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Development and validation of UHPLC–ESI-MS/MS method for the determination of selected cardiovascular drugs, polyphenols and their metabolites in human urine

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ABSTRACT

A sensitive ultra-performance liquid chromatography tandem mass spectrometry method with electrospray ionisation (UHPLC–ESI-MS/MS) was developed for the simultaneous determination of 52 compounds: β-blockers, polyphenols (antioxidants) and their metabolites in mixture of standards and after addition the 52 standard solutions to human urine samples. The analyses of urine samples obtained from patients treated with β-blockers were also carried out.

The separation of analytes was performed on a Hypersil GOLD™ column (100 mm × 2.1 mm, 1.9 μm) using a gradient elution profile for 10 min and mobile phase consisting of 0.1% formic acid in water and acetonitrile. In these conditions, some of the tested compounds were not separated, but this was compensated by the use of MS/MS detection. The drugs, polyphenols and their metabolites were detected with a tandem mass spectrometer after being ionised positively or negatively (depending on the molecule) using an electrospray ionisation (ESI) source. The MS system was operated in the selected reaction monitoring (SRM) mode, where one quantitation and one confirmation transition was done for each analyte.

The quantitative method was validated for selectivity, linearity, low limits of quantitation, accuracy, precision, recovery, matrix effect and analyte stability. The LLOQ varied from 0.01 to 0.40 ng mL^{-1} for β-blockers and from 0.05 to 40.0 ng mL^{-1} for polyphenols. The linear range was 0.08–1000 ng mL^{-1} for the drugs and 0.10–2300 ng mL^{-1} for the polyphenols. Intra-day and inter-day precision was less than 8%, and the accuracy ranged from −4.40 to 2.23% for all analytes. The average recoveries for all compounds analysed were better than 90%.

The developed method can be successfully used to monitor cardiovascular drugs and their metabolites in urine samples of patients treated with β-blockers and can also be used to study the effect of polyphenols on the metabolism of drugs.

1. Introduction

The aetiology and pathophysiology of cardiovascular diseases is complex, but it is known that major risk factors include unhealthy lifestyles and behaviours and a complex interaction between environmental and genetic factors. Cardiovascular diseases are increasing in almost all developing countries as the population ages and lifestyles change rapidly [1].

β-Blockers play a crucial role in the treatment of cardiovascular disease, and they are also recommended as a primary therapy for other diverse medical conditions that, present problems with other treatments [2]. Interest in the possible health benefits of polyphenols has increased due to their potent antioxidant and free-radical scavenging activities observed *in vitro*. However, epidemiological studies exploring the role of polyphenols in human health have been inconclusive. Some studies support a protective effect of polyphenol in cardiovascular disease and cancer, while other studies demonstrate no effect, and a few studies actually suggest that polyphenols can potentially be harmful [3–5]. There is growing evidence from human studies suggesting that the absorption and bioavailability of specific polyphenols is much higher than originally believed [6].

The use of dietary supplements enriched with antioxidants is becoming increasingly popular, which raises concerns about the possible interactions of polyphenols with therapeutic drugs, because both are xenobiotics and share at least partially the same metabolic pathways. A number of *in vitro* studies have shown the effects of polyphenols on cytochrome P450 monooxygenases,

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phase II conjugation enzymes, and membrane transporters involved in drug excretion [7,8]. Some investigations have also reported that flavonoids and drug–flavonoid interactions can change drug bioavailability. Thus, the uncontrolled intake of antioxidants in the form of dietary supplements or plant extracts is a serious concern for consumer safety [9]. Because there are many biological activities attributed to the polyphenols, some of which could be beneficial or detrimental depending on specific circumstances, further laboratory and clinical studies are warranted.

Therefore, there is a need for a rapid and sensitive analytical method that can simultaneously quantify both β-blockers and polyphenols in biological samples.

Different analytical methods have been reported for the analysis of some cardiovascular drugs in biological samples such as plasma, urine, and body tissue. These methods include high performance liquid chromatography (HPLC) coupled to mass spectrometry (MS) [10–12]; tandem MS/MS [13–15]; diode array detection (DAD) [16,17]; fluorimetric (FLD) [17] and electrochemical detection (ECD) [18]; gas chromatography (GC) although this technique often requires derivatisation [19,20]; and capillary electrophoresis (CE) [21].

There are several reports of analytical techniques used for the quantification of polyphenols in biological samples. HPLC with UV [22,23] or electrochemical [24,25] detection has been used, in addition to capillary electrochromatography for the determination of trace polyphenols in biological samples [26]. Gas chromatography–mass spectrometry (GC–MS) has been used as a sensitive analytical technique for polyphenol analysis [27], but LC–MS/MS has become the method of choice due to its high sensitivity, selectivity and easy sample preparation [28–30].

Initially a quantitative ultra-performance liquid chromatography method with UV detection (UHPLC–UV) for the simultaneous determination of 5 β-blockers, 6 isoflavones and 11 metabolites [31] and gas chromatography method with MS detection (GC–MS) [32] was developed, as an alternative analytical method for determination of drugs, polyphenols and metabolites. However, this method is not sufficient as an ever-increasing number of compounds need to be analysed in the laboratory.

Therefore, the aim of this study is the development of an analytical method for the simultaneous determination of drugs and polyphenols and their metabolites in human urine. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) was selected as the analytical method of choice due to the need for sensitivity and selectivity and the fact that LC–MS/MS offers convenient sample preparation without derivatisation. In this study a rapid UHPLC–MS/MS method for the simultaneous quantification of 52 compounds in urine was described, making this assay suitable for the high-throughput analysis of β-blockers and polyphenols and their metabolites.

2. Experimental

2.1. Chemicals and reagents

The polyphenols used were chrysin (CHS) (internal standard; IS), (±)-catechin hydrate ((±)-CA), (−)-epicatechin ((−)-EC), rutin (RUT), hesperetin (HST), quercetin dihydrate (QUE), quercitrin (QUR), (±)-naringenin ((±)-NAR), hesperidin (HSD), neohesperidin (NHSD), kaempferol (KAM), apigenin (AP), isorhamnetin (ISO), (−)-epigallocatechin gallate (EGCG), (−)-epicatechin gallate (ECG), pinocembrin (PIN), myricetin (MYR), puerarin (PUR), genistein (GT), daidzein (DA), biochanin A (BIO), glycitin (GLY), 3-hydroxybenzoic acid (3-HBA), benzoic acid (BA), caffeic acid (CA), 3,4-dihydroxybenzoic acid (3,4-DHBA), hippuric acid (HA), α-hydroxyhippuric acid (α-HHA), 4-hydroxybenzoic acid (4-HBA), 3,4-dihydroxy-phenylacetic acid (DOPAC), 3-hydroxyphenylacetic acid (3-HPA), *p*-coumaric acid (*p*-COA), vanillic acid (VA), 4-hydroxy-3-methoxyphenylacetic acid (HVA), and ferulic acid (FA); these were purchased from Sigma Chemicals (St. Louis, MO, USA) and Aldrich Chemicals (Milwaukee, WI, USA). The β-blockers used were milrinone (MIL), sotalol hydrochloride (IS) ((±)-SOT), metoprolol (+)-tartrate salt ((±)-MET), propranolol hydrochloride ((±)-PRO) and β-glucuronidase/sulfatase (crude solution from *Helix pomatia*, type HP-2, G7017), and they were purchased from Sigma Chemicals (St. Louis, MO, USA) and Aldrich Chemicals (Milwaukee, WI, USA). Carvedilol (CAR) and the β-blocker metabolites 4-hydroxypropranolol hydrochloride (4-HPRO), α-hydroxymetoprolol (α-HMET), *O*-desmethylmetoprolol (*O*-DMMET), *O*-desmethylcarvedilol (*O*-DMCAR), and 5′-hydroxycarvedilol (5′-HCAR) were purchased from Toronto Research Chemicals (North York, Canada). Glycitein (GLC) and some metabolites, including dihydrogenistein (DHGT), dihydrobiochanin A (DHBIO), dihydrodaidzein (DHDA), desmethylglycitein (DMGLC), 8-hydroxydaidzein (8-HDA), 8-hydroxygenistein (8-HGT), 2′-hydroxybiochanin A (2′-HBIO), and daidzein-7,4′-diglucoside (Glu-DA) were purchased from PLANTECH (UK, England). The sodium acetate buffer (pH 4.66), ascorbic acid, phosphate buffer (pH 2.4), formic acid, hypergrade acetonitrile, water and methanol for LC–MS were obtained from Merck (Darmstadt, Germany).

2.2. Preparation of the standard solution and quality control samples

Standard stock solutions at a concentration of 1 mg mL^{-1} were prepared in methanol for each analyte separately. From these stock solutions, a working solution containing adequate concentrations of all the analytes in methanol was spiked into the urine samples.

The calibration standards (CS) and quality control (QC) samples were prepared by spiking a pool of drugs and polyphenols (the working solution) into human urine and diluting it several times to make standards covering the desired concentration range. The calibration standards were made at a concentration of 0.08–1000 ng mL^{-1} for the drugs and 0.1–2300 ng mL^{-1} for the polyphenols. The quality controls (QC) were prepared at four different concentration levels: the lower limit of quantification (LLOQ) (0.01–0.40 ng mL^{-1} for the drugs and 0.05–40.0 ng mL^{-1} for the polyphenols), the low concentration quality control (LQC) (0.1–0.8 ng mL^{-1} for the drugs and 0.8–80.0 ng mL^{-1} for the polyphenols), the medium concentration quality control (MQC) (10.0–60.0 ng mL^{-1} for the drugs and 60.0–300.0 ng mL^{-1} for the polyphenols) and the high concentration quality control (HQC) (250.0–500.0 ng mL^{-1} for the drugs and 500.0–1000.0 ng mL^{-1} for the polyphenols).

2.3. Instrumentation and analytical conditions

The analysis was performed using a Dionex UHPLC system (Dionex Corporation, Sunnyvale, CA, USA) consisting of an UltiMate 3000 RS (Rapid Separation) pump, an UltiMate 3000 autosampler, an UltiMate 3000 column compartment with a thermostable column area, and an UltiMate 3000 variable wavelength detector, all of which were operated using the Dionex Chromeleon™ 6.8 software. Chromatographic separations were performed on a Hypersil GOLD™ (Thermo Scientific) column (100 mm × 2.1 mm, 1.9 μm) with a guard column of the same material (4 mm × 2.1 mm). The column was thermostated at 25 °C, and samples were kept at 5 °C in the autosampler. The mobile phase consisted of acetonitrile (solvent A) and 0.1% aqueous formic acid (solvent B) using a gradient elution with the following parameters: 0–1 min, 5–15% A (0.65 mL min^{-1}); 1–2.5 min, 25% A (from 0.65 to 0.7 mL min^{-1});

2.5–3 min, 25% A (from 0.7 to 0.65 mL min^{-1}); 3–4 min, 30% A (0.65 mL min^{-1}); 4–5 min, 35% A (0.65 mL min^{-1}); 5–6 min, 38% A (from 0.65 to 0.7 mL min^{-1}); 6–8 min, 70% A (0.7 mL min^{-1}); or 8–10 min, 5% A (from 0.7 to 0.65 mL min^{-1}).

The UHPLC system was connected to a 4000 Q TRAP triple quadrupole linear ion trap mass spectrometer (Applied Biosystems/MDS SCIEX, Foster City, CA, USA), controlled by the Analyst 1.4 software. The TurboIonSpray source system equipped with an electrospray ionisation source (ESI) was operated in negative- and positive-ion mode.

The operating parameters of the ion source, including the source-dependent and the compound-dependent ones, were optimised to obtain the best performance from mass spectrometer for the analysis of drugs, antioxidants and metabolites. The source-dependent parameters for all analysed compounds were consisted of the nebulizer gas, the curtain gas, the collision gas, the ionspray voltage, and the temperature of the heater gas. The detector conditions were as follows: ion spray voltage at 4000 V, source temperature at 650 °C, ion source gas 1 (GS1) at 90 psi and ion source gas 2 (GS2) at 60 psi, and curtain gas at 10 psi. High-pressure nitrogen was used as the ion source gas, curtain gas, and collision gas. The collision activated dissociation (CAD) gas was set at medium using nitrogen as the collision gas. The compound-dependent parameters were also tuned for individual analytes to achieve the highest instrument response. Compound-dependent parameters were established using the injection of various standard solutions at a concentration of 300 ng mL^{-1} into the ion source using a Harvard syringe pump at a flow rate of 10 μL min^{-1}. Continuous mass spectra were obtained by scanning from 50 to 800 *m*/*z*. To get good sensitivity and peak shape, a product ion was selected for the optimisation of the MS parameters (i.e., declustering potential (DP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP)).

Analysis was performed in selected reaction monitoring mode (SRM), using the precursor ions and the corresponding product ions. Three transitions were chosen for each drug, polyphenol and metabolite according to the European Union (EU) criteria of one precursor ion and two product ions. One of two product ions is the quantitative ion and the other is the qualitative ion for each compound.

2.4. Sample preparation

Urine samples were obtained from 32 patients who were on a diet rich in antioxidants and who were treated with propranolol or metoprolol. This study was approved by the Institutional Review Board of Regional Specialist Hospital (Wroclaw, Poland). The blank urine sample was collected before the patients had taken the drugs. Urine samples were stored in the freezer at – 20 °C.

2 mL sample of the human urine was transferred to a 10 mL Eppendorf cup and spiked with 10 μL of 2.5 μg mL^{-1} IS solution (sotalol and chrysin). Protein precipitation was carried out using 1.5 mL acetonitrile and 1.5 mL methanol, and a universal centrifuge Z 323 K (Hermle Labortechnik GmbH, Germany) was used for centrifugation of the urine samples. After vortexing, mixing, and centrifugation for 15 min at 6500 rpm at room temperature, the supernatant was transferred to a glass vial and incubated with a mixture of 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 [23]. Next, the sample was diluted with 0.5 mL phosphate buffer (pH 2.4) and filtered through a 0.2-μm membrane filter. Afterwards, the sample was transferred to a 5 mL volumetric flask, filled with a mixture of 0.1% formic acid:acetonitrile (95:5, v/v) to the mark and vortexed. From this solution, 5 μL of aliquots were injected into the UHPLC–ESI-MS/MS system for analysis.

2.5. Method validation

A full validation according to FDA guidelines and matrix effect were performed for the assay in human urine [33,34].

To investigate the selectivity of this method, blank urine samples from six volunteers were pre-treated and analysed. The chromatogram from each blank urine sample was compared with that of the corresponding urine sample spiked with drugs, polyphenols and metabolites. For method to be sensitive enough, the response from the different analytes in the spiked urine samples at the LLOQ concentration should be at least five times that of the baseline noise in the blank urine samples.

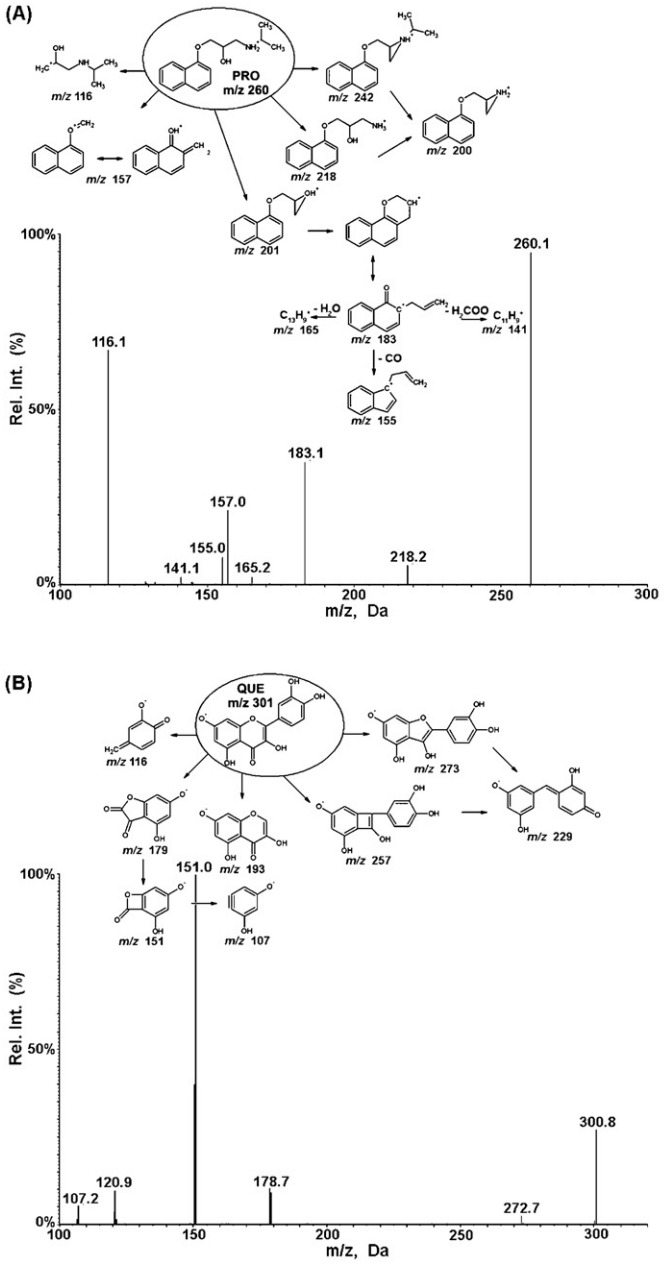

Fig. 1. Proposed structures for ions formed during MS/MS experiments on: (A) propranolol (PRO) [30] and (B) quercetin (QUE) [31].

Linearity was assessed by assaying calibration curves in urine at eight concentration levels (concentration of β-blockers and their metabolites in range: (1) 1000–800 ng mL^{-1}, (2) 700–600 ng mL^{-1}, (3) 500–400 ng mL^{-1}, (4) 250–200 ng mL^{-1}, (5) 100–50 ng mL^{-1}, (6) 10–5 ng mL^{-1}, (7) 1–0.5 ng mL^{-1}, and (8) 0.15–0.08 ng mL^{-1}; concentration of polyphenols and their metabolites in range: (1) 2300–2000 ng mL^{-1}, (2) 1900–1500 ng mL^{-1}, (3) 1400–800 ng mL^{-1}, (4) 500–250 ng mL^{-1}, (5) 100–40 ng mL^{-1}, (6) 20–5.0 ng mL^{-1}, (7) 3–0.8 ng mL^{-1}, and (8) 0.7–0.1 ng mL^{-1}). Calibration curves were built by plotting the corrected areas (analyte area/IS area) for each concentration level versus the nominal concentration of each calibration standard. Considering the large concentration ranges for several analytes, a $1/x$ statistical weight was applied to obtain the most reliable calibration curves. The acceptance criteria for a calibration curve were a correlation coefficient (r^2) of 0.992 or better and each

Table 1
Selected reaction monitoring (SRM) analysis conditions for the determination of drugs and polyphenols and their metabolites.

Analyte	t_r[a] (min)	Q1[b] (m/z)	Q3[c] (m/z)	DP[d] (V)	EP[e] (V)	CE[f] (V)	CXP[g] (V)
Drugs and their metabolites							
MIL	0.61	209.9	165.6	−125	−5	−38	−9
SOT (IS)	1.69	273.1	255.0	56	−3	17	8
α-HMET	2.19	284.1	116.0	71	−5	27	8
O-DMMET	2.28	254.0	116.1	66	−5	27	8
MET	3.36	268.1	116.1	61	−5	27	8
4-HPRO	3.49	275.8	116.0	41	−3	25	8
PRO	5.06	259.9	116.1	66	−3	27	8
5′-HCAR	5.20	423.0	99.9	86	−5	43	6
O-DMCAR	5.86	393.0	209.9	81	−5	31	10
CAR	6.40	407.1	99.9	76	−5	43	6
Polyphenols and their metabolites							
3,4-DHBA	1.51	152.9	108.9	−45	−3	−20	−7
α-HHA	1.68	193.9	72.7	−25	−5	−12	−1
DOPAC	1.82	166.9	122.7	−25	−5	−12	−9
Glu-DA	2.07	613.0	414.7	−60	−5	−26	−9
4-HBA	2.15	136.9	92.8	−45	−3	−18	−5
(±)-CA	2.25	288.9	108.8	−70	−5	−34	−3
HA	2.30	177.9	133.7	−50	−3	−16	−7
VA	2.39	166.8	122.7	−50	−3	−16	−7
CA	2.46	178.9	135.2	−60	−3	−20	−3
PUR	2.47	415.4	294.8	−60	−5	−32	−3
HVA	2.57	180.8	136.8	−35	−5	−10	−9
3-HBA	2.58	136.9	92.6	−55	−3	−18	−5
3-HPA	2.60	150.9	107.7	−25	−5	−10	−5
(−)-EC	2.64	288.9	108.8	−70	−5	−34	−5
(−)-EGCG	2.69	456.9	168.7	−75	−5	−26	−3
GLY	2.93	445.0	281.5	−80	−9	−36	−5
p-COA	3.01	162.9	118.6	−50	−5	−18	−5
RUT	3.21	609.0	299.7	−70	−5	−56	−5
FA	3.26	192.9	134.2	−45	−5	−22	−7
(−)-EGC	3.34	441.0	168.6	−75	−5	−26	−3
8-HDA	3.37	268.9	168.8	−70	−5	−34	−5
DHDA	3.39	254.9	148.8	−70	−5	−28	−1
QUR	3.73	446.9	300.2	−70	−5	−36	−5
BA	3.78	120.9	76.8	−40	−15	−16	−1
HSD	3.84	609.0	300.7	−70	−5	−38	−7
MYR	3.98	316.8	150.7	−60	−5	−36	−7
NHSD	4.02	609.0	300.6	−80	−7	−40	−7
DMGLC	4.05	268.9	239.9	−60	−5	−40	−5
8-HGT	4.13	284.9	240.8	−70	−5	−26	−9
DA	4.64	252.9	132.3	−60	−3	−46	−3
GLC	4.92	282.9	267.7	−70	−5	−26	−5
QUE	5.06	300.9	150.7	−70	−5	−32	−5
GT	5.85	268.9	132.7	−60	−3	−42	−3
(±)-NAR	5.87	270.9	118.7	−60	−10	−28	−3
DHGT	5.88	270.9	164.7	−70	−5	−32	−7
AP	5.91	269.0	116.6	−60	−5	−48	−3
KAM	6.03	284.8	117.1	−60	−3	−38	−3
HST	6.14	300.9	163.7	−70	−10	−34	−3
ISO	6.16	314.9	299.6	−60	−5	−32	−3
2′-HBIO	6.77	298.9	283.6	−70	−5	−30	−9
DHBIO	7.74	284.9	150.9	−70	−5	−36	−9
CHS (IS)	7.80	252.9	142.8	−60	−5	−40	−3
PIN	7.91	254.6	150.7	−65	−3	−30	−3
BIO	7.98	282.9	267.9	−70	−5	−32	−3

[a] Retention time.
[b] Precursor ion.
[c] Fragment ion.
[d] Declustering potential.
[e] Entrance potential.
[f] Collision energy.
[g] Cell exit potential.

back-calculated standard concentration being within 15% deviation from the nominal value except at the LLOQ concentration, for which the maximum acceptable deviation was set at 20%.

The sensitivity was expressed as the LLOQ and defined as the lowest concentration on the calibration curve. The signal to noise ratio corresponding to the LLOQ was required to be higher than 10.

Accuracy and precision were assessed by analysing QC samples using six replicates at the LLOQ and low, middle and high concentration levels (LLOQ: 0.01–0.40 ng mL^{-1} for the drugs and 0.05–40.0 ng mL^{-1} for the polyphenols, LQC: 0.1–0.8 ng mL^{-1} for the drugs and 0.8–80.0 ng mL^{-1} for the polyphenols, MQC: 10.0–60.0 ng mL^{-1} for the drugs and 60.0–300.0 ng mL^{-1} for the polyphenols, HQC: 250.0–500.0 ng mL^{-1} for the drugs and 500.0–1000.0 ng mL^{-1} for the polyphenols). Precision is expressed by the relative standard deviation (RSD) between the replicate measurements. Accuracy is defined as the relative error (RE), which is calculated using the formula RE(%) = [(measured value − theoretical value)/theoretical value] × 100. Intra-day precision and accuracy were assessed by processing the aforementioned samples (n = 6) and analysing them on the same day. Inter-day precision and accuracy were assessed by processing the aforementioned samples (n = 6) on five different days.

Extraction recoveries of the analytes at the LLOQ and the three QC levels (low, medium, and high – the same concentration levels which were used for determining the accuracy and precision) were evaluated by determining the peak area ratios of the analytes in the post-extraction spiked samples to those acquired from the pre-extraction spiked samples. Matrix effects were measured by comparing the peak areas of the analytes dissolved in the pretreated blank urine with those from the pure standard solution containing equivalent amounts of the analytes. The LLOQ sample and the three QC samples of different compounds were evaluated by analysing six samples at each level for both the extraction recovery and the matrix effect.

The stability of the analytes in human urine was assessed by analysing QC samples at four concentration levels under four different conditions. The short-term stability was determined by analysing untreated QC samples stored for 12 h at room temperature. The long-term stability was assessed after the untreated QC samples were stored at −20 °C for 30 days. The freeze–thaw stability was determined after three freeze–thaw cycles (−20 °C to room temperature as one cycle). The post-preparative stability was measured by analysing QC samples kept under the autosampler conditions (5 °C) for 24 h. All the stability studies were done with six replicates.

3. Results and discussion

3.1. Optimisation of UHPLC–MS/MS condition

UHPLC with MS/MS detection was selected as the method of choice for assaying drugs and polyphenols and their metabolites simultaneously in human urine. Main challenge was developing a simple and reproducible method that gave good peak shapes with low baseline noise and a high recovery. To meet this challenge, the mass spectrometry parameters and chromatographic conditions had to be adjusted.

The mass spectrometric behaviour of all analytes was studied using both positive- and negative-ion ESI. Optimisation of the mass spectrometric conditions was carried out by injecting the standard solutions and manually increasing the sample collision energy to achieve maximised sensitivity for the molecular ions $[M-H]^-$ of polyphenols and $[M-H]^+$ of drugs. On average, positive-ion ESI showed 2–3 times better sensitivity than negative-ion ESI for of β-blockers and their metabolites; thus, the positive-ion mode was used for the detection of these drugs. In this experiment, was discovered that the negative-ion mode was more sensitive for the detection and analysis of polyphenols and their metabolites. The positive-ion mode was also tested, but the sensitivity obtained was not satisfactory for all polyphenols and their metabolites. Polyphenols do not contain nitrogen atoms, and for this reason, the formation of protonated molecules $[M+H]^+$ is lower in positive-ion ESI than in negative-ion ESI.

Crucial parameters such as temperature, voltage, ionisation mode, nebulizer gas, heater gas, declustering potential, entrance potential, collision energy (CE) and collision cell exit potential (CXP) were optimised by flow injection analysis (FIA) to obtain better ionisation. The selected reaction monitoring mode (SRM) was used to enhance the sensitivity and selectivity of detection and to monitor each compound. Table 1 shows the optimum MS/MS conditions for all analytes.

As an example, the fragmentation pattern of PRO is shown in Fig. 1A. Under conditions two of the most abundant product ions observed were m/z 116 and m/z 183, with the former corresponding to the side chain with the loss of 1-naphthol and the latter resulting from neutral losses of water and C_3H_9N, the chemical formula of isopropylamine. Further fragmentation of PRO leads to the formation of ions with m/z 165, m/z 155 and m/z 141, corresponding to the loss of water, carbon monoxide and ketene, respectively. Another product ion from the fragmentation of the unlabeled PRO ion had a m/z of 157, indicating that the C3 of the side chain and the ether oxygen must be retained in this fragment [35].

The MS/MS product ions obtained from the $[M-H]^-$ ion of QUE are presented in Fig. 1B. Losses of carbon monoxide

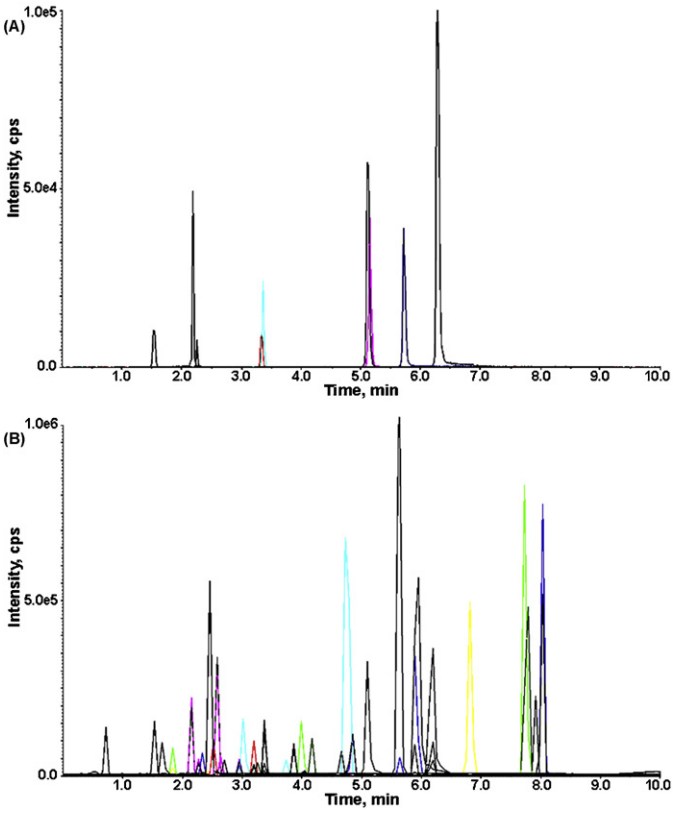

Fig. 2. UHPLC–MS/MS chromatograms obtained from a mixed standards solution containing: (A) drugs and their metabolites and (B) polyphenols and their metabolites, based on quantifying MS–MS transitions. Positive (A) and negative (B) ion electrospray ionisation tandem mass spectrometry was applied for detection of selected analytes in mixture 52 compounds (retention times of all analytes were placed in Table 1).

$[M-H-CO]^-$ (m/z 273) and carbon dioxide $[M-H-CO_2]^-$ (m/z 257) were observed. Further losses of CO_2 and CO from $[M-H-CO]^-$ and $[M-H-CO_2]^-$, respectively, gave rise to the resonance-stabilised ion $[M-H-CO_2-CO]^-$ (m/z 229). For QUE, the proposed fragmentation scheme shows that the retrocyclisation pathway affects bonds 1 and 2, leading to $^{1,2}A^-$ and $^{1,2}B^-$ fragments at 179 and 121 m/z. This $^{1,2}A^-$ diagnostic ion undergoes further loss of CO, giving rise to a $^{1,2}A^-$ –CO ion at an m/z of 151. Another further loss of CO_2 leads to a $^{1,2}A^-$ –CO–CO_2 ion at an m/z of 107 [36].

Different dwell times (from 50 to 250 ms) were used to find the best detection parameters for obtaining a sufficient number of data points across the peak. Negligible differences were found

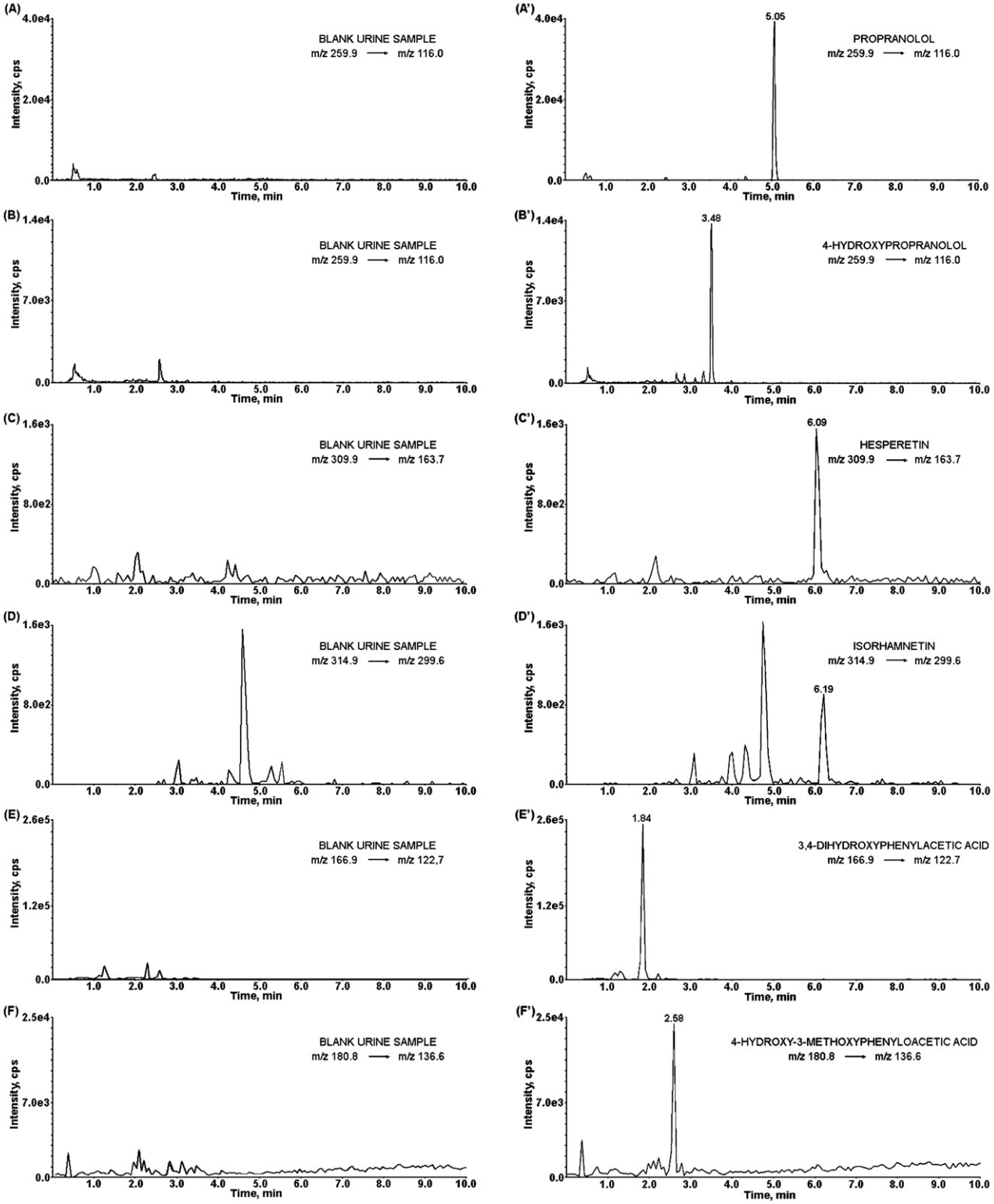

Fig. 3. Typical SRM chromatograms of: (A–F) blank urine sample and (A′–F′) analytes in human urine samples collected after the oral administration of propranolol and of antioxidants with diet and supplements.

at low dwell times. An optimum dwell time of 75 ms was selected, resulting in at least 10 points per peak, and using this dwell time gave reproducible results for determination and confirmation. Fig. 2 shows a representative chromatogram obtained from the mixture of 52 standards, using the conditions described in Section 2. The positive ion mode was used for the detection of selected drugs and their metabolites (Fig. 2A), while negative ion mode for detection of antioxidants and their metabolites (Fig. 2B) in the mixture. Complete resolution between the β-blockers, and polyphenols and their metabolites was not achieved in the chromatogram, but this can be resolved using MS/MS detection due to its high specificity.

To separate the selected compounds and allow for an optimum response during MS/MS detection, a gradient elution was used, using acetonitrile and an aqueous solution of formic acid (0.1%) as mobile phase components. Concentrations of formic acid additive from 0.05% to 0.30% (v/v) were evaluated. When formic acid is less than 0.1% (v/v), the formation of $[M+formate]^-$ adducts is difficult. However, higher formic acid concentrations can cause ion-masking and suppress the ionisation efficiency. Mobile phases consisting

Table 2
Calibration curves and the LLOQs, precision (intra-day and inter-day), accuracy (intra-day and inter-day), recovery (Rec.), and matrix effects (M.E.) for the determination of drugs and antioxidants and their metabolites in urine (values obtained from three different concentrations and shown as the average ± SD[a]; $n = 6$).

Analyte	Linear range (ng mL^{-1})	LLOQ (ng mL^{-1})	Intra-day		Inter-day		Rec. (%)	M.E. (%)
			RSD[b] (%)	RE[c] (%)	RSD[b] (%)	RE[c] (%)		
Drugs and their metabolites								
MIL	0.50–1000	0.40	4.06 ± 1.86	−2.65 ± 1.13	4.43 ± 0.76	−1.71 ± 1.39	92.69 ± 1.02	11.70 ± 4.76
α-HMET	0.08–500	0.01	2.82 ± 1.74	−0.67 ± 0.41	4.08 ± 3.32	−2.20 ± 4.91	95.13 ± 3.42	4.56 ± 2.02
O-DMMET	0.10–500	0.03	3.35 ± 0.48	0.83 ± 3.12	3.48 ± 0.59	−1.49 ± 2.93	94.10 ± 3.30	−0.80 ± 1.27
MET	0.08–400	0.01	2.46 ± 0.71	−0.23 ± 1.66	3.02+0.58	1.99 ± 5.56	97.50 ± 3.51	11.47 ± 0.05
4-HPRO	0.15–650	0.04	2.89 ± 0.42	−1.83 ± 2.76	3.48 ± 2.56	1.04 ± 2.47	96.68 ± 1.74	13.54 ± 2.34
PRO	0.08–450	0.02	3.20 ± 0.19	1.52 ± 1.74	3.45 ± 0.80	1.53 ± 1.58	96.90 ± 0.80	−7.47 ± 0.62
5′-HCAR	0.15–650	0.03	2.76 ± 1.08	1.61 ± 1.54	3.96 ± 2.45	1.73 ± 1.77	96.13 ± 1.76	11.86 ± 0.45
O-DMCAR	0.12–600	0.02	2.04 ± 1.29	1.35 ± 1.30	2.75 ± 0.88	1.29 ± 1.24	102.72 ± 4.48	9.02 ± 0.13
CAR	0.10–500	0.02	4.92 ± 0.73	0.55 ± 2.25	5.14 ± 1.83	2.23 ± 2.31	102.18 ± 2.78	0.57 ± 1.30
Polyphenols and their metabolites								
3,4-DHBA	0.80–1100	0.60	2.18 ± 1.11	−1.05 ± 1.28	4.22 ± 2.55	0.59 ± 3.21	91.76 ± 1.11	3.67 ± 1.26
α-HHA	8.0–1900	5.00	4.59 ± 2.21	−2.19 ± 1.54	4.79 ± 0.76	−4.21 ± 1.69	94.65 ± 1.31	−3.10 ± 2.56
DOPAC	20.0–2000	10.00	3.46 ± 2.57	−0.91 ± 0.79	5.00 ± 0.13	−1.06 ± 1.20	91.98 ± 2.01	0.29 ± 2.42
Glu-DA	0.90–1500	0.60	3.09 ± 1.54	−0.08 ± 1.76	4.08 ± 1.90	−0.89 ± 0.37	93.06 ± 1.86	6.03 ± 2.05
4-HBA	8.00–1000	5.00	3.83 ± 1.89	−2.70 ± 2.07	4.50 ± 0.61	−1.81 ± 0.68	91.77 ± 0.62	5.21 ± 0.23
(±)-CA	0.40–1100	0.30	2.44 ± 1.12	−1.45 ± 1.92	5.16 ± 0.31	−4.07 ± 1.51	92.25 ± 2.17	14.70 ± 2.58
HA	1.00–1200	0.80	4.84 ± 1.96	−1.94 ± 1.87	4.76 ± 0.94	−2.71 ± 1.35	92.61 ± 1.81	1.13 ± 0.89
VA	60.0–2100	40.00	3.52 ± 2.03	−2.60 ± 1.58	6.33 ± 0.88	−2.12 ± 0.77	91.54 ± 0.11	0.11 ± 1.78
CA	1.20–1000	1.00	3.55 ± 2.13	−1.46 ± 1.81	4.62 ± 2.34	−2.28 ± 2.32	91.80 ± 0.81	2.07 ± 0.17
PUR	0.16–1200	0.12	3.24 ± 1.84	−1.99 ± 1.83	7.86 ± 5.22	−1.88 ± 3.85	93.82 ± 0.62	5.80 ± 1.38
HVA	20.0–2300	15.00	3.00 ± 1.09	−1.66 ± 0.60	4.02 ± 0.24	−1.52 ± 1.09	90.29 ± 0.17	0.17 ± 3.35
3-HBA	1.00–1000	0.80	3.53 ± 2.21	−2.22 ± 0.38	4.31 ± 1.84	−1.65 ± 2.23	92.16 ± 2.05	2.02 ± 0.18
3-HPA	60.0–2200	40.00	2.37 ± 1.01	−1.35 ± 0.99	5.54 ± 0.33	1.57 ± 4.86	91.34 ± 0.81	0.32 ± 4.76
(−)-EC	0.16–1000	0.10	1.89 ± 0.61	0.14 ± 1.96	4.13 ± 0.96	−0.10 ± 1.77	91.80 ± 2.74	5.84 ± 1.78
(−)-EGCG	0.50–1300	0.40	2.13 ± 0.81	−0.76 ± 1.94	4.27 ± 1.42	−0.37 ± 2.00	92.42 ± 0.65	−3.44 ± 4.08
GLY	0.50–1200	0.40	4.10 ± 2.70	−2.07 ± 2.77	5.10 ± 2.43	−1.28 ± 1.21	90.87 ± 0.38	14.98 ± 1.05
p-COA	8.00–1000	5.00	4.43 ± 2.65	−2.80 ± 2.46	4.75 ± 2.06	−2.79 ± 2.77	92.72 ± 2.09	3.10 ± 1.58
RUT	0.16–1000	0.10	1.93 ± 1.36	−0.23 ± 2.67	4.82 ± 2.53	−2.19 ± 0.52	92.64 ± 1.73	3.11 ± 1.17
FA	0.40–1300	0.20	4.01 ± 1.84	−1.13 ± 1.40	5.29 ± 1.56	−1.37 ± 1.97	92.91 ± 0.66	3.43 ± 0.87
(−)-EGC	0.50–1400	0.40	3.30 ± 2.11	−2.66 ± 2.86	4.54 ± 2.05	−1.60 ± 3.11	91.53 ± 1.45	−7.14 ± 0.76
8-HDA	0.30–1000	0.20	3.32 ± 1.17	−1.06 ± 0.48	4.18 ± 1.60	−1.08 ± 3.02	92.18 ± 1.01	6.35 ± 0.53
DHDA	0.10–900	0.08	4.79 ± 2.28	−2.76 ± 2.17	5.99 ± 2.29	−4.40 ± 0.92	92.91 ± 0.94	1.92 ± 0.58
QUR	0.16–1000	0.12	2.47 ± 1.82	0.12 ± 0.97	4.49 ± 1.12	−0.93 ± 0.59	92.89 ± 0.59	7.02 ± 1.23
BA	1.00–1700	0.80	3.70 ± 2.59	−2.62 ± 2.68	4.83 ± 2.35	−1.59 ± 1.33	90.26 ± 0.18	2.75 ± 1.56
HSD	0.12–1000	0.08	3.37 ± 1.34	0.20 ± 1.61	5.60 ± 1.34	−0.17 ± 1.74	93.23 ± 1.32	3.23 ± 0.56
MYR	0.40–1000	0.30	2.29 ± 0.98	−3.23 ± 2.30	4.32 ± 1.52	−3.12 ± 1.58	91.94 ± 0.48	−8.97 ± 0.48
NHSD	0.12–1000	0.08	2.56 ± 0.90	−1.13 ± 1.22	5.48 ± 1.70	−0.38 ± 3.23	94.27 ± 1.83	2.43 ± 0.97
DMGLC	0.70–1500	0.50	4.61 ± 1.87	−2.31 ± 1.62	5.45 ± 0.90	−2.49 ± 2.30	90.93 ± 1.35	6.68 ± 1.17
8-HGT	0.50–1100	0.30	3.46 ± 2.46	0.25 ± 1.06	4.71 ± 1.56	−0.26 ± 2.35	91.07 ± 1.05	13.18 ± 1.08
DA	0.20–1100	0.15	3.41 ± 1.65	−1.62 ± 3.32	3.94 ± 1.73	−2.38 ± 4.09	91.46 ± 1.50	2.86 ± 1.60
GLC	0.80–1200	0.60	4.27 ± 1.76	−1.72 ± 0.94	4.69 ± 1.42	−2.70 ± 2.22	92.12 ± 2.44	10.61 ± 2.41
QUE	0.40–1000	0.25	1.98 ± 0.90	−0.07 ± 2.73	3.44 ± 0.34	−0.25 ± 3.06	94.73 ± 0.92	9.54 ± 1.06
GT	0.10–1000	0.08	3.30 ± 2.84	−3.33 ± 2.23	5.17 ± 2.39	−1.90 ± 1.68	91.09 ± 0.67	9.36 ± 0.14
(±)-NAR	0.10–950	0.05	3.13 ± 1.63	−2.41 ± 1.61	5.76 ± 1.47	−1.42 ± 2.39	93.60 ± 1.98	2.82 ± 1.15
DHGT	0.16–850	0.12	3.05 ± 2.15	−0.30 ± 2.89	3.89 ± 2.16	0.34 ± 2.01	92.36 ± 0.67	1.21 ± 0.33
AP	0.12–1000	0.08	3.79 ± 2.38	−1.11 ± 1.88	4.01 ± 2.12	−0.94 ± 1.93	92.67 ± 1.14	6.68 ± 0.30
KAM	0.16–1000	0.10	2.86 ± 1.27	−1.79 ± 2.24	4.41 ± 2.76	−1.42 ± 1.34	92.58 ± 0.74	4.31 ± 0.14
HST	0.16–1000	0.12	2.83 ± 1.79	−2.32 ± 3.36	3.76 ± 1.62	−1.84 ± 2.67	96.05 ± 1.13	11.20 ± 0.17
ISO	0.30–1100	0.20	3.41 ± 1.41	−2.29 ± 2.80	4.49 ± 1.46	−3.33 ± 1.93	93.12 ± 1.50	16.53 ± 0.72
2′-HBIO	0.12–1000	0.08	4.85 ± 2.93	−2.42 ± 1.83	5.37 ± 2.53	−1.04 ± 0.64	91.52 ± 0.95	5.54 ± 0.09
DHBIO	0.10–800	0.07	4.40 ± 2.23	0.66 ± 1.39	5.00 ± 2.34	0.60 ± 2.09	91.50 ± 1.36	2.08 ± 0.24
PIN	0.12–1000	0.08	3.12 ± 1.74	−2.07 ± 1.37	5.18 ± 3.15	−0.15 ± 1.38	93.24 ± 0.76	4.41 ± 2.27
BIO	0.10–800	0.05	3.84 ± 1.23	−2.76 ± 1.15	4.07 ± 1.31	−2.98 ± 0.90	91.38 ± 0.43	7.84 ± 6.55

[a] Standard deviation.
[b] Relative standard deviation.
[c] Relative error.

of methanol, acetonitrile and different proportions of methanol and acetonitrile were tested. A significantly higher response and sharper peak shapes were obtained with an acetonitrile/water mobile phase than with a methanol/water mobile phase. Therefore, a mixture of acetonitrile and water containing 0.1% (v/v) formic acid was selected as the mobile phase. Other parameters such as flow rate, column temperature, and injection volume were optimised to achieve fast and reliable separation. A flow rate of 0.65–0.75 mL min^{-1} was used, with 25 °C as the column temperature and 5 μL as the injection volume. Under these conditions, retention times of the compounds in urine matrices were constant, ranging from 0.61 min for milrinone to 7.98 min for biochanin A, with a relative standard deviation lower than 0.25%.

3.2. Method validation

Selectivity was studied by analysing six blank urine samples from healthy volunteers. Method selectivity was determined by comparing the SRM chromatograms of blank samples with those from the spiked samples. The chromatograms did not show interfering signals within the retention time of the chromatographic peaks of the analytes and the internal standards, which could be misinterpreted as the target compounds or could affect the bias of the method (Fig. 3A). The results indicated that the method exhibited good specificity and selectivity.

The calibration curve was linear over the concentration range of 0.08–1000 ng mL^{-1} for β-blockers and their metabolites and

Table 3
Stability of drugs and antioxidants and their metabolites (values obtained from three different concentrations and shown as the average ± SD[a], $n = 6$).

Analyte	RE[b] (%)			
	Autosampler	Short-term	Long-term	Freeze–thaw cycles
Drugs and their metabolites				
MIL	−0.65 ± 4.19	−3.52 ± 3.08	−5.04 ± 1.79	−4.78 ± 2.13
α-HMET	−1.20 ± 0.92	−2.43 ± 0.51	−1.68 ± 3.60	−0.86 ± 2.59
O-DMMET	−1.03 ± 1.50	−1.35 ± 1.59	−3.85 ± 3.30	−2.80 ± 0.48
MET	−0.42 ± 3.05	−1.68 ± 1.99	−4.70 ± 2.32	−1.86 ± 0.89
4-HPRO	0.21 ± 0.70	−0.42 ± 0.94	−4.21 ± 2.83	−1.71 ± 1.91
PRO	−0.24 ± 1.74	−1.79 ± 0.34	−4.99 ± 1.12	−4.12 ± 1.49
5′-HCAR	0.89 ± 1.23	−1.04 ± 3.08	5.10 ± 2.50	−1.17 ± 1.56
O-DMCAR	−1.13 ± 1.93	1.22 ± 1.82	−2.65 ± 2.09	1.29 ± 1.67
CAR	1.58 ± 2.50	−0.88 ± 3.20	−4.64 ± 3.42	1.89 ± 6.37
Polyphenols and their metabolites				
3,4-DHBA	−1.62 ± 3.36	−2.78 ± 1.20	−6.99 ± 1.58	−5.82 ± 0.80
α-HHA	−3.19 ± 1.43	−3.50 ± 1.92	−4.21 ± 1.09	−4.50 ± 1.34
DOPAC	−1.17 ± 2.11	−1.90 ± 3.74	−2.85 ± 0.34	−3.84 ± 0.78
Glu-DA	−1.87 ± 3.67	−4.16 ± 3.33	−6.12 ± 1.28	−6.49 ± 1.47
4-HBA	−1.06 ± 1.96	−2.78 ± 1.49	−6.44 ± 1.45	−6.76 ± 1.39
(±)-CA	−3.72 ± 1.24	−2.50 ± 2.74	−7.93 ± 3.05	−7.39 ± 1.42
HA	−1.11 ± 2.14	−1.92 ± 2.94	−7.40 ± 0.36	−3.98 ± 0.87
VA	−1.63 ± 1.72	−2.33 ± 0.11	−5.24 ± 1.83	−6.53 ± 2.07
CA	−1.30 ± 4.32	−1.52 ± 0.57	−6.03 ± 1.87	−3.99 ± 1.88
PUR	−3.36 ± 1.89	−3.49 ± 2.85	−3.81 ± 2.05	−6.40 ± 0.23
HVA	−2.25 ± 3.18	−3.13 ± 0.30	−6.27 ± 0.61	−5.68 ± 1.99
3-HBA	−1.19 ± 3.32	−3.17 ± 2.18	−3.38 ± 0.79	−4.90 ± 1.18
3-HPA	0.17 ± 3.06	−4.92 ± 1.20	−5.86 ± 0.61	−4.56 ± 1.74
(−)-EC	−2.79 ± 1.66	−0.95 ± 3.78	−5.66 ± 1.21	−6.37 ± 1.77
(−)-EGCG	1.18 ± 1.98	1.93 ± 3.07	−5.65 ± 3.38	−7.17 ± 1.43
GLY	−2.25 ± 1.20	−2.61 ± 1.98	−5.06 ± 0.53	−5.13 ± 0.45
p-COA	−0.13 ± 2.56	−0.32 ± 2.07	−6.42 ± 0.95	−6.06 ± 1.96
RUT	−2.88 ± 2.57	−2.62 ± 2.55	−4.27 ± 0.95	−7.83 ± 0.66
FA	−1.73 ± 1.20	−2.10 ± 0.53	−5.86 ± 2.54	−4.17 ± 1.68
(−)-EGC	0.93 ± 4.58	−4.30 ± 2.56	−4.39 ± 0.82	−5.83 ± 1.87
8-HDA	−1.76 ± 0.43	−2.07 ± 0.34	−2.82 ± 2.04	−5.48 ± 1.61
DHDA	−1.27 ± 3.19	−3.83 ± 1.99	−6.65 ± 2.87	−4.66 ± 2.53
QUR	0.52 ± 3.03	1.65 ± 4.67	−3.45 ± 0.94	−6.86 ± 1.56
BA	−2.30 ± 4.81	−3.64 ± 2.02	−6.71 ± 1.36	−4.50 ± 1.64
HSD	−3.10 ± 2.94	−2.63 ± 3.27	−7.80 ± 1.81	−5.76 ± 1.56
MYR	−2.12 ± 1.30	−2.94 ± 0.45	−6.64 ± 2.01	−6.04 ± 1.08
NHSD	1.01 ± 2.75	−1.07 ± 2.20	−5.31 ± 0.73	−7.30 ± 0.72
DMGLC	−1.67 ± 5.18	−2.39 ± 3.46	−4.21 ± 1.71	−5.38 ± 2.18
8-HGT	0.69 ± 2.65	−2.76 ± 2.59	−4.65 ± 2.17	−5.06 ± 1.87
DA	−0.68 ± 3.22	−0.77 ± 3.76	−4.44 ± 2.39	−7.22 ± 1.40
GLC	−1.94 ± 3.71	−3.23 ± 2.51	−4.19 ± 2.53	−4.68 ± 2.21
QUE	−2.56 ± 3.11	−1.27 ± 2.80	−2.60 ± 2.00	−5.81 ± 0.61
GT	0.86 ± 2.63	−1.98 ± 1.11	−4.64 ± 1.78	−6.39 ± 0.59
(±)-NAR	0.60 ± 4.61	1.51 ± 4.45	−5.80 ± 3.60	−7.53 ± 1.95
DHGT	0.56 ± 4.58	−5.06 ± 2.28	−6.88 ± 1.49	−5.03 ± 2.27
AP	0.72 ± 2.24	2.59 ± 4.13	−3.26 ± 1.85	−6.20 ± 1.96
KAM	−0.87 ± 2.43	0.96 ± 1.86	−7.19 ± 1.58	−6.47 ± 2.46
HST	−1.38 ± 3.19	−1.51 ± 3.59	−3.95 ± 1.13	−6.59 ± 2.40
ISO	−3.13 ± 1.63	−3.38 ± 2.79	−3.71 ± 1.16	−6.31 ± 1.05
2′-HBIO	−2.26 ± 1.68	−4.76 ± 1.76	−8.48 ± 0.95	−7.56 ± 1.67
DHBIO	−1.18 ± 2.35	−2.78 ± 3.44	−4.39 ± 0.34	−3.38 ± 1.91
PIN	−0.90 ± 2.75	−2.05 ± 1.37	−5.91 ± 2.37	−6.14 ± 1.13
BIO	−0.91 ± 2.89	−3.62 ± 2.62	−4.86 ± 2.61	−5.10 ± 1.86

[a] Standard deviation.
[b] Relative error.

0.10–2300 ng mL^{-1} for polyphenols and their metabolites. The correlation coefficients (r^2) of the calibration curves were greater than 0.993. The LLOQ, defined as the lowest concentration analysed, had an accuracy of 15% and precision of 15%. The results for the LLOQ sample and the linearity of calibration curves for drugs and polyphenols and their metabolites in human urine are summarised in Table 2.

The intra-day precision ranged from 1.89 to 4.92%, and the mean accuracy values ranged from −3.33 to 1.61% at the four concentration levels of the analysed compounds. The inter-day precision and accuracy for the QC samples ranged between 2.75 and 7.86% and −4.40 and 2.23%, respectively. The results obtained for the intra-day and inter-day accuracy (RE) and precision (RSD) are summarised in Table 2 as the average values with the corresponding standard deviations obtained for the four different concentration levels. The data indicated that developed method has satisfactory accuracy, precision and reproducibility.

The extraction recovery was determined in six replicates by comparing the peak areas of the extracted urine at the LLOQ and the low, medium and high concentrations with those obtained from a direct injection of the standard solutions at the same concentrations without extraction. The extraction recoveries of drugs and their metabolites varied from 92.69 to 102.72%, and that of the polyphenols and their metabolites ranged from 90.26 to 96.05%. These data are summarised in Table 2. The extraction recovery of β-blockers and polyphenols and their metabolites in human urine was consistent, precise and reproducible.

At the LLOQ and low, medium and high concentration levels, the matrix effects on the analytes ranged from −8.97 to 16.53%. The matrix effects from urine on each analyte are listed in Table 2. These results indicated that there were minor to moderate matrix effects for the analysis of compounds in urine.

The stability of drugs and polyphenols and their metabolites in human urine at different temperatures for different durations was evaluated. QC samples were subjected to short-term room temperature conditions, long-term storage conditions (−20 °C), and three freeze–thaw cycles. All the stability studies were conducted at four different concentration levels with six replicates each. The stability results are summarised in Table 3. The data showed that there was no significant degradation of the analysed compounds in urine at room temperature for 12 h, during the three freeze–thaw cycles and during storage in the autosampler for 24 h at 5 °C. However, β-blockers and polyphenols and their metabolites were only stable in urine for approximately 30 days at −20 °C. In all cases, the concentrations of stored samples deviated from freshly prepared samples by less than 8.5%.

3.3. Application to real samples

Following the optimisation and validation of the UHPLC–MS/MS method, it was successfully used to analyse of cardiovascular drugs and polyphenols and their metabolites in human urine samples collected from 32 patients treated with metoprolol or propranolol and after the ingestion of a food containing antioxidants. The samples were analysed six times.

The metabolism of β-blockers and polyphenols 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, enzymatic hydrolysis with β-glucuronidase/sulfatase (from *H. pomatia*) in an acetate buffer (pH 4.66) was chosen.

Concentrations of the analysed compounds were calculated using the relative calibration curves. The mean concentrations of propranolol and metoprolol in human urine were 25.72 ± 0.04 ng mL^{-1} and 55.38 ± 0.04 ng mL^{-1}, respectively. The urinary excretions for the metabolites of propranolol were 26.56 ± 9.88 ng mL^{-1} for 4-HPRO and for the metabolites of metoprolol were 16.46 ± 7.76 ng mL^{-1} for α-HMET and 23.85 ± 1.99 ng mL^{-1} for *O*-DMMET. The urine samples were also tested for antioxidants, which are components of supplements and soy products, fruits, vegetables, tea. The mean concentrations of polyphenols were summarised in Table 4. Representative SRM chromatograms of an extract of a urine sample obtained after the oral dosing of propranolol (10 mg) to a human are shown in Fig. 3.

Table 4
Concentration values for urine samples obtained from patients who were treated with cardiovascular drugs and who consumed a food containing antioxidants (shown as the average ± SD[a], $n = 6$).

Concentration ± SD[a] (ng mL^{-1})			
Drugs		Their metabolites	
PRO	25.72 ± 0.041	4-HPRO	26.6 ± 9.9
MET	55.38 ± 0.038	α-HMET	16.5 ± 7.8
		O-DMMET	23.8 ± 2.0
Polyphenols		Their metabolites	
PUR	1.31 ± 0.089	8-HDA	54.0 ± 1.2
GT	0.18 ± 0.011	8-HGT	3.32 ± 0.15
DA	0.21 ± 0.012	Glu-DA	2.50 ± 0.17
(±)-CA	18.17 ± 0.40	3-HBA	35.0 ± 1.1
(−)-EC	21.85 ± 0.14	CA	23.1 ± 1.2
HST	1.22 ± 0.041	3,4-DHBA	34.3 ± 2.0
QUE	12.76 ± 0.12	*p*-COA	4.92 ± 0.16
ISO	7.27 ± 0.012	FA	27.32 ± 0.20
		α-HHA	7.90 ± 0.35
		BA	930 ± 29
		HA	$447.8 \times 10^2 \pm 4.4 \times 10^2$
		4-HBA	278 ± 12
		3-HPA	$67.9 \times 10^2 \pm 1.9 \times 10^2$
		VA	1183 ± 92
		DOPAC	198 ± 11
		HVA	479 ± 30

[a] Standard deviation.

These results demonstrate the importance of the developed LC–MS/MS method for the quantification of β-blockers and flavonoids and their metabolites in urine and can be used in large population-based studies. The developed UHPLC–ESI-MS/MS method can be used to monitor bioavailability of any of the studied drugs in the presence of antioxidants, which are components of a diet and supplements. This method might provide a convenient index of metabolism of compounds in urine and could be used to explore the effect of dietary antioxidants on pathways involved in drug metabolism.

4. Conclusions

In the present study, a UHPLC–ESI-MS/MS method has been established, optimised, and validated for the reliable determination of β-blockers and polyphenols and their metabolites in human urine. After optimisation of the MS/MS and chromatographic conditions, the 52 compounds were separated and determined in less than 10 min, and the developed method gave narrow peaks with good peak symmetry. This method was successfully applied to the detection and quantitation of the studied analytes in urine samples obtained from patients treated with of cardiovascular drugs who have a diet rich in polyphenols and took orally administrated polyphenol tablets.

Of course there is no need for the simultaneous determination of all studied cardiovascular drugs because they are not simultaneously administered to the patient. However, thanks to this method in the laboratory there will be no need to use different methods, because application of this method makes possible

of separation and determination of several cardiovascular drugs, polyphenols and metabolites in the mixture in the same chromatography system. The developed UHPLC–ESI-MS/MS method appears to be the first direct method for the analysis of the studied β-blockes and antioxidants and their metabolites. This method can be applied in clinical and toxicological studies. Furthermore, this new UHPLC–MS/MS method may be extended to determine the pharmacokinetics of drugs and polyphenols and also to examine the interaction of β-blockes and polyphenols in combination therapy. Further study of these subjects is essential and will be performed in laboratory in the future.

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