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Simultaneous determination of clozapine and its *N*-desmethyl and *N*-oxide metabolites in plasma by liquid chromatography/electrospray tandem mass spectrometry and its application to plasma level monitoring in schizophrenic patients.

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Abstract

A liquid chromatography tandem mass spectrometry (LC-MS-MS) assay method for the simultaneous determination of clozapine and its *N*-desmethyl (norclozapine) and *N*-oxide metabolites in human plasma is described. The compounds were extracted from plasma by a single step liquid-liquid extraction procedure and analyzed using a high performance liquid chromatography electrospray tandem mass spectrometer system. The compounds were eluted isocratically on a C-18 column, ionized using positive ion atmospheric pressure electrospray ionization method by a TurboIonspray source and analyzed using multiple reaction monitoring mode. The ion transitions monitored were m/z 327 $\rightarrow$ m/z 270 for clozapine, m/z 313 $\rightarrow$ m/z 192 for norclozapine, m/z 343 $\rightarrow$ m/z 256 for clozapine-*N*-oxide and m/z 421 $\rightarrow$ m/z 201 for internal standard. The standard curves of clozapine, norclozapine and clozapine-*N*-oxide were linear over the range of 1 ng/ml to 1000 ng/ml when 0.5 ml of plasma was used for the analysis ($r^2 > 0.998$). Three pooled plasma samples collected from patients who were treated with clozapine were used as long-term quality control samples to check the validity of spiked standard curve samples made at various times. The intra- and inter-assay variations for the spiked standard curve and quality control samples were less than 14%. These variations for the long-term patient quality control samples were less than 11%. The LC-MS-MS assay for simultaneous determination of clozapine, norclozapine and clozapine-*N*-oxide reported here is highly specific, sensitive, accurate and rapid. This method is currently being used for the plasma level monitoring of clozapine and its *N*-desmethyl and *N*-oxide metabolites in patients treated with clozapine. The plasma levels of clozapine, norclozapine and clozapine-*N*-oxide varied widely within and among patients. The data revealed that the norclozapine and clozapine *N*-oxide metabolites were present at about 58% $\pm$ 14% and 17% $\pm$ 6% of clozapine concentrations in plasma, respectively.

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1. Introduction

Clozapine (CLZ), a dibenzodiazepine derivative (Fig. 1), is an atypical antipsychotic agent. It has a complex pharmacological profile, which includes activity at multiple receptors [1–3]. CLZ is an effective antipsychotic with a low incidence of extrapyramidal symptoms (EPS) and good antipsychotic efficacy in schizophrenic patients [4]. However, CLZ can cause dose dependent epileptic seizures [5] and reversible, but potentially fatal, agranulocytosis [6]. Studies indicate that metabolism, immunological and toxicological mechanisms are probably involved in causing these side effects [7–9]. However, by implementing suitable monitoring programs where blood cell counts were regularly monitored [10], CLZ has been effectively used in treating treatment-resistant schizophrenic patients to improve positive as well as negative symptoms with low incidence of side effects [11].

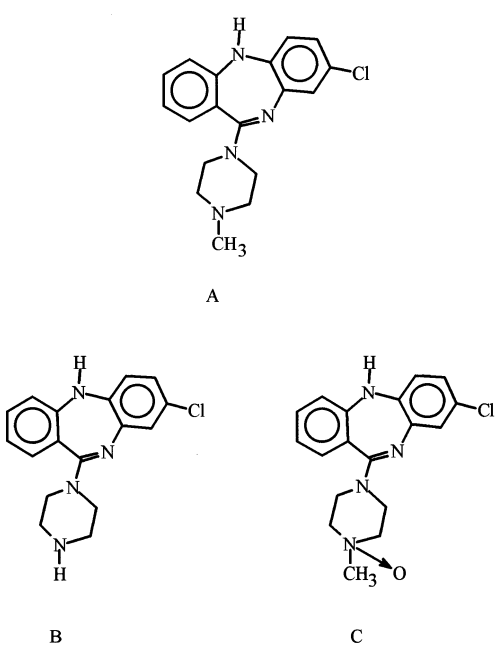

Fig. 1. Chemical structures of clozapine (A, molecular weight 327), norclozapine (B, molecular weight 313,) and clozapine *N*-oxide (C, molecular weight 343).

Orally administered CLZ is extensively metabolized by hepatic microsomal enzyme systems to several metabolites in animals and humans [12,13]. Two of these metabolites, *N*-desmethyl clozapine, (norclozapine, NorCLZ) and clozapine *N*-oxide (CLZ-*N*-oxide), are major circulating metabolites (Fig. 1). Both are pharmacologically less active than CLZ [14,15]. Although studies have provided evidence that toxic side effects might be associated with CLZ metabolism [16], it remains to be substantiated whether or not the high blood levels of CLZ and/or its metabolites are related to the incidences of CLZ's side effects. However, plasma level monitoring of CLZ and its major metabolites may be useful in assisting clinicians in monitoring and managing the risks of side effects.

Plasma level monitoring of antipsychotic drugs in the treatment of schizophrenic symptoms has been used in many instances, such as with fluphenazine determined by RIA [17] and haloperidol determined by HPLC-ECD [18]. In the case of CLZ, several analytical methods with limited sensitivity and specificity for the determination of CLZ and one or both of its major metabolites, including analysis by gas chromatography [19], radioimmunoassay [20], HPLC-UV [21–30]) and GC-MS [31,32] have been reported. Gas chromatography and GC-MS methods may not be suitable for the assay of CLZ-*N*-oxide because CLZ-*N*-oxide is thermally unstable and decomposes under experimental conditions involving high temperature [31,32]. Methods with milder experimental conditions, such as HPLC-UV method [21–30] and HPLC with photodiodearray detector (PDA) ([27], M. Aravagiri et al., unpublished, refer section 2.8), have been used to assay CLZ and it metabolites including CLZ-*N*-oxide but with limited sensitivity and long analysis time.

The current report describes a specific and sensitive liquid chromatography-electrospray tandem

mass spectrometry (LC-MS-MS) method developed for the first time to determine CLZ, NorCLZ and CLZ-*N*-oxide simultaneously. This method showed improved specificity, sensitivity and had shorter sample analysis time as compared to existing HPLC method with photodiodearray (PDA) detector ([27], Aravagiri et al., unpublished, refer section 2.8). This LC-MS-MS method is being used for the plasma level monitoring in patients with schizophrenia who are treated with CLZ.

2. Experimental

2.1. Chemicals and reagents

CLZ was generously donated by the Sandoz Research Institute (E Hanover, NJ, USA). The metabolites, NorCLZ and CLZ-*N*-oxide were a gift from the Drug Metabolism and Disposition Group, College of Pharmacy, University of Saskatchewan (Saskatoon, Canada). The internal standard was a congener of risperidone (INS) obtained as a gift from Janssen Pharmaceutical (Beerse, Belgium). All solvents and chemicals were HPLC grade procured from Fisher Scientific Company (Tustin, CA.) and used without further purification. Deionized high purity water was produced in the lab by reverse osmosis using ROpure-Nanopure water purification system (Barnstead Company, MA). All centrifugations were carried out using a refrigerated centrifuge (Centra GP 8R, IEC, Fisher Scientific) at 18°C at $1725 \times g$.

2.2. Standard curve samples

Stock solutions of CLZ, NorCLZ and CLZ-*N*-oxide were made in methanol by dissolving accurately weighed amount of compounds (Cahn Ultra Micro Balance, Thomas Scientific). The spiked standard curve and quality control (QC) samples were made from stock solution by serial dilution in blank pooled plasma from drug free volunteers. A typical standard curve consisted of at least 8 standard curve plasma samples containing CLZ, NorCLZ and CLZ *N*-oxide at concentrations ranging from 1 to 1000 ng/ml. Four spiked QC samples containing 300, 60, 12 and 1.2 ng/ml of CLZ, NorCLZ and CLZ-*N*-oxide (QC-1, QC-2, QC-3, and QC-4, respectively) were made to check the reliability of standard curve. Approximately 80 ml of plasma was pooled from patients who were taking ~ 350, ~ 450 and above 500 mg/day of clozapine and arbitrarily assigned Pt-QC 1, 2, and 3. Sufficient number (36 tubes for each Pt-QC) of 0.5 ml aliquots of these samples were pipetted out into 125×16 mm borosilicate glass tubes and stored at -70°C to avoid frequent thawing and freezing. These samples were used as long-term patient quality control (Pt-QC) samples to establish the validity of the spiked standard curve and QC samples prepared at various times.

At each instance of making standard curve samples, the freshly prepared plasma standards and QCs were tested with frozen long-term Pt-QCs and frozen spiked QCs of previous standard to verify that the freshly made standard was similar to the previous standard curve samples and to confirm that there were no mistakes made in sample weighing and sample dilutions for spiked standards. Once the validity of newly made standard and QC samples was established, sufficient number (7–9 sets) of aliquots of 0.5 ml from the spiked standards and QCs were pipetted out into 125×16 mm tubes and stored at -70°C to avoid frequent thawing and freezing during analysis of each batch of unknown samples. On each day of the assay one set of standard, QC and Pt-QC samples were assayed along with unknown samples.

2.3. Patient's plasma samples

Twelve patients diagnosed as having symptoms of schizophrenia and who met the DSM-IV criteria for Schizophrenia (*American Psychiatric Association*, 1994) were treated with CLZ. All patients gave informed consent for venipuncture and plasma concentration determination. The patients were treated with daily oral doses, varied from 350 to 600 mg/day, of CLZ. At the discretion of physician, biweekly blood samples were collected in the morning approximately 12 h after the last

dose and just before the next morning dose, by venipuncture in heparinized Vacutainer blood collection tubes (Becton and Dickinson, Rutherford, NJ). Patients were treated with a constant daily dose of CLZ at least for two weeks before the blood collection. The blood samples were immediately centrifuged for 10 min at 4°C at $1725 \times g$, and plasmas were separated and stored at -70°C until analysis.

2.4. Sample preparation

CLZ, NorCLZ and CLZ-*N*-oxide were extracted from plasma samples by a simple one- step extraction procedure. Briefly, to an aliquot of 0.5 ml of the plasma sample (unknown, QC, standard curve samples) taken in a borosilicate glass tube (12×125 mm, 15 ml capacity), 100 ng of INS (0.1 ml of 1 μg/ml solution of INS in methanol:water mixture (1:9)) and 0.5 ml of an aqueous solution of saturated sodium carbonate were added (pH ~ 10, not adjusted) [31]. The contents of the tubes were mixed and extracted with 7 ml of a mixture of ethyl acetate, methylene chloride and pentane (50:30:20, v/v/v) by shaking in a test tube shaker for 10 min, followed by centrifugation for 10 min at 18°C ($1725 \times g$). The supernatant organic layer was pipetted out in to a borosilicate glass tube (16×100 mm, 10 ml capacity) and dried at 60°C in a dry bath under a slow stream of nitrogen. The residue was reconstituted in 250 μl of mobile phase and an aliquot was injected into the HPLC system.

2.5. Extraction recovery

The absolute extraction recovery of CLZ, NorCLZ, CLZ-*N*-oxide and the INS was determined by analyzing four 0.5 ml aliquots of spiked standard curve plasma samples for CLZ, NorCLZ and CLZ-*N*-oxide containing 100 ng of INS by the method described above. The absolute recovery was calculated by comparing the peak-heights obtained for spiked standard curve samples and peak-heights obtained from direct injections of known amounts of CLZ, NorCLZ, CLZ-*N*-oxide and the INS.

2.6. LC-MS-MS system and sample analysis

A triple quadrupole tandem mass spectrometer (API2000, Perkin Elmer Sciex, Foster City, CA, USA) was interfaced with a HPLC system consisting of Perkin Elmer Micro200 pumps and a Micro 200 autosampler fitted with 50 μl sample loop (Perkin Elmer). The HPLC system, mass spectrometer and data acquisition were controlled by a PC microcomputer using data capture and data analysis software (Analyst, PE Sciex). The compounds were separated on a C-18 column (5 μ particle size, 50×4.6 mm, Phenomenex). The mobile phase consisted of ammonium acetate (60 mM, pH 7, not adjusted), methanol and acetonitrile (5:45:50, v/v/v) degassed by filtering through a 0.25 Nylon filter (Fisher Scientific) under vacuum. The compounds were eluted isocratically with a flow rate of 0.4 ml/min.

The mass spectrometer was operated in the positive ion electrospray ionization mode. The eluted compounds from the C-18 column were introduced into the mass spectrometer using the TurboIonspray ion source LC-MS-MS interface. The ion source temperature was kept at 300°C. Nitrogen was used as the nebulizer gas (Gas 1), auxiliary gas (Gas 2), curtain gas and the gas for collision-activated dissociation (CAD). The nitrogen flow for the above gases was kept at the API2000 instrumental settings of 25, 30, 25 and 3, respectively. The detection and quantitation of compounds were performed by positive ion multiple reaction monitoring (+ MRM) mode. The ion transitions monitored were m/z $327 \rightarrow 270$ for CLZ, m/z $313 \rightarrow 192$ for NorCLZ, m/z $343 \rightarrow 256$ for CLZ-*N*-oxide and m/z $421 \rightarrow 201$ for INS. These ion transitions were selected based on predominant fragmentation pathways of CLZ, NorCLZ, CLZ-*N*-oxide and the INS and their intensity as observed in their product ion spectra (Fig. 2). The dwell time for each transition was set at 250 ms with a inter channel pause time of 20 ms to provide optimal sampling of each peak of interest. The total scan time was 1.08 s. The CAD was ensured with nitrogen ($\sim 177 \times 10^{13}$ molecules /cm^2) in the collision cell. The optimum collision energy for the selected transitions were found to be -31, -53, -27 and -37V for

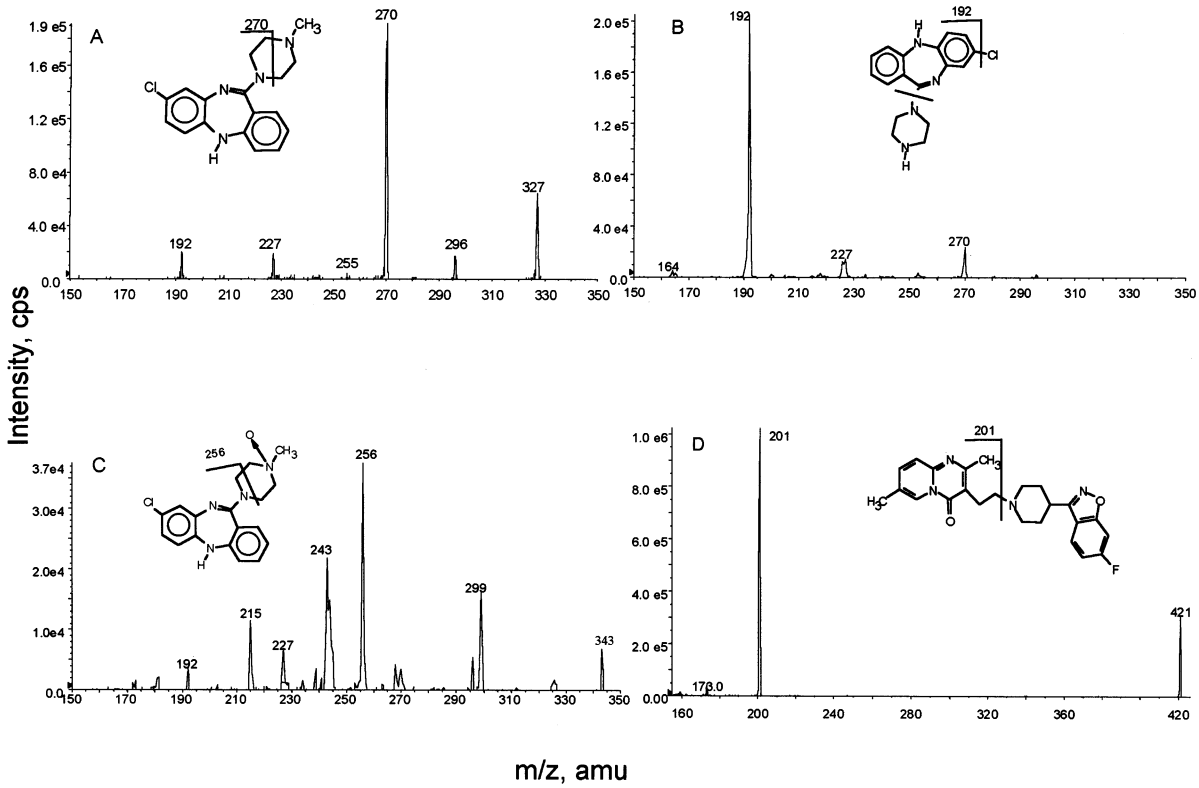

Fig. 2. Product ion mass spectrum of clozapine (A), norclozapine (B), clozapine *N*-oxide (C) and INS (D).

CLZ, NorCLZ, CLZ-*N*-oxide and the INS, respectively. The mass spectrometer, LC system, mass calibration, data acquisition, data representation and post acquisition quantitative analyses were carried out using a suite of Perkin Elmer Sciex application software (Analyst).

2.7. Calibration curve and data analysis

Calibration curve was constructed by plotting the peak area ratios of CLZ or NorCLZ or CLZ-*N*-oxide to INS against the concentrations of CLZ or NorCLZ or CLZ-*N*-oxide, respectively. The linear regression line was constructed covering the concentration range of 1 to 1000 ng/ml of CLZ or NorCLZ and CLZ-*N*-oxide ($r^2 > 0.998$). The concentration of these compounds in unknown plasma samples from schizophrenic patients was calculated by comparing their peak area ratios with that of spiked calibration curve standards and expressed in terms of ng/ml.

The statistical and graphical analyses were accomplished using instrument specific (Analyst, PE Sciex) and commonly available commercial software packages (Microsoft Office, Microsoft Corp.; Prism, GraphPad Software Inc., San Diego, CA).

2.8. HPLC-PDA method

The HPLC system consisted of a solvent delivery pump (Schimadzu model 590AD) and an auto injector with 200 μl sample loop (Schimadzu, SIL 9AD). The compounds were eluted isocratically using an Ultrasphere cyano column (4.6 × 250 mm, 5 μ particle size, Beckman, Fullerton, CA, USA). The mobile phase consisted of an aqueous solution of ammonium acetate (56 mM. pH not adjusted), methanol and acetonitrile (20:6:74, v/v/

v) and the flow rate was 0.8 ml/min. The analytical column was heated in a column heater and the temperature was maintained at 37°C. Eluted compounds were detected by a photodiode array (PDA) detector (ThermoQuest, UV6000, San Jose, CA, USA), which was set at 254 nm wave length. The chromatography data was collected and analyzed by a chromatographic software system (ChromQuest®, ThermoQuest Inc., San Jose, CA, USA).

The extraction procedure was the same as used in the LC-MS-MS method, but 100 ng of prochlorperazine was used as the internal standard. The calibration curves were constructed by plotting peak height ratios of the analytes to internal standard and they were linear over the range of 10–1000 ng/ml for CLZ and NorCLZ and CLZ-*N*-oxide ($r^2 > 0.99$) when 0.5 ml plasma samples were used for analysis. The retention time was ~7, 10, 14 and 12 min for CLZ and NorCLZ, CLZ-*N*-oxide and INS, respectively. The analysis time for each sample was 25 min long in order to elute some of the late eluting peaks of plasma components.

3. Results and discussion

3.1. LC-MS-MS conditions

Fig. 2 shows the typical product ion mass spectra of CLZ (A), NorCLZ (B), CLZ-*N*-oxide (C) and INS (D). Attempts to use compounds with clozapine-like chemical structures such as olanzapine and ethyl olanzapine were not successful. Their use as an internal standard was not feasible because ethyl olanzapine showed similar molecular weight and product ions as clozapine. Olanzapine could not be used in patients taking both medications. Furthermore, olanzapine molecular ion was similar to NorCLZ. Therefore, a derivative of risperidone (INS) was used as an internal standard.

The nebulizer gas, auxiliary gas, curtain gas and CAD gas flows were optimized to maximize the intensity of the selected product ions of CLZ, NorCLZ and CLZ-*N*-oxide. They were similar for these compounds. The collision energy for each ion transition was then optimized to produce the highest intensity of the selected ion peak. The typical LC-MS-MS extracted ion chromatograms of the blank plasma (A), the plasma standard spiked with CLZ, NorCLZ and CLZ-*N*-oxide (B), and the plasma from a patient receiving a dose of 300 mg of CLZ per day (C) are shown in Fig. 3. All compounds were eluted within 3 min. The compounds exhibited required retention on a relatively short C-18 column (5 cm). The compounds were not chromatographically fully resolved, as it was not essential to completely resolve them for accurate quantitation by LC-MS-MS method. Also, the ion source temperature of 300°C did not result in any noticeable decomposition of CLZ-*N*-oxide in the electrospray ion source. When an extract of CLZ-*N*-oxide spiked plasma sample was analyzed by the above LC-MS-MS method, there were no detectable amounts of decomposition products of CLZ-*N*-oxide such as CLZ.

The compounds were stable when samples were stored in the freezer at −70°C. The concentrations of Pt-QCs determined over a period of six months indicated that the analytes including CLZ-*N*-oxide were stable in the freezer. As indicated in Table 1, the variations were less than 14%. Similarly, the overall variations of parent to metabolite ratios in these samples were less than 15% for both NorCLZ and CLZ-*N*-oxide, indicating that the frozen samples were stable for over 6 months.

3.2. Assay precision and accuracy

The validation of the LC-MS-MS assay was assessed by the precision and accuracy of the method and evaluated by the determination of intra- (within day) and inter- (between-days) assay variations. Both variations were assessed as the percentage of the coefficient of variation (CV%) of the spiked plasma sample concentrations determined by the assay. The intra-assay variance was determined by analyzing four 0.5 ml aliquots of each of the four spiked quality control plasma samples containing 300, 60, 12 and 1.2 ng ml^{-1} of CLZ, NorCLZ and CLZ-*N*-oxide and four 0.5 ml aliquots of each of the three Pt-QC samples. These concentrations were determined from the

standard curve samples analyzed on the same day. The inter-assay variation was determined by analyzing 0.5 ml aliquots of the four spiked quality control plasma and the three long term Pt-QC plasma samples on 8 different days and determining their concentration from the standard curve samples analyzed on each day of analysis. The standard curves were linear and had highly reproducible correlation coefficients (>0.998), slopes (<0.0128) and intercepts (<-0.0005). The intra- and inter-assay variations for CLZ, NorCLZ and CLZ-*N*-oxide are given in Table 1. For CLZ, NorCLZ and CLZ-*N*-oxide determinations, the intra- and inter-assay variations for the spiked QCs and the long term Pt-QC were less than 11% and for spiked standards the variations were less

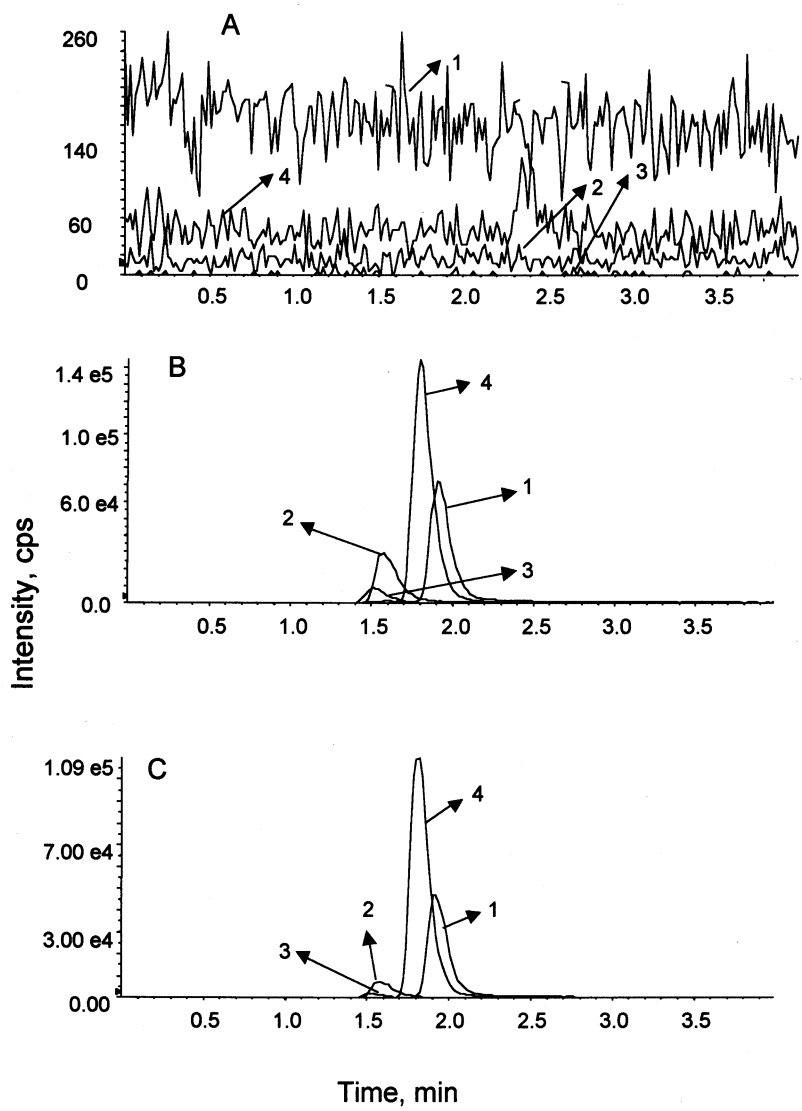

Fig. 3. LC-MS-MS + MRM extracted ion chromatograms (XIC) of blank plasma (A) and the spiked plasma standard (B) and the plasma from a patient receiving 300 mg of clozapine daily oral dose (C). The compounds are labeled as clozapine (1), norclozapine (2), clozapine *N*-oxide (3) and INS (4).

Table 1
Intra- (within-day) and inter- (between days) assay variations for the determination of CLZ, NorCLZ and CLZ-*N*-oxide in plasma by the LC-MS-MS method

Samples	Mean concentration determined by LC-MS-MS, ng/ml			CV		
	CLZ	NorCLZ	CLZ-*N*-oxide	CLZ	NorCLZ	CLZ-*N*-oxide
Inter-assay variation[a]						
Plasma QC[b] *(spiked)*						
QC-1 (300 ng/ml)	290.0	296.4	289.7	4.9%	7.8%	7.4%
QC-2 (60 ng/ml)	64.0	57.3	64.5	6.6%	11.3%	9.0%
QC-3 (12 ng/ml)	13.0	11.8	12.6	6.3%	7.8%	6.9%
QC-4 (1.2 ng/ml)	1.4	1.4	1.2	12.5%	13.0%	13.2%
Patient QC[c]						
Pt-QC-1	379.7	195.9	115.0	6.9%	7.0%	10.4%
Pt-QC-2	915.8	283.5	188.1	7.9%	10.4%	10.6%
Pt-QC-3	380.3	235.4	102.2	4.5%	10.3%	8.5%
Intra-assay variation[d]						
Plasma QC (spiked)						
QC-1 (300 ng/ml)	283.1	300.3	265.7	4.2%	4.6%	4.9%
QC-2 (60 ng/ml)	59.1	54.2	61.6	3.2%	1.6%	2.1%
QC-3 (12 ng/ml)	12.4	11.2	12.6	3.2%	6.2%	1.9%
QC-4 (1.2 ng/ml)	1.3	1.4	1.4	10.4%	12.1%	13.8%
Patient QC						
Pt-QC-1	353.5	209.3	101.4	2.3%	5.8%	5.6%
Pt-QC-2	919.5	278.8	175.4	0.9%	1.2%	1.5%
Pt-QC-3	364.8	213.1	98.6	1.1%	4.3%	1.7%

[a] Intra- (within-day) assay variations were calculated as coefficient of variation (CV%, SD/Mean) from the determined concentrations of four aliquots of each of four spiked QC samples and three Pt-QC samples assayed in a single day.

[b] Spiked quality control samples QC-1, QC-2, QC-3 and QC-4 (300, 60, 12 and 1.2 ng ml^{-1} of CLZ, NorCLZ and CLZ-*N*-oxide, respectively) were prepared by spiking pooled blank plasma from drug free volunteers with known amounts of CLZ, NorCLZ and CLZ-*N*-oxide.

[c] Patient plasma QC (Pt-QC-1, Pt-QC-2 and Pt-QC-3) were pooled plasma samples collected from patients treated with CLZ and used as a long-term QC to validate standard curves prepared at various times.

[d] Inter- (between-days) assay variations were calculated as coefficient of variation (CV%) from the determined concentrations of QC samples assayed on eight different days.

than 14%. The overall extraction recovery, by the extraction procedure described here, was 84, 89, 76% and 41% for CLZ, NorCLZ, CLZ-*N*-oxide and INS, respectively. Although the recovery of this INS was lower than the analytes, it was consistent and did not create any quantitation problem as indicated by the acceptable inter- and intra assay variations (Table 1).

3.3. Plasma level monitoring in schizophrenic patients

Plasma concentrations of CLZ, NorCLZ and CLZ-*N*-oxide were determined as part of an on-going study where plasma levels were routinely monitored for compliance and to adjust the CLZ dose to achieve the optimum clinical outcome. The plasma concentrations of CLZ, NorCLZ and CLZ-*N*-oxide in schizophrenic patients treated with various doses of CLZ are given in Table 2. The plasma levels of CLZ, NorCLZ and CLZ-*N*-oxide varied widely within and among patients. Plasma levels of NorCLZ were lower than the CLZ levels. The plasma concentration data revealed that the NorCLZ metabolite was present at about $58\% \pm 14\%$ of CLZ concentrations in

Table 2
Mean ± SD plasma concentrations of CLZ, NorCLZ and CLZ-*N*-oxide in patients treated with daily oral doses of CLZ determined by the LC-MS-MS method[a]

Dose ng/ml	*n* of samples (*n* of patients)	Mean concentration			Mean ratio% of		CV% of plasma levels		
		CLZ	NorCLZ	CLZ-*N*-oxide	NorCLZ/CLZ	CLZ-*N*-oxide/CLZ	CLZ	NorCLZ	CLZ-*N*-oxide
350	20 (1)	664.2	435.9	63.0	66.3	9.6	27.1	24.6	30.6
400	13 (4)	314.5	175.4	74.9	59.3	24.0	34.5	30.9	49.0
500	46 (6)	559.4	266.5	93.9	49.1	17.8	38.9	36.6	34.4
550	9 (3)	359.9	233.4	58.9	66.6	16.1	20.9	19.8	34.5
600	8 (3)	396.6	263.1	69.4	75.1	17.0	61.5	34.1	70.4

[a] All plasma samples were collected in the morning 12 h after last dose and before giving the next dose

plasma. The *N*-oxide levels in plasma were lower than the NorCLZ. The CLZ-*N*-oxide metabolite was present in plasma approximately $17 \pm 6\%$ of that of CLZ concentrations. Although, NorCLZ and CLZ-*N*-oxide metabolites were less active than CLZ, their presence in substantial amounts in plasma is important. Determination of NorCLZ and CLZ-*N*-oxide, along with CLZ, may be a useful tool in the therapeutic drug monitoring of schizophrenic patients treated with CLZ to achieve optimum clinical outcome and possibly to avoid side effects.

4. Conclusions

In conclusion, a LC-MS-MS method has been developed and validated for the simultaneous determination of CLZ, NorCLZ and CLZ-*N*-oxide in plasma samples. The method reported here is simple, selective and highly sensitive with a lower limit of determination of 1 ng/ml of CLZ, NorCLZ and CLZ-*N*-oxide when 0.5 ml of plasma is used for the analysis. The sample analysis time was less than 4 min for each sample with the LC-MS-MS method as compared to ~ 25 min in the HPLC-PDA method. The LC-MS-MS method is more sensitive than previously reported HPLC methods and the HPLC-PDA method (Aravagiri et al., unpublished, refer section 2.8). The plasma levels determined by LC-MS-MS method were similar to those values obtained for the same samples by HPLC-PDA method ($r^2 = 0.94$, 0.87 and 0.55 for CLZ, NorCLZ and CLZ-*N*-oxide) and did not differ significantly ($P < 0.001$). The LC-MS-MS method described here is currently being used in the plasma level monitoring of CLZ and its metabolites in patients who are treated with CLZ for the symptoms of schizophrenia.

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References

[1] G. Stille, H. Lauener, E. Eichenberger, Il. Farmaco Ed. Pr. 26 (1971) 603–625.
[2] D.E. Casey, Psychopharmacology 99 (1989) 47–53.
[3] Clozapine Study Group, Br. J. Psychiatry 163 (1993) 150–154.
[4] M. Ackenheil, Psychopharmacology 99 (1989) S32–S37.
[5] G.M. Simpson, T.A. Cooper, Am. J. Psychiatry 135 (1978) 99–100.
[6] P. Krupp, P. Barnes, Br. J. Psychiatry 160 (suppl 17) (1992) 38–40.
[7] M. Hummer, B. Sperner-Unterweger, G. Kemmler, M. Falk, M. Kurz, H. Oberbauer, W.W. Fleischhaker, Psychopharmacology 124 (1996) 201–204.
[8] Z.C. Liu, J.P. Uetrecht, J. Pharmacol. Exp. Ther. 275 (1995) 1476–1483.
[9] V. Fisher, J.A. Haar, L. Greiner, R.V. Lloyed, R.P. Mason, Mol. Pharmacol. 40 (1991) 846–853.
[10] B. Bastani, L.D. Alphs, H.Y. Meltzer, Development of the clozaril patient management system, Psychopharmacology 99 (1989) S122–S125.
[11] R.J. Baldessarini, F.R. Frankenburg, New Engl. J. Med. 324 (1991) 746–754.
[12] R. Gauch, W. Michaelis, Il. Farmaco Ed. Pr. 26 (1971) 667–681.
[13] J.G. Dain, J. Nicoletti, F. Ballard, Drug Metab. Dispos. 25 (1997) 603–609.
[14] W.W. Jann, Pharmacotherapy 1 (1991) 179–195.
[15] L.H. Lindstrom, Acta Psychiatr. Scand. 71 (1988) 176–185.
[16] V. Fisher, J.A. Haar, L. Greiner, R.V. Lloyd, R.P. Mason, Mol. Pharmacol. 40 (1991) 846.
[17] S.R. Marder, T. Van Putten, M. Aravagiri, E.M. Hawes, J.W. Hubbard, G. McKay, J. Mintz, K.K. Midha, Psychopharmacol. Bull. 29 (1990) 256–259.
[18] T. Van Putten, B.D. Marshall, R.P. Liberman, J. Mintz, T.G. Kuenel, L. Bowen, M. Aravagiri, S.R. Marder, Psychopharmacol. Bull. 29 (1993) 315–320.
[19] R. Heipertz, H. Pilz, W. Beckers, Arch. Toxicol. 37 (1977) 313–318.
[20] J. Rosenthaler, F. Nimmerfall, R. Sigrist, H. Munzer, Eur. J. Biochem. 80 (1977) 603–609.
[21] C. Haring, C. Humpel, B. Auer, A. Saria, C. Barnas, W. Leischhacker, H. Hinterrhuber, J. Chromatogr. B. 428 (1988) 160–166.
[22] D. Wilbelm, A. Kemper, J. Chromatogr. B. 525 (1990) 218–224.
[23] M. Lopdahl, P.J. Perry, D.D. Miller, Ther. Drug Monit. 13 (1991) 69–72.

[24] H. Weigmann, C. Hiemke, J. Chromatogr. B. 583 (1992) 209–216.
[25] O.V. Olesen, B. Poulsen, J Chromatogr. B. 622 (1993) 39–46.
[26] Mc. Chung, S.K. Lin, W.H. Chang, J Chromatogr. B. 613 (1993) 168–173.
[27] S.A. Volpicelli, F. Centarrino, P.R. Puopolo, J. Kando, F.R. Frankenburg, R.J. Baldessarini, J.G. Flood, Clin. Chem. 39 (1993) 1656–1659.
[28] C. Guitton, G. Facciolà, G.M. Campo, A. Fazio, E. Spino, J Chromatogr. B. 714 (1998) 299–308.
[29] C. Guitton, J.M. Kinowski, M. Abbar, P. Chabrand, F. Bressolle, J. Clin. Pharmacol. 39 (1999) 721–728.
[30] C. Guitton, J.M. Kinowski, M. Abbar, P. Chabrand, F. Bressolle, J. Clin. Psychopharmacol. 18 (1998) 470–476.
[31] G. Lin, G. McKay, J.W. Hubbard, K.K. Midha, J. Pharm. Sci. 83 (1994) 1412–1417.
[32] G. Lin, G. McKay, K.K. Midha, J. Pharm. Biomed. Anal. 14 (1996) 1561–1577.