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Simultaneous determination of the antipsychotic drugs levomepromazine and clozapine and their main metabolites in human plasma by a HPLC-UV method with solid-phase extraction

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Abstract

A HPLC method with UV detection has been developed for the simultaneous determination of levomepromazine, clozapine and their main metabolites: *N*-desmethyl-levomepromazine, levomepromazine sulphoxide, *O*-desmethyl-levomepromazine, *N*-desmethylclozapine and clozapine *N*-oxide. The analytes were separated on a C8 reversed-phase column using a mobile phase composed of acetonitrile and a pH 2.0, 34 mM phosphate buffer containing 0.3% triethylamine (29:71, v/v). Loxapine was used as the internal standard. A reliable biological sample pre-treatment procedure by means of solid-phase extraction on C1 cartridges was implemented, which allows to obtain good extraction yields (>91%) for all analytes and appropriate sample purification from endogenous interference. The method was validated in terms of extraction yield, precision and accuracy. These assays gave RSD% values for precision always lower than 4.9% and mean accuracy values higher than 92%. The method is suitable for the therapeutic drug monitoring (TDM) of patients undergoing polypharmacy with levomepromazine and clozapine.

Keywords: Levomepromazine; Clozapine; Metabolites; Human plasma; Liquid chromatography; Solid-phase extraction

1. Introduction

The treatment of severe forms of schizophrenia often requires the simultaneous use of two antipsychotic agents in order to achieve sufficient control of psychotic symptoms [1]. Usually, the polypharmacy is carried out with antipsychotics belonging to different classes, i.e., a "classical" neuroleptic (levomepromazine, chlorpromazine, haloperidol) complemented with an "atypical" antipsychotic (clozapine, olanzapine, risperidone) [2]. This approach should allow to take advantage of the different mechanisms of action of the two drugs [3].

Levomepromazine or methotrimeprazine (Nozinan, 2-methoxy-*N*,*N*,β-trimethyl-10*H*-phenothiazine-10-propanamine, LMP, Fig. 1) is a classical phenothiazine neuroleptic. It is used in the treatment of schizophrenia, paranoia, mania, toxic psychoses and mental organic syndromes associated with delirium. It is only active against positive symptoms of schizophrenia and its administration is often associated with the onset of extrapyramidal side effects and hyperprolactinemia. It is also used in the treatment of nausea in advanced cancer patients [4] and as a sedative in terminal care and burn patients [5].

Plasma half-life is widely variable (15–78 h) [6]. LMP is extensively metabolised; the main metabolites formed are *N*-desmethyl-levomepromazine (NDML) and levomepromazine sulphoxide (LMSO); minor metabolites are *O*-desmethyl-levomepromazine (ODML), 3-hydroxylevomepromazine and 7-hydroxylevomepromazine (the respective chemical structures are reported in Fig. 1). Among these, only NDML seems to possess an antipsychotic activity similar to that of the parent drug, while LMSO does not appear to contribute to the therapeutic effect but could be involved in the onset of autonomic side effects [7].

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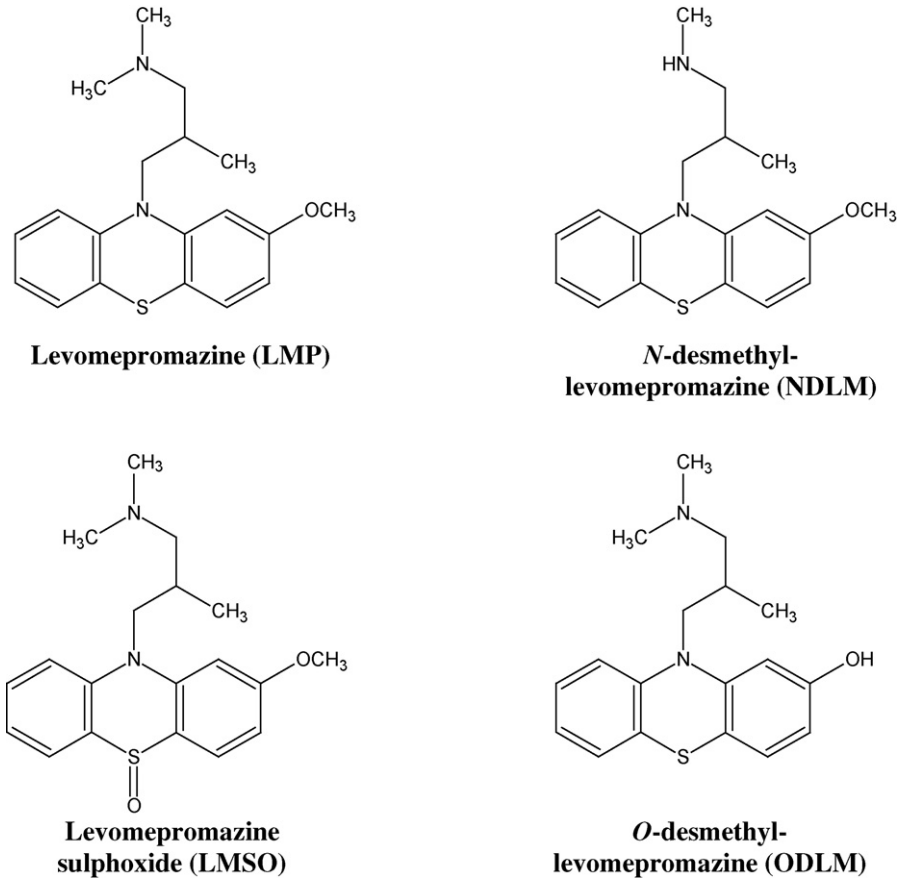

Fig. 1. Chemical structures of levomepromazine and its metabolites.

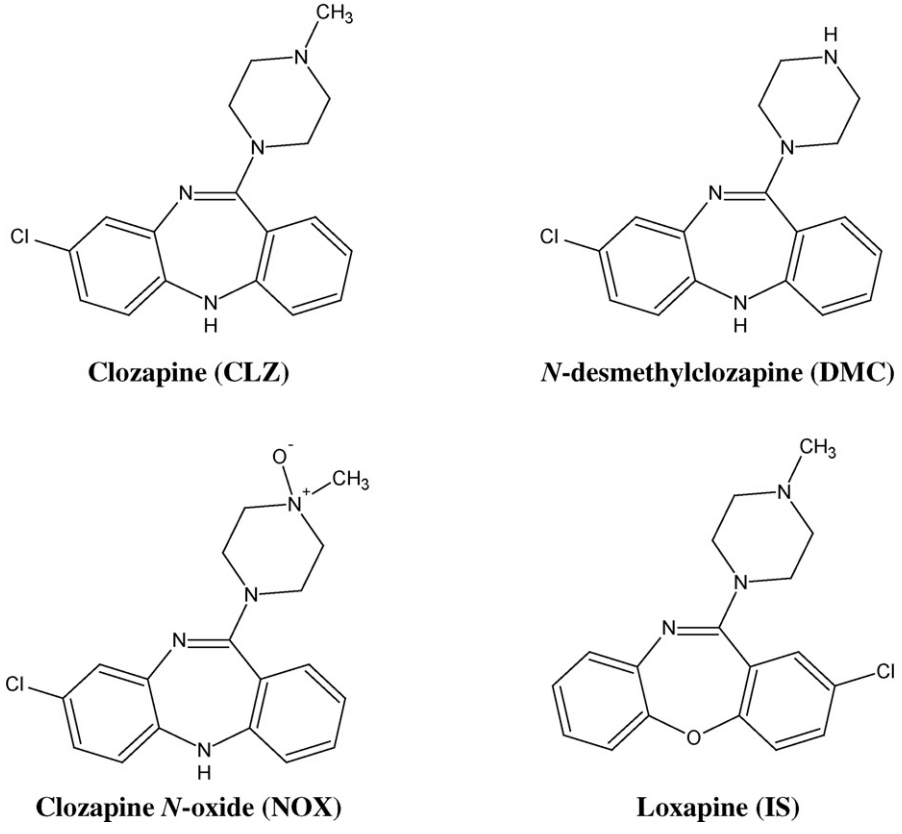

Fig. 2. Chemical structures of clozapine and its metabolites.

Clozapine (Clozaril or Leponex, 8-chloro-11-(4-methyl-1-piperazinyl)-5*H*-dibenzo[*b*,*e*][1,4]diazepine, CLZ, Fig. 2) was the first "atypical antipsychotic" introduced in therapy. It is a serotonergic (5-HT_2) antagonist and a dopaminergic (D_2) antagonist, with affinity also toward histamine, adrenergic and cholinergic receptors [8]. Clozapine has some advantages with respect to classical neuroleptics; it causes a lower rate of extrapyramidal side effects [9] and hyperprolactinemia [10], however it can cause severe agranulocytosis [11].

A therapeutic response threshold has been found for CLZ at about 350 ng/ml plasma concentration [12,13]; it has been also noted that the risk of delirium, confusion and seizures increases above 800 ng/ml [14].

The two main metabolites of CLZ are *N*-desmethylclozapine (DMC) and clozapine *N*-oxide (NOX), which have shorter and weaker pharmacological activity and also reach lower plasma levels than CLZ: 50–70% for DMC and 10–20% for NOX [15] (the respective chemical structures are reported in Fig. 2).

The aim of this research was to develop a method for the analysis of LMP, CLZ and their metabolites based on HPLC with UV detection in order to carry out the therapeutic drug monitoring (TDM) of patients undergoing polypharmacy with the two drugs and in particular to monitor possible pharmacological interactions between them.

To our best knowledge no analytical method has been described in the literature which simultaneously determines these two analytes with their metabolites, even though several methods exist, which separately determine either LMP and its metabolites [16–19] or CLZ and its metabolites [13,20–26]. Other papers report the analysis of LMP and CLZ together with many other Central Nervous System (CNS) drugs for toxicological and forensic screening, however the methods are not validated for quantitative purposes [27,28] and metabolites are either not considered [27] or only qualitatively identified [28].

2. Materials and methods

2.1. Chemicals

Levomepromazine, *N*-desmethyl-levomepromazine, levomepromazine sulphoxide and *O*-desmethyl-levomepromazine were kindly provided by Sanofi–Aventis (Paris, France), clozapine, *N*-desmethylclozapine and clozapine *N*-oxide by Novartis Italia (Origgio, Italy). Methanol, acetonitrile (HPLC grade), 85% (w/w) phosphoric acid and triethylamine were from Carlo Erba (Milan, Italy). Loxapine used as the Internal Standard (IS, Fig. 2) was a kind donation of Lederle Laboratories (Gosport, Hampshire, England). Ultrapure water (18.2 MΩ cm) was obtained by means of a MilliQ apparatus by Millipore (Milford, Mass., USA).

2.2. Solutions

Stock solutions of the analytes (1 mg/ml) were prepared by dissolving suitable amounts of each pure substance in methanol. Standard solutions were prepared by diluting stock solutions with the mobile phase. Stock solutions were stable for at least two months when stored at −20 °C (as assessed by HPLC); standard solutions were prepared afresh every day.

2.3. Chromatographic apparatus and conditions

The chromatographic system consisted of a Jasco (Tokyo, Japan) PU-980 isocratic pump and a Jasco UV-975 spectrophotometric detector set at 254 nm. The C8 reversed-phase column (150 mm × 4.6 mm I.D., 5 μm; Phenomenex, Torrance, CA, USA) was supplemented with a C8 cartridge precolumn. The mobile phase was a mixture of acetonitrile and a phosphate buffer (34 mM, pH 2.0) containing 0.3% triethylamine (29:71, v/v). Flow rate was 1.0 ml/min and injections were carried out though a 20 μL loop.

2.4. Human plasma sampling

Blood samples were drawn from patients subjected to simultaneous therapy with LMP and CLZ. Both drugs were administered twice daily, at 8.00 a.m. and at 8.00 p.m., and the samples were taken 12 hours after the last drug administration (i.e., usually at 8.00 a.m., immediately before the morning dose). The samples were put into test tubes containing EDTA as the anticoagulant and centrifuged at 1400 × *g* for 15 min (*T* = 4 °C). The supernatant plasma was transferred into 1.5 ml polypropylene test tubes and frozen at −20 °C until analysis, which was usually carried out within two weeks.

"Blank" plasma was obtained from the blood of healthy volunteers and subjected to the same procedure as reported above for patients' plasma.

2.5. Solid-phase extraction procedure (SPE)

Plasma samples were pre-treated by means of SPE with Varian (Walnut Creek, USA) BondElut C1 cartridges (100 mg, 1 ml). The cartridges were equilibrated with 1 ml of methanol 5 times, then conditioned with 1 ml of ultrapure water 5 times. An aliquot of 250 μL of plasma was diluted with 250 μL of water, then 50 μL of IS (loxapine) were added; the resulting mixture was loaded onto the cartridge. Washing was carried out with 1 ml of water 3 times, then 1 ml of 20% methanol aqueous solution two times, then 50 μL of methanol. The analytes were eluted with 1 ml of methanol, then the eluate was dried under vacuum, redissolved with 125 μL of mobile phase and finally injected into the HPLC system.

2.6. Method validation

2.6.1. Calibration curves

Aliquots of 50 μL of analyte standard solutions at seven different concentrations containing the IS at a constant concentration were added to 250 μL of blank plasma. The resulting plasma concentration ranges were: LMP 9–200 ng/ml; NDLM 10–150 ng/ml; LMSO 5–500 ng/ml; ODLM 7–150 ng/ml; CLZ 20–2500 ng/ml; DMC 15–1000 ng/ml; NOX 10–200 ng/ml; IS 200 ng/ml (constant). The mixtures were subjected to the previously described SPE procedure and analysed by HPLC. The

procedure was carried out in triplicate for each concentration. The obtained analyte/IS peak area ratios (pure numbers) were plotted against the corresponding concentrations of the analytes (expressed as ng/ml) and the calibration curves were set up by means of the least-square method. One stock solution was used for each replicate; different working solutions were prepared from the stock solutions and added to the blank plasma samples to obtain the different concentrations.

The values of limit of quantification (LOQ) and limit of detection (LOD) were calculated according to USP XXVIII [29] and "Crystal City" [30] guidelines as the analyte concentrations which give rise to peaks whose heights are 10 and 3 times the standard deviation of the baseline noise, respectively.

2.6.2. Extraction yield (absolute recovery)

The procedure was the same as that described under "Calibration Curves", above, except the points were at 3 different concentrations, corresponding to the lower limit, middle point and upper limit of each calibration curve. The analyte peak areas were compared to those obtained injecting standard solutions at the same theoretical concentrations and the extraction yield values were calculated.

2.6.3. Precision

The assays described under "Extraction yield" were repeated six times within the same day to obtain the repeatability (intra-day precision) and six times over different days to obtain the intermediate precision (inter-day precision) [8] of analyte/IS peak area ratios, expressed as RSD% values.

2.6.4. Selectivity, stability, carry-over

Selectivity was assessed both by injecting 6 different blank plasma samples and by injecting sample solutions of different compounds active on the CNS. Long-term frozen stability, room temperature stability and carry-over from previous runs were also evaluated. Freeze–thaw cycles were not carried out since the samples were subdivided into small aliquots and frozen immediately after plasma separation and thus were not frozen again after the first thawing.

2.6.5. Accuracy

Accuracy was evaluated by means of recovery assays. The assays described under "Extraction yield" were carried out adding standard solutions of the analytes and the IS (loxapine) to real plasma samples taken from patients subjected to treatment with LMP and CLZ. The assays were repeated three times during the same day to obtain mean recovery and standard deviation data.

3. Results and discussion

3.1. Preliminary experiments

Some patients undergoing treatment of CLZ, for which TDM was carried out, were treated with LMP as well. This prompted us to develop an analytical method for the simultaneous analysis of these drugs and their most significant metabolites. The starting point of this assay was the method previously developed by us for the analysis of CLZ and metabolites, based on HPLC with amperometric detection [20]. The method uses a C18 reversed-phase column (150 mm × 4.6 mm i.d., 5 μm) with a mobile phase composed of acetonitrile, methanol and 10.4 mM, pH 1.9 phosphate buffer (17.5:20:62.5, v/v/v), containing 0.25% (v/v) triethylamine. The detection voltage was +800 mV and the column was thermostatted at 31 °C. The method, however, was not suitable for the determination of LMP and metabolites. In fact, the NDML peak overlapped that of LMP, and the run time was too long (about 30 min); ODML was detected at about 10 min, while LMSO cannot be detected (it is not oxidisable under these conditions). Moreover, sensitivity was limited with regard to LMP and its detectable metabolites. So it was decided that a new method, based on HPLC with UV detection, was needed in order to carry out the simultaneous determination of all analytes.

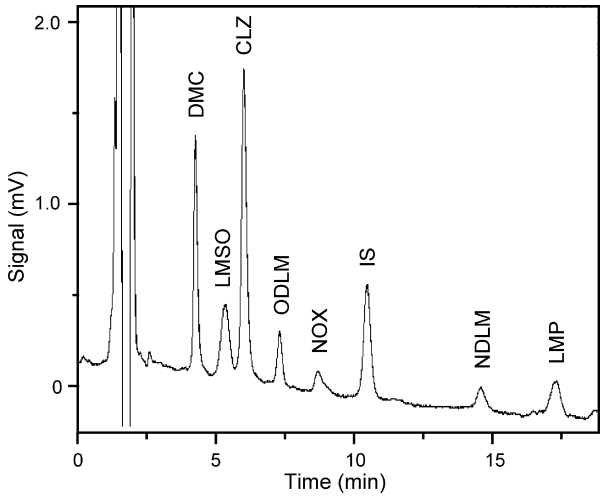

Fig. 3. Chromatograms of a standard solution of the analytes. Analyte concentrations: DMC, 200 ng/ml; LMSO, 100 ng/ml; CLZ, 400 ng/ml; ODLM, 50 ng/ml; NOX, 100 ng/ml; IS, 200 ng/ml; NDLM, 100 ng/ml; LMP, 100 ng/ml.

3.2. Chromatographic conditions

Different ratios of acetonitrile to phosphate buffer (containing triethylamine and adjusted to acidic pH values) were tested for their suitability for the analysis. The organic/aqueous ratio was varied between 50/50 (v/v) and 15/85 (v/v) in order to obtain baseline separation of all analytes while keeping run times to a minimum. Best results were obtained with a 29/71 (v/v) ratio: lower percentages of organic modifier lengthen the analysis, while higher percentages cause overlapping of the less retained analytes (i.e., DMC, LMSO and CLZ).

Under the finally selected conditions, all seven analytes were baseline separated within a 18 min chromatographic run (Fig. 3). Loxapine was used as the internal standard (IS) and the spectrophotometric detector was set at 254 nm.

3.3. Solid-phase extraction procedure

Other authors [16] have previously reported that weakly lipophilic (C2) cartridges are suitable for the pre-treatment of

Table 1
Extraction yield and precision results

Compound	Concentration (ng/mL)	Mean extraction yield, %[a]	Repeatability, RSD%[a]	Intermediate precision, RSD%[a]
LMP	10	93.9	2.5	4.6
	100	96.9	1.8	3.5
	200	98.7	1.0	2.8
NDLM	10	101.0	4.7	4.9
	75	98.6	3.9	3.8
	150	97.6	2.7	2.8
LMSO	5	99.1	2.3	2.5
	250	95.0	1.3	2.1
	500	98.5	1.1	1.7
ODLM	10	97.9	2.1	4.0
	75	94.5	1.3	3.7
	150	91.7	1.1	2.0
CLZ	20	95.1	1.8	4.9
	1000	95.6	1.7	4.5
	2500	93.7	0.7	1.9
DMC	15	91.2	4.4	4.8
	500	91.1	3.3	4.0
	1000	94.2	1.1	1.8
NOX	10	94.4	4.5	4.9
	100	94.7	4.2	4.7
	200	93.9	3.0	3.5
IS	200	94.3	2.8	3.1

[a] $n = 6$.

plasma samples containing LMP and metabolites. Since CLZ and its metabolites are mostly less lipophilic than LMP and metabolites, a C1 sorbent was tried, and it allowed to obtain good extraction yields for all analytes. Furthermore, other improvements were made to the procedure. Firstly, the plasma volume loaded was lowered from 600 μL to 250 μL in order to spare plasma sample. The cartridges were then washed with water and a water/methanol mixture: preliminary assays showed that the highest percentage of methanol which allowed to retain the analytes while eluting most endogenous compounds was 20%. An aliquot of 50 μL of methanol was added as the final washing step since this volume of eluate did not contain any detectable amount of the analytes. A single elution step with 1 ml of pure methanol allowed to obtain high extraction yields of all analytes. The eluate was then dried and redissolved with 125 μL of the mobile phase. From the complete procedure described in the Experimental section, it can be seen that 50 μL of standard solution are always added to the plasma samples before loading. Since 50 μL of standard solution on 250 μL of plasma represent a substantial dilution of the sample, preliminary assays were carried out using smaller volumes of standard solution (10 μL and 25 μL). The analytical results obtained with the smaller volumes were not significantly different from those obtained when spiking the samples with 50 μL (standard error lower than 1%). Thus, 50 μL were chosen for ease of use.

Using the procedure developed, good purification of the biological matrix and satisfactory extraction yield results were obtained (Table 1). As an example, the chromatograms of a blank plasma sample and of the same plasma sample spiked with the analytes are reported in Fig. 4. As can be seen, a few small peaks from endogenous plasma compounds are still present, however none of them causes any interference with the analytical determination.

3.4. Method validation

Having thus assured the suitability of the SPE procedure, calibration curves were set up on blank plasma by adding to the plasma standard solutions of the analytes at different concentrations (and of the IS at a constant 200 ng/ml concentration) and subjecting the resulting mixture to the SPE procedure. The complete details of linearity assays are reported in Table 2: as can be seen, good linearity ($r^2 > 0.9940$) was obtained for all the analytes. LOQ values are suitable for the reliable determination of the analytes and the values of LOD confirm the practical applicability of the method.

Extraction yield (absolute recovery) and precision assays were carried out on blank plasma spiked with analyte concentrations corresponding to the lower limit, middle point and upper limit of the calibration curves. The results of these assays are reported in Table 1. As one can note, mean extraction yields were always higher than 91% for all analytes (94.3% for the IS). Precision results were also satisfactory: RSD values for repeatability were always lower than or equal to 4.7% (2.8% for the IS); RSD values for intermediate precision were lower than or equal to 4.9% (3.1% for the IS).

Both long-term frozen stability and room temperature stability assays were carried out. Plasma samples stored at −20 °C for 2 months and then analysed reproduced the original results with

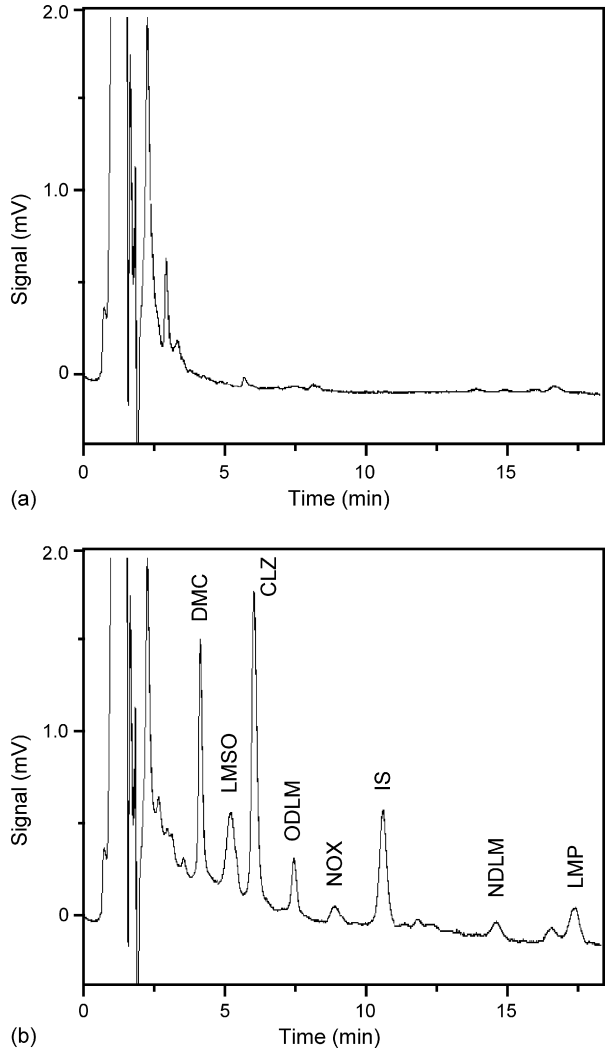

Fig. 4. Chromatograms of (a) a blank plasma sample and (b) the same plasma sample to which standard solutions of the analytes were added. Analyte concentrations added: DMC, 100 ng/ml; LMSO, 50 ng/ml; CLZ, 200 ng/ml; ODLM, 25 ng/ml; NOX, 50 ng/ml; IS, 200 ng/ml; NDLM, 50 ng/ml; LMP, 50 ng/ml (plasma concentrations).

a mean standard error of 3%; samples kept at room temperature for up to 8 h gave reproducible results (RSD = 2%).

Carry-over from previous injections was also evaluated after injecting plasma samples; it was noted that no potentially interfering peak is detected for a span of time corresponding to two consecutive analytical runs (i.e., for 40 min after the end of the first run).

3.4.1. Selectivity

Selectivity was evaluated by injecting into the HPLC "blank" plasma samples drawn from six different volunteers not subjected to any pharmacological therapy. None of them produced peaks which could interfere with the analysis. Further proof of selectivity (with respect to exogenous compounds) was obtained from the injection of standard solutions of several drugs (mainly CNS drugs: antipsychotics, antidepressants and antiepileptics) which can be coadministered with LMP and/or CLZ in psychiatric clinics. The complete list of these drugs is reported in Table 3. As can be seen, none of the tested drugs had a retention time similar to those of the analytes or the IS. Therefore, selectivity was deemed satisfactory. Those drugs which were detected within a 30 min run were also tested for extraction with the proposed SPE prodedure. Amisulpride, chlorpromazine, haloperidol, risperidone and carbamazepine gave extraction yield results higher than 80% (SD = 2–5%) and can thus at least semiquantitatively be determined in plasma samples.

3.5. Analysis of patient plasma samples

After validating the method, it was applied to the analysis of plasma samples from some schizophrenic patients of Mental Health Departments (Bologna, Italy) undergoing simultaneous therapy with LMP and CLZ. The chromatogram of a plasma sample from a patient treated with 200 mg/day of LMP and 450 mg/day of CLZ (as well as 1200 mg/day of amisulpride) is shown in Fig. 5. Peak shapes and resolution are very similar to those obtained using spiked blank plasma and no interference is apparent. Analyte concentrations found in this real sample were: DMC 78 ng/ml; LMSO 161 ng/ml; CLZ 84 ng/ml; ODLM 45 ng/ml; NOX 60 ng/ml; NDLM 73 ng/ml; LMP 116 ng/ml. The analysis was carried out on a plasma sample from another patient treated with 50 ng/ml of LMP

Table 2
Linearity parameters

Compound	Concentration range (ng/mL)[a]	Equation coefficients, $y = a + bx$[b]		r^2	LOQ (ng/mL)[a]	LOD (ng/mL)[a]
		a	b			
LMP	9–200	−0.0124	0.0784	0.9969	9	3
NDLM	10–150	0.0320	0.0651	0.9950	10	3
LMSO	5–500	0.0554	0.1039	0.9978	5	2
ODLM	7–150	0.0190	0.0870	0.9959	7	3
CLZ	20–2500	−0.0117	0.0666	0.9980	20	7
DMC	15–1000	−0.0953	0.0901	0.9981	15	5
NOX	10–200	−0.0784	0.0549	0.9944	10	3

[a] The reported values are plasma concentrations; due to the SPE procedure, the concentrations of the injected solutions can be found by multiplying these values by 2.

[b] y = analyte/IS peak area ratio (pure number); x = analyte concentration, ng/mL; a = analyte/IS peak area ratio (pure number); b = 1/analyte concentration, mL/ng.

Table 3
Drugs tested for interference

Therapeutic class	Compound	t_R (min)
Analytes	*N*-desmethylclozapine	4.2
	Levomepromazine sulphoxide	5.1
	Clozapine	5.9
	O-Desmethyl-levomepromazine	7.4
	Clozapine *N*-oxide	8.6
	Loxapine (IS)	10.2
	N-Desmethyl-levomepromazine	14.3
	Levomepromazine	17.3
Antidepressants	Amitriptyline	18.0
	Amoxapine	15.2
	Imipramine	7.9
	Mirtazapine	16.1
Antipsychotics	Amisulpride	2.9
	Chlorpromazine	21.1
	Haloperidol	19.0
	9-hydroxyrisperidone	2.8
	Risperidone	3.3
	Thioridazine	22.4
Antiepileptics	Carbamazepine	20.1
	10,11-Dihydro-10,11-epoxycarbamazepine	12.4
	10,11-Dihydro-10,11-dihydroxycarbamazepine	8.1
Other	Amiloride	2.2
	Indomethacin	12.5
	Triprolidine	9.4

Table 4
Accuracy and precision results

Compound	Concentration (ng/mL)	Mean recovery (%)[a]	Precision, SD[a]
LMP	9	94.7	3.5
	50	95.3	3.0
	100	95.1	2.8
NDLM	10	96.1	4.7
	50	96.4	3.9
	100	97.8	2.7
LMSO	5	97.3	4.7
	125	97.1	4.3
	250	99.0	3.1
ODLM	7	94.4	4.0
	50	95.4	3.3
	100	95.2	3.1
CLZ	20	93.8	2.8
	500	95.6	2.1
	1000	95.4	1.7
DMC	15	92.5	4.4
	250	93.5	2.5
	500	93.0	1.8
NOX	10	92.7	4.3
	50	93.1	4.0
	100	93.8	3.2

[a] $n = 3$.

and 600 ng/mL of CLZ, giving the following results: DMC 107 ng/ml; LMSO 111 ng/ml; CLZ 207 ng/ml; ODLM 27 ng/ml; NOX 40 ng/ml; NDLM 21 ng/ml; LMP 33 ng/ml. It can be noted that plasma levels of CLZ and metabolites are quite low with respect to the usual levels found with this dose [31], while plasma levels of LMP and metabolites are normal [32]. The SPE procedure used for the sample pre-treatment allows to extract amisulpride as well: the drug is visible as a neat chromatographic peak at $t_R = 2.9$ min. While formal method validation was not carried out for amisulpride, a semiquantitative estimate of this compound was possible: its concentration in this sample was about 500 ng/ml.

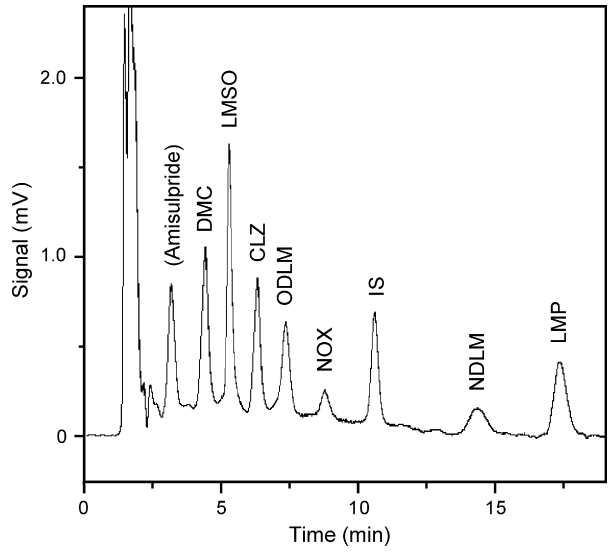

Fig. 5. Chromatogram of a plasma sample from a patient undergoing therapy with 200 mg/day of LMP and 450 mg/day of CLZ (as well as 1200 mg/day of amisulpride).

Accuracy was evaluated by means of recovery assays. Standard solutions of the analytes at three different concentrations were added to plasma samples containing known amounts of LMP, CLZ and metabolites (i.e., samples which had been already analysed). Then, the recovery of the analytes was calculated, as well as the standard deviation of the assays. As can be seen from Table 4, method accuracy is satisfactory, in fact mean recovery ± SD values were: 95.0 ± 3.1 for LMP, 96.8 ± 3.8 for NDLM, 97.8 ± 4.0 for LMSO, 95.0 ± 3.5 for ODLM, 94.9 ± 2.2 for CLZ, 93.9 ± 2.9 for DMC and 93.2 ± 3.8 for NOX.

3.6. Concluding remarks

A HPLC-UV method is described for the simultaneous determination of LMP and CLZ including their main metabolites. It has been developed for the TDM of schizophrenic patients undergoing polypharmacy with these drugs. Separation was carried out on a C8 column; the mobile phase was a phosphate buffer containing triethylamine and adjusted to acidic pH and containing 29% (v/v) acetonitrile as the organic modifier. Baseline separation of the 7 analytes (and the IS) was achieved in less than 20 min. The analytes were preconcentrated by the aid of SPE on C1 columns, obtaining good extraction yield results (>91%). The method was validated according to internationally accepted guidelines, demonstrating satisfactory precision

(RSD < 4.9%) and good selectivity. The method has been successfully applied to the analysis of plasma samples from patients subjected to treatment with LMP and CLZ, providing excellent accuracy results (recovery > 92%).

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