Research Article

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Optimization of chromatographic systems for analysis of selected psychotropic drugs and their metabolites in serum and saliva by HPLC in order to monitor therapeutic drugs

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Abstract: Retention, separation selectivity and system efficiency of selected basic psychotropic drugs (clozapine, aripiprazole, vortioxetine and zolpidem) and drug metabolites (desmethylclozapine, clozapine N-oxide and dehydroaripiprazole) on Hydro RP, Phenyl-Hexyl and Polar RP columns were studied. Mobile phases containing methanol or acetonitrile as organic modifiers, acetate buffer at pH 3.5 and addition of diethylamine (DEA) as a silanol blocker were applied. Significant differences in the retention, peak shapes and systems' efficiency of the investigated compounds were obtained depending on the tested chemically bonded stationary phases with various ligands. Based on the obtained results the Phenyl-Hexyl column was selected for analysis of the drugs and their metabolites in human serum and saliva samples. Solid phase extraction (SPE) was applied for sample pretreatment. The best SPE-HPLC-DAD procedure was used for simultaneous analysis of clozapine, aripiprazole and their metabolites in body fluids. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) method was applied for confirmation of the presence of the investigated compounds in biological samples. The lower limit of quantification (LLOQ) of clozapine obtained using the proposed method was 10 ng/mL. The validated method for determining the presence of clozapine and its main metabolite was successfully applied in therapeutic drug monitoring.

Keywords: Optimization of chromatographic systems; HPLC-DAD; HPLC-MS/MS; SPE; psychotropic drugs; therapeutic drug monitoring.

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1 Introduction

Monitoring of psychotropic drugs in clinical studies is significant and establishing methodologies for analysing these drugs in biological matrices is essential for patients' safety. To determine the concentrations of psychotropic drugs and their metabolites several methods have been developed. The majority of methods for analysing psychotropic drugs in different biological samples are based on combining a very efficient separation technique, for example high-performance liquid chromatography (HPLC), gas chromatography (GC) and capillary electrophoresis (CE), with a sensitive detection method. HPLC is one of the most efficient and robust separation techniques due to its convenience, simple operation, strong separation ability and wide sample application. HPLC with Ultraviolet-Visible (UV-VIS) [1], fluorescence [2], diode array (DAD) [3] mass spectrometry (LC-MS)

[4] or tandem mass spectrometry (LC–MS/MS) [3, 5-10] detection methods were often applied for analysis of different psychotropic drugs in various biological samples such as: serum [5, 6], plasma [4], urine [2], saliva [3], breast milk [7]. Most often HPLC analysis of psychotropic drugs was performed on octadecyl (C18) or octyl (C8) columns with mobile phases containing a mixture of organic modifier, water with addition of formic or acetic acid [5, 11, 12], acidic buffer [1, 6, 8], basic buffer [4], ammonium salts (acetate or formate) [13, 14] or amine [15]. Sometimes psychotropic drugs were analyzed on various columns, e.g., phenyl column [3].

Most of the published methods allow quantification of a single compound and sometimes with their related metabolite. Simultaneous quantification of various psychotropic drugs have also been published especially for the determination of drugs from the same therapeutic class. Rarely methods for simultaneous determination of drugs from different therapeutic classes were described. For example aripiprazole, atomoxetine, duloxetine, clozapine, olanzapine, sertindole, venlafaxine and their active metabolites dehydroaripiprazole, norclozapine, dehydrosertindole and O-desmethylvenlafaxinen were determined in human plasma [4], agomelatine, asenapine, amisulpride, iloperidone, zotepine, melperone, ziprasidone, vilazodone, aripiprazole and its metabolite dehydro-aripiprazole were quantified in human serum [6]. Simultaneous analysis of olanzapine, fluoxetine, and norfluoxetine in human plasma was also performed by HPLC-MS [16].

Aripiprazole, (7-{4-[4-(2,3-dichlorophenyl)piperazin-1-yl] butoxy}-3,4-dihydroquinolin-2(1H)-one), is a D_2/5-HT_{1A}/5- HT_{2C} partial agonist and a $D_{3/4}$/5-HT_{2A}/5-HT_7 antagonist, with moderate serotonin transporter inhibitory, antihistaminic H_1, and adrenolytic $_{a1}$ activities [17].

Aripiprazole is mainly metabolized in the liver through the cytochrome 450 (CYP) 3A4 and 2D6 isoenzymes to dehydroaripiprazole, an active metabolite possessing the same affinity for dopamine receptors but a lower antipsychotic activity than the parent drug.

Clozapine, as a newer atypical antipsychotic drug with distinct curative effect, is commonly used to treat schizophrenia and was determined by HPLC with UV detection in plasma and urine samples [12], in serum by HPLC with UV detection [15] and in hair and nails was quantified by UHPLC-MS/MS [18].

The aim of this work was to investigate selected psychotropic drugs and their metabolites on C18, Phenyl-Hexyl and Polar RP columns using aqueous eluents containing methanol (MeOH) or acetonitrile (MeCN) and diethylamine as silanol blocker to obtain sufficient selectivity of separation, system efficiency, and peak symmetry. The use of the double protection (use of stationary phase with π-π ligands and mobile phase containing addition of silanol blocker against interaction between aromatic basic solutes and residual silanol groups) allows symmetrical peaks and good system efficiency. The effect of using MeOH or MeCN as an organic modifier of mobile phases and the influence of different types of chemically bonded stationary phase on chromatographic parameters of investigated drugs were examined. Most selective chromatographic systems were used for qualitative and quantitative determination of clozapine and aripiprazole and their metabolites: desmethylclozapine, clozapine N-oxide and dehydroariprazol in human plasma and saliva samples. To the best of our knowledge for the first time the simultaneous quantification of clozapine, aripiprazole and their metabolites in human saliva is described. Our method was also successfully applied in therapeutic drug monitoring.

2 Materials and Methods

2.1 Chemicals

Standards of clozapine, zolpidem, vortioxetine, aripiprazole, drug metabolites (desmethylclozapine, clozapine N-oxide and dehydroaripiprazole) were purchased from EGIS Pharmaceuticals PLC (Budapest, Hungary), Polpharma (Starogard Gdański, Poland), Lundbeck (Copenhagen, Denmark), Otsuka Pharmaceutical Europe Ltd. (Wexham, Great Britain) and Sigma Aldrich (Saint Louis, USA) respectively. Zolpidem and vortioxetine were used as internal standards in methods for quantification of clozapine, aripiprazole and their metabolites in biological samples. Methanol (MeOH) and acetonitrile (MeCN) HPLC-grade and diethylamine (DEA), acetic acid, sodium acetate, ammonia (25%) and ammonium chloride were purchased from Merck (Darmstadt, Germany). Water used for preparation of mobile phases was double distilled.

2.2 Apparatus and HPLC conditions

The HPLC system (LaChrom Elite, Merck) was equipped with an autosampler, column oven L-7350, solvent degasser L-7612 and DAD detector. The chromatographic

analyses were performed at 22°C with an eluent flow rate of 1.0 mL min^{-1}. The chromatographic separations were carried out on Hydro RP 150 mm × 4.6 mm, 4 µm particle (Phenomenex), XSELECT Phenyl-Hexyl (150 mm x 4.6 mm, 5 µm) and Polar RP 80A 150 mm × 4.6 mm, 4 µm particle (Phenomenex) columns. Mobile phase compositions and gradient programs applied for HPLC-DAD analysis are shown in **Table 1**. The DAD detector was set in the 200–400 nm range. Data acquisition and processing were carried out with an EZchrom Elite software.

2.3 LC-MS/MS conditions

The confirmation of the identity of the compound of interest has been performed by UHPLC-MS/MS experiments using a HPLC system (Dionex UltiMate 3000 UHPLC) equipped with the Hypersil Gold analytical column (Thermo Scientific). The column was thermostated at 40 °C. 10 µl of samples were injected into the LC-MS/MS system.

Mass spectrometry was carried out on linear ion trap mass spectrometer (Thermo Scientific Velos Pro) equipped with an atmospheric pressure chemical ionization (APCI) source operating in positive ion mode under the following conditions: source heater temperature of 400°C, capillary temperature of 260°C, collision energy was 35V, current corona discharge of 5 µA, the flow of gases (N_2) surrounding– 50 ARB, auxiliary gas – 5 ARB. MS full scan (m/z 50–650) was used. APCI-MS/MS precursor ions parameters and retention time for analysed compounds are summarised in **Table 2**.

The mobile phase consisted of acetonitrile (eluent A) and 25 mM ammonium formate in water, pH 4.5 (eluent B). The gradient profile is as shown in Table 1. Elution was performed at flow rate of 0.4 mL/min.

2.4 Serum and saliva sample collection

Serum and saliva samples were collected from psychiatric patients at Clinical Hospital No. 1 in Lublin (Poland). Samples of 5 ml venous blood were collected from patients treated by clozapine, and simultaneously by clozapine and aripiprazole. The samples were collected in steady state in the morning before the next dose. After blood coagulation the samples were centrifuged for 10 minutes at 1500 × g. The serum was separated and stored at –20°C until analysis. The saliva samples were collected using Salivette kit (Sarstedt, Nümbrecht, Germany). After centrifugation the saliva samples were separated and stored at –20°C until analysis. The study was approved by Bioethical Commission at Medical University of Lublin No KE-0254/254/2017.

2.5 Sample pre-treatment

Samples were prepared by solid phase extraction on BAKERBOND™ SPE Octadecyl (C18) J.T. Baker (Phillipsburg, USA) cartridges (100 mg m^{-1}). Extraction was carried out in a SPE chamber – Baker SPE – 12 G J.T. Baker (Philipsburg, USA).

The sample pre-treatment SPE methods are described in our earlier work [3] as well as appropriate modified procedures developed and used in the comparative analysis in this work were applied, as described below.

2.5.1 Preparation of serum samples

2.5.1.1 Procedure I

1mL of the serum sample was diluted with 1 mL of ammonium buffer at pH 8.6. C18 SPE columns were initially activated using 1 mL of methanol and then conditioned using 1 mL of a mixture containing water and ammonium buffer at pH 8.6 (5:1, *v/v*). The ammonium buffer at pH 8.6 was used to suppress the dissociation of basic analytes allowing stronger retention on the C18 SPE columns. After washing and conditioning steps, the serum sample was introduced to the SPE columns at a speed of 1 mL/min. The columns were prewashed with 1 mL of a solution containing MeOH and water (20:80, *v/v*) and the dried by applying vacuum for 3 minutes. Next, the extracted compounds were eluted twice with 1 mL of solution of MeOH and acetic acid (98:2, *v/v*). The sample was then evaporated to dryness and dissolved in 0.5 mL of HPLC mobile phase (Table 1). 20 µL of the sample was injected directly into the HPLC system.

2.5.1.2 Procedure II

1mL of the serum sample was diluted with 1 mL of water. C18 SPE columns were activated with 1 mL of methanol and conditioned using 1 mL of water. After loading at speed 1 mL/min, the columns were prewashed with 1 mL of solution containing MeOH and water (20:80, *v/v*) and dried by applying vacuum for 3 minutes. Then, the analytes were eluted with 1 mL of a mixture containing methanol and acetic acid (98:2, *v/v*). The sample was evaporated to dryness and dissolved in 0.5 mL of HPLC mobile phase (Table 1). 20 µL of the eluate was injected directly into the HPLC system.

Table 1: HPLC-DAD and HPLC-MS/MS conditions.

Gradient I				
Column	**XSELECT Phenyl-Hexyl (150 mm x 4.6 mm, 5 µm)**			
Injection volume	20 µl			
Temperature	22 °C			
Components of the mobile phase	A – Methanol + 0.025 M DEA B – Water + 0.025 M DEA C – acetate buffer pH 3.5 + 0.025 M DEA			
Gradient profile	Time (minute)	% A	% B	%C
	0	40	40	20
	15	45	35	20
	35	60	20	20
	50	60	20	20
Gradient II				
Column	XSELECT Phenyl-Hexyl (150 mm x 4.6 mm, 5 µm)			
Injection volume	55 µl			
Temperature	22 °C			
Components of the mobile phase	A – Methanol + 0.025 M DEA B – Water + 0.025 M DEA C – acetate buffer pH 3.5 + 0.025 M DEA			
Gradient profile	Time (minute)	% A	% B	%C
	0	40	40	20
	15	40	40	20
	35	65	15	20
	45	65	15	20
Gradient III				
Column	Hypersil Gold, 3.0 x 125 mm; 5 µm			
Injection volume	10 µl			
Temperature	40 °C			
Components of the mobile phase	A – acetonitrile B – 25 mM ammonium formate in water, pH 4.5			
Gradient profile	Time (minute)	% A	% B	Time (minute)
	0	5	95	0
	1	5	95	1
	16	70	30	16
	17.5	100	0	17.5
	19.5	100	0	19.5
	21.0	5	95	21.0
	24.5	5	95	24.5

Table 2: APCI-MS/MS parameters and retention time for selected compounds.

Compound name	Precursor ion	Retention time [minute]
Clozapine	327	12.65
Desmethylclozapine	313	12.18
Clozapine N-oxide	343	13.82
Aripiprazole	448	14.04
Dehydroaripiprazole	446	13.65

2.5.2 Preparation of saliva samples

2.5.2.1 Procedure I

0.2 mL of ammonium buffer at pH 8.6 was added to 1 mL of saliva sample. Before loading the sample at speed 1 mL/min the SPE C18 extraction column was activated with 1 mL of methanol and conditioned with 1 mL of a mixture containing water and ammonium buffer at pH 8.6 (5:1, *v/v*). The sample was passed through the SPE column and then the column was prewashed with 1 mL of solution containing MeOH and water (20:80, *v/v*). The SPE column was dried for 3 minutes (under vacuum). The analytes were eluted twice with 1 mL of mixture containing methanol and acetic acid (98:2, *v/v*). The sample was evaporated to dryness and dissolved in 0.5 mL of HPLC mobile phase (Table 1). 20 µL of the final sample was injected into the HPLC system.

2.5.2.2 Procedure II

In this procedure, the C18 extraction column was activated with 1 mL of methanol and conditioned with 1 mL of water. The saliva sample was then passed through the SPE column at a speed 1 mL/min. After sample loading, the column was prewashed with 1 mL of solution containing MeOH and water (20:80, *v/v*) and dried by applying vacuum for 3 minutes. The extracted compounds were eluted twice with 1 mL of mixture containing methanol and acetic acid (98:2, *v/v*). The sample was evaporated to dryness. The dry residue was reconstituted in 0.5 mL of mobile phase (Table 1). 20 µL of the sample was injected into the HPLC system.

2.6 Method validation

Before quantitative analysis the methods were validated taking into account evaluating the linearity, limit of detection (LOD), lower limit of quantification (LLOQ), selectivity, matrix effects, extraction recovery, process efficiency, precision and accuracy.

The limit of detection (LOD) is the lowest concentration of an analyte in a sample that can be detected, but not necessarily quantified under the stated experimental conditions and was defined as the lowest concentration of the calibration curve based on the signal-to-noise ratio of 3.

The lower limit of quantification (LLOQ) value is the lowest concentration of calibration standard used in the daily calibration curve which meets the acceptance criteria of 20% precision and accuracy.

The matrix effect was also examined (Table 2). No significant matrix effect ≤ 10% was observed for the investigated compounds. The matrix effect was in the range from 102.83 to 108.19% for investigated compounds. The higher matrix effect at 20 ng/mL was 106.58 and 108.19% for Clozapine and N-desmethylclozapine respectively. The lower matrix effect at 800 ng/mL was 103.58 and 102.83% for Clozapine and N-desmethylclozapine respectively.

2.6.1 Intraday precision and accuracy

Intraday precision and accuracy of the method were determined by analyzing six replicates at three concentrations: 20 ng/mL, 200 ng/mL and 800 ng/mL in the same day. Investigation of the potential interferences with the analyte peaks was performed by analyzing the saliva and serum samples from different sources.

2.6.2 Extraction recoveries

Extraction recoveries, extraction efficiencies and matrix effects were calculated at three concentration levels (20 ng/mL, 200 ng/mL and 800 ng/mL) based on the following formulas:

$$\text{Recovery (\%)} = CB \times 100 \quad (1)$$

$$\text{Extraction Efficiency (\%)} = CA \times 100 \quad (2)$$

$$\text{Matrix effect (\%)} = BA \times 100 \quad (3)$$

where: A = external solution peak area, B = post-extraction sample peak area, C = extracted matrix peak area.

2.7 Practical application

The various chromatographic systems have been applied for the separation and analysis of various combinations of selected drugs and their metabolites in the serum and saliva samples obtained from psychiatric patients using the HPLC-DAD and LC-MS/MS methods. The optimal SPE procedure and chromatographic system was chosen for the determination of clozapine, aripiprazole and their active metabolites in human serum and saliva samples by HPLC-DAD. Optimal conditions for sample preparation and LC-MS/MS analysis was also applied for determination of clozapine and its active metabolite N-desmethylclozapine in the serum collected from patients treated with clozapine and aripiprazole for therapeutic drug monitoring.

Quantification of clozapine and its active metabolite N-desmethylclozapine was performed in the serum collected from patients treated with clozapine and aripiprazole based on the previously published method applied for determination of clozapine [3] after appropriate modification. Currently, the method allows determination of N-desmethylclozapine with the required accuracy and precision. This analytical method was successfully applied in routine therapeutic drug monitoring.

3 Results and discussion

3.1 Optimization of SPE procedure

This work is focused on the development of HPLC analytical method to achieve an efficient separation of selected psychotropic drugs and metabolites for the purpose of their analysis in human serum and saliva samples. Method development started with the optimization of sample preparation conditions. Psychotropic drugs and their metabolites from serum and saliva samples were extracted by SPE. In order to maximize extraction recovery and sample purification, the loading, washing, elution, evaporation and reconstitution steps of the sample preparation used during the SPE process were optimized and two optimum procedures in terms of extraction efficiency and sample purification were selected for the preparation of samples from patients.

The comparison of extraction efficiency obtained using the two selected extraction procedures of investigated drugs and metabolites from saliva samples is presented in **Figure 1A**. Both procedures were performed by SPE on Octadecyl C18 columns. In procedure I the SPE columns were conditioned by methanol followed by an ammonium buffer and in procedure II columns were conditioned by methanol followed by water. The remaining steps of the procedures were the same and as detailed in the Experimental section. For all investigated compounds higher extraction efficiency from saliva samples were obtained when procedure II was applied and therefore it was selected for the extraction of drugs from saliva samples from patients. The obtained results indicated that the composition of solvents applied for conditioning the SPE columns have a significant influence on extraction efficiency.

The two sample preparation procedures were also successfully applied for extraction of the investigated compounds from human serum samples. For dehydroaripiprazole and vortioxetine a higher extraction efficiency for serum was obtained using procedure I, whereas for aripiprazole, clozapine, clozapine N-oxide and zolpidem a higher extraction efficiency was found for procedure II (**Figure 1B**). Similar extraction efficiencies were obtained for procedures for N-desmethylclozapine. On the basis of these experiments procedure II (without alkalization) was also used for extraction of drugs and their metabolites from serum samples, with the exception of samples containing only clozapine and its metabolite for which procedure I was applied.

3.2 Optimization of chromatographic condition

In the next step of investigation, the chromatographic conditions were optimized through several steps in order to achieve good separation selectivity of analytes and matrix components. The optimization was performed for clozapine, aripiprazole and their metabolites: desmethylclozapine, clozapine N-oxide and dehydroaripiprazole and also for zolpidem and vortioxetine applied as internal standards. Symmetry of analyte peaks and systems efficiency were also taken into account. Octadecyl (C18) reversed phase columns are frequently used for analysis of basic compounds in biological samples but the residual silanols on the silica-based bonded stationary phases provide ion exchange sites. This results in excessive retention and peak tailing of basic compounds through a mixed-mode retention mechanism thus affecting the reproducibility and accuracy of the analytical results. Many approaches have been developed to mitigate the adverse effects of the residual silanols, one of them is the simultaneous application of stationary phases with preferable π-π interactions and mobile phases with addition of silanol blocking

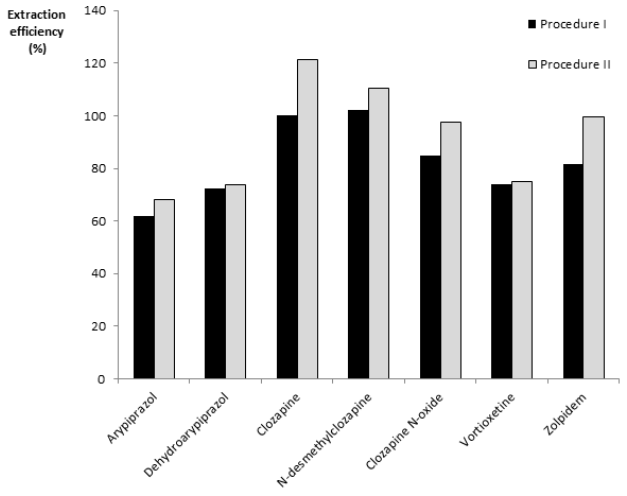

Figure 1A: Comparison of extraction efficiency obtained for saliva samples by SPE procedures I or II (Details see section 2.5.1).

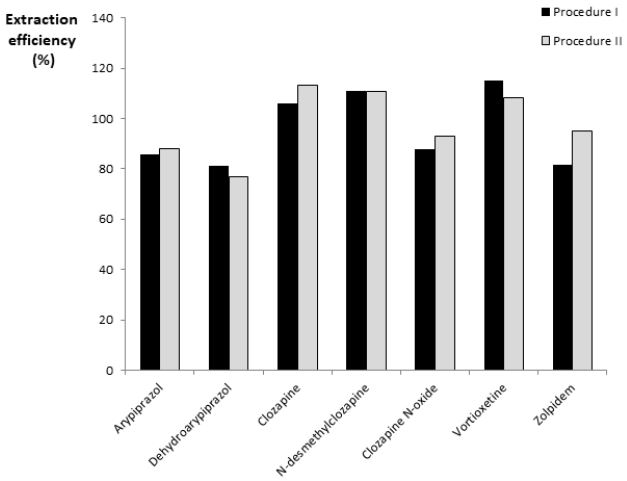

Figure 1B: Comparison of extraction efficiency obtained for serum samples by SPE procedures I or II (Details see section 2.5.1).

reagents. In order to determine the optimum HPLC-DAD and HPLC-MS/MS conditions, HPLC columns: C18 with alkyl bonded stationary phase and Polar RP or Phenyl-Hexyl columns with stationary phases containing phenyl substituents were tested. Various elution conditions with methanol or acetonitrile as organic modifier and different concentrations of DEA were examined for the determination of the investigated drugs and metabolites. The mobile phase composition in addition to the kind of stationary phase is one of the most important variables in the control of retention and system efficiency in HPLC. In the present study, the mobile phases containing methanol or acetonitrile, acetic buffer at pH 3.5 and 0.025 M DEA were selected. Comparison of the retention, the peak shape and the theoretical plate number per meter (N/m) representing the chromatographic column efficiency obtained for the investigated compounds on all applied columns was performed.

The strongest retention for most of the investigated analytes was observed on the Polar RP column and the weakest on the Phenyl-Hexyl column in both eluent systems (Table 3). For the octadecyl column for all investigated compounds stronger retention was obtained in eluent system with acetonitrile. Similar results were observed for the Phenyl-Hexyl column – strongest retention for all compounds was obtained in the eluent containing acetonitrile, whereas on Polar RP column dehydroaripiprazole and zolpidem were more strongly retained in the system with methanol. The best separation selectivity for the majority of investigated compounds was achieved on the Polar RP column in both eluent systems.

The symmetry of the peaks changed on all tested columns (Table 3). Better peak shape for most of the compounds was achieved in the system with the mobile phase containing methanol on phenyl stationary phases. For the Polar RP and Phenyl-Hexyl columns six out of seven compounds had values in the acceptable range. For the Phenyl-Hexyl column in the system with acetonitrile four compounds with symmetrical peaks were obtained. In the other systems only two compounds had values that were in the acceptable range. Taking into account the results obtained on all applied columns more symmetrical peaks were obtained in systems containing methanol as the organic modifier.

The effect of various columns with the same mobile phases was also compared in terms of efficiency (N/m values). As it can be seen in **Figure 2** differences were observed in values of efficiency. In the system with methanol for five of the investigated compounds had the highest N/m values for the Polar RP column, for aripiprazole on Phenyl-Hexyl and for vortioxetine on Hydro RP column.

3.3 Qualitative analysis of clozapine, aripiprazole and their metabolites in serum and saliva samples

Currently, polytherapy is often used to treat schizophrenic patients. Therefore, there is a need to develop methods for the simultaneous determination of two or more drugs and their metabolites in samples from patients. The method

Table 3: t_R (min) and As values for drugs and metabolites obtained on Hydro RP, Phenyl-Hexyl and Polar RP columns in eluent: 60% MeOH, acetic buffer at pH 3.5 and 0.025M DEA or 30% MeCN, acetic buffer at pH 3.5 and 0.025M DEA.

Name of compound	C18 stationary phase				Phenyl stationary phases							
	Hydro RP column				Phenyl-Hexyl column				Polar RP column			
	MeOH		MeCN		MeOH		MeCN		MeOH		MeCN	
	t_R	As	t_R	As	t_R	As	t_R	As	t_R	As	t_R	As
Arypiprazol	24.21 ± 0.08	1.65 ± 0.03	31.3 ± 0.04	1.86 ± 0.06	20.48 ± 0.11	1.24 ± 0.02	28.12 ± 0.09	1.35 ± 0.05	35.45 ± 0.10	1.25 ± 0.01	34.17 ± 0.08	1.4 ± 0.02
Dehydroarypiprazol	25.06± 0.07	1.56± 0.06	26.12 ± 0.10	2.92 ± 0.11	18.91 ± 0.09	1.20 ± 0.02	20.52 ± 0.11	1.49 ± 0.06	26.39 ± 0.10	1.26 ± 0.03	30.52 ± 0.12	1.64 ± 0.07
Clozapine	6.88 ± 0.02	1.54 ± 0.05	8.54 ± 0.05	1.81 ± 0.09	6.85 ± 0.06	1.42 ± 0.03	8.88 ± 0.09	1.87 ± 0.07	10.97 ± 0.12	1.42 ± 0.03	8.79 ± 0.09	1.52 ± 0.04
N-desmethylclozapine	3.98± 0.01	1.05. ± 0.01	5.08 ± 0.08	1.52 ± 0.05	5.84 ± 0.10	1.70 ± 0.06	7.05 ± 0.09	1.48 ± 0.03	8.21 ± 0.10	1.31 ± 0.02	5.84 ± 0.07	1.59 ± 0.03
Clozapine N-oxide	7.07± ± 0.02	1.73± 0.08	14.78 ± 0.10	3.53 ± 0.12	6.68 ± 0.08	1.28 ± 0.03	9.44 ± 0.10	2.44 ± 0.12	16.02 ± 0.11	1.89 ± 0.10	10.58 ± 0.04	2.49 ± 0.08
Vortioxetine	14.36± 0.10	1.94 ± 0.08	27.9 ± 0.14	1.25 ± 0.01	13.15 ± 0.12	1.37 ± 0.03	27.96 ± 0.11	1.61 ± 0.07	32.66 ± 0.15	1.36 ± 0.01	15.56 ± 0.08	1.97 ± 0.05
Zolpidem	5.61 ± 0.05	1.02 ± 0.02	8.54 ± 0.05	1.19 ± 0.01	5.54 ± 0.03	1.1 ± 0.01	6.64 ± 0.06	1.26 ± 0.02	10.02 ± 0.05	1.03 ± 0.01	12.59 ± 0.04	1.28 ± 0.02

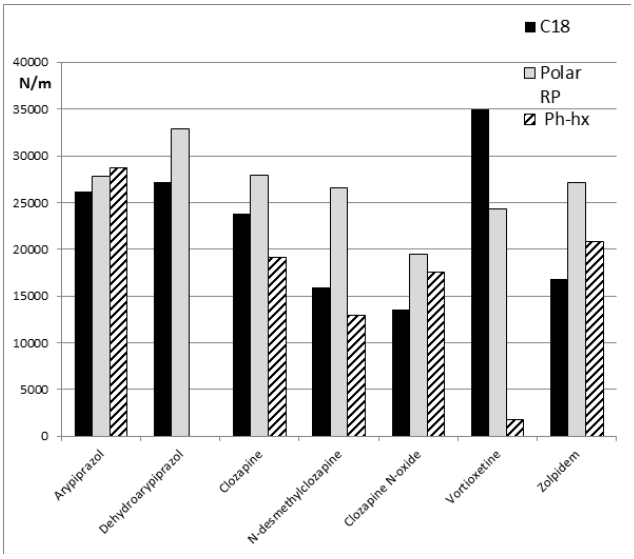

Figure 2: Comparison of N/m values obtained on C18, Polar RP and Phenyl-Hexyl columns with mobile phase containing 60% MeOH, acetic buffer at pH 3.5 and 0.025 M DEA.

optimization summarised in the previous section allows a choice in the chromatographic conditions for analysis of clozapine, aripiprazole and their metabolites in serum and saliva samples from psychiatric patients. Taking into account the results in terms of separation, selectivity of analytes, selectivity of separation in relation to the matrix components, peaks symmetry and system efficiency, the Phenyl-Hexyl column with mobile phase containing methanol, an acetate buffer pH 3.5 and DEA was selected for determining the analytes in biological samples. The gradient elution program is presented in **Table 1** (program I). Metabolic pathways of aripiprazole and clozapine leading to the formation of their major metabolites are shown in **Figure 3**. An example showing a chromatogram obtained from serum from a patient treated with clozapine and aripiprazole is presented in **Figure 4**. Analytes were fully separated, and no interference was observed with the blank serum samples.

Saliva samples obtained from the same patient treated by clozapine and aripiprazole were also analysed. Both drugs and their metabolites were also determined in saliva samples. The presence of clozapine and aripiprazole and their metabolites in serum and saliva samples from patients were confirmed by interpretation of their MS spectra (**Figure 5**). The gradient elution program is presented in Table 1 (program III). It confirms that saliva is a potential material that can be used for determination of investigated compounds, including therapeutic drug monitoring as an alternative to a blood draw, with the primary advantage being the ease of sample acquisition following adequate patient instruction.

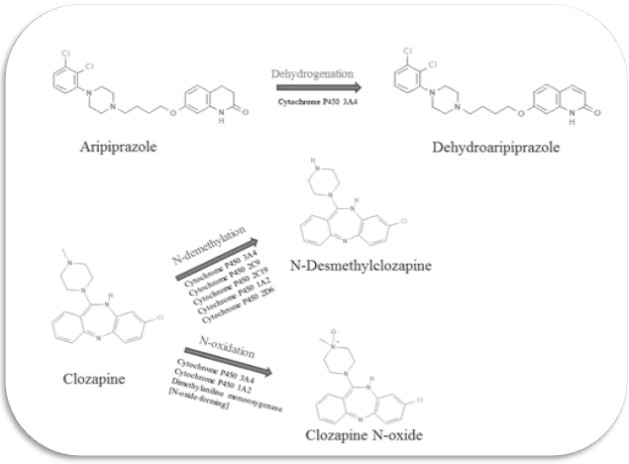

Figure 3: Metabolic pathways of aripiprazole and clozapine leading to the formation of their major metabolites [19].

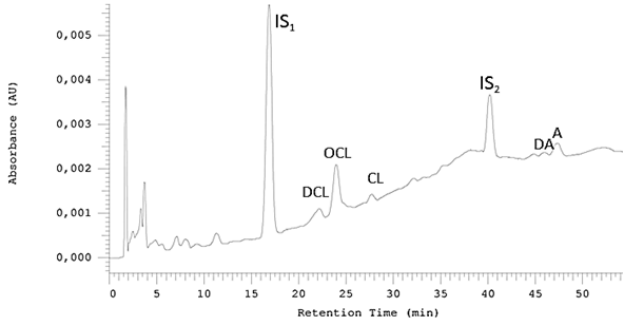

Figure 4: HPLC-DAD chromatogram obtained for serum sample received from patient treated with clozapine (CL) and aripiprazole (A) on Phenyl-Hexyl column with mobile phase containing MeOH, acetate buffer pH 3.5 (20%), DEA (0.025 M/L) using gradient I (see Table 1); DCL: N-desmethylclozapine, OCL: clozapine n-oxide, DA-dehydraripiprazole, IS1 zolpidem, IS2 –vortioxetine.

3.4 Quantitative analysis of clozapine, and their main metabolites in serum and saliva samples

Following the optimization of chromatographic systems we proposed a method using the Phenyl-Hexyl column and gradient elution program II (see Table 1) for analysis of clozapine and its active metabolite in body fluids due to the best selectivity of separation in relation to the matrix components, good peak shape and high system efficiency.

The method allows the determination of clozapine and N-desmethylclozapine with the required accuracy and precision. No significant interfering peaks were detected at similar retention times of the tested compounds when blank samples were evaluated (**Figure 6A**). For quantitative determination of clozapine in human serum a calibration curve was constructed by analyzing serum samples at six concentrations, ranging from 10 to 1000 ng/mL (r>0.9993). The analytical wavelength was 250 nm and the LLOQ was set at 10 ng/mL. The extraction recoveries were above 95% with precision below 8% CV. Validation parameters for clozapine and its main metabolite, N-desmethylclozapine, identified in samples from patients are presented in Table 4. Although HPLC-DAD provides valuable information for the separation and quantification of compounds present in biological samples, the use of conventional approaches based on absorption spectra and retention time is often limited when samples contain very similar compounds. Modern high-performance chromatographic techniques combined with other detectors are applied for the structural profiling and elucidation of active compounds. These techniques (such as MS) are often necessary for structure identification. For this purpose the presence of drugs and metabolites in samples from patients was confirmed by HPLC-MS/MS (**Figure 6B**). Detection conditions in mass spectrometer were also optimized to gain a better abundance of all analytes (Table 2).

3.5 Application of the method in therapeutic drug monitoring

The developed method was also applied in therapeutic drug monitoring. An example of application of the method for monitoring the concentration of clozapine and the therapeutic effect is presented below. Blood samples were collected from patients at various time intervals (within approximately 5 weeks) at steady state prior to the next dose of the drug. A good correlation between the dose and the drug concentration in serum was obtained. The therapeutic effect was assessed with the Positive and Negative Syndrome Scale (PANSS). The relationships between dose, serum concentration of the drug and the therapeutic effect were also determined. An example of the obtained results is shown in **Figure 7**. The samples were collected from a woman (22 years old, weight: 74 kg, high: 179 cm) suffering from paranoid schizophrenia who had no liver and kidney problems (appropriate laboratory tests have been carried out). The patient was treated with clozapine. Good correlation between serum drug concentration, applied dose and decreasing number of points on the PANSS scale was observed which indicates a linear dependence of the dose - therapeutic effect in this patient. The results indicate that by controlling the drug concentration in the serum the appropriate therapeutic dose can be easily selected. In the sample obtained

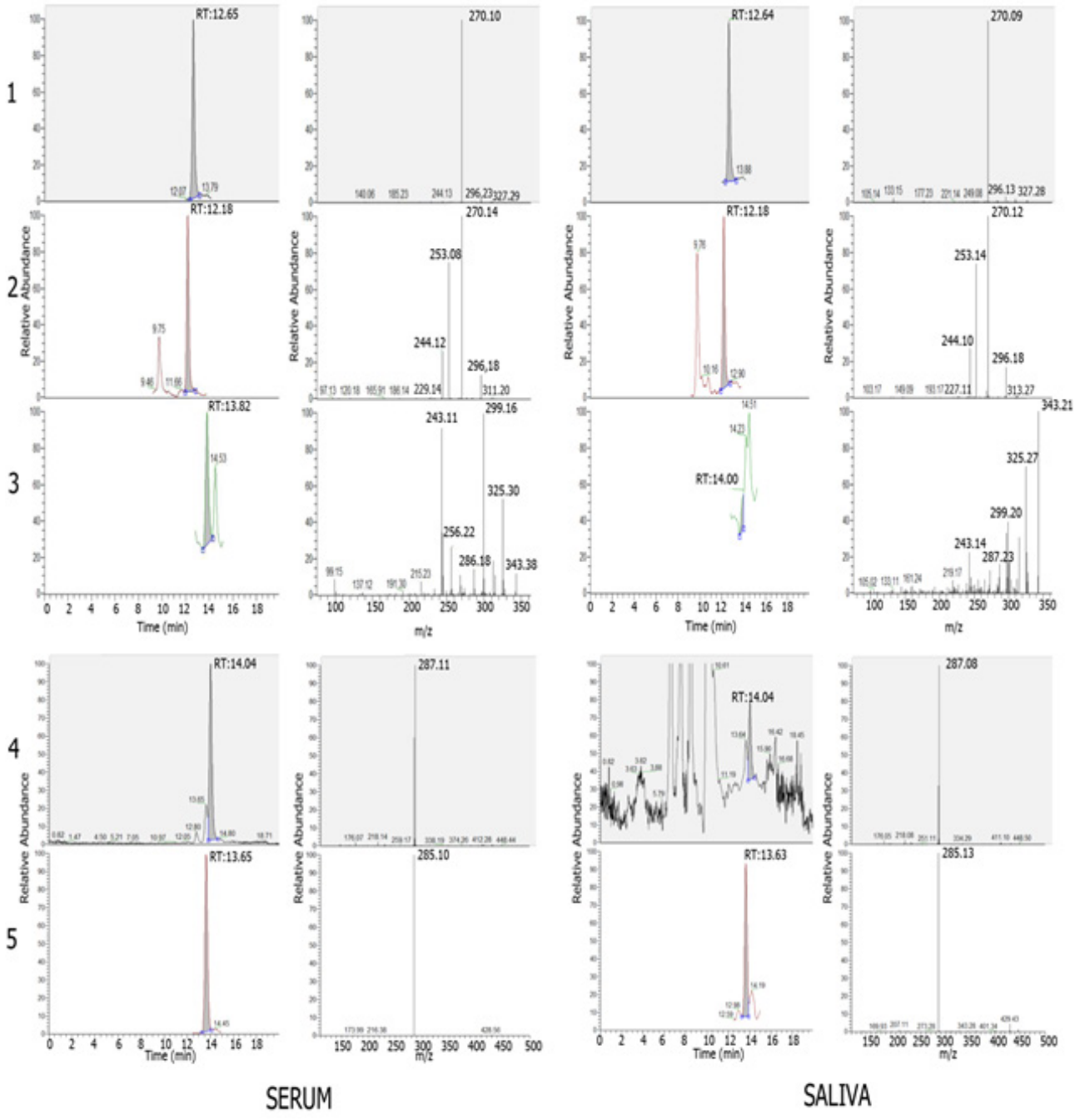

Figure 5: MS spectra obtained for selected drugs and their metabolites in serum and saliva samples on Hypersil Gold column with mobile phase containing acetonitrile and 25 mM ammonium formate in water, pH 4.5 using gradient III (see Table 1); 1) clozapine, 2) desmethylclozapine, 3) clozapine N-oxide, 4) aripiprazole, 5) dehydroaripiprazole.

from the patient after administration of the 300 mg per day dose the highest serum concentration of clozapine was determined as 196 ng/mL. The active metabolite N-desmethylclozapine was also determined in serum samples obtained from the patient. The procedure allows having results for clinical decisions in a short period of time.

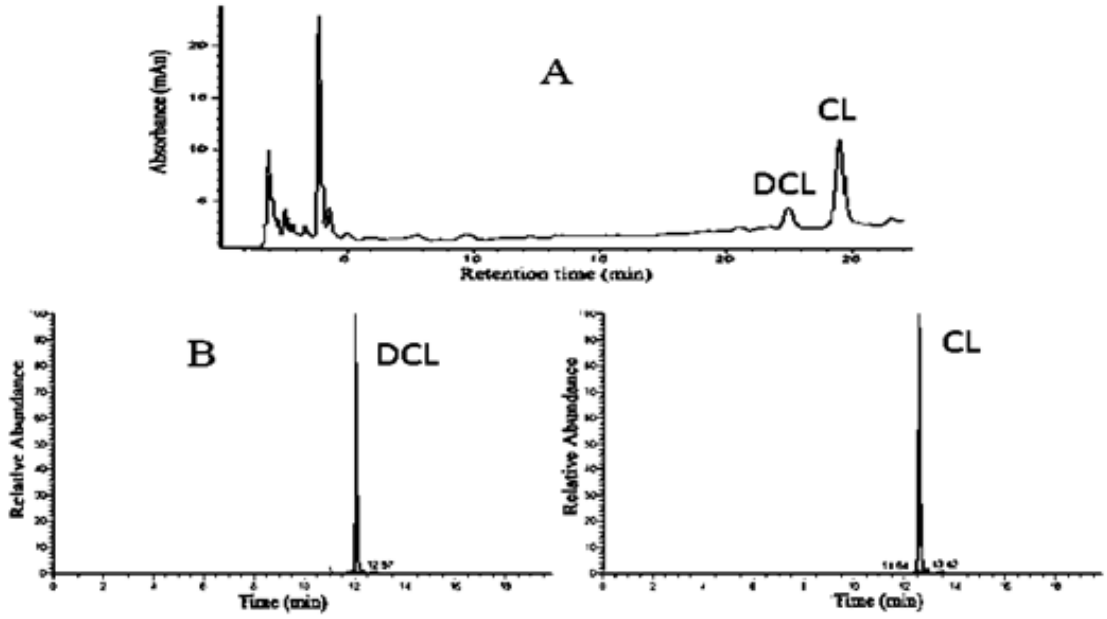

Figure 6: Chromatograms obtained using the HPLC-DAD (A) and HPLC-MS (B) methods for serum sample obtained from a patient treated with clozapine in chromatographic systems: A) Phenyl-Hexyl column; mobile phase containing MeOH, acetate buffer pH 3.5 (20%), DEA (0.025 M/L); gradient II (see Table 1); B) Hypersil Gold column; mobile phase containing acetonitrile and 25 mM ammonium formate in water, pH 4.5; gradient III (see Table 1).

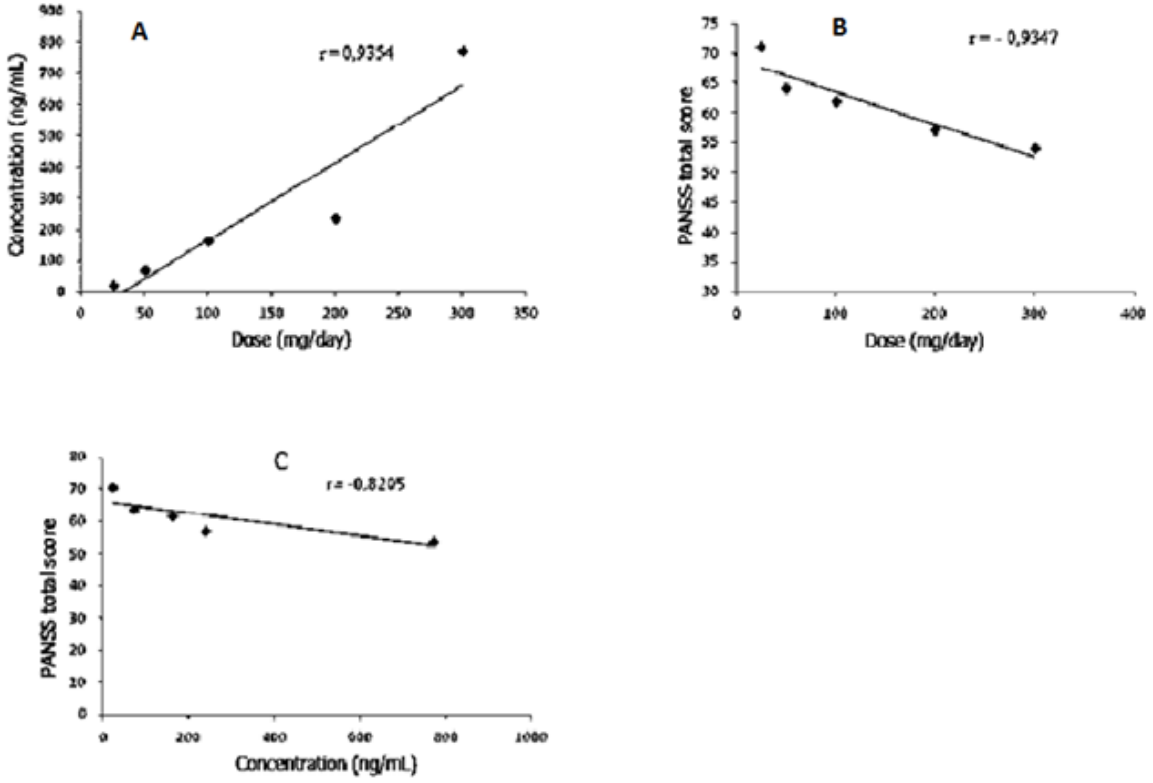

Figure 7: The graphs presented relationship between dose and PANSS total score (A) or clozapine concentration (B) as well as correlation between clozapine concentration and PANSS total score (C).

Table 4: Validation parameters obtained for Clozapine and N-desmethylclozapine.

Name of compound	Concentration added (ng/mL)	Recovery (%)	Matrix effect (%)	Proces efficiency (%)	Accurency (%)	Precision (%CV)
Clozapine	20	98.50 ± 1.94	106.58 ± 3.06	104.98 ±2.06	92.54	7.92
	200	95.07 ± 5.65	102.95 ± 4.39	97.88 ± 5.82	100.02	7.61
	800	98.74 ± 4.36	103.58 ±2.05	102.28 ± 4.51	97.33	4.73
N-desmethylclozapine	20	95.09 ± 1.96	108.19 ± 3.1	102.87 ± 2.12	111.99	7.85
	200	96.04 ± 5.71	103.97 ± 2.1	99.85 ±5.93	107.02	6.59
	800	96.27 ± 4.25	102.83 ± 1.74	98.99 ± 4.37	96.01	3.81

4 Conclusions

Significant differences in retention, peak shapes and system efficiency of the investigated psychotropic drugs and their metabolites were obtained on chemically bonded stationary phases with alkyl and phenyl ligands. In mobile phases containing methanol or acetonitrile the highest N/m values for nearly all compounds were obtained on the Polar RP column while the most symmetrical peaks were obtained on Phenyl-Hexyl and C18 columns.

The most symmetrical peaks were obtained on all tested columns in eluent systems containing methanol as organic modifier, but higher system efficiency was found for all columns and the target compounds in mobile phases containing acetonitrile.

In the current study the HPLC analytical quantification method was developed, validated and demonstrated to be selective, linear, precise, accurate and robust, being useful for analysis of aripiprazole, clozapine and their metabolites in human serum and saliva samples. The Phenyl-Hexyl column was selected for analysis of the clozapine, aripiprazole and their metabolites in body fluids due to the best selectivity of separation from the matrix components and high system efficiency.

The SPE process effectively removes interfering substances from the matrix and the optimal HPLC procedure allows the analysis of clozapine, aripiprazole and their metabolites in serum and saliva samples from psychiatric patients. The comparison of two SPE procedures indicated that SPE column conditioning is significant for recoveries of investigated drugs and metabolites.

The proposed method has been successfully applied for determining the presence of the investigated drugs and their metabolites in serum and saliva samples obtained from patients.

Aripiprazole and its metabolites were previously determined in serum [5, 6], serum and saliva samples [3]. Clozapine was determined in human plasma [1,4,14], human serum [15], urine [14], hair and nail [18]. This is the first report describing simultaneous quantification of clozapine, aripiprazole and their metabolites in human saliva. The use of saliva compared to serum or plasma for determination of psychotropic drugs has been shown to be an attractive alternative for therapeutic drug monitoring in psychiatric patients because its collection is simpler, non-invasive and painless.

The HPLC method for determining clozapine and its main metabolite was successfully applied in therapeutic drug monitoring and demonstrated a good correlation between applied dose, serum drug concentration, and number of points on the PANSS scale obtained in a patient for whom this relationship was studied. The method can be applied for routine human serum therapeutic drug monitoring.

Implementation of monitoring therapy for patients medicated with clozapine and their active metabolite can facilitate the treatment objective which is to establish and maintain in individual patients the minimum concentration of the drug, which ensures effectiveness of the treatment while causing the least expressed side-effects. Monitoring therapeutic concentrations of clozapine and its active metabolite can significantly contribute to personalized drug therapies in psychiatry. The method follows new trends in the treatment of

psychological diseases and it is an interesting tool for monitoring drugs, which is important in clinical practice.

Conflict of interest All authors declare that there is no conflict of interest.

Ethical statement All procedures involving humans in this study were approved by the Bioethical Commission at Medical University of Lublin No KE-0254/254/2017 and confirm to the Declaration of Helsinki. Serum and saliva samples were provided by the Department of Clinical Neuropsychiatry Medical University of Lublin. Informed consent was obtained from all individual participants included in the study.

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