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UHPLC method for the simultaneous determination of β-blockers, isoflavones and their metabolites in human urine

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ABSTRACT

A rapid-resolution ultra high-performance liquid chromatography separation method (UHPLC) for the simultaneous determination of the following β-blockers: milrinone, sotalol, metoprolol, propranolol and carvedilol, and their metabolites: 5′-hydroxylphenyl-carvedilol, *O*-desmethylcarvedilol, 4-hydroxypropranolol, α-hydroxy-metoprolol, *O*-desmethyl-metoprolol; the following isoflavones: genistein, daidzein, glycitin, glycitein, puerarin and biochanin A; as well as their metabolites: dihydrogenistein, desmethylglycitein, 8-hydroxygenistein, daidzein-7,4′-diglucoside, 8-hydroxydaidzein, dihydrobiochanin A in human urine was optimized. The analysed compounds were extracted from human urine by means of solid phase extraction (SPE). The effective UHPLC separation of the examined compounds was applied on a Hypersil GOLD^{TM} (50 mm × 2.1 mm, 1.9 μm) column with a gradient mobile phase system and a UV detector. The complete separation of all analytes was achieved within 8.0 min. The method was validated for the determination of the aforementioned substances in human urine. The linear ranges, limits of detection (LOD) and limits of quantification (LOQ) for β-blockers, isoflavones and their metabolites were determined. The intra- and inter-day precision (%C.V.) was less than 4.48%, and the intra-day and inter-day accuracy was less than 4.74%. The tested SPE sorbent proved that appropriate absolute recoveries can be obtained for Oasis HLB (Waters). The mean recovery of the analytes, using the new SPE procedure, amounted from 70.14% to 99.85%. The present paper reports, for the first time, the method for the determination of β-blockers, isoflavones and their metabolites in human urine samples. The newly developed method was suitably validated and successfully applied for the analysis of the certain of the aforementioned analytes in human urine samples obtained from the patients suffering cardiovascular disease.

1. Introduction

Cardiovascular disease poses a major health problem in the world, and therefore an accurate diagnosis and pharmacological therapy is critically important for successful treatment. β-Blockers play a crucial role in the progression of cardiovascular disease, moreover they are also recommended as a primary therapy in other diverse medical conditions, which present treatment problems. Furthermore, these are the drugs used in the treatment of hypertension, angina pectoris and arrhythmia. Most β-blockers are metabolised to a different extent and are excreted as a variety of different metabolites, but some are not metabolised and are excreted unchanged [1,2].

Cardiovascular disease is often caused by the accumulation of unhealthy habits, moreover, a number of the effects of bad habits can be prevented by through lifestyle changes. Numerous major clinical studies conducted in the last two decades have shown that isoflavones exert positive influence on health, and, notably, a diet rich in these compounds alleviates and prevents manifold serious diseases. The high level of isoflavones in the diet has been associated with a lowered risk for hormone dependent diseases, including breast and prostate cancers, osteoporosis and cardiovascular disease. Theoretical underpinnings for the efficacy of isoflavones as antioxidants *in vivo* stem from the inhibition of low-density lipoprotein (LDL) oxidation. The inhibition of LDL oxidation and platelet aggregation by isoflavones points on the fact that the regular consumption of food or beverages containing these compounds may protect against atherosclerosis and a tendency to thrombosis. A variety of pills and capsules, containing isoflavones originating from soy or red clover extracts, are commercially available, and getting high levels of isoflavones without changing the original diet poses no major problem [3–5]. The metabolism of isoflavones in humans has been studied to a certain extent, however, detailed studies are lacking. As the literature collected in

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this paper presents, it can be concluded that the metabolism of isoflavones in humans is diverse. After isoflavone supplementation several isoflavone metabolites are formed by deglycosylation, reduction, oxidation, methylation and demethylation, and conjugation with glucuronic and/or sulfonic acids prior to excretion [6].

The recommendation, given to patients, for taking isoflavones as medicine or as the ingredients of a diet rich in isoflavones undoubtedly supports a β-blockers therapy. Therefore, in the following research we are suggesting an analytical method, which is within the bounds of possibility of monitoring both drugs and antioxidants in human urine.

Nevertheless, several methods have been reported for the β-blockers determination in biological fluids. Separation has been achieved with either conventional LC [7–16] or ultrahigh performance liquid chromatography (UPLC) [17]. Moreover, several methods utilizing low resolution MS have been published [7–16]. A UV–vis detector, particularly those in a diode-array configuration [18–24], fluorescence detector [24–30] and electrochemical detector [31] are the most widely utilised detectors with HPLC. Exclusively, one HPLC–UV method for the determination of milrinone was developed [32]. The simultaneous analysis of β-blockers and their metabolites in biological samples was carried out by capillary electrophoresis (CE) [33,34], GC–MS [35], HPLC with a fluorescence detector [36–40].

Because of the importance of isoflavones to human health, the analysis, identification and structural determination of these compounds in biological fluids are of the uttermost importance in various areas of science. Likewise, the analysis of isoflavones is often accomplished via GC–MS involving the derivatization of the analytes with a number of reagents [41–43]. Nevertheless, the application of HPLC with a UV, fluorescence, electrochemical or mass spectrometry detector to the detection and identification of isoflavones in biological fluids is rapidly becoming the method of choice for laboratories [44–50]. The most commonly used analytical method for the simultaneous detection and identification of isoflavones and their metabolites has been HPLC–MS and HPLC–UV [51–55].

The reviews on this subject have recently appeared, though no article related to the simultaneous determination of drugs and isoflavones in biological samples using UHPLC has ever been mentioned in literature. It was decided to establish a single method to extract and identify β-blockers, isoflavones and their metabolites through a single extraction and analysis procedure. The aim of our study was to develop a rapid and sensitive UHPLC method for the analysis of urine samples.

2. Experimental

2.1. Chemicals and standards

Isoflavones, including genistein (GT), daidzein (DA), glycitin (GLY), puerarin (PUR) and biochanin A (BIO), as well as β-blockers: milrinone (MIL), (±)-sotalol hydrochloride (SOT), (±)-metoprolol (+)-tartrate salt (MET), (±)-propranolol hydrochloride (PRO) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA) and Aldrich Chemicals Co. (Milwaukee, WI), except carvedilol (CAR), 5′-hydroxyphenyl-carvedilol (5′-HCAR), *O*-desmethylcarvedilol (*O*-DMCAR), 4-hydroxypropranolol hydrochloride (4-HPRO), α-hydroxymetoprolol (α-HMET), *O*-desmethylmetoprolol (*O*-DMMET) which were purchased from Toronto Research Chemicals (TRC). Glycitein (GLC) and metabolites, including dihydrogenistein (DHGT), desmethylglycitein (DMGLC), 8-hydroxygenistein (8-HGT), daidzein-7,4′-diglucoside (GLU-DA), 8-hydroxydaidzein (8-HDA), dihydrobiochanin (DHBIO) were purchased from Plantech (U.K., England). As internal standards (IS) were used hesperetin (HST) and paracetamol (PAR) (Sigma Chemicals Co. (St. Louis, MO, USA) and Aldrich Chemicals Co. (Milwaukee, WI)). HPLC-grade acetonitrile, water and trifluoroacetic acid were purchased from Merck (Darmstadt, Germany). Analytical-grade methanol, acetone, sodium acetate buffer (pH 4.66), phosphate buffer (pH 2.4) and formic acid were purchased from POCH S.A. (Gliwice, Poland). β-Glucuronidase/sulfatase (crude solution from *Helix pomatia*, type HP-2, G7017) and ascorbic acid were purchased from Sigma (St. Louis, MO, USA). The stock standard solutions of β-blockers, isoflavones and their metabolites at 1 mg/ml were prepared in methanol and stored in the dark at 4 °C. The working standard solutions were prepared daily by dilution of the stock solutions with 0.05% trifluoroacetic acid (TFA) in water. The IS (HST and PAR) solutions were prepared in the same way as described above at a concentration of 100 μg/ml.

Urine samples were obtained from a healthy volunteer who was on a diet rich in isoflavones, orally-administrated tablets of isoflavones and was treated with pill of PRO (80 mg) and MET (50 mg) two times daily. Human urine samples, after drugs administration, were collected during 4 h and 6 h. The dietary supplements contained following isoflavones: PUR, GT, and GLC. The drug- and isoflavone-free human urine samples were collected for a method validation. Human urine samples taken from patients treated with β-blockers were obtained from the Silesian Centre of Heart Disease, Medical University in Katowice (Poland). The urine samples were not personally identifiable and were collected by medical personnel on the occasion of other studies. In this publication were used only to confirm the applicability of the analytical method for the analysis of real samples.

The structures of the analysed β-blockers, isoflavones and their metabolites are presented in Table 1 .

2.2. Chromatographic conditions

The UHPLC system (Merck Hitachi, Germany) was equipped with a pump Model L-2160U, absorbance detector Model L-2400U, autosampler Model L-2200U, thermostated column compartment Model L-2350U and a degasser module.

Separation was performed on a Hypersil GOLD™ (50 mm × 2.1 mm, 1.9 μm) (Thermo Fisher Scientific Inc.) column applying a linear gradient consisting of 0.05% trifluoroacetic acid (TFA) in water (solvent A) and acetonitrile (solvent B). The gradient program of the optimum elution profile and the mobile phase flow rate are presented in Table 2. The temperature of the column oven was set to 12 °C. The detector wavelengths were set at 227, 240, 254, 280, and 324 nm. The injection volume was 2 μl.

The individual compounds were identified by the retention time comparison and the identification was verified by the method of standard addition. Data acquisition and integration were performed using an EZ Chrom Elite System Manager.

2.3. Sample preparation

A urine sample (3 ml) was mixed with 60 μl IS solutions (HST and PAR) and incubated under continuous shaking with 100 μl 1 M sodium acetate buffer (pH 4.66), 100 μl 0.1 M ascorbic acid and 50 μl β-glucuronidase/sulfatase (crude preparation from *H. pomatia*) for 18 h at 37 °C. The hydrolyzed urine sample was diluted with 2 ml phosphate buffer (0.1 M, pH 2.4) and centrifuged using a Universal Centrifuge Z 323 K (Hermle Labortechnik GmbH, Germany) at 6500 rpm for 15 min at room temperature (ca. 22 °C).

Extraction was performed with the BAKERBOND spe-12G system (J.T. Baker Inc., Deventer, Netherlands). The hydrolyzed sample was applied to the extraction Oasis HLB cartridges (6 ml, 500 mg, Waters), preconditioned successively with 6 ml methanol and 6 ml 0.1% formic acid, and allowed to run through.

Table 1
Structures of the examined compounds.

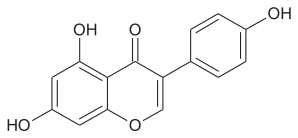
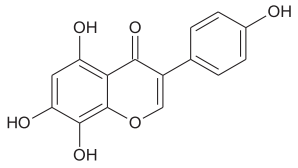
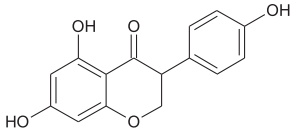
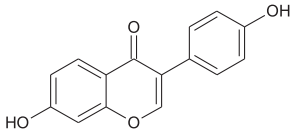
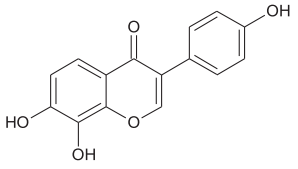
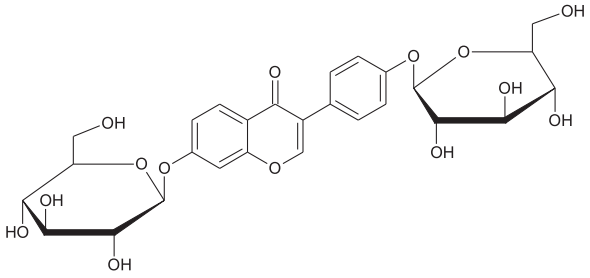
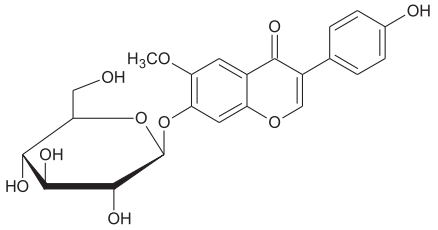
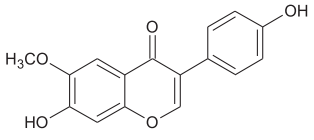
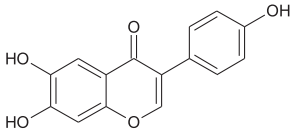
Isoflavones and their metabolites	
GT	
8-HGT	
DHGT	
DA	
8-HDA	
GLU-DA	
GLY	
GLC	
DMGLC	

Table 1 (*Continued*)

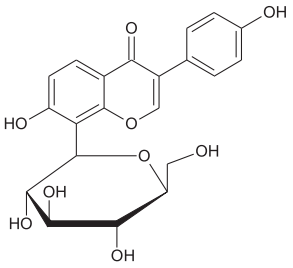
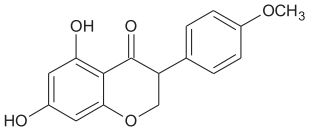
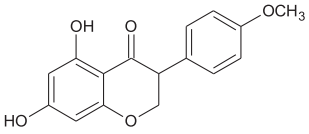
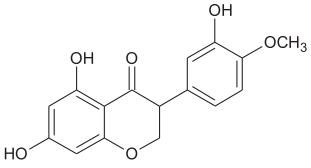
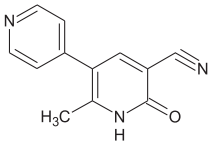
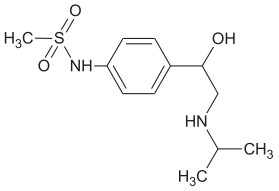
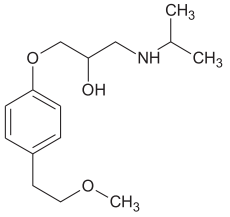
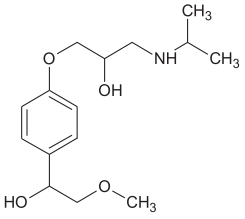
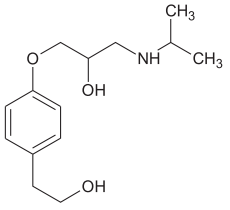
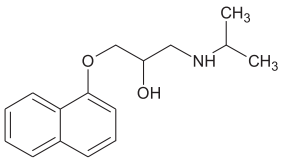
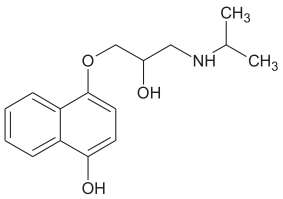
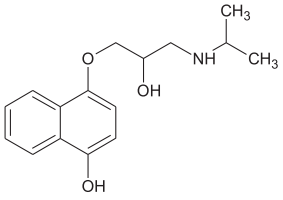
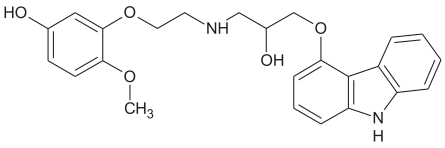
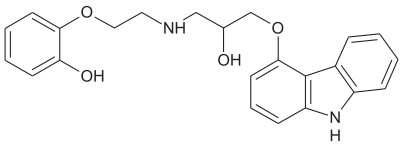
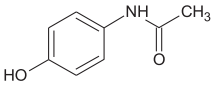
PUR	
BIO	
DHBIO	
HST (IS)	
β-Blockers and their metabolites	
MIL	
SOT	
MET	
α-HMET	
O-DMMET	

Table 1 (*Continued*)

PRO	
4-HPRO	
CAR	
5′-HCAR	
O-DMCAR	
PAR (IS)	

Isoflavones, β-blockers and their metabolites were eluted with 5 ml methanol:acetone:formic acid (4.5:4.5:1; v/v/v) and the eluate was evaporated to dryness under nitrogen stream. Finally, the residue was redissolved in 1 ml 0.05% trifluoroacetic acid (TFA) in water, filtered through a 0.20-μm membrane filter and a 2 μl volume was injected into the chromatographic system for quantification. The analysis was repeated six times.

The urine samples spiked with standards underwent the same procedure. The concentrations of the analysed compounds were determined by the calculation the ratio of analyte area to IS and the interpolation of the respective calibration curve.

Table 2
Optimum gradient elution profile.

Time (min)	Solvent		Flow rate (ml/min)
	A (%)	B (%)	
0.0	95	5	0.3
2.0	80	20	0.6
2.5	75	25	0.7
4.0	72	28	0.4
5.0	72	28	0.5
6.0	60	40	0.8
7.0	40	60	0.8
8.0	95	5	0.3

Solvent A: 0.05% TFA in water. Solvent B: acetonitrile.

2.4. Method validation

The assay was validated with respect to system suitability, linearity, the limit of detection (LOD), limit of quantification (LOQ), accuracy, precision, extraction efficiency (expressed as recovery) and stability.

Having optimized the efficacy of a chromatographic separation the quality of the chromatography was monitored by applying following system suitability tests: theoretical plates, selectivity and resolution. The system suitability method acceptance criteria set in each validation run were: theoretical plates >2000, selectivity >1.0 and resolution >1.5.

A six-point linearity curve was constructed for each analyte. The calibration curves were run on each analysis day and the coefficient of determination r^2 was used to judge linearity. The calibration curve data were generated by injecting calibration samples of different concentrations that all contain the same concentration of IS. The ratio of analyte area to IS area was calculated and plotted as the *y*-value against the concentration of the calibrator. The obtained data was submitted to a regression analysis and coefficients of determination were calculated for each isoflavone, β-blocker and metabolite using Excel®.

The LOD and LOQ were calculated according to ICH Q2R1 recommendations using the following equation [54]

$$\mathrm{LOD} = \frac{3.3S_a}{b} \quad \text{and} \quad \mathrm{LOQ} = 3\mathrm{LOD}$$

Table 3
Properties of the developed method ($n = 6$).

No.	Compound	Detection wavelengths (nm)	Retention time of standard (min)	Intra-day		Inter-day		N[c]	α[d]	R_s[e]	LOD (ng/ml)	LOQ (ng/ml)	LOD (fmol)
				S.D.[a] (min)	C.V.[b] (%)	S.D.[a] (min)	C.V.[b] (%)						
1	MIL	324	0.77	0.0005	0.07	0.0056	0.73	3645	-	-	22.0	66.1	208.5
2	SOT	227	2.30	0.0194	0.84	0.0212	0.92	17432	6.60	7.89	12.8	38.4	94.1
3	GLU-DA	254	2.58	0.0076	0.29	0.0123	0.47	74871	1.19	3.53	26.5	79.6	91.8
4	α-HMET	227	2.73	0.0088	0.32	0.0189	0.69	71596	1.07	3.89	37.9	113.8	267.6
5	*O*-DMMET	227	2.83	0.0077	0.27	0.0087	0.30	68121	1.04	2.26	37.9	113.7	299.6
6	PUR	254	2.89	0.0077	0.27	0.0087	0.30	71797	1.05	1.72	19.9	59.6	95.5
7	GLY	254	3.21	0.0068	0.21	0.0085	0.26	86348	1.14	3.64	29.1	87.3	130.4
8	8-HDA	254	3.53	0.0067	0.19	0.0099	0.28	44595	1.11	3.03	39.7	119.2	293.9
9	MET	227	3.63	0.0061	0.17	0.0095	0.26	76043	1.05	1.74	28.8	86.3	215.2
10	4-HPRO	227	3.79	0.0041	0.11	0.0113	0.30	67283	1.05	2.80	21.8	65.3	158.2
11	DMGLC	254	4.05	0.0072	0.18	0.0126	0.31	37334	1.08	3.67	63.2	189.7	467.9
12	8-HGT	254	4.19	0.0067	0.16	0.0189	0.45	23611	1.04	1.85	66.9	200.7	467.4
13	DA	254	4.57	0.0050	0.11	0.0140	0.31	47223	1.10	3.00	14.2	42.6	111.8
14	GLC	254	4.67	0.0046	0.10	0.0120	0.26	53631	1.02	1.64	78.6	235.9	553.2
15	PRO	227	5.19	0.0031	0.06	0.0073	0.14	73093	1.12	6.46	14.8	44.4	114.0
16	5′-HCAR	227	5.37	0.0032	0.06	0.0103	0.19	53999	1.04	2.24	28.4	85.3	134.7
17	DHGT	227	5.58	0.0024	0.04	0.0105	0.19	45610	1.04	2.19	44.9	134.6	329.6
18	GT	254	5.89	0.0015	0.03	0.0068	0.11	36354	1.06	2.67	13.0	38.9	96.0
19	*O*-DMCAR	227	6.30	0.0014	0.02	0.0068	0.10	73279	1.07	3.75	29.6	89.0	151.1
20	CAR	227	6.63	0.0014	0.02	0.0068	0.10	159924	1.06	4.19	13.8	41.4	67.9
21	DHBIO	227	7.14	0.0019	0.03	0.0092	0.13	104107	1.08	6.39	54.6	164.0	381.8
22	BIO	254	7.43	0.0083	0.11	0.0163	0.22	68161	1.05	2.62	10.7	32.2	75.6

[a] Standard deviation of retention times.
[b] Coefficient of variation of retention times.
[c] $N = 16(t_R/W)^2$.
[d] $\alpha = t_{R2} - t_m/t_{R1} - t_m$.
[e] $R_s = 2(t_2 - t_1)/W_1 + W_2$.

where S_a—the standard deviation of the intercept of the calibration curve and b—the slope of the calibration curve.

Three quality control (QC) samples were prepared by adding the appropriate working standard solutions to a drug, isoflavone and metabolite-free human urine. The concentrations of drugs, isoflavones and their metabolites were in range 0.16–0.48 μg/ml, 0.60–1.80 μg/ml and 2.00–6.00 μg/ml in human urine to represent low, middle and high QC, respectively.

Precision, expressed as a percent coefficient of variation (%C.V.), was determined by the back-calculation of concentrations from the respective calibration curves. Accuracy was determined by comparing the calculated concentrations from the calibration curves with the known concentrations. Accuracy, expressed as the percent error, was measured by determining the concentration of drugs, isoflavones and their metabolites measured in each sample relative to the amount of each compound added. The precision and accuracy of the assay were determined by the replicate analyses ($n = 6$) of the QC samples on the same day (intra-day) and also on three consecutive days (inter-day).

In order to calculate the recovery of the extraction procedure, QC samples were extracted according to the procedure described above and analysed. Each QC sample was analysed for three concentrations in six replicates and the concentrations were calculated using the calibration curves. Furthermore, recovery was calculated by comparing the determined amounts for extracted urine samples with the known amounts added.

The short-term stabilities of drugs, isoflavones and their metabolites were assessed by determining QC urine samples kept at room temperature for 12 h, which exceeded the routine preparation time of samples. The long-term stability was evaluated by determining QC urine samples kept at low temperature (−20 °C) for 30 days. The post-preparative stability was measured by determining QC samples kept under the autosampler conditions (12 °C) for 24 h. The freeze and thaw stability was tested by analyzing QC urine samples undergoing three freeze (−20 °C) and thaw (room temperature) cycles on consecutive days. Subsequently, concentrations of the drugs, isoflavones and their metabolites were measured compared to freshly prepared samples.

Dilution integrity exercise was carried out to ensure the integrity of analyte in those samples which are beyond upper limit of the standard curve and need to be diluted. A fresh stock of drugs, isoflavones and their metabolites was prepared and spiked in urine to get a concentration of analysed compounds 5 times higher than the highest concentration in the calibration measurements. It was then diluted 5 times and 10 times with the same urine. Six aliquots of both dilutions were processed along with freshly spiked calibration standards and analysed by back calculation using regression equation obtained.

Control human urine obtained from six volunteers, was assessed using the procedure described above and compared with results from respective urine samples to evaluate the selectivity of the method. The resulting chromatograms were examined to determine the presence of any peak that could interfere with the analysis of β-blockers, isoflavones and metabolites.

3. Results and discussion

3.1. Development of UHPLC method

In this study an UHPLC method with UV detection is reported for quantifying β-blockers, isoflavones and their metabolites in human urine.

We have studied the optimization of the proposed procedure and examined conditions which could affect the results. The selec-

tion of suitable mobile phase relied, as well, on choosing organic acids, pH and column temperature which could enable separation of the analysed compounds. Chromatographic separation was performed by gradient elution, since it improves the chromatographic peak shapes, increases the response and shortens the elution time. A number of gradient compositions and flow rates were tested for the best and shortest separation of the analytes. The best resolution of the investigated compounds was obtained using, as a mobile phase, a mixture of 0.05% TFA in water (pH 2.5) and acetonitrile. The gradient programme of the optimum elution profile and the mobile phase flow rate are presented in Table 2. The aforementioned gradient programme was selected to achieve the maximum separation and sensitivity.

Owing to the use of the short column and small particles, which has lower separation impedance comparing to the particulate packings, considerably faster separations are possible and the productivity of chromatographic processes can be increased by at least one order of magnitude as compared to traditional chromatographic columns packed with porous particles. Accordingly, the chromatographic elution step is undertaken in a short time (less than 8 min) with high resolution. The better chromatographic separation of the analytes was achieved using the column temperature of 12 °C. The eluate was monitored by the UV detector with wavelengths (227, 254 and 324 nm) specified for each analysed compound (Table 3). UV detection of IS solutions was performed at 240 nm for PAR and 280 nm for HST.

A typical chromatogram of the mixed standard solutions is shown in Fig. 1. The retention times of the compounds in the developed system are presented in Table 3. The variations in retention times were negligible with intra-assay and inter-assay %C.V. was less than 1.00% for the analysed compounds.

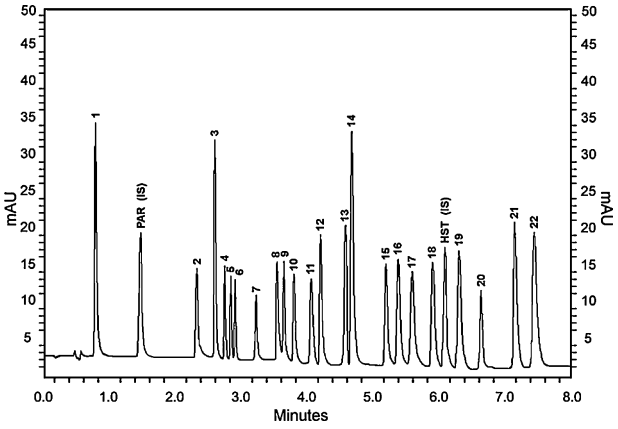


Fig. 1. The chromatogram of isoflavones: PUR (6), GLY (7), DA (13), GLC (14), GT (18), BIO (22) and their metabolite: GLU-DA (3), 8-HAD (8), DMGLC (11), 8-HGT (12), DHGT (17), DHBIO (21), β-blockers: MIL (1), SOT (2), MET (9), PRO (15), CAR (20) and their metabolites: α-HMET (4), *O*-DMMET (5), 4-HPRO (10), 5′-HCAR (16), *O*-DMCAR (19) obtained using the UHPLC method described.

3.2. Method validation

The method performance was evaluated by the determination of linearity, precision, accuracy, LOD and LOQ. The validation studies were applied for isoflavones, including GT, DA, GLY, GLC, PUR, as well as β-blockers: MIL, MET, PRO, CAR and metabolites, including 5′-HCAR, *O*-DMCAR, 4-HPRO, α-HMET, *O*-DMMET, DHGT, DMGLC, 8-HGT, GLU-DA, 8-HDA, DHBIO. Their theoretical plate numbers, selectivity and resolution values were within an acceptable criterion (Table 3).

The linear regression analysis was carried out by plotting the ratio of analyte area to IS area "*y*" against the concentrations "*x*"

Table 4
Data on regression equations for determination of all examined analytes ($n = 6$).

Compound	Linear dynamic range [a] (μg/ml)	Slope[b]	C.V.[c] (%)	Intercept[b]	C.V.[d] (%)	S_{xy}	C.V.[e] (%)	Coefficient of determination r^2 ($n = 6$)[f]
Isoflavones and their metabolites								
PUR	0.15–6	0.1399	0.38	−0.0020	1.02	0.0049	1.98	0.9998
GLY	0.15–6	0.1049	0.56	−0.0016	1.49	0.0054	2.91	0.9995
DMGLC	0.30–12	0.1253	0.31	−0.0192	1.68	0.0141	3.27	0.9994
DA	0.10–4	0.5856	0.62	−0.0152	1.10	0.0148	2.16	0.9997
8-HDA	0.25–10	0.3019	0.28	−0.0303	1.25	0.0213	2.44	0.9997
GLU-DA	0.15–6	0.4262	0.52	−0.0142	1.37	0.0201	2.68	0.9996
GLC	0.25–10	0.4196	0.44	0.0247	2.09	0.0522	3.64	0.9991
GT	0.15–6	0.1677	0.24	0.0107	0.64	0.0039	1.24	0.9999
8-HGT	0.30–12	0.1054	0.33	−0.0090	1.74	0.0125	3.39	0.9993
DHGT	0.25–10	0.1625	0.31	0.0010	1.36	0.0129	2.66	0.9996
BIO	0.20–8	0.8587	0.43	−0.0710	1.50	0.0580	2.94	0.9995
DHBIO	0.25–10	0.2152	0.41	−0.0164	1.82	0.0209	3.54	0.9994
β-Blockers and their metabolites								
MIL	0.08–8	0.3336	0.29	0.0021	0.95	0.0146	2.08	0.9998
SOT	0.10–10	0.1440	0.34	0.0104	1.63	0.0140	2.86	0.9994
MET	0.20–10	0.1373	0.29	0.0121	1.06	0.0070	2.06	0.9997
α-HMET	0.25–10	0.0748	0.26	−0.0042	1.18	0.0050	2.29	0.9997
O-DMMET	0.25–10	0.0673	0.26	−0.0025	1.17	0.0045	2.28	0.9997
PRO	0.10–4	0.3995	0.64	−0.0067	1.14	0.0105	2.23	0.9997
4-HPRO	0.15–6	0.1796	0.41	0.0045	1.09	0.0069	2.13	0.9997
CAR	0.15–6	0.1844	0.25	0.0181	0.66	0.0045	1.29	0.9999
5′-HCAR	0.15–6	0.3094	0.53	−0.0113	1.40	0.0149	2.74	0.9996
O-DMCAR	0.20–8	0.2747	0.33	−0.0210	1.17	0.0144	2.28	0.9997

S_{xy}: residual standard deviation of regression coefficient.

[a] Calibration curves were fitted to the linear regression equation $y = ax + b$, where "*y*" represents the ratio of peak areas, "*a*", and "*b*" are constants, and "*x*" is the concentration of analysed compounds.

[b] Values are mean ± S.D. of calibration curves.

[c] Coefficient of variation of slope.

[d] Coefficient of variation of intercept.

[e] Coefficient of variation of regression coefficient.

[f] Number of points in calibration curves.

Table 5
Intra-day and inter-day precision, accuracy of all examined analytes in human urine and absolute recoveries from spiked human urine ($n = 6$).

Compound	Added (μg/ml)	Intra-day					Inter-day				
		Found (μg/ml)	S.D.[a] (μg/ml)	C.V.[b] (%)	Accuracy (%)	Absolute recovery (%)	Found (μg/ml)	S.D.[a] (μg/ml)	C.V.[b] (%)	Accuracy (%)	Absolute recovery (%)
Isoflavones and their metabolites											
PUR	3.00	3.02	0.0304	1.00	0.81	80.24	3.02	0.0331	1.10	0.77	80.86
	0.90	0.93	0.0146	1.57	3.39	80.04	0.90	0.0335	3.71	0.46	80.40
	0.24	0.24	0.0009	0.37	−1.57	84.36	0.24	0.0102	4.17	2.35	86.35
GLY	3.00	3.06	0.0894	2.92	2.09	82.38	3.02	0.0767	2.54	0.58	82.86
	0.90	0.87	0.0327	3.77	−3.74	86.72	0.87	0.0222	2.56	−3.69	83.52
	0.24	0.25	0.0029	1.18	4.02	82.09	0.25	0.0105	4.20	3.85	85.25
DA	2.00	1.99	0.0174	0.87	−0.34	86.30	2.00	0.0122	0.61	−0.22	86.12
	0.60	0.57	0.0168	2.94	−4.74	94.02	0.57	0.0114	1.99	−4.70	89.60
	0.16	0.16	0.0034	2.14	−0.54	82.56	0.16	0.0060	3.65	2.63	84.73
8-HDA	5.00	5.03	0.0958	1.90	0.56	71.12	5.03	0.0681	1.35	0.68	71.60
	1.50	1.50	0.0212	1.41	−0.09	70.74	1.46	0.0460	3.15	−2.85	68.73
	0.40	0.40	0,0029	0.72	1.13	70.44	0.41	0.0063	1.54	2.42	72.14
GLU-DA	3.00	2.94	0.0188	0.64	−1.99	79.87	2.96	0.0257	0.87	−1.47	78.70
	0.90	0.88	0.0366	4.15	−2.07	80.95	0.88	0.0265	3.03	−2.74	78.74
	0.24	0.24	0.0015	0.62	−1.36	80.46	0.25	0.0104	4.26	2.23	82.25
GLC	5.00	5.03	0.0584	1.16	0.65	84.25	5.07	0.0598	1.18	1.48	85.50
	1.50	1.48	0,0062	0.42	−1.26	85.55	1.43	0.0506	3.53	−4.46	81.74
	0.40	0.40	0.0060	1.51	−1.15	86.50	0.40	0.0041	1.03	−1.03	85.61
DMGLC	6.00	5.98	0.1290	2.16	−0.40	78.15	5.96	0.0938	1.58	−0.71	77.59
	1.80	1.79	0.0066	0.37	−0.57	73.23	1.73	0.0690	4.00	−4.10	70.22
	0.48	0.48	0.0033	0.68	0.95	71.15	0.50	0.0120	2.42	3.20	73.43
GT	3.00	2.97	0.0236	0.80	−1.03	75.39	3.00	0.0378	1.26	0.03	75.42
	0.90	0.90	0.0047	0.52	−0.16	75.29	0.89	0.0175	1.97	−1.43	74.21
	0.24	0.24	0.0019	0.81	−0.94	80.77	0.25	0.0096	3.91	2.68	82.94
8-HGT	6.00	5.84	0.0790	1.35	−2.64	78.15	5.96	0.1330	2.23	−0.74	77.57
	1.80	1.80	0.0040	0.22	−0.05	74.86	1.74	0.0664	3.82	−3.38	72.33
	0.48	0.48	0.0107	2.25	−0.48	73.54	0.49	0.0173	3.53	2.17	75.14
DHGT	5.00	4.97	0.1612	3.24	−0.55	71.51	4.93	0.1244	2.52	−1.40	74.81
	1.50	1.48	0.0099	0.67	−1.42	71.38	1.45	0.0402	2.77	−3.34	71.63
	0.40	0.40	0.0048	1.21	0.04	73.05	0.41	0.0147	3.56	3.31	72.70
BIO	4.00	3.94	0.0642	1.63	−1.60	88.88	3.99	0.0703	1.76	−0.32	88.59
	1.20	1.22	0.0090	0.73	2.05	80.25	1.18	0.0493	4.17	−1.64	78.93
	0.32	0.32	0.0007	0.23	0.37	81.27	0.31	0.0089	2.82	−1.94	79.69
DHBIO	5.00	5.08	0.0797	1.57	1.55	70.36	5.01	0.1095	2.18	0.22	70.52
	1.50	1.51	0.0122	0.81	0.54	70.31	1.50	0.0243	1.62	−0.26	70.13
	0.40	0.39	0.0058	1.49	−1.89	73.36	0.40	0.0146	3.59	1.38	74.38
β-Blockers and their metabolites											
MIL	4.00	3.85	0.0188	0.49	−3.80	93.43	3.96	0.0735	1.88	−2.36	91.22
	1.20	1.15	0.0115	1.00	−4.13	90.37	1.15	0.0084	0.73	−4.01	86.75
	0.32	0.31	0.0063	2.06	−3.92	95.23	0.32	0.0120	3.79	−0.67	94.59
SOT	5.00	4.81	0.0115	0.24	−3.72	90.89	4.85	0.0694	1.43	−2.96	88.20
	1.50	1.47	0.0230	1.56	−1.73	90.68	1.54	0.0689	4.48	2.50	92.95
	0.25	0.24	0.0060	2.51	−3.87	87.42	0.24	0.0102	4.18	−2.36	85.35
MET	4.00	3.94	0.0674	1.71	−1.37	96.32	3.99	0.0676	0.02	−1.69	96.00
	1.20	1.18	0.0119	1.00	−1.29	92.15	1.20	0.0201	0.02	−1.68	91.92
	0.32	0.31	0.0138	4.40	−1.62	90.15	0.32	0.0129	0.04	−4.00	90.97
α-HMET	5.00	4.85	0.0800	1.65	−3.03	82.01	4.78	0.0887	1.85	−4.28	78.50
	1.50	1.44	0.0252	1.75	−3.81	88.63	1.49	0.0583	3.90	−0.34	78.46
	0.40	0.39	0.0023	0.60	−3.39	88.63	0.40	0.0126	3.18	−0.64	86.03
O-DMMET	5.00	5.00	0.0105	0.21	−0.06	90.07	4.95	0.0518	1.05	−1.00	89.17
	1.50	1.48	0.0236	1.59	−1.26	99.69	1.48	0.0210	1.42	−1.58	98.11
	0.40	0.40	0.0026	0.66	−0.90	99.85	0.40	0.0093	2.32	0.18	100.04
PRO	2.00	2.03	0.0135	0.66	1.34	84.15	2.01	0.0160	0.79	0.75	84.78
	0.60	0.60	0.0179	2.98	0.00	84.57	0.59	0.0169	2.88	−1.85	83.00
	0.16	0.16	0.0010	0.64	−2.10	88.58	0.16	0.0060	3.74	0.89	89.37
4-HPRO	3.00	2.98	0.0466	1.57	−0.75	81.87	2.98	0.0327	1.10	−0.79	81.22
	0.90	0.89	0.0057	0.64	−0.86	76.55	0.87	0.0202	2.30	−2.79	74.41
	0.24	0.24	0.0082	3.45	−1.44	80.06	0.24	0.0068	2.84	0.15	80.18
CAR	3.00	2.96	0.0184	0.62	−1.22	95.97	2.99	0.0373	1.25	−0.42	98.36
	0.90	0.89	0.0126	1.41	−1.16	99.40	0.88	0.0111	1.25	−1.87	96.99
	0.24	0.24	0.0026	1.11	−0.53	99.63	0.25	0.0087	3.53	2.74	102.20
5′-HCAR	3.00	2.98	0.0375	1.26	−0.52	75.87	3.02	0.0466	1.54	0.70	76.40
	0.90	0.87	0.0102	1.17	−2.87	74.63	0.87	0.0114	1.30	−2.84	72.51
	0.24	0.24	0.0043	1.80	−1.00	75.43	0.24	0.0095	3.90	1.20	76.33
O-DMCAR	4.00	4.06	0.0662	1.63	1.47	70.14	4.02	0.0704	1.75	0.54	70.52
	1.20	1.19	0.0050	0.42	−0.58	70.45	1.20	0.0159	1.33	−0.19	70.31
	0.32	0.33	0.0060	1.84	2.50	70.72	0.32	0.0063	1.95	1.44	71.74

[a] Standard deviation of concentrations found.
[b] Coefficient of variation of concentrations found.

of β-blockers, isoflavones and their metabolites. The slopes, intercepts and coefficient of determination for the calibration curves of the analysed compounds are shown in Table 4. By examining the calibration curves and the table, it can be deduced that the relationship between peak area ratio and concentration is linear within the studied concentration range. The high coefficients of determination (r^2) of all the calibration curves were between 0.9991 and 0.9999.

The LOD and LOQ scores of all the analysed compounds are listed in Table 3. In the following table, the LODs, evaluated after recalculation to account for the sample injection (2 μl volume), are presented as well.

Intra- and inter-day precision and accuracy were determined by QC samples at various concentrations as described in Section 2. The intra-day precision (%C.V.) was found to be less than 4.40% ($n = 6$) and accuracy ranged from 0.05 to 4.74% for each analyte. The assay method was validated with inter-day precision (%C.V.) less than 4.48% and accuracy ranged from 0.03 to 4.70%. The results for intra- and inter-day precision (%C.V.) and accuracy are summarized in Table 5. Conclusively, the results demonstrate that the precision and accuracy of this assay were acceptable.

The enzymatic hydrolyzed and SPE procedure has been successfully applied to the extraction of the analysed compounds from human urine. The extraction recoveries (absolute recovery) of the examined compounds from human urine samples were determined at different concentrations. These samples were subjected to the enzymatic hydrolyzed and SPE procedure, described previously, and injected into the UHPLC system. The extraction recoveries of analysed compounds from spiked human urine were evaluated at the low, medium and high concentration. The highest recoveries were obtained for CAR (from 95.97% to 99.63%) and *O*-DMMET (from 90.07% to 99.85%). The lowest recoveries were obtained for *O*-DMCAR (from 70.14% to 70.72%) and 8-HDA (from 70.44% to 71.12%). The summary of the absolute recovery results is given in Table 5.

Table 6 summarizes the results of the short-term, long-term, post-preparative and freeze–thaw stabilities of β-blockers, isoflavones and their metabolites. The data showed the reliable stability behavior of each compound under the conditions tested.

In dilution integrity study, the % accuracy of 5 and 10 times diluted samples ranged from 96.3 to 101.6% of the nominal concentration for β-blockers, isoflavones and their metabolites. These results conclude that the dilution of the concentrated urine sample up to four times maintains legibility and integrity of β-blockers, isoflavones and their metabolites concentration.

Selectivity experiments were carried out using human urine samples that did not contain the target compounds. Chromatograms obtained by UHPLC from extracted blank urine sample and extracted urine samples enriched with analyte concentrations at the LOQ and LOD are shown in Fig. 2. There were no interfering peaks at retention times corresponding to the analysed β-blockers, isoflavones and their metabolites. There are some additional unidentified peaks in the chromatogram from the human urine samples, but these peaks do not interfere with the β-blockers, isoflavones and their metabolites of interest.

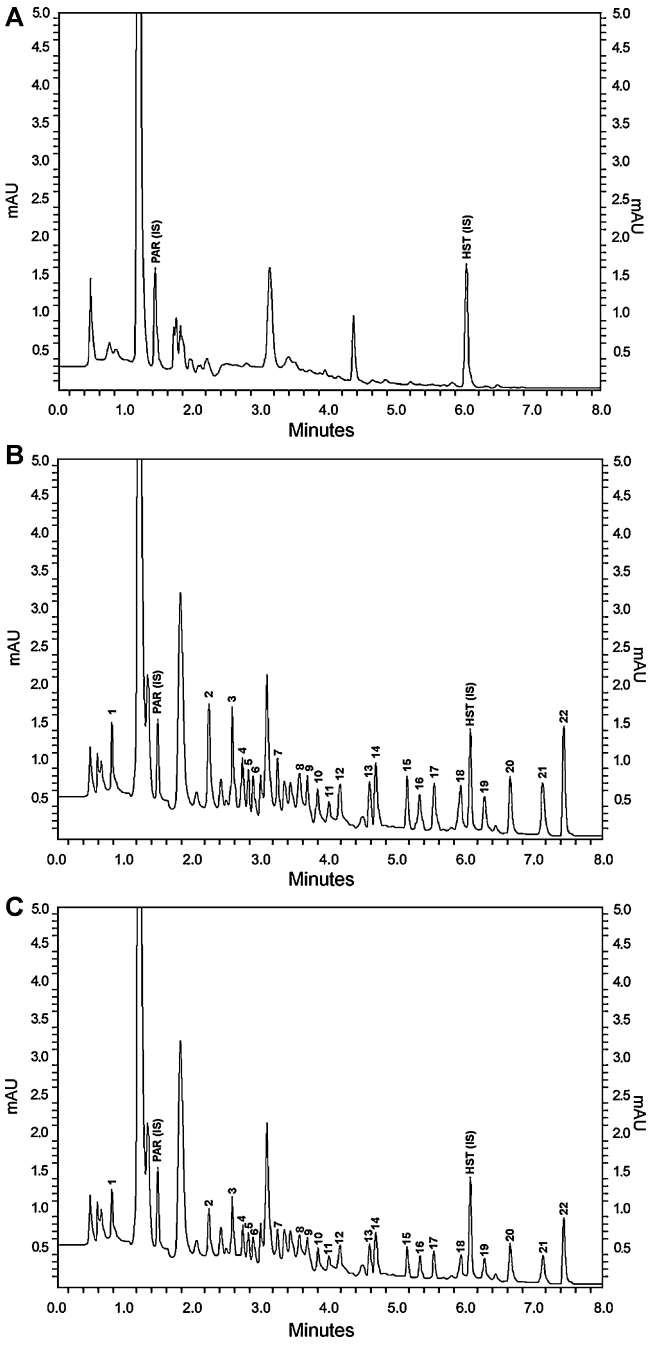


Fig. 2. The chromatograms of: (A) extracted blank urine sample, (B) extracted urine sample enriched with analyte concentrations at the LOQ and (C) extracted urine sample enriched with analyte concentrations at the LOD obtained using the UHPLC method described.

4. Application of the method to patient urine samples

The proposed gradient reversed-phase UHPLC method was successfully applied to the analysis of urine samples in the study of isoflavones, β-blockers and their metabolites. The identification was performed according to the standard addition method and by the comparison of retention times. The isoflavone, β-blocker and their metabolite content of the urine samples was determined using calibration curves.

For the further evaluation of the applicability of the method, a subset of urine samples from an intervention study, which involved patients on a diet rich in an isoflavone content and treated with metoprolol (50 mg) and propranolol (80 mg) twice a day, was analysed.

The metabolism of isoflavones involves the action of intestinal microflora (hydrolysis; demethylation) as well as the modification by conjugating enzymes (phase II) and/or phase I enzymes (reduction, hydroxylation). To obtain the free form of the phase II metabolites present in the human urine samples, hydrolyses were carried out. For this step, we chose enzymatic hydrolysis with pure β-glucuronidase/sulfatase (preparations from *H. pomatia*) in an acetate buffer (pH 4.66) as used previously for the urine

Table 6
The stability of β-blockers, isoflavones and their metabolites in urine under tested conditions.

Compound	Added (μg/ml)	Short-term stability			Long-term stability			Post-preparative stability			Freeze–thaw stability		
		Found (μg/ml)	C.V.[a] (%)	Accuracy (%)	Found (μg/ml)	C.V.[a] (%)	Accuracy (%)	Found (μg/ml)	C.V.[a] (%)	Accuracy (%)	Found (μg/ml)	C.V.[a] (%)	Accuracy (%)
Isoflavones and their metabolites													
PUR	3.00	2.90	1.12	−3.36	2.89	1.38	−3.62	2.92	1.19	−2.66	2.89	1.37	−3.63
	0.90	0.88	1.75	−2.71	0.85	5.31	−5.50	0.89	0.76	−1.43	0.87	1.80	−2.94
	0.24	0.24	6.63	−2.04	0.23	4.71	−3.63	0.24	5.95	−1.57	0.23	4,87	−3.69
GLY	3.00	2.91	1.43	−2.99	2.79	4.46	−6.81	2.91	2.06	−3.07	2.83	3.28	−5.72
	0.90	0.86	0.62	−4.02	0.84	3.29	−6.90	0.86	0.23	−4.34	0.84	2.05	−6.05
	0.24	0.23	2.69	−2.66	0.23	3.05	−4.74	0.23	1.15	−2.33	0.22	7.31	−8.55
DA	2.00	1.98	0.98	−1.07	1.97	0.93	−1.36	1.98	0.24	−0.77	1.94	2.41	−2.94
	0.60	0.59	1.88	−1.77	0.59	1.92	−1.87	0.59	1.58	−1.27	0.60	1.19	−0.79
	0.16	0.15	1.25	−3.25	0.15	1.69	−3.88	0.16	1.70	−2.93	0.16	1.87	−2.57
8-HDA	5.00	4.90	1.79	−2.02	4.83	3.20	−3.34	4.98	0.59	−0.44	4.85	2.73	−2.91
	1.50	1.48	1.19	−1.02	1.48	1.37	−1.26	1.48	1.05	−1.25	1.44	3.43	−4.29
	0.40	0.39	0.68	−1.69	0.39	2.28	−2.73	0.39	0.36	−1.27	0.38	3.32	−4.26
GLU-DA	3.00	2.88	1.88	−3.87	2.90	2.33	−3.32	2.93	1.19	−2.38	2.88	2.01	−3.83
	0.90	0.86	1.28	−3.92	0.86	2.72	−4.84	0.87	0.29	−2.94	0.88	4.81	−2.61
	0.24	0.23	2.43	−2.89	0.23	3.18	−4.72	0.23	2.35	−2.29	0.23	2.45	−5.01
GLC	5.00	5.00	1.31	−0.01	4.97	0.59	−0.52	4.96	0.49	−0.87	4.85	2.35	−2.96
	1.50	1.49	2.00	−0.86	1.46	3.96	−2.31	1.51	0.64	0.64	1.47	3.23	−2.19
	0.40	0.39	4.77	−1.29	0.39	6.22	−2.32	0.40	5.96	0.93	0.39	7.17	−1.42
DMGLC	6.00	6.00	1.58	−0.02	5.80	3.35	−3.29	5.97	2.15	−0.55	5.82	2.99	−2.94
	1.80	1.80	1.89	−0.15	1.71	4.51	−5.09	1.77	1.60	−1.34	1.76	1.33	−1.94
	0.48	0.47	1.28	−1.28	0.47	1.56	−2.09	0.47	1.72	−1.81	0.47	1.78	−2.41
GT	3.00	2.94	2.03	−2.09	2.94	1.95	−2.01	2.99	0.44	−0.23	2.94	1.78	−1.82
	0.90	0.88	1.44	−2.37	0.87	2.34	−3.27	0.89	0.18	−1.22	0.88	1.53	−2.49
	0.24	0.23	3.42	−2.85	0.23	3.06	−2.48	0.24	2.63	−0.65	0.24	2.29	−1.50
8-HGT	6.00	5.95	1.95	−0.75	5.98	1.93	−0.32	5.98	2.76	−0.30	5.89	2.54	−1.90
	1.80	1.76	2.77	−2.18	1.72	4.79	−4.29	1.79	2.57	−0.25	1.73	4.28	−3.78
	0.48	0.46	4.58	−4.48	0.46	3.32	−3.56	0.47	2.46	−1.88	0.49	5.79	3.18
DHGT	5.00	4.91	1.32	−1.76	4.83	2.97	−3.47	4.96	1.17	−0.88	4.81	3.26	−3.77
	1.50	1.46	1.24	−2.38	1.44	2.84	−4.02	1.48	0.30	−1.46	1.44	3.00	−4.14
	0.40	0.39	1.44	−2.26	0.38	2.60	−3.88	0.39	0.70	−1.81	0.39	1.44	−3.01
BIO	4.00	3.80	5.07	−4.89	3.82	4.64	−4.52	3.99	0.35	−0.32	3.81	4.79	−4.65
	1.20	1.15	1.14	−4.06	1.12	4.97	−6.33	1.16	0.22	−3.25	1.12	3.31	−6.19
	0.32	0.31	2.60	−3.47	0.30	4.97	−5.60	0.32	1.13	−1.23	0.30	4.78	−5.45
DHBIO	5.00	4.81	2.30	−3.86	4.88	0.60	−2.28	4.90	0.78	−2.01	4.67	5.20	−6.58
	1.50	1.42	1.00	−4.99	1.42	1.41	−5.57	1.43	0.93	−4.72	1.39	3.05	−7.19
	0.40	0.39	0.56	−3.36	0.39	2.96	−1.38	0.39	0.01	−3.49	0.38	1.70	−4.71
β-Blockers and their metabolites													
MIL	4.00	3.94	1.52	−1.62	3.90	4.41	−2.45	3.96	1.20	−0.94	3.77	5.71	−5.77
	1.20	1.18	4.21	−1.65	1.17	6.08	−2.18	1.20	5.36	−0.17	1.14	6.78	−5.03
	0.32	0.31	7.42	−1.79	0.31	5.97	−2.88	0.32	6.94	−0.70	0.31	8.53	−3.38
SOT	5.00	4.76	5.76	−4.77	4.67	7.65	−6.68	4.97	0.36	−0.57	4.76	4.82	−4.89
	1.50	1.48	4.49	−1.31	1.45	6.49	−3.24	1,49	4.68	−0.72	1.43	5.25	−4.37
	0.25	0.25	5.47	−1.24	0.24	4.65	−4.43	0.25	3.80	−1.02	0.24	5.12	−3.92
MET	4.00	3.87	2.90	−3.20	3.78	5.42	−5.52	3.97	0.23	−0.66	3.81	4.66	−4.85
	1.20	1.16	2.43	−3.08	1.15	3.78	−4.33	1.19	1.50	−1.11	1.16	2.48	−2.99
	0.32	0.31	3.82	−3.41	0.30	3.92	−5.43	0.31	3.45	−3.19	0.31	2.66	−3.91
α-HMET	5.00	4.93	1.76	−1.47	4.83	5.39	−3.31	5.00	1.06	−0.02	4.78	4.89	−4.39
	1.50	1.45	2.21	−3.28	1.45	3.06	−3.48	1.48	1.17	−1.43	1.46	3.90	−2.50
	0.40	0.39	6.66	−2.93	0.39	4.28	−3.42	0.40	4.01	−0.57	0.38	4.43	−3.72
O-DMMET	5.00	4.98	1.29	−0.31	4.95	0.94	−1.00	4.97	1.22	−0.58	4.96	0.85	−0.75
	1.50	1.46	1.66	−2.29	1.47	2.05	−2.25	1.48	1.94	−1.36	1.44	3.43	−3.97
	0.40	0.38	2.41	−3.92	0.39	1.30	−3.08	0.39	0.16	−2.20	0.38	5.27	−4.14
PRO	2.00	1.98	1.36	−1.07	1.95	2.56	−2.57	1.99	1.23	−0.35	1.94	2.77	−2.77
	0.60	0.59	2.17	−1.18	0.59	2.75	−1.89	0.60	1.17	0.52	0.57	6.24	−5.05
	0.16	0.16	3.17	−1.74	0.16	2.18	−1.11	0.16	1.55	0.66	0.15	4.98	−3.76
4-HPRO	3.00	2.92	1.91	−2.58	2.88	1.69	−3.95	2.92	0.03	−2.47	2.94	0.46	−2.15
	0.90	0.88	1.93	−2.52	0.87	2.73	−3.50	0.89	1.40	−1.35	0.87	3.04	−3.32
	0.24	0.23	4.62	−3.87	0.23	4.67	−3.93	0.24	0.08	−1.53	0.23	4.14	−4.07
CAR	3.00	3.00	3.11	0.10	2.98	0.85	−0.66	3.00	0.27	0.09	2.96	1.33	−1.39
	0.90	0.91	3.53	0.76	0.90	2.45	−0.24	0.91	2.32	0.82	0.86	0.41	−4.64
	0.24	0.24	4.64	−0.25	0.24	2.27	−0.34	0.24	2.52	0.91	0.23	0.12	−3.25
5′-HCAR	3.00	3.00	1.19	0.16	3.02	1.59	0.53	3.01	0.61	0.24	3.02	0.76	0.83
	0.90	0.89	1.02	−0.80	0.89	1.43	−1.34	0.90	1.24	−0.26	0.89	1.07	−0.78
	0.24	0.24	2.78	−1.15	0.24	1.99	−0.79	0.24	1.13	0.83	0.24	1.77	−0.59
O-DMCAR	4.00	3.85	3.84	−3.84	3.86	3.43	−3.48	3.98	0.01	−0.36	3.84	4.02	−3.99
	1.20	1.19	2.72	−0.58	1.20	1.86	0.09	1.20	0.69	0.45	1.18	3.53	−1.97
	0.32	0.31	2.89	−2.05	0.31	2.68	−1.86	0.32	0.99	0.48	0.30	7.09	−4.45

[a] Coefficient of variation of concentrations found.

samples containing isoflavone and β-blocker conjugates. After hydrolysis, the extraction of the aglycones (free aglycones plus aglycones released from the sulfate and glucuronide conjugates) from the matrix was necessary to order to avoid interference from other components within the urine sample. Solid-phase extraction was chosen for the extraction and purification of the analytes due to high selectivity, the simple, yet efficient, speed of extraction, the potential for automation, and, additionally, the fact that much lower volumes of organic solvents are required than for liquid-liquid extraction. The tested SPE sorbent proved that appropriate absolute recoveries can be obtained for Oasis HLB (Waters), after a proper optimization of conditions, such as volume and the amount of sorbent. Hydrolyzing urine samples with enzyme and then extracting the aglycones with SPE get the high recoveries of the examined compounds.

For the analysis of β-blockers, isoflavones and their metabolites in the urine by UHPLC, these compounds were exhibited as well-separated peaks using a Hypersil GOLD™ column. As an example of the application of this method, the urine concentrations of a drug, isoflavone and their metabolite were analysed from samples taken from patients treated with β-blockers and after the ingestion of a food containing isoflavones. Table 7 shows the levels of β-blockers, isoflavones and their metabolites found in the samples of urine. The representative chromatograms of the extract of a urine sample obtained 4 h after the oral dosing of propranolol and metoprolol to a human are shown in Fig. 3.

5. Conclusions

In the following paper we have developed a simple and reliable procedure for the determination of isoflavones, β-blockers and metabolites by gradient UHPLC with UV detection. An efficient SPE method was developed for extraction and the clean-up process. An UHPLC technique with the excellent precision, accuracy, linearity and recovery has been developed and validated for the simultaneous separation and determination of twenty-two compounds in human urine samples within time no longer than 8.0 min. The best sensitivity makes this method a valuable tool in clinical and basic research on the metabolism of β-blockers and isoflavones and their role in the treatment of a cardiovascular disease. The present method can be applied to the pharmacokinetic study after administering drugs and isoflavones to humans.

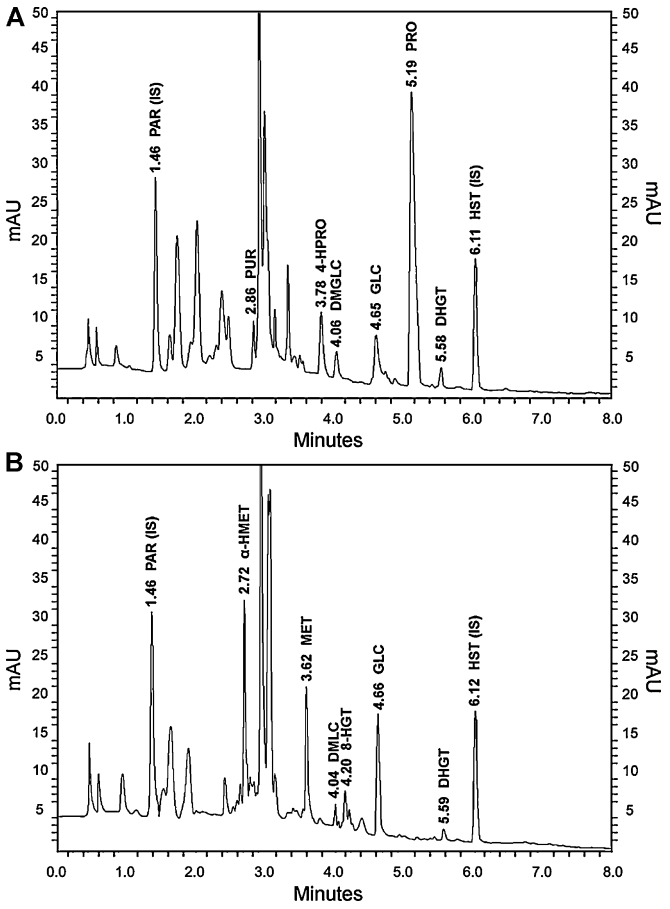


Fig. 3. The chromatograms of urine sample from patients on a diet rich in isoflavones and treated with (A) propranolol and (B) metoprolol (collected 4 h after administration) obtained using the developed UHPLC method.

This method is well suited for a routine application in a clinical laboratory because of the speed of an analysis and simple extraction procedure. What is more, a high percentage of recovery indicates that the method can be successfully used. To the best of our knowledge, the UHPLC method described herein is the first procedure allowing the simultaneous detection

Table 7
β-Blockers, isoflavones and their metabolites content of urine samples ($n = 6$).

	Urine 4 h (MET)			Urine 6 h (MET)		
	Concentration (μg/ml)	S.D.[a] (μg/ml)	C.V.[b] (%)	Concentration (μg/ml)	S.D.[a] (μg/ml)	C.V.[b] (%)
MET	6.14	0.0054	0.09	2.72	0.0023	0.08
α-HMET	21.42	0.0909	0.42	0.85	0.0018	0.21
O-DMMET	–	–	–	5.30	0.0306	0.58
8-HGT	0.64	0.0100	1.55	0.12	0.0005	0.41
DHGT	0.10	0.0020	2.00	0.32	0.0024	0.76
GLC	1.19	0.0003	0.02	0.08	0.0005	0.62
DMGLC	0.16	0.0008	0.53	–	–	–
	Urine 4 h (PRO)			Urine 6 h (PRO)		
	Concentration (μg/ml)	S.D.[a] (μg/ml)	C.V.[b] (%)	Concentration (μg/ml)	S.D.[a] (μg/ml)	C.V.[b] (%)
PRO	26.18	0.0586	0.22	0.68	0.0039	0.58
4-HPRO	2.53	0.0157	0.62	3.50	0.0013	0.04
DHGT	0.36	0.0011	0.31	0.31	0.0004	0.12
GLC	0.50	0.0003	0.05	0.46	0.0001	0.03
DMGLC	0.75	0.0060	0.44	0.38	0.0022	0.58
PUR	1.16	0.0188	1.61	1.07	0.0074	0.69
8-HDA	–	–	–	0.20	0.0005	0.26

[a] Standard deviation of concentrations found.
[b] Coefficient of variation of concentrations found.

and quantification of β-blockers, isoflavones and their metabolites.

References

[1] S. Bangalore, F.H. Messerli, J.B. Kostis, C.J. Pepine, JACC 50 (2007) 563.
[2] M. Packer, AJH 11 (1998) 23S.
[3] T. Walle, Free Radical Biol. Med. 36 (2004) 829.
[4] M.J. Tikkanen, H. Adlercreutz, Biochem. Pharmacol. 60 (2000) 1.
[5] T.B. Clarkson, J. Nutr. 132 (2002) 566S.
[6] S.M. Heinonen, K. Wähälä, H. Adlercreutz, Phytochem. Rev. 1 (2002) 175.
[7] B. Buszewski, T. Welerowicz, E. Tęgowska, T.F. Krzemiński, Anal. Bioanal. Chem. 393 (2009) 263.
[8] R.D. Johnson, R.J. Lewis, Forensic Sci. Int. 156 (2006) 106.
[9] S. Kawano, H. Murakita, E. Yamamoto, N. Asakawa, J. Chromatogr. B 792 (2003) 49.
[10] D.W. Jeong, Y.H. Kim, H.Y. Ji, Y.S. Youn, K.C. Lee, H.S. Lee, J. Pharm. Biomed. Anal. 44 (2007) 547.
[11] M. Josefsson, A. Sabanovic, J. Chromatogr. A 1120 (2006) 1.
[12] K.V. Gowda, U. Mandal, P.S. Selvan, W.D.S. Solomon, A. Ghosh, A.K. Sarkar, S. Agarwal, T.N. Rao, T.K. Pal, J. Chromatogr. B 858 (2007) 13.
[13] A.K. Sarkar, D. Ghosh, A. Das, P.S. Selvan, K.V. Gowda, U. Mandal, A. Bose, S. Agarwal, U. Bhaumik, T.K. Pal, J. Chromatogr. B 873 (2008) 77.
[14] E. Yang, S. Wang, J. Kratz, M.J. Cyronak, J. Pharm. Biomed. Anal. 36 (2004) 609.
[15] Y. Alnouti, K. Srinivasan, D. Waddell, H. Bi, O. Kavetskaia, A.I. Gusev, J. Chromatogr. A 1080 (2005) 99.
[16] R.S. Kadam, U.B. Kompella, J. Chromatogr. B 877 (2009) 253.
[17] S.A.C. Wren, P. Tchelitcheff, J. Pharm. Biomed. Anal. 40 (2006) 571.
[18] I. Baranowska, P. Markowski, J. Baranowski, Anal. Chim. Acta 570 (2006) 46.
[19] G. Venkatesh, S. Ramanathan, S.M. Mansor, N.K. Nair, M.A. Sattar, S.L. Croft, V. Navaratnam, J. Pharm. Biomed. Anal. 43 (2007) 1546.
[20] V.P. Ranta, E. Toropainen, A. Talvitie, S. Auriola, A. Urtti, J. Chromatogr. B 772 (2002) 81.
[21] M.V.S. Varma, N. Kapoor, M. Sarkar, R. Panchagnula, J. Chromatogr. B 813 (2004) 347.
[22] C. Mišl'anova, M. Hutta, J. Chromatogr. B 765 (2001) 167.
[23] P. Chiap, A. Ceccato, B.M. Buraglia, B. Boulanger, P. Hubert, J. Crommen, J. Pharm. Biomed. Anal. 24 (2001) 801.
[24] O. Rbeida, B. Christiaens, P. Chiap, P. Hubert, D. Lubda, K.S. Boos, J. Crommen, J. Pharm. Biomed. Anal. 32 (2003) 829.
[25] P. Ptáček, J. Macek, J. Klíma, J. Chromatogr. B 789 (2003) 405.
[26] M. Saito, J. Kawana, T. Ohno, M. Kaneko, K. Mihara, K. Hanada, R. Sugita, N. Okada, S. Oosato, M. Nagayama, T. Sumiyoshi, H. Ogata, J. Chromatogr. B 843 (2006) 73.
[27] A. Zarghi, S.M. Foroutan, A. Shafaati, A. Khoddam, J. Pharm. Biomed. Anal. 41 (2006) 1433.
[28] A. Zarghi, S.M. Foroutan, A. Shafaati, A. Khoddam, J. Pharm. Biomed. Anal. 44 (2007) 250.
[29] P.D. Martin, G.R. Jones, F. Stringer, I.D. Wilson, J. Pharm. Biomed. Anal. 35 (2004) 1231.
[30] R. Rathod, L.P.C. Prasad, S. Rani, M. Nivsarkar, H. Padh, J. Chromatogr. B 857 (2007) 219.
[31] M. Machida, M. Watanabe, S. Takechi, S. Kakinoki, A. Nomura, J. Chromatogr. B 798 (2003) 187.
[32] A.Q.N. Nguyen, Y. Théorêt, C. Chen, A. Denault, F. Varin, J. Chromatogr. B 877 (2009) 657.
[33] H.K. Lim, P.T. Linh, C.H. Hong, K.H. Kim, J.S. Kang, J. Chromatogr. B 755 (2001) 259.
[34] C. Pak, P.J. Marriott, P.D. Carpenter, R.G. Amiet, J. Chromatogr. A 793 (1998) 357.
[35] S.W. Myung, C.H. Jo, J. Chromatogr. B 822 (2005) 70.
[36] I. Baranowska, A. Wilczek, Anal. Sci. 25 (2009) 769.
[37] B. Mistry, J.L. Leslie, N.D. Eddington, J. Chromatogr. B 758 (2001) 153.
[38] P.M. Cerqueira, V.B. Boralli, E.B. Coelho, N.P. Lopes, L.F.L. Guimarães, P.S. Bonato, V.L. Lanchote, J. Chromatogr. B 783 (2003) 433.
[39] T.W.B. Gehr, D.M. Tenero, D.A. Boyle, Y. Qian, D.A. Sica, N.H. Shusterman, Eur. J. Clin. Pharmacol. 55 (1999) 269.
[40] B. Mistry, J. Leslie, N.E. Eddington, J. Pharm. Biomed. Anal. 16 (1998) 1041.
[41] P.B. Grace, J.I. Taylor, N.P. Botting, T. Fryatt, M.F. Oldfield, S.A. Bingham, Anal. Biochem. 315 (2003) 114.
[42] K.D.R. Setchell, M.S. Faughnan, T. Avades, L. Zimmer-Nechemias, N.M. Brown, B.E. Wolfe, W.T. Brashear, P. Desai, M.F. Oldfield, N.P. Botting, A. Cassidy, Am. J. Clin. Nutr. 77 (2003) 411.
[43] S. Moors, M. Blaszkewicz, H.M. Bolt, G.H. Degen, Mol. Nutr. Food Res. 51 (2007) 787.
[44] P.B. Grace, N.S. Mistry, M.H. Carter, A.J.C. Leathem, P. Teale, J. Chromatogr. B 853 (2007) 138.
[45] B. Klejdus, J. Vacek, V. Adam, J. Zehnálek, R. Kizek, L. Trnková, V. Kubá, J. Chromatogr. B 806 (2004) 101.
[46] C.L. Holder, M.I. Churchwell, D.R. Doerge, J. Agric. Food Chem. 47 (1999) 3764.
[47] T. Nurmi, H. Adlercreutz, Anal. Biochem. 274 (1999) 110.
[48] Z. Ma, Q. Wu, D.Y.W. Lee, M. Tracy, S.E. Lukas, J. Chromatogr. B 823 (2005) 108.
[49] P.B. Grace, J.I. Taylor, N.P. Botting, T. Fryatt, M.F. Oldfield, N. Al-Maharik, S.A. Bingham, Rapid Commun. Mass Spectrom. 17 (2003) 1350.
[50] J. Maubach, M.E. Bracke, A. Heyerick, H.T. Depypere, R.F. Serreyn, M.M. Mareel, D.D. Keukeleire, J. Chromatogr. B 784 (2003) 137.
[51] M. Richelle, S. Pridmore-Merten, S. Bodenstab, M. Enslen, E.A. Offord, J. Nutr. 132 (2002) 2587.
[52] J.K. Prasain, K. Jones, N. Brissie, R. Moore, J.M. Wyss, S. Barnes, J. Agric. Food Chem. 52 (2004) 3708.
[53] C.O. Cimino, S.R. Shelnutt, M.J.J. Ronis, T.M. Badger, Clin. Chim. Acta 287 (1999) 69.
[54] L. Chen, X. Zhao, L. Fang, D.E. Games, J. Chromatogr. B 1154 (2007) 103.
[55] N. Fang, S. Yu, T.M. Badge, J. Agric. Food Chem. 50 (2002) 2700.