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Rapid assays of clozapine and its metabolites in dried blood spots by liquid chromatography and microextraction by packed sorbent procedure

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ABSTRACT

A novel analytical approach has been developed for the determination of clozapine and its metabolites in dried blood spots on filter paper, using a chromatographic method coupled with a microextraction by packed sorbent procedure. The analytes were separated on a RP-C18 column using a mobile phase composed of 20% methanol, 16% acetonitrile and 64% aqueous phosphate buffer. Coulometric detection was used, setting the guard cell at +0.050 V, the first analytical cell at −0.200 V and the second analytical cell at +0.500 V. Clozapine and its metabolites were extracted from dried blood spots with phosphate buffer and, then, a microextraction by packed sorbent procedure for the sample clean-up was implemented obtaining good extraction yields. The calibration curve was linear over the 2.5–1000 ng mL^{-1} blood concentration ranges for all the analytes. The method validation gave satisfactory results in terms of sensitivity, precision, selectivity and accuracy. The analytical method was successfully applied to dried blood spots from several psychiatric patients for therapeutic drug monitoring purpose.

1. Introduction

In the last few decades, several drugs for the treatment of mental illness such as schizophrenia have been introduced in therapy, notably improving the quality of life of psychiatric patients [1]. A breakthrough in the treatment of schizophrenia was achieved with the introduction of clozapine (8-chloro-11-(4′-methyl-1-piperazinyl)-5Hdibenzo[b,e]-[1,4]-diazepine, CLZ, Fig. 1a) the parent drug of the so-called "atypical" antipsychotics. CLZ is very effective against both the negative and positive symptoms of schizophrenia and has been successfully used in patients who are "*non-responder*" to the classical neuroleptic drugs [2]. Moreover, the off-label use of CLZ has been reported in the treatment of psychosis in L-Dopa treated patients [1]. The main active metabolite of CLZ, formed upon interaction with cytochrome P450, is N-desmethylclozapine (DMC, Fig. 1b), while the other metabolite clozapine N-oxide (CLZ-NO, Fig. 1c) appears to be generated in the brain by a flavin monooxygenase. CLZ usually has a higher tolerability with respect to classical antipsychotics such as chlorpromazine or haloperidol; in particular it rarely causes extrapyramidal side effects or hyperprolactinemia. However, CLZ can cause severe agranulocytosis and, to a lesser extent, sedation, orthostatic hypotension, myocarditis and hypersalivation [3]. Measurement of CLZ and its metabolites concentrations can help to assess patient compliance, optimize dosage, and minimize the risk of dose-related toxicity. Thus, an accurate therapeutic drug monitoring (TDM) of CLZ should be carried out in patients treated with this drug. Being whole blood is the optimal matrix for the drug analysis, the use of dried blood spot (DBS) technique could be an attractive approach for TDM. In fact, the collection of blood samples on filter paper is an established technique to screen for neonatal metabolic diseases [4,5]. Recently, a number of papers have been reported on the use of DBS for the analysis of a variety of compounds including hormones, lipids and drugs [6–9]. The use of DBSs affords numerous advantages, such as the elimination of the venous blood withdrawal as well as the absence of post-collection processing. Moreover, the technique poses a low biohazard risk and cost and it is also feasible for sample storage and transport.

Several analytical methods are reported in the literature regarding the analysis of CLZ and its metabolites in plasma and serum [10–32]; some papers include high performance liquid chromatography (HPLC) with UV or Diode Array (DAD) [12,14,15,18,20–22,25,27,28], fluorimetric [23] and amperometric [30–32] detection and with mass spectrometry [10,16,17,19,24,26]. Sample pretreatment is usually carried out by means of liquid–liquid [12,19,20,22,23,26,28], solid phase extraction (SPE) [10,14,15,17,21,30–32] and deproteinization [24] procedures.

To the best of our knowledge no paper has been published for the analysis of CLZ and its metabolites in DBSs. Thus, the present study is the first example of DBSs having been chosen as a represen-

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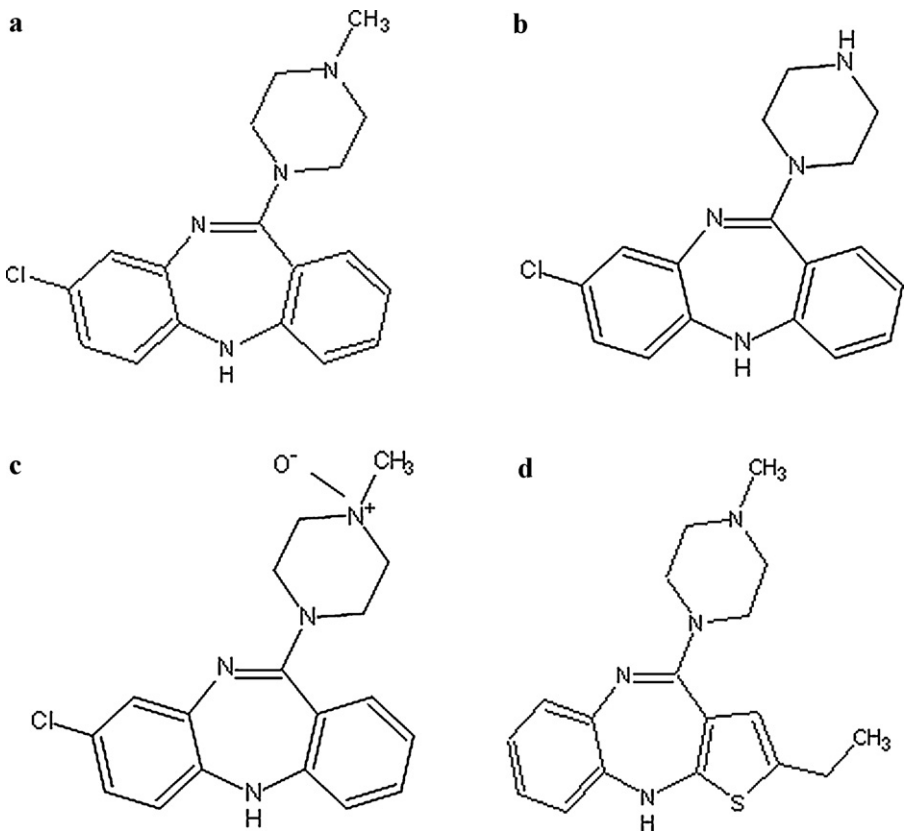

Fig. 1. Chemical structures of (a) clozapine (CLZ), (b) N-desmethylclozapine (DMC), (c) clozapine N-oxide (CLZ-NO), and (d) 2-methylolanzapine, used as the internal standard (IS).

tative of the entire blood, with great advantages for the patients and management procedure in terms of feasibility and easiness in storage and shipment. Moreover, an original analytical method based on the use of HPLC with coulometric detection and a microextraction by packed sorbent (MEPS) technique has been developed and validated.

2. Experimental

2.1. Chemicals

Clozapine (CLZ, Fig. 1a), N-desmethylclozapine (DMC, Fig. 1b) and clozapine N-oxide (CLZ-NO, Fig. 1c) were kindly provided by Novartis Pharma (Basel, Switzerland); 2-methylolanzapine, used as the Internal Standard (IS, Fig. 1d) was kindly provided by Eli Lilly (Indianapolis, IN, USA). Potassium phosphate monobasic, 2 M sodium hydroxide, 37% (w/w) hydrochloric acid, 85.0% (w/w) phosphoric acid, triethylamine, acetonitrile and methanol were from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water (18.2 MΩ cm) from a MilliQ apparatus by Millipore (Milford, MA, USA) was used.

2.2. Equipment

Filter papers (type 41, 55 mm ⌀) for blood spotting were supplied by Whatman (Sanford, NC, USA). The sample pretreatment procedure was carried out by means of MEPS on a BIN (Barrel Insert and Needle Assembly) containing 4 mg of solid phase silica-C8 material, inserted into a 250 μL gas-tight syringe from SGE Analytical Science (Melbourne, VIC, Australia). A Crison (Barcelona, Spain) MicropH 2000 pHmeter, a Universal 32 R centrifuge from Hettich (Tuttlingen, Germany), an Elma (Berlin, Germany) Transsonic T310 ultrasonic bath and a vortex agitator were also used.

2.3. Chromatographic conditions

The HPLC apparatus consisted of a Jasco (Tokyo, Japan) PU-1580 chromatographic pump and an ESA (Milford, MA, USA) Coulochem III coulometric detector which is equipped with cells having porous graphite working electrodes and α-hydrogen/palladium reference electrodes. The conditioning cell was set at +0.050 V; in the analytical cell, detector 1 (E1) was set at −0.200 V and detector 2 (E2) at +0.500 V, with a range of 200 nA and an output of +1.00 V. The analytes were monitored in oxidation at the analytical detector 2. Data were handled by means of Software Chromatography Station (CSW 32 v. 1.4) from DataApex (Prague, Czech Republic).

The chromatographic separation was achieved by isocratic elution on a Gemini C18 reversed-phase column (150 × 4.6 mm I.D., 5 μm) equipped with a C18 cartridge pre-column (4 × 3 mm I.D., 5 μm) from Phenomenex (Torrance, CA, USA) and kept at room temperature (25 ± 3 °C). The mobile phase was a mixture (20:16:64, v/v/v) of methanol, acetonitrile and a 50.0 mM, pH = 2.1 phosphate buffer. The flow rate was 1.0 mL min^{-1} and the samples were injected by means of a 20 μL loop. Prior to use, the mobile phase was filtered through Varian nylon filters (47 mm diameter, 0.2 μm pore size) and degassed with sonication.

2.4. Preparation of solutions

The stock solutions of CLZ, DMC, CLZ-NO and IS were 1 mg mL^{-1} and were prepared by dissolving 3 mg of the pure substance in 3 mL of methanol. These solutions were stable for at least three months when stored at −20 °C, as assessed by HPLC assays. Working standard solutions of the analytes and the IS were prepared daily by diluting the primary stock solutions with mobile phase.

Calibration standards were prepared by diluting the appropriate working solutions with blank whole human blood from healthy volunteers. The concentrations of calibrants were 2.5, 50, 100, 500, 750 and 1000 ng mL^{-1} of the analytes in whole blood.

2.5. Dried blood spots collection and processing

The venous blood samples (3 mL each) were drawn between 8.00 a.m. and 10.00 a.m. from healthy volunteers and psychiatric patients treated with CLZ and put into test tubes containing EDTA as anticoagulant. The study was approved by local review board and informed consent was obtained for experimentation with human subjects.

DBSs were collected applying calibrated pipette aliquots (25 μL) of venous blood onto the sampling paper; they were dried at room temperature for at least 3 h and, then, the spots were stored at room temperature (25 ± 3 °C with 45 ± 5% of relative humidity) in a sealed plastic bag containing a suitable desiccant (i.e. silica gel).

One dried blood spot (25 μL) from calibration standards or patient samples was cut into small pieces and placed in polypropylene tubes. Twenty-five microlitres of the IS (on column concentration of 50 ng mL^{-1}) and 225 μL of phosphate buffer (50 mM; pH = 7.4) were added in the order as presented. The tubes were shaken on an ultrasonic bath for 5 min and, then, centrifuged at 4000 rpm (1780 × *g*) for 5 min at 4 °C. An aliquot of the supernatant (150 μL) was used for the MEPS procedure (see the section below). The eluate was directly injected into the HPLC system.

2.6. MEPS procedure

The packed syringe for MEPS was activated with 3 × 100 μL of methanol and then conditioned with 3 × 100 μL of water before being used. Methanol and water were picked up and then discarded every time at a flow rate of 20 μL s^{-1}.

An aliquot of 150 μL of the extract from DBS processed (see the section 2.5) was drawn up and down through the syringe 10 times (at a flow rate of 5 μL s^{-1}) without discarding it. The sorbent was washed once with water (100 μL) and once with a mixture of water and methanol (95:5, v/v) to remove biological interference. Then, the analytes were eluted with 150 μL of mobile phase and injected into the HPLC system. All MEPS steps including activation, loading, washing and elution were carried out manually. After each extraction, the cleaning of the sorbent was done with 3 × 100 μL of methanol followed by 3 × 100 μL of water. This step not only decreased memory effects, but also acted as the conditioning step for the next extraction. The same packing bed was used for about 50 extractions; then it was discarded due to both the low analyte extraction yields and clogging of the sorbent.

2.7. Method validation

Method validation procedures were carried out according to USP XXVIII [33] and Crystal City [34] guidelines.

2.7.1. Extraction yield (absolute recovery) and precision

For these studies, blood samples were prepared at three different concentrations of CLZ, DMC and CLZ-NO whole blood (2.5, 500 and 1000 ng mL^{-1}). After spotting on the filter papers and drying, DBSs were treated as reported in the section above (*Dried Blood Spots collection and processing*), subjected to the MEPS procedure and injected into the HPLC.

The peak areas of analytes obtained in this way were compared to those obtained from standard solutions at the same theoretical concentration, and the percentage extraction yield was calculated.

The assays described above were repeated six times within the same day to obtain repeatability (*intraday precision*) and six times over six different days to obtain intermediate precision (*interday precision*), both expressed as percentage relative standard deviation values (RSD%).

2.7.2. Linearity, limit of quantitation, limit of detection

Calibration standards were prepared in triplicate for each point. The analyte/IS peak area ratios obtained were plotted against the corresponding concentrations of the analytes (expressed as ng mL^{-1}) in the 2.5–1000 ng mL^{-1} blood range for all the analytes. The calibration curves were constructed by means of the least-square method. One stock solution was used for each replicate.

The values of limit of quantitation (LOQ) and limit of detection (LOD) were calculated according to official guidelines [33,34] as the analyte concentrations which give rise to peaks whose heights are 10 and 3 times the baseline noise, respectively.

2.7.3. Selectivity

The method selectivity was evaluated by injecting into HPLC system standard solutions of several compounds usually co-administered in psychiatric practice.

Blank DBSs derived from 6 healthy volunteers not receiving CLZ treatment were processed in the absence of the internal standard.

2.7.4. Stability

Stability was tested in stock solutions of all the analytes by comparing the chromatographic peak area ratios (analyte to internal standard) of a standard solution of 50 ng mL^{-1} prepared from stock solutions stored for 3 months at −20 °C with those obtained from the fresh stock samples (n = 3).

Stability assays were also carried out in DBS samples. Human blood aliquots (n = 3) from 4 patients were spotted on filter papers and stored at room temperature (25 ± 3 °C with 45 ± 5% of relative humidity) for fifty days. DBSs were kept in the dark and in plastic bags with a suitable desiccant (i.e. silica gel). After the extraction of the analytes (see sections above), the measured concentrations within fifty days were compared to those of the same samples extracted and analysed immediately after initial spotting and drying.

2.7.5. Accuracy

Analyte standard solutions at three different concentrations (in order to obtain analyte additions of 50, 100 and 250 ng mL^{-1}) were added to DBS samples from patients treated with CLZ whose analytes concentrations were previously analysed; then, the mixture were subjected to the MEPS procedure described above. Recovery values were calculated according to the following formula: 100 × ([after spiking]−[before spiking])/[added].

2.7.6. Bland–Altman plot

The Bland and Altman plot [35] is a statistical method to compare two measurements techniques in clinical chemistry for establishing if they agree sufficiently and therefore, if the new can replace the old. The plot represents a graphical method in which the differences between the two measurements (y-axis) are plotted against the average of the two values (x-axis). If the differences between measurements using the two assay methods lie within the limits of agreement of the Bland–Altman test 95% of the time, this indicates that the two methods are not producing different results.

3. Results and discussion

3.1. Optimization of the chromatographic conditions

The choice of appropriate chromatographic conditions was made starting from an analytical method [32] previously developed

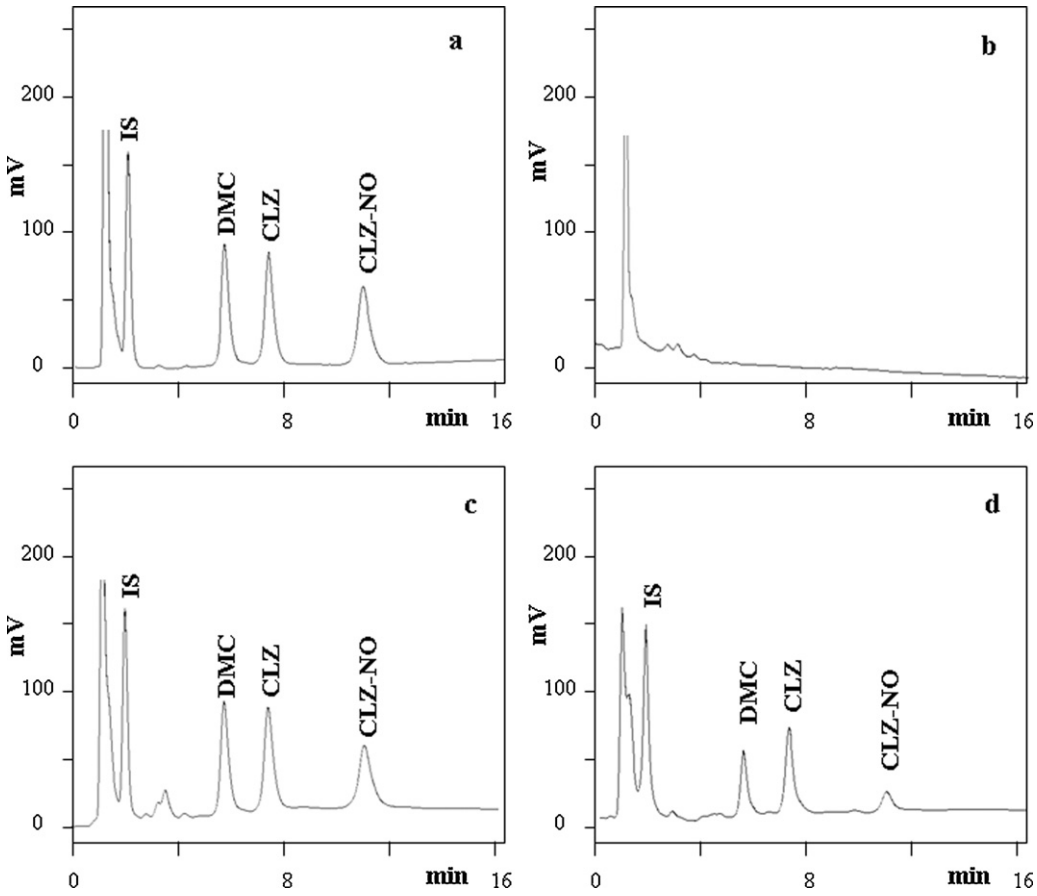

Fig. 2. Chromatograms of (a) a standard solution containing 50.0 ng mL^{-1} of CLZ, DMC, CLZ-NO and IS; (b) a blank DBS sample; (c) a blank DBS sample spiked with 500 ng mL^{-1} of CLZ, DMC, CLZ-NO and IS; and (d) a DBS sample from a psychotic patient treated with 500 mg day^{-1} of CLZ.

for the analysis of CLZ and its metabolites by HPLC with amperometric detector. In order to obtain a better resolution of all analytes within a reasonable run time, the percentages of the aqueous phase and acetonitrile were changed to 64% and 16%, respectively, and the C8 column replaced by a C18 one.

Several trials were carried out to find the best working electrochemical conditions for the coulometric detector, in terms of sensitivity and selectivity. An oxidation potential of +0.500 V in a range from +0.200 to +0.600 V was chosen for E2, obtaining a good sensitivity. For E1, used as the screening electrode a reduction potential of −0.200 V was chosen because of a satisfactory cut-off of biological interference.

The next step was to find a suitable internal standard. Thus, some electroactive compounds such as melatonin, promethazine and 2-methylolanzapine were tested. Among these the most suitable was found to be 2-methylolanzapine which had a chromatographic behaviour similar to those of the analytes and was eluted in a reasonable time.

The chromatogram of a standard solution containing 50 ng mL^{-1} of CLZ, DMC, CLZ-NO and IS is reported in Fig. 2a. As can be seen, the peaks are neat and well resolved. Retention times (t_R) are: CLZ, t_R = 7.8 min (RSD = 1.6%); DMC, t_R = 5.9 min (RSD = 1.5%); CLZ-NO, t_R = 11.7 min (RSD = 1.2%); IS, t_R = 2.0 min (RSD = 1.8%).

3.2. Optimization of the extraction from DBSs

In contrast to the determination in plasma, whole blood is a heterogeneous and complex matrix in which the analytes are distributed between plasma and blood cells according to a partition coefficient. Moreover, hematocrit, which has a notable effect on blood viscosity, may affect flux and diffusion properties of the blood on filter paper. Despite the specific nature of DBS, after drying the blood spots at room temperature for at least 3 h, the main problem was to find a suitable solvent for the extraction of CLZ and its metabolites from the DBSs. The spots were cut into small pieces so as to increase the contact surface with the solvent as well as the extraction yield of the analytes. Organic and aqueous solvents including methanol, acetonitrile and borate, citrate and phosphate buffers were tested. The organic solvents did not allow a good extraction of the analytes and a small amount of the analytes was further lost as a result of the step of drying necessary before loading onto MEPS device. It was decided to use an aqueous buffer with a suitable pH for the quantitative extraction of CLZ, DMC and CLZ-NO from the filter paper. Phosphate buffer (50 mM, pH 7.4) was finally chosen as the extraction solvent which enhanced the absolute recovery of all the analytes (Fig. 3).

3.3. Development of a MEPS procedure

In the analytical field, the choice of the suitable pretreatment of a complex biological matrix like blood is certainly a delicate step. Among the possible options of sample pretreatment (LLE, SPE and microextraction techniques) it was decided to use the microextraction by packed sorbent. The MEPS technique has several advantages over other methods of clean-up. First, its use saves a significant amount for the time of preparing of the biological sample (i.e. 15 min of pretreatment *vs* 45 min of the SPE) and in the amount of solvents used. Moreover, a less sorbent phase (4 mg *vs* 30–100 mg used for SPE) reduced the matrix and carry-over effects, allowing for a good clean-up of the biological matrix.

Table 1
Linearity parameters.

Compound	Linearity range (ng mL^{-1})	Equation coefficients, $y = ax + b$[a]		r[b]	LOQ (ng mL^{-1})[c]	LOD (ng mL^{-1})[c]
		a	b			
DMC	2.5–1000	0.0206	−0.0200	0.9998	0.25	0.08
CLZ	2.5–1000	0.0212	−0.0127	0.9993	0.25	0.08
CLZ-NO	2.5–1000	0.0191	0.0047	0.9996	0.25	0.08

[a] y = analyte/IS peak area ratio; x = analyte concentration, ng mL^{-1}.
[b] r = correlation coefficient.
[c] On-column concentrations.

For the analysis of CLZ and its metabolites a sorbent containing a BIN C8 was used. The loading, washing and elution steps were carefully investigated.

3.3.1. The effect of the number of extraction cycles and flow rate on extraction efficiency

The retention of the analytes to the sorbent phase is affected by the number of extraction cycles performed and the speed applied. Practically, an aliquot of the volume of the sample can be drawn up and down through the syringe, once or several times (cycles) without discarding it. Assays showed that sample response increased as the applied extraction number and sample volume increased. The mean absolute recovery increased from 50% to 93% using a sample volume from 5 × 50 μL to 10 × 100 μL (Fig. 4a). Moreover, during method development, it is important to evaluate the effect of different sample flow rates on the efficiency of analyte adsorption and desorption. An increase of 15% in the response (10 × 100 μL sample volume with C8 sorbent) was observed with a flow rate of 5 μL s^{-1} as compared to a flow rate of 20 μL s^{-1}.

3.3.2. Nature and volume of washing and elution solutions

Considering the complexity of a biological matrix like blood, the washing with only ultrapure water did not allow a good clean-up of the biological sample, however, the addition of a further aliquot of a mixture of ultrapure water and methanol (95:5, v/v) allowed to achieve a reduction of the presence of biological interfering with satisfactory extraction yields of all analytes (Fig. 2b).

Regarding the elution step, the use of the mobile phase as the eluent gave good yields by allowing the direct injection of the eluate into the HPLC system. An aliquot of 150 μL of the mobile phase among those tested (Fig. 4b) gave the best results.

The chromatogram from a DBS sample from a healthy volunteer spiked with the analytes and the IS shows neat and symmetric peaks and no interference from the matrix (Fig. 2c).

3.4. Method validation

Calibration curves were set up for CLZ, DMC and CLZ-NO and good linearity (r > 0.9993) was found in the concentration ranges studied (Table 1). The on column limit of quantitation (LOQ) and the limit of detection (LOD) for CLZ, DMC and CLZ-NO were 0.25 ng mL^{-1} and 0.08 ng mL^{-1}, respectively. Extraction yield and precision assays were carried out at three different concentration levels, corresponding to the lowest level, highest level and middle point of each calibration curve. The results of these assays are reported in Table 2. As one can see, the results are satisfactory, being the extraction yield values are higher than 90.0%. The mean extraction yield of the IS was 93.1%. The precision was also satisfactory, with RSD values always lower than 4.1% for all analytes.

3.5. Stability

The mean difference in peak area ratios (analyte to internal standard) between a standard solution of the analytes (50 ng mL^{-1}) from stock solutions after storage and the standard solution from freshly prepared stock solutions was −0.2%, indicating that all the analytes were stable in methanol when stored at −20 °C for at least 3 months.

When the analytes in DBS samples on filter paper from 4 patients were stored for fifty days at room temperature (see storage condi-

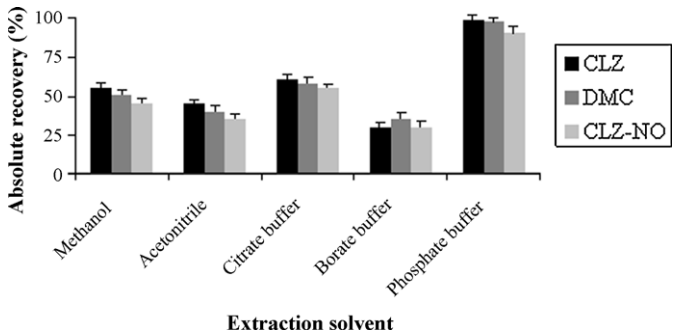

Fig. 3. Solvents tested for the extraction of CLZ, DMC and CLZ-NO from DBSs.

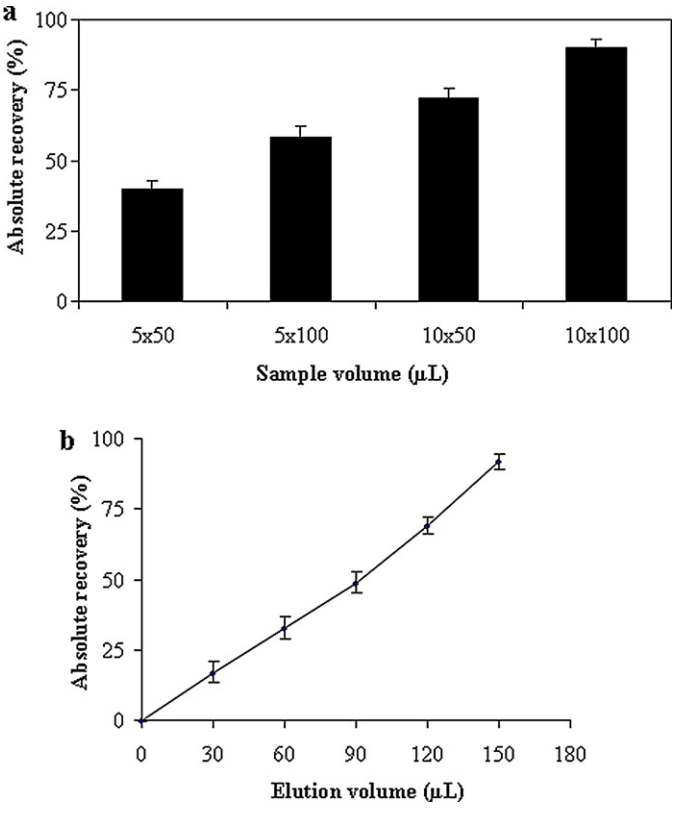

Fig. 4. Mean absolute recovery (±SD) of CLZ, DMC and CLZ-NO as a function of (a) applied sample volume; and (b) elution volume. MEPS conditions are as described in Section 2.

Table 2
- Validation parameters.

Analyte	Amount added (ng mL^{-1})	Extraction yield (%)[a]	Repeatability (RSD%)[a]	Interday precision (RSD%)[a]
DMC	2.5	94.1	2.84	2.86
	500.0	92.1	2.39	2.42
	1000.0	91.4	2.15	2.29
CLZ	2.5	94.6	3.00	3.18
	500.0	92.1	2.80	3.05
	1000.0	90.2	2.63	2.74
CLZ-NO	2.5	96.1	3.71	4.11
	500.0	92.8	3.00	3.30
	1000.0	90.0	2.94	3.07

[a] $n = 6$.

Table 3
- Compounds tested for interference.

Drug	Retention time (min)	Drug	Retention time (min)
Antipsychotics		Sedative-hypnotics	
Aripiprazole	n.d.[a]	Clonazepam	n.d.
Levosulpiride	n.d.	Flurazepam	n.d.
Olanzapine	n.r.[b]	Lorazepam	n.d.
Ziprasidone	n.d.	Antiepileptic agents	
Antidepressants		Valproic acid	n.d.
Trazodone	n.d.	Lamotrigine	n.d.
Fluoxetine	n.d.	Oxcarbamazepine	n.d.
Sertraline	n.d.	Antiparkinson drugs	
Imipramine	20	Orphenadrine	n.d.
Reboxetine	n.d.	Pramipexole	n.r.
Fluvoxamine	n.d.	Ropinirole	n.d.

[a] n.d. = not detected within 30 min.
[b] n.r. = not retained.

tions in Section 2.7.4), the comparison with the fresh spot samples revealed a mean difference of −9%, −11% e−13% in CLZ, DMC and CLZ-NO concentrations, respectively, indicating that the analytes were almost stable under the conditions of storage. In fact, the percentages of loss of the analytes are not significant if one considers the long time of storage (fifty days).

3.6. Selectivity

The method selectivity was evaluated injecting under the optimal chromatographic conditions standard solutions of several compounds usually co-administered in clinical practice, such as antidepressants and sedative-hypnotics. Retention times were compared with those of the analytes. The compounds tested for possible interference are reported in Table 3. As one can see, none of the tested compounds interfered with the chromatographic peaks of the analytes within 30 min.

The analysis of blank DBS samples showed no evidence of unacceptable interferences from endogenous compounds at the retention times of CLZ, DMC, CLZ-NO and the IS (Fig. 2b).

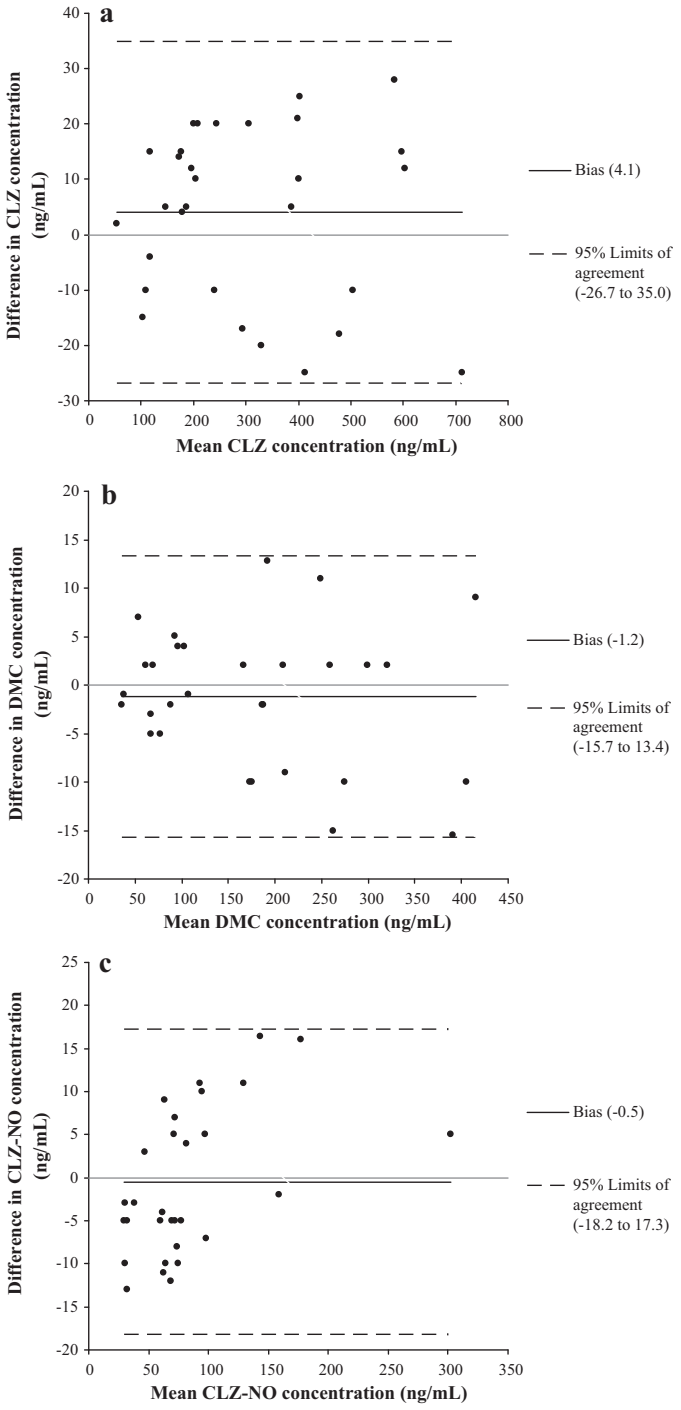

Fig. 5. Bland–Altman plot for (a) CLZ; (b) DMC; and (c) CLZ-NO. The average discrepancy between the two measures of the analyte concentration is acceptable lying between the mean difference ± 2SD.

Table 4
– Results from Bland–Altman test.

Analyte	Bias (ng mL^{-1})[a]	SE (%)[b]	SD (ng mL^{-1})[c]	95% Limit of agreements (ng mL^{-1})	
				Lower	Upper
CLZ	4.1	2.9	15.8	−26.7	35.0
DMC	−1.2	1.4	7.4	−15.7	13.4
CLZ-NO	−0.5	1.7	9.1	−18.2	17.3

[a] Bias represents the mean difference in analyte concentrations between plasma and DBS samples.
[b] SE = standard error.
[c] SD = standard deviation.

3.7. Application of the DBS assay to the analysis of real samples

The method was applied to the analysis of DBS samples ($n = 3$) from several psychiatric patients ($n = 30$). As an example, the chromatogram of a DBS sample from a psychotic patient treated with 500 mg day^{-1} of CLZ is reported in Fig. 2d. The haematic levels found were: 350 ± 5 ng mL^{-1} for CLZ, 227 ± 4 ng mL^{-1} for DMC and 90 ± 7 ng mL^{-1} for CLZ-NO. Results from DBS samples from 30 patients were compared to those from plasma samples from the same patients, analysed by means of another established assay [32]. We note a significant difference in all the analyte concentrations between plasma and blood spot samples for the influence of hematocrit on plasma/whole-blood distribution of the analytes. Therefore, we converted the analyte measurements from DBS to plasma concentrations by multiplying by 1.8, assuming that hematocrit is normally about 40–54% for men and 36–46% for women (mean 45%). The correction factor of 1.8 was calculated according to the following formula: (100/[100−45]). Thus, the analyte hematic levels of the patient from Fig. 2d have to be multiplied by 1.8 to obtain the respective plasmatic levels (630 ± 9 ng mL^{-1} for CLZ, 409 ± 7 ng mL^{-1} for DMC and 162 ± 12 ng mL^{-1} for CLZ-NO); the patient was considered *responder* by clinicians, because the CLZ level was in the plasmatic therapeutic range (300–700 ng mL^{-1}).

The statistical analysis showed a good agreement between the concentrations of all the analytes obtained on DBS samples and those on plasma samples. The Bland–Altman plots are shown in Fig. 5a–c, for CLZ, DMC and CLZ-NO, respectively; while the mean difference or *Bias* (plasma concentration *minus* converted spot concentration) and the 95% limits of agreement of Bland–Altman are reported in Table 4. All differences lay between the mean difference ± 2 SD.

3.7.1. Accuracy

Method accuracy was evaluated by means of recovery studies at three different concentration levels for the analytes ($n = 3$ for each level), as reported in Section 2 above. Results were satisfactory. In fact, mean recovery values were always 92.0% (±2%).

4. Conclusions

An original analytical method based on the use of an HPLC system with coulometric detector and a microextraction by packed sorbent technique for the determination of CLZ and its metabolites in DBSs from psychiatric patients has been developed. The coulometric detector combines better sensitivity and selectivity if compared with other detectors such as UV or DAD or amperometric ones.

The main novelty of the present work deals with the analysis of CLZ and metabolites in DBSs with significant advantages with regards to sampling (minimally invasive), transport and storage of biological materials (in plastic bags at room temperature), requirements for special biohazard arrangements and volume of blood used (25 μL). In addition, the development of an original procedure for the clean-up of the biological sample by means of MEPS (extraction yields > 90.0%) represents another improvement of the method in comparison with those previously published. In fact, MEPS is a technique for miniaturized solid-phase extraction which reduces the sample volume and the time necessary for the analysis. Moreover, the MEPS procedure developed was simpler and faster than conventional extraction techniques (liquid–liquid and SPE) and limited the consumption of organic solvents thus also reducing the testing cost. Good results were obtained in terms of precision (RSD < 4.1%), sensitivity (LOQ = 0.25 ng mL^{-1} for all the analytes) and accuracy (mean recoveries = 92.0%). The selectivity was also satisfactory.

Thus, the proposed method is suitable for the analysis of CLZ and its metabolites in DBSs from psychiatric patients for therapeutic drug monitoring purpose.

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References

[1] M.A. Raggi, Curr. Med. Chem. 9 (2002) 1397.
[2] M.A. Raggi, R. Mandrioli, C. Sabbioni, V. Pucci, Curr. Med. Chem. 11 (2004) 279.
[3] S.C. Sweetman, Martindale – The Complete Drug Reference, 36th ed., Pharmaceutical Press, London, 2009, p. 981.
[4] R. Guthrie, A. Susi, Pediatrics 32 (1963) 338.
[5] J.V. Mei, J.R. Alexander, B.W. Adam, W.H. Hannon, J. Nutr. 131 (2001) 1631S.
[6] S. AbuRuz, J. Millership, J. McElnay, J. Chromatogr. B 832 (2006) 202.
[7] D. Blessborn, S. Ro¨msing, A. Annerberg, D. Sundquist, A. Bjo¨rkman, N. Lindegardh, Y. Bergqvist, J. Pharm. Biomed. Anal. 45 (2007) 282.
[8] O.M. Minzi, A.Y. Massele, L.L. Gustafsson, O. Ericsson, J. Chromatogr. B 814 (2005) 179.
[9] N. Spooner, R. Lad, M. Barfield, Anal. Chem. 81 (2009) 1557.
[10] E. Choong, S. Rudaz, A. Kottelat, D. Guillarme, J.L. Veuthey, C.B. Eap, J. Pharm. Biomed. Anal. 50 (2009) 1000.
[11] J. Hermida, E. Paz, J.C. Tutor, Ther. Drug Monit. 30 (2008) 41.
[12] M. Rosland, P. Szeto, R. Procyshyn, A.M. Barr, K.M. Wasan, Drug Dev. Ind. Pharm. 33 (2007) 1158.
[13] S. Mennickent, A. Sobarzo, M. Vega, C.G. Godoy, M. de Diego, J. Sep. Sci. 30 (2007) 2167.
[14] L. Mercolini, M. Grillo, C. Bartoletti, G. Boncompagni, M.A. Raggi, Anal. Bioanal. Chem. 388 (2007) 235.
[15] L. Mercolini, F. Bugamelli, E. Kenndler, G. Boncompagni, L. Franchini, M.A. Raggi, J. Chromatogr. B 846 (2007) 273.
[16] H. Kirchherr, W.N. Kühn-Velten, J. Chromatogr. B 843 (2006) 100.
[17] H.A. Niederländer, E.H. Koster, M.J. Hilhorst, H.J. Metting, M. Eilders, B. Ooms, G.J. de Jong, J. Chromatogr. B 834 (2006) 98.
[18] J. Sachse, J. Köller, S. Härtter, C. Hiemke, J. Chromatogr. B 830 (2006) 342.
[19] Z. Zhou, X. Li, K. Li, Z. Xie, Z. Cheng, W. Peng, F. Wang, R. Zhu, H. Li, J. Chromatogr. B 802 (2004) 257.
[20] L. Garay Garcia, I. Forfar-Bares, F. Pehourcq, C. Jarry, J. Chromatogr. B 795 (2003) 257.
[21] C. Frahnert, M.L. Rao, K. Grasmäder, J. Chromatogr. B 794 (2003) 35.
[22] K. Titier, S. Bouchet, F. Péhourcq, N. Moore, M. Molimard, J. Chromatogr. B 788 (2003) 179.
[23] R. Waschgler, M.R. Hubmann, A. Conca, W. Moll, P. König, Int. J. Clin. Pharmacol. Ther. 40 (2002) 554.
[24] M. Kollroser, C. Schober, Rapid Commun. Mass Spectrom. 16 (2002) 1266.
[25] H. Weigmann, S. Härtter, S. Maehrlein, W. Kiefer, G. Krämer, G. Dannhardt, C. Hiemke, J. Chromatogr. B 759 (2001) 63.
[26] M. Aravagiri, S.R. Marder, J. Pharmaceut. Biomed. 26 (2001) 301.
[27] Y.Y. Liu, L.J. van Troostwijk, H. Guchelaar, J. Biomed. Chromatogr. 15 (2001) 280.
[28] A. Llerena, R. Berecz, M.J. Norberto, A. de la Rubia, J. Chromatogr. B 755 (2001) 349.
[29] G. Dumortier, A. Lochu, A. Zerrouk, V. Van Nieuwenhuyse, P. Colen de Melo, D. Roche Rabreau, K. Degrassat, J. Clin. Pharm. Ther. 23 (1998) 35.
[30] M.A. Raggi, F. Bugamelli, R. Mandrioli, D. De Ronchi, V. Volterra, Chromatographia 47 (1998) 8.
[31] M.A. Raggi, F. Bugamelli, R. Mandrioli, D. De Ronchi, V. Volterra, Chromatographia 49 (1999) 75.
[32] M.A. Raggi, F. Bugamelli, C. Sabbioni, D. De Ronchi, S. Pinzauti, V. Volterra, Chromatographia 51 (2000) 147.
[33] United States Pharmacopeia, United States Pharmacopeial Convention, Rockville MD, 32th ed., 2009, p. 734.
[34] V.P. Shah, K.K. Midha, J.W.A. Findlay, H.M. Hill, J.D. Hulse, I.J. McGilveray, G. McKay, K.J. Miller, R.N. Patnaik, M.L. Powell, A. Tonelli, C.T. Viswanathan, A. Yacobi, Pharm. Res. 17 (2000) 1551.
[35] J.M. Bland, D.G. Altman, Lancet 1 (1986) 307.