of clozapine and its metabolite, N-desmethylclozapine, in serum microsamples by high-performance liquid chromatography and its application to pharmacokinetics in rats

Fang Ma, Chyan E. Lau*

Department of Psychology, Rutgers University, New Brunswick, NJ, USA

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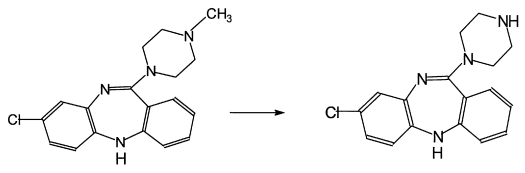
Abstract

A single solvent extraction step high-performance liquid chromatographic method is described for quantitating clozapine and its metabolite, N-desmethylclozapine, in rat serum microsamples (50 μl). The separation used a 2.1-mm I.D. reversed-phase Symmetry C_{18} column with an isocratic mobile phase consisting of methanol–acetonitrile–28.6 m*M* sodium acetate buffer, pH 2.6 (10:20:70, v/v/v). The detection limit was 2.5 ng/ml for all the compounds using an ultraviolet detector operated at 230 nm. The method was used to study the pharmacokinetics of clozapine after an intravenous bolus dose (2.5 mg/kg).

Keywords: Clozapine; N-Desmethylclozapine

1. Introduction

Clozapine (Fig. 1), a piperidine derivative of the dibenzodiazepine family, is an atypical antipsychotic agent with proven efficacy in the management of refractory schizophrenia [1]. Clozapine is metabolized to N-desmethylclozapine and clozapine-N-oxide in humans [2,3]. N-Desmethylclozapine is found to be pharmacologically active [4]. The other metabolite, clozapine-N-oxide is pharmacologically inactive and is present mainly in patients' urine samples [3,4]. While serum clozapine monitoring is often done to maximize treatment effectiveness in humans, the serum clozapine concentration–time profile is seldom examined in research on animals



Clozapine N-Desmethylclozapine

Fig. 1. Chemical structures of clozapine and N-desmethylclozapine.

*Corresponding author. Department of Psychology, Rutgers, The State University of NJ, 152 Frelinghuysen Road, Piscataway, NJ 08854-0820 USA.

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despite the fact that brain neurochemistry and behavioral effects have been addressed [5–7].

A number of gas chromatographic and high-performance liquid chromatographic (HPLC) methods have been described for clozapine and its metabolites in serum or plasma [3,8–21]. The aim of the present study was to develop a rapid and sensitive microsample (50 μl) HPLC method for the determination of the concentration–time profile of clozapine and its active metabolite, N-desmethylclozapine, in rats following a moderate, nontoxic, clozapine dose. No attempt was made to analyze clozapine-N-oxide concentrations because a negligible amount of this metabolite was found in rat serum samples even after i.v. 20 mg/kg clozapine administration [11]. Sample size is critical when the animal species used is small, especially when repeated blood sampling is necessary to trace the temporal changes in drug levels in individual animals. The convenience of our method is facilitated by its use of a single solvent extraction procedure and the commercially available 2-mm I.D. column. An added advantage of using the 2-mm I.D. column is a reduction in solvent consumption by up to 80%, compared to that of the 4.6-mm I.D. column. This method is hereby applied to evaluate the pharmacokinetics of clozapine after a single i.v. bolus dose.

2. Experimental

2.1. Instrumentation

The HPLC system consisted of a Perkin Elmer 200 LC pump coupled to an autosampler ISS-200 (Norwalk, CT, USA), and a 785A programmable absorbance UV detector with a detector cell volume of 12 μl, operated at 230 nm (Applied Biosystems Instruments, Foster City, CA, USA). The separation was performed at room temperature on a Symmetry C_{18} column, 150×2.1 mm I.D., 5 μm particle size (Waters Associates, Milford, MA, USA) with a 2-μm Rheodyne precolumn filter (Cotati, CA, USA). The data were collected using a PE Nelson 900 series interface, TURBOCHROM 4.1 software (Perkin Elmer) and an IBM-type pentium microcomputer workstation.

2.2. Reagents and standards

Clozapine was obtained from Sandoz (E. Hanover, NJ, USA). N-Desmethylclozapine was purchased from Sigma (St. Louis, MO, USA). α-Hydroxymidazolam was supplied by Hoffmann-La Roche (Nutley, NJ, USA). HPLC-grade methanol, acetonitrile, chloroform and sodium acetate were purchased from Fisher Scientific (Springfield, NJ, USA). The 1 *M* borate–sodium carbonate–potassium chloride buffer (pH 9.0) was prepared by the method of de Silva and Puglisi [22]. All other chemicals were reagent grade.

Clozapine, N-desmethylclozapine, and α-hydroxymidazolam were dissolved in methanol individually to make 1 mg/ml stock base solution. Dilutions of the 1 mg/ml standards, clozapine and N-desmethylclozapine, were used to make the working standards (25, 50, 100, 250, 500 and 1000 ng/ml) containing the two compounds. The internal standard, α-hydroxymidazolam, was diluted and used at concentration of 1.5 μg/ml.

The HPLC analyses were performed using an isocratic mobile phase consisting of methanol–acetonitrile–28.6 m*M* sodium acetate buffer (adjusted to pH 2.6 with 40% phosphoric acid), (10:20:70, v/v/v). Mobile phases were degassed and filtered through a solvent filtration apparatus (Alltech Associates, Deerfield, IL, USA). The flow-rate was set at 0.3 ml/min and normally operated at a pressure of 104 bar (1500 p.s.i.).

2.3. Sample preparation

Standards and serum samples were prepared as previously described [23,24]. Briefly, a 25-μl volume of the internal standard (α-hydroxymidazolam, 1.5 μg/ml) and 50 μl working serum standard were added to a 15-ml conical centrifuge tube. Borate buffer (1 *M*, pH 9.0, 100 μl) was added and the solution was mixed well. A 1 ml volume of chloroform was added and the sample mixture was vortex-mixed for 1 min and centrifuged for 5 min at 1100 *g*. The 1.15-ml sample mixture rose to 2 cm below the rim of the 15-ml conical centrifuge tube during vortex mixing, a procedure which ensured vigorous mixing for the extraction of alkalized clozapine and N-desmethylclozapine to the organic solvent. The

organic layer was carefully transferred to a 5-ml conical centrifuge tube and evaporated to dryness in an evaporator (Pierce, Rockford, IL, USA) at 40°C under nitrogen. The residue was resuspended in 50 μl of the mobile phase, and 20 μl was injected onto the column by the autosampler. Samples for serum drug analysis were prepared identically except that standards were not added.

2.4. Extraction recovery

The assay recoveries of clozapine and N-desmethylclozapine were assessed at concentrations of 50, 100, 250, 500 and 1000 ng/ml. Six replicates of each concentration, containing the two compounds, were extracted according to the method described above. Six replicates of each concentration were computed using the following equation:

Recovery = (peak height extract)/
(mean peak height direct injection) × 100%

2.5. Clozapine administration and blood sampling

One male, albino, virus-free Sprague-Dawley rat from HSD (Indianapolis, IN, USA), held to 80% of its normal, adult starting weight, 382 g, was used. Right jugular vein cannulation and blood sampling have been described previously [25,26]. The catheter was flushed with 0.9% saline containing 30 units of heparin/ml and sealed with fishing line when not in use.

Clozapine, 2.5 mg, was dissolved in 25 μl of 1.2 *M* HCl and was further diluted to working concentration with 0.9% NaCl solution. The animal was allowed to recover for 2 days and then received an i.v. bolus 2.5 mg/kg clozapine. Injection was given in a volume of 1 ml/kg. Blood samples (100 μl) from the jugular catheter were collected at 5, 10, 15, 20, 30, 45, 60, 90 and 120 min and were centrifuged for 10 min at 13 700 *g* and stored frozen until analysis. Experiments were executed in accordance with the guide for the care and use of laboratory animals (National Institute of Health Publ. No. 85-23, revised 1985).

2.6. Pharmacokinetic analysis

Pharmacokinetic analysis was performed using SAAM II [27]. The data were described by an open two-compartment model for clozapine and fit to the following equation:

$$C_p = Ae^{\alpha t} + Be^{\beta t}$$

where, C_p is the total serum drug concentration at time t, the terms A and B are the extrapolated zero intercepts, and α and β represent the apparent first-order distribution and elimination rate constants, respectively. The halflife ($t_{1/2}$) for the distribution or elimination phase and volume of distribution for the central compartment (V_c) were calculated by the following equations: $t_{1/2} = 0.693/\alpha$ or β and $V_c = \text{Dose}/(A+B)$. The area under the serum drug concentration–time curve ($AUC_{0-\infty}$) was calculated by the following equation: $AUC_{0-\infty} = A/\alpha + B/\beta$. Total clearance (Cl) was then defined as $\text{Dose}/AUC_{0-\infty}$.

3. Results and discussion

3.1. Method evaluation

Fig. 2 shows chromatograms of a serum blank containing no interfering peaks, a spiked serum sample containing a working standard (500 ng/ml) which was extracted by liquid–liquid extraction, and a representative rat serum sample (50 μl) obtained 10 min following i.v. 2.5 mg/kg clozapine administration.

The internal standard method was used in the calibration and evaluation of the unknown samples. Table 1 shows the within-day and between-day precisions and accuracy of clozapine and N-desmethylclozapine, which were established at five different concentrations (50, 100, 250, 500 and 1000 ng/ml) by the addition of these two compounds to blank serum. Both within-day and between-day precisions for clozapine were high as indicated by the coefficients of variation (C.V.s), which ranged from 2.69% to 7.31% and from 3.02% to 7.58%, respectively; whereas these values for N-desmethylclozapine ranged from 2.46% to 13.55% and from 6.15% to 14.87%, respectively. The precision and accuracy of the present method at the limit of quantification are within the recommended value of

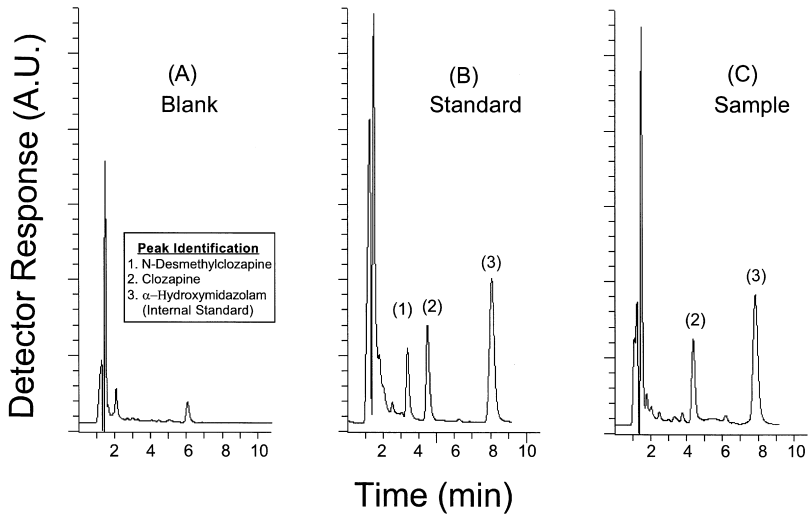

Fig. 2. Chromatograms of (A) serum blank, (B) serum containing 500 ng/ml N-desmethylclozapine, clozapine, and α-hydroxymidazolam, and (C) a 50-μl rat serum sample obtained 10 min after i.v. 2.5 mg/kg clozapine administration.

±20% for a valid analytical method used in bioavailability and pharmacokinetic studies [28].

Calibration curves for clozapine and N-desmethylclozapine are linear within the ranges (25, 50, 100, 250, 500 and 1000 ng/ml) examined. For each of the six regression lines, the correlation coefficients are 0.9993 and 0.9986 for clozapine and N-desmethylclozapine, respectively. The C.V.s of the slopes ($n=6$) of the regression lines ranged from 6.99 to 7.28 with intercepts all close to zero (Table 2). The detection limit was 2.5 ng/ml for the two compounds.

The extraction recoveries (mean±S.D.) for clozapine and at the five concentrations (50, 100, 250, 500 and 1000 ng/ml) were in the range 68.17–83.56%, whereas those for N-desmethylclozapine were markedly lower, 46.23–57.53% (Table 3). It has been reported that solid-phase extraction attained higher recoveries for clozapine [8,11,12,18,21] and N-desmethylclozapine [18,21]. Although recoveries

Table 1
Precision and accuracy data for clozapine and N-desmethylclozapine in serum

Compound	Within-day ($n=6$)		Accuracy (%)	Between-day ($n=5$)		Accuracy (%)
	Concentration (mean±S.D.) (ng/ml)	C.V. (%)		Concentration (mean±S.D.) (ng/ml)	C.V. (%)	
Clozapine	49.97±3.65	7.31	99.94	50.31±3.69	7.34	100.62
	100.24±5.67	5.66	100.24	101.54±3.06	3.02	101.54
	250.46±14.06	5.62	100.18	255.39±19.35	7.58	102.16
	498.97±13.42	2.69	99.79	510.82±35.70	6.99	102.16
	981.84±31.75	3.23	98.18	1025.56±40.64	3.96	102.56
N-Desmethylclozapine	50.23±1.89	3.75	100.46	47.86±7.12	14.87	95.73
	99.89±13.53	13.55	99.89	104.14±13.92	13.37	104.14
	256.23±24.32	9.49	102.49	247.80±17.63	7.11	99.12
	509.76±36.80	7.22	101.95	489.14±30.09	6.15	97.83
	995.75±24.53	2.46	99.58	1064.54±102.28	9.61	106.45

Table 2
Mean of calibration equations for clozapine and N-desmethylclozapine over the concentration range 25–1000 ng/ml

Compound	Equation	Correlation coefficient	C.V. of slope (%)
Clozapine	$y=0.0024(\pm0.0002)x-0.0444(\pm0.0130)$	0.9993	6.99
N-Desmethylclozapine	$y=0.0012(\pm0.0001)x-0.0330(\pm0.0037)$	0.9986	7.28

y = Ratio of peak height of clozapine or N-desmethylclozapine over that of internal standard, x = concentration of each compound.

were moderate for clozapine and somewhat low for N-desmethylclozapine in the present study, these values were consonant with those found with other HPLC methods [10,14,20]. However, the primary aim of the present study was to demonstrate the capability of this method for the determination of concentrations across time for clozapine and its metabolite, N-desmethylclozapine, in rat serum microsamples by using a 2.1-mm microbore column despite the relative lower recoveries observed for these agents.

3.2. Clozapine pharmacokinetics

Fig. 3 shows the serum clozapine and its metabolite, N-desmethylclozapine, concentration–time profiles after i.v. 2.5 mg/kg clozapine administration for the rat. The concentrations of N-desmethylclozapine were <80 ng/ml during the formation of the metabolite (first 30 min) and progressively decreased to 2.5 ng/ml for the duration of the blood sampling. The low concentration profile of N-desmethylclozapine found in the present study corresponded to the results reported for rats after i.v. 20 mg/kg clozapine administration [11]. V_c and clearance for clozapine were 1.28 l/kg and 5.1 l/h/kg, respectively. The distribution and terminal elimination halflives for clozapine were 2.2 and 41.9 min, respectively. This study is the first to determine clozapine phar-

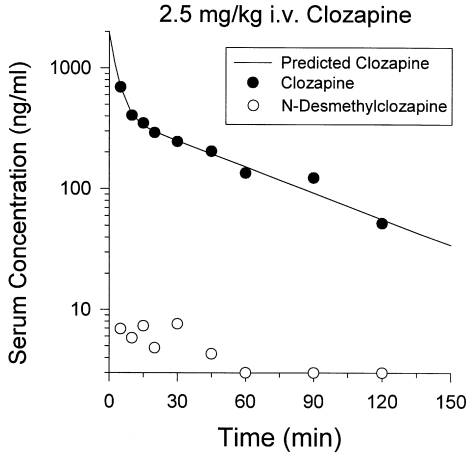

Fig. 3. Serum clozapine and N-desmethylclozapine concentration–time profiles after i.v. bolus 2.5 mg/kg clozapine administration for the rat.

Table 3
Recovery of clozapine and N-desmethylclozapine

Compound	Concentration (ng/ml)	Recovery (mean±S.D.)(%)	C.V. (%)
Clozapine	50	77.67±5.54	7.13
	100	68.17±6.04	8.86
	250	70.52±6.36	9.01
	500	73.49±5.24	7.17
	1000	83.56±5.87	7.03
N-Desmethylclozapine	50	57.53±7.90	13.73
	100	53.84±4.44	8.24
	250	46.23±4.56	9.87
	500	49.92±0.99	1.99
	1000	48.88±3.26	6.66

macokinetic parameters with the use of pharmacokinetic modeling and a repeated blood sampling to trace the temporal changes in drug levels in a rat. Consequently, comparison could not be made with other studies; however, a terminal elimination halflife of 1.5–1.6 h was reported after i.p. 10 mg/kg clozapine administration [29]. Nevertheless, clozapine is much shorter-lived in rats than in humans ($t_{1/2}$, 10.3–15.8 h) [30,31]. Similar species differences in halflives have been found for benzodiazepines, e.g., flurazepam [32].

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