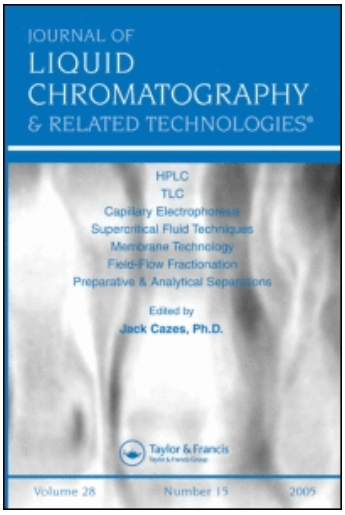

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A RAPID UHPLC METHOD FOR THE SIMULTANEOUS DETERMINATION OF SELECTED B-BLOCKERS, NSAIDS, AND THEIR METABOLITES IN HUMAN URINE AND WATER SAMPLES

Irena Baranowska[a]; Andrzej Wilczek[a]
[a] Department of Analytical Chemistry, Chemical Faculty, Silesian University of Technology, Gliwice, Poland

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A RAPID UHPLC METHOD FOR THE SIMULTANEOUS DETERMINATION OF SELECTED B-BLOCKERS, NSAIDS, AND THEIR METABOLITES IN HUMAN URINE AND WATER SAMPLES

Irena Baranowska and Andrzej Wilczek

Department of Analytical Chemistry, Chemical Faculty, Silesian University of Technology, Gliwice, Poland

□ *A rapid Ultra High Performance Liquid Chromatography (UHPLC) method for the simultaneous determination of β-blockers (sotalol SOT, metoprolol MET, propranolol PRO), Non-steroidal Anti-inflammatory Drugs (NSAIDs) (paracetamol PAR, ketoprofen KET, salicylic acid SAL), and their metabolites (paracetamol sulfate PAR-S, paracetamol glucuronide PAR-G, ketoprofen glucuronide KET-G, o-desmethylmetoprolol D-MET, α-hydroxymetoprolol MET-H, 4′-hydroxypropranolol sulphate PRO-S) is presented in this paper. The separation of twelve determined substances was conducted by means of a C_{18} Fast Gradient monolithic column (50 mm × 2 mm) in no longer than 7.5 min. The amounts of analytes introduced onto the column varied from 0.09 ng to 0.69 ng in a 1-μL injection. The method was validated for the determination of these substances in human urine and tap water, according to Solid Phase Extraction (SPE) procedures. The linearity ranges for the analyzed substances were (μg/mL): 0.60–40 for PAR-G; 0.69–44 for PAR-S; 0.60–42 for PAR; 0.33–40 for SOT; 0.30–35 for MET-H; 0.27–40 for DMET; 0.40–45 for SAL; 0.33–40 for PRO-S; 0.40–36 for MET; 0.45–42 for PRO, 0.36–46 for KET, and for 0.42–40 KET-G. The described method was successfully applied to water samples and to urine from patients who were administered the aforementioned drugs.*

Keywords beta blockers, drugs, metabolites, NSAIDs, solid phase extraction, UHPLC-UV

INTRODUCTION

The detection of drugs and their metabolites is necessary for monitoring the state of patients and in clinical toxicology and forensic medicine. The determination of drug and/or metabolite quantities can provide information about the time of administration, dosage, or route of drug metabolism.

Address correspondence to Prof. Irena Baranowska, Department of Analytical Chemistry, Chemical Faculty, Silesian University of Technology, Strzody 7 44-100, Gliwice, Poland. E-mail: irena.baranowska@polsl.pl

Despite interest in Ultra High Performance Liquid Chromatography (UHPLC), there are few methods allowing for the simultaneous determination of drugs from different therapeutic groups and their metabolites in urine. These methods are indispensable, especially with polypragmasy, as numerous side effects may result in taking different drugs simultaneously.[1]

Many non-prescribed pharmaceuticals that contain drugs such as paracetamol and aspirin are readily available for consumption. A person with cardiological problems, who is taking a β-blocker drug, can also easily take a non-prescribed NSAID to reduce an ache or fever.

Therefore, fast methods that allow for the simultaneous determination of β-blockers, NSAIDs, and their metabolites in patients' body fluids need to be developed. Within the past few years, methods for the determination of drugs from different therapeutic groups and other biologically active compounds based on thin-layer chromatography, HPLC,[2–6] and voltamperometry[7,8] have been evaluated in our research.

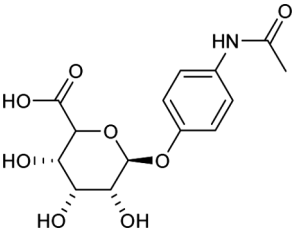
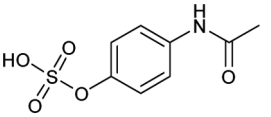
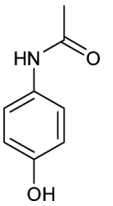
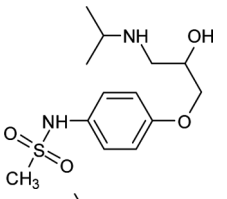
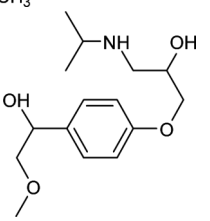
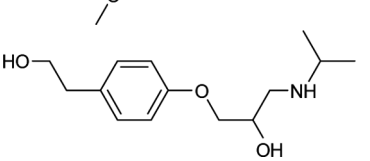
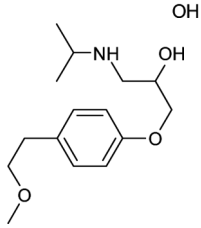
In this study, we focused on the rapid simultaneous determination of β-blockers, NSAIDs, and their metabolites in human urine.

Drugs are continuously released to the environment in both their base form and as their metabolites. These substances are introduced to surface waters as a result of manufacturing processes, outdated pharmaceutical disposal, and the excretion of body fluids. Sewage treatment plants are unable to remove these small quantities from effluent waters. Therefore, the presence of pharmaceuticals in surface water has become an acute analytical problem.[9] The continual introduction of pharmaceuticals to the environment can lead to their accumulation in ecosystems. A European Union directive (2000/60/EC) imposed continuous water monitoring for the detection of pharmaceuticals and their metabolites.

Determination of the following drugs was considered in this study: paracetamol (PAR), ketoprofen (KET), aspirin (ASP), sotalol (SOT), metoprolol (MET), and propranolol (PRO). Aspirin residues were determined as salicylic acid (SAL), because of the faster hydrolysis of ASP in water. The metabolites investigated were: paracetamol sulfate (PAR-S) and glucuronide (PAR-G) metabolites, α-hydroxymetoprolol (MET-H), o-demethylmetoprolol(D-MET), propranolol sulfate (PRO-S), and ketoprofen glucuronide (KET-G). The structures are presented in Table 1.

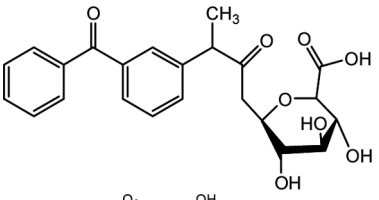
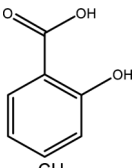
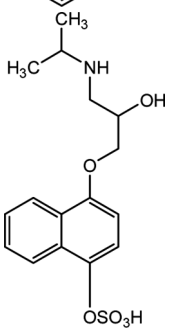
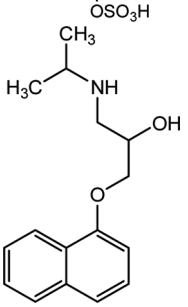
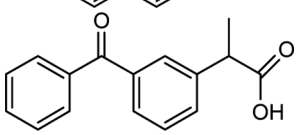
A literature survey revealed that there is no analytical method allowing for the simultaneous separation of all of these substances by UHPLC in one chromatographic system. Rather, the analysis of these biologically active ingredients is based mostly on HPLC, GC, or CE with different types of detectors. However, UHPLC has been used for the determination of some NSAIDs[10,11] and β-blockers[12] in urine. Some of the methods were

TABLE 1 Structures and Log P Values for Analysed Substances

Drug	Structure	Log P Value[a] (Octanol/Water)
PAR-G		−1.04
PAR-S		0.43
PAR		0.91
SOT		1.01
MET-H		1.06
D-MET		1.12
MET		1.76

(*Continued*)

TABLE 1 Continued

Drug	Structure	Log P Value[a] (Octanol/Water)
KET-G		1.96
SAL		2.13
PRO-S		2.31
PRO		3.48
KET		3.89

[a]Calculated with ChemAxon Marvin Calculator.

utilized to determine antibiotics or amphetamine-like stimulants in water samples.[13–16] Nevertheless, a method for the determination of selected β-blockers and NSAIDs along with their metabolites has not yet been published and will be helpful where fast analysis of the aforementioned substances is required.

EXPERIMENTAL

Chemicals and Solutions

Paracetamol (PAR) (99% purity) was obtained from Fluka BioChemika (Darmstadt, Germany); paracetamol sulfate sodium salt (PAR-S), paracetamol glucuronide sodium salt (PAR-G), sotalol hydrochloride (SOT), salicylic acid (SAL), ketoprofen (KET), metoprolol tartrate salt (MET), and propranolol (PRO) were purchased from Sigma-Aldrich (Schnelldorf, Germany) (98–99% purity). α-Hydroxymetoprolol (MET-H), o-desmethylmetoprolol (D-MET), 4′-hydroxypropranolol sulfate (PRO-S) and ketoprofen acyl-beta-D-glucuronide sodium salt (KET-G) (98% purity) were bought from Toronto Research Chemicals (North York, Canada). Acetonitrile (ACN), methanol (MeOH), water, and trifluoroacetic acid (TFA) (HPLC grade) were purchased from Merck (Darmstadt, Germany).

Stock Solutions

Stock solutions of PAR, PAR-G, PAR-S, SOT, MET, PRO, KET, SAL, GENT, D-MET, and MET-H were prepared by dissolving 10 mg of each drug in 10 mL of a methanol-water mixture (50:50 v/v). Stock solutions for KET-G and PRO-S were prepared in methanol as mentioned previously. After preparation, stock solutions were stored at 4°C and were stable for at least one month. Working standard solutions were prepared daily by mixing individual stock solutions and diluting these mixtures with specified amounts of the methanol-water mixture (1:9 v/v). The working standard solutions were stable for at least 3 days at 4°C.

Apparatus and Conditions

A reversed phase Ultra HPLC system containing a UV Detector Model L-2400U (Hitachi), L-2350 column oven (Hitachi, Merck), two L-2160U pumps, and a reversed phase Chromolith® Fast Gradient monolithic C_{18}e column (50 mm × 2 mm) (Merck) was used. Samples were injected by an L-2200U autosampler (Hitachi). The human urine was centrifuged using a HERMLE 323 K (Germany) centrifuge. Solid phase extraction procedures were performed using J.T. Baker SPE-12 G (Deventer, Netherlands) equipment with different extraction columns (Bond Elut C18 and Oasis HLB columns).

Analyses were carried out at 20°C with gradient elution. The best gradient program evaluated for the determination using the C_{18} monolithic column was received with 0,05% trifluoroacetic acid in water (solvent A) and

TABLE 2 Gradient Elution Program for UHPLC Equipment. Solvents: A – 0.05% TFA in Water; B–Acetonitrile

Time (min)	Solvent A (%)	Solvent B (%)	Flow (mL/min)
0.0	100	0	1.5
2.0	98	2	1.0
4.0	90	10	0.9
5.0	90	10	1.5
10.0	10	90	1.5

acetonitrile (solvent B). The gradient elution program is presented in Table 2. The eluate was monitored by UV detection at wavelengths specified for each drug; PAR, PAR-G, PAR-S, and SAL were measured at $\lambda = 241$ nm; SOT, MET MET-H, D-MET, PRO-H, and PRO at $\lambda = 227$ nm; and KET and KET-G at $\lambda = 254$ nm. The analytical wavelengths and retention times are presented in Table 3.

Sample Preparation Procedure for Urine Measurements

Urine samples were collected from patients who took the drugs of interest. The urine (1 mL, acidified to pH = 2 with 1 M hydrochloric acid) was centrifuged at 6500 rpm for 15 min. A clear supernatant was collected and the Solid Phase Extraction (SPE) procedures were carried out.

In addition, blank urine (for the accuracy and recovery tests) was collected from three volunteers who had not taken any of the analyzed drugs in the previous 72 hr.

TABLE 3 Monitoring Wavelengths and Retention Times for Analysed Substances (n = 6)

Analytes	Monitoring Wavelength (nm)	Retention Time, Rt (min)	S.D. of Rt (min)	R.S.D. (%)
PAR-G	241	0.670	0.0058	0.13
PAR-S	241	1.009	0.0042	0.10
PAR	241	1.672	0.0099	0.23
SOT	227	2.566	0.0090	0.20
MET-H	227	4.313	0.0057	0.13
DMET	227	4.564	0.0110	0.27
SAL	241	4.698	0.0063	0.15
PRO-S	227	5.111	0.0035	0.09
MET	227	5.762	0.0310	0.73
PRO	227	6.543	0.0052	0.12
KET-G	254	6.830	0.0041	0.10
KET	254	7.221	0.0059	0.13

Solid Phase Extraction Procedure for PAR-G and PAR-S From Urine

The C18 SPE columns (250 mg, 6 mL, J.T. Baker) were conditioned with 3 mL of methanol and 3 mL of H_2O acidified to pH = 2. Next, 0.5 mL of the clear (or spiked) supernatant (prepared as described previously) was diluted with distilled water to 5 mL and loaded on the column. After drying, the analytes were eluted from the column with 2 mL of MeOH. Methanol was evaporated to dryness under a nitrogen stream and the residues were dissolved in 1 mL of a MeOH:H_2O mixture (1:9 v/v), of which 1 μL was introduced to the chromatographic column.

Solid Phase Extraction Procedure for PAR, SOT, MET-H, D-MET, SAL, PRO-S, MET, PRO, KET, and KET-G From Urine

Oasis HLB columns (500 mg (6 mL), Waters) were conditioned with 6 mL of methanol and 6 mL of distilled water (pH = 7). Next, 0.5 mL of the clear (or spiked) supernatant (at pH = 7) was diluted with H_2O to a volume of 5 mL and continuously transferred through the SPE column. Afterwards, the column was washed with 5% methanol in water. The analytes were eluted, after column drying, with 2 mL of MeOH. Methanol was evaporated to dryness under a gentle nitrogen stream and the residues were dissolved in 1 mL of a methanol-water mixture (1:9 v/v).

Sample Preparation Procedure for Tap Water Measurements

Tap water samples were collected in different places (Poland). Before the extraction procedure, samples were put through a 0.2-μm filter to remove suspended solids and other contaminants. Following this step, one liter of filtered tap water was transferred to a previously conditioned (6 mL MeOH, 6 mL H_2O) HLB extraction column. The sample flow rate was set to 8 mL/min. After sample throughput and the washing step (5% methanol in water), the column was dried to remove all water remaining on the bed. After elution with 2 mL of MeOH, methanol was dried under a gentle nitrogen stream. The residues were dissolved in 1 mL of a methanol-water mixture (1:9 v/v) and injected onto the column. The injection volume used for the analysis was 1 μL.

RESULTS AND DISCUSSION

A new Ultra HPLC analytical method for the determination of PAR-G, PAR-S, PAR, SOT, D-MET, MET-H, SAL, PRO-S, MET, PRO, KET, and

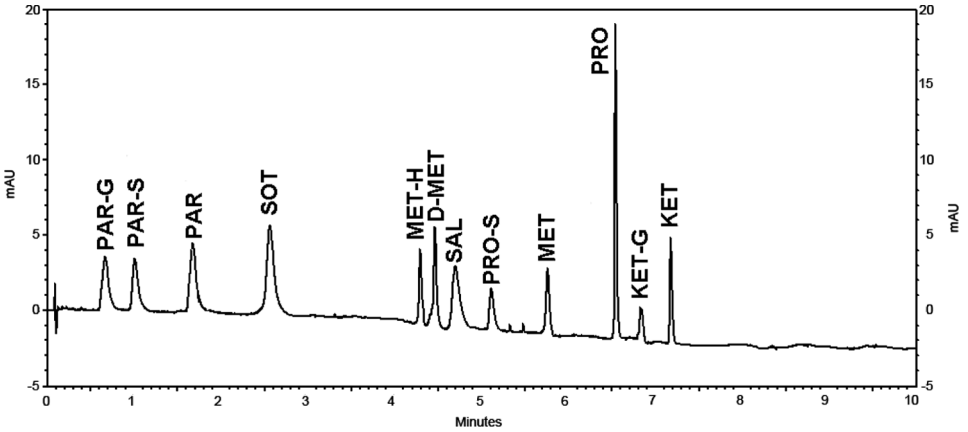


FIGURE 1 An example of an UHPLC chromatogram obtained for standard solutions of determined drugs with the gradient elution program presented in Table 1. Drug peaks were recorded at the proper wavelength (Table 3) and the concentration level was 5 μg/mL. The injection volume was 1 μL.

KET-G is proposed herein. Various gradient compositions and flow rates were tested for the best and shortest separation of the analytes. Acetic acid, trifluoroacetic acid, and formic acid were tested as mobile phase modifiers. The chromatographic system based on a C_{18}e monolithic column and a two-component (0.05% TFA and ACN) mobile phase allowed for the optimal separation of the analytes. Under the presented conditions, no interferences with matrix peaks were observed. The analysis time was quite short, as all of the twelve determined substances were eluted from the column in no longer than 7.5 min. By comparison, these compounds are separated on a conventional HPLC (C_{18} monolithic column) in 25 minutes. Figures 1–4 present examples of UHPLC chromatograms obtained from standards and spiked urine samples. The presented analytical procedure was successfully applied to patients' urine samples.

Calibration Curves

Calibration curves were determined as a linear function $y = ax + b$, where y is the peak area measured on the UV detector and x is the drug concentration (μg/mL). The values a and b are constant. The limits of detection (LOD) were calculated with a signal-to-noise ratio (S/N) of 3. The limits of quantification values were determined based on the S/N ratio being multiplied by 10 ($LOQ = 10 \times S/N$). The linearity ranges, LOD and LOQ values, and calibration curve parameters are detailed in Table 4.

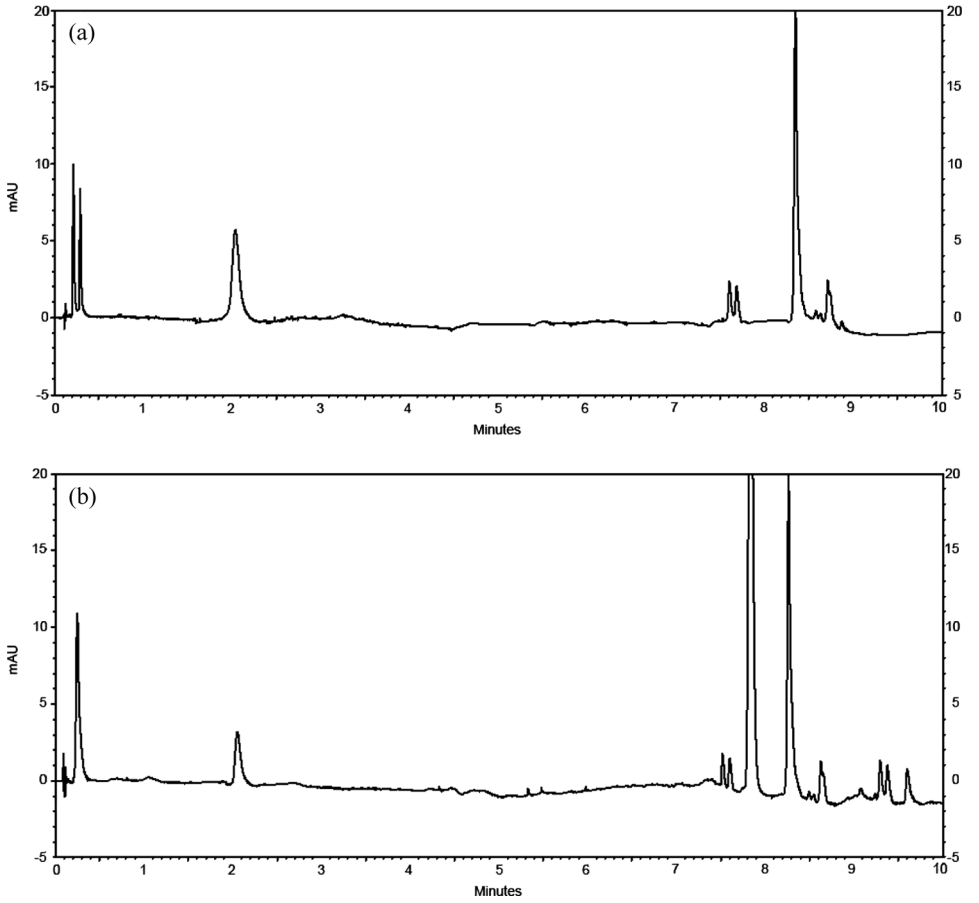


FIGURE 2 An example of an UHPLC chromatogram of blank human urine after the sample preparation procedure using Oasis HLB extraction cartridges (best chromatogram). (a) Human urine from a healthy volunteer. (b) Human urine from a volunteer with kidney inflammation who had taken only an antibiotic drug (norfloxacin). Injection volume was 1 μL.

Recoveries and Accuracy

Two SPE procedures were evaluated for the preconcentration of the analytes from urine and one from tap water. The samples, which were spiked with all 12 drugs, were extracted. Each extract was injected onto a column, and the drug recoveries are presented in Table 5.

Recoveries From Urine

The recoveries of the determined substances were established for blank human urine samples spiked with known amounts of drugs (5 μg/mL). The

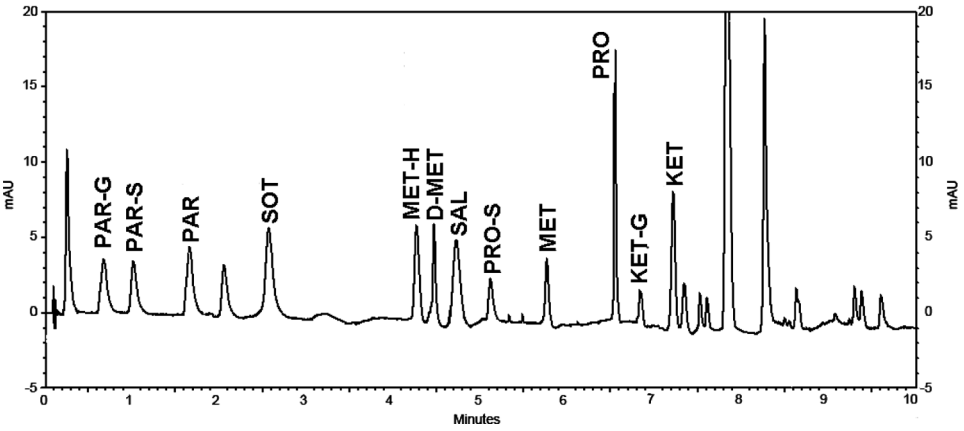


FIGURE 3 An example chromatogram of human urine spiked with a known amount of drugs (5 μg/mL). Spiking was performed after the solid phase extraction procedure on the extraction cartridges (Oasis HLB) (best chromatogram). The urine was collected from a person with kidney inflammation.

recoveries for the Oasis HLB and C_{18} extraction columns are presented in Table 5. The highest recoveries for PAR-G and PAR-S extraction from urine were achieved after solid phase extraction on a C_{18} column at pH 2. The other drugs had higher recoveries on HLB columns. Although the preconcentration method on the HLB column can be adapted to almost all of the analytes, we recommend using a C_{18} solid phase extraction procedure for PAR-G and PAR-S (even in the presence of other drugs in a sample) and the HLB procedure for the remainder of the drugs.

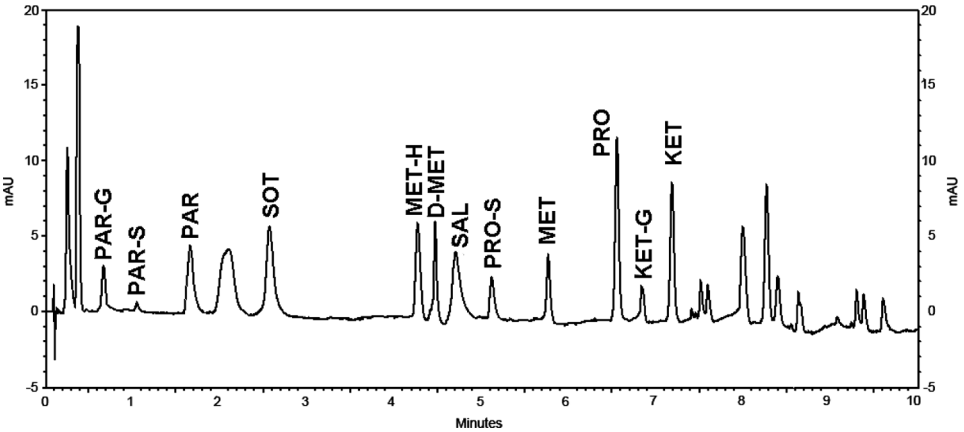


FIGURE 4 An example of an UHPLC chromatogram of human urine spiked with drugs (5 μg/mL) and extracted with the extraction procedure using HLB cartridges (injection volume 1 μL). Each drug was monitored with the specified wavelength (Table 3).

TABLE 4 Linearity Ranges, Calibration Curves Parameters, LOD, and LOQ Values (n = 6)

Analytes	Linearity Range (μg/mL)	Calibration Curves Parameters					LOD (μg/mL)	LOQ (μg/mL)	LOQ_{Method} (μg/mL)
		Slope (a)	Sa[a]	Intercept (b)	Sb[b]	R^2			
PAR-G	0.60–40	17942	279	2787	5532	0.9990	0.18	0.60	0.62
PAR-S	0.69–44	21076	498	5787	9870	0.9977	0.21	0.69	0.92
PAR	0.60–42	17468	424	18584	11157	0.9997	0.18	0.60	0.60
SOT	0.33–40	35271	580	−18447	11480	0.9989	0.099	0.33	0.33
MET-H	0.30–35	13769	365	−4131	8196	0.9972	0.10	0.30	0.35
DMET	0.27–40	8681	125	1184	3001	0.9992	0.08	0.27	0.27
SAL	0.40–45	16395	149	−1001	2958	0.9996	0.108	0.36	0.36
PRO-S	0.33–40	6028	30	493	566	0.9999	0.10	0.33	0.40
MET	0.40–36	7297	156	191257	3644	0.9981	0.12	0.40	0.42
PRO	0.45–42	24513	625	10684	7662	0.9980	0.13	0.45	0.45
KET-G	0.42–40	12343	218	128099	2672	0.9990	0.13	0.42	0.42
KET	0.36–46	18950	578	13509	7083	0.9970	0.108	0.36	0.36

[a]Standard deviation of a slope.
[b]Standard deviation of intercept.

The accuracy was measured for urine on three concentration levels (1 μg, 10 μg, and 30 μg per mL) to cover the expected concentration range of the determined substances. For measuring the accuracy of PAR, SOT, MET-H, D-MET, PRO-S, MET, PRO, SAL, KET-G, and KET blank urine was previously prepared by extraction on Oasis HLB cartridges. For PAR-G and PAR-S, blank urine samples were extracted using C_{18} cartridges. The average measured concentrations and accuracies are presented in Table 6.

TABLE 5 Recoveries of Analyzed Substances for the Evaluated Spe Procedures (n = 6)

Analytes	HLB (Urine)		C_{18} (Urine)		HLB (Tap Water)	
	Spiking with 5 μg of Drugs				Spiking with 2 μg of Drugs	
	Recovery (%)	SD (%)	Recovery (%)	SD (%)	Recovery (%)	SD (%)
PAR-G	34.5	3.1	**95.7**	3.3	9.1	2.4
PAR-S	12.6	1.3	**72.5**	2.7	5.3	1.9
PAR	103.5	3.6	75.0	5.0	95.7	4.1
SOT	98.6	1.5	95.6	1.9	94.3	3.3
MET-H	81.4	2.8	79.9	2.0	79.15	1.5
DMET	111.6	3.0	30.2	4.5	105.4	3.7
SAL	78.1	2.2	108.4	3.6	75.2	1.3
PRO-S	79.7	3.1	45.2	1.1	78.1	2.5
MET	95.0	3.2	58.4	0.9	89.6	3.0
PRO	116.0	1.9	60.4	2.2	104.25	2.4
KET-G	99.6	3.6	106.4	3.1	90.2	2.1
KET	86.3	4.1	110.8	2.1	85.2	3.3

TABLE 6 Accuracy Values for Determined Drugs (n = 6)

Analytes	Concentration Added (μg/mL)	Concentration Measured (μg/mL)	Average Accuracy (%)
PAR-G[a]	1	1.12 ± 0.15	110.25
	10	10.05 ± 0.11	100.72
	30	29.58 ± 0.49	98.62
PAR-S[a]	1	0.91 ± 0.05	91.70
	10	9.85 ± 0.12	99.26
	30	29.82 ± 0.42	99.41
PAR	1	0.93 ± 0.026	94.20
	10	10.04 ± 0.05	100.27
	30	29.96 ± 0.20	99.86
SOT	1	1.15 ± 0.09	115.26
	10	10.2 ± 0.14	101.33
	30	29.64 ± 0.17	98.81
MET-H	1	1.01 ± 0.09	101.35
	10	10.08 ± 0.08	100.79
	30	30.1 ± 0.11	100.31
DMET	1	1.08 ± 0.014	107.63
	10	10.14 ± 0.14	100.96
	30	30.11 ± 0.07	100.36
SAL	1	1.05 ± 0.049	104.75
	10	9.89 ± 0.07	98.34
	30	28.82 ± 0.52	96.08
PRO-S	1	0.982 ± 0.03	98.03
	10	10.04 ± 0.15	100.32
	30	30.34 ± 0.64	101.13
MET	1	1.03 ± 0.04	102.90
	10	10.15 ± 0.23	101.37
	30	30.05 ± 0.05	100.17
PRO	1	1.04 ± 0.045	103.96
	10	9.91 ± 0.11	98.89
	30	30.14 ± 0.04	100.47
KET-G	1	0.96 ± 0.02	95.96
	10	10.06 ± 0.03	103.27
	30	29.89 ± 0.08	99.62
KET	1	1.03 ± 0.05	102.76
	10	10.18 ± 0.10	100.87
	30	30.02 ± 0.07	100.09

[a]blank human urine extracted on C_{18} cartridges.

Tap Water

For the tap water analysis, a procedure using HLB cartridges was utilized. One liter of spiked (2 μg/1 L of each substance) tap water was used for the analysis. The obtained recoveries were similar to those achieved for urine and are presented in Table 5. The HLB cartridges have favorable properties for the preconcentration of the analytes from water samples. Due to the balanced hydrophilic and lipophilic properties, HLB cartridges showed good recoveries for almost all of the determined substances, except for PAR-G and PAR-S.

Urine Sample Measurement

After validation, human urine samples, which came from patients taking propranolol (together with paracetamol because of infection) or metoprolol, were analyzed. The compounds of interest were identified by the standard addition method.

Figure 5 presents example chromatograms of human urine samples collected 6 hr after drug administration. Certain displacements of the analytes on the elution curves can be observed; however, the differences in retention time values were within the range estimated by standard deviation of standards. The concentration range in urine samples for metoprolol was 0.84–1.35 μg/mL. For the metoprolol metabolites, the concentration ranges were 5.30–9.80 μg/mL for MET-H and 1.13–4.35 μg/mL for D-MET. Propranolol concentrations were within the range of

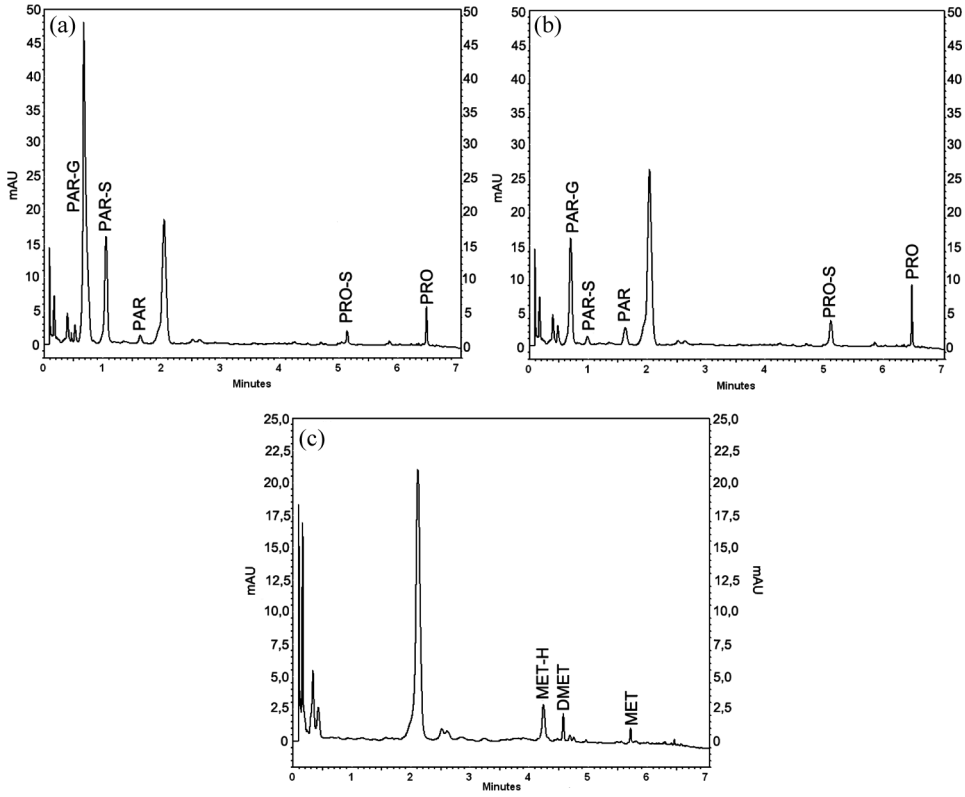


FIGURE 5 Examples of UHPLC chromatograms of human urine 6 h after administration: (a) propranolol (10 mg) and paracetamol (500 mg) – extracted on C18 spe cartridges; (b) propranolol (10 mg) and paracetamol (500 mg) – extracted on HLB cartridges; and (c) metoprolol (50 mg) – extraction on HLB cartridges. Each drug was monitored with the specified wavelength: 241 nm for PAR-G, PARS, and PAR; 227 nm for MET, MET-H, D-MET, PRO, and PRO-S.

1.72–7.95 μg/mL and 10.68–18.94 μg/mL for its sulfate metabolite (PRO-S). The concentration ranges of paracetamol and its metabolites were: 5.76–27.32 μg/mL for PAR, 81.7–145.23 μg/mL for PAR-G, and 51.25–73.25 μg/mL for PAR-S.

CONCLUSION

The UHPLC technique was applied for the determination of the selected *β*-blockers, NSAIDs, and their metabolites in human urine and tap water. The substances were separated with good resolution in no longer than 7.5 min. The sharp peaks obtained using the UHPLC equipment led to superior sensitivity and lower limits of detection. The evaluated solid phase extraction procedures on C_{18} and HLB cartridges allowed for the determination of low quantities of drugs.

The following method may be further adapted for determination in other body fluids and water samples. If a new extraction method allowing for simultaneous extraction of the aforementioned 12 drugs is proposed, this chromatographic system can be utilized.

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