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Determination of clozapine, and its metabolites, *N*-desmethylclozapine and clozapine N-oxide in dog plasma using high-performance liquid chromatography

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Abstract

Clozapine and its two major metabolites, *N*-desmethylclozapine and clozapine N-oxide were quantified using a high-performance liquid chromatographic method with UV detection in dog plasma following a single dose of clozapine. The analysis was performed on a 5-μm Hypersil CN (CPS-1; 250×4.6 mm) column. The mobile phase consisted of acetonitrile–water–1 *M* ammonium acetate (50:49:1, v/v/v), which was adjusted to pH 5.0 with acetic acid. The detection wavelength was 254 nm. A liquid–liquid extraction technique was used to extract clozapine and its metabolites from dog plasma. The recovery rates for clozapine, *N*-desmethylclozapine, and the internal standard (I.S.) were close to 100% using this method. The recovery rate for clozapine N-oxide (62–66%) was lower as expected because it is more polar. The quantitation limits for clozapine, clozapine N-oxide, and *N*-desmethylclozapine were 0.11, 0.05 and 0.05 μ*M*, respectively. Intra-day reproducibility for concentrations of 0.1, 1.0 and 5.0 μ*M* were 10.0, 4.4 and 4.2%, respectively, for N-oxide; 11.2, 4.3 and 4.9%, respectively, for *N*-desmethylclozapine; and 10.8, 2.2 and 4.9%, respectively, for clozapine. Inter-day reproducibility was <15% for clozapine N-oxide, <8% for *N*-desmethylclozapine and <19% for clozapine. This simple method was applied to determine the plasma concentration profiles of clozapine, *N*-desmethylclozapine and clozapine N-oxide in dog following administration of a 10 mg/kg oral dose of clozapine.

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

1. Introduction

Clozapine, an ''atypical'' antipsychotic drug, antagonizes 5-$HT_{2A/2C}$ and has a low affinity for D_2 receptors. This leads to reduced incidence of extrapyramidal side effects and greater efficacy for reducing negative symptoms of schizophrenia compared to the ''typical'' antipsychotic drugs [1,2]. While several metabolites of clozapine have been previously identified [3], clozapine's two primary metabolites are *N*-desmethylclozapine and clozapine N-oxide [4,5]. One third of clozapine N-oxide can be converted back to clozapine in schizophrenic patients. This reversible metabolism of N-oxide to clozapine may partially account for the wide interpatient variability seen in clozapine plasma concentrations

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[6]. Monitoring of therapeutic plasma levels of antipsychotics may be necessary because of their narrow therapeutic windows. It is also necessary to monitor metabolite levels because metabolites of antipsychotics often have different pharmacokinetic properties and pharmacological properties than the parent drug, which can enhance therapeutic effect or lead to harmful side effects [7,8].

Clozapine at doses that are well tolerated in schizophrenic patients may cause adverse side effects (e.g. syncope, cardiac arrest) in healthy volunteers due to differences in tolerability, even only after a single oral dose of clozapine [9]. Because of the risk of developing agranulocytosis [10], only those schizophrenic patients that are treatment-resistance, intolerant to other neuroleptic drugs or suffering from severe extra-pyramidal side effects or tardive dyskinesia are considered for clozapine treatment [11–14]. This criterion narrows the pool of schizophrenics available for participation in a clozapine study. Thus, dogs will be used to conduct detailed studies on the pharmacokinetics and drug interactions involving clozapine. The metabolic breakdown of clozapine is similar in dogs and humans. For example, the amount of N-oxide present in the urine is approximately twice the amount of clozapine in both humans and dogs. Whereas, the amount of the *N*-desmethylclozapine present in human urine is approximately the same as clozapine levels, the quantity of *N*-desmethylclozapine present in dog urine is substantially less [15].

HPLC is frequently used in therapeutic drug monitoring and pharmacokinetic studies and has been used to investigate the relationship between plasma concentrations of clozapine and its metabolites and therapeutic response [16], the influence of gender on steady-state plasma levels of clozapine [17], the effects of clozapine overdose [18] and identification of individual cytochrome P450 enzymes involved in clozapine metabolism [19].

HPLC methods that are available to determine the concentration of clozapine and its metabolites in plasma are often laborious [20–27]. The objective for developing this method was to have a simple and accurate means available to quantify the concentration of clozapine and its metabolites in dog plasma.

2. Experimental

2.1. Chemicals

Solvents used for extractions and for the preparation of mobile phase were of HPLC grade (BDH Toronto, Ontario, Canada). Clozapine, *N*-desmethylclozapine, and clozapine N-oxide were purchased from Sigma–Aldrich (Ontario, Canada). Loxapine was obtained from Research Biochemicals International (Natick, MA, USA). All chemicals purchased were of the highest grade commercially available.

2.2. Animal

The female beagle, 8 years of age, obtained from Marshall Farms, New York, was housed in a hutch with indoor/outdoor dog runs during the summer months (Animal Care Unit, Western College of Veterinary Medicine, Saskatoon, Saskatchewan, Canada). The animal was normally fed twice, morning and afternoon (Science Diet, canine maintenance) and had fresh water available at all times. Blood was collected from the jugular veins for the intensive bleeds (up to 24 h) and from the cephalic vein in leg for the remaining samples (48 and 72 h).

2.3. Extraction procedure (standards)

The procedure involved transferring 270 μl of human plasma into a glass culture tube (100×16 mm). A 100-μl volume of saturated Na_2CO_3, 20 μl of loxapine and 20 μl of clozapine/clozapine N-oxide/*N*-desmethylclozapine mixture were added to each test tube. Each tube was shaken to enhance mixing. After the addition of 4 ml of butenol–2:cyclohexane (15:85, v/v), the tubes were shaken for 5 min with a vortex (IKA-Vibrax-VXR) at 1800 rpm and then centrifuged (Beckman AccuspinFR) for 2 min at 2000 rpm. The organic layer from each test tube was manually pipetted to another test tube containing 150 μl 0.1 *M* HCl and then vortexed and centrifuged as previously described. The organic phase was discarded, whereas the acidic aqueous phase containing the drugs was used for HPLC analysis.

2.4. Instrument analysis

Chromatography was performed using a Waters HPLC system consisting of a Model 510 pump, a WISP 710B autosampler and a 2487 Dual λ absorbance detector set at 254 nm. Signals from the UV detector were collected and processed by a Waters system interface module and Maxima 810 Baseline chromatography workstation. The analytical column was a Hypersil CN column (CPS-1 5 μm, 250×4.6 mm). A SecurityGuard™ guard column (CN, 4×3.0 mm) was used between the injector and the analytical column. The injection volume was 50 μl. The mobile phase consisted of acetonitrile (50%), double distilled water (49%) and ammonium acetate (1%, 1 M). The mobile phase was adjusted to pH 5.0 with acetic acid (pH meter radiometer Copenhagen PHM 62, Bach-Simpson, London, Ont. Canada). The flow-rate was 1.0 ml min^{-1}.

2.5. Blood sample collection

The bleeding schedule consisted of a prebleed before the capsule was administered, followed by blood being taken at the following intervals (after the capsule was taken): 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 48 and 72 h. The dog fasted the night before the experiment. A single dose of clozapine (10 mg/kg) was given to the dog in a capsule.

2.6. Sample preparation

Collected blood samples were centrifuged (Beckman AccuspinFR) for 20 min at 2000 rpm to separate the plasma from the red blood cells. The plasma was manually pipetted to appropriately labeled Eppendorf centrifuge vials. The bulk of the samples were stored at −20 °C until all the samples were collected.

2.7. Extraction procedure (dog plasma)

Samples from the same experiment were extracted together on the same day. The plasma samples were assayed for levels of clozapine and its metabolites within 1 week of collection. The procedure involved transferring 270 μl of dog plasma into a glass culture tube (100×16 mm). A 100-μl volume of saturated Na_2CO_3 and 20 μl of loxapine was added to each test tube. This mixture was then extracted and analyzed as described above.

3. Results

3.1. Chromatography

A chromatogram of pure standards of clozapine, clozapine N-oxide, *N*-desmethylclozapine and loxapine in 0.1 *M* HCl is shown in Fig. 1A. The peaks show good separation.

Fig. 1B is a chromatogram of extracted dog plasma 2 h after the administration of clozapine (10 mg/kg). The compounds are well resolved and there is no interference from biological impurity. The peaks are symmetrical except for clozapine N-oxide which has a slight tail. A chromatogram of a blank plasma is shown in Fig. 1C.

3.2. Recovery and linearity

Plasma samples containing two different concentrations of clozapine, *N*-desmethylclozapine, clozapine N-oxide, and an internal standard (I.S.) loxapine were extracted according to the procedure described above, prior to HPLC analysis. The percent recovery for each drug was determined by comparing peak heights of each drug in the extracted samples with those obtained from direct injection of the nonextracted (standard) drugs. The average percent recovery was calculated for each compound (see Table 1). The recoveries of clozapine, *N*-desmethylclozapine and loxapine were around 100%. Clozapine N-oxide recoveries were 62 and 66% for concentrations of 0.069 and 0.69 μ*M*, respectively. The lower recovery rate for clozapine N-oxide was expected because it is more polar than the other compounds.

Other published methods for extraction of clozapine and its metabolites use ethyl acetate as a solvent [17,22,23,28]. We have found that some brands of ethyl acetate had components that can oxidize clozapine to clozapine N-oxide and can

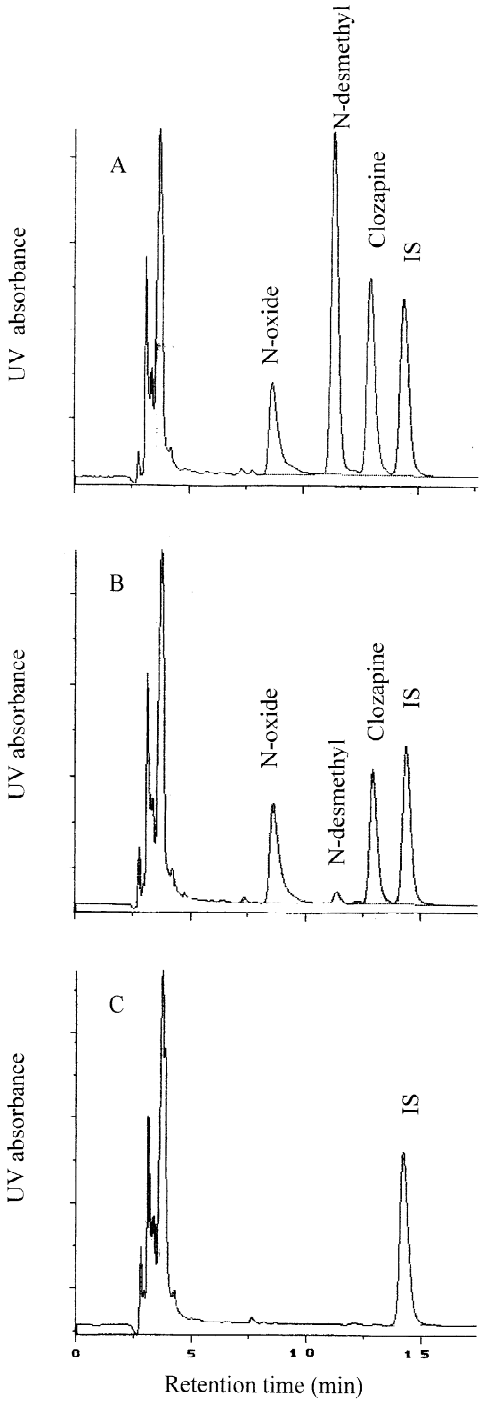

Fig. 1. (A) Chromatogram of clozapine, clozapine N-oxide, *N*-desmethylclozapine and loxapine solution, dissolved in 0.1 *M* HCl and injected without extraction. (B) Chromatogram of dog plasma (2 h) after extraction. (C) Chromatogram obtained from the analysis of blank plasma (0 h) after extraction.

Table 1
Percent recovery for clozapine N-oxide, *N*-desmethylclozapine, clozapine and loxapine (I.S.)

Drug	Conc. (μ*M*)	Mean	SD
Clozapine N-oxide	0.069	62.0	2.2
	0.69	66.3	5.8
N-Desmethylclozapine	0.069	102.3	6.9
	0.69	101.7	3.1
Clozapine	0.069	108.3	6.1
	0.69	107.7	10.2
I.S.	0.069	110.7	7.1
	0.69	104.7	9.7

Data are mean±SD from three independent experiments based on peak height ratio.

result in errors in the assay. Thus, our method avoided the use of ethyl acetate as a solvent.

A standard calibration curve ($n=6$), ranging from 0.05 to 1.72 μ*M*, was constructed. The peak height of clozapine and each of its metabolites were divided by the peak height of loxapine (internal standard) to attain a peak height ratio. The standard curves were analyzed using linear regression. The average correlation coefficients for clozapine, clozapine N-oxide and *N*-desmethylclozapine were 0.996, 0.998 and 0.997, respectively.

Table 2
Intra-day reproducibility

Conc. (μ*M*)	Mean (peak height ratio)	SD	Error (%)
Clozapine N-oxide			
0.1	0.35	0.04	10.0
1.0	2.08	0.09	4.4
5.0	26.91	1.12	4.2
N-Desmethylclozapine			
0.1	1.01	0.11	11.2
1.0	5.37	0.23	4.3
5.0	67.56	3.30	4.9
Clozapine			
0.1	0.73	0.08	10.8
1.0	4.48	0.10	2.2
5.0	59.03	2.90	4.9

Data taken from seven samples prepared on the same day using peak height ratio were used to determine intra-day reproducibility.

Table 3
Inter-day reproducibility

Conc. (μ*M*)	Mean	SD	Error (%)
Clozapine N-oxide			
1.72	0.543	0.032	4.1
0.86	0.274	0.035	9.1
0.43	0.125	0.013	7.8
0.22	0.064	0.003	5.5
0.11	0.024	0.001	4.1
0.05	0.013	0.001	14.1
N-Desmethylclozapine			
1.72	2.061	0.155	0.1
0.86	1.074	0.070	2.4
0.43	0.457	0.034	7.6
0.22	0.193	0.002	7.1
0.11	0.065	0.003	7.4
0.05	0.023	0.002	7.4
Clozapine			
1.72	1.208	0.091	0.7
0.86	0.553	0.029	3.8
0.43	0.165	0.014	8.3
0.22	0.053	0.010	16.3
0.11	0.013	0.003	18.3
0.05	0.005	0.002	40.3

Data based on peak height ratio was taken from samples collected on 3 separate days.

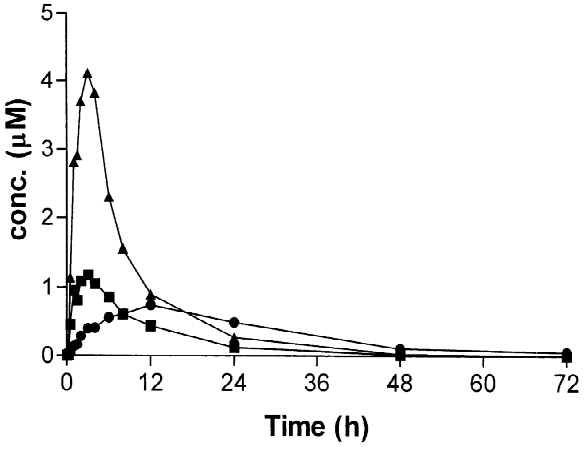

Fig. 2. Plasma concentration profile of clozapine and its metabolites clozapine N-oxide and *N*-desmethylclozapine following a single dose of clozapine (10 mg/kg) in a dog. Legend: circles, *N*-desmethylclozapine, triangles, clozapine N-oxide, and squares, clozapine.

3.3. Reproducibility

Clozapine and its metabolites were stable in human plasma for at least 3 days (data not shown). The literature indicates that clozapine in suspension remains stable for up to 18 days [29]. Intra-day reproducibilities for clozapine and each of its metabolites were <12% at a concentration of 0.1 μ*M* and <5% at concentrations of 1.0 and 5.0 μ*M* (see Table 2).

Data taken from samples prepared on 3 different days was used to determine inter-day reproducibility (see Table 3). Clozapine, *N*-desmethylclozapine and clozapine N-oxide were analyzed at concentrations ranging from 0.05 to 1.72 μ*M*. The quantitation limits for clozapine, clozapine N-oxide and *N*-desmethylclozapine were 0.11, 0.05 and 0.05 μ*M*, respectively.

3.4. Pharmacokinetics of a single dose of clozapine

The concentrations of clozapine, clozapine N-oxide and *N*-desmethylclozapine in the dog plasma were determined. Samples from each time point were analyzed in duplicate and the average concentration was used to plot the graph (see Fig. 2).

4. Discussion

The present report describes a sensitive and simple HPLC assay for the determination of clozapine and its two main metabolites in dog plasma. Reproducibility for this method was good. Intra-day reproducibility was within acceptable ranges (clozapine: 2.2–10.8%; clozapine N-oxide: 4.2–10.0%; *N*-desmethylclozapine: 4.3–11.2%). Inter-day reproducibility was <15% for clozapine N-oxide and <8% for *N*-desmethylclozapine for concentrations ranging from 0.05 to 1.72 μ*M* and <19% for clozapine for concentrations ranging from 0.05 to 1.72 μ*M*.

This method resulted in total recoveries of clozapine and its metabolite *N*-desmethylclozapine (recovery above 100% is due most likely to experimental error). Commonly used extraction meth-

ods for clozapine and its metabolites include solid phase extraction [21,24,27], and liquid-liquid extraction with a back extraction step [22,25,28, 30,31]. Reported recovery rates range between 72–97% for clozapine, 37–96% for desmethylclozapine, and 43–92% for clozapine N-oxide.

In conclusion, this simple method used to quantify clozapine and its two major metabolites, has good reproducibility, sensitivity, and results in good recovery rates. It would be useful in therapeutic drug monitoring and pharmacokinetic studies of clozapine and its metabolites.

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