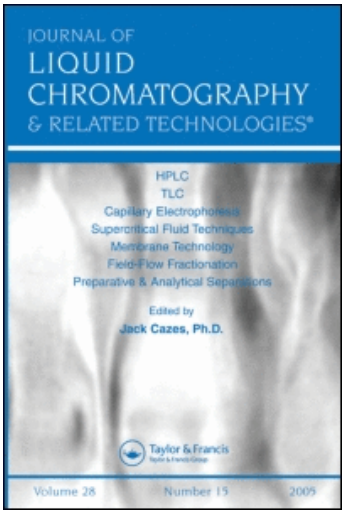

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Study of Separation and Extraction Conditions for Five Neuroleptic Drugs by an LLE-HPLC-DAD Method in Human Plasma

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Abstract: Separation and extraction conditions for four atypical neuroleptics (clozapine, N-desmethylclozapine (active metabolite of clozapine), olanzapine, and quetiapine) and one classical neuroleptic (perazine) in human plasma have been studied. Reverse phase chromatography (RP-18) with the mobile phase consisted of: A. aq. orthophosphoric acid with addition of N,N,N′,N′-tetramethylethylendiamine (TEMED), pH = 5.0, and B. acetonitrile, in gradient flow, was selected. As the optimal liquid-liquid extraction conditions, sample pH = 11.6, extraction system, ethyl acetate/n-hexane/isopropanol (16:3:1, v/v/v), and back extraction into 0.01% orthophosphoric acid were chosen.

Keywords: Antipsychotic drugs, Atypical neuroleptics, High performance liquid chromatography, Human plasma, Liquid–liquid extraction, Phenothiazine neuroleptics

INTRODUCTION

In clinical practice, pharmacological treatment of schizophrenia and similar psychotic disorders is usually carried out with antipsychotic drugs. Nowadays, approximately thirty different antipsychotic drugs

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are available, which can be classified in two main groups: classical and atypical neuroleptics.

Atypical neuroleptics (e.g., clozapine, risperidon) in relation to classical antipsychotic drugs (e.g., neuroleptic phenothiazines, haloperidol), demonstrate a more advantageous profile of activity. These new antipsychotics are better tolerated and accepted by patients, and are less toxic and safer than classical neuroleptic drugs. The main advantage of these drugs is low occurrence of extrapyramidal adverse symptoms like dystonic reactions, akathesie, Parkinsonian syndrome, tremor, tardive dyskinesias, and sexual and endocrine side effects.[1]

Although neuroleptic drugs (especially atypical neroleptics), taken in appropriate dosages, seem to be relatively safe, they should be monitored in patients' blood due to inter-individual variations in compliance and in activities of drug metabolizing enzymes. Therapeutic drug monitoring (TDM) of a number of classical[2] and atypical[3] antipsychotics improves response rates, minimizes side effects, and reduces the risk of relapses under chronic treatment.

Poisonings by overdosing of atypical neuroleptic drugs, such as: clozapine,[4] olanzapine,[5] quetiapine,[6] or classical neuroleptic phenothiazines, such as perazine and promazine,[7] have been also reported. For this reason, appropriate screening procedures for antipsychotics in biological fluids (e.g., urine or blood) are also required in toxicological, clinical, and forensic laboratories.

There is a number of chromatographic methods for determination of one or a few antipsychotic drugs, including their metabolites.[8] However, to our knowledge, only one paper[9] was focused on simultaneous determination of quetiapine, clozapine, N-desmethylclozapine, olanzapine, and perazine in human serum by an automated HPLC-UV with a column–switching technique as a sample preparation method.

The aim of this work was to find optimal experimental conditions for extraction and separation of five commonly used antypsychotic compounds (quetiapine, clozapine, N-desmethylclozapine, olanzapine, and perazine) in human plasma using low cost and robust techniques such as liquid–liquid extraction and high performance liquid chromatography with spectrophotometric detection (DAD).

EXPERIMENTAL

Apparatus and Experimental Conditions

The chromatographic system, Merck-Hitachi LaChrom, consisting of an L-7100 pump and an L-7455 programmable diode array detector (Darmstadt, Germany) was used. Separations of the drugs tested were

performed on a thermostatted column LiChroCART (250 mm × 4.6 nm i.d.) packed with LiChrospher 100 RP-18, 5 μm particle size (Merck, Germany). Chromatographic analyses were carried out using gradient conditions, and as well as isocratic conditions. The mobile phase consisted of aqueous orthophosphoric acid with the amine modifier (phase A), and acetonitrile (phase B), mixed in different ratios. Phase A was prepared by diluting of 85% orthophosphoric acid with doubly deionised water and addition of an appropriate amine (N,N,N′,N′-tetramethylethylendiamine or diethylamine). Optimization of the gradient profile was performed by varying of phase A and phase B ratio, and flow rate in the time of analysis. The flow rate of the mobile phase [water-acetonitrile-N,N,N′,N′-tetramethylethylenediamine (63:37:0.4; v/v/v), adjusted to pH 6.5 by acetic acid (95%)], under isocratic conditions, was 1 mL/min. The drugs were detected by UV-light absorption at 254 nm.

Reagents

Acetonitrile and methanol of HPLC-gradient grade were supplied by Merck (Germany). Diethylamine (DEA) and N,N,N′,N′-tetramethylethylendiamine (TEMED) were purchased from Sigma-Aldrich (Germany). The reagents: 85% orthophosphoric acid, 30% sodium hydroxide, n-hexane, isopropanol, ethyl acetate, diethyl ether and isoamyl alcohol, all of analytical grade, were purchased from POCH (Poland). Doubly deionised water (<1.0 μS/cm) was used throughout.

Examined Drugs and Materials

Standard substances of clozapine, N-desmethylclozapine (the active metabolite of clozapine), olanzapine, and quetiapine were purchased from Sigma-Aldrich (Germany). Standards of perazine and promazine (IS) were obtained from the pharmaceutical factory Galena (Poland). A stock solution of each drug (10 mg/mL) was prepared in methanol and stored in a refrigerator (4°C). Control human plasma was obtained from the local blood bank (Kraków, Poland). Working drug solutions (concentration of each drug usually was 0.5 μg/mL and in some cases of analysis, concentration of N-desmethylclozapine was 0.05 μg/mL) were prepared by appropriate dilution of the stock drug solutions with an appropriate medium (a mixture of water phase (A) and acetonitrile phase (B); water phase (A); and 0.01% orthophosphoric acid). In order to obtain control plasma samples, this material was spiked with water diluted standard drugs.

General Extraction Procedure

One mL of spiked plasma sample (the concentration of each drug was 0.5 μg/mL) was placed in 22-mL extraction vessel and alkalized with an appropriate amount of 0.6 M or 2 M NaOH to achieve sample pH = 11.47; 11.60 or 11.90. Next, 5 mL of an extraction solvent was added and the vessel was closed with PTFE cap. The vessel was gently shaken for 15 minutes and centrifuged at 3500 rpm for 10 minutes. 4 mL of upper (organic) phase was successively transferred to an Ependorff type tube and evaporated at 35°C in nitrogen atmosphere. The obtained residue was reconstituted with 250 μL of extraction solvent and then re-extracted for 2 minutes into 50 μL or 100 μL of 0.01% orthophosphoric acid. After centrifugation (10 min, 4500 rpm), the aqueous phase was directly injected onto the chromatographic column.

RESULTS

Examination of Chromatographic Separation Conditions

In order to find optimal separation conditions for five neuroleptics, the influence of pH of the mobile phase, column temperature, gradient profile of mobile phase flow, as well as kind of amine (added into the mobile phase as the modificator) on drug peaks resolution, was investigated. In this part of examinations all experiments were carried out using aqueous drug standard solutions.

In preliminary experiments isocratic chromatographic conditions were applied using a mobile phase: water-acetonitrile-N,N,N′,N′-tetramethylethylenediamine (63:37:0.4, v/v/v), adjusted to pH 6.5 by acetic acid (95%).[9] Temperature of the column was 24°C. Under the applied conditions poor separation of the tested compounds was observed and retention times of the drugs were long (the first drug was eluted over 15 min), thus the analysis was broken. Hence, optimization process of chromatographic separation, based on the one independent variable method, was performed. In the first step, the influence of pH changes of the mobile phase on drugs separation efficiency was tested. The experiments were carried out changing pH values (4.0, 4.5, 4.75, 5.0, and 5.1) of aqueous phase A, using appropriate additions of 85% orthophosphoric acid. In Fig. 1, the selected chromatograms at pH = 4.75 and 5.0 were compared. At pH = 5.0 all the chromatogram peaks were completely separated, but retention time of the last peak corresponding to perazine was still long (c.a. 40 min). In order to reduce the analysis time, the column temperature was elevated to 34°C and then to 42°C. Approaching higher column temperatures, better peak shapes were

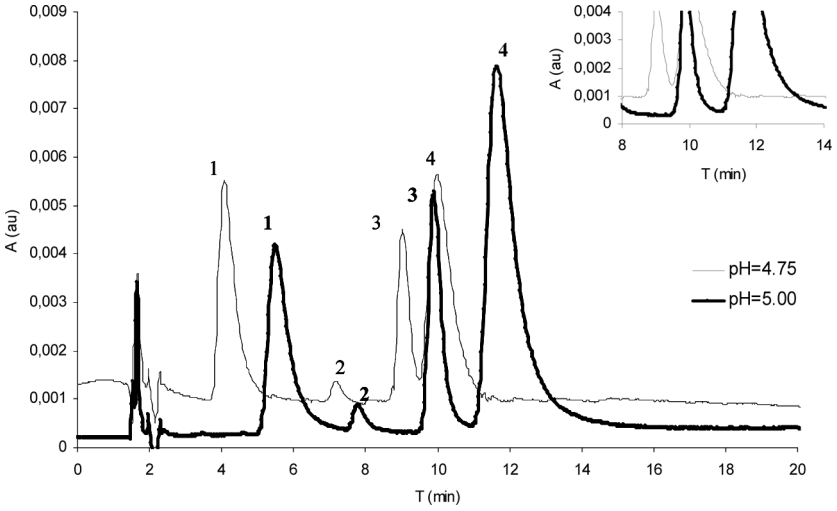

Figure 1. Selected chromatograms of the examined drugs at two values of pH of the mobile phase. 1 – olanzapine (OLA), 2 – N-desmethylclozapine (NOR), 3 – quetiapine (QUE), 4 – clozapine (CLO). Concentrations of the drugs (in the standard solution): OLA, QUE, CLO and NOR were 5 μg/mL and 0.5 μg/mL, respectively.

obtained, but separation efficiency was becoming worse (Fig. 2). For improved peak separation, gradient conditions (gradient of mobile phase rate and phase composition) were applied and optimized. The influence

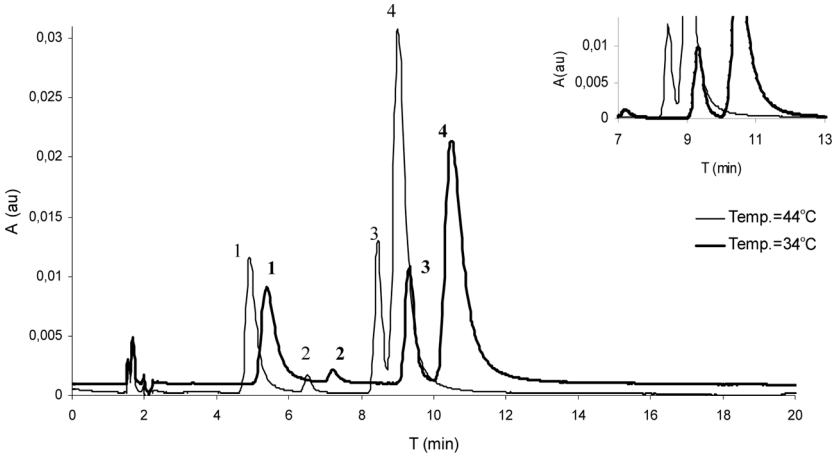

Figure 2. Selected chromatograms of the examined drugs at two values of column temperatures. The rest data are the same as in Fig. 1.

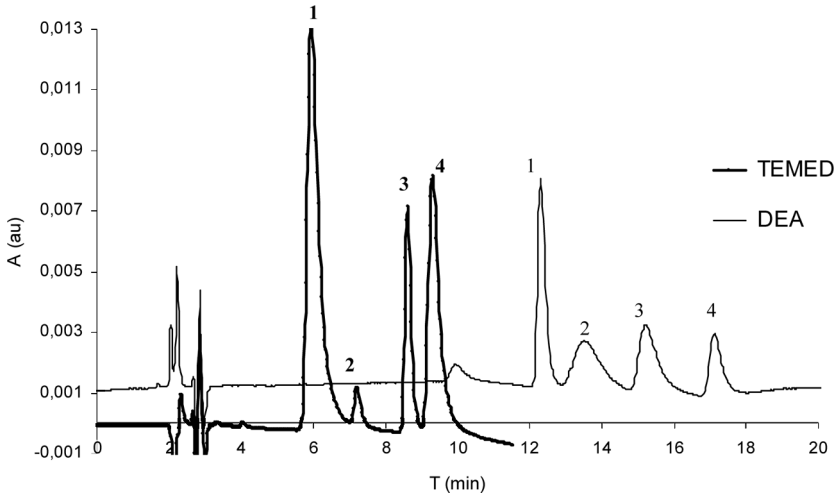

Figure 3. Influence of the kind of amine (used as the modificator of the mobile phase) on chromatographic properties of the studied drugs. The rest data are the same as in Fig. 1.

of another amine (diethylamine) on chromatographic behaviour of the drugs tested was also checked, but this amine worsened peak shapes and separation quality; also, retention times of the drugs were much longer (Fig. 3).

On the basis of the performed series of experiments, these final separation conditions were selected: water with the addition of TEMED and orthophosphoric acid; pH = 5.0 (A) and acetonitrile (B) as the mobile phase, column temperature = 38°C, and gradient conditions of mobile phase flow (Table 1). At the optimized chromatographic

Table 1. The optimal gradient profile of elution

	Mobile phase A:B (%, v/v)		
Time (min)	A (water phase)	B (acetonitryl phase)	Flow rate (mL/min)
0.00	60	40	0.7
10.00	48	52	0.9
14.00	48	52	1.5
17.00	48	52	1.5
19.00	60	40	0.7
22.00	60	40	0.7

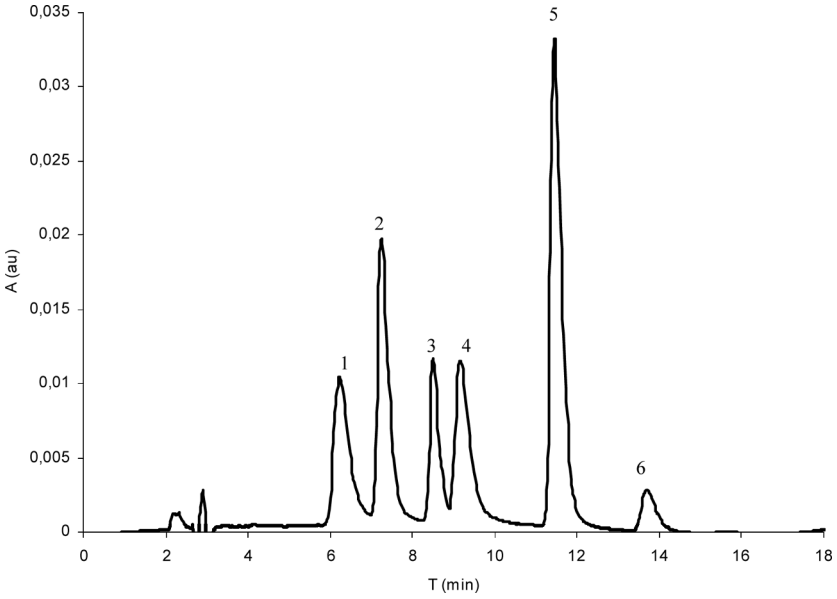

Figure 4. Separation of the studied drugs under the optimalized chromatographic conditions. 1 – olanzapine (OLA), 2 – N-desmethylclozapine (NOR), 3 – quetiapine (QUE), 4 – clozapine (CLO), 5 – chlorpromazine (IS), 6 – perazine (PER). Concentration of all drugs tested in the standard solution was 5 μg/mL.

conditions, five examined compounds were completely separated within 15 min (Fig. 4).

Promazine (neuroleptic phenothiazine) was chosen as the internal standard (IS). Identification parameters (absolute retention time and relative retention time) of the examined five antipsychotics are given in Table 2.

Table 2. Absolute and relative retention times of the studied drugs

Drug	Absolute retention time (min)	Relative retention time
Olanzapine (OLA)	6.24	0.52
N-desmethylclozapine (NOR)	7.25	0.61
Clozapine (CLO)	8.72	0.73
Quetiapine (QUE)	9.49	0.79
Promazine (PRO, IS)	11.92	1.00
Perazine (PER)	14.66	1.22

Examination of Liquid–Liquid Extraction Conditions

In this part of the study, all experiments were carried out using the optimized separation conditions achieved in the first part of the examinations. All drugs tested were present in human plasma samples at a concentration of 0.5 μg/mL, which corresponds to their high therapeutic or low toxic concentration levels in this material.

The influence of sample pH, kind of extraction system, and volumes of extraction and reextraction solvents on extraction recovery and purity of the obtained extracts using a single independent variable method, was examined.

In the next step, using n-hexane:isoamyl alcohol (99:1, v/v) as the extractant (extraction system I) and 0.01% orthophosphoric acid as the reextractant, pH of sample solution, type, and volume of extraction solvent, and volume of reextraction solvent were optimized. The following values of sample pH (11.47; 11.60 and 11.90), extractant volume (5 and 7 mL) and reextractant volume (50 and 100 μL) were tested. As the

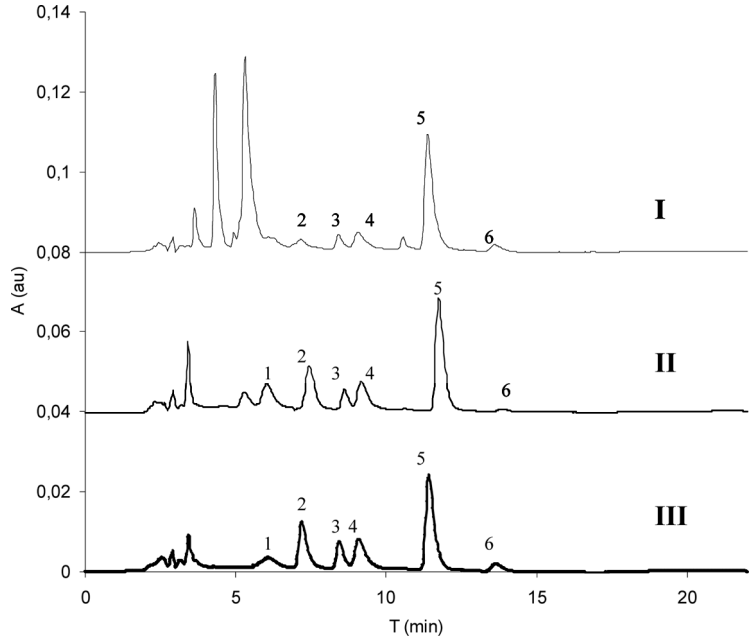

Figure 5. Comparison of the extraction results obtained by use of the selected extraction systems: I – n-hexane: isoamyl alcohol (99:1, v/v), II – diethyl ether, and III – ethyl acetate: n-hexane: isopropanol; (16:3:1, v/v/v). 1 – olanzapine (OLA), 2 – N-desmethylclozapine (NOR), 3 – quetiapine (QUE), 4 – clozapine (CLO), 6 – perazine (PER). Concentration of all drugs extracted from human plasma was 0.5 μg/mL.

Table 3. Extraction recovery and repeatability of the studied drugs from human plasma using the selected extraction systems and ultrasonic energy (extraction system III)

	Extraction recovery (%) (n = 4)					Extraction repeatability (n = 4)				
Extraction system	OLA	NOR	QUE	CLO	PER	OLA	NOR	QUE	CLO	PER
I: n-hexane/isoamyl alcohol (99:1, v/v)	–[a]	13.8	34.4	63.2	65.6	–[a]	1.4	1.8	1.8	2.7
II: diethyl ether	43.7	68.1	46.8	80.6	14.9	2.8	4.2	4.2	6.2	0.9
III: ethyl acetate/n-hexane/isopropanol (16:3:1, v/v/v)	56.1	82.2	69.6	93.3	60.1	12.0	2.7	2.8	4.4	8.9
	45.8[b]	50.0[b]	64.7[b]	52.1[b]	36.4[b]	2.6[b]	3.0[b]	3.7[b]	4.5[b]	5.1[b]

[a]contaminations extracted from isoamyl alcohol did not enable to read olanzapine data (first peak on the chromatogram, Fig. 5).
[b]LLE process was assisted by ultrasonic energy. The abbreviations of the drugs studied are the same as in Table 2.

best conditions: pH = 11.60, extractant volume = 5 mL and reextractant volume = 100 μL were chosen.

Hence, three other extraction systems: diethyl ether (extraction system II), ethyl acetate: n-hexane: isopropanol; 16:3:1, v/v/v (extraction system III), and n-hexane (extraction system IV) were employed for isolation of the tested drugs from standard solutions. On the basis of the obtained results, extraction system IV was excluded due to creation of an emulsion.

Moreover, two other solvents: 1. water phase (A) and 2. mixture of water phase (A) and acetonitrile phase (B) [1:1, v/v] were tested as the final media for the drugs in regard to quality of the obtained drug peaks. The comparison of the chromatograms showed that the obtained peaks were a little worse than those using 0.01% orthophosphoric acid. Thus, 0.01% orthophosphoric acid was selected as the final back extraction solution.

Three selected extraction systems (I, II, and III) and 0.01% orthophosphoric acid as the back extraction solvent (extractions with extraction solvents I and III) were then used for isolation of the examined neuroleptics from plasma samples. For each drug tested, yield and repeatability of the performed extractions were determined (Fig. 5, Table 3).

Finally, as the optimal extraction system, the mixture of ethyl acetate, n-hexane, and isopropanol (16:3:1, v/v/v) was chosen. Furthermore, in order to improve extraction efficiency, ultrasonic energy during the extraction process with the selected extraction system was applied. Although extraction repeatability was improved, the extraction recovery was considerably decreased (Table 3).

DISCUSSION AND CONCLUSIONS

Chromatographic and extraction conditions for four atypical antipsychotic compounds: 1. clozapine, 2. N-desmethylclozapine - pharmacological active metabolite of clozapine, 3. olanzapine and 4. quetiapine, and one classical neuroleptic phenothiazine-perazine, have been studied.

The applied isocratic chromatographic conditions (similar to those used in the paper[9]) with the mobile phase: water-acetonitrile-N,N,N″,N′-tetramethylethylenediamine; pH 6.5 (63: 37: 0.4, v/v/v) and the ambient column temperature (24°C) were not satisfied. Thus, optimization of the separation condition for the drugs tested were carried out. At the optimized chromatographic conditions: addition of TEMED as the modifier of the mobile phase, pH of the mobile phase = 5.0, gradient conditions of elution (Table 1), and column temperature = 38°C, all examined compounds were baseline separated within 15 min (Fig. 4). The replacement of N,N,N′,N′-tetramethylethylendiamine (TEMED) for diethylamine

(DEA) decreased chromatographic properties of the tested compounds (Fig. 3).

In the second part of our examinations, optimal exatraction conditions for the antipsychotics were studied. Considering extraction recovery and purity of the obtained extracts, extraction system: ethyl acetate/n-hexane/isopropanol (16:3:1, v/v/v), pH of sample solution = 11.6, extractant volume = 5 mL, and reekstractant (0.01% orthophosphoric acid) volume = 100 μL were selected. A trial with ultrasonic energy did not improve extraction efficiency, but just the opposite, it made it worse. However, in the cases of olanzapine and perazine, extraction repeatability was improved (Table 3).

Under optimal conditions, we obtained the following extraction drug recoveries from plasma samples: olanzapine 56.1 (±12.0)%; N-desmethylclozapine 82.2 (±2.7)%; quetiapine 69.6 (±2.8)%; clozapine 93.3 (±4.4)% and perazine 60.1 (±8.9)%.

The obtained extraction recoveries and repeatabilities were not quite satisfactory for all examined drugs, especially for olanzapine and perazine. Our results were compared with the achievements of other authors. It may be stated that the extraction recoveries obtained by us for clozapine were similar[10,11] or better.[12,13] However, in the case of the active metabolite of clozapine (N-desmethylclozapine), and especially in the case of olanzapine and quetiapine, lower recoveries were obtained. From the reported results in the literature, it seems that the use of an SPE technique for olanzapine,[14] quetiapine,[11] and also for N-desmethylclozapine[11,14] is more effective. Using the SPE technique, the extraction recoveries for olanzapine, quetiapine, and N-desmethylclozapine were above 90%. Application of liquid–liquid extraction twice also improved isolation efficiency of clozapine and N-desmethylclozapine from plasma samples, i.e., 93.0%.[10]

According to the authors of this work, extraction efficiency, as well as repeatability of recovery, of all studied drugs could be improved by assistance of the proposed LLE method with microwave energy. This may be anticipated on the basis of the extraction results achieved for structurally similar tricyclic antidepressant drugs isolated from human serum samples by use of microwave-assisted liquid–liquid extraction.[15]

The developed procedure for detection and identification of five studied antipsychotic drugs in human plasma involves widely used analytical techniques such as liquid–liquid extraction and high performance liquid chromatography with spectrophotometric detection (DAD). This procedure may be extended over other drugs from the groups of: atypical antipsychotics and phenothiazine neuroleptics. It can be a useful tool for toxicological screening analysis in forensic and clinical laboratories, but an appropriate validation study should be performed.

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