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GC–MS method for the simultaneous determination of β-blockers, flavonoids, isoflavones and their metabolites in human urine

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ABSTRACT

A sensitive and selective method based on gas chromatography hyphenated to mass spectrometry (GC–MS) for the screening of 23 different compounds including β-blockers, flavonoids, isoflavones and metabolites in human urine sample was developed and validated. The present paper reports, for the first time, the method for the simultaneous determination of β-blockers, isoflavones, flavonoids and metabolites in human urine samples. When flavonoids are ingested in combination with drugs that have a narrow therapeutic range, interactions between flavonoids and drugs should be investigated.

Substances of interest were extracted from urine samples by solid-phase extraction (SPE) employing a mixture of *tert*-butyl methyl ether:methanol:formic acid (4.5:4.5:1; v/v/v) as a mobile phase and Oasis HLB (Waters) as a stationary phase. Before extraction, urine samples were incubated with β-glucuronidase/sulfatase in order to achieve enzymatic hydrolysis. Before GC–MS analysis the analytes had to be derivatized with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) into their trimethylsilyl derivatives by incubating for 60 min at 60 °C. Statistical central composite design and response surface analysis were used to optimize the derivatization reagent. These multivariate procedures were efficient in determining the optimal separation condition, using peak areas as responses.

The calibration curves were indicative of high linearity ($r^2 \geq 0.9992$) in the range of interest for each analyte. LODs (S/N = 3) ranged between 0.6 and 9.7 ng/ml. Intra-day and inter-day precision (CV, %) was less than 4.96%, accuracy between 0.01 and 4.98% and recovery was found in the range from 70.20 to 99.55%.

The developed method can be applied to the routine determination of examined compounds' concentrations in human urine. Moreover the method is suitable for detecting pharmaceutical compounds containing β-blockers, isoflavones and flavonoids in urine after administration to humans.

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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 [1].

Cardiovascular disease is often caused by the accumulation of unhealthy habits; moreover, a number of the effects of bad habits can be prevented by lifestyle changes. Numerous major clinical studies conducted in the last two decades have shown that flavonoids and isoflavones exert positive influence on health, and, notably, a diet rich in these compounds alleviates and prevents manifold serious diseases [2]. The high level of flavonoids and isoflavones in the diet has been associated with a lowered risk for hormone dependent diseases, breast, prostate cancers, and osteoporosis [3]. In order to achieve the proposed health benefits of polyphenols, it is nowadays possible to supplement the diet with

Abbreviations: AP, apigenin; BIO, biochanin A; BSTFA, *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide; (±)-CA, (±)-catechin; CCD, central composite design; CE, capillary electrophoresis; CEC, capillary electrochromatography; CHS, chrysin; CV, coefficient of variation; DA, daidzein; DMGLC, desmethylglycitein; *O*-DMMET, *O*-desmethylmetoprolol; DHBIO, dihydrobiochanin A; DHDA, dihydrodaidzein; DHGT, dihydrogenistein; EI, electron impact; EIC, extract ion current; (−)-EC, (−)-epicatechin; GC, gas chromatography; GT, genistein; GLC, glycitein; HST, hesperetin; HLB, hydrophilic lipophilic balanced; α-HMET, α-hydroxymetoprolol; HPLC, high performance chromatography; 2′-HBIO, 2′-hydroxybiochanin A; 8-HDA, 8-hydroxydaidzein; 4-HPRO, 4-hydroxypropranolol; IS, internal standard; LC, liquid chromatography; LOD, limit of detection; LOQ, limit of quantification; MECK, micellar electrokinetic chromatography; MET, metoprolol; MIL, milrinone; MS, mass spectrometry; MSTFA, *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide; MYR, myricetin; QC, quality control; QUE, quercetin; r-DA, retro-Diels–Alder; PEL, pelargonidin; PRO, propranolol; SD, standard deviation; S/N, signal-to-noise; SOT, sotalol; SPE, solid-phase extraction; TIC, total ion current; TMCS, trimethylchlorosilane; TMS, trimethylsilyl; UPLC, ultrahigh performance liquid chromatography; UV, ultraviolet–visible.

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polyphenols-rich dietary supplements. The use of dietary supplements enriched with flavonoids is becoming increasingly popular. This raises concerns about possible interactions of flavonoids with therapeutic drugs, because both are xenobiotics and, thus, share at least partially the same metabolic pathways [4]. A number of *in vitro* studies have shown effects of flavonoids on enzymes involved in xenobiotic metabolism, like cytochrome P450 monooxygenases and phase II conjugation enzymes, or on membrane transporters involved in drug excretion [5]. Several investigations have also reported changes of drug bioavailability by certain flavonoids and pharmacokinetic interactions drug–flavonoid [4]. Thus, the uncontrolled intake of flavonoids in the form of dietary supplements or plant extracts is a serious concern for consumer safety [5].

Therefore, there is a need for the analytical methods which allow a rapid and sensitive simultaneous measuring of drugs and polyphenols in biological samples.

Nevertheless, several methods have been reported for the determination of β-blockers in biological fluids. Separation has been achieved with either conventional liquid chromatography (LC) [6–9] or ultrahigh performance liquid chromatography (UPLC) [10]. Moreover, several methods utilizing low resolution mass spectrometry (MS) have been published [6–9]. An ultraviolet–visible (UV) detector, particularly those in a diode-array configuration [11–14], fluorescence detector [14–18] and electrochemical detector [19] are the most widely utilized detectors with high performance liquid chromatography (HPLC). Exclusively, one HPLC-UV method for the determination of a β-blocker, milrinone, was developed [20]. The analysis of individual β-blockers and their metabolites in biological samples was carried out by capillary electrophoresis (CE) [21], GC–MS [22,23] and HPLC with a fluorescence detector [24,25].

Because of the importance of flavonoids and 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 and flavonoids is often accomplished via GC–MS involving the derivatization of the analytes with a number of reagents [26]. HPLC, with various types of detectors, for example UV [27–32], fluorescence [32], and MS [33–36], is the most common method used for determination of flavonoids and isoflavones in biological fluids. Flavonoids and isoflavones have been quantified in the presence of other antioxidants using electrochemical detection by Bolarinwa and Klejdus [37]. Papers have been published on flavonoid separation using different mobile phases; most separations have been accomplished on C8 or C18 reversed-phase columns. The most commonly used analytical methods for the simultaneous detection and identification of isoflavones and their metabolites have been HPLC [38]. In addition, capillary electrophoresis (CE), capillary electrochromatography (CEC) and micellar electrokinetic chromatography (MECK) were proposed for the separation of flavonoids and isoflavones [39].

In this study we are suggesting an analytical method, which is within the bounds of possibility of monitoring both drugs and antioxidants in human urine. The development of new and highly effective techniques for the determination of drugs, isoflavones, flavonoids and metabolites is very important to reported changes of flavonoid effects on pathways involved in drug metabolism. As far as we are aware, there is no literature related to the simultaneous analysis of drugs, isoflavones, flavonoids and metabolites in biological samples.

2. Experimental

2.1. Reagents and chemicals

The flavonoids CHS (≥97% purity) (internal standard; IS), PEL chloride (≥98% purity), (±)-CA hydrate (≥98% purity), (−)-EC (≥97% purity), QUE dihydrate (≥98% purity), HST (≥98% purity), AP (≥95% purity), and MYR (≥96% purity), the isoflavones GT (≥98% purity), DA (≥98% purity), and BIO (≥98% purity), the β-blockers: MIL (≥97% purity), (±)-SOT hydrochloride (IS) (≥98% purity), (±)-MET (+)-tartrate salt (≥98% purity), (±)-PRO hydrochloride (≥99% purity), β-glucuronidase/sulfatase (crude solution from *Helix pomatia*, type HP-2, G7017), and the derivatization reagents: *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) and *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS), were purchased from Sigma Chemicals (St Louis, MO, USA) and Aldrich Chemicals (Milwaukee, WI, USA). The metabolites of β-blockers 4-HPRO hydrochloride, α-HMET, and *O*-DMMET (≥98% purity) were purchased from Toronto Research Chemicals (North York, Canada). GLC and metabolites, including DHGT, DHBIO, DHDA, DMGLC, 8-HDA, 2′-HBIO (≥98% purity) were purchased from PLANTECH (U.K., England). Sodium acetate buffer (pH 4.66), ascorbic acid, phosphate buffer (pH 2.4), analytical-grade methanol, and *tert*-butyl methyl ether were obtained from Merck (Darmstadt, Germany).

2.2. Preparation of the standard solution and quality control samples

The appropriate amounts of β-blockers, flavonoids, isoflavones and metabolites 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 final mixed standard solutions containing β-blockers, flavonoids, isoflavones and metabolites. A series of working solutions of these analytes were freshly prepared by diluting the mixed standard solution in methanol to obtain the necessary multicomponent working solutions for spiking the urine samples. Calibration standards were prepared by spiking a drug, flavonoid, isoflavone and metabolite-free human urine with the working solutions.

To validate this method, three concentration levels of standard solutions containing flavonoids, isoflavones, β-blockers and metabolites were used to prepare the quality control (QC) urine samples. Three QC samples were prepared by adding the appropriate working standard solutions to a drug, flavonoid, isoflavone and metabolite-free human urine. The concentrations of drugs and their metabolites, polyphenols and their metabolites were in the range of 0.005–0.020 μg/ml, 0.500–3.500 μg/ml and 2.500–15.000 μg/ml in human urine to represent low, middle and high QC, respectively.

2.3. Gas chromatography–mass spectrometry

Analyses were carried out with an Agilent 6890N gas chromatograph coupled to a 5973 mass selective detector (70 eV, electron impact mode, Agilent, Waldbronn, Germany). The system was equipped with a CombiPAL autosampler from CTC Analytics. Chromatographic separation was achieved on an HP-5-MS capillary column (Agilent Technologies) (30 m × 0.25 mm inner diameter; 0.25 μm film thickness). A gooseneck splitless liner (78.5 mm × 6.5 mm, 4 mm) from Restek Corporation (PA, USA) was used and the helium gas flow rate was set at constant 1.2 ml/min. The electron impact (EI) ion source, quadrupole mass analyzer, and the interface temperature were maintained at 230, 150 and 280 °C, respectively. Injector temperature was 250 °C and 1 μl sample was injected. The oven temperature was set initially at 160 °C (maintained for 1 min), followed by a gradient of 30 °C/min up to 190 °C (maintained for 10 min), and then programmed to 250 °C at 3 °C/min (maintained for 5 min) and finally to 270 °C at a rate of 10 °C/min (maintained for 8 min). The mass range scanned was 50–800 u at a rate of 0.99 scans/s and solvent delay was 5 min. Four characteristic ions for each compound were used

for peak-identification, while one ion underlined was selected for quantification (Table 2).

2.4. Central composite design (CCD) [40]

Experiment was designed by CCD using STATISTICA 6.0 software (StatSoft, Tulsa, OK, USA). CCD is composed of a 2^n 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 Eq. (1):

$$N = 2^n + 2n + m \quad (1)$$

where 2 – number of levels per design variable; n – number of design variables.

The central composite design investigated changes in derivatization time and temperature. Levels varied from 20 to 90 min for time and between 20 and 80 °C for applied derivatization 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. Peak area was chosen as the response variable. Since it resulted in acceptable statistical models permitting an adequate assessment of the quality of peak separation for all 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:

$$y = b_0 + b_1x_1 + b_2x_2 + b_{12}x_1x_2 + b_{11}x_1^2 + b_{22}x_2^2 \quad (2)$$

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.

2.5. Sample preparation

Urine samples were obtained from healthy volunteers who were on a diet rich in isoflavones and flavonoids, orally administrated tablets of flavonoids and isoflavones and was treated with one pill of propranolol (10 mg). Urine samples were collected at every sampling point with intervals of time (36 h), and then stored in the freezer at −20 °C. The blank urine sample was collected before the volunteer had taken propranolol tablet.

The first step of sample preparation was an enzymatic hydrolysis. A urine sample (1 ml) and SOT and CHS (IS) were incubated with 100 μl 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 0.5 ml phosphate buffer (pH 2.4).

After cooling urine samples the solid phase extraction was performed. The hydrolyzed sample was applied to Oasis HLB extraction cartridges (1 ml, 30 mg, Waters, Milford, MA, USA), preconditioned successively with 2 ml methanol:formic acid (9:1; v/v) and 2 ml water, and allowed to run through. Analytes were eluted with 2 ml *tert*-butyl methyl ether:methanol:formic acid (4.5:4.5:1; v/v/v) and the eluate was evaporated to dryness in a vacuum concentrator (Concentrator 5301, Eppendorf AG, Hamburg, Germany).

Derivatization (silylation/acetylation) of the analytes was carried out using MSTFA or BSTFA containing 1% TMCS. The dry residue was combined with 100 μl derivatization reagent. The mixture was vortex mixed, and then incubated at 60 °C for 60 min. The mixture was cooled and an aliquot of 1 μl was injected onto the GC–MS system.

2.6. Method validation

The newly developed GC–MS method was validated for linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy, selectivity, recovery and stability.

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, flavonoids, isoflavones and metabolites.

Calibration standards were prepared by adding appropriate amounts of standard solutions to β-blocker, flavonoid, isoflavone and metabolite-free human urine, followed by performing serial dilutions with additional blank urine sample to obtain different concentrations. After SPE procedure six replicates of these samples were injected on to the GC–MS system and the analyses were carried out as described in Section 2.3. The calibration curves were made from the peak area ratio of analyzed compounds to the IS (SOT, CHS) and the regression equation was calculated for each curve. The data obtained were submitted to regression analysis and correlation coefficients were calculated for each compound using Excel (Microsoft).

The LOD and the LOQ were determined as the concentrations with a signal-to-noise ratio of 3 and 10, respectively.

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). Precision was expressed as relative standard deviation (CV, %). Accuracy was determined by comparing the calculated concentrations from the calibration curves with the known concentrations.

Absolute recovery was calculated by investigating urine spiked with the known amounts of each examined compound and IS. The recoveries were tested at low, medium, and high concentrations. The spiked urine samples were extracted using the described SPE method, followed by derivatization and GC–MS analysis. Furthermore, the concentrations of compounds were calculated using the calibration curves. The recovery was calculated by comparing the determined amounts for the extracted urine samples with the known amounts added.

The short-term stabilities of drugs, flavonoids, isoflavones and metabolites were assessed by determining QC samples of low, middle and high concentrations 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 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, flavonoids, isoflavones and metabolites were measured compared to freshly prepared samples.

3. Results and discussion

3.1. Optimization of derivatization

In this experiment the derivatization reaction was optimized against reagents, temperature, and reaction time. The derivatization with MSTFA was compared to derivatization with the BSTFA containing 1% TMCS.

The derivatization with BSTFA included in these experiments produced two derivatization products for DHGT, DHDA, DHBIO (Fig. 1). The silylation of DHGT, DHDA, DHBIO leads to two different TMS ethers, one from the keto form and one from the enol form of the molecule. The GC-peak of the keto form gives rise to a weak

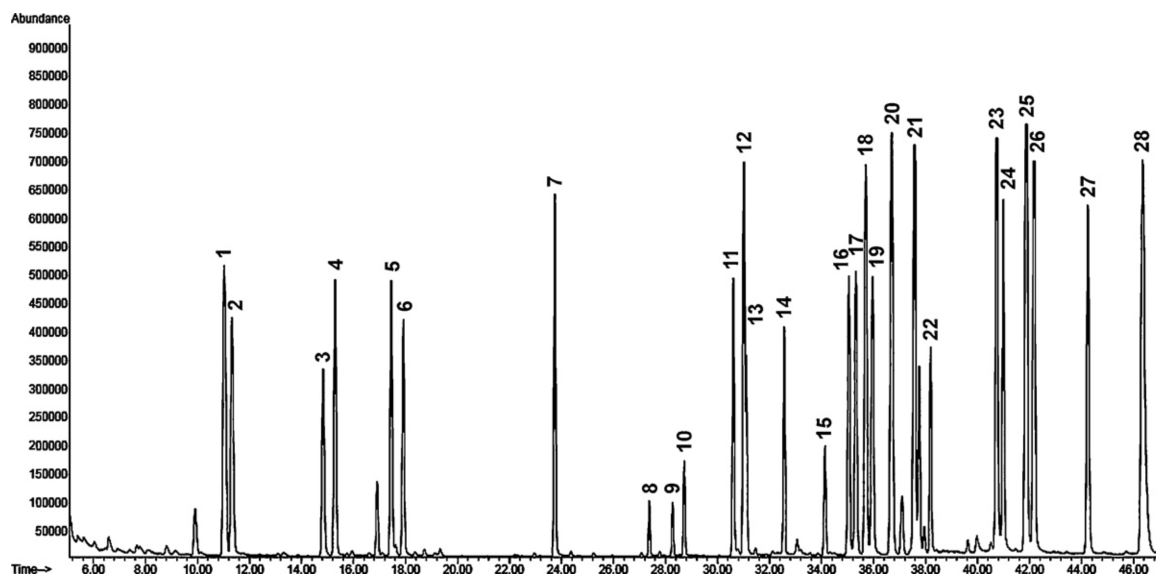

Fig. 1. TIC chromatogram obtained from a mixed standard containing β-blockers, flavonoids, isoflavones and metabolites using the GC–MS method and BSTFA derivatization reagent at 60 °C for 60 min; (1) MIL; (2) MET; (3) PRO; (4) *O*-DMMET; (5) α-HMET; (6) SOT (IS); (7) 4-HPRO; (8) DHBIO (enol); (9) DHDA (enol); (10) DHGT (enol); (11) DHDA (keto); (12) CHS (IS); (13) DHBIO (keto); (14) DHGT (keto); (15) PEL; (16) BIO; (17) 2′-HBIO; (18) (±)-CA; (19) DA; (20) (−)-EC; (21) GT; (22) HST; (23) GLC; (24) 8-HDA; (25) AP; (26) DMGLC; (27) QUE; (28) MYR.

signal of the molecular ion (m/z = 488) as well as to the $[M-15]^+$ fragment (m/z = 473) arising from the methyl radical loss. The keto tautomer undergoes an intense rDA fragmentation (Fig. 2a). The mass spectrum of the GC peak of the enol form shows a dominating molecular ion $[M]^+$ (m/z = 560) and a corresponding rDA (retro-Diels–Alder) reaction product at m/z = 369 (Fig. 2b). In the chromatogram were three additional peaks for DHGT (keto form) 30.65 min, DGBIO 31.10 min and DHGT 32.60 min. The signal for DHBIO (keto form) interfered with peak for internal standard CHS (IS).

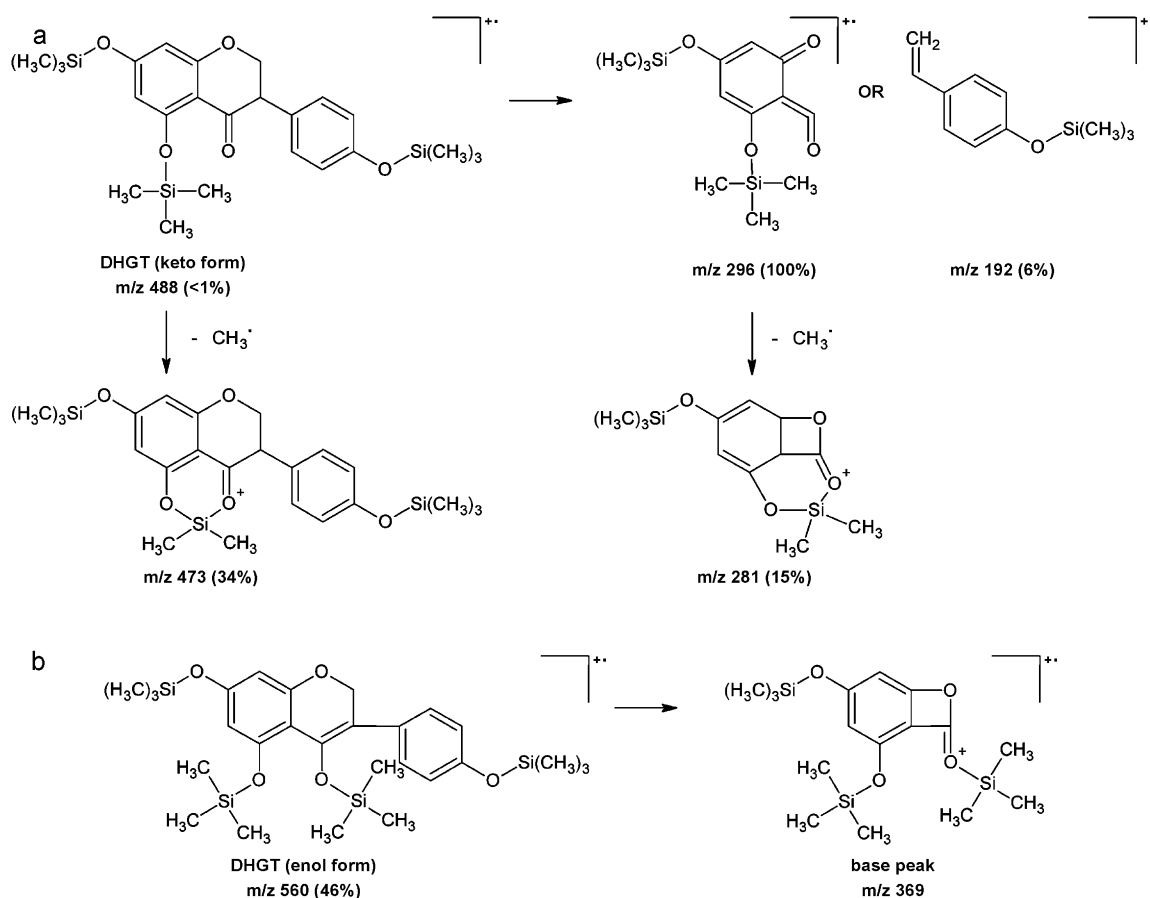

Fig. 2. Main fragmentation of two different silyl ethers of the dihydrogenistein (DHGT) tautomers in the keto form (a) and the enol form (b).

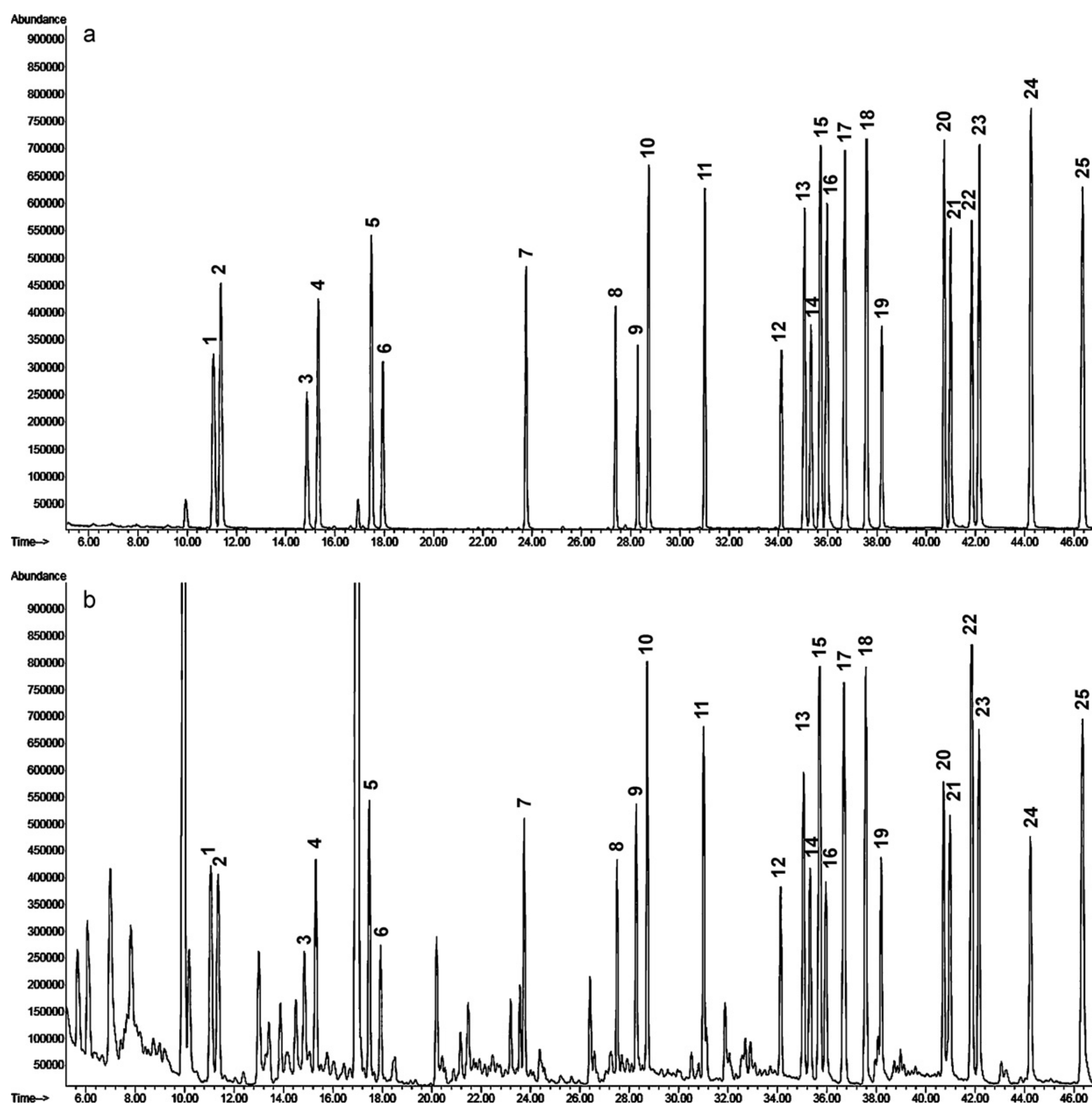

Fig. 3. TIC chromatogram obtained from a mixed standard containing β-blockers, flavonoids, isoflavones and metabolites using the GC–MS method and MSTFA derivatization reagent: (a) at 60 °C for 60 min; (b) at 90 °C for 60 min; (1) MIL; (2) MET; (3) PRO; (4) *O*-DMMET; (5) α-HMET; (6) SOT (IS); (7) 4-HPRO; (8) DHBIO (enol); (9) DHDA (enol); (10) DHGT (enol); (11) CHS (IS); (12) PEL; (13) BIO; (14) 2′-HBIO; (15) (±)-CA; (16) DA; (17) (−)-EC; (18) GT; (19) HST; (20) GLC; (21) 8-HDA; (22) AP; (23) DMGLC; (24) QUE; (25) MYR.

MSTFA was the only derivatization agent that gave one satisfying derivatization product for all the substances tested. Using MSTFA the highest signal intensity was obtained for more analytes (Fig. 3a). Consequently, it was decided to use MSTFA in the further experiments.

Having chosen the derivatization reagent, the derivatization temperature and time were then optimized. A CCD, using surface response, was applied to the selection of the derivatization conditions. Twelve experiments were performed (Table 1).

The effect of increasing the derivatization temperature from 20 °C to 90 °C was evaluated using the MSTFA as the optimum derivatization reagent. When the derivatization temperature was varied, sensitivity varies for most analytes. The results show that in general higher incubation temperature increases derivatization efficiency. Temperatures higher than 60 °C had a negative effect on the separation because extraneous peaks were observed in the chromatograms. The observation of note is that at temperatures higher than 60 °C, all sample vials experienced some degradation of their septa. This degradation was believed to be the source of extraneous peaks observed in the chromatograms (Fig. 3b). These peaks co-eluted with either of the model analytes or internal standard. We prefer better chromatographic separation (i.e., no co-eluting peaks) to improved peak height (sensitivity); thus we decided to operate at approximately 60 °C.

Several reaction times (Table 1) were studied and it was concluded that a step of 60 min derivatization yielded best efficiency in the least time for the formation of the trimethylsilyl derivatives for all the compounds. Increasing the reaction time from 10 to 90 min had several positive effects on the sensitivity; we observed increased peak heights (22%) of drugs, flavonoids, isoflavones and metabolites. Between 60 and 90 min the increase was negligible (2%) and furthermore not characteristic for all analytes. Therefore, the derivatization conditions were further studied using 60 min, as

Table 1
Exemplary central composite design (CCD).

Observation	Variable 1	Time (min)	Variable 2	T (°C)	Peak area	
1	−1	20	−1	20.0	512565	2^n fractional design
2	−1	20	1	80.0	1252657	
3	1	90	−1	20.0	645475	
4	1	90	1	80.0	1654413	
5	−1.414	5.5	0	50.0	240023	2n start points
6	1.414	104	0	50.0	767035	
7	0	55	−1.414	7.6	737245	
8	0	55	1.414	92.4	2111487	
9	0	55	0	50.0	953611	m replication of the center point
10	0	55	0	50.0	965453	
11	0	55	0	50.0	944578	
12	0	55	0	50.0	956796	

the optimal duration of the process, as well as the time in which we obtained a satisfactory intensity.

The response surface graphs were drawn as a function of derivatization temperature and derivatization time (Fig. 4). From the partial derivatives of the equation describing the curve, it was possible to extract the critical values for the investigated factors. The values of 60 °C and 60 min were obtained for temperature and time, respectively. Furthermore, the model was generated and based on the experimental multiple responses. Conclusively, the model can be represented by the following equations:

$$R_s = 275705 + 20404x_1 - 15742x_2 + 64x_1x_2 - 173x_1^2 + 276x_2^2 \quad (3)$$

where x_1 – derivatization temperature; x_2 – derivatization time.

3.2. Chromatographic separation

GC–MS conditions were optimized such that baseline resolution was achieved between all analytes while also keeping an adequate run time (47 min) so as to maximize the throughput of samples. Because of the similar polarity exhibited by all of the derivatized β-blockers, flavonoids, isoflavones and metabolites, the mix of compounds was separated using a slow temperature program (3 °C/min) in the GC–MS oven. Fig. 3a shows the separation of the twenty three compounds and two internal standards. Good chromatographic separation was obtained with the temperature program reported in Section 2. As can be observed in this figure, the MSTFA derivatives eluted in an order depending on the number of trimethylsilyl groups substituted, and the

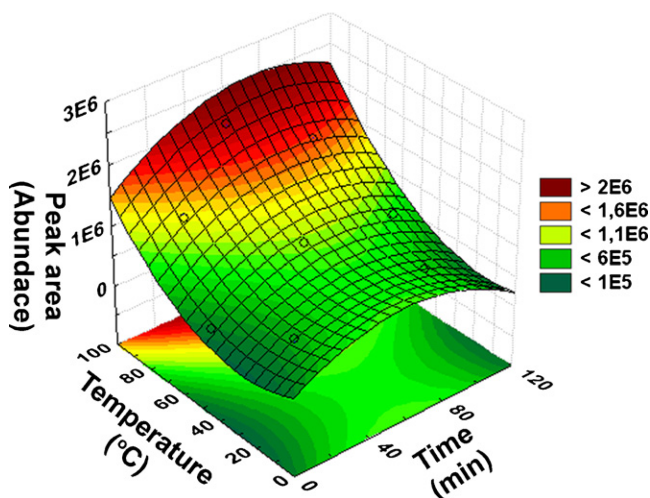

Fig. 4. Response surface graph functions of derivatization temperature and derivatization time – peak area.

position of other groups in the molecule such as methoxy and carbonyl groups. In general, the compounds eluted in the order of 1, 2, and 3 trimethylsilyl groups substituted. β-Blockers and their metabolites eluted first because they have only one or two trimethylsilyl groups. GLC was the other exception being the last eluting compound of the isoflavone since the ortho/meta position of the trimethylsilyl and the methoxy groups gives this molecule a highly hydrophobic nature. CHS, with two trimethylsilyl groups eluted first of flavonoids, followed by PEL and (±)-CA, (−)-EC and QUE with four and five trimethylsilyl groups, and finally, myricetin with six trimethylsilyl groups, which was the last one of this group of three member ring phytoestrogens. In the case of HST, similarly as GLC, the ortho/meta position of the trimethylsilyl and the methoxy groups gives this molecule a highly hydrophobic nature.

Table 2 shows retention time obtained in GC–MS system used in this work and the m/z ratio characteristic for each standard. The derivatization of an alcohol function to a trimethylsilylated function increases molar mass by 72. In some cases the fragmentation of the trimethylsilyl group is involved such as for GT. In other cases either a methyl or a methyl and a carbonyl group are lost and rearrangement of the structure occurs. Among the diverse fragment ions, two abundant peaks (or base peak) at m/z 72 and m/z 144, and one minor $[M-15]^+$ ion were found to be very selective for the detection of β-blockers with aryloxypropanol and isopropyl amine moieties except for SOT, PRO and MET. The fragment ion at m/z 72 corresponding to $[CH_2NHCH–(CH_3)_2]^+$ constituted the base peaks for most of the β-blockers.

From an analytical point of view it is of primary importance to select those fragment ions which can be used for quantitative and confirmatory purposes that typically do not involve the loss of the TMS (trimethylsilyl) group. For this reason and based on the relative abundance, four selective fragment ions were chosen for each analyte. One of them (having the highest abundance) was used for quantitation purposes (shown in Table 2) and the second one was used as a qualifier ion for identification and confirmatory purposes.

Urine matrix effects were examined with urine extracts prepared from blank urine. The typical chromatogram of blank urine sample showing a few peaks in its low background (Fig. 5) verified that the present method was very selective for all analyzed compounds. The spiked twenty five compounds (including SOT and CHS as IS) were simultaneously detected with good sensitivity and excellent selectivity from urine.

3.3. Method validation

The method was evaluated for selectivity, linear range, LOD, LOQ, precision, accuracy, recovery and stability.

Selectivity experiments were carried out using human urine samples that did not contain the target compounds. A chromatogram obtained by GC–MS from a blank sample of urine is

Table 2
Mass spectral parameters for silylated standard compounds.

No.	Compound	Retention time (min)	Molecular weight	TMS groups	TMS derivatized molecular weight	Characteristic ions	Ion selected for quantification
β-Blockers and their metabolites							
1	MIL	11.06	211	1	283	134, 243, 268, 283	268
2	MET	11.36	267	1	339	72, 101, 223, 324	72
3	PRO	14.86	259	1	331	72, 101, 144, 215	72
4	*O*-DMMET	15.32	253	2	397	72, 103, 281, 382	72
5	α-HMET	17.48	283	2	427	72, 188,311, 412	72
6	SOT (IS)	17.94	272	1	344	72, 250, 344, 401	72
7	4-HPRO	23.76	276	2	420	72, 188, 216, 232	232
Flavonoids, isoflavones and their metabolites							
8	DHBIO (enol)	27.39	286	3	502	73, 369, 487, 502	369
9	DHDA (enol)	28.29	256	3	472	73, 281, 457, 472	281
10	DHGT (enol)	28.73	272	4	560	73, 369, 545, 560	369
11	CHS (IS)	31.03	254	2	398	73, 184, 311, 383	383
12	PEL	34.14	272	4	559	73, 193, 306, 559	559
13	BIO	35.07	284	2	428	73, 199, 370, 413	413
14	2′-HBIO	35.32	300	3	516	73, 383, 427, 501	501
15	(±)-CA	35.70	290	5	650	73, 267, 355, 368	368
16	DA	35.98	254	2	398	73, 184, 383, 398	398
17	(−)-EC	36.70	290	5	650	73, 267, 355, 368	368
18	GT	37.58	270	3	486	73, 228, 399, 471	471
19	HST (enol)	38.20	302	4	590	73, 273, 545, 575	575
20	GLC	40.74	284	2	428	73, 398, 413, 428	428
21	8-HDA	41.00	270	3	486	73, 383, 471, 486	486
22	AP	41.85	270	3	486	73, 228, 399, 471	471
23	DMGLC	42.16	270	3	486	73, 383, 471, 486	486
24	QUE	44.26	287	5	647	73, 487, 559, 647	647
25	MYR	46.35	318	6	750	73, 147, 647, 735	735

shown in Fig. 5a. There were no interfering peaks at the retention times corresponding to the analyzed compounds. There are some additional unidentified peaks in the chromatogram from the human urine samples, but these peaks do not interfere with the β-blockers, flavonoids, isoflavones and metabolites of interest.

A six-point linearity curve was constructed for each analyte. The linear relationships between peak areas and concentrations were observed with the correlation coefficients (r^2) greater than 0.9992 and linearity ranges are listed in Table 3.

The LODs for the analytes were estimated under GC–MS conditions (SCAN mode) by considering the lowest concentration of each analyte that could be detected (Table 3). In this table, the LODs, evaluated after re-calculation to account for the sample injection (1 μl volume), are also presented.

Under optimized conditions, intra-day and inter-day precision and accuracy were determined by QC samples at three concentrations as described in Section 2. Both intra-day and inter-day precision, given by the relative standard deviation (CV, %), were lower than 4.96% at each tested concentration level. The intra-day accuracy of the method was acceptable at each concentration level: 0.28–3.86% for the low concentration level, 0.01–4.33% for the medium concentration level, and 0.02–3.10% for the high concentration level. The inter-day accuracy ranged from 0.03 to 4.98%. These results indicated that the present method has an acceptable accuracy and precision.

The percent recoveries for all analyzed compounds from spiked human urine were evaluated at a low (0.005–0.020 μg/ml), medium (0.500–3.500 μg/ml) and high (2.500–15.000 μg/ml) concentrations. The overall mean recoveries calculated for the analyzed compounds are in range from 70.20% (for (±)-CA) to 99.55% (for 4-HPRO). A high percent recovery indicates that the method can be successfully used for the determination of analyzed β-blockers, flavonoids, isoflavones and metabolites in human urine samples.

All analytes were found to be stable in blank sample for 12 h at room temperature (0.32–8.48% for β-blockers and their metabolites, 0.27–9.87% for polyphenols and their metabolites). Both drugs and polyphenols were shown to be stable in urine for 30 days when stored at −20 °C in range 1.07–9.83% and 0.10–9.98%, respectively. The three freeze–thaw cycle stability of the analytes in the study was also excellent. Generally, after three freeze–thaw cycles the percentage degradation of the compounds was less than 10% for all analytes. The data showed the reliable stability behavior of each compound under the condition tested.

3.4. Application to real human urine sample analysis

The developed GC–MS method was applied to the investigation of isoflavones, flavonoids, β-blockers and metabolites in real human urine under the optimum conditions. A volunteer was treated with one pill of propranolol tablet (10 mg) and was on a diet high in isoflavone and flavonoid content. The total ion current (TIC) chromatograms and extracted ion current chromatograms (EIC, m/z are 72; 232; 398; 471; 575; 428) of positive urine and blank urine samples using optimized parameters are shown in Fig. 5. It could be seen that the extract of positive PRO urine sample after SPE was markedly distinguished from blank urine and sensitively detected by GC–MS.

The study reported here presents a method to detect β-blockers in human urine and to follow the kinetics of their excretion after normal supplementation. The possible influence of supplements' intake on the bioavailability and metabolism of oral PRO – nonselective beta adrenoceptor antagonist, was examined by determinations of the drug concentrations in urine of healthy subject, taking single doses of the drugs both on an empty supplement and together with a taken supplement. A real-time monitoring in urine samples within 36 h of a volunteer on a single-dose administration of PRO was executed.

The results indicate that dietary supplements enhance the urinary excretion of PRO. Among the various human CYP enzymes that metabolise xenobiotics as well as endogenous substrates, the isoforms 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4 and 3A5 are of special significance to drug metabolism and, thus, are most likely to be involved in drug–flavonoid interactions. A number of studies have demonstrated that flavonoids from several classes are potent inhibitors of this isoform *in vitro*. Indeed, the pre-treatment of

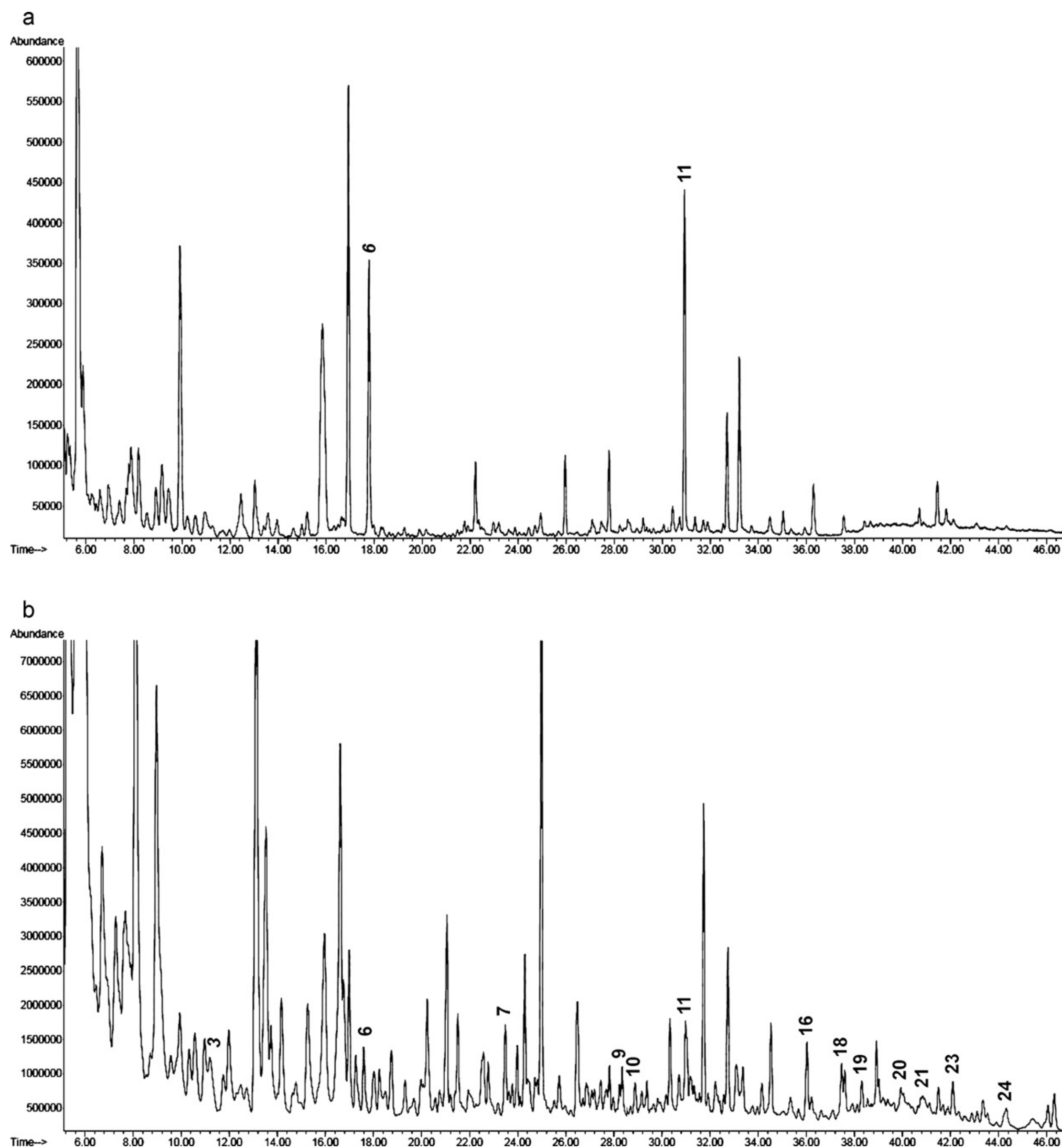

Fig. 5. TIC chromatogram of (a) blank urine and (b) real urine sample of PRO (after 7 h), flavonoids, isoflavones and metabolites (TIC in SCAN mode); (3) PRO; (6) SOT (IS); (7) 4-HPRO; (9) DHDA (enol); (10) DHGT (enol); (11) CHS (IS); (16) DA; (18) GT; (19) HST; (20) GLC; (21) 8-HDA; (23) DMGLC; (24) QUE.

isoflavones and flavonoids decreased CYP1A2 activity in the study subject [5].

Administration of flavonoids and isoflavones increased the urinary excretion of PRO and decreased urinary excretion of its metabolite – 4-HPRO. Because the PRO is mainly metabolised by hepatic CYP1A2, pre-treatment of flavonoids and isoflavones most probably inhibited this CYP isoform. The increase in urinary excretion and the elimination time can be explained by inhibition of CYP1A2 isoform. Mean concentrations of PRO in human urine were 25.72 ± 0.04 ng/ml before supplementation and 36.20 ± 0.36 ng/ml after supplementation. The mean concentration of 4-HPRO decreased from 146.72 ± 0.45 ng/ml to 85.43 ± 0.48 ng/ml. Urine PRO concentrations increased in response to ingestion of isoflavones and flavonoids. The maximum urinary excretion for the drug was determined at 9 h post dosing of PRO before and 7 h after supplementation. The minimum drug excretion was at 36 h after administration of PRO both before and after supplementation. Pharmacokinetic analysis of the urine concentration–time curves showed that the elimination $t_{1/2}$ was 3.99 h before supplementation and $t_{1/2}$ was 5.44 h after supplementation Urinary concentrations and the cumulative urinary excretion of PRO after and before intake of supplements are presented in Fig. 6.

The urine samples were also tested for isoflavones, flavonoids and metabolites, which are components of supplements and soy products, fruits, vegetables, tea. Analysis of the 36-h urinary excretion of DA, GT, GLC and HST demonstrated that the cumulative amounts gradually increased during the collection period after oral administration of these isoflavones and flavonoids to the subject. The mean urine concentration–time profile of HST is shown in Fig. 7.

The flavonoid and isoflavone concentrations reached a maximum between 12 and 24 h after dosing with a level

Table 3
Analytical parameters of calibration curves of all examined compounds ($n = 6$).

Compound	Linear dynamic range (μg/ml)	Correlation coefficient r^2 ($n = 6$)[a]	LOD (ng/ml)	LOQ (ng/ml)	LOD (fmol)
β-Blockers and their metabolites					
MIL	0.020–5.00	0.9999	1.3	4.1	6.4
MET	0.020–5.00	0.9994	3.6	10.8	13.4
PRO	0.020–5.00	0.9995	6.2	18.7	23.8
O-DMMET	0.020–5.00	0.9995	2.9	8.8	11.4
α-HMET	0.020–5.00	0.9996	2.8	8.6	10.1
4-HPRO	0.020–5.00	0.9997	4.1	12.5	14.9
Flavonoids, isoflavones and their metabolites					
DHBIO (enol)	0.020–2.5	0.9993	3.8	11.6	13.4
DHDA (enol)	0.020–5.0	0.9996	6.4	19.5	25.1
DHGT (enol)	0.010–2.5	0.9998	1.9	5.9	7.2
PEL	0.050–15.0	0.9994	9.7	29.3	35.5
BIO	0.005–2.5	0.9998	0.8	2.3	2.6
2′-HBIO	0.015–5.0	0.9992	1.4	4.3	7.0
(±)-CA	0.020–5.0	0.9998	2.2	6.7	7.6
DA	0.020–5.0	0.9996	3.0	9.2	12.0
(−)-EC	0.020–5.0	0.9999	3.1	9.4	10.7
GT	0.005–5.0	0.9994	0.7	2.2	2.7
HST (enol)	0.005–5.0	0.9995	0.6	1.8	1.9
GLC	0.010–2.5	0.9998	1.3	4.0	4.6
8-HDA	0.020–5.0	0.9999	2.5	7.7	9.4
AP	0.020–15.0	0.9998	3.3	10.0	12.2
DMGLC	0.015–5.0	0.9998	1.7	5.3	6.4
QUE	0.010–10.0	0.9998	1.4	4.2	4.8
MYR	0.010–10.0	0.9996	2.1	6.3	6.6

[a] Number of points in calibration curves.

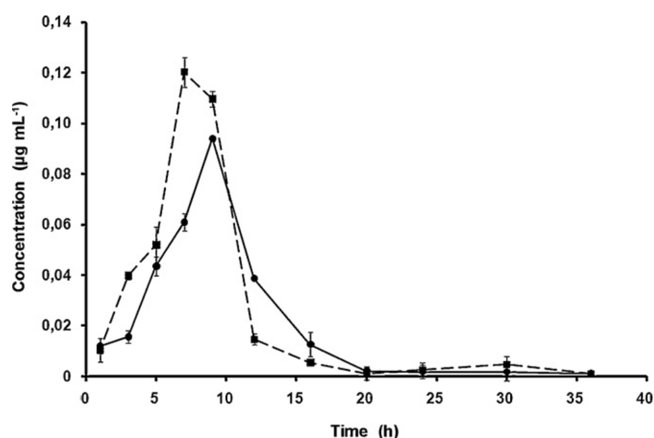

Fig. 6. The mean urine concentration–time profiles (±SD) of PRO before (–) and after (- -) oral administration of supplements with isoflavones and flavonoids.

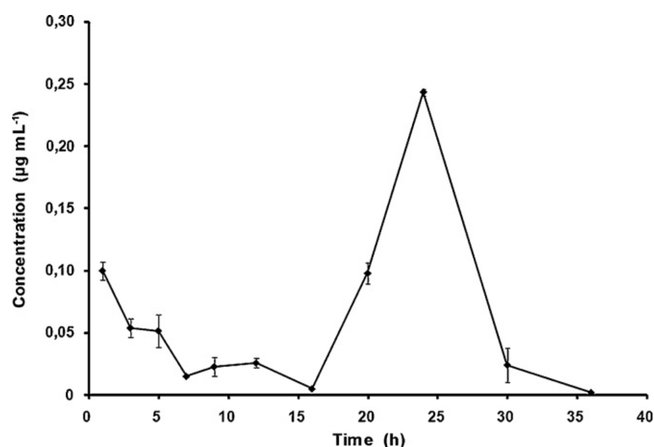

Fig. 7. The mean urine concentration–time profile (±SD) of HST after oral administration of supplements.

of 702.33 ± 9.78 ng/ml for GT, 328.69 ± 4.27 ng/ml for GLC, 3367.18 ± 27.00 ng/ml for DA, and 244.45 ± 1.72 ng/ml for HST. Pharmacokinetic analysis of the urine concentration–time curves showed that the elimination $t_{1/2}$ was 10.65 h for GT, 9.28 h for DA, 7.15 h for GLC and 11.8 h for HST. The maximum urinary excretion for the metabolites was determined between 20 and 20 h post dosing of supplements with a level of 2679.56 ± 9.88 ng/ml for DHDA, 165.46 ± 7.76 ng/ml for DHGT, 232.85 ± 1.99 ng/ml for 8-HDA, 23.61 ± 0.23 ng/ml for DMGLC.

The urine samples were also tested for (±)-CA, (−)-EC, QUR, and AP, which are components of a diet rich in fruits, vegetables and tea. The mean concentrations of these flavonoids were: 4.91 ± 0.01 ng/ml for (−)-EC, 126.03 ± 3.02 ng/ml for QUE, and 10.22 ± 0.35 ng/ml for AP. (±)-CA was detected in urine, but not quantified. The relationship between the concentrations of these compounds and the collection times of the urine samples is not shown.

The results show, that the GC–MS method could be effectively used for the analysis of drugs, flavonoids, isoflavones and metabolites in real urine samples. This method might provide a convenient index of metabolism of compounds in urine and could be used to explore the effect of dietary polyphenols on pathways involved in drug metabolism.

4. Conclusion

In the present study, a GC–MS method was developed and validated for simultaneous identification and determination of β-blockers: milrinone, metoprolol, propranolol and their metabolites: 4-hydroxypropranolol, α-hydroxymetoprolol, *O*-desmethylmetoprolol; polyphenols: pelargonidin, (±)-catechin, (−)-epicatechin, quercetin, hesperetin, apigenin, myricetin, genistein, daidzein, glycitein, biochanin A and their metabolites: dihydrogenistein, dihydrobiochanin A, dihydrodaidzein, desmethylglycitein, 8-hydroxydaidzein, 2′-hydroxybiochanin A in human urine. The proposed GC–MS method was fully validated and showed an appropriate specificity, linearity, sensitivity and precision for all the analytes studied. A simple and convenient sample

preparation procedure makes this method more feasible for the bioanalysis of β-blockers, flavonoids, isoflavones and metabolites. The central composite design, response surface analysis and the Derringer–Suich multicriteria method were used to optimize the derivatization conditions of all compounds from human urine providing maximum sensitivity.

The developed method appears to be the first direct method for the simultaneous analysis of the studied compounds. It is expected that this method can be applied to clinical and toxicological studies. This method can also be applied to study the pharmacokinetic parameters of drugs and polyphenols in healthy human volunteers. Finally, this new GC–MS method may be extended to determine the pharmacokinetics of drugs and polyphenols and also to examine the drug–polyphenols interaction in combination therapy.

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