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UPLC–MS/MS quantification of total hesperetin and hesperetin enantiomers in biological matrices

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ABSTRACT

Hesperidin (hesperetin-7-*O*-rutinoside), a flavonoid affecting vascular function, is abundant in citrus fruits and derived products such as juices. After oral administration, hesperidin is hydrolyzed by the colonic microbiota producing hesperetin-7-*O*-glucoside, the glucoside group is further cleaved and the resulting hesperetin is absorbed and metabolized. Flavanones have a chiral carbon generating (R)- and (S)-enantiomers, with potentially different biological activities. A rapid UPLC–MS/MS method for the analysis of (R)- and (S)-hesperetin enantiomers in human plasma and urine was developed and validated. Biological matrices were incubated with β-glucuronidase/sulfatase, and hesperetin was isolated by solid-phase extraction using 96-well plate mixed-mode cartridges having reversed-phase and anion-exchange functionalities. Racemic hesperetin was analyzed with a UPLC HSS T3 reversed phase column and hesperetin enantiomers with a HPLC Chiralpak IA-3 column using H_2O with 0.1% CHOOH as solvent A and acetonitrile with 0.1% CHOOH as solvent B. The method was linear between 50 and 5000 nM for racemic hesperetin in plasma and between 25 and 2500 nM for (S)- and (R)-hesperetin in plasma. Linearity was achieved between 100 and 10,000 nM for racemic hesperetin in urine and between 50 and 5000 nM for (S)- and (R)-hesperetin in urine. Values of repeatability and intermediate reproducibility for racemic hesperetin and enantiomers in plasma and urine were below 15% of deviation in general, and maximum 20% for the lowest concentrations. In addition, the method was applied for the quantification of total hesperetin and of hesperetin enantiomers in human plasma and urine samples, obtained after oral ingestion of purified hesperetin-7-*O*-glucoside. In conclusion, the developed and validated method was sensitive, accurate and precise for the quantification of enantiomers of hesperetin in biological fluids.

1. Introduction

Hesperidin (hesperetin-7-*O*-rutinoside) is a flavonoid abundant in citrus fruits and derived products such as juices [1]. Hesperidin belongs to the class of flavanones, it has a chiral carbon generating (R)- and (S)-enantiomers (Fig. 1) and it is naturally predominant in citrus fruits ((R)-/(S)-ratio of 8/92 in fresh orange juice) [2].

When hesperidin is consumed by humans, the rhamnose part of the rutinoside group is hydrolyzed by the colonic microbiota producing hesperetin-7-*O*-glucoside and hesperetin [3]. Hesperitin can then be absorbed by the colonocytes [4]. In addition, hesperetin-7-*O*-glucoside can be hydrolysed in the small intestine by lactase phlorizin hydrolase [3] to produce hesperitin. Once absorbed, hesperetin is extensively metabolized by UDP-glucuronosyltransferases and sulfotransferases producing glucuronidated and/or sulfated compounds [5].

Hesperidin exhibits potential health benefits. In animal models, the consumption of hesperidin could reduce the risk of osteoporosis [6,7] and could be causally linked to the vascular protective effects of orange juice in human [8]. Interestingly, it was recently demonstrated that hesperetin enantiomers have different transport and bioactivity [9]. Therefore, in order to further investigate the mechanisms of action of the enantiomers of hesperetin, it is necessary to understand which compounds are present at the site of action. Consequently, there is an increased demand for sensitive analytical methods to quantify hesperetin and evaluate the chirality of metabolites present in biological fluids.

The previously published methods for assessing hesperetin and hesperetin enantiomers in urine [10,11] did not allow extracting hesperetin enantiomers from plasma at concentration of about 25 nM with a precision (CV(*r*) and CV(iR)) lower than 15%.

The aim of this study was to develop a rapid method for assessing hesperetin and hesperetin enantiomers in human urine and

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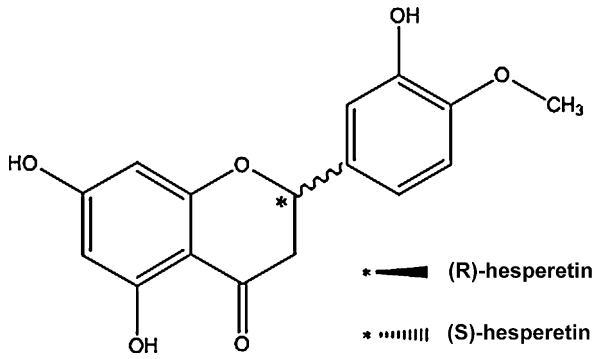

Fig. 1. Structure of hesperetin (3′,5,7-trihydroxy-4′methoxyflavanone) and its two chiral forms.

plasma samples within a clinical trial. Biological matrices were incubated with β-glucuronidase/sulfatase and hesperetin was isolated by solid-phase extraction and quantified by UPLC–MS/MS. The validated method was very sensitive and feasible to be used with a large number of samples. Finally, the validated method was used to quantify hesperetin and its enantiomers in a pilot human clinical trial.

2. Experimental

2.1. Chemicals and reagents

Food grade hesperetin-7-*O*-glucoside was produced by enzymatic treatment of natural hesperidin isolated from dried bitter oranges as described before [3]. Hesperetin and (−)-homoriodictyol authentic standards were obtained from Extrasynthese SAS (Genay, France). β-Glucuronidase, Type H-1 from *Helix pomatia* and ammonium hydroxide, 28% NH_3, 99.99+%, were purchased from Sigma–Aldrich (St. Louis, MO, USA). Gamylo 300L glucosidase solution from *Aspergillus niger* was purchased from AB Enzymes GmbH (Darmstadt, Germany). Acetonitrile Ultra Gradient HPLC grade was purchased from J.T. Baker Europe (Deventer, The Netherlands). HPLC grade water was prepared using a Millipore Milli-Q purification system (Bedford, MA, USA). Acetic acid (glacial), Lichrosol® methanol gradient grade for liquid chromatography, sodium acetate anhydrous for analysis and formic acid 98–100%, GR for analysis were purchased from Merck (Darmstadt, Germany).

2.2. Instrumentation and chromatographic conditions

Hesperetin was analyzed by reversed-phase ultra-performance liquid chromatography using Acquity UPLC HSS T3 2.1 mm × 100 mm, 1.8 μm column (Waters, Switzerland) equipped with HSS T3 VanGuard precolumn (Waters, Switzerland). Hesperetin enantiomers were separated using DAICEL Chiralpak® IA-3 2 mm × 150 mm, 3 μm column (Chiral Technologies, France).

The chromatography system consisted of a Waters Acquity UPLC system, including a column manager for both analytical columns, coupled with Quattro micro API mass spectrometer detector equipped with an electron spray ionisation source (Waters AG, Baden, Switzerland).

The chromatography was performed at 30 °C for racemic hesperetin and at 40 °C for hesperetin enantiomers. Solvent A was composed of H_2O with 0.1% CHOOH and solvent B of acetonitrile with 0.1% CHOOH. Gradient conditions for hesperetin analysis were as follows: time = 0 min 33% solvent B; time = 0.5 min 33% solvent B; time = 5 min 40% solvent B; time = 5.1 min 100% solvent B; time = 6 min 100% solvent B; time = 6.1 min 33% solvent B; time = 8 min 33% solvent B. The injection volume was 2 μL and the flow rate was 0.4 mL/min. Gradient conditions for hesperetin enantiomer analysis were as follows: time = 0 min 45% solvent B; time = 1 min 45% solvent B; time = 6 min 55% solvent B; time = 7 min 45 solvent B; time = 9 min 45 solvent B. The injection volume was 5 μL and the flow rate was 0.3 mL/min.

Data were collected and processed using MassLynx software. The best signals and resolution were achieved at the following MS conditions: capillary 3.00 kV, cone −37.00 V, source temperature 130 °C, desolvation temperature 400 °C, desolvation gas flow 300 L/h, collision energy 25 eV. The molecular transition m/z 301.0 to m/z 163.8 and to m/z 150.8 were used to determine hesperetin and the internal standard (−)-homoeriodictyol, respectively. The column manager allowed running the two chromatographic conditions one after the other, injecting the same samples. For all standards and samples, duplicate injections were routinely made with both analytical methods.

2.3. Identification of hesperetin and hesperetin enantiomers

Hesperetin was identified by comparison of retention time with an authentic external standard. Hesperetin enantiomers were first isolated using preparative supercritical fluid chromatography (SFC). Briefly, 33 mg of racemic hesperetin were injected on a DAICEL 30 mm × 250 mm Chiralpak IA 5 μm preparative column flushed with carbon dioxyde:methanol (7:3), 25 °C, 150 bar with a flow rate of 120 mL/min. These chromatographic conditions, monitored by UV detection (210 nm), allowed to recover 15 mg of a first fraction with an enantiomeric purity of >99.5% and 16 mg of a second with an enantiomeric purity of >98.5%. In order to confirm the identity of these fractions, optical rotary dispersion and comparison with the reported circular dichroism spectra [12] were performed.

Circular dichroism (CD) spectra of the two enantiomers were recorded on a Jasco J-715 spectropolarimeter equipped with a Jasco PTC-348WI temperature controller fixed at 40 °C. Experiments were carried out using a 0.1 cm path length quartz cuvette. Spectra were recorded between 250 and 400 nm at a resolution of 0.2 nm with a time response of 1 s. Each spectrum represents an average of 10 consecutive scans subtracted from the background. Molar ellipticity $[\Theta]$ in deg cm^2 d mol^{-1} was calculated as follows

$$[\Theta] = [\Theta]_m(MW/10lc)$$

where $[\Theta]_m$ is the ellipticity in millidegrees; MW is the molecular weight of the compound (302.29 g/mol); l is the optical path length of the cuvette (1 cm) and c is the compound concentration in ethanol 0.6% DMSO (0.025 g/dm^3). Optical rotations of the two enantiomers were also measured using a JASCO P-1020 digital polarimeter, equipped with a 10 cm microcell.

2.4. Stock and working standard solution

Stock solution of racemic hesperetin was prepared by dissolving appropriate amounts of the compound in DMSO to achieve a final concentration of 10 mM. A DMSO solution of (−)-homoeriodictyol (internal standard) was prepared similarly at concentration of 10 mM. These solutions were divided into aliquots of 10 μL, protected from light and stored at −20 °C for maximum 3 months.

Working solutions were prepared daily by dissolving the stock solutions in an appropriate amount of methanol to achieve a concentration of 0.5 mM racemic hesperetin and of homoeriodictyol. Calibration curves were prepared daily in mobile phase A (100 μL), plasma (200 μL) and urine (100 μL) to yield concentrations corresponding to 5, 2.5, 1, 0.5, 0.25, 0.125, 0.05 μM racemic hesperetin in plasma and to 10, 5, 2, 1, 0.5, 0.25, 0.1 μM racemic hesperetin in urine. The concentration of homoeriodictyol was kept constant (1 μM) and 0.1% formic acid was used to adjust to the final volume.

2.5. Quality control samples

Flavanone-free human plasma and urine samples were freshly spiked with an appropriate volume of working solutions to obtain quality control (QC) samples containing 125, 500 and 3750 nM of racemic hesperetin for plasma and 250, 1000 and 7500 nM of racemic hesperetin for urine. The concentration of homoeriodictyol was kept constant (1 μM) and 0.1% formic acid was used to adjust to the final volume.

These samples were used as quality controls with the purpose of checking recovery of racemic hesperetin and hesperetin enantiomers.

2.6. Sample preparation

Sample preparation was performed according to Kanaze et al. [13] with slight modifications. Briefly, flavanone-free plasma and urine samples, spiked with hesperetin and internal standard, were mixed with 10% acetic acid solution (10 μL) and incubated under continuous shaking with 100 μL of 0.1 M sodium acetate buffer (pH 5.5) and 5 μL of β-glucuronidase/sulfatase (crude preparation from *H. pomatia*) overnight at 37 °C. The hydrolyzed samples were diluted with 300 μL 0.1% of formic acid solution and loaded on Waters Oasis® MAX 96-Well Plate 30 μm (30 mg) solid phase extraction cartridges (Waters, Switzerland) preconditioned with 500 μL of methanol and 500 μL of water. After loading, the wells were washed with 1000 μL of 2% ammonium hydroxide solution and 1000 μL of methanol and purged with air until complete drying of the aqueous phase. Hesperetin and internal standard were eluted with 1000 μL of 2% of formic acid solution in methanol and the eluate was evaporated to dryness at room temperature under a flow of nitrogen. Finally, the residue was dissolved in 100 μL of mobile phase (40% acetonitrile and 0.1% CHOOH in water) and injected into the UPLC–MS/MS system for total hesperetin and quantification of hesperetin enantiomers.

2.7. Clinical trial treatment

In order to estimate the proportion of each hesperetin enantiomer, the aqueous preparation of 1.36 mM hesperetin-7-*O*-glucoside was analyzed. Briefly, 100 μL of this treatment was incubated under continuous shaking with 450 μL of 0.1 M sodium acetate buffer (pH 5.5) and 50 μL of Gamylo 300L glucosidase (preparation from *A. niger*) overnight at 50 °C in triplicate. After incubation the samples were treated according to the solid phase extraction procedure described above (Section 2.6) and the (R)-/(S)-hesperetin ratio determined using the DAICEL Chiralpak® IA-3 2 mm × 150 mm, 3 μm column. Quantitation of each enantiomer was achieved using the two standards obtained from preparative SFC.

3. Method validation

3.1. Linearity

The linearity of the method was assessed by analyzing different concentrations of racemic hesperetin in plasma (5, 2.5, 1, 0.5, 0.25, 0.125, 0.05 μM) and of racemic hesperetin in urine (10, 5, 2, 1, 0.5, 0.25, 0.1 μM). The concentration of homoeriodictyol was kept constant (1 μM) and 0.1% formic acid was used to adjust to final volume.

The calibration curves were plotted as peak areas of racemic hesperetin and hesperetin enantiomers (y) *vs* concentrations of the working solutions (x) using a weighted linear regression model, i.e. calibration points were weighted by the factor $1/x$.

3.2. Limit of quantification (LoQ)

The LoQ can be determined as the mean value of 10 blank plasma measurements plus 10 standard deviation (SD). However, this can generate a LoQ value which is likely to be artificially low.

FDA defines the LoQ as the lowest concentration of an analyte in a sample that can be quantitatively determined with suitable precision and accuracy [14].

According to the FDA, the lowest concentration of the calibration curve should be accepted as the LoQ if the analyte response is at least 5 times higher than the blank response and if this response is reproducible with a precision of at least 20%.

3.3. Trueness

Recovery of added standard racemic hesperetin was studied at 3 levels. 10 mg of racemic hesperetin was accurately weighed and mixed with 3.308 mL of DMSO. Aliquots of standard solution were added to flavanone-free plasma and urine in order to obtain final concentrations of 125, 500 and 3750 nM of racemic hesperetin in plasma and 250, 1000 and 7500 nM of hesperetin in urine. The concentration of homoeriodictyol was kept constant (1 μM) and 0.1 formic acid was used to adjust to final volume. A *t*-test was performed to check if recovery was significantly different from 100%.

3.4. Precision

The precision of the method was evaluated by calculating the repeatability (r) and the intermediate reproducibility (iR). Repeatability represents the variability of independent results obtained in the same laboratory, with the same analyst, on the same equipment, in a short interval of time. Intermediate reproducibility represents the variability of independent results obtained in the same laboratory, on different days, with different analysts, different calibrations, and different equipment (within laboratory variation). Repeatability and intermediate reproducibility were calculated by analyzing spiked samples in duplicate, on three different days, by two analysts, with the same equipment and with different solution preparations.

4. Study design

The validated analytical method was used to quantify hesperetin and its enantiomers in human plasma and urine samples after the consumption of purified hesperetin-7-*O*-glucoside. The Independent Ethics Committee of the Medical Faculty of the Eberhard-Karls-University of Tübingen and the Bundesinstitut für Arzneimittel und Medizinprodukte approved and authorized the study protocol. Three male volunteers (42 ± 3 years of age; 25 ± 1 mean body mass index) healthy, nonsmokers, and not on any medication, gave their written consent and participated in the study. A diet low in polyphenols and phenolic compounds, which excluded fruits and vegetables, high-fiber products, and beverages such as tea, coffee, fruit juices, and wine, for two days before the study, was followed. After 12 h fasting, volunteers orally consumed 30 mL of aqueous preparation containing 19 mg of hesperetin-7-*O*-glucoside.

Blood samples were collected using EDTA tubes at baseline, 30, 60, 90 min, 2, 3, 4, 6, 8, 24 h. Immediately after collection, blood samples were placed on ice and centrifuged for 10 min at 3000 × *g* at 4 °C. Plasma samples were spiked with 20 μL stabilization buffer (0.4 M NaH_2PO_4, 20% ascorbic acid and 0.1% EDTA at pH 3.6) and kept at −80 °C. Urine samples were collected at baseline and over a 24 h period using pre-weighed tubes containing 1 g ascorbic acid, 25 mg EDTA and 10 mL 1 M HCl. After collection, urine samples

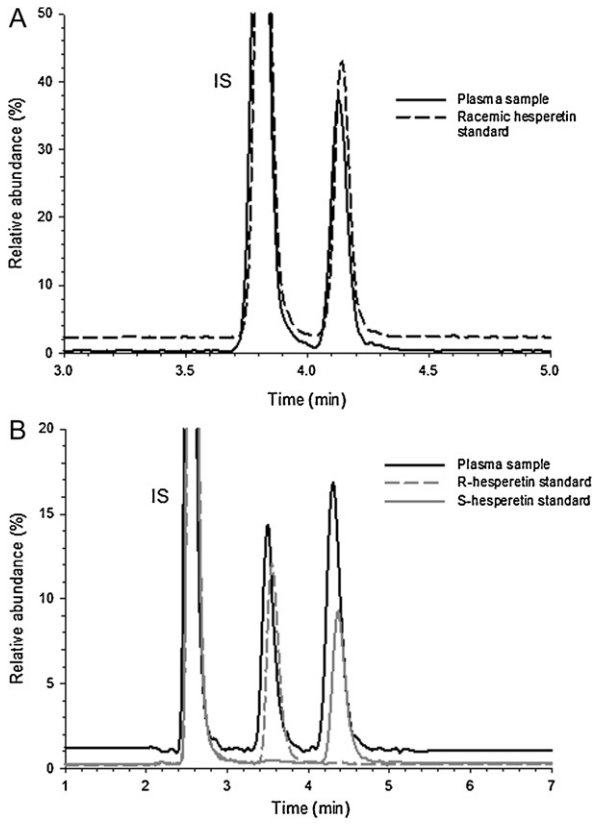

Fig. 2. UPLC–MS/MS chromatograms (MRM experiment) of racemic hesperetin and (−)-homoeriodictyol (IS) (A) separated on a UPLC HSS T3 column and of purified hesperetin R- and S-enantiomers and IS (B) separated on a DAICEL Chiralpak® IA-3 column. Dot line and solid light line represent standard molecules. Solid dark lines represent plasma samples.

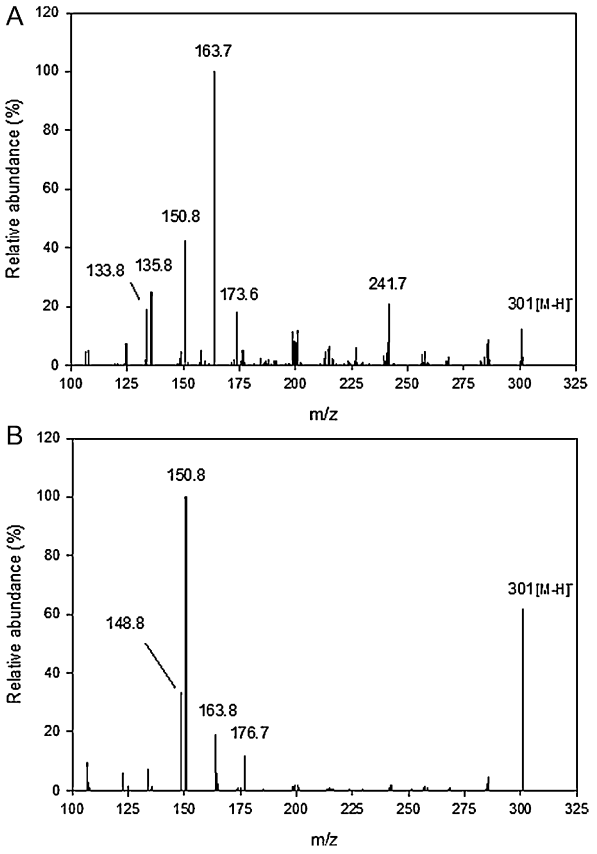

Fig. 3. MS/MS spectra of racemic hesperetin standard solution (A) and (−)-homoeriodictyol (IS) solution (B) obtained in negative ion mode.

were stored at −80 °C until sample preparation. Hesperetin and enantiomers were extracted and quantified as described above.

5. Results and discussion

5.1. Chromatography separation

The aim of this work was to develop a rapid method to quantify hesperetin and hesperetin enantiomers in human plasma and urine. Fig. 2 shows a chromatogram of hesperetin and its enantiomers separated on a Acquity UPLC HSS T3 equipped with HSS T3 VanGuard precolumn (Fig. 2A) and on a DAICEL Chiralpak® IA column (Fig. 2B), respectively. The racemic solution of hesperetin and the hesperetin enantiomers were well separated from each other and from the internal standard with the above described UPLC–MS/MS conditions. Hesperetin and its enantiomers were eluted within 10 min.

The MS spectra of the racemic hesperetin solution standard showed an intense deprotonated molecule at *m*/*z* 301.0, $[M-H]^-$. The fragmentation of the precursor at *m*/*z* 301.0 showed product ion at *m*/*z* 150.8 (Fig. 3A). The MS spectra of the internal standard solution showed an intense deprotonated molecule at *m*/*z* 301.0 $[M-H]^-$ whose fragmentation led to the product ion at *m*/*z* 163.8 (Fig. 3B).

5.2. Identification of hesperetin enantiomers

In order to confirm the identity of hesperetin enantiomers, optical rotary dispersion and comparison with the reported circular dichroism spectra were performed. Fig. 4 shows the CD spectra of both fractions obtained by preparative SFC. They are close mirror images of each other showing their opposing enantiomeric nature. The molecular ellipticity [Θ] of the first eluted hesperetin enantiomer fraction at 289 nm was found to be +46,500 while the one of the second eluted enantiomer fraction was −35,000.

It has been described and confirmed experimentally by Gaffield [12] that flavanones of 2S configuration exhibit a positive Cotton effect due to a $n \rightarrow \pi^*$ transition observed at lower energy wavelength (325–330 nm) and a negative Cotton effect due to a $\pi \rightarrow \pi^*$ transition observed at maximum absorption wavelength (285–290 nm).

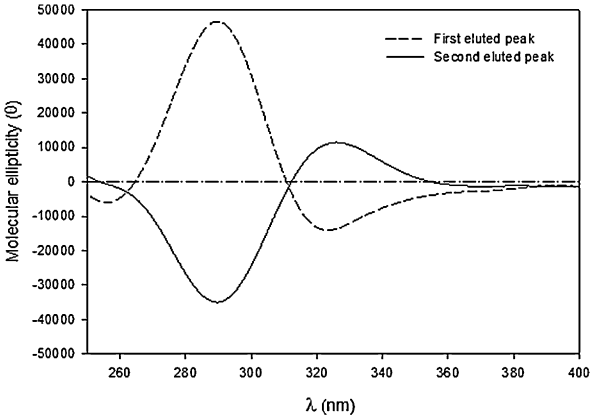

Fig. 4. Circular dichroism spectra (ethanol 0.6% DMSO, 40 °C) of each enantiomer of hesperetin obtained by preparative SFC.

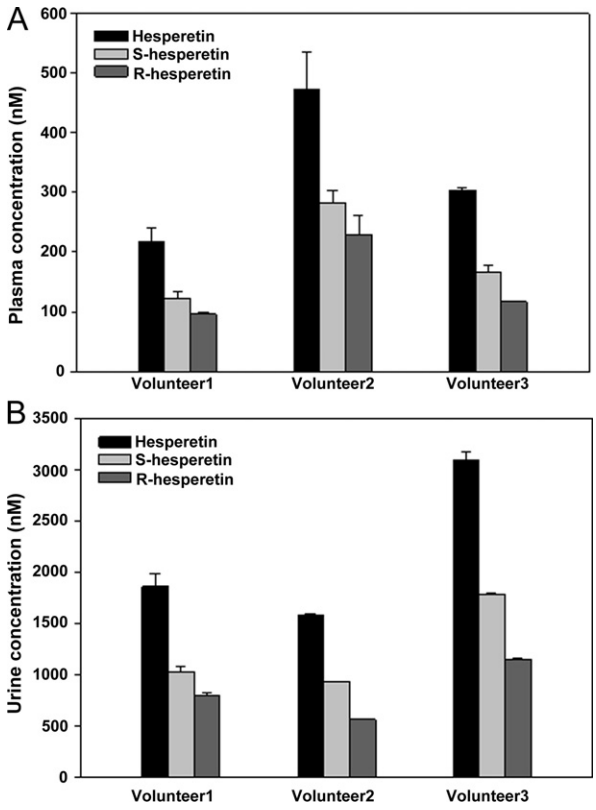

Fig. 5. Concentrations of racemic hesperetin and hesperetin enantiomers in human plasma at C_{max} (A) and urine (B) for three volunteers fed with 19 mg of hesperetin-7-glucoside.

As a consequence, we can definitively assign absolute configuration (R)- to the first eluted hesperetin enantiomer fraction and (S)- to the second eluted hesperetin enantiomer fraction.

Furthermore, specific rotation $[\alpha]^{25} = +46.7$ (c 0.25, EtOH 6% DMSO) was measured for the first hesperetin enantiomer fraction, while the second one gave an experimental $[\alpha]^{25} = -57.3$ (c 0.25, EtOH 6% DMSO). Therefore, the (+)/(R) correlation and the (−)/(S) correlation assigned by Gaffield for flavanones and more recently done theoretically by quantitative *ab initio* calculation of the CD spectrum of (−)-naringenin [15] is confirmed.

5.3. Method validation

Due to the presence of endogenous substances and the low level of analytes, biological matrices needed to be processed by a clean-up and a concentration step prior to further analysis. Recovery rates using different sorbents and elution protocols were tested (results not shown). Waters Oasis® MAX 96-Well Plate 30 μm (30 mg) solid phase extraction cartridges yielded the highest recovery rates and the best precision results.

The method was validated in terms of linearity, precision and accuracy essays. In order to establish the best regression model to quantify racemic hesperetin and its enantiomers, the response (at least triplicate determinations) of concentrations at 5, 2.5, 1, 0.5, 0.25, 0.125, 0.05 μM racemic hesperetin in plasma and at 10, 5, 2, 1, 0.5, 0.25, 0.1 μM racemic hesperetin in urine was measured. The heterogeneity of variance, i.e. dispersion of results at high concentrations (results not shown), indicated the inaccuracy of a non-weighted model to evaluate results, therefore a weighted linear model was used to quantify racemic hesperetin and hesperetin enantiomers.

As this measurement meets the acceptance criteria, the LoQ was considered as the lowest concentration of the calibration curve. LoQ was decided at 50 nM for racemic hesperetin and at 25 nM for hesperetin enantiomers in plasma; at 100 nM for racemic hesperetin and at 50 nM for hesperetin enantiomers in urine.

The trueness of the method was evaluated by spiking flavanone-free plasma and urine with the racemic hesperetin standard solution as described in Section 2. Recovery was calculated by analyzing spiked samples in duplicate, on three different days, by two analysts and with the same equipment. Recovery values (Rec) were compared with reference values and a t-test was performed to check if recovery was significantly different from 100% (results not

Table 1
Median, recovery (Rec), standard deviation of repeatability (SD(r)), relative standard deviation of repeatability (CV(r)), standard deviation of intermediate reproducibility (SD(iR)), and relative standard deviation of intermediate reproducibility (CV(iR)) of racemic hesperetin and hesperetin enantiomers in human plasma and urine. Results are expressed in nM and calculated for three different spiked concentrations (125, 500, 3750 nM in plasma; 250, 1000, 7500 nM in urine).

	Median[a]	Rec	SD(r)	CV (r)%	SD(iR)	CV(iR)%
Human plasma						
Hesperetin	120.7	96.6	3.2	2.6	8.6	7.1
	493.0	98.6	6.0	1.2	13.2	2.7
	3805.2	101.5	44.0	1.2	421.7	11.1
R-hesperetin	64.5	102.3	4.9	7.6	5.7	8.8
	241.7	96.7	16.7	6.9	17.9	7.4
	1917.2	103.1	38.7	2.0	319.1	16.6
S-hesperetin	62.7	100.3	3.3	5.2	6.5	10.4
	229.7	91.9	5.3	2.3	20.7	9.0
	1941.6	103.6	27.9	1.4	407.4	21.0
Human urine						
Hesperetin	232.4	99.9	22.1	9.5	17.2	7.4
	970.6	97.1	13.9	1.4	56.5	5.8
	7492.0	93.0	155.1	2.1	315.1	4.2
R-hesperetin	121.6	99.6	3.3	2.7	15.2	12.5
	471.9	94.4	29.5	6.3	46.4	9.8
	3734.9	97.3	27.4	0.7	296.5	7.9
S-hesperetin	122.7	97.3	12.1	9.8	8.9	7.2
	488.0	97.6	17.7	3.6	65.6	13.4
	3648.8	98.2	56.3	1.5	287.0	7.9

[a] Values given are means of 6 independent determinations.

shown). The recovery ranged between 96.6 and 101.5 in human plasma and between 93.0 and 99.9 in human urine (Table 1). In both plasma and urine, the recovery was not significantly different from 100%.

The precision of the method was evaluated by calculating the simple repeatability and the intermediate reproducibility. Standard deviation of repeatability (SD(r)) and intermediate reproducibility (SD(iR)), and relative standard deviation of repeatability (CV(r)) and intermediate reproducibility (CV(iR)) are listed in Table 1. In human plasma, CV(r) and CV(iR) values ranged between 1.2 and 2.6% and between 2.7 and 11.1%, respectively for racemic hesperetin and from 1.4 to 7.6% and from 7.4 to 21.0%, respectively for hesperetin enantiomers. In human urine, CV(r) and CV(iR) values ranged between 1.4 and 9.5% and between 4.2 and 7.4%, respectively for racemic hesperetin and between 0.7 and 9.8% and between 7.2 and 13.4%, respectively for hesperetin enantiomers. Values of 20% for CV(r) and CV(iR) were considered acceptable with respect to bioanalytical UPLC–MS/MS method (FDA guidelines [14]).

Therefore, this method was suitable for the absolute quantification of racemic hesperetin and its enantiomers in plasma at concentrations ranging between 120 and 3800 nmol/L and 62 and 1940 nmol/L, respectively and in urine at concentrations ranged between 230 and 7490 nmol/L and 120 and 3640 nmol/L, respectively.

5.4. Quantitative analyses of racemic hesperetin and hesperetin enantiomers in human plasma and urine

The validated method described above was used to analyze plasma and urine samples obtained from three healthy volunteers who received 19 mg of hesperetin-7-glucoside exhibiting a 61/39 (±0.4) in S/R ratio moieties. It was previously demonstrated [3] that the *in vitro* digestion of the natural hesperidin into hesperetin-7-glucoside improves the total absorption of hesperitin in humans. Fig. 5A shows the plasma concentration of total hesperetin and hesperetin enantiomers at the time point of maximum concentration (C_{max}). Mean plasma (S)-hesperetin levels at C_{max} were 191 ± 82 nM while mean plasma (R)-hesperetin levels were 147 ± 72 nM. Plasma (S)-hesperetin and (R)-hesperetin levels were quantifiable up to 3 h of sample collection (data not shown) while after that the levels were under the LoQ. Total 24 h urinary excretion of total hesperetin, (S)-hesperetin and (R)-hesperetin was 2.2 ± 0.8, 1.3 ± 0.5, 0.8 ± 0.3 μM, respectively (Fig. 5B).

Therefore, we supported the previous results [3] showing that hesperetin-7-*O*-glucoside absorption occurs predominantly in the small intestine.

6. Conclusions

A new quantitative LC–MS/MS method for the measurement of racemic and hesperetin enantiomers in human plasma and urine was described. The method was validated and found sensitive, selective, accurate and repeatable for the quantification of these compounds. Furthermore, the application of the current method for analyzing biological samples obtained after the ingestion of a single dose of purified hesperetin-7-glucoside shows the feasibility of using it for large human clinical trials.

It is important to mention, that in this study only aglycone forms were quantified. It has been shown that hesperitin metabolites are the biological bioactive compounds [16]. However, the current unavailability of commercial hesperitin enantiomers metabolites is an important limitation for the assessment of such compounds in biological samples. Therefore, upon standards availability, further investigation should be performed for identification and quantification of hesperetin enantiomer metabolites.

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