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Short Communication

Determination of clozapine and its major metabolites in human serum and rat plasma by liquid chromatography using solid-phase extraction and ultraviolet detection

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Introduction

Clozapine (8-chloro-11-(4-methyl-1-piperazinyl)-5*H*-dibenzo)[b,e]-[1,4]diazepine (Fig. 1) is an atypical neuroleptic drug which has been used extensively for the treatment of schizophrenic disorders [1, 2]. Clozapine (CLP) is clinically regarded as an atypical antipsychotic agent because of its low propensity to produce extrapyramidal effects and its lack of stimulatory effects on prolactin secretion. The major routes of metabolism are *N*-demethylation and *N*-oxidation to yield the *N*-desmethylclozapine (NCLP) and clozapine-*N*-oxide (OCLP; Fig. 1) [3]. Progress in understanding the pharmacokinetics and pharmacodynamics of CLP and its major metabolites has been hindered by lack of a simple and precise method of analysis. Analytical techniques that have been used to measure CLP and/or its metabolites include: GLC [4], GC [5], GC–MS [6], TLC [7], RIA [8] and HPLC [9–16]. The majority of these methods [4, 5, 8, 11–13] assayed only CLP while others assayed CLP and one metabolite [6, 9, 14, 15]. The TLC technique [7] assayed CLP and the two metabolites but required large sample volumes (3–6 ml) because of its low sensitivity. There are two LC methods that reported the ability to assay CLP and its metabolites. However, one of these methods [10] did not report data for the metabolites while the second method [16], which used automated solid-phase extraction, required a comparatively large volume of the sample (1 ml) and, therefore, cannot be applied to animal studies. The objective of this investigation was to develop a rapid, simple and sensitive method for the simultaneous determination of clozapine and its major metabolites in small volumes of human serum or rat plasma.

Experimental

Chemicals

Methanol and acetonitrile were HPLC grade (J.T. Baker Inc., Phillipsburg, NJ, USA). All other reagents were analytical reagent grade. Liquid chromatography quality water was obtained by passing distilled water through a Millipore-Q Water System purification unit. CLP, NCLP and OCLP were gifts from Sandoz Research Industries (East Hanover, NJ) while *N*-methylspiperone (IS) was obtained from Dr Robert Dannals (Division of Nuclear Medicine, Johns Hopkins Medical School, Baltimore, MD, USA).

Equipment

Isocratic chromatography was performed using Waters Model 6000A solvent delivery system, Kratos Spectraflow 783 detector set at

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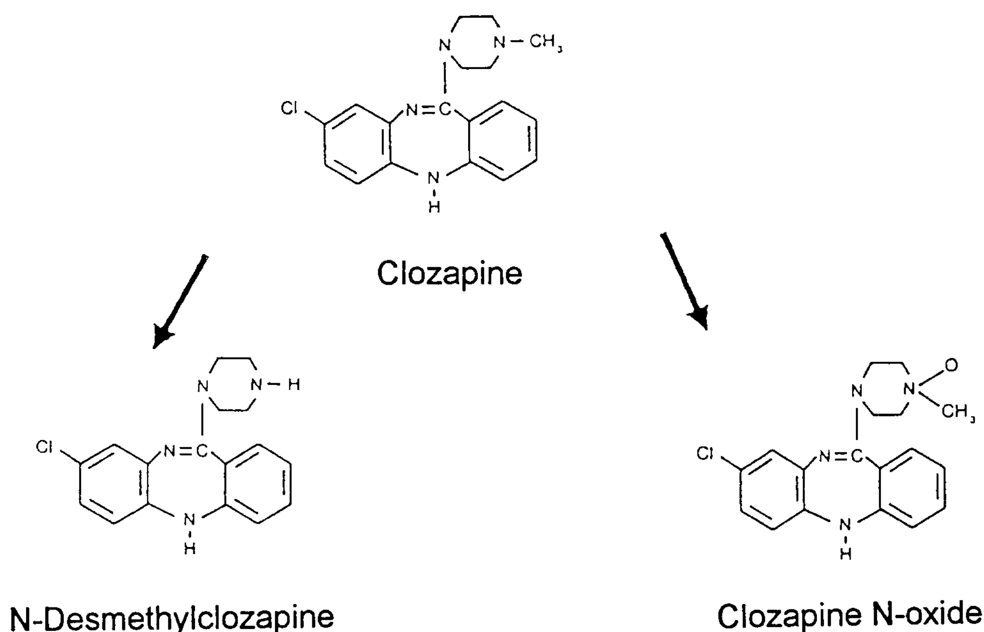

Figure 1
Structures of clozapine and its major metabolites.

254 nm, Waters Intelligence Sample Processor 712 and a Hewlett–Packard 3392A Integrator. Separation was accomplished using a μBondapak C18 column (300 × 3.9 mm i.d.) obtained from Phenomenex with a Waters C18 Guard Column (50 mm) and a flow rate of 1 ml min^{-1}.

Reagents

The mobile phase buffer was prepared by dissolving 7.8 g of dibasic potassium phosphate in 500 ml of water. The pH was adjusted to pH 4.0 with phosphoric acid and the volume was made up to 1 l with water (0.045 M). Acetonitrile was then mixed with the buffer (360:640, v/v) adjusted to pH 3.7 with 10% phosphoric acid, filtered (0.45 μm filter) and degassed before use.

The conditioning buffer for the solid-phase extraction was prepared by dissolving 7.8 g of dibasic potassium phosphate in water and the volume was made up to 1 l (pH 9.0, 0.045 M). The wash solution was prepared by diluting 28 ml of acetonitrile to 100 ml with the buffer. The eluting solution was prepared by diluting 0.7 ml of triethylamine to 100 ml with methanol (50 mM).

Stock solutions of drugs and internal standard (CLP, OCLP, NCLP and IS) were prepared in methanol to a concentration of 1 mg ml^{-1} and stored at −20°C.

Extraction

One millilitre of methanol was passed through the solid-phase extraction (SPE) column (Bond Elut^R C18, Varian, CA, USA, 1 cm^3) followed by 1 ml of buffer. The sample, standard or control plasma or serum (100 μl — rat; 250 μl — human) and 100 μl of the IS solution (5 μg ml^{-1}) were added to the SPE column and aspirated through the column using a Vac-Elut (Analytichem International). The SPE column was washed twice with 1 ml of the wash solution. The compounds were eluted with 1 ml of the eluting solution, the elute evaporated to dryness under nitrogen at 50°C and the residue reconstituted in 100 μl of mobile phase.

At the start of each day, standards in the range of 60–800 ng ml^{-1} of the drug and its metabolites in rat plasma or 15–800 ng ml^{-1} in human serum were processed to check linearity. Thereafter, samples were extracted and chromatographed with 125, 500 and 750 ng ml^{-1} quality control (QC) standards with the total number of samples and standards not to exceed 10 (the capacity of the Vacelut module used) in any one run.

Analysis

Fifty-five microlitres of the reconstituted extract was injected into the equilibrated LC system. For each run the mean response factor

(peak height ratio/concentration) for the three QC standards was calculated and used to calculate the concentrations of the three compounds in the samples processed in the same run.

Results and Discussion

Baseline separation of CLP, NCLP and OCLP was obtained using a mobile phase consisting of acetonitrile–phosphate buffer (360:640, v/v; pH 3.7). Optimum washing of the SPE column was obtained using a solution consisting of 28% acetonitrile in methanol. Increasing the concentration of acetonitrile in the wash solution drastically reduced the recovery of the three compounds, particularly OCLP. The use of methanol alone as the eluting solution gave reproducible recovery (RSD <5%; $n = 5$) of the three analytes from the SPE column but failed to give reproducible internal standard recovery. The addition of 50 mM triethylamine to the methanol provided reproducible recovery for the internal standard analyte (RSD <5%; $n = 5$).

The internal standards that have been used in previously published methods include diazepam, nitrazepam, oxazepam, protriptyline, nortriptyline and amitriptyline. Although these compounds were separated from the three compounds of interest under the LC conditions used in this study, their recovery from the SPE

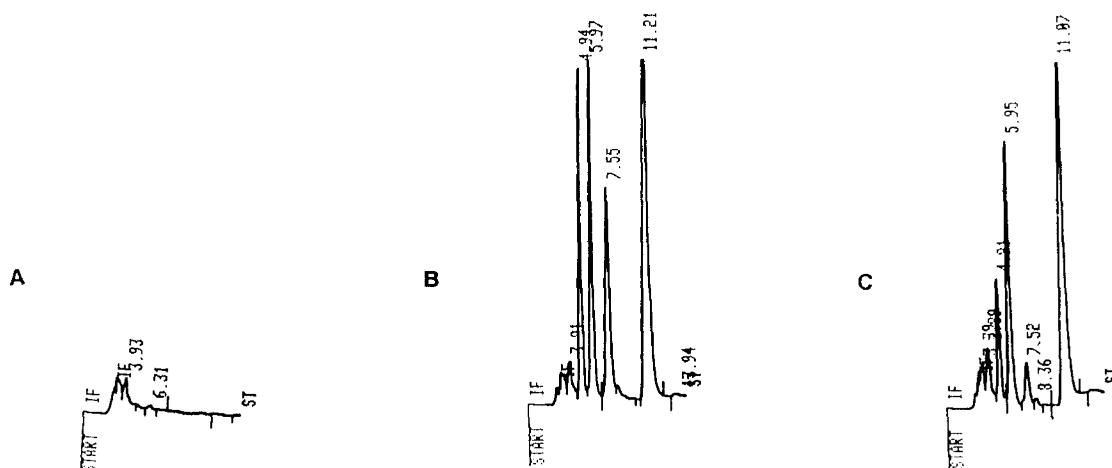

Figure 2
Typical chromatograms obtained from human plasma. A, blank human plasma; B, human plasma spiked with CLP, NCLP and OCLP (500 ng ml^{-1}); C, plasma sample from a subject 4 h after oral administration of 300 mg of CLP. Retention times: NCLP, 5.0 min; CLP, 6.0 min; OCLP, 7.5 min; IS, 11.2 min.

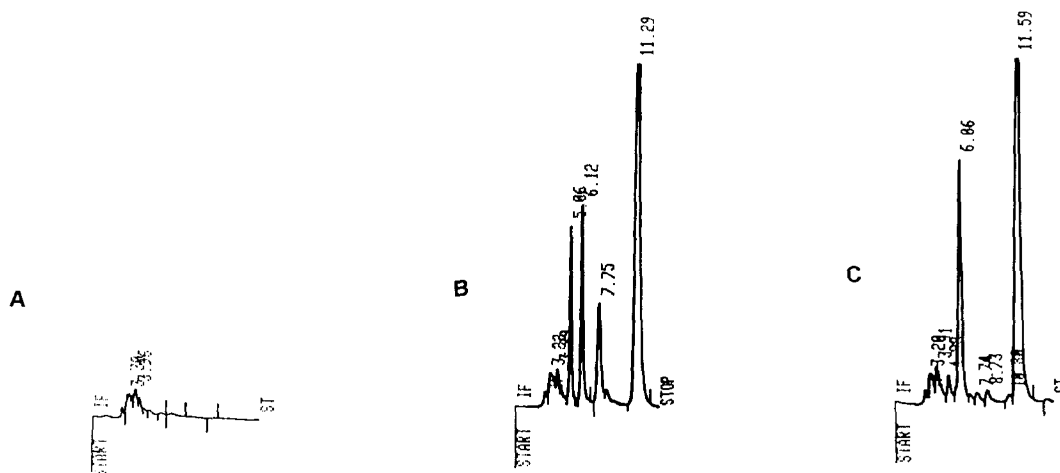

Figure 3
Typical chromatograms obtained from rat plasma. A, blank rat plasma; B, rat plasma spiked with CLP, NCLP and OCLP (800 ng ml^{-1}); C, plasma sample from a rat 30 min after intravenous administration of CLP (20 mg kg^{-1}). Retention times: NCLP, 5.1 min; CLP, 6.1 min; OCLP, 7.8 min; IS, 11.3/11.6 min.

column was not reproducible. The low reproducibility of the recovery of these basic compounds could be due to a strong secondary retention process resulting from hydrogen bonding or ion-exchange silanol interactions with these compounds in the reversed-phase system [17]. The most effective solution to such secondary retention effects is the addition of amine modifiers that will preferentially interact with and block the silanol groups. Amine additives such as triethylamine are commonly used for this purpose at concentrations of 1–50 mM [17]. Concentrations of triethylamine >50 mM in methanol were found to adversely affect the chromatography for the three compounds of interest. The use of 50 mM of triethylamine dramatically improved the reproducibility of recovery of potential internal standards, from the SPE column with the best results obtained from the *N*-methylspiperone internal standard (RSD <5%; $n = 5$ for *N*-methylspiperone compared with RSD = 10–15%; $n = 5$ for the others).

Typical chromatograms obtained with extracts from human serum and rat plasma are shown in Figs 2 and 3. Analysis of blank serum and plasma shows that no endogenous substances interfered with NCLP, CLP, OCLP and IS at 5.0, 6.0, 7.5 and 11.3 min, respectively. Standard curves for CLP, NCLP and OCLP fitted a linear model over the concentration range of 60–800 ng ml^{-1} in rat plasma and 15–800 ng ml^{-1} in human serum with correlation coefficients >0.999. The typical inter-run variability (RSD) of the mean response factor for the three QC samples (n = 4) of 125, 500 and 750 ng ml^{-1} of the three analytes was <5%. The intra- and inter-day RSDs for the three compounds was <20% at concentrations >15 ng ml^{-1} in human serum (Table 1) and <10% at concentrations >60 ng ml^{-1} in rat plasma. The precision and accuracy of the method at the limit of quantification are within the recommended value of ±20% for a valid analytical method used in bioavailability, bioequivalence and pharmacokinetics studies [18]. The mean accuracy ($n = 6$) of the method at the low and high concentrations was >95% (Table 2). Under the optimized HPLC conditions the mean absolute recovery rates for 250 ng ml^{-1} were 95.0, 70.0 and 65.0% for CLP, NCLP and OCLP, respectively. Figures 4 and 5 show the CLP, NCLP and OCLP

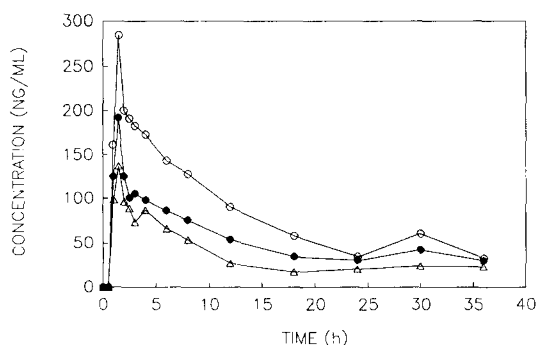

Figure 4
Concentration–time curves for CLP, NCLP and OCLP for a human subject that received an oral dose of 300 mg CLP. ○, CLP; ●, NCLP; △, OCLP.

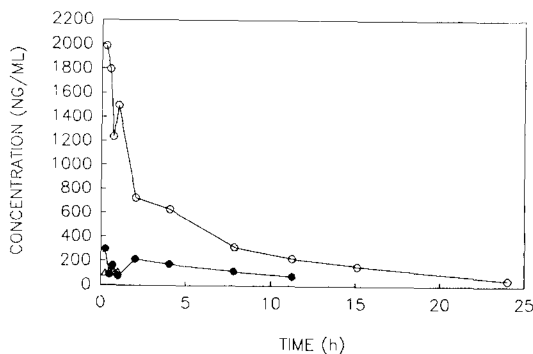

Figure 5
Concentration–time curves for CLP, NCLP and OCLP for a rat that was given an intravenous dose of CLP (20 mg kg^{-1}). ○, CLP; ●, NCLP; △, OCLP.

Table 1
Intra- and inter-day precision ($n = 6$) of the assay for CLP, NCLP and OCLP in human serum

CLP			NCLP			OCLP		
Conc. (ng ml^{-1})	RSD* (%)	RSD† (%)	Conc. (ng ml^{-1})	RSD* (%)	RSD† (%)	Conc. (ng ml^{-1})	RSD* (%)	RSD† (%)
16.6	18.5	18.7	15.5	17.6	18.7	16.5	17.8	18.5
66.6	7.0	6.6	61.9	8.4	8.5	66.1	8.7	8.9
133.2	3.7	1.9	123.8	4.6	1.1	132.2	4.9	3.6
532.5	3.0	1.6	495.0	4.2	2.4	528.5	2.7	2.3
798.8	2.4	2.9	742.5	4.4	2.9	792.8	2.4	2.0

*Intra-day RSD (%).
†Inter-day RSD (%).

Table 2
Accuracy ($n = 6$) of the assay for CLP, NCLP and OCLP in human serum and rat plasma

CLP			NCLP			OCLP		
Conc.* (ng ml^{-1})	Human†	Rat‡	Conc.* (ng ml^{-1})	Human†	Rat‡	Conc.* (ng ml^{-1})	Human†	Rat‡
16.6	96.4 ± 18.7	—	15.5	101.3 ± 18.7	—	16.5	102.2 ± 18.4	—
66.6	96.1 ± 6.7	101.2 ± 8.6	61.9	100.1 ± 8.5	95.4 ± 8.5	66.1	105.6 ± 8.7	96.5 ± 4.2
133.2	101.0 ± 3.8	100.5 ± 2.7	123.8	101.6 ± 4.6	103.7 ± 4.4	132.2	102.2 ± 5.0	100.8 ± 2.1
532.5	98.1 ± 3.0	100.9 ± 2.8	495.0	102.9 ± 4.2	103.2 ± 2.7	528.5	96.7 ± 2.7	99.8 ± 2.2
798.8	101.0 ± 2.4	101.7 ± 1.5	742.5	102.1 ± 2.9	100.4 ± 2.2	792.8	102.6 ± 2.5	101.6 ± 2.2

*Concentration of drug added to rat plasma/human serum.
†% Concentration of drug found in human serum (Mean ± SD).
‡% Concentration of drug found in rat plasma (Mean ± SD).

concentrations for a human subject who received a single 300 mg oral dose of CLP and a rat which was given an intravenous dose of 20 mg kg^{-1} CLP.

Conclusions

The method described in this report is more sensitive than the previously published LC method which used automated solid-phase extraction; the precision of the two methods are comparable [16]. The method is simple, rapid and sensitive enough for routine application to pharmacokinetic studies of CLP and its metabolites in clinical and preclinical research.

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References

[1] A. Fitton and R.C. Heel, *Drugs* **40**, 722–747 (1990).
[2] M.W. Jann, S.R. Grimsley, E.C. Gray and W.H. Chang, *Clin. Pharmacokinet.* **24**, 161–176 (1993).
[3] R. Gauch and W. Michaelis, *Farmaco.* **26**, 667–681 (1971).
[4] R. Heipertz, H. Pilz and W. Beckers, *Arc. Toxicol.* **37**, 313–318 (1977).
[5] K. Richter, *J. Chromatogr.* **434**, 465–468 (1988).
[6] U. Bondesson and L.H. Lindstrom, *Psychopharmacol.* **95**, 472–475 (1988).
[7] U. Breyer and K. Villumsen, *J. Clin. Pharmacol.* **9**, 457–465 (1976).
[8] J. Rosenthaler, F. Nimmerfall, R. Sigrist and H. Munzer, *Eur. J. Biochem.* **80**, 603–609 (1977).
[9] J. Meier, *Brit. J. Pharmacol.* **53**, 440 (1975).
[10] W. Zeren, L. Minglian, X. Peipei, Z. Yanlin and Z. Yanlia, *Biomed. Chromatogr.* **1**, 53–57 (1986).
[11] C. Haring, C. Humpel, B. Auer, A. Saria, C. Barnas, N. Fleischhacker and H. Hinterhuber, *J. Chromatogr.* **428**, 160–166 (1988).
[12] C. Humpel, C. Haring and A. Saria, *J. Chromatogr.* **491**, 235–239 (1991).
[13] D. Wilhelm and A. Kemper, *J. Chromatogr.* **525**, 218–224 (1990).
[14] M.C. Chung, S.K. Lin, W.H. Chang and M.W. Jann, *J. Chromatogr.* **613**, 168–173 (1993).
[15] M.J. Lovdahl, P.J. Perry and D.D. Miller, *Ther. Drug Monit.* **13**, 69–72 (1991).
[16] H. Weigmann and C. Hiemke, *J. Chromatogr.* **583**, 209–216 (1992).
[17] J.W. Dolan and L.R. Snyder, *Troubleshooting LC Systems*, pp. 404–416. Humana, Clifton, NJ (1989).
[18] V.P. Shah, K.K. Midla, S. Dighe, I.J. McGilvery, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman and S. Spector, *J. Pharm. Sci.* **81**, 309–312 (1992).

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