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Determination of some psychotropic drugs in serum and saliva samples by HPLC-DAD and HPLC MS

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ABSTRACT

A simple, rapid and sensitive HPLC-DAD method has been developed and validated for the simultaneous determination of seven psychotropic drugs (risperidone, citalopram, clozapine,quetiapine, levomepromazine, perazine and aripiprazole) in human serum or saliva samples. The chromatographic analyses were performed on a XSELECT CSH Phenyl-Hexyl column with a mobile phase containing methanol, acetate buffer at pH 3.5 and 0.025 mL^{-1} diethylamine. The influence of concentration of methanol in injection samples and injection volume on peak symmetry and system efficiency was examined.The full separation of all investigated drugs, good peaks' symmetry and simultaneously high systems efficiency were obtained in applied chromatographic system. The method is suitable for the analysis of investigated drugs in human plasma or saliva for psychiatric patients for control of pharmacotherapy, particularly in combination therapy. HPLC–MS was applied for verification of the presence of drugs and their metabolites in serum and saliva samples from patients.

1. Introduction

Therapeutic drug monitoring is a useful tool for the clinical management of patients receiving a pharmacotherapy, particularly in psychiatry. Therapeutic drug monitoring of antidepressants is necessary for an optimal supervision of patient drug regimen to avoid medical complications, intoxication, non responsiveness or noncompliance. A significant percentage of psychiatric patients who are treated with psychotropic drugs are treated with more than one drug. Thus, it is advantageous to use a rapid and reliable assay that is suitable for determination of multiple antipsychotic drugs in biological samples in a single run. The concentrations of the drugs in biological samples are often very low. For these reasons, to monitor psychoactive drugs, the analytical methods have to be highly sensitive and selective for accurate and precise quantification.

The general methods for analyzing psychotropic drugs in different biological fluids are based on a combination of efficient separation method with a sensitive detection technique. At present, numerous separation techniques, including high-performance liquid chromatography (HPLC) [1], gas chromatography (GC) [2,3] and capillary electrophoresis (CE) [4,5], have been employed for the analysis of psychotropic drugs. Among those methods, HPLC has been considered as the most efficient and robust specific technique to some advantages including convenience, high separation ability and simple operation.

Various detection methods have also been applied to accurate determination of psychotropic drugs in different samples such as: voltammetry, chemiluminescence [6], fluorescence [7], UV adsorption [8–11], and mass spectrometry [12–20]. Nowadays most HPLC methods are developed with mass spectrometric detection techniques.

Psychotropic drugs were quantified in different biological samples e.g., in plasma [7,11,21,22], serum [10,13,23,24], whole blood [16], urine [1,7], saliva [3,25].

Most of the published methods allow quantification of a single drug, sometimes with its related metabolites [6,11,18]. Simulta-

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neous quantification of various psychotropic drugs have also been published [1,9,10], mostly in the same therapeutic class. In recent years there is a trend in clinical and forensic toxicology toward simultaneous quantification of a various compounds in one analytical run [16].

In most cases psychotropic drugs separation and determination were performed on alkyl bonded stationary phase (C18, rarely C8) [1,8,10,12,13,15,16,21]. Protonated basic psychotropic drugs can interact with residual silanol groups of the stationary phases. Thus, besides the reversed phase retention mechanism also an ion-exchange retention mechanism occurs, which often results in asymmetry of peaks, irreproducible retention, poor efficiency and worse separation. The silanol ion-exchange interaction can be reduced by using mobile phase with buffer at low pH, when the silanol ionization is suppressed, mobile phase with buffers at high pH to suppress solutes ionization, addition of an anionic ion-pairing reagents, making neutral associates or addition of organic amines as silanol blockers. Good results were also obtained by selecting a stationary phase to minimize the interaction between analyte and residual silanols. The introduction of hydrophobic π–π active aromatic moieties to the common *n*-alkyl chain RP-sites generates a concerted π–π reversed-phase retention mechanism, which diversivies the common RP-interaction properties. Analysis of some psychotropic drugs was also successfully performed on CN [26], Phenyl [27], CycloHexyl [28], Polar RP [16] columns.

Described in literature mobile phases applied to analysis of basic psychotropic drugs often contained organic modifier and addition of salts e.g., ammonium acetate [12], ammonium formate [17,18], acids [9,15,16,21], buffer at acidic pH [9,15,16,21,24], buffer at basic pH [29]. Mobile phases with addition of ion-pairing reagents [7] or amines e.g., triethylamine, tetramethylethylenediamine as silanol blockers were also used [8,11].

In most cases, owing to complex matrix such as biological fluids interference and insufficient instrumental detection limit for trace psychotropic drugs in real biological samples, direct HPLC determination of those compounds is difficult.Therefore, a separation from matrices and preconcentration is often required prior to chromatographic analysis of the analytes. Different sample preparation methods for analysis of psychotropic drugs in various biological samples e.g., protein precipitation [14,16,20,21], liquid–liquid extraction (LLE) [8,9,17], dispersive liquid–liquid microextraction (DLLME) [1], Ultrasound-assisted emulsification microextraction [30], solid-phase extraction (SPE) [11,12,18,24], stir bar sorptive extraction (SBSE) [7], some methods using on-line SPE with a column-switching system or methods with a simple protein precipitation have also been developed.

The aim of this study was to develop and validate a method for the simultaneous quantification of some psychotropic drugs in serum and saliva. The HPLC-DAD method was fully validated including function response, linearity, limit of detection and quantification, recovery, matrix effects, process efficiency, repeatability and intermediate precision. The presence of some investigated drugs in samples from patients receiving medication were confirmed by HPLC–MS.

2. Experimental

2.1. Chemicals

Drug standards: Risperidone, Quetiapine, Clozapine, Levomepromazine, Perazine, Citalopram were obtained respectively from Janssen-Cilag International NV (Beerse, Belgium), Adamed Sp. z o.o. (Pieńków, Poland), EGIS Pharmaceuticals PLC (Budapest, Hungary), PROTERAPIA Sp z o.o. (Warszawa, Poland), HASCO-LEK S.A. (Wrocław, Poland), Ranbaxy Sp. z o. o. (Warszawa, Poland), Otsuka Pharmaceutical Europe Ltd. (Wexham, Great Britain). Methanol (MeOH) of chromatographic quality and diethylamine (DEA), acetic acid, sodium acetate, phosphoric acid, ammonium (25%), ammonium chloride were purchased from Merck (Darmstadt, Germany). Water was double distilled.

2.2. Apparatus and HPLC-DAD conditions

There were used BAKERBOND^{TM} speOctadecyl (C18) J.T. Baker (Phillipsburg, USA) cartridges (100 mg/1 mL) and SPE chamber – Baker SPE – 12 G J.T. Baker (Philipsburg, USA) for sample preparation.

Chromatographic analysis was performed using liquid chromatograph LaChrom Elite (Merck) equipped with an autosampler, column oven L-7350, solvent degasser L-7612 and DAD detector. The chromatographic measurements were carried out at 22 °C with an eluent flow rate of 1.0 mL/min. The chromatographic separation was performed on Phenyl-Hexyl column from Phenomenex (5 μm,150 mm × 4.6 mm). The following eluents were used: eluent (A) H_2O and 0.025 mL^{-1} DEA, eluent (B) MeOH and 0.025 mL^{-1} DEA, eluent (C) acetate buffer at pH 3.5 and 0.025 mL^{-1} DEA. Samples were injected onto HPLC column and eluted with following gradient elution program: 40% B from 0 to 15 min, from 40 to 45% B from 15 to 35 min, from 45 to 65% B from 35 to 45 min. The concentration of the eluent C was constant and was 20% during the whole analysis.

The DAD detector was set in the 200–400 nm range. The chromatographic data was acquired and processed with EZchrom Elite software.

2.3. HPLC–MS conditions

Determination of target compounds was carried out using an HPLC system equipped with the Phenyl-Hexyl analytical column. The column was maintained at 20 °C. The injected sample volume was 20 μL, while the mobile phase was composed of MeOH, acetate buffer at pH 3.5 and 0.025 mL^{-1} mixed and dosed at a flow rate of 0.6 mL/min.The mass spectral analysis was performed on a 6410 triple quadrupole mass spectrometer from Agilent (Santa Clara, USA) equipped with an ESI interface operating in positive ion mode, with the following set of operation parameters: capillary voltage, 4000 V; nebulizer pressure, 40 psi; drying gas flow, 9 L/min; drying gas temperature, 310 °C; LC–MS mass spectra were recorded across the range 50–1000 *m*/*z*. Quadrupole 1 was fixed at a set parent ion, quadrupole 2 was used as a collision chamber to induce fragmentation, and quadrupole 3 was fixed at a set daughter ion. The HPLC–MS data were collected and processed by MassHunter software (Agilent). The data were further processed using Microsoft Excel.

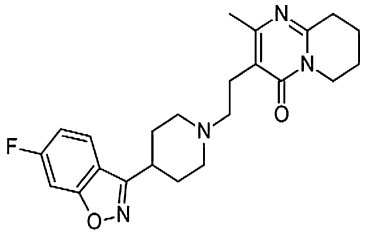
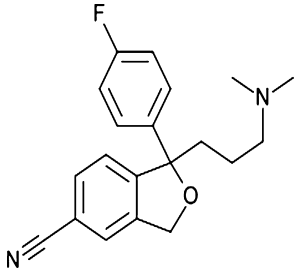
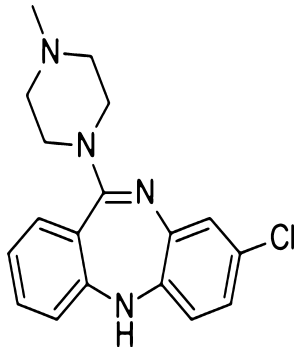
The instrument was operated in selected ions monitoring mode (SIM) and multiple reaction monitoring (MRM) as well. The monitored pseudomolecular ions $[M+H]^+$ are presented in Table 1 .

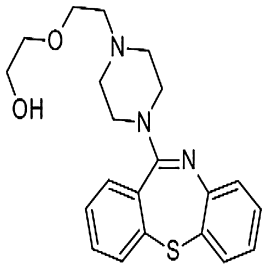
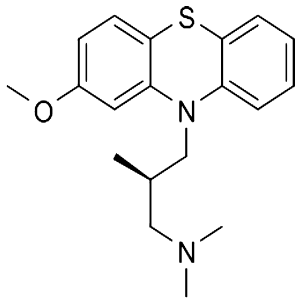
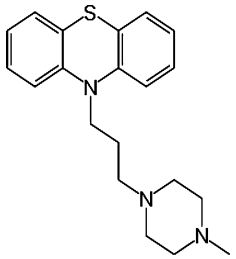
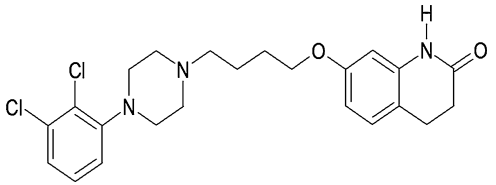
2.4. Serum and saliva sample collection

Serum and saliva samples for SPE procedure optimization and validation were taken from healthy volunteers. After blood coagulation the samples was centrifuged for 10 min at 1500 × *g*. The serum was separated and stored at −20 °C until analysis. The saliva was also stored at the same temperature.

Human body fluid samples were collected from psychiatric patients at Autonomous Public Clinical Hospital No. 1 in Lublin (Poland). The study was approved by Bioethical Commission. The

Table 1
MRM transitions and conditions for the measurement of target compounds.

Name of compound	Abbreviation	Chemical structures	Precursorion *m/z*	Production *m/z*	Fragmentor [V]	Collision energy [eV]
Risperidone	R		411	191	105	29
Citalopram	Ci		325	109	105	35
Clozapine	Cl		327	270	135	22

Quetiapine	Q		384	253	115	29
Levomepromazine	L		329	100	105	35
Perazine	P		340	141	135	15
Aripiprazole	A		448	285	105	35

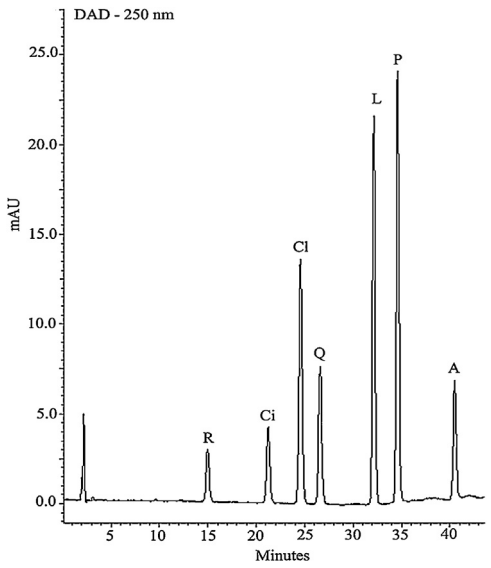


Fig. 1. Chromatogram obtained for separation of psychotropic drug stnadards' mixture.

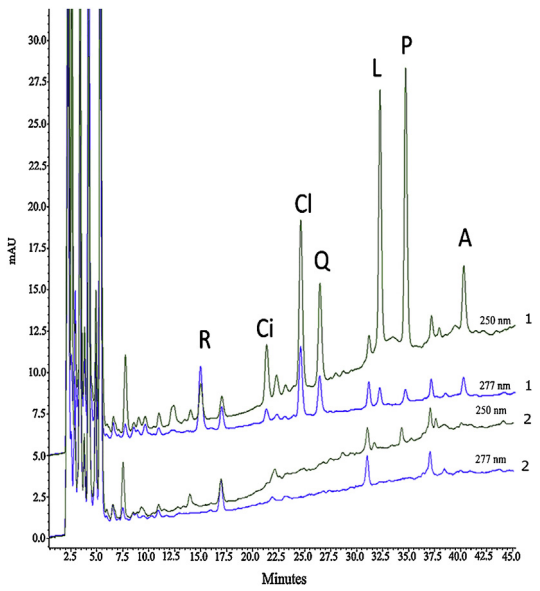


Fig. 2. Chromatograms obtained for human serum spiked of 200 ng of seven psychotropic drugs (1) and for blank human serum (2). λ = 250 and 277 nm.

samples were collected two hours after drug administration and next were prepared and stored as mentioned above.

2.5. Extraction procedure for isolation of investigated drugs from serum and saliva

Solid-phase extraction (SPE) was carried out using BAKERBOND^{TM} spe Octadecyl J.T. Baker cartridges, 100 mg/1 mL on a Baker spe-12 G apparatus.

2.5.1. Preparation of serum samples

Blank serum samples (1.5 mL) were spiked with appropriate amount of drugs. Then, the serum sample was diluted with 1.5 mL of ammonium buffer at pH 8.6.

C18 extraction columns were activated with 1 mL of methanol and conditioned with 1 mL of mixture containing water and ammonium buffer at pH 8.6 (5:1). Then, the serum containing the investigated drug was introduced to the columns at speed 1 mL/min. The columns were prewashed with 1 mL of MeOH—water solution (1:4) and dried applying vacuum for 3 min. The extracted drugs were eluted twice with 1 mL of mixture containing 98% methanol and 2% acetic acid. The sample was evaporated to dryness and dissolved in 0.5 mL of mixture containing 40% (v/v) MeOH, 40% (v/v) double distilled water, 20% (v/v) acetate buffer at pH. 3.5 and 0.025 mol/L DEA. 95 μL of the eluate was injected directly into the HPLC column.

2.5.2. Preparation of saliva samples

1.5 mL of human saliva was spiked with appropriate amount of drugs and was filtered through a 0.22 μm filter. Next 0.3 mL of ammonium buffer at pH 8.6 was added to the sample. SPE C18 extraction column were activated with 1 mL of methanol and conditioned with 1 mL of mixture containing water and ammonium buffer at pH 8.6 (5:1). Then, the saliva sample was introduced to the column at speed 1 mL/min. The column was prewashed with 1 mL of MeOH—water solution (1:4) and dried applying vacuum for 3 min. The extracted drugs were eluted twice with 1 mL of mixture containing 98% methanol and 2% acetic acid. The sample was evaporated to dryness and dissolved in 0.5 mL of mixture containing 40% (v/v) MeOH, 40% (v/v) double distilled water, 20% (v/v) acetate buffer at pH. 3.5 and 0.025 mol/l DEA. 95 μL of the eluate was injected directly into the HPLC column.

2.6. Method validation

The validation study was performed using spiked human saliva and serum according to the recommendations of the international guidelines and include evaluation of selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ), matrix effects, extraction recovery, process efficiency, precision and accuracy.

2.6.1. Selectivity

Selectivity was evaluated by analyzing the saliva and serum samples from different sources to investigate the potential interferences with the signals of analytes. Extent of interferences originated by endogenous plasma components at the specific retention time of each analyte was evaluated through a comparison of blank human body fluids' samples with the fortified serum or saliva samples.

2.6.2. Linearity

Method linearity was studied by spiking blank plasma or saliva with suitable amounts of drug standards. Samples were prepared by SPE and determined by HPLC method described above. Calibration curves were constructed by analyzing fortified samples at six concentrations, ranging from 10 to 1000 ng/mL in six replicates. The calibration curves were obtained by means of the least square method.

2.6.3. Limit of detection (LOD) and limit of quantification (LOQ)

Limit of detection (LOD) and limit of quantification (LOQ) values were calculated by determination of signal/noise ratio. Signal/noise ratio 3:1 and 10:1 responds to LOD and LOQ respectively.

Table 2
Comparison of process efficiency obtained for psychotropic drugs by various extraction procedures from human serum samples. Nominal concentration of investigated drugs in spiked serum samples was 200 ng/mL.

Name of drugs	Process efficiency (%)					
	Procedure 1	Procedure 2	Procedure 3	Procedure 4	Procedure 5	Procedure 6
Risperidone	99.35	98.52	87.52	96.68	81.53	83.98
Citalopram	119.25	106.21	100.22	97.73	37.53	97.64
Clozapine	69.79	87.64	95.24	100.78	31.67	88.46
Quetiapine	134.93	119.27	115.74	149.41	83.55	88.07
Levomepromazine	85.15	94.81	81.84	82.90	4.648	83.36
Perazine	34.41	51.50	18.04	46.04	7.272	78.46
Aripiprazole	100.95	82.08	104.67	128.16	9.685	82.60

Table 3
Matrix effect obtained for investigated psychotropic drugs in serum and saliva samples. Values were measured at 250 or 270 nm (marked *).

Name of compound	Matrix effect for serum samples			Matrix effect for saliva samples		
	20 ng/mL	200 ng/mL	800 ng/mL	20 ng/mL	200 ng/mL	800 ng/mL
Risperidone	72.69 98.27*	90 98.42*	95.74 99.84*	99.66	102.65	100.9
Citalopram	120.29	97.16	103.04	124.2	105.48	102.01
Clozapine	101.93	102.9	99.48	105.5	104.72	100.74
Quetiapine	113.76	93.46	97.41	103.7	102.9	97.84
Levomepromazine	105.84	98.62	99.68	100.6	103.4	101.4
Perazine	118.93	95.02	100.69	110.5	103.59	101.73
Apiprazole	102.71	99.46	98.12	94.79	101.97	100.57

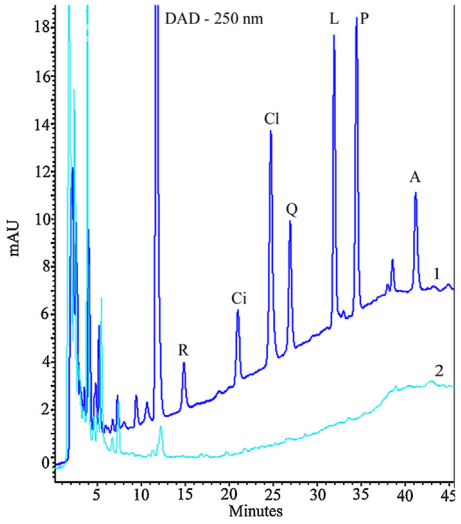


Fig. 3. Chromatograms obtained for human saliva spiked of 200 ng of seven psychotropic drugs (1) and for blank human serum (2). λ = 250 nm.

2.6.4. Matrix effects, extraction recovery, and process efficiency

Matrix effects, extraction recovery, and process efficiency were evaluated at three concentration levels (low, medium and high) according the following formulas:

$$\text{Matrix effect}\,(\%) = \frac{B}{A} \times 100$$

$$\text{Recovery}\,(\%) = \frac{C}{B} \times 100$$

$$\text{ExtractionEfficiency}\,(\%) = \frac{C}{A} \times 100$$

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

2.6.5. Precision and accuracy

Intraday precision and accuracy were performed by analyzing six replicates at three concentration: 20 ng/mL, 200 ng/mL and 800 ng/mL in the same day. For interday precision and accuracy, six replicates of blank saliva or serum samples spiked at low, medium and high concentrations were analyzed in three different days.

3. Results and discussion

3.1. Optimization of chromatographic condition

Psychotropic drug standards (Table 1) were chromatographed on Pheny-Hexyl column by the use of aqueous mobile phases containing MeOH, acetate buffer at pH 3.5, and addition of 0.025 M DEA. The chromatographic system was examined in terms of retention, separation selectivity, peak shape and system efficiency in respect of their usefulness for analysis of investigated drugs in human serum or saliva. Fig. 1 shows a typical chromatogram of standard solution. As can be seen obtained peaks are fully separated and symmetrical which is caused by the use of the double protection against interaction between basic psychotropic drugs and free silanol groups, when are simultaneously applied phenyl-hexyl stationary phase with π-π ligands and mobile phases containing addition of DEA as silanol blocker. Application of such chromatographic system lets to obtain very symmetrical peaks (all As values in range 0.9–1.2) and system efficiency was very high (N/m > 50 000) for all investigated psychotropic drugs. The high performance chromatographic system allows for separation of the analytes from the matrix components and decreases the limit of their detection and quantification.

The effect of methanol concentration in the injected psychotropic drug standards' samples (containing mixture of methanol and water) on system efficiency and peaks' symmetry was investigated. For most investigated compounds N/m were not significantly

Table 4
Intra-day and inter-day validation parameters obtained for spiked serum samples ($n = 6$).

Name of compound	Concentration added (ng/mL)	Intra-day				Inter-day			
		Average concentration founded(ng/mL)	Standard deviation SD	Accuracy (%)	Precision (%CV)	Average concentration founded(ng/mL)	Standard deviation SD	Accuracy (%)	Precision (%CV)
Risperidone	20	21.95	0.72	109.76	3.26	21.97	0.87	109.83	3.97
	200	197.22	14.07	98.61	7.13	197.29	11.44	98.64	5.80
	800	785.45	21.46	98.18	2.73	785.27	18.07	98.16	2.30
Citalopram	20	22.32	4.12	111.58	18.48*	21.22	3.89	106.11	18.33
	200	204.70	11.72	102.35	5.72	202.93	10.03	101.46	4.94
	800	822.26	43.86	102.78	5.33	821.29	40.51	102.66	4.93
Clozapine	20	20.63	0.62	103.13	3.03	20.86	0.97	104.31	4.63
	200	191.95	13.49	95.98	7.03	196.10	11.83	98.05	6.03
	800	778.81	32.86	97.35	4.22	786.75	30.78	98.34	3.91
Quetiapine	20	21.13	1.19	105.66	5.61	21.25	1.05	106.26	4.96
	200	186.43	8.62	93.22	4.62	191.21	11.20	95.60	5.86
	800	775.94	29.11	96.99	3.75	779.09	24.09	97.39	3.09
Levomepromazine	20	21.59	2.18	107.96	10.08	21.68	1.98	108.42	9.15
	200	191.83	14.17	95.92	7.39	193.51	12.48	96.75	6.45
	800	792.71	37.99	99.09	4.79	802.33	33.00	100.29	4.11
Perazine	20	21.88	2.30	109.39	10.53	21.88	1.91	109.42	8.73
	200	178.20	6.42	89.10	3.60	181.39	7.38	90.69	4.07
	800	794.92	39.31	99.37	4.95	796.02	31.09	99.50	3.91
Aripiprazole	20	22.76	2.48	113.81	10.90	22.70	1.81	113.49	7.96
	200	207.87	5.04	103.93	2.43	205.21	8.05	102.60	3.92
	800	819.38	51.70	102.42	6.31	831.35	40.03	103.92	4.81

Table 5
Parameters of calibration curves for quantitative analysis of psychotropic drugs in human serum or saliva samples: calibration curves' equations, regression coefficient (r), limit of detection (LOD), limit of quantitation (LOQ). Concentration range 10–1000 ng/mL. Analytical wavelength was 277 nm for risperidone and 250 nm for other drugs.

Name of compounds	Serum samples				Salivasamples			
	Equation of calibration curve	r	LOD	LOQ	Equation of calibration curve	r	LOD	LOQ
Risperidone	$y = 1821x - 8273$	0.9998	4.78	15.93	$y = 850.97x + 452$	0.9999	4.00	13.35
Citalopram	$y = 1140.1x + 9532.9$	0.9994	5.60	18.68	$y = 1181.3x + 8903.3$	0.9991	5.48	18.25
Clozapine	$y = 3790x - 9759.5$	0.9995	3.15	10.51	$y = 3848.4x + 5624.7$	0.9993	5.65	18.83
Quetiapine	$y = 2218.1x - 2563$	0.9992	5.23	17.44	$y = 2325.1x + 1656.3$	0.9994	2.77	9.24
Levomepromazine	$y = 4630.1x - 7054.9$	0.9999	2.40	7.99	$y = 4706.5x - 26302$	0.9999	5.63	18.76
Perazine	$y = 5046.4x + 17551$	0.9995	1.41	4.72	$y = 5147.8x - 14383$	0.9999	2.87	9.57
Aripiprazole	$y = 1370.7x + 971.9$	0.9995	5.29	17.63	$y = 1697.3x + 507.9$	0.9996	4.97	16.58

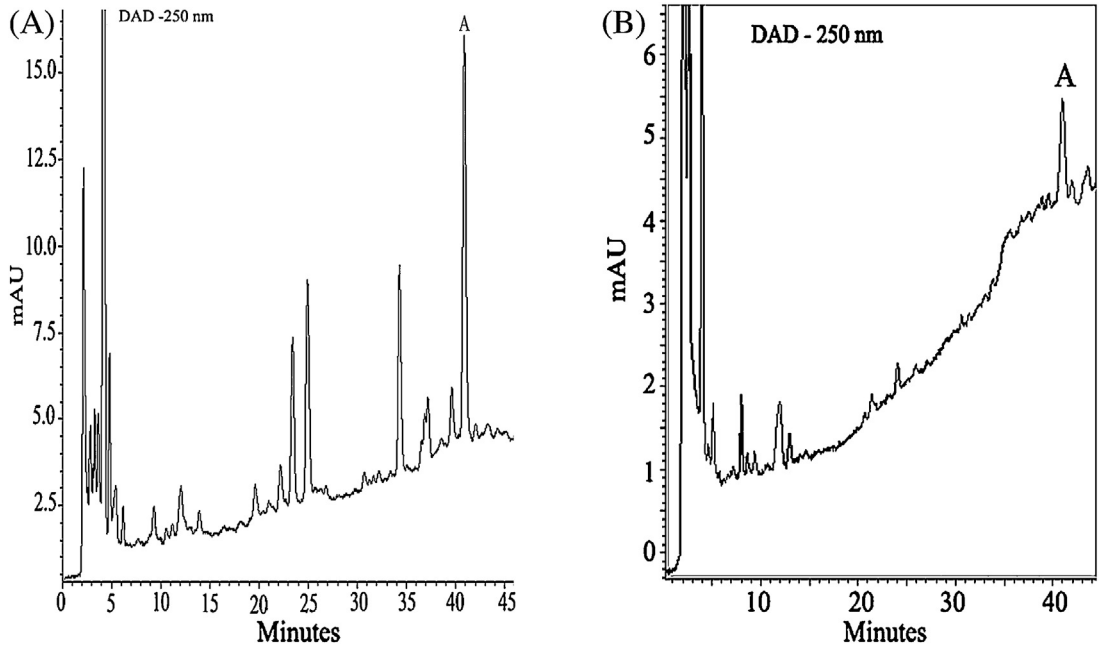


Fig. 4. (A) Chromatogram obtained for serum sample obtained from patient treated by 30 mg of aripriprazole. λ = 250 nm. (B) Chromatogram obtained for saliva sample obtained from patient treated by 30 mg of aripriprazole. λ = 250 nm.

changed in range of 0–40% of methanol and little increased in range 40–80% of methanol concentrations. However, symmetry of peaks significantly changed with increased methanol concentration in injected samples. In range 0–60% of methanol asymmetry factors were similar (in range from 1.1 to 1.4) for all injected samples, however with further increase of methanol concentration As values decreased (for 80% of methanol $0.7 < As < 1.2$). For most investigated compounds most symmetrical peaks were obtained when concentration of methanol in injected sample was 60% of methanol ($1.1 < As < 1.2$).

The influence of the injection volume on system efficiency and symmetry of peaks was also investigated. For samples dissolved in methanol increase of injection volume caused significant decrease of N/m values obtained for most investigated drugs especially for apiprazole, perazine and levomepromazine. When injection volume was 10 μL N/m values were in range 50,000–450,000 for all investigated compounds, but for 100 μL samples N/m were lower (10,000–110,000). Great differences were also observed in As values. Most symmetrical peaks were obtained for samples volume 10 or 20 μL, all peaks were of excellent symmetry ($0.9 < As > 1.2$), but for 100 μL samples As for all compounds were out of the optimal range.

In the next part of experiments psychotropic drug standards were dissolved in mobile phase of the composition such as initial gradient elution mobile phase composition.The increase of the sample injection volume did not cause changes in the number of theoretical plates obtained for all test drugs. As values were also similar in whole range of injected sample volumes ($1.0 < As < 1.3$). Based on obtained results, the serum or saliva fortified samples and samples from patients, after proper preparation, were dissolved in the mobile phase.

3.2. Optimization of SPE conditions

Different extraction procedures were tested for isolation of investigated psychotropic drugs from both biological fluids—serum and saliva. Extraction was performed on BAKERBOND™ speOctadecyl J.T. Baker cartridges (100 mg/1 mL or 500 mg/mL) with application of different solvents, pH, volume of solvents and different concentration of solvent mixtures. The aim of this study was selection an extraction procedure to obtain the best recovery and process efficiency, the possible lowest matrix effect and good purification from endogenous compounds. In this procedure SPE columns were activated with methanol and conditioned with mixture containing water and ammonium buffer at pH 8.6. Then, the samples containing investigated drugs were introduced to the columns which were prewashed with MeOH —water solution. After drying the extracted drugs were eluted twice with mixture containing 98% methanol and 2% acetic acid.

Table 6
Intra-day and inter-day validation parameters obtained for spiked saliva samples ($n = 6$).

Name of compound	Concentration added (ng/mL)	Intra-day				Inter-day			
		Average concentration found(ng/mL)	Standard deviation SD	Accurancy (%)	Precision (%CV)	Average concentration found(ng/mL)	Standard deviation SD	Accurancy (%)	Precision (%CV)
Risperidone	20	17.00	0.80	84.98	4.69	16.77	0.85	83.84	5.05
	200	212.25	7.94	106.13	3.74	208.90	10.58	104.45	5.07
	800	797.53	38.96	99.69	4.88	811.31	34.88	101.41	4.30
Citalopram	20	20.72	3.49	103.62	16.82	19.95	3.80	99.77	19.07
	200	216.00	10.71	108.00	4.96	214.44	9.82	107.22	4.58
	800	825.75	48.06	103.22	5.82	815.42	48.91	101.93	6.00
Clozapine	20	17.57	0.43	87.83	2.47	17.51	0.93	87.55	5.29
	200	222.25	16.43	111.13	7.39	219.78	12.69	109.89	5.77
	800	830.47	45.42	103.81	5.47	831.31	35.55	103.91	4.28
Quetiapine	20	18.30	1.60	91.48	8.73	17.58	1.56	87.92	8.89
	200	209.96	2.11	104.98	1.00	219.78	12.69	109.89	5.77
	800	775.07	29.11	96.88	3.76	211.16	4.16	105.58	1.97
Levomepromazine	20	21.53	0.38	107.63	1.77	21.54	0.84	107.70	3.89
	200	195.52	2.60	97.76	1.33	195.92	3.51	97.96	1.79
	800	793.99	38.10	99.25	4.80	800.26	31.37	100.03	3.92
Perazine	20	22.21	0.96	111.06	4.34	22.15	0.99	110.75	4.45
	200	193.41	3.64	96.71	1.88	192.88	3.85	96.44	2.00
	800	801.08	33.80	100.14	4.22	797.98	27.02	99.75	3.39
Aripiprazole	20	17.50	1.51	87.51	8.62	18.25	1.96	91.25	10.76
	200	204.83	8.22	102.42	4.01	203.90	14.39	101.95	7.06
	800	816.16	39.46	102.02	4.83	805.26	40.20	100.66	4.99

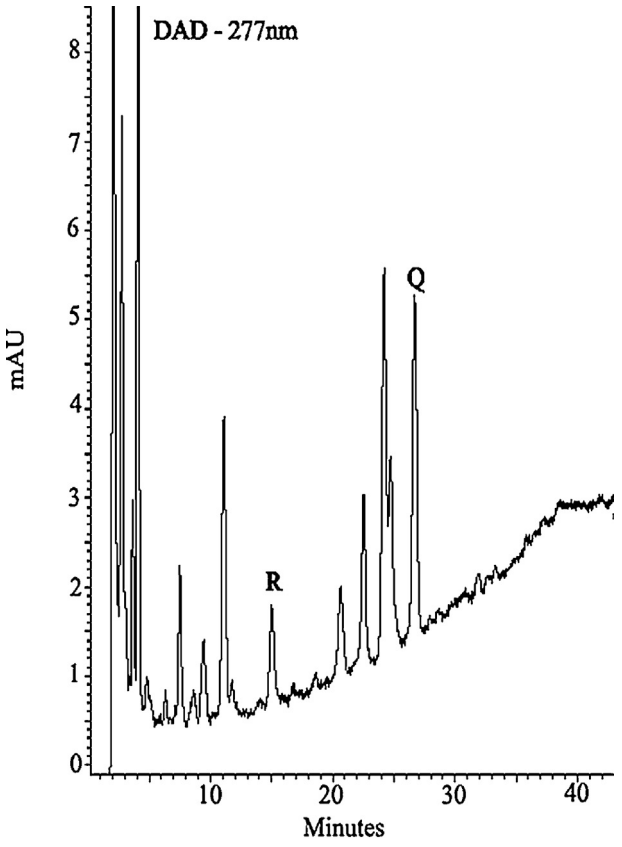


Fig. 5. Chromatogram obtained for serum sample obtained from patient treated by 3 mg of risperidone and 400 mg of quetiapine. λ = 277 nm.

Table 2 presents comparison of process efficiency obtained for psychotropic drugs by various extraction procedures from human serum samples. The optimal values of process efficiency for most investigated drugs were obtained when procedure 6 was applied. The procedure was chosen for extraction of investigated drugs from serum samples of patients treated with therapeutic doses of the psychotropic drugs. Extraction recovery, process efficiency and %CV for psychotropic drugs in human serum samples spiked by 2000 and 800 ng/mL were also investigated. The extraction recovery was in range from 94.23 to 101.21%, process efficiency in range 94.65–121.74% and %CV in range 3.46–10.85 for samples spiked of 20 ng/mL of psychotropic drugs. For serum samples spiked by 200 ng/mL of investigated drugs extraction recovery values were obtained from 80.10 to 93.66%, process efficiency values were in range 78.60–95.72% and CV in range 2.51–4.82% and for samples spiked by 800 ng/mL of drugs extraction recoveries were from 82.157 to 100.49%, process efficiency values were in range 78.46–97.64% and CV in range 2.42–7.450%.

Optimization of SPE procedure for psychotropic drugs in saliva has also been carried out. The use of saliva for determination of psychotropic drugs has the advantage of being non-invasive and easily acquired. Different solvents, pH, volume of solvents and different concentration of solvent mixtures were tested. Matrix effect was lower for most investigated compounds (excepted clozapine and quetiapine) for following SPE procedure: columns were conditioned by MeOH, mixture of water and ammonium buffer at pH 8.6 was applied for column pre-equilibration, sample was mixed with ammonium buffer at pH 8.6, 20% MeOH in water was used for washing of adsorbent and mixture containing 98% MeOH and acetic acid was applied for elution of drugs. Recovery and process efficiency obtained by the procedure was higher for all investigated drugs, for this reason, the above procedure was used for extraction of psychotropic drugs from patient samples.

The matrix effect was also investigated at different wavelength. Based on the obtained results the optimum wavelength for most investigated drugs in serum and saliva samples was 250 nm, only for risperidone in serum lowest matrix effect was at λ = 227 nm. Values of % matrix effect is presented in Table 3. All drugs in saliva and most drugs except risperidone in serum samples from patients were quantified at 250 nm. Risperidone in serum samples was analyzed at 227 nm.

3.3. Analysis of fortified human fluid samples

Fig. 2 presents chromatograms obtained for blank human serum and for serum samples fortified by seven psychotropic drugs at two wavelengths. All peaks of analytes are symmetrical and fully separated from components of matrix.

Validation of the method was performed for human serum samples spiked by 20, 200 or 800 ng/mL of all investigated psychotropic drugs. Data on intra- and inter-assay validation parameters are summarized in Table 4. The values of the intra-day accuracy were obtained in range 109.39–113.81% for samples spiked by 20 ng/mL of psychotropic drugs and in range 89.10–103.93% or 96.99–102.78% for samples spiked by 200 or 800 ng/mL respectively. The inter-day accuracy were between 104.31–113.49%; 90.69–102.60% and 97.39–103.92% for serum samples fortified by psychotropic drugs at 20, 200 and 800 ng/mL concentrations. Intra-day CV with low level drugs were from 3.03 to 18.48% and inter-day from 3.97 to 18.33%. For serum samples containing addition of 200 or 800 ng/mL intra-day CV were in range 2.43–7.39% and 2.73–6.31 respectively. Inter-day CV were from 3.92–6.45 for samples spiked by 200 ng/mL and from 2.30 to 4.93 for samples spiked by 800 ng/mL of each psychotropic drugs.

To evaluate linearity, calibration curves with six concentration points for each drug were prepared. The calibration curves were constructed by analyzing human serum samples containing different concentrations of the target drugs previously treated by the optimized SPE procedure. Equation of calibration curves, regression coefficient, limit of detection (LOD) and limit of quantitation (LOQ) were calculated (Table 5). For all drugs in investigated range of concentrations linear dependencies were obtained. The lowest LOD and LOQ values in serum samples were obtained for perazine (LOD = 1.41 and LOQ = 4.72 ng/mL).

The procedure for determination of seven psychotropic drugs in saliva samples was also developed. Typical chromatograms of blank saliva and spiked saliva samples (after SPE) are given in Fig. 3. HPLC procedure for quantification of psychotropic drugs in saliva samples was validated in a similar manner as serum samples. Intra-day accuracy were in range 84.98–107.63% for samples spiked by 20 ng/mL of drugs, 96.71–111.13% and 96.88–103.81% for samples fortified by 200 and 800 ng/mL respectively (Table 6). Inter-day accuracy were 83.84–107.70%, 96.44–109.89% and 99.75–105.58% for samples spiked by 20,200 and 800 ng/mL of drugs. Intra-day %CV were in range 1.77–16.82, 1.00–7.39 and 3.76–5.82, inter-day %CV were from 3.89 to 19.07, 1.79–7.06 and 1.97–6.00 for saliva samples spiked at three concentration levels. For saliva samples linearity was also obtained for all tested concentration levels (10–1000 ng/mL). The lowest LOD and LOQ in saliva were obtained for quetiapine (2.87 and 9.24 ng/mL).

Table 7
Drugs and their metabolites founded in human serum and saliva samples.

Sample	Drugs detected by HPLC–MS	Dose (mg)	Measured concentration by HPLC-DAD (ng/m	Metabolites detected by MS (precursor ion *m*/*z*-product ion *m*/*z*)
Serum A	Quetiapine	750	334.62	Norquetiapine: (296–210) (7-hydroxy-quetiapine: 400–269) 7-hudroxy, *N*-desalkyl quetiapine: (312–226) Quetiapine sulfoxide: (400–221)
	Clozapine	100	–	Norclozapine: (313–192) Clozapine *N*-oxide: (343–256)
Saliva A	Quetiapine	750	107.16	Norquetiapine: (296–210) (7-hydroxy-quetiapine: 400–269) 7-hudroxy, N-desalkyl quetiapine: (312–226) Quetiapine sulfoxide: (400–221)
	Clozapine	100	14.23 (<LOQ)	Norclozapine: (313–192) ClozapineN-oxide: (343–256)
Serum B	Aripiprazole	30	742	Dehydroaripiprazole: (446–285)
Saliva B	Aripiprazole	30	93.06	Dehydroaripiprazole: (446–285)
Serum C	Clozapine	150	86.51	Norclozapine: (313–192) Clozapine N-oxide (343–256)
Saliva C	Clozapine	150	50.53	Norclozapine: (313–192) Clozapine N-oxide: (343–256)
Serum D	Clozapine	150	97.35	Norclozapine: (313–192) Clozapine N-oxide: (343–256)
	Risperidone	3	16.36	9-Hydroxyrisperidone: (427–207) 7-Hydroxyrisperidone
Serum E	Perazine	150	32.63	–
Serum F	Aripiprazole	15	78.48	Dehydroaripiprazole: (446–285)
Serum G	Risperidone	3	54.85	9-Hydroxyrisperidone: (427–207) 7-Hydroxyrisperidone
	Quetiapine	400	304.95	Norquetiapine: (296–210) (7-hydroxy-quetiapine: (400–269) 7-hudroxy, N-desalkyl quetiapine: (312–226) Quetiapine sulfoxide: (400–221)
Saliva H	Aripiprazole	30	93.06	Dehydroaripiprazole: (446–285)
Saliva I	Quetiapine	400	107.5	Norquetiapine: (296–210) 7-Hydroxy-quetiapine: (400–269) 7-Hudroxy, N-desalkyl quetiapine: (312–226) Quetiapine sulfoxide: (400–221)
Saliva J	Citalopram	40	34,23	Desmethylcitalopram: (311–109) N,N-Didesmethylcitalopram: (297–109)
	Risperidone	4	1,1 (<LOD)	9-Hydroxyrisperidone: (427–207) 7-Hydroxyrisperidone
Saliva K	Quetiapine	100	64.72	Norquetiapine: (296–210) (7-Hydroxy-quetiapine: 400–269) 7-Hudroxy, N-desalkyl quetiapine: (312–226) Quetiapine sulfoxide: (400–221)
Saliva L	Quetiapine	400	9.69 (<LOQ)	(7-Hydroxy-quetiapine: (400–269) 7-Hudroxy, N-desalkyl quetiapine: (312–226) Quetiapine sulfoxide: (400–221)
	Aripiprazole	15	–	Dehydroaripiprazole: (446–285)
Saliva M	Risperidone	3	54.85	9-Hydroxyrisperidone (427–207) 7-Hydroxyrisperidone

Lower LOD and LOQ values were obtained for risperidone, citalopram, quetiapine and aripiprazole when were analyzed in saliva samples, for clozapine, levomepromazine and perazine in serum samples.

3.4. Analysis of fluid samples from patients

The proposed method was successfully applied to determine of the psychotropic drugs in human serum and saliva samples from patients receiving medication. For identification of psychotropic drugs in samples from patients HPLC–MS method was used. The presence of drugs in samples from patients were additionally confirmed by MS spectra. Patients were treated with different doses of one or two psychotropic drugs. Sample chromatograms obtained for serum and saliva from the same patient treated by 30 mg of aripriprazole (both samples obtained 2 h after administration) are presented in Fig. 4A and B. Fig. 5 presents chromatogram obtained for saliva sample from patient treated by 3 mg of risperidone and 400 mg of quetiapine obtained also two hours after administration. There was no significant interference for any of the analytes from matrix components in the real serum and saliva samples. In some analyzed samples main metabolites of investigated drugs were also

identified. Results of quantification of some psychotropic drugs and determination of their metabolites in real serum and saliva samples are summarized in Table 7.

4. Conclusions

A simple HPLC-DAD method was developed and validated for the quantification of seven basic psychotropic drugs in human serum and saliva. The SPE process allows to efficiently remove interfering substances from serum and saliva matrices while the HPLC-DAD method permits the quantification of investigated drugs and some their metabolites over the wide concentration range typically measured in psychiatric patients. Optimized HPLC–MS method can be used for identification of drugs and metabolites in samples of serum and saliva from medicated patients.

The use of the double protection against interaction between basic compounds and free silanols, when are simultaneously applied stationary phase with π–π ligands and mobile phase containing addition of DEA as silanol blocker, allows to obtain symmetrical peaks and good system efficiency. Further improvement of peaks symmetry and system efficiency was obtained by dissolving of injection samples in mobile phase.

Proposed method has been successfully applied for quantification of investigated drugs in real serum and saliva samples. The method allows for therapeutic drug monitoring, not only for a single compound but also for mixtures. The good sensitivity, precision and the high accuracy of the assay of psychotropic drugs make the methods a useful tool in clinical laboratories for therapeutic drugs monitoring as well as in forensic laboratories for their determination at therapeutic and higher levels in human fluids. The use of saliva as opposed 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 of its collection are simpler, non-invasive and painless.

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