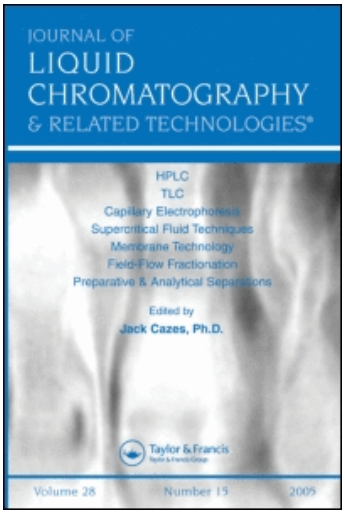

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Determination of Clozapine and Its Two Major Metabolites in Human Serum by Liquid Chromatography Using Ultraviolet Detection

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DETERMINATION OF CLOZAPINE AND ITS TWO MAJOR METABOLITES IN HUMAN SERUM BY LIQUID CHROMATOGRAPHY USING ULTRAVIOLET DETECTION

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ABSTRACT

A new, simple, reverse phase, and highly reproducible HPLC-UV method that has all the comprehensive features of good column, mobile phase, internal standard and extraction has been developed for the assay of clozapine (CLZ), norclozapine (NCLZ) and clozapine-N-oxide (CLZNO) in human serum. The method is very easy to adapt, overcomes the problems of earlier methods and is very economical. Amoxapine is the internal standard. All three analytes are extracted from alkaline serum using ethyl acetate and the absolute extraction efficiency is ~95%. The UV detector is set at 230 nm. The mobile phase is a mixture of phosphate buffer (0.05M, pH 2.7), acetonitrile and methanol (62:20:18 v/v) and contains 2.5 mL triethylamine per liter of solution. The detection limit of the method is 2 ng/mL for CLZ and NCLZ and 4 ng/mL for CLZNO. The mean CV for the method is 5%.

INTRODUCTION

A typical antipsychotic agent, clozapine (CLZ, Clozaril) is commonly given to schizophrenic patients refractory to conventional neuroleptic agents. The drug has minimal extrapyramidal side effects, but can cause agranulocytosis and dose dependent seizures.[1] Norclozapine (NCLZ), and clozapine-N-oxide (CLZNO) are the two major metabolites of clozapine in humans. The two compounds NCLZ and CLZNO have minor antipsychotic activity. A strong correlation between serum concentrations of analytes and CLZ dose has been shown, but the correlation between serum CLZ concentration and its antipsychotic or side effects are not as yet clearly defined.[1-3]

Although there are many methods for the analysis of CLZ alone, or both CLZ and NCLZ simultaneously, there are only four HPLC methods for the simultaneous analysis of all three compounds.[4-7] These methods however have one or more of the following defects: difficult to adapt, cost ineffective, require column heating, poor choices of internal standard, analytical column, and the UV detector wavelength setting, long assay times and poor extraction efficiencies of all compounds. We have developed an isocratic, reverse phase, and ambient temperature HPLC method that overcomes most of the above problems. We have validated our method, by determining accuracy and precision data using quality control samples, and the steady state concentrations of CLZ, NCLZ, and CLZNO, in 80 samples from 43 schizophrenic patients.

MATERIALS AND METHODS

Analytical grade clozapine, norclozapine and clozapine-N-oxide, were gifts from Sandoz Pharmaceutical Corporation, E. Hanover, NJ. Amoxapine, was obtained from Research Biochemicals International, Natick, MA.

The mobile phase is a mixture of aqueous buffer (0.05 M sodium monobasic phosphate, contains 2.5 mL triethylamine per liter of solution and adjusted to pH 2.7 using phosphoric acid), acetonitrile and methanol in the ratio 62:20:18 v/v. The mobile phase flow rate was 1.3 mL/min.

Working standards (CLZ : NCLZ : CLZNO), of the following concentration (μg/L) triplets, were prepared in drug free serum: 25/25/12.5, 150/150/75, 300/300/150, 500/500/250, 800/800/400 and 1000/1000/500. The standard solutions were aliquoted in 2 mL polypropylene tubes and kept frozen at -20°C. The frozen standard solutions were quite stable for at least 6 months. The working internal standard solution contained 200 ng of amoxapine per 50 μL of 0.1 M hydrochloric acid solution.

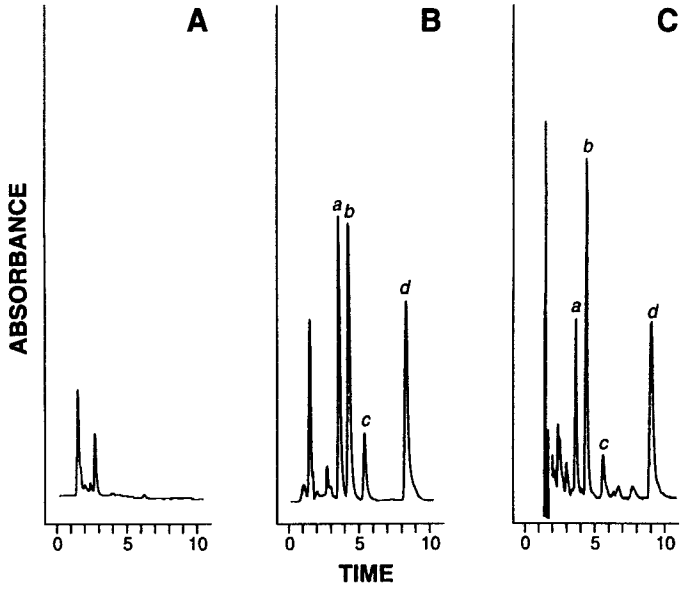

Figure 1. Chromatograms of human serum extracts. A - Blank drug free serum. B - Serum standard containing 150 µg/L each of NCLZ(a), and CLZ(b), and 75 µgL of CLZNO(c). C - Patient sample containing 212 µg/L of NCLZ(a), 290µg/L of CLZ(b), and 52 µg/L of CLZNO(c). Peak (d) represents 200 ng of amoxapine

Equipment

The HPLC system consisted of a pump (Waters, Model 510, Milford, MA), an autosampler (Waters, Model 717), a self packed C_8 guard column (Upchurch, Oak harbor, WA, Model C130-B, containing Perisorb RP-8), an analytical column (Supelco C_8-DB, 3 µm, 100 X 4.6 mm; Supelco Inc., Bellefonte, PA), a variable wavelength ultraviolet detector (Waters, Model 480, with a 1 cm pathlength flow cell) and an integrator (Hitachi, San Jose, CA, Model D-2500). The ultraviolet detector was set at 230 nm.

Extraction

0.5 mL of standard, quality control or patient's serum, 50 µL (200 ng) of internal standard, 400 µL of 1M sodium hydroxide and 6 mL of ethyl acetate were all pipetted into a 10 mL Teflon tube. The tubes were screw capped and shaken for 15 min in an Eberbach shaker, and centrifuged at 4000 X g for 10

min. The top organic layer was transferred to another Teflon tube and the analytes back extracted with 3 mL of 0.05 M hydrochloric acid. In the last step, the top organic layer was transferred to another tube and the ethyl acetate was evaporated under nitrogen in a water bath at 45°C. The residue was dissolved in 120 μL of mobile phase and 30 μL injected into the HPLC system.

RESULTS AND DISCUSSION

Figure1 illustrates typical chromatograms of extracts of human serum: A) a blank - drug free serum, B) drug free serum standard of clozapine and its metabolites, C) a patient sample in this study. There are no interfering peaks in the blank. The peaks are baseline resolved, amoxapine elutes well away from all the clozapine metabolites peaks, and the run time per sample is about 9 min. Serial standardization was performed on each analysis day. The good reproducibility (CV 5%) of the response factors, in about 30 assays so far in this study, clearly points to the robustness of the assay.

Table 1 summarizes the precision, recovery, and accuracy data for the assay. Two somewhat different solid phase extraction methods for CLZ, NCLZ, and CLZNO from serum have been reported,[4,7] but the absolute extraction efficiency of the metabolites by both methods is low. In our experience, our liquid-liquid extraction method has uniform and very high absolute extraction efficiency (~90%, n=6) for all three compounds (Table 1).

The choice of an internal standard for this HPLC assay has been a difficult one for many workers. Volpicelli,[5] "after extensive research," chose triprolidine as her internal standard. Schulz,[6] who had the highest extraction efficiency and sensitivity of all published methods, had an assay run time of 25 min per sample perhaps due to their internal standard ----- imipramine. Fadiran[7] chose n-methylspiperone as the internal standard, so that all the analytes will have uniform extraction efficiency by their solid phase extraction method. Numerous different compounds have been used as internal standards in the assay. We chose amoxapine as the internal standard, and it eluted at about 9 min and away from clozapine and all its metabolites. Amoxapine is very stable in solution and extracted very well (95 ± 3% absolute recovery; n=6). The good reproducibility and accuracy of our method (Table 1) is, at least, partially due to our choice of amoxapine as the internal standard.

A variety of columns (C_6, C_8, C_{18}, phenyl, cyano, cation exchange and silica) have been used in the assay. The nature and extent of reports on the normal phase and cation exchange columns, do not indicate that these methods are simple and did not meet our objective to develop a simple and easy to adapt method. C_8 columns are the choice lately for separating basic drugs.

Table 1

Precision and Recovery Data for all CLZ, NCLZ and CLZNO in Drug Free Human Serum

Spiked Concn*	Measured Concentration* CLZ	NCLZ	CLZNO*
	Mean, CV%, Recovery %		
	Inter-assay (n = 6)		
40	42, 7.0, 105	36, 4.3, 90	18, 5.3, 90
80	74, 6.0, 93	78, 5.1, 98	38, 4.5, 95
400	375, 3.6, 94	379, 2.8, 95	179, 4.1, 90
800	745, 1.9, 93	761, 1.5, 95	378, 2.5, 95
	Intra-assay (n = 6)		
40	44, 5.4, 110	37, 1.0, 92	21, 4.4, 105
80	74, 4.1, 92	80, 2.6, 100	38, 8.0, 95
400	388, 2.5, 97	380, 1.6, 95	182, 3.8, 91
800	744, 1.5, 93	761, 1.0, 95	382, 4.3, 96

* concn. μg/L,, CLZNO concns. in all control samples are half of that for CLZ.

The Supelco C_8-DB, 3 μm (100 X 0.46 cm) has been quite reliable for our assays of ketamine, midazolam, thiothixene, etc. The mobile phase (refer experimental section for composition) for the work, was quickly optimized and it contains the least amount of acetonitrile (20%, which is the most expensive of the solvents) compared to other works. The mobile phase flow rate was 1.3 mL per min and, as such, the method is one of the most cost effective. Ion-pairing agents, like heptanesuphonic acid increased the retention times of the compounds at the low pH mobile phase. Addition of triethylamine decreased the retention times of peaks.

Different workers have chosen different ultraviolet wavelengths (215, 230, and 254 nm) for detection. Greater assay sensitivity results when the detector is set at 215 nm.[8] But, the extraction method, the nature of the analytical column used, and the composition of the mobile phase, influence the selection of detector wavelength. Detectors set at 254 nm[7] clearly have poor detection limits.

Table 2

Daily Dose Levels and Plasma Concentrations of CLZ, NCLZ and CLZNO Obtained by the Method

Patient ID	Dose mg/Day	Concentrations in μg/L CLZ	NCLZ	CLZNO
1	600	486	344	71
2	600	891	474	207
3	400	392	312	152
4	500	388	254	176
5	500	468	574	152
6	125	284	215	48
7	300	398	338	31
8	600	550	489	140
9	250	145	85	73
10	150	140	78	57

In this work, ethyl acetate extraction did not affect the detection at 230 nm. While there are distinct advantages in using photodiode array detector, particularly in eliminating interferences, good interference studies can still be cost effectively done using an UV detector. According to Beer's law, higher pathlength flow cell increases the sensitivity of the method. The Waters (Model 480) UV detector with a 1 cm pathlength flow cell, enabled us to achieve very good sensitivities (2 ng/mL for CLZ and NCLZ and 4 ng/mL for CLZNO). The noise to signal ratio was 1 to 5 during these sensitivity studies.

Patient Results

Table 2 gives dosage and plasma concentrations of all the three analytes, CLZ, NCLZ, and CLZNO, for a few patients included in the study. As noted in earlier studies,[4-8] there is a lot of interpatient variability. In a few instances, metabolite concentrations are greater than those of the parent drug.

In the following discussions, the results of six patients who were taking the drugs fluoxetine, or dilantin or valproic acid, which are known to affect the metabolism of clozapine,[7] were excluded. The mean daily dose for the remaining 37 patients was 463 ± 165 mg/day (range 125-900). The mean serum concentrations of CLZ, NCLZ, and CLZNO were 517 ± 317, 304 ± 199 and 122 ± 72 μg/L respectively.

Table 3

Drugs Checked for Possible Interference and their Relative (to Norclozapine) Retention Times

Drug	Relative Retention Times
Atenolol	0.50
Norlclozapine	1.00 (5.5 min)
Bupropion	1.13
Clozapine	1.19
Paroxetine	1.49
Clozapine-N-oxide	1.50
Trazadone	1.50
Sertraline	1.85
Inderal	2.10
Dilantin	2.26
Doxepin	2.29
Medazepam	2.35
Amoxapine	2.60
Carbamazepine	2.70
Desmethylsertraline	2.80
Haloperidol	3.10
Loxapine	3.11
Verapamil	3.70
Imipramine	3.89
Oxazepam	3.91
Thiothixene	3.96
Valproic acid	4.55
Desipramine	4.90
Cogentin	4.98
Nortryptiline	6.00
Amitryptiline	6.10
Diazepam	6.94
Trifluoperazine	8.50
Fluoxetine	8.60

The correlations between daily dose and plasma concentrations of CLZ, NCLZ, and CLZNO were $r = 0.6244$, $r = 0.6482$, and $r = 0.6995$ respectively. The mean ratio in individuals NCLZ/CLZ = 0.662 ± 0.202, and CLZNO/CLZ = 0.2779 ± 0.151. These results are very similar to values reported by Volpicelli[5] and McCarthy.[8]

Interference Studies

The specificity of the assay was determined by injecting the drugs that our patient population were taking along with clozapine. Table 3 gives the retention times of these drugs checked in our method. About 200 ng of the drug was injected into the HPLC system. The drug bupropion could interfere with clozapine; paroxetine and trazodone interfere with clozapine-N-oxide; carbamazepine and desmethylsertraline coelute with amoxapine. We noted, that the patients in this study were not given carbamazepine, but either Dilantin or valproic acid. Although, Dilantin itself does not interfere in the assay, its metabolites were found to coelute with CLZ and NCLZ. Similar other drug metabolites could interfere in the assay and caution should be exercised in interpreting the results.

CONCLUSIONS

Our HPLC method for the simultaneous assay of CLZ, NCLZ, and CLZNO, offers many distinct advantages over the existing four similar methods.[4-7] It has nearly all the comprehensive salient features for a successful HPLC method. These include the analytical column, mobile phase, internal standard, extraction procedure and the UV detector with a long path length flow cell and set at the sensitive wavelength of 230 nm. Optimizing these features, make any HPLC assay simple, sensitive, easy to adapt, reliable and cost effective. Briefly, in our view, the analytical columns tried in earlier methods[4-8] have been a handicap, requiring a higher percentage of acetonitrile in the mobile phase, column heating, and perhaps precluded the choice of a good internal standard.

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REFERENCES

1. R. J. Baldessarini, F. R. Frankenburg, N. Engl. J. Med., **324**, 746-754 (1991).

2. M. W. Jann, Pharmacotherapy, **11**, 179-195 (1991).

3. M. W. Jann, S. R. Grimsley, E. C. Gray, W. H. Chang, Clin. Pharmacokinet., **24,** 161-176 (1993).

4. H. Weigman, C. Heimke, J. Chromatogr., **582**, 209-216 (1992).

5. S. A. Volpicelli, F. Centorrino, P. R. Puopolo, J. Knado, F. R. Frankenburg, R. J. Baldessarini, J. G. Flood, Clin. Chem., **39**, 1656-1659 (1993).

6. E. Shultz, C. Fleishhaker, H. Remschmidt, Pharmacopsychiat., **28**, 20-25 (1995).

7. E. O. Fadiran, J. Leslie, M. Fossler, D. Young, J. Pharm. and Biomed. Anal., **13**, 185-190 (1995).

8. P. T. McCarthy, S. Hughes, C. Paton, Biomed. Chromatogr., **9**, 36-41 (1995).

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