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Stereospecific high-performance liquid chromatographic analysis of hesperetin in biological matrices

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Abstract

A method of analysis of hesperetin (+/−-3,5,7-trihydroxy-4′-methoxyflavanone) in biological fluids is necessary to study the kinetics of in vitro and in vivo metabolism and tissue distribution. A simple high-performance liquid chromatographic method was developed for simultaneous determination of hesperetin enantiomers in rat serum, and rat and human urine. Serum and urine (0.1 ml) were precipitated with cold acetonitrile after addition of the internal standard, 7-methoxycoumarin. Separation was achieved on a Chiralpak AD-RH column with UV detection at 298 nm. The calibration curve was linear ranging from 0.5 to 100 μg/ml for each enantiomer. The mean extraction efficiency was >98%. Precision of the assay was <5% (CV), and was within 5% at the limit of quantitation (0.5 μg/ml). Bias of the assay was lower than 5%, and was within 5% at the limit of quantitation. The assay was applied successfully to the urinary excretion of hesperetin in rats and humans.
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Keywords: Reversed-phase HPLC; UV-detection; Stereospecific; Enantiomer; Flavonoid

1. Introduction

Hesperidin [+/−-3,5,7-trihydroxy-4′-methoxyflavanone 7-rhamnoglucoside] (Fig. 1) is a chiral flavanone-7-*O*-glycoside consumed in oranges and other citrus fruits and herbal products. The rutinose sugar moiety is rapidly cleaved off the parent compound to leave the aglycone bioflavonoid hesperetin (+/−-3,5,7-trihydroxy-4′-methoxyflavanone) also a chiral flavonoid (Fig. 2). There is current interest in the medical use of bioflavonoids including hesperetin in the treatment of a variety of cancers and vascular diseases [1].

Hesperetin has been previously quantified utilizing a variety of methods [2–5]. All of these other methods have overlooked the fact that hesperetin is a chiral compound. There are, however, two reports demonstrating that multiple-ureido-covalent bonded methylated β-cyclodextrin columns supported on silica gel [6] and that multiple microcrystalline cross-linked acetylcellulose (CTA) columns [7] can separate hesperetin enantiomers although baseline resolution and separation were poor. Unfortunately, some of these columns are not commercially available, and separation and quantification were not validated in biological matrices or applied to pharmacokinetics studies.

To our knowledge, no study has been published characterizing the separation of hesperetin enantiomers in pharmacokinetic studies as there are no validated direct methods of stereospecific analysis of hesperetin in the literature. The analytical column used was the Chiralpak AD-RH column which is packed with tris(3,5-dimethylphenylcarbamate) derivative of amylose and can be utilized in the reverse phase. The present study describes a simple stereoselective, isocratic, reversed-phase high performance liquid chromatography (HPLC) method for the determination of the enantiomers of hesperetin and its application to in vivo kinetic studies.

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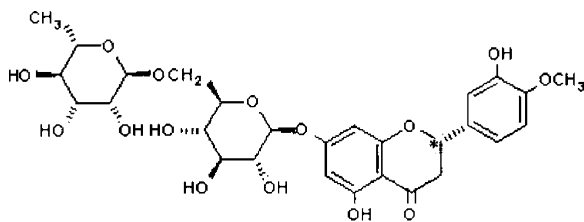

Fig. 1. Structure of hesperidin. (*) Denotes chiral centre.

2. Experimental

2.1. Chemicals and reagents

Racemic hesperetin, hesperidin, 7-ethoxycoumarin, β-glucuronidase Type IX A and *H. pomatia* Type-HP-2 were purchased from Sigma Chemicals (St. Louis, MO, USA). HPLC grade acetonitrile and water were purchased from J.T. Baker (Phillipsburg, NJ, USA). Phosphoric acid was from Aldrich Chemical Co. Inc. (Milwaukee, WI, USA). Minute Maid® Orange Juice was purchased from a local grocery. Rats were obtained from Charles River Laboratories. Ethics approval for animal experiments was obtained from Washington State University. Human experiments were conducted with written informed consent according to the principles of the Declaration of Helsinki.

2.2. Chromatographic system and conditions

The HPLC system used was a Shimadzu HPLC (Kyoto, Japan), consisting of an LC-10AT VP pump, a SIL-10AF auto injector, a SPD-M10A VP spectrophotometric diodearray detector, and a SCL-10A VP system controller. Data collection and integration were accomplished using Shimadzu EZ Start 7.1.1 SP1 software (Kyoto, Japan). The analytical column used was Chiralpak AD-RH column (150 mm × 4.6 mm i.d., 5 μm particle size, Chiral Technologies Inc., Exton, PA, USA). The mobile phase consisted of acetonitrile, water and phosphoric acid (42:58:0.01, v/v/v), filtered and degassed under reduced pressure, prior to use. Separation was carried out isocratically at ambient temperature (25 ± 1 °C), and a flow rate of 0.8 ml/min, with ultraviolet (UV) detection at 298 nm.

2.3. Stock and working standard solutions

Twenty-five milligram of racemic hesperetin was accurately weighed on an analytical balance (AG245, Mettler) and dissolved with methanol in a 25 ml volumetric flask to make a stock standard solution in methanol with a racemic concentration of 1 mg/ml. A methanolic stock solution of 7-ethoxycoumarin (internal standard) was prepared similarly with a concentration of 1 mg/ml. This solution was diluted with methanol to make a working internal standard solution of 25 μg/ml. These solutions were protected from light and stored at −20 °C between uses, for no longer than 3 months. Calibration standards in serum were prepared daily from the stock solution of hesperetin by sequential dilution with blank rat serum or human and rat urine, yielding a series of concentrations namely, 0.5, 1.0, 5.0, 10.0, 50.0 and 100.0 μg/ml of each enantiomer in three replicates.

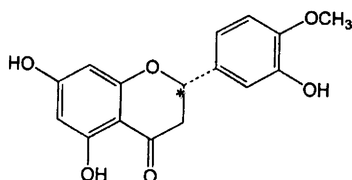

Fig. 2. Structure of hesperetin. (*) Denotes chiral centre.

Quality control (QC) samples were prepared from the stock solution of hesperetin by dilution with blank biological fluid to yield target concentrations of 0.5, 1.0, 5.0, 10.0, 50.0 and 100.0 μg/ml. The QC samples were divided into 0.1 ml aliquots in micro centrifuge tubes and stored at −70 °C before use.

2.4. Sample preparation

To the working standards or samples (0.1 ml) were added 25 μl of internal standard solution (25 μg/ml), and 1 ml of cold acetonitrile in 2.0 ml Eppendorf tubes. The mixture was vortexed for 1 min (Vortex Genie-2, VWR Scientific, West Chester, PA, USA), and centrifuged at 15,000 rpm for 5 min (Beckman Microfuge centrifuge, Beckman Coulter Inc., Fullerton, CA, USA). The organic phase supernatant was collected into culture tubes (10 mm × 75 mm) and evaporated to dryness using a HetoVac concentrator (Heto-Holten, DK-3450 Allerød, Denmark). The residue was reconstituted with 200 μl of mobile phase, vortexed for 1 min and centrifuged at 5000 rpm for 5 min. The supernatant was transferred to HPLC vials and 20 μl of the injected into the HPLC system.

2.5. Precision and accuracy

The within-run precision and accuracy of the replicate assays ($n = 6$) were tested by using six different concentrations of hesperetin enantiomers, namely 0.5, 1.0, 5.0, 10.0, 50.0 and 100.0 μg/ml. The between-run precision and accuracy of the assays were estimated from the results of six replicate assays of QC samples on six different days within 1 week. The precision was evaluated by the relative standard deviation (R.S.D.). The accuracy was estimated based on the mean percentage error of measured concentration to the actual concentration [8].

2.6. Recovery

Recovery for hesperetin enantiomers from biological fluids was assessed ($n = 6$) at 0.5, 1.0, 5.0, 10.0, 50.0 and 100 μg/ml and the recovery of the internal standard 7-ethoxycoumarin was evaluated at the concentration used in sample analysis (25 μg/ml). A known amount of hesperetin or 7-ethoxycoumarin was spiked into 0.1 ml biological fluid

to give the above concentrations. The samples were treated as described under section 2.4 and analysed by HPLC. The extraction efficiency was determined by comparing the peak areas of hesperetin or 7-ethoxycoumarin to those of hesperetin or 7-ethoxycoumarin solutions of corresponding concentration injected directly in the HPLC system without extraction.

2.7. Freeze-thaw and bench-top stability of hesperetin samples

The freeze-thaw stability of hesperetin enantiomers was evaluated at three concentrations 1.0, 5.0 and 50 μg/ml, using QC samples. These samples were analysed in triplicate without being frozen at first, and then stored at −70 °C and thawed at room temperature (25 ± 1 °C) for three cycles.

The stability of hesperetin in reconstituted extracts during run-time in the HPLC auto-injector was investigated using pooled extracts from QC samples of three concentration levels 1.0, 5.0, and 50.0 μg/ml. Samples were kept in the sample rack of the auto-injector and injected into HPLC system every 4 h, from 0 to 24 h at the temperature of auto-injector (26 ± 1 °C).

2.8. Urinary excretion of hesperetin in human and rat

After 3 days of a citrus-free diet and an overnight fast a healthy 21 year old male subject (73 kg) drank three bottles of Minute Maid® Orange Juice (236 ml each). The hesperetin enantiomer content in the orange juice was quantified and it was determined that the total ingested dose by the healthy volunteer was 69.13 mg of *R*-hesperidin, 468.13 mg of *S*-hesperidin, 1.52 mg of *R*-hesperetin and 15.85 mg of *S*-hesperetin. Urine was collected at intervals between 0 and 24 h post-dose and stored at −70 °C until analysed.

A male Sprague-Dawley rat (200 g) was placed in a metabolic cage, and fasted for 12 h before dosing. On the day of experiment, the rat was dosed orally with 200 mg/kg racemic hesperidin in polyethylene glycol 400 via oral gavage with a curved feeding needle attached to a syringe. This dose of hesperidin has previously been shown to demonstrate anti-inflammatory and analgesic activity in rats [9]. Urine was collected 0–4, 4–8, 8–24 h and stored at −70 °C until analysis. Urine samples (100 μl) were run in duplicate with or without the addition of 40 μl of 500 U/ml β-glucuronidase IX-A and incubated in a shaking water bath at 37 °C with for 2 h to liberate any glucuronide conjugates [10].

2.9. Data analysis

Quantification was based on calibration curves constructed using peak area ratio (PAR) of hesperetin to internal standard, against hesperetin concentrations using unweighted least squares linear regression.

3. Results and discussion

3.1. Chromatography

Separation of hesperetin enantiomers and the internal standard in biological fluids was achieved successfully. There were no interfering peaks co-eluted with the compounds of interest (Fig. 3A and C). The order of elution was determined by taking a 150 μl aliquot of lemon juice and subjecting it to 200 μl of *H. pomatia* Type-HP-2 enzyme [11]. The predominant enantiomer of hesperidin in lemon juice is in the *S*(−)configuration [12–13]. The retention times of *R*- and *S*-hesperetin were approximately 15 and 24 min, respectively (Fig. 3B). The internal standard eluted at approximately 12 min.

The performance of the HPLC assay was assessed using the following parameters, namely peak shape and purity, interference from endogenous substances in biological fluid, linearity, limit of quantitation (LOQ), freeze-thaw stability, stability of reconstituted extracts, precision, accuracy and recovery. Various compositions of mobile phase were tested to achieve the best resolution between hesperetin enantiomers.

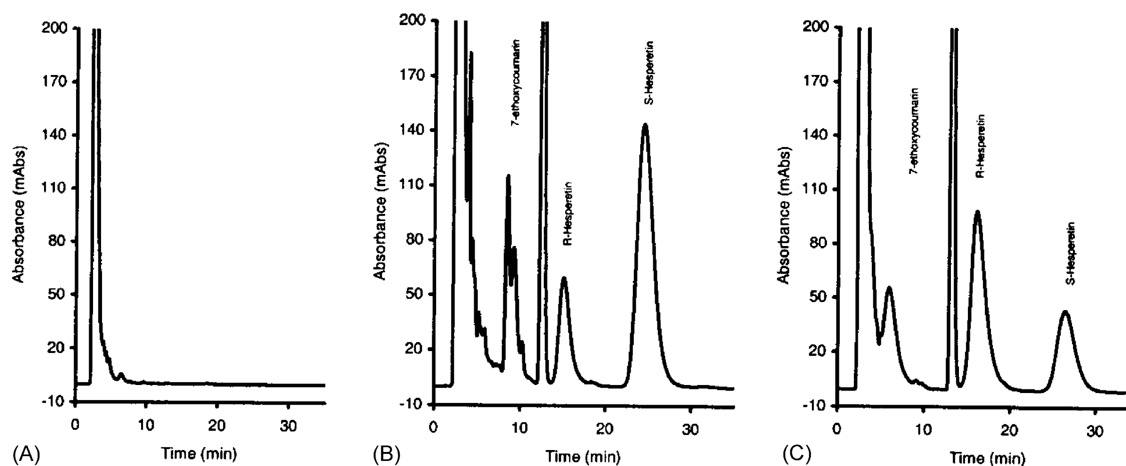

Fig. 3. Representative chromatograms, of (A) drug-free urine, (B) urine containing hesperetin (HP) enantiomers each with concentration of 1.0 μg/ml and the internal standard (IS), and (C) 6 h human urine sample containing hesperetin enantiomers and the IS.

Table 1
Within- and between-day precision and accuracy of the assay for hesperetin (HT) enantiomers in human urine ($n = 3$, mean ± S.D.)

Enantiomer concentration (μg/ml)												
Added	Observed				R.S.D. (%)				Bias (%)			
	Within-day		Between-day		Within-day		Between-day		Within-day		Between-day	
	R-HT	*S*-HT	*R*-HT	*S*-HT	*R*-HT	*S*-HT	*R*-HT	*S*-HT	*R*-HT	*S*-HT	*R*-HT	*S*-HT
0.5	0.49	0.49	0.49	0.49	4.51	4.43	5.12	5.21	2.45	2.49	4.23	4.55
1.0	1.00	1.01	1.02	1.01	3.21	3.21	3.25	3.33	1.81	1.82	3.31	3.32
5.0	5.01	5.02	5.01	5.10	2.10	2.11	2.01	4.02	0.50	0.52	0.74	0.75
10	9.97	9.99	9.99	10.01	1.71	1.54	1.51	2.94	3.30	3.31	1.05	1.08
50	24.89	25.00	24.92	25.03	1.92	1.80	2.21	2.43	3.32	3.34	2.15	2.12
100	50.01	49.98	50.01	49.98	3.41	3.62	3.51	3.52	0.05	0.05	0.04	0.04

For instance, the addition of phosphoric acid (0.01%) leaded to an improvement on the shape and sharpness of the peaks compared to a mobile phase with any acid added. Then, the amount of phosphoric acid was increased to 0.1%, doing so reduced the sharpness and resolution of the peaks. Also different mobile phase compositions were tested, the starting mobile phase was acetonitrile, water and phosphoric acid (40:60:0.01, v/v/v). Increasing the polarity through the addition of water to the mobile phase increased retention time. Thus, more acetonitrile was added in order to reduce the retention time to minimize run time, obtaining a desirable mobile phase constituted of acetonitrile, water and phosphoric acid (42:58:0.01, v/v/v). Acetonitrile content was not increased to avoid the internal front of the mobile phase overlapping with the internal standard. The retention times of the analytes were very sensitive to small changes in mobile phase composition on the Chiralpak AD-RH column.

There are no other stereospecific assays of hesperetin published in the literature. The present assay is practical to use in pre-clinical and clinical applications of hesperetin where small sample volumes are obtained.

3.2. Linearity and LOQ

Excellent linear relationships ($r^2 = 0.999$) were demonstrated between PAR of *R*- and *S*-hesperetin to the internal standard and the corresponding serum concentrations of hesperetin enantiomers over a range of 0.5–100 μg/ml. The mean regression lines from the validation runs were described by *R*-hesperetin (μg/ml) = $0.0392x + 0.311$ and *S*-hesperetin (μg/ml) = $0.0397x + 0.028$.

The LOQ of this assay was 0.5 μg/ml in biological fluids with the corresponding between-day relative standard deviation of 5.12 and 5.21% for *R*- and *S*-hesperetin respectively, and bias of 4.23 and 4.55% for *R*- and *S*-hesperetin, respectively. The back-calculated concentration of QC samples was within the acceptance criteria.

3.3. Precision, accuracy and recovery

The within- and between-run precision (R.S.D.) calculated during replicate assays ($n = 6$) of hesperetin enantiomers in human urine was <5% over a wide range of concentrations (Table 1). The intra- and inter-run bias assessed during the replicate assays for hesperetin enantiomers varied between 0.04 and 4.6% (Table 1). These data indicated that the developed HPLC method is reproducible and accurate. The mean extraction efficiency for hesperetin enantiomers from biological fluids varied from 98.2 to 98.7% (Table 2). In addition, the recovery of 7-ethoxycoumarin was 97.1% at its concentration used in the assay. High recovery from biological fluids suggested that there was negligible loss of hesperetin enantiomers and 7-ethoxycoumarin during the protein precipitation process. Additionally the efficiencies of extraction of hesperetin enantiomers and 7-ethoxycoumarin were comparable.

3.4. Stability of hesperetin samples

No significant degradation was detected after the samples of racemic hesperetin in biological fluids following three freeze-thaw circles. The recoveries of *R*- and *S*-hesperetin were respectively from 98.5 to 99.9% and 98.3 to 99.7% following three freeze-thaw cycles for hesperetin QC samples of hesperetin or 7-ethoxycoumarin. There was no significant decomposition observed after the reconstituted extracts of racemic hesperetin were stored in the auto-injector at room temperature for 24 h. The measurements were from 98.3 to 99.9% of the initial value for extracts of racemic hesperetin in biological fluids of 0.5, 1.0, 5.0, 10.0, 50.0 and 100.0 μg/ml respectively, during the storage in the auto injector at room temperature for 24 h.

Table 2
Recovery of hesperetin enantiomers from human urine ($n = 6$)

Concentration (μg/ml)	Recovery (%) (mean ± S.D.)	
	R-hesperetin	*S*-hesperetin
1.0	98.2 ± 1.1	98.6 ± 1.3
5.0	98.5 ± 1.2	98.5 ± 1.2
50.0	98.7 ± 1.3	98.7 ± 0.9

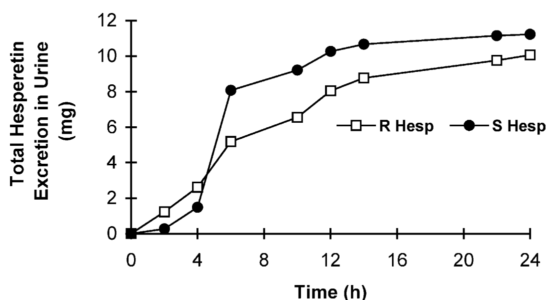

Fig. 4. Cumulative urinary excretion profile of hesperetin enantiomers following the administration of orange juice orally to a healthy volunteer.

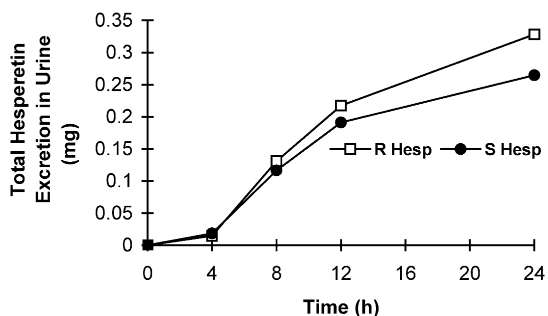

Fig. 5. Cumulative urinary excretion profile of hesperetin enantiomers following the administration of racemic hesperidin 200 mg/kg orally to a rat.

3.5. Urinary excretion of hesperetin in human

The HPLC method has been applied to the determination of hesperetin enantiomers in the urinary excretion study in a human and rat. Hesperetin has previously been demonstrated to be excreted into urine after consumption of hesperidin in human and rat studies [14–16]. Following oral administration of orange juice, or racemic hesperidin marked stereoselective disposition was observed for the enantiomer excretion in urine (Figs. 4 and 5). Our laboratory has recently conducted pharmacokinetic studies in three different species after administration of orange juice, hesperetin or hesperidin and also examined the stereospecific concentrations of these flavonoids in various citrus fruits [17]. In the three species there is a marked stereoselective disposition in their urinary excretion and a marked stereoselective in the content of hesperetin in all the citrus fruit juices.

In summary, the developed HPLC assay is stereospecific, reproducible and accurate. It has been successfully applied to a urinary excretion study of hesperetin enantiomers in humans and rats and studies are currently ongoing.

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