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Validated GC/MS method for the simultaneous determination of clozapine and norclozapine in human plasma. Application in psychiatric patients under clozapine treatment

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ABSTRACT

A sensitive and specific GC/MS method for the determination of clozapine (CLZ) and its major metabolite norclozapine (NCLZ), in plasma has been developed, optimized and validated. Specimen preparation includes solid-phase extraction of both analytes using Bond-Elut Certify cartridge and further derivatization with TFAA. Clozapine-d8 was used as internal standard for the determination of CLZ and NCLZ. Limits of detection were 0.45 ng/mL for CLZ and 1.59 ng/mL for NCLZ, while limits of quantification were 1.37 ng/mL for CLZ and 4.8 ng/mL for NCLZ, as calculated by the calibration curves. The calibration curves were linear up to 600 ng/mL for CLZ and NCLZ. Absolute recovery ranged from 82.22% to 95.35% for both analytes. Intra- and interday accuracy was less than 7.13% and −12.52%, respectively, while intra- and interday precision was between 9.47% and 12.07%, respectively, for CLZ and NCLZ. The method covers all therapeutic range and proved suitable for the determination of CLZ and NCLZ not only in psychiatric patients but also in forensic cases with clozapine implication.

1. Introduction

Clozapine (CLZ), a tricyclic dibenzodiazepine derivative, is classified as an atypical antipsychotic drug [1] due to its complex pharmacologic properties [2] and different effects that produce comparing to those exhibited by more typical antipsychotic drugs [2]. CLZ is efficacious for the treatment of patients with schizophrenia who fail to show an acceptable response to standard antipsychotic drugs [3]. However, the usage of CLZ has been restricted as a consequence of its side effects such as agranulocytosis, seizures and sedation [4]. CLZ is rapidly absorbed and, prior to excretion, extensively metabolised [3] in two major metabolites, N-desmethyl clozapine (norclozapine: NCLZ) and clozapine-N-oxide (CLZNO). CLZNO is present in low amounts in the plasma of patients receiving CLZ [1], conversely NCLZ is present in significant amounts and usually determined along with CLZ [1].

Most patients respond sufficiently to dosages between 300 and 500 mg/day [5]. If improvement has not occurred, the dosage can be gradually increased to 900 mg/day [6]. It is suggested that therapeutic response is correlated with clozapine plasma levels between 350 and 600 ng/mL.

Although the precisely toxic range of clozapine is not known, its side effects are considered to be dose-related. Clozapine needs to be closely monitored when its concentration is higher than 600 ng/mL in plasma [7]. On the other hand, despite indications that NCLZ does not possess any clinical outcome [8], a recent review [9], suggests that higher NCLZ/CLZ ratios may be associated with greater symptom improvement, particularly based on its muscarinic M1 agonism. These findings propose that, the complex interaction between clozapine and NCLZ at receptor sites makes it necessary for a validated method able to monitor not only CLZ but also NCLZ in clinical trials as well as in overdose and forensic cases.

The aim of this study was to develop and validate a GC/MS specific method for the determination of clozapine and norclozapine in human plasma covering all therapeutic and toxic levels. A recent review provides sufficient information regarding the published methods in the literature for the determination of CLZ and NCLZ [10]. Three LC methods [11–13] have been reported using single quadruple MS detector for the determination of these analytes. Comparing to them, the proposed GC/MS method offers the advantage of identification and sufficient sensitivity, although the chromatographic run time is higher. Single MS detector is known to provide in some cases non-adequate fragmentation [14] failing thus to meet EU identification criteria [15] which is critical in forensic samples. Furthermore, LC–MS/MS [16–19] comparing to GC/MS

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technique, possess the disadvantage of high cost making this instrumentation not affordable for all laboratories. On the other hand, the reported GC/MS methods determine the concentrations of analytes by using long chromatographic separation (127 min) [20], employing semi-quantification technique with lower sensitivity [22], or plotting a standard curve (from 1 to 128 ng/mL) without covering therapeutic range [21]. This article is the first report for a validated GC/MS method to determine the concentrations of clozapine and norclozapine in human plasma with the standard curve covering the clinical therapeutic range.

2. Materials and methods

2.1. Materials

CLZ and NCLZ of pharmaceutical purity grade were kindly donated by Novartis (Basel, Switzerland) while, clozapine-d8 (internal standard) of pharmaceutical purity, was purchased from LGC-Promochem (Molsheim, France). Dichloromethane and isopropanol of HPLC grade were purchased from Merck (Darmstadt, Germany). Ammonium solution, trifluoroacetic acid anhydride (TFAA) and disodium hydrogen phosphate of analytical grade were obtained by Fluka (Sigma Aldrich). Water was deionized and further purified by means of a Milli-Q Plus water purification system, Millipore SA (Molsheim, France). For solid-phase extraction, Bond-Elut Certify columns, a mixed-mode bonded silica SPE extraction support containing hydrophobic chains (octylsilane, n-C8) and strong cation-exchange moieties (benzenesulphonylpropylsilane), were used. Phosphate buffer (pH 9.0, 1 M) was prepared by dissolving the appropriate amount of sodium-dihydrogen phosphate dihydrate in water and accurately adjusting the pH 9.0 with the addition of sodium hydroxide (0.1N). For the preparation of spiked samples, human plasma from different units was obtained from blood bank of Aghia Sophia Pediatric Hospital (Athens, Greece).

2.2. Equipment

The analysis of the extracts was performed on a Hewlett Packard (HP) model 5890 gas chromatographer interfaced with HP 5970 MSD detector and equipped with HP-5MS column (30 m × 0.25 mm i.d., 0.25 μm film thickness). Helium was used as carrier gas at 1.0 mL/min flow rate. Injections of 1 μL were carried out in the splitless mode using a HP AOC autosampler system. The MS was operated in electron impact (EI) ionization and selective ion monitoring (SIM) mode for the quantitation of clozapine and norclozapine. A vortex (Chiltern, Model MT 19) set at speed 4, was used for the mixing of samples and standards. The digital pH-meter used was a 691 digital model pH-meter (Metrohm, Switzerland) with a glass combination electrode. Evaporation of the samples was performed using an evaporating device (Reacti-Vap Pierce, Model 18780, Rochford, IL) under gas nitrogen was used. Centrifugation was performed using a centrifuge (Sigma 4K10, Germany).

2.3. Chromatographic conditions

The developed GC method was optimized for column temperature program, flow rate of carrier gas and temperatures of injector, ion source, and interface. The optimized GC conditions were as follows: initial temperature of 50 °C for 3 min, increased to 250 °C at 70 °C/min and held for 30 min giving a total run time of 35 min. Injector port and detector temperature were set at 240 °C and 250 °C, respectively. Analytes were quantified at the base peaks of *m*/*z* 70, 323 and 352 for clozapine, *m*/*z* 352, 323 and 355 for norclozapine and *m*/*z* 75, 90 and 355 for clozapine-d8. For clozapine, *m*/*z* 70 was used as quantifier ion while *m*/*z* 323 and 352 were used as qualifier ions. For norclozapine, *m*/*z* 352 was used as quantifier ion while *m*/*z* 323 and 355 were used as qualifier ions. The maximum permitted tolerances for the above ion ratios have been established to be ±50% for *m*/*z* 70 → 352, *m*/*z* 70 → 323, *m*/*z* 352 → 355 and ±10% for *m*/*z* 352 → 323.

2.4. Calibrators and quality control samples

Two separate stock solutions containing 1.0 mg/mL of clozapine and norclozapine were prepared in methanol and stored at −20 °C. Appropriate dilutions with methanol yielded the working standard solutions containing both analytes. Combined working solutions containing both clozapine and norclozapine were prepared with appropriate dilutions from stock standard solutions in absolute methanol. The concentrations of these solutions ranged between 30 and 6000 ng/mL for each compound. Drug-free human plasma was screened prior to use to ensure that it was free of endogenous interference at the retention time of the analyte. A 100 μL aliquot of the appropriate mixed working standard solution along was added to a 900 μL aliquot of human plasma. Calibration samples were prepared freshly every day over a final concentration range of 3, 10, 50, 100, 300, 500, 600 ng/mL for CLZ and 5, 10, 50, 100, 300, 500, 600 ng/mL for NCLZ. The upper concentration level of the calibration curve was set at 600 ng/mL close to the upper therapeutic concentration of clozapine, while the lower concentration level was set at the lower limit of quantitation (LLOQ) of the proposed method. Quality control samples were prepared in human plasma at four concentration levels (10, 15, 300 and 450 ng/mL). Calibration standard solutions and quality control samples were prepared from separate stock solutions prepared with separate weighing of the analyte.

2.5. Sample preparation

In each calibration and quality control sample, 50 μL of CLZ-d8 (2500 ng/mL) as the internal standard, was added. The pH value of the plasma sample was alkalized by the dropwise addition of 3.00 mL disodium hydrogen phosphate buffer (1 M, pH 9.0), while the sample was vortex mixing. After centrifugation at 3000 rpm for 5 min, the supernatant layer was applied to the extraction columns preconditioned with 3 mL of each methanol, deionized water and phosphate buffer 1 M (pH 9.0). The samples were passed through the cartridges at a flow rate of approximately 1.0 mL/min. The SPE columns were then washed with 3 mL phosphate buffer 1 M (pH 9.0), 50 μL butyl acetate, and subsequently draining at maximum vacuum for 15 min. The analytes were eluted twice with 2.0 mL of freshly prepared mixture of dichloromethane:isopropanol:ammonium solution (90:10:2, v/v/v). The elution solvent was evaporated to dryness under a gentle stream of N_2. Dry extracts were derivatized with 80 μL TFAA under 52 °C for 45 min. The derivative was finally evaporated to dryness and reconstituted with 100 μL of dichloromethane. A 1 μL aliquot of the resulting solution was injected into the chromatographic system.

2.6. Method validation

The following criteria were used to evaluate the GC/MS method: selectivity, specificity, limit of detection (LOD), limit of quantification (LOQ), linearity, precision, accuracy, recovery, robustness and stability. All parameters were validated through nine analytical runs on nine different days.

Selectivity of the method was examined for six different plasma sources while specificity for thirty-three other drugs (Table 1). All compounds were assayed independently by injecting spiked solutions at 250 ng/mL in the GC/MS system using the same chromatographic conditions as the proposed method.

Table 1
Compounds studied for interferences (C = 250 ng/mL).

Nitrazepam	Midazolam	THCA	Penfluridole
Oxazepam	Diltiazepam	codeine	Perphenazine
Flurazepam	Venlafaxine	morphine	Thioridazine
Temazepam	Phenobarbital	Haloperidol	Trifluoperazine
Alprazolam	Cocaine	Pipamperone	Paracetamol
Clobazam	ECME	Carbamazepine	Mirtazapine
Nordiazepam	Benz	Sulpiride	
Chlordiazepoxide	6-Monoacetylmorpine	Olanzapine	
7-Amino-flunitrazepam	THC	Chlorpromazine	

The LOD and LOQ for each analyte were determined as the lowest concentration yielding a signal-to-noise ratio of at least 3:1 and 10:1, respectively.

Four calibration graphs of the recovered standards were prepared to establish linearity and reproducibility of the GC/MS system. Graphs were constructed correlating the peak area ratio of each analyte with the internal standard versus each analyte concentration. A weighting factor of $1/x^2$ was used.

Within-run precision and accuracy were determined by extracting plasma supplemented with clozapine and norclozapine at 10, 300 and 450 ng/mL (n = 6). Between-run assays were performed in six different days (n = 6).

Extraction recovery was calculated at three levels of spiked plasma samples (10, 300 and 450 ng/mL). Extraction recovery was determined by comparing the ratio of peak areas between the analytes and the internal standard from extracted samples with those obtained from a direct injection of the corresponding unextracted standards dissolved in methanol (n = 6). Recovery was tested by adding the internal standard before and after the extraction procedure.

Robustness of the entire method was studied by slightly altering experimental parameters on extraction procedure and chromatographic separation.

In order to test the usefulness of the method in forensic cases of suspected clozapine poisoning, six samples at each one of two concentration levels were spiked and further analyzed. The concentrations were selected to be three times higher than the quality control levels (900 and 1350 ng/mL), falling thus into the therapeutic range after appropriate dilution with plasma and pretreated according to the proposed extraction method.

2.7. Application in clinical samples

The proposed method was further applied to plasma samples of psychiatric patients. Prior to this method, the dose for these patients was adjusted according only to their clinical outcome and not to any other existing analytical method. Plasma samples were obtained after centrifugation of blood at 3500 rpm for 10 min and further analyzed according to the proposed method. The extracted data enable the sufficient quantitation of CLZ and NCLZ in all patients who undergo clozapine treatment for a long period in order to prevent adverse reactions and failure to treatment. Furthermore, the ratio of NCLZ to total clozapine (the sum of CLZ plus NCLZ:%NCLZ) was calculated according to the following equation as an indicator of the metabolic conversion of clozapine [23]:

$$\%\mathrm{NCLZ} = 100 \times \frac{\mathrm{NCLZ}}{\mathrm{CLZ} + \mathrm{NCLZ}}$$

3. Results and discussion

The simultaneous determination of CLZ and NCLZ would help to elucidate the effects of both substances on schizophrenia symptoms, clinical outcome, physiologic/metabolic side effects, and fatal incidents. Of interest, antipsychotics are some of the most frequently encountered drugs not only in clinical but also in forensic cases [24]. Thus, measurement of plasma CLZ and NCLZ concentrations can help to optimize dose, minimize the risk of toxicity [25] and may provide useful guidance for monitoring of patients, who concomitantly receive other medication that potentially interferes with CLZ metabolism. It has been reported that higher NCLZ/CLZ ratios may be associated with a higher rate of side effects such as sedation, which may counter any advantages in this domain. In addition, its metabolic side effects may be more considerable than those of clozapine. Importantly, the muscarinic activity of norclozapine has been also demonstrated in an experimental study by Gray et al. [26]. Olianas et al. [27] also showed that at clinical relevant concentrations NCLZ behaves as an efficacious agonist at δ-opioid receptors present in human frontal cortex membranes. These data support the idea that δ-opioid receptors may constitute an additional molecular target of NCLZ in the brain.

3.1. Method development and optimization

During the optimization of extraction procedure a variety of SPE columns with similar affinity with both analytes were tested (Bond-Elut Certify, Nexus, HCX, C18 and MCX). All cartridges provided matrix with more interferences and lower recovery than Bond-Elut Certify columns. When plasma samples were applied onto Bond-Elut Certify columns, the flow rate was regular and there were no problems of matrix effects. The washing and elution steps of the sample pretreatment, during the SPE procedure, were systematically investigated. Different washing procedures were tested using water, phosphate buffer (pH 9.0, 1 M), a mixture of phosphate buffer (pH 9.0, 1 M) and methanol, acetonitrile, methanol, alkalized methanol, ethyl acetate, butyl acetate, acetone, acetic acid and formic acid. A consequent washing with phosphate buffer (pH 9.0, 1 M) and butyl acetate provided the best recovery for all analytes with the lower matrix effect. The extraction efficiency during SPE was also improved by testing different elution solvents separately and mixtures of them. Dichloromethane:isopropanol:ammonium solution (90:10:2, v/v/v) provided the best recovery for all analytes. Although a satisfactory outcome of SPE process was gained, an extra step of protein precipitation was tested prior to SPE procedure using different organic solvents such as acetonitrile, methanol and acetone in spiked plasma samples. No significant improvement was observed.

A sensitive gas chromatography–mass spectrometric method was developed and validated for the simultaneous quantification of CLZ and NCLZ, in plasma samples. The column temperature program, flow rate of carrier gas, and temperatures of injector and interface were optimized, in order to maximize the intensity of the selected ions, to gain sufficient asymmetry factor and resolution between analytes, and to avoid matrix effects. Separation of the analytes of interest and their internal standard was achieved within 26 min, and the total chromatographic run time was under 35 min. The retention times of clozapine, norclozapine and clozapine-d8 were 19.5, 25.4, and 19.4 min, respectively.

Derivatization procedure, prior to chromatographic analysis, may be used to produce volatile derivatives of involatile substances, to render them amenable to separation by gas chromatography. Polar N–H groups of CLZ and its metabolite result in involatile compounds. Therefore, in this study, a derivatization process was necessary in order to produce less polar and thus more volatile molecules suitable for gas chromatographic system. Several derivatization reagents (PFPA, HFBA, TFAA, TFPA, TMAH-DMSO, BSTFA, BSTFA-TMS, MTBSTFA and MSTFA) and in different derivatization conditions (temperature and time of derivatization) were tested, in order to conclude to a sensitive procedure. TFAA was selected as

Table 2
Intraday and interday accuracy and precision data for clozapine (CLZ) and norclozapine (CLZ) in spiked plasma samples.

C_a (ng/mL)	CLZ			NCLZ		
	C_f (ng/mL)	%R.S.D.	%Er	C_f (ng/mL)	%R.S.D.	%Er
Intraday ($n=6$)						
10	10.28 ± 0.97	9.47	2.84	10.71 ± 0.73	6.83	7.13
300	308.49 ± 5.28	1.71	2.83	286.49 ± 22.44	7.83	−4.50
450	467.10 ± 5.01	1.07	3.80	436.89 ± 22.58	5.17	−2.91
Interday ($n=6$)						
10	10.30 ± 0.54	5.30	2.98	10.43 ± 1.26	12.07	4.30
300	295.27 ± 4.09	1.40	−1.58	268.92 ± 7.49	2.78	−10.36
450	431.16 ± 19.31	4.50	−4.19	393.67 ± 5.81	1.48	−12.52

C_a: concentration added; C_f: concentration found.

the most appropriate due to (a) its total derivatization comparing to other reagents due to its smaller size and thus the avoidance of strereochemical blockage and (b) its less sensitivity in deterioration and moisture than other reagents.

3.2. Method validation

Plasma samples from six different sources and all thirty-five drugs did not yield any interference at the elution time for the detection of clozapine and norclozapine suggesting the selectivity and specificity of the method.

The peak areas ratios were linearly related to plasma concentrations of clozapine (3–600 ng/mL) and norclozapine (5–600 ng/mL). The average regression equation and slope of four calibration curves of clozapine in plasma, prepared in four different days is Sclz = 0.007Ccl ($\pm 1 \times 10^{-3}$) to 0.002 ($\pm 1 \times 10^{-3}$), standard error (Sr) < 3.1×10^{-4}, $n=4$, $r=0.999$ Sclz, corresponds to peak area ratio of clozapine and clozapine-d8, while Cclz, corresponds to plasma concentration of clozapine (ng/mL). The correlation coefficients square (r^2) for each standard curve constructed invariably exceeded 0.996 and intercepts were all close to zero and not statistically significant. %R.S.D. of the slopes between the different calibration curves for clozapine was 0.89%. The average regression equation and slope of four calibration curves of norclozapine in plasma, prepared in four different days is Snclz = 0.002Cnclz ($\pm 1 \times 10^{-3}$) + 0.001 ($\pm 1 \times 10^{-3}$), standard error (Sr) < 5.6×10^{-5}, $n=4$, $r=0.997$ Snclz, corresponds to peak area of norclozapine and Cnclz, corresponds to plasma concentration of norclozapine (ng/mL). The correlation coefficients square (r^2) for each standard curve constructed invariably exceeded 0.990 and intercepts were all close to zero and not statistically significant. %R.S.D. of the slopes between the different calibration curves for norclozapine was 6.89%. The lower limit of detection was 0.45 ng/mL for clozapine and 1.59 ng/mL for norclozapine, while the lower limit of quantification was 1.37 ng/mL for clozapine and 4.8 ng/mL for norclozapine based on S/N > 3 and S/N > 10, respectively.

Table 3
Recovery data for clozapine and norclozapine in human plasma ($n=6$).

C_a (ng/mL)	% Recovery (±S.D.)	%R.S.D.
CLZ		
10	82.22 ± 6.45	7.84
300	90.23 ± 0.23	0.26
450	89.77 ± 0.07	0.07
NCLZ		
10	90.65 ± 7.32	8.51
300	83.22 ± 0.31	0.39
450	95.35 ± 0.09	1.23

C_a: concentration added.

Intraday precision and error were found to be less than 9.5% and 3.8% for clozapine, 7.8% and 7.1% for norclozapine, respectively. Interday precision and error in four different days were found to be less than 5.3% and −4.19% for clozapine, 12.07% and −12.52% for norclozapine, respectively (Table 2).

Extraction recovery from plasma spiked with both analytes was higher than 82.22% and 79.81% in all levels of quality control samples when the internal standard was added before (Table 3) and after the extraction procedure, respectively.

No significant difference was found for the concentration of each analyte when the robustness was studied by using (a) pH 8.45 and 9.55 instead of 9.00, (b) a different ratio of elution mixture solvent (85/15/2 and 75/25/2 instead of 80/20/2, v/v/v, dichloromethane/isopropanol/ammonium solution), (c) column temperature rate 65 °C/min and 75 °C/min instead of 70 °C/min and (d) injector port temperature 230 °C and 250 °C instead of 240 °C.

In order to test the usefulness of the method in forensic cases of suspected clozapine poisoning, six samples at two concentration levels were spiked with three times higher concentrations than the quality control levels (900 and 1350 ng/mL), appropriately diluted with plasma and pretreated according to the proposed extraction method. Representative chromatogram obtained from spiked plasma sample with both analytes at LOQ is shown in Fig. 1A. Fig. 1B represents a typical chromatogram

Table 4
Concentrations of clozapine and desmethylclozapine in seven psychiatric patients under clozapine treatment.

Patient	Dosing schedules (mg/day)	Therapy duration	Cclz (ng/mL)	Cnclz (ng/mL)
1	500	Five years	435.16	94.02
2	500	Five years	691.51	181.45
3	500	Three years	440.19	307.85
4	500	Ten months	475.83	237.15
5	550	Five years	422.91	149.19
6	400	One year	152.73	78.96
7	550	Two years	565.66	335.97

Cclz: concentration of clozapine; Cnclz: concentration of desmethylclozapine.

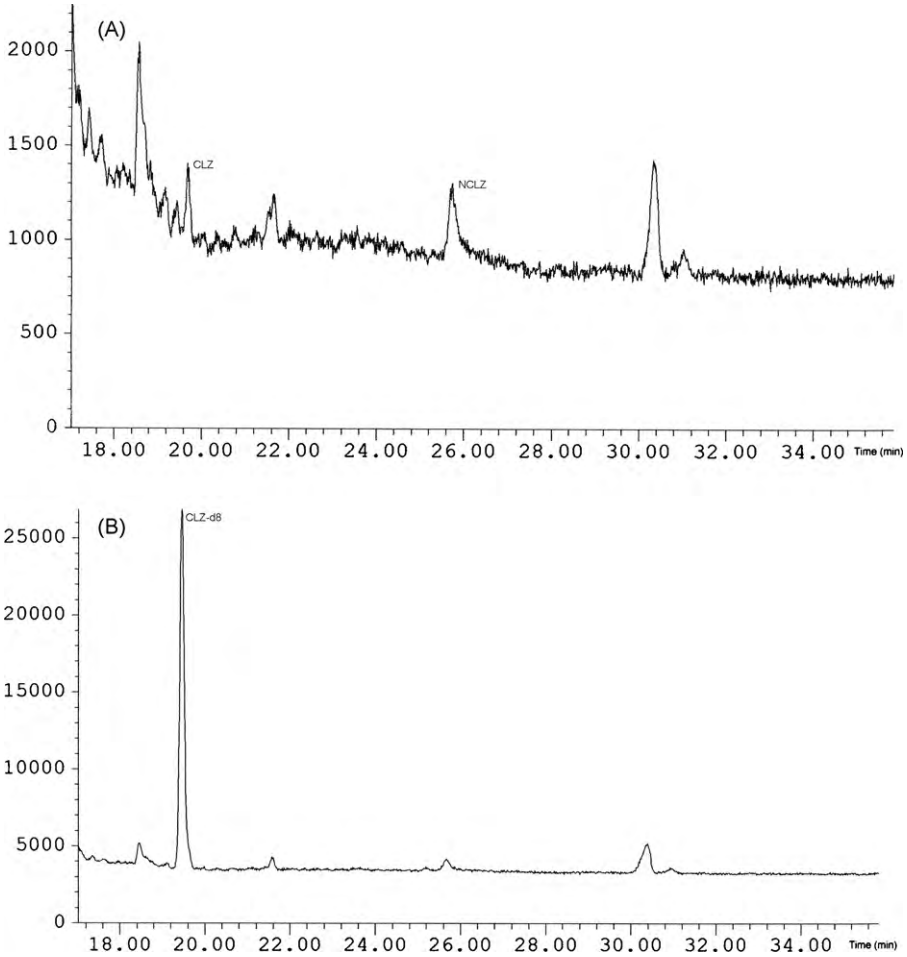

Fig. 1. Representative SIM chromatograms of human plasma samples. (A) Spiked at the limit of quantitation for CLZ (3 ng/mL) and NCLZ (5 ng/mL) and (B) spiked with internal standard (clozapine-d8).

of plasma sample spiked with the internal standard (clozapine-d8).

3.3. Application in plasma clinical samples of psychiatric patients

Plasma samples from seven psychiatric patients were assayed according to the proposed method. All patients were in clozapine long term treatment and thus steady-state clozapine and norclozapine levels were expected. Five patients were indeed between 400 and 600 ng/mL concentration range. One patient provided clozapine concentration at 691.5 ng/mL and required dosage adjustment in order to prevent side effects. Controversy, another patient provided clozapine concentration below the therapeutic range (169.68 ng/mL) that matched with his failure in clinical outcome. An increase of dosage helped to reach the appropriate response. The ratio of NCLZ to total clozapine was normally distributed, with most values in the range of 20–50% (Table 4). Samples of all patients were stored at −20 °C and analyzed for a second time after three

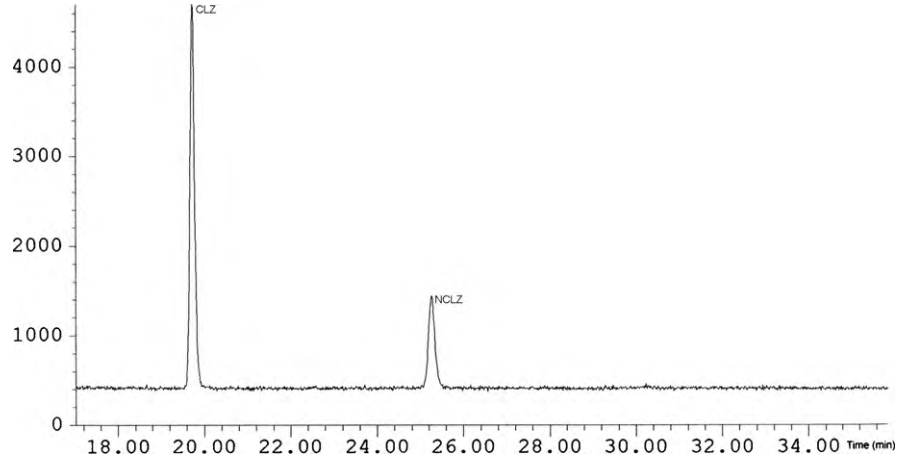

Fig. 2. Representative SIM chromatogram of a psychiatric patient plasma sample at steady-state levels of clozapine.

months. The provided results were very similar to the first measurements indicating the stability of the samples. Representative chromatogram obtained from a clinical plasma sample of a psychiatric patient is shown in Fig. 2.

4. Conclusions

This paper describes a GC–MS method for the simultaneous determination of clozapine and norclozapine in human plasma. The use of SPE procedure provides minimal matrix effect and no interference from commonly used drugs. To our knowledge and in comparison with previously reported HPLC-UV/Vis methods, LC–MS or LC–MS–MS methods and GC–MS methods, this is the first validated GC/MS method which characterizes by the usage of low cost instrumentation comparing to LC/MS/MS. GC combined with MS is the most common analytical technique used by toxicological laboratories and is available worldwide. In addition, the chromatographic separation allows the analysis of both analytes at a sufficient run time which is important for routine analysis. The method coves the expected concentration range for clinical or forensic purposes.

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