UHPLC Method for the Simultaneous Determination of β-Blockers, Isoflavones, and Flavonoids in Human Urine

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Abstract

A simple method using solid-phase extraction (SPE) and ultra high-performance liquid chromatography (UHPLC) for the simultaneous determination of β-blockers, isoflavones, and flavonoids in human urine is developed. A statistical central composite design and response surface analysis is used to optimize the separation of the analytes. These multivariate procedures are efficient in determining the optimal separation condition using resolutions and retention time as responses. A gradient elution using a mobile phase consisting of 0.05% trifluoroacetic acid in water and acetonitrile is applied on a Hypersil GOLD column within a short analysis time of 4.5 min. UV detection was used to monitor the analytes. The suggested method was linear in a concentration range from 0.04–20.00 µg/mL, depending on the compound. The limits of detection ranged from 8.9 to 66.2 ng/mL. The precision was lower than 2.74%, and the accuracy was between 0.01–3.65%. The Oasis HLB column, with the highest recoveries, is selected for the pre-concentration step. This present paper reports, for the first time, a method for the simultaneous determination of β-blockers, isoflavones, and flavonoids in human urine samples. Furthermore, the developed method can also be applied to the routine determ-ination of examined compounds concentrations in human urine.

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. Cardiovascular disease includes several conditions of the heart and blood vessels, such as stroke, high blood pressure, angina, and rheumatic heart disease. β-Blockers reduce the mortality of patients after an acute myocardial infarction; this effect appears to be particularly marked in patients with post-infarction heart failure. The results of several trials suggest that long-term treatment with β-blockers can improve symptoms and reduce the frequency of hospitalizations for heart failure. Most recently, carvedilol has apparently turned out to reduce the risk of all-cause mortality by 65% in patients with either an ischemic or nonischemic cardiomyopathy. β-Blockers play a significant role in the progression of cardiovascular disease; what is more, the drugs are recommended as a primary therapy in other diverse medical conditions, which present certain treatment problems (1,2).

Cardiovascular disease is often caused by the accumulation of unhealthy habits; moreover, a number of the effects of the bad habits can be prevented through lifestyle changes. Numerous major clinical studies conducted in the last two decades have shown that flavonoids and isoflavones exert a positive influence on health, and notably, a diet rich in these compounds alleviates and prevents many serious diseases. Flavonoids and isoflavones, the groups of phenolic compounds originally found in fruit, vegetables, nuts, flowers, and seeds, are an integral part of the human diet. They have been reported to exhibit a wide range of biological effects, including antiischemic, antiplatelet, antineoplastic, antiinflammatory, antiallergic, antilipoperoxidant, or gastroprotective actions. Furthermore, flavonoids and isoflavones are potent antioxidants, free radical scavengers and metal chelators, and inhibit lipid peroxidation. The oxidative modification of low-density lipoproteins (LDLs) is believed to play a crucial role in atherogenesis. The inhibition of LDLs oxidation and platelet aggregation by phenolic compounds suggests that the regular consumption of food or beverages containing flavonoids and isoflavones may protect against atherosclerosis and a tendency to thrombosis. Epidemiological studies have indicated that the consumption of fruit and vegetables, and regular red wine consumption is related to a reduced risk of cardiovascular diseases (3–6).

The recommendation given to patients for taking flavonoids and isoflavones as medicine, or as the ingredients of a diet rich in flavonoids and isoflavones, undoubtedly supports β-blocker therapy. Due to the importance of drugs and phenolic compounds in biomedicine, new isolation, purification, and separation techniques are developed in order to increase the speed of isolation and, additionally, simplify the identification of the compounds in biological fluids. There is a need for an analytical methods which allows a rapid and sensitive measuring of these compounds in biological samples generated in large epidemiological studies.

Traditionally, the most commonly used analytical method for the detection and identification of β-blockers has been gas chromatography–mass spectrometry (GC–MS) (7). However, the GC–MS analysis often requires a time-consuming derivatization process. This methodology has been largely superseded in recent

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years by high-performance liquid chromatography–mass spectrometry (HPLC–MS or HPLC–MS–MS) techniques, which offer greater versatility and robustness (8–16). The UV-vis detectors, particularly those in a diode-array configuration (17–24), fluorescence detectors (22–30), and electrochemical detectors (31) are the most widely used detectors with HPLC. One method exclusively developed for milirinone, employed HPLC–UV (32). Recent methods, such as ultra-performance liquid chromatography with mass spectrometry (UPLC–MS), have been utilized to increase sample throughput and decrease analysis time (33).

The most common methods were applied in the study of the pharmacokinetic parameters, absorption, metabolization, and excretion of a whole spectrum of flavonoids and isoflavones. In biological fluids (serum, plasma, and urine), flavonoids exist as glucuronide and sulphate conjugates. In most cases, only the total aglycone content is determined; therefore, a hydrolysis step is utilized. Numerous analytical methods have been reported for the quantification of total flavonoids and isoflavones concentrations in biological samples obtained after the enzymatic hydrolysis of conjugated metabolites. The aforementioned analytical methods included LC–MS (34–38), LC–MS–MS (39–44), HPLC with UV detection (45–51), HPLC with fluorescent detection (51), and HPLC with electrochemical detection (52–55). The flavonoids and isoflavones analyzed in the biological fluids after the derivatization also used a GC–MS method (56). In addition, capillary electrophoresis, capillary electrochromatography, and micellar electrokinetic chromatography were proposed for the separation of flavonoids and isoflavones (57).

Reviews on this subject have recently been published; however, no article related to the simultaneous determination of drugs, flavonoids, and isoflavones in biological samples has ever been mentioned in literature. In the following paper, a central composite design, response surface analysis, and the Derringer-Suich desirability function were applied to the separation of the selected β-blockers, flavonoids, and isoflavones in human urine using UHPLC. The chromatographic conditions were optimized to yield the best separation in the shortest time and highest sensitivity. The aim of this paper was also the development of the new procedures for the pre-concentration of the selected drugs, flavonoids and isoflavones, using different SPE cartridges. The resulting analytical method was applied to monitoring the content of the examined compounds in human urine samples taken from patients suffering cardiovascular disease.

Experimental

Chemicals and standards

Flavonoids [including (±)-catechin ((±)-CA), (-)-epicatechin ((-)-EC), rutin (RUT), quercitrin (QUR), hesperidin (HSD), neohesperidin (NHSD), and hesperetin (HST)], isoflavones [including genistein (GT), daidzein (DA), glycitin (GLY), puerarin (PUR), and biochanin A (BIOA) (IS)], as well as β-blockers [milirinone (MIL), sotalol (SOT) (IS), metoprolol (MET), and propranolol (PRO)] were purchased from Sigma Chemicals (St. Louis, MO) and Aldrich Chemicals (Milwaukee, WI), except carvedilol (CAR), which was purchased from Toronto Research Chemicals (TRC). HPLC-grade acetonitrile, methanol, water, and trifluoroacetic acid were purchased from Merck (Darmstadt, Germany). Analytical-grade methanol, acetone, formic acid, 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 (a crude solution from Helix pomatia, type HP-2, G7017) was purchased from Sigma Chemicals (City, State).

Human urine was obtained from patients treated with β-blockers, being on a diet rich in flavonoids, and orally-administrated tablets of flavonoids and isoflavones. The samples were stored in the freezer at –20°C.

The data analysis of the results (chemometric approaches, the study of the regression models, etc.) was performed using Statistica 9.0 software. The structures and IUPAC names of the analysed β-blockers, flavonoids and isoflavones are presented in Table I.

Preparation of the standard solution and quality control samples

The appropriate amounts β-blockers, flavonoids, and isoflavones were separately weighed and dissolved in methanol to make stock solutions (1 mg/mL) and stored in the dark at 4°C. These stock solutions were then mixed and diluted with the same diluent to prepare the final mixed standard solutions containing 10 µg/mL of β-blockers, flavonoids, and isoflavones. A series of working solutions of these analytes were freshly prepared by diluting the mixed standard solution in 0.05% TFA–acetonitrile (95:5, v/v) to obtain the necessary multicomponent working solutions for spiking the urine samples. Calibration standards were prepared by spiking pool urine with the working solutions.

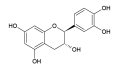
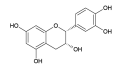
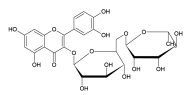
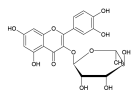
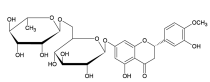
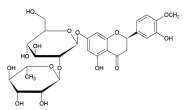
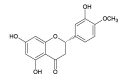
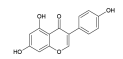
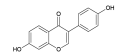
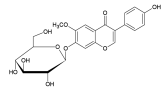
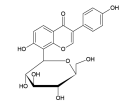
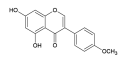
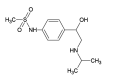
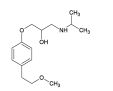
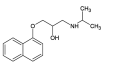
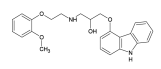
To validate this method, three concentration levels of standard solutions containing flavonoids, isoflavones, and β-blockers were used to prepare the quality control (QC) urine samples. QC samples were prepared by spiking blank urine with the proper volume of one of the aforementioned working solutions to produce a final concentration equivalent in range 0.1–1.0 µg/mL (low level), 0.6–3.0 µg/mL (middle level), and 1.4–10.0 µg/mL (high level) of drugs, flavonoids, and isoflavones.

Chromatographic conditions

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

The compounds were separated on a Hypersil GOLD (50 × 2.1 mm, 1.9 µm) column from Thermo Fisher Scientific, Inc (Waltham, MA). As Eluent A, 0.05% trifluoroacetic acid (TFA) in water was used; as Eluent B, acetonitrile was used. The gradient profile was: 0–1.0 min, from 5% to 20% solvent B (flow rate from 0.3 to 0.8 mL/min); 2.0 min, 25% solvent B (flow rate 0.8 mL/min); 2.5 min 35% solvent B (flow rate 0.8 mL/min); 3.5 min, 70% solvent B (flow rate 0.6 mL/min); 4.0 min, 95% solvent B (flow rate 0.8 mL/min); 4.5 min, 5% solvent B (flow rate 0.8 mL/min). The temperature of the column oven was set to 18°C. The detector wavelengths were set at 227, 254, 280, and 324 nm. The injection volume was 2 µL. Data acquisition and integration were performed using an EZ Chrom Elite System Manager.

Table I. Structures and IUPAC Names of the Examined Compounds

	Structure	IUPAC name
Flavonoids		
(±)-CA		*trans*-2-(3,4-dihydroxyphenyl)-3,4-dihydro-1(2H)-benzopyran-3,5,7-triol
(-)-EC		(2R,3R)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-1(2H)-benzopyran-3,5,7-triol
RUT		3-[[6-O-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4H-1-benzopyran-4-one
QUR		3-[(6-deoxy-α-L-mannopyranosyl)oxy]-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4H-benzopyran-4-one
HSD		(S)-7-[[6-O-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-2,3-dihydro-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4H-1-benzopyran-4-one
NHSD		(S)-7-(((2-O-6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl)oxy)-2,3-dihydro-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4H-1-benzopyran-4-one
HST		3′,5,7-trihydroxy-4-methoxyflavanone
Isoflavones		
GT		5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one
DA		7-hydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one
GLY		7-(β-D-glucopyranosyloxy)-3-(4-hydroxy-phenyl)-6-methoxy-4H-1-benzopyran-4-one
PUR		8-(β-D-glucopyranosyl-7-hydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one
BIOA (IS)		5,7-dihydroxy-3-(4-methoxyphenyl)-4H-1-benzopyran-4-one
β-Blockers		
MIL		2-methyl-6-oxo-1,6-dihydro-3,4′-bipyridine-5-carbonitrile
SOT (IS)		N-[4-[1-hydroxy-2 (isopropylamino) ethyl]phenyl] methanesulfonamide
MET		(±)1-(isopropylamino)-3-[p-(β-methoxyethyl) phenoxy]-2-propanol
PRO		(±)-1-isopropylamino-3-(1-naphthyloxy)-2-propanol
CAR		1-(9H-carbazol-4-yloxy)-3-((2-(2-methoxy-phenoxy)ethyl)amino)-2-propanol

Central composite design

The Central Composite Design (CCD) is composed of a 2n factorial design (cube levels), 2n so-called star point design (α-levels), and m replications of the center point (0-level). The number of required experiments N needed for CCD can be calculated with the Equation 1:

$$N = 2^n + 2n + m \qquad \text{Eq. 1}$$

where 2 is the number of levels per design variable, and n is the number of design variables.

The quantity N of experiments for a CCD with three or more design variables is considerably smaller than for a 3n factorial design, due to the combination of the 2n design and the star point design. Figure 1A shows a systematic graph of the CCD for two and three design variables. The replications of the center point (0-level) increases the precision of the CCD and thus minimizes the model error. The five levels of the CCD are coded as $-\alpha, -1, 0, +1, +\alpha$. An exemplary CCD is illustrated in Table II. The levels are needed for the statistical safeguard of the edges of the created model. The distance between the star-point (± α-level) and the center point can be chosen randomly. In order to get a rotatable design the coded interval has to be calculated according to the Equation 2:

$$\alpha = (2^n)^{1/4} \qquad \text{Eq. 2}$$

If the CCD is rotatable, all cube and star point levels are located within the same distance from the center point (Figure 1A). The rotatability of a design leads to a better statistical balance and thereby to equal precision for all equidistant points from the center point. The settings for the center point (A_0) of every variable can be chosen freely; likewise, so can the settings for the cube levels, as long as the variation A between +1 level and center point and –1 level and center point are equal (Figure 1B). For a rotatable design the ± α levels must be determined in the following way:

$$+\alpha = A_0 + A \times (2^n)^{1/4}$$

$$-\alpha = A_0 - A \times (2^n)^{1/4} \qquad \text{Eq. 3}$$

The large number of variables to be considered in the UHPLC separation would imply an extremely complicated experimental design. Therefore, in order to reduce the number of experiments, some of these variables were studied by means of traditional methodology "one variable at a time" (OVAT) and were fixed prior to the use of the experimental design. The central composite design investigated changes in pH of the mobile phase and column temperature. Levels varied from 2.5 to 6.5 for pH and between 15°C and 45°C for applied column temperature. The design center point was executed in four times resulting in a total of 12 experiments, which were executed in random order. Each design experiment was replicated three times. Elementary resolution, Rs, was chosen as the response variable. Because it resulted in acceptable statistical models, permitting an adequate assessment of the quality of the peak separation for all the design experiments, alternative response variables were not investigated.

Response surface mapping was an effective way to find the optimum condition. The design fitting with a full quadratic model is provided below:

$$y = b_0 + b_1x_1 + b_2x_2 + b_{12}x_1x_2 + b_{11}x_{12} + b_{22}x_2^2 \qquad \text{Eq. 4}$$

where y represents the experimental response, x the independently evaluated factors (in coded variables), b_0 the intercept and b_n the parametric coefficients of the model obtained by multiple regression.

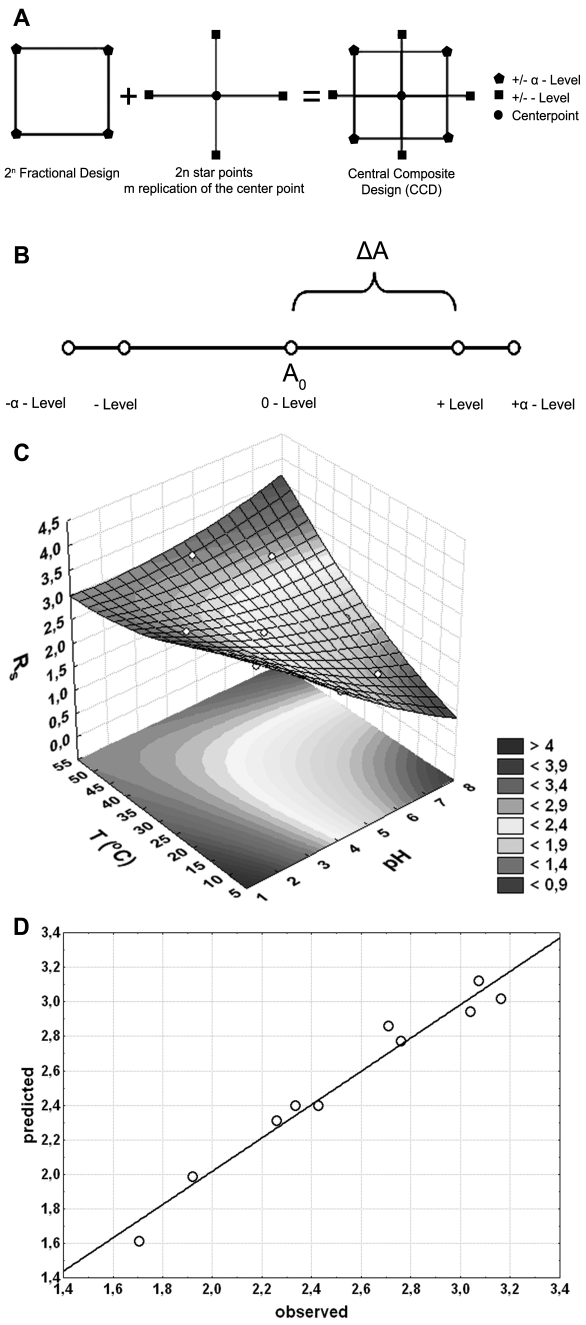


Figure 1. Central Composite Design (CCD): (A) Two dimensional constitution of the CCD; (B) Schematically exposition of 5 levels; (C) Response surface graph functions of pH of the mobile phase and column temperature; (D) Correlation plot of the predicted vs. the observed.

Sample preparation

An urine sample (3 mL) was incubated under continuous shaking with 100 µL 1 mol/L sodium acetate buffer (pH 4.66), 100 µL 0.1 mol/L ascorbic acid, and 40 µL β-glucuronidase–sulfatase (a crude preparation from Helix pomatia) for 18 h at 37°C after the addition of the appropriate IS solutions. The hydrolyzed urine sample was diluted with 2 mL phosphate buffer (0.1 M, pH 2.4).

The sample is cooled, and then the solid-phase extraction (SPE) was performed with the Bakerbond SPE-12G system (J.T. Baker Inc., Deventer, Netherlands). Four SPE cartridges were tested using the same extraction procedure for the urine samples. These columns included an Oasis HLB column (6 mL, 500 mg, Waters, Milford, MA), a Nexus column (6 mL, 200 mg, Varian, Cary, NC), and Bond Elut columns (Varian): a PPL column (6 mL, 500 mg), an ENV column (6 mL, 500 mg). Each column was conditioned with 6 mL methanol and 6 mL 0.1% formic acid. Subsequently, 3 mL urine was passed through and then dried. The retained analytes were eluted with 5 mL methanol–acetone–formic acid (4.5:4.5:1, v/v/v) and evaporated to dryness. Next, reconstituted in 1 mL 0.05% TFA–acetonitrile (95:5; v/v), filtered through a 0.45-µm membrane filter and 2 µL obtained extracts was injected through the autosampler into the UHPLC system for quantification.

Method Validation

The method was validated for selectivity, linearity, precision, accuracy, recovery, and stability according to the US Food and Drug Administration (USFDA) guidelines (60).

The system suitability was assessed by six replicate analyses of the drugs, flavonoids and isoflavones. Their retention times, capacity factors, resolutions, theoretical plate numbers, and peak asymmetries were estimated.

The selectivity of the method was investigated by analyzing blank urine from ten different volunteers without addition of

Table II. Exemplary Central Composite Design (CCD)*

Observation	Variable 1	pH	Variable 2	T (°C)	Rs
1	–1	2.5	–1	15	3.07
2	–1	2.0	1	30	3.16
3	1	6.5	–1	15	1.70
4	1	6.5	1	45	2.76
5	–1.414	2.0	0	30	3.16
6	1.414	7.3	0	30	1.92
7	0	4.5	–1.414	9	2.26
8	0	4.5	1.414	51	3.04
9	0	4.5	0	30	2.34
10	0	4.5	0	30	2.43
11	0	4.5	0	30	2.38
12	0	4.5	0	30	2.37

* Observation 1–4: 2n Fractional Desing
Observation 5–8: 2n start points
Observation 9–12: m replication of the center point

internal standard, then with addition of the internal standard or β-blockers, flavonoids, isoflavones. The resulting chromatograms were examined to determine the presence of any endogenous constituents which might potentially interfere with the compounds of interest.

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 curves were plotted with the peak area ratio of drugs, flavonoids, and isoflavones on y-axis and concentration on the *x*-axis and the regression equation was calculated for each curve. The obtained data were submitted to the regression analysis and correlation coefficients were calculated for each flavonoid, isoflavone and β-blocker using Microsoft Excel.

The limits of detection and quantitation (LOD and LOQ) were calculated using equations 5 and 6, respectively. The calculated theoretical detection and quantification limits were confirmed by the analysis of these concentrations using the developed method:

$$\text{LOD} = (\text{SD} \times 3.3) / \text{slope of calibration curve} \quad \text{Eq. 5}$$

$$\text{LOQ} = (\text{SD} \times 10) / \text{slope of calibration curve} \quad \text{Eq. 6}$$

where SD is the standard deviation of the intercept.

Three validation batches, each containing six replicates of QC samples at low, medium and high concentration levels, were assayed to assess the precision and accuracy. Assay precision and accuracy was assessed by the calculation of the inter-day and intra-day variability of quality control samples. The precision was calculated as a percent coefficient of variation (% CV) for the repeated measurement. Accuracy was calculated as the ratio of the measured concentration to the nominal concentration multiplied by 100%. The intra-day precision and accuracy were determined by analyzing six sets of spiked urine samples of the examined compounds at each QC level (low, middle, high) within a day. The inter-day precision and accuracy were determined by analysing six sets of spiked urine samples of drugs, flavonoids, and isoflavones at each QC level (low, middle, high) in three consecutive days. The USFDA recommended acceptance criterion (percentage of deviation between theoretical and back-calculated concentrations less than ±15%) was used in this study (60).

Recoveries were tested at low, medium and high concentration levels (n = 6). A mixture containing the analytes including IS at concentrations resulting in the low, medium, and high levels, respectively, were spiked to 3.0 mL of blank urine. The spiked urine samples were extracted and analysed according to the procedure described above. Furthermore, the concentrations of drugs, flavonoids, and isoflavones were calculated using the calibration curves. Recovery was calculated by comparing the determined amounts for extracted urine samples with the known amounts added.

Short-term stability, long-term stability, autosampler stability, and freeze–thaw cycle stability were assessed by analyzing three QC levels over six replicates. The short-term stabilities of flavonoids, isoflavones, and the drugs 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 assessed by analyzing the extracted QC urine samples kept in autosampler vials at 18°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. Mean peak areas obtained from the analysis of the stored samples were compared to those obtained from the analysis of freshly prepared urine samples. Deterioration of each analyte was defined as a greater than 15% difference in the tested sample versus the control at the nominal sample concentration.

Results and Discussion

UHPLC method development and optimization

In order to develop and validate a simple and sensitive UHPLC method which is suitable for permeability studies of drugs, flavonoids, and isoflavones in human urine, the following parameters were optimized. The optimum wavelength for detecting all the analytes with an adequate sensitivity was ascertained and found to be 227, 254, 280, and 324 nm.

The best separation, peak symmetry, and reproducibility were obtained on the Hypersil GOLD column. When experiments were performed with C18 column all the analytes were eluted at later retention to the presence of high organic content in the optimized mobile phase. A two-solvent gradient elution programme was used to obtain the best separations for all tested compounds on the Hypersil GOLD column. Acetonitrile was used as an organic solvent. When experiments were performed with methanol instead of acetonitrile as the organic solvent in the mobile phase, compounds were unresolved and retention time increased. Higher separation efficiency was observed in experiments with acetonitrile in the case of the most complicated group of the examined compounds.

The nature of the studied compounds requires the use of acidic mobile phase for satisfactory separation and peak shapes. Therefore, in our research 0.05% TFA in water was chosen. To optimize the UHPLC separation of the mixture of β-blocker, flavonoids, and isoflavones using CCD, preliminary two factor central composite design was devised to map out the space of the experimental conditions setting and to estimate the limits of factors. The preliminary CCD included: pH of the mobile phase in the range 2.5–6.5 and column temperature in the range 15–45°C.

According to the results, the limits of factors were adjusted, and the CCD was proposed. The pH of aqueous component of the mobile phase was excluded from this CCD because it was observed that pH influences mainly the time of the analysis. The time of the analysis decreased with the decreasing value of pH while the resolution slightly changed. The optimum conditions were achieved at pH 2.0. However, at pH 2.0 the resolution between PUR and (–)-EC was found to be poor, but the other analytes were well separated with good peak shapes. Therefore, pH was increased to 2.5 to ensure excellent separation for all the analytes. Thus, the limit pH value for the given column equal to 2.5 was chosen.

The influence of temperature on analytes retention was optimized either. As the temperature of the column increased, the capacity factors (k') for studied compounds decreased. The best resolution was obtained using 18°C. The final optimum conditions for chromatographic separation were 0.05% TFA in water (pH 2.5) and acetonitrile with gradient elution and 18°C column temperature. In the aforementioned conditions, the separation of seventh compounds was completed within 4.5 min (Figure 2).

A new optimization analysis was performed following selection of the statistically significant factors. A CCD using surface response was applied to the selection of analytical conditions. The factors studied were pH of mobile phase and column temperature. Twelve experiments were performed (Table II). The multiple response data, obtained by normalization of the resolution (R_s) was used to generate a response surface for the system studied. The response surface graphs were drawn as a function of pH and temperature (Figure 1C). From the partial derivatives of the equation describing the curve, it was possible to extract the critical values for the factors investigated. The values of 2.5 and 18°C were obtained for pH and column temperature, respectively. The model was generated based on the experimental multiple response. The model can be represented by the equation:

$$R_s = 5.2873 - 0.7242x_1 - 0.069x_2 + 0.0118x_1x_2 + 0.0188x_1^2 + 0.0005x_2^2 \quad \text{Eq. 7}$$

A graphical plot of the predicted and observed values demonstrates excellent performance of the model (Figure 1D).

Method validation

The validation studies were applied for flavonoids, including (±)-CA, (–)-EC, RUT, HSD, NHSD, QUR, HST, isoflavones including GT, DA, GLY, PUR, as well as β-blockers MIL, MET, PRO, and CAR. Using the optimized gradient, the mean selectivity factor (α), resolution (R_s), and the number of theoretical plates (N) were 1.41, 3.82, and 90987, respectively.

Selectivity experiments were carried out using human urine samples that did not contain the target compounds. The studied

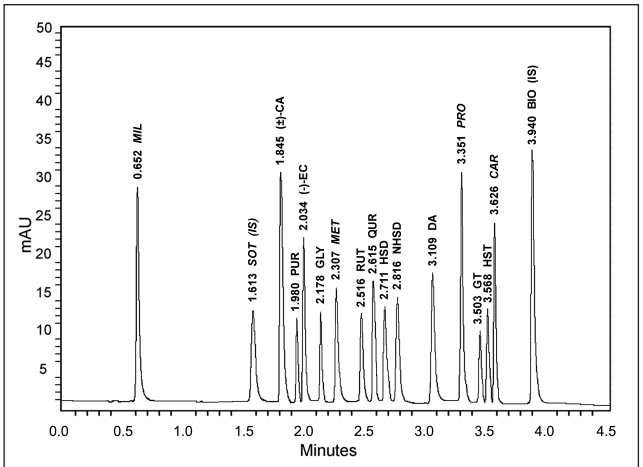


Figure 2. Chromatogram obtained from a mixed standard containing β-blockers: MIL, SOT (IS), MET, PRO, CAR; flavonoids: (±)-CA, (–)-EC, RUT, QUR, HSD, NHSD, HST, and isoflavones: PUR, GLY, DA, GT, BIOA (IS) by use the UHPLC method.

Table III. Properties of the Method Developed (n = 6)

Compound	Detection wavelengths (nm)	Retention time of standard (min)	Linear dynamic range* (µg/mL)	Slope	CV† (%)	Intercept	CV‡ (%)	Sxy	CV§ (%)	Correlation coefficient (r^2) (n = 6)††	LOD (ng/mL)	LOQ (ng/mL)
Flavonoids												
(±)-CA	280	1.847	0.2–20	0.0855	0.18	0.0068	1.53	0.0152	3.09	0.9994	66.2	198.5
(–)-EC	280	2.032	0.1–10	0.0782	0.26	0.0139	1.16	0.0055	2.34	0.9997	26.2	78.6
RUT	254	2.516	0.2–20	0.2243	0.19	0.0005	1.60	0.0429	3.31	0.9993	61.4	184.2
QUR	254	2.616	0.2–20	0.3759	0.18	–0.0066	1.57	0.0711	3.25	0.9993	61.0	183.1
HSD	280	2.684	0.1–10	0.2089	0.28	0.0113	1.18	0.0135	2.41	0.9997	31.4	94.3
NHSD	280	2.785	0.1–5	0.2404	0.51	0.0079	1.30	0.0108	2.31	0.9995	25.3	75.9
HST	280	3.553	0.04–4	0.4345	1.11	–0.0154	2.09	0.0204	3.74	0.9992	13.1	39.4
Isoflavones												
PUR	254	1.977	0.04–4	0.6769	0.71	0.0047	1.29	0.0212	2.36	0.9996	12.8	38.5
GLY	254	2.174	0.04–4	0.6262	0.51	–0.0030	0.85	0.0115	1.75	0.9999	8.9	26.7
DA	254	3.085	0.04–4	1.4592	1.14	–0.0328	1.90	0.0585	3.90	0.9993	13.0	39.0
GT	254	3.487	0.04–4	1.2510	0.72	–0.0141	1.23	0.0368	2.55	0.9996	10.7	32.1
β-Blockers												
MIL	324	0.652	0.06–6	0.5142	0.34	0.0025	0.93	0.0174	1.68	0.9998	15.9	47.8
MET	227	2.302	0.08–8	0.2232	0.33	–0.0107	1.23	0.0124	2.18	0.9997	23.7	71.0
PRO	227	3.335	0.04–4	0.6249	0.50	0.0165	0.86	0.0133	1.78	0.9998	10.3	30.9
CAR	227	3.616	0.04–4	0.4064	0.92	–0.0187	1.68	0.0159	3.08	0.9993	12.9	38.6

* Calibration curves were fitted to the linear regression equation y = ax+b, where "y" represents the ratio of the peak areas, "a", and "b" are constants, and "x" is the concentration of the analyzed compounds
† Coefficient of variation of slope
‡ Coefficient of variation of intercept
§ Coefficient of variation of regression coefficient
†† Number of points in calibration curves

blanks did not show area values higher than 20% of the LOQ's areas at the analyte's retention times, nor higher than 5% of the IS area at its corresponding retention time. There are some additional unidentified peaks in the chromatogram from the human urine samples, but these peaks do not interfere with the flavonoids, isoflavones and drugs of interest.

The calibration curves were determined using linear regression: $y = ax + b$, where y is the peak area, a is the slope, x is the respective concentration, and b is the intercept. The exact parameters of the obtained calibration curves were calculated (Table III). By examining the calibration curves and the table, it can be noticed that the relationship between the peak area ratio and the concentration is linear within the studied concentration range. The correlation coefficients (r^2) obtained from the least square regression analysis of calibration curve was more than 0.9992 for all the analytes.

The LOD and LOQ values were determined using the parameters of the obtained calibration curves. The calculated values included all steps introduced to the analytical procedure. The LOD and LOQ scores of all analyzed compounds are listed in Table III.

Intra-day and inter-day precision and accuracy were determined by QC samples at three concentrations as described in the Experimental section. The intra-day precision of the method, expressed by the coefficient of variation (% CV) was lower than 2.74% at each tested concentration level and the inter-day precision (% CV) was found to be less than 2.15%. The intra-day accuracy of the method was acceptable at each concentration level: 0.01–3.45% for the low concentration level, 0.79–3.53% for the medium concentration level, and 0.18–2.72% for the high concentration level. The inter-day accuracy ranged from 0.05 to 3.65%. The results for intra-day and inter-day precision (% CV) and accuracy are summarized in Table IV. These results indicate that the present method has an acceptable accuracy and precision.

Absolute recoveries obtained for Oasis HLB cartridge at three different concentration levels are shown in Table IV. Extraction recoveries of β-blockers, isoflavones and flavonoids ranged

Table IV. Intra-day and Inter-day Precision, Accuracy and Absolute Recovery of Drugs, Flavonoids and Isoflavones in Human Urine Samples (n = 6)

		Intra-day				Inter-day				
Compound	Added (µg/mL)	Found (µg/mL)	SD* (µg/mL)	CV† (%)	Accuracy (%)	Found (µg/mL)	SD* (µg/mL)	CV† (%)	Accuracy (%)	Absolute recovery (%)
Flavonoids										
(±)-CA	10.00	9.74	0.0369	0.38	–2.58	9.66	0.0907	0.94	–3.34	84.12
	3.00	3.03	0.0026	0.09	0.93	3.03	0.0337	1.11	0.94	82.43
	1.00	0.98	0.0034	0.35	–1.43	0.97	0.0163	1.68	–2.77	81.85
(–)-EC	5.00	4.90	0.1009	2.06	–2.00	4.91	0.0861	1.76	–1.88	90.94
	1.50	1.55	0.0094	0.60	3.53	1.54	0.0256	1.66	2.73	85.37
	0.25	0.25	0.0025	1.02	0.09	0.25	0.0037	1.46	0.68	90.37
RUT	10.00	9.74	0.1288	1.32	–2.63	9.78	0.1829	1.87	–2.22	70.95
	3.00	3.02	0.0403	1.33	0.79	3.02	0.0507	1.68	0.83	71.00
	1.00	1.00	0.0106	1.05	0.20	1.00	0.0149	1.48	0.24	70.35
QUR	10.00	10.02	0.0465	0.46	0.22	9.98	0.0785	0.79	-0.18	80.62
	3.00	3.02	0.0265	0.88	0.85	3.02	0.0186	0.62	0.70	81.56
	1.00	1.00	0.0205	2.06	–0.42	1.00	0.0203	2.03	–0.10	77.89
HSD	5.00	4.89	0.0357	0.73	–2.20	4.88	0.0430	0.88	–2.29	73.35
	1.50	1.53	0.0388	2.54	1.83	1.53	0.0285	1.87	1.96	76.98
	0.50	0.50	0.0022	0.43	0.29	0.50	0.0030	0.60	–0.05	75.41
NHSD	5.00	4.98	0.0235	0.47	–0.35	5.00	0.0304	0.61	0.05	82.48
	1.50	1.45	0.0033	0.23	–3.30	1.45	0.0041	0.28	–3.11	84.11
	0.50	0.52	0.0059	1.14	3.45	0.52	0.0068	1.31	3.65	80.09
HST	2.00	1.97	0.0016	0.08	–1.19	1.95	0.0261	1.34	–2.35	80.06
	0.60	0.59	0.0023	0.39	–0.94	0.58	0.0102	1.74	–2.38	79.56
	0.10	0.10	0.0018	1.79	2.83	0.10	0.0021	2.04	1.56	75.89
Isoflavones										
PUR	2.00	2.00	0.0071	0.35	–0.18	1.97	0.0267	1.35	–1.38	85.27
	0.60	0.62	0.0092	1.49	3.42	0.62	0.0068	1.10	3.21	82.58
	0.20	0.21	0.0057	2.74	3.44	0.20	0.0042	2.04	2.60	86.19
GLY	1.40	1.38	0.0003	0.02	–1.53	1.38	0.0035	0.26	–1.34	81.07
	0.60	0.60	0.0012	0.19	0.88	0.61	0.0089	1.45	1.93	81.10
	0.20	0.20	0.0007	0.36	0.54	0.20	0.0006	0.28	0.52	95.18
DA	1.40	1.36	0.0278	2.04	–2.72	1.37	0.0198	1.44	–2.13	83.22
	0.60	0.62	0.0065	1.04	3.48	0.62	0.0067	1.09	2.90	89.21
	0.20	0.20	0.0047	2.31	1.69	0.20	0.0037	1.82	1.42	96.09
GT	2.00	1.96	0.0040	0.21	–2.21	1.95	0.0072	0.37	–2.49	73.11
	0.60	0.61	0.0019	0.31	1.89	0.60	0.0102	1.69	0.35	73.40
	0.20	0.20	0.0047	2.30	1.75	0.20	0.0044	2.15	2.20	85.10
β-Blockers										
MIL	3.00	3.02	0.0198	0.65	0.73	3.02	0.0395	1.31	0.59	89.81
	1.00	0.96	0.0120	1.24	–3.52	0.98	0.0159	1.62	–2.30	98.08
	0.30	0.30	0.0036	1.19	–0.01	0.30	0.0023	0.77	0.12	95.30
MET	4.00	3.96	0.0491	1.24	–0.89	3.96	0.0491	1.24	–0.89	94.45
	1.20	1.22	0.0076	0.62	1.77	1.22	0.0076	0.62	1.77	90.60
	0.40	0.39	0.0013	0.34	–2.25	0.39	0.0013	0.34	–2.25	86.97
PRO	2.00	2.00	0.0045	0.22	0.26	2.01	0.0070	0.35	0.44	87.03
	0.60	0.61	0.0010	0.17	1.49	0.60	0.0093	1.54	0.81	98.62
	0.20	0.20	0.0042	2.14	–1.66	0.20	0.0031	1.58	–2.30	95.70
CAR	2.00	1.95	0.0071	0.36	–2.38	1.96	0.0101	0.52	–1.97	93.74
	0.60	0.59	0.0054	0.91	–0.87	0.59	0.0050	0.85	–0.99	98.61
	0.20	0.20	0.0007	0.33	2.33	0.20	0.0006	0.29	2.51	96.03

* Standard deviation of concentrations found.
† Coefficient of variation of concentrations found.

between 70.35% and 98.62%. These values showed no significant differences at different concentration levels, for most of the analytes. The recovery value of the IS were determined at a single concentration of 0.50 µg/mL for SOT (93.84%) and of 0.60 µg/mL for BIOA (96.58%).

Stability studies were investigated at low, middle, and high QC levels. All the analytes were found to be stable in the blank sample for 12 h at room temperature (0.32–4.63% for β-blockers, 0.84–8.31% for isoflavones, and 0.14–4.63% for flavonoids). Drugs, isoflavones, and flavonoids were shown to be stable in urine for 30 day when stored at –20°C in the range of 0.24–6.88%, 0.10–9.17%, and 1.56–9.26%, respectively. The autosampler stability of the analytes in the study was also excellent. Generally, after 24 h in storage at 18°C, the percentage degradation of the compounds was less than 5% for all analytes. The analyzed compounds were found to degrade in a range of 1.07–7.85% for β-blockers, 0.39–9.74% for isoflavones, and 0.23–7.59% for flavonoids after three freeze–thaw cycles in human urine. The data showed the reliable stability behavior of each compound under the condition tested.

Method Application

As a typical application of the developed gradient reversed-phase UHPLC method, urinary excretion of isoflavones, flavonoids, and β-blockers from the patients was investigated. To further evaluate the applicability of the method, a subset of urine samples from an intervention study that involved patients on a diet high in flavonoid content, with supplementation of isoflavones and orally received metoprolol (50 mg) and propranolol (80 mg) twice a day was analyzed. The urine samples were collected at 4 and 6 h after the oral administration.

The metabolism of isoflavones and flavonoids involves the action of intestinal microflora (hydrolysis; demethylation), as well as 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, hydrolysis was carried out. For this step, enzymatic hydrolysis was choosen with a pure β-glucuronidase–sulfatase (preparations from *Helix pomatia*) in acetate buffer (pH 4.66), as used previously for urine samples containing isoflavone, flavonoids, and β-blocker conjugates. After hydrolysis, the extraction of the aglycones (free aglycones plus aglycones released from sulfate and glucuronide conjugates) from the matrix was necessary to avoid interference from other components within the urine sample. After sample hydrolysis with the β-glucuronidase–sulfatase enzyme, glucosides of isoflavones and flavonoids were transformed to their aglycones. Subsequently, solid-phase extraction was chosen for extraction and purification of the analytes due to high selectivity, simple, yet the efficient, speed of the extraction, the potential for automation, and also the fact that much lower volumes of organic solvents are required than for liquid-liquid extraction. The tested SPE sorbent showed that appropriate absolute recoveries can be obtained for the Oasis HLB (Waters) cartridge, after a proper optimization of conditions, such as volume and amount of sorbent. The application of Oasis HLB cartridge enables to concentrate all the examined compounds with the acceptable recoveries ≥70.0%. The received results prove that the cartridges can be successfully used for the determination of the analyzed drugs, flavonoids, and isoflavones in human urine. The recoveries of drugs, flavonoids, and isoflavones using four tested sorbents are presented in Figure 3.

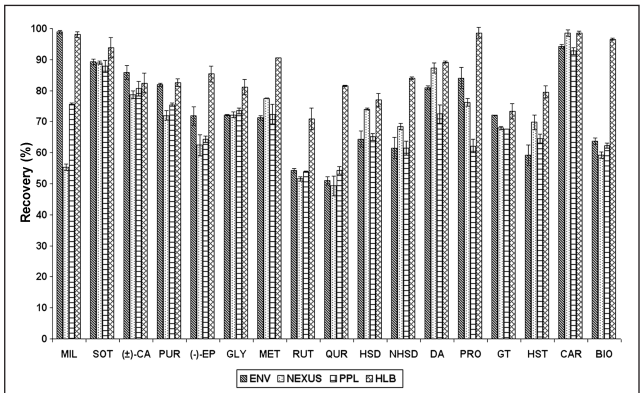


Figure 3. Recoveries of tested compounds extracted from human urine spiked at the standards mixture (middle level) using various SPE cartridges.

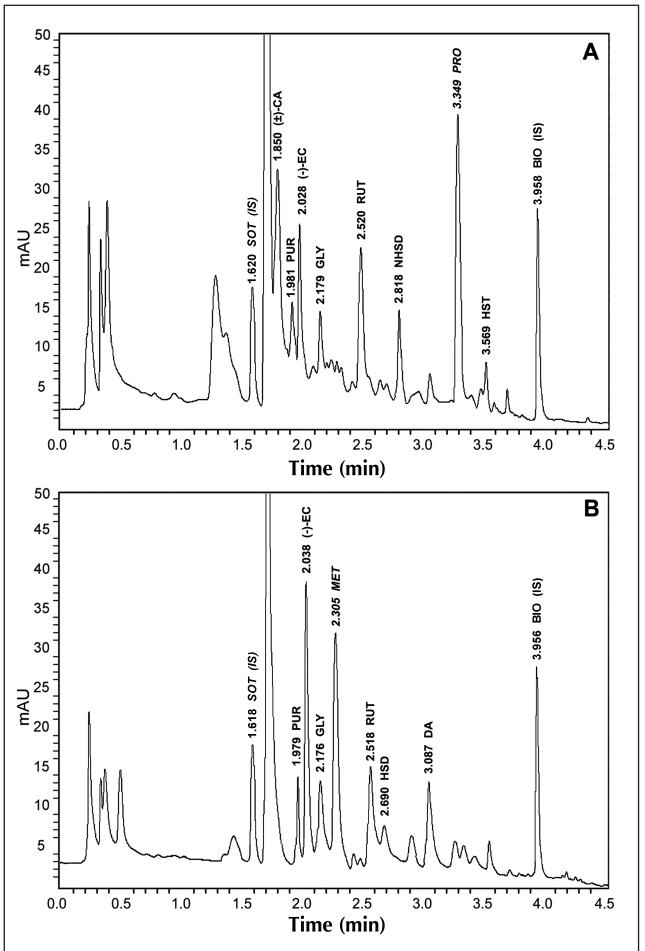


Figure 4. Chromatograms obtained by use of UHPLC method from a urine samples obtained from patients treated with propranolol (A) and metoprolol (B) (collected 4 h after administration) and being on a diet rich in isoflavones and flavonoids.

Finally, the UHPLC system was used to compare the levels of isoflavones, flavonoids and β-blockers in human urine. Table V shows the levels of the compounds measured in urine samples from patients treated with β-blockers and after ingestion of food containing isoflavones and flavonoids.

Isoflavone, flavonoid, and β-blocker identification was performed according to retention time by comparison with standard solutions and by addition of the standard solution. The isoflavone, flavonoid, and β-blocker content of the urine samples were determined using calibration curves. The samples with concentrations outside the linear range of the calibration curve were successfully analysed after diluting the urine sample with a suitable amount of 0.05% TFA in water. Representative chromatograms of the extract of a urine sample obtained 4 h after oral dosing of propranolol and metoprolol to human are shown in Figure 4.

Conclusions

Conclusively, a reversed-phase UHPLC assay using SPE with Oasis HLB cartridges, for the simultaneous determination of flavonoids, isoflavones, and β-blockers in urine samples incubated with β-glucuronidase–sulfatase was developed and validated. The CCD, response surface analysis, and the Derringer-Suich multicriteria method were used to optimize the chromatographic separation of all the compounds from human urine, providing maximum resolution between peaks and a shorter time of analysis.

Table V. β-Blockers, Isoflavones, and Flavonoids Content of Urine Samples (*n* = 6)

	Urine 4 h (MET)			Urine 6 h (MET)		
	Conc. (μg/mL)	SD* (μg/mL)	CV† (%)	Conc. (μg/mL)	SD* (μg/mL)	CV† (%)
PUR	1.45	0.0012	0.07	1.46	0.0147	1.01
(-)-EC	3.57	0.0207	0.58	2.78	0.0303	1.10
GLY	0.60	0.0004	0.09	1.32	0.0132	1.00
MET	4.36	0.0444	1.02	2.73	0.0086	0.32
RUT	1.26	0.0011	0.42	0.62	0.0012	0.20
HSD	0.81	0.0012	0.15	0.76	0.0120	1.60
DA	1.01	0.0038	0.37	0.06	0.0009	1.46
	Urine 4 h (PRO)			Urine 6 h (PRO)		
PUR	0.47	0.0002	0.05	0.57	0.0041	0.71
(±)-CA	14.14	0.1291	0.91	12.23	0.1613	1.32
(-)-EC	9.51	0.2144	2.26	6.31	0.1612	2.57
GLY	0.41	0.0004	0.11	–	–	–
RUT	4.60	0.0032	0.07	0.46	0.0123	2.70
NHSD	3.97	0.0576	1.45	1.65	0.0040	0.24
PRO	26.16	0.6938	2.65	2.37	0.0180	0.76
GT	–	–	–	5.42	0.0440	0.81
HST	0.26	0.0004	0.15	–	–	–

* Standard deviation of concentrations found.
† Coefficient of variation of concentrations found.

The final conditions consist of using a Hypersil GOLD column, a mobile phases consisting of 0.05% trifluoroacetic acid in water and acetonitrile. The separation of 17 compounds occurred within 4.5 min. The validation study showed that the method met with the official requirements for the quantification of β-blockers, flavonoids, and isoflavones in urine samples. The additional advantages of this method include small sample volume and good extraction recovery from urine samples. Finally, the following method will be applicable for β-blockers, flavonoids, and isoflavones level monitoring during pharmacokinetic studies because of the easy sample preparation and utilization of commonly available chromatographic instrument.

It should be stated that the patient does not take all the examined drugs simultaneously. However, this developed chromatographic method can be applied regardless of which β-blocker the patient was treated with.

Acknowledgements

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