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Stereospecific high-performance liquid chromatographic analysis of eriodictyol in urine

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Abstract

A stereospecific method of analysis of eriodictyol [5,7,3′,4′-tetrahydroxyflavanone] in biological fluids is necessary to study the kinetics of *in vitro* and *in vivo* metabolism, and tissue distribution in fruits and humans. A simple high-performance liquid chromatographic method was developed for the stereospecific determination of eriodictyol in rat and human urine. Separation was achieved on a Chiralpak OJ-RH column with UV detection at 288 nm. The stereospecific calibration curves were linear ranging from 0.5 to 100 μg/ml. The mean extraction efficiency was >98.8%. Precision of the assay was <15% (CV), and was within 12% at the limit of quantitation (0.5 μg/ml). Bias of the assay was lower than 8%, and was within 6% at the limit of quantitation. The assay was applied successfully to the urinary excretion of eriodictyol in rats and humans, and to the stereospecific quantification of eriodictyol in raw lemon juice, conventional and organic lemonade.

Keywords: Reversed-phase HPLC; UV-detection; Stereospecific; Epimer; Flavonoid

1. Introduction

Eriocitrin [5,7,3′,4′-tetrahydroxyflavanone-7-*O*-ruinoside] (Fig. 1A) is a flavanone-7-*O*-glycoside with a stereogenic center present in lemons, tamarinds and other citrus fruits, as well as in mint, oregano, fennel, thyme, and rose hip [1–7]. After consumption, the neohesperidose sugar moiety is rapidly cleaved off the parent compound in the gastrointestinal tract and liver to leave the aglycone bioflavonoid eriodictyol [5,7,3′,4′-tetrahydroxyflavanone] (Fig. 1B). The proposed metabolism of eriodictyol in the gastrointestinal tract and liver is presented in Fig. 1.

Eriodictyol has been previously quantified utilizing a variety of methods including liquid chromatography–electrospray-ionisation–mass spectrometry (LC–ESI–MS) [3], nano-electrospray-ionisation mass spectrometry (ESI–MS) [1,3]. High-performance liquid chromatography-1,1-diphenyl-2-picrylhydrazyl (HPLC-DPPH) [4], normal phase silica gel and Al_2O_3 chromatography, IR, MS and NMR [5–8]. All of these methods have overlooked the fact that eriodictyol has a stereogenic center. However, there is a recent study that employed the commercially available Chiralpak AS-H, which has an amylose-derived stationary phase, for the stereospecific separation of eriodictyol under normal-phase HPLC, the authors obtained baseline resolution but the method was not validated in biological matrices [9]. The method depicted here is the only validated method for the stereospecific separation of eriodictyol using reversed-phase HPLC.

We have also utilized this novel method to examine racemization of eriodictyol. It has been previously reported that flavanones such as eriodictyol can racemize depending on the substituted functional groups around the stereogenic center [10]. Furthermore, flavanones with a free hydroxyl group in the carbon 4′ such as naringenin and eriodictyol have the potential to racemize easier than flavanones with a methoxy group on that position such as hesperetin and isosakuranetin [11]. The racemization process reaches an equilibrium between the two antipodes and is facilitated with by high temperatures, moisture, solvent, and pH, among other factors [10].

Our method is a stereoselective, isocratic, reversed-phase high-performance liquid chromatography (HPLC) method that

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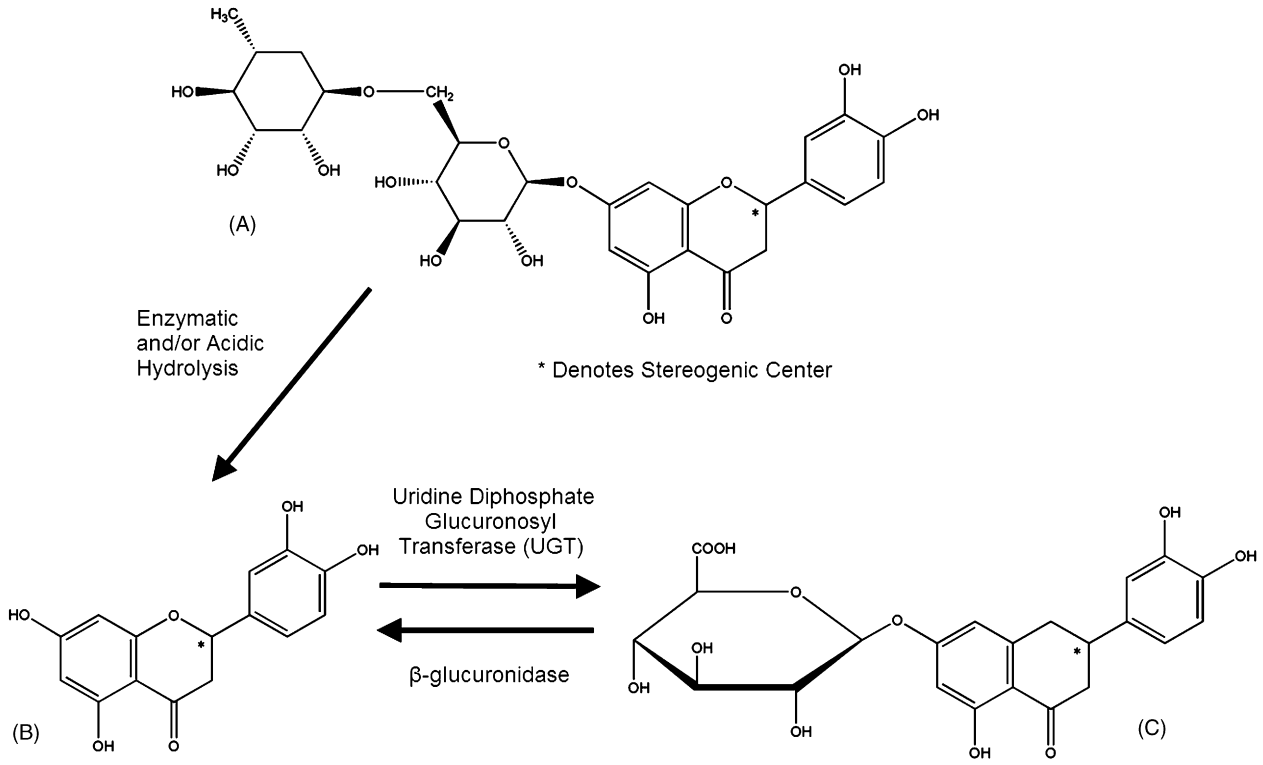

Fig. 1. Eriodictyol metabolism. Structure of eriocitrin (A), structure of eriodictyol (B), and structure of 7-*O*-eriodictyol glucuronide (C). *Stereogenic center.

has been successfully applied for the stereospecific determination of eriodictyol and its application to examine racemization, *in vivo* kinetic studies, quantification of fruits and fruit juices, and to stereospecifically isolate eriodictyol for further pharmacological testing.

2. Experimental

2.1. Chemicals and reagents

Eriocitrin and eriodictyol were purchased from Indofine Chemical Company (Hillsborough, NJ, USA). Etoposide, β-glucuronidase Type IX A, *Helix pomatia* β-glucuronidase type-HP-2, and tert-butylmethylether were purchased from Sigma Chemicals (St. Louis, MO, USA). HPLC grade acetonitrile, methanol, and water were purchased from J.T. Baker (Phillipsburg, NJ, USA). Phosphoric acid was purchased from Aldrich Chemical Co. Inc. (Milwaukee, WI, USA). Raw lemons, commercial conventional lemonade (Minute Maid®), commercial organic lemonade (Santa Cruz®), fresh thyme (*Thymus vulgaris*) and fresh peanut hulls (*Arachis hypogea*) were obtained from a local Safeway grocery store. Rats were obtained from Charles River Laboratories. Ethics approval for animal experiments was obtained from Washington State University.

2.2. Chromatographic system and conditions

The HPLC system used was a Shimadzu HPLC (Kyoto, Japan), consisting of an LC-10ATVP 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 OJ-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 (20:80:0.04, 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 0.7 ml/min, with ultraviolet (UV) detection at 288 nm.

2.3. Stock and working standard solutions

Twenty-five milligram of eriodictyol was accurately weighed on an analytical balance (AG245, Mettler) and dissolved with methanol in a 250 ml volumetric flask to make a stock standard solution with a racemic concentration of 100 μg/ml. A methanolic stock solution of the internal standard (etoposide) was prepared similarly to a final concentration of 100 μg/ml. These solutions were protected from light and stored at −20 °C between uses, for no longer than 3 months. Stereospecific calibration standards in urine were prepared daily from the stock solution of eriodictyol by sequential dilution with blank rat urine, yielding a series of concentrations namely, 0.5, 1.0, 5.0, 10.0, 50.0 and 100.0 μg/ml in three replicates.

Quality control (QC) samples were prepared from the stock solution of eriodictyol by dilution with blank rat urine 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), 100 μl of internal standard solution (100 μg/ml) was added into 2.0 ml Eppendorf tubes. The mixture was vortexed for 1 min (Vortex Genie-2, VWR Scientific, West Chester, PA, USA), and centrifuged at 5000 rpm for 5 min (Beckman Microfuge centrifuge, Beckman Coulter Inc., Fullerton, CA, USA). The supernatant was collected into 2.0 ml Eppendorf tubes and evaporated to dryness under compressed nitrogen gas. The residue was reconstituted with 400 μl of mobile phase, vortexed for 1 min and centrifuged at 5000 rpm for 5 min, the supernatant was transferred to HPLC vials and 150 μl of it was injected into the HPLC system.

2.5. Precision and accuracy

The stereospecific within-run precision and accuracy of the replicate assays ($n = 6$) were tested by using six different concentrations of eriodictyol, 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 6 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 [12].

2.6. Recovery

The stereospecific recovery for eriodictyol 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 was evaluated at the concentration used in sample analysis (100 μg/ml). A known amount of eriodictyol or etoposide was spiked into 0.1 ml blank rat urine to give the above concentrations. The samples were treated as described under Section 2.4 and analyzed by HPLC. The extraction efficiency was determined by comparing the peak areas of eriodictyol or etoposide to those of eriodictyol or etoposide solutions of corresponding concentration injected directly in the HPLC system without extraction.

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

The stereospecific freeze-thaw stability of eriodictyol was evaluated at three concentrations 1.0, 5.0 and 50 μg/ml, using QC samples. These samples were analyzed 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 eriodictyol 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 eriodictyol in human and rat

After 3 days of a citrus free diet and an overnight fast a healthy 24-year old male subject (73 kg) drank 1000 ml of Conventional Lemonade (Minute Maid®). The stereospecific eriocitrin and eriodictyol content in the lemonade was quantified and it was determined that the total ingested dose by the healthy volunteer was 103.25 mg of (2R)-eriocitrin, 299.94 mg of (2S)-eriocitrin, 9.39 mg of R(+)-eriodictyol and 9.90 mg S(−)-eriodictyol. Urine was collected in acid washed containers at intervals between 0 and 24 h post-dose and stored at −70 °C until analyzed. Eriocitrin epimers were indirectly quantified by taking a 150 μl aliquot of lemonade and subjecting it to 1 ml HPLC-grade H_2O, 110 μl 0.78 M sodium acetate acetic acid buffer (pH 4.8), 100 μl 0.1 M ascorbic acid, and 200 μl crude preparation of *H. pomatia* (type HP-2, Sigma Chemical Co.) followed by incubation for 17–24 h at 37 °C [13]. Then, 25 μl of internal standard (etoposide) were added followed by 1 ml cold acetonitrile to precipitate proteins. The mixture was vortexed for 1 min (Vortex Genie-2, VWR Scientific, West Chester, PA, USA), and centrifuged at 5000 rpm for 5 min (Beckman Microfuge centrifuge, Beckman Coulter Inc., Fullerton, CA, USA). The supernatant was collected into 15 ml poly-propylene tubes and evaporated to dryness under compressed nitrogen gas. The residue was reconstituted with 400 μl of mobile phase, vortexed for 1 min and centrifuged at 5000 rpm for 5 min, the supernatant was transferred to HPLC vials and 150 μl of it was injected into the HPLC system. *H. pomatia* Type-HP-2 is a β-glucuronidase that cleaves specifically the glycosylated sugar moiety of eriocitrin and other flavanones as previously described [13]. Therefore, the samples without enzymatic hydrolysis (free samples) were utilized to determine the concentration of eriodictyol (aglycone), whereas the samples with enzymatic hydrolysis (total samples) were utilized to determine the concentration of eriodictyol originally present plus the concentration of eriocitrin converted to eriodictyol by the cleavage action of the enzyme. Finally, by subtracting the free sample eriodictyol concentration from the total sample, the concentration of eriocitrin epimers can be calculated.

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 intravenously 10 mg/kg eriodictyol in polyethylene glycol 400 via a jugular vein cannula. This dose of eriodictyol has previously been demonstrated to exert pharmacological effects in preclinical studies [14,15]. Urine was collected 0–4, 4–8, 8–24 h and stored at −70 °C until analysis. Urine samples (0.1 ml) 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 for 2 h to liberate any glucuronide conjugates [16]. The samples were evaporated to dryness under compressed nitrogen gas. The residue was reconstituted with 400 μl of mobile phase, vortexed

for 1 min and centrifuged at 5000 rpm for 5 min, the supernatant was transferred to HPLC vials and 150 μl of it was injected into the HPLC system. β-Glucuronidase IX-A cleaves specifically the glucuronidated metabolite (eriodictyol-7-*O*-glucuronide) back to eriodictyol. Therefore, the samples without enzymatic hydrolysis (free samples) were utilized to determine the concentration of eriodictyol (aglycone), whereas the samples with enzymatic hydrolysis (total samples) were utilized to determine the concentration of eriodictyol originally present plus the concentration of the major metabolite eriodictyol-7-*O*-glucuronide converted to eriodictyol by the cleavage action of the enzyme. Finally, by subtracting the free sample eriodictyol concentration from the total sample, the stereospecific concentration of eriodictyol-7-*O*-glucuronide can be calculated.

2.9. Quantification of lemon juice and lemonades

Raw lemons, commercial conventional lemonade (Minute Maid®), and commercial organic lemonade (Santa Cruz®) were purchased from a local grocery store. Eriodictyol and eriocitrin were stereoselectively quantified as described in Section 2.8.

2.10. Quantitation of thyme and peanut hulls

One gram of dried peanut hulls (*A. hypogea*) was extracted with 20 ml HPLC-grade methanol or 20 ml tert-butylmethylether for thyme (*T. vulgaris*) at ambient temperature according to previous methods [17]. The extracts were evaporated to dryness under compressed nitrogen gas at room temperature. The peanut hull sample was dissolved in 1 ml mobile phase, while the thyme sample was dissolved in 3 ml mobile phase, vortexed for 1 min and centrifuged at 5000 rpm for 5 min, the supernatant was filtered through a 13 mm syringe filter (Pall Corporation, Ann Arbor, MI). Both solutions were further diluted 10-fold, transferred to HPLC vials, and 150 μl injected into the HPLC system.

2.11. Racemization of eriodictyol

One gram of dried peanut hulls (*A. hypogea*) and one gram of dried thyme (*T. vulgaris*) were extracted with 1 ml 25% aqueous methanol for 1 h at 70 °C according to previous methodology [17]. The extracts were evaporated to dryness under compressed nitrogen gas at room temperature, the residues were reconstituted with 400 μl of mobile phase, vortexed for 1 min and centrifuged at 5000 rpm for 5 min, the supernatant was transferred to HPLC vials and 150 μl of sample was injected into the HPLC system.

2.12. Data analysis

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

3. Results and discussion

3.1. Chromatography

Stereospecific separation of eriodictyol enantiomers and the internal standard in biological fluids was achieved successfully. There were no interfering peaks co-eluting with the compounds of interest (Fig. 2A–C). The order of elution was determined by using the circular dichroism data reported by Caccamese et al. [9]. Under normal-phase conditions, the first enantiomer to be eluted is S(−)-eriodictyol [18]. Since we utilized reverse-phase conditions, the order of elution was reversed with the R(+)-eriodictyol being eluted first. To confirm this elution order, peanut hulls (*A. hypogea*) and thyme (*T. vulgaris*) were analyzed since it has been previously reported that these two plants contain stereochemically enriched S(−)-eriodictyol [17]. As shown in Fig. 3A, thyme contains predominantly S(−)-eriodictyol (96.35%) and this is the predominant second peak in order of elution. Therefore, the retention times of R- and S-eriodictyol were approximately 56 and 60 min, respectively. The internal standard eluted at approximately 16 min (Fig. 2B). It was also observed that peanut hulls contain predominantly S(−)-eriodictyol (97.16%).

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 stereospecific resolution of eriodictyol.

The final mobile phase constitution is acetonitrile, water and phosphoric acid (20:80:0.04, v/v/v), this concentration was chosen because it was possible to attain baseline separation and no co-eluting peaks in fruit juices or biological matrices were evident. An optimization process was followed to attain this final mobile phase constitution. The first mobile phase to be examined was acetonitrile water (50:50, v/v), but eriodictyol and etoposide did not adequately resolve. Subsequently, the amount of acetonitrile was reduced to 35% and two broad peaks were observed around 6 and 7 min without baseline resolution. As the two eriodictyol peaks were too close to the solvent front, and etoposide was not resolved further optimization was necessary. An increase in the polarity of the mobile phase, by increasing the concentration of water was employed to extend the elution time. Thus, the amount of water was increased from 65% to 80% and 90%. The initial mobile phase attempt simultaneously eluted etoposide, R(+)-, and S(−)-eriodictyol at 16, 56, and 60 min, respectively. The second mobile phase also eluted etoposide, R(+)-, and S(−)-eriodictyol, but the elution times were higher: 29, 76, and 85 min, respectively. The retention times of the analytes were very sensitive to small changes in mobile phase composition on the Chiralpak OJ-RH column. It was observed that with both mobile phases the peaks were broad. For practical purposes and to reduce sample run-time, the first mobile phase was chosen. However, the peaks were still broad, thus the use of an acid was proposed as previously demonstrated for other flavonoids [19–21]. It was proposed to test 0.15% triflu-

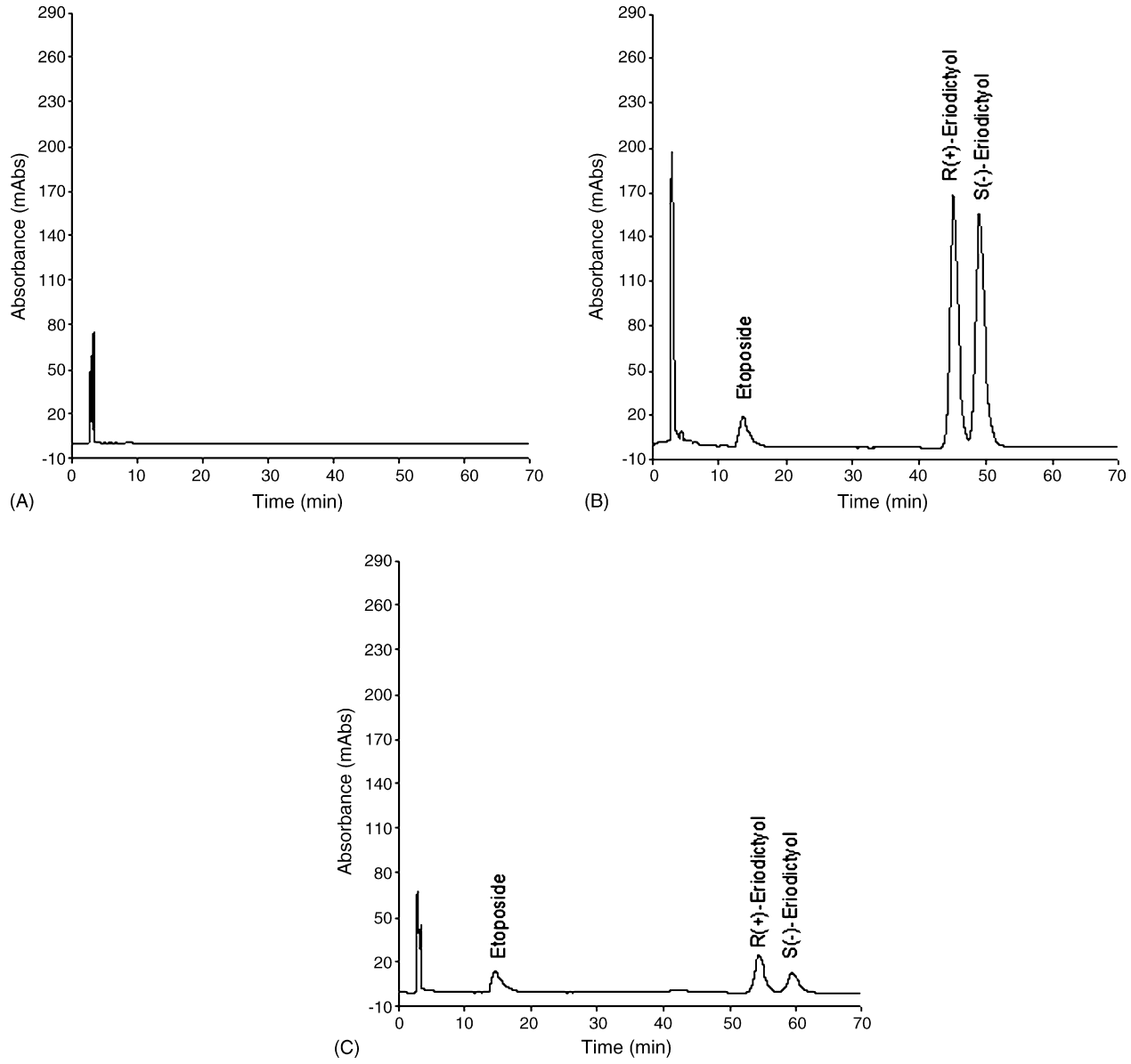

Fig. 2. Representative chromatograms of (A) drug-free urine, (B) urine containing eriodictyol with concentration of 10 μg/ml and the internal standard (IS), and (C) 10 h human urine sample containing eriodictyol and the IS after oral ingestion of lemonade (1000 ml).

oroacetic acid (TFA) and 0.15% phosphoric acid. Phosphoric acid improved the sharpness of the peaks to a greater extent than TFA. Therefore, phosphoric acid was explored in greater detail and ultimately its concentration was reduced to 0.1% and 0.04%. Phosphoric acid (0.04%) provided the best sharpness of the peaks of interest. There was baseline resolution, and there were no interfering co-eluting peaks observed with biological samples.

The choice of etoposide as internal standard was determined after extensive attempts with many other compounds such as naringenin and kaempferol. In the end we chose this internal standard was preferred because of the large difference in retention times which enables baseline separation of etoposide and eriodictyol and facilitates other peaks of interest in fruits and vegetables to elute without interfering with the peaks of interest. In addition, in some tissues and other biological matrices many interfering peaks make other internal standards impossible to use. Naringenin originally offered good potential as internal standard, but this was also found to be present in many fruit samples and interfering peaks in urine were noted. Another suitable alternative was kaempferol, which was found to be present in the majority of fruit samples and eluted after S(−)-eriodictyol (around 79 min) making run times even longer.

The present assay is practical to use in pre-clinical applications of eriodictyol 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(−)-eriodictyol to the internal standard and the corresponding urine concentrations of eriodictyol over a range of 0.5–100 μg/ml. The mean regres-

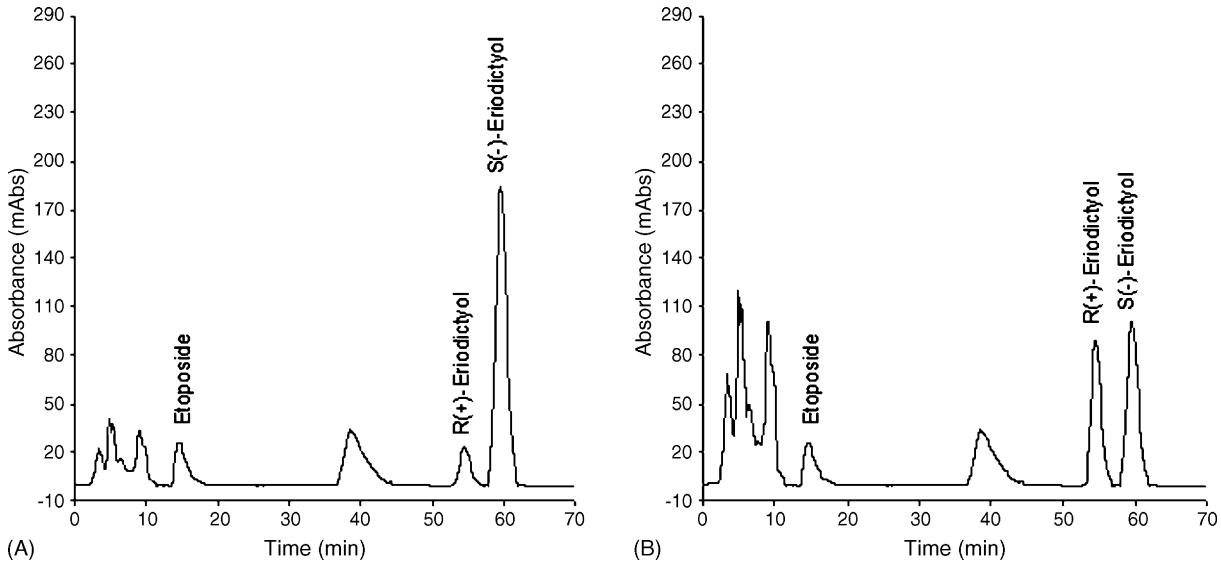

Fig. 3. Representative chromatograms of (A) thyme (*T. vulgaris*) containing predominantly S(−)-eriodictyol and (B) thyme (*T. vulgaris*) after racemization (1 h heat treatment at 70 °C in 25% methanol).

sion lines from the validation runs were described by R(+)-eriodictyol (μg/ml) = 0.0331*x* − 0.0080 and S(−)-eriodictyol (μg/ml) = 0.0433*x* − 0.0049.

The LOQ of this assay was 0.5 μg/ml in biological fluids with the corresponding between day relative standard deviation of 4.72% and 2.61% for R(+)- and S(−)-eriodictyol, respectively, and bias of 3.39% and −7.94% for R(+)- and S(−)-eriodictyol, respectively. The back-calculated concentration of QC samples was within the acceptance criteria.

3.3. Precision, accuracy and recovery

The stereospecific within- and between-run precision (R.S.D.) calculated during replicate assays ($n = 6$) of eriodictyol in human urine was <12% over a wide range of concentrations (Table 1). The stereospecific intra- and inter-run bias assessed during the replicate assays for eriodictyol varied between −7.94% and 5.58% (Table 1). These data indicated that the developed HPLC method is reproducible and accurate. The mean extraction efficiency for eriodictyol from biological fluids varied from 88.3% to 97.1% (Table 2). In addition, the recovery of etoposide was 99.3% at its concentration used in the assay. High recovery from biological fluids suggested that there was negligible loss of eriodictyol and during the protein precipitation process. Additionally the efficiencies of extraction of eriodictyol and etoposide were comparable.

3.4. Stability of eriodictyol samples

No significant degradation was detected after the samples of eriodictyol in biological fluids following three freeze-thaw circles. The recoveries of R(+)- and S(−)-eriodictyol were, respectively, from 98.81% to 101.75% and 99.41% to 103.71% following three freeze-thaw cycles for eriodictyol QC samples of eriodictyol or etoposide. There was no significant decomposition observed after the reconstituted extracts of eriodictyol were stored in the auto-injector at room temperature for 24 h. The measurements were from 99.22% to 99.80% of the initial value for extracts of eriodictyol 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.

3.5. Stereospecific quantitation and racemization of eriodictyol in thyme (T. vulgaris) and peanut hulls (A. hypogea)

The HPLC method has been applied to the stereospecific quantification of eriodictyol in thyme (*T. vulgaris*) and peanut hulls (*A. hypogea*). These two plants have being reported previously to contain stereochemically enriched S(−)-eriodictyol [17]. As shown in Fig. 3A, thyme contains predominantly S(−)-eriodictyol (96.35%), while peanut hulls contain predominantly S(−)-eriodictyol (97.16%) (chromatogram not shown).

Eriodictyol contains a free hydroxyl group in the carbon 4′, which increases its potential to racemize easier than other flavanones with a methoxy group on that position such as hesperetin and isosakuranetin [11]. Therefore, the racemization process of eriodictyol under heated 25% methanol was studied. It was observed that because of racemization, the content of S(−)-eriodictyol decreased from 96.35% to 60% as evident in Fig. 3B. Therefore, the content of R(+)-eriodictyol increased to 40%. Similar results were observed for peanut hulls, whereas the content of S(−)-eriodictyol decreased from 97.16% to 63.32% and the content of R(+)-eriodictyol increased to 36.68%. These results parallel previous observations with naringenin where the racemization reaches an equilibrium between the two antipodes [10].

3.6. Urinary excretion of eriodictyol in human and rat

The HPLC method has been applied to the stereospecific determination of eriodictyol in the urinary excretion study in a human and rat.

Table 1
Stereospecific within- and between-day precision and accuracy of the assay for eriodictyol in rat urine ($n = 6$, mean, R.S.D., and bias)

Eriodictyol concentration (μg/ml) added	Observed				R.S.D. (%)				Bias (%)			
	Within-day		Between-day		Within-day		Between-day		Within-day		Between-day	
	R(+)-eriodictyol	S(−)-eriodictyol	R(+)-eriodictyol	S(−)-eriodictyol	R(+)-eriodictyol	S(−)-eriodictyol	R(+)-eriodictyol	S(−)-eriodictyol	R(+)-eriodictyol	S(−)-eriodictyol	R(+)-eriodictyol	S(−)-eriodictyol
0.5	0.53	0.47	0.52	0.46	11.37	1.80	4.72	2.61	5.58	−6.24	3.39	−7.94
1.0	1.03	1.02	1.01	1.01	6.30	2.78	2.80	4.13	2.98	1.77	1.04	1.07
5.0	5.06	4.99	5.02	5.11	0.40	0.19	0.91	3.51	1.13	−0.29	0.49	2.24
10	10.05	9.98	9.97	9.95	0.19	2.31	1.00	0.42	0.51	−0.20	−0.28	−0.47
50	49.64	50.28	49.91	49.96	0.61	0.95	0.50	1.21	−0.72	0.56	−0.17	−0.09
100	100.3	99.91	100.0	100.1	0.18	0.23	0.20	0.29	0.25	−0.09	0.03	0.09

Table 2
Stereospecific recovery of eriodictyol from rat urine ($n = 6$)

Concentration (μg/ml)	Recovery(%) (mean ± S.D.)	
	R(+)-eriodictyol	S(−)-eriodictyol
0.5	9.71 ± 3.3	88.3 ± 0.8
1.0	104.8 ± 4.7	96.0 ± 1.7
5.0	101.0 ± 2.2	98.6 ± 0.7
10	100.1 ± 0.3	99.5 ± 2.9
50	98.9 ± 1.1	101.7 ± 0.3
100	100.3 ± 0.2	99.5 ± 0.3

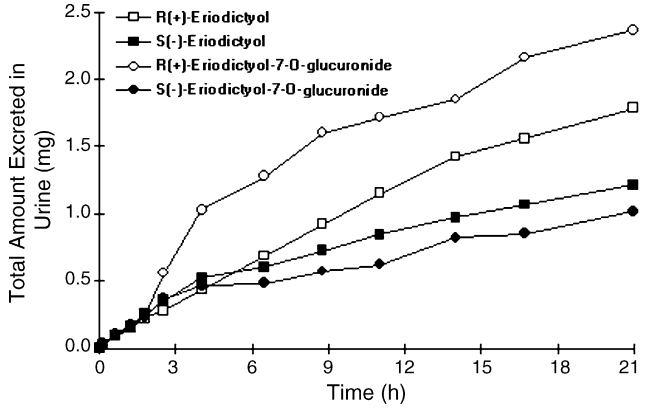

Fig. 4. Cumulative stereoselective urinary excretion profile of eriodictyol and eriodictyol-7-*O*-glucuronide following the administration of lemonade orally to a healthy volunteer.

Eriodictyol has previously been demonstrated to be excreted into urine after consumption of lemonade in human and rat studies [22]. Following oral administration of lemonade to a human and eriodictyol to a rat, apparent enantioselective renal excretion was observed for eriodictyol and its major metabolite eriodictyol-7-*O*-glucuronide (Figs. 4 and 5).

3.7. Stereospecific quantification of eriocitrin and eriodictyol in lemon juice and lemonades

The HPLC method has been applied to the stereospecific quantification of eriodictyol in raw lemon juice, conventional and organic lemonade.

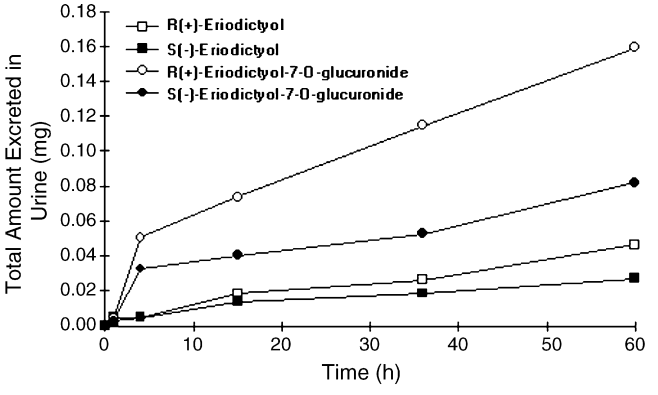

Fig. 5. Cumulative stereoselective urinary excretion profile of eriodictyol and eriodictyol-7-*O*-glucuronide following the administration of the eriodictyol 10 mg/kg intravenously (IV) to a rat.

Table 3
Stereospecific eriocitrin and eriodictyol concentrations in lemonade ($n = 4$)

Citrus foodstuff	(2R)-eriocitrin (mg/100 g)	(2S)-eriocitrin (mg/100 g)	Total eriocitrin (sum of 2R- and 2S-eriocitrin) (mg/100 g)	Total eriocitrin USDA value (mg/100 g) [23]
Lemon juice, raw	11.9	21.3	33.2	N/R
Lemon juice, bottled	10.3	30.0	40.3	N/R
Organic lemon juice, bottled	15.8	29.9	45.7	N/R
Citrus foodstuff	**R(+)-eriodictyol (mg/100 g)**	**S(−)-eriodictyol (mg/100 g)**	**Total eriodictyol (sum of R(+)- and S(−)-eriodictyol) (mg/100 g)**	**Total eriodictyol USDA value (mg/100 g) [23]**
Lemon juice, raw	0.4	0.4	0.80	4.9
Lemon juice, bottled	0.9	1.0	1.9	11.6
Organic lemon juice, bottled	0.2	0.3	0.5	N/R

N/R, not reported.

Eriodictyol in raw and processed lemon juice (lemonade) has been reported previously in the United States Department of Agriculture (USDA) Database for the Flavonoid Content of Selected Foods [23]. This database reports only the non-stereospecific concentration of eriodictyol, also there is no report of the stereogenic glycoside eriocitrin, and no reports on the organic lemon juice. This novel HPLC method allowed us to successfully stereoselectively quantify eriocitrin and eriodictyol. It was observed that (2S)-eriocitrin and S(−)-eriodictyol were predominant in all the juices examined. The conventional and organic lemonade had higher concentrations of eriocitrin epimers than raw lemon juice, except for (2R)-eriocitrin, which was the lowest for conventional lemonade. Conventional lemonade had the highest concentration of the aglycone eriodictyol followed by raw lemon juice, and organic lemonade (Table 3).

Each stereogenic flavonoid that we have examined thus far demonstrate different stereoselective pharmacokinetic disposition patterns [19–21].

In summary, the developed HPLC assay is stereospecific, reproducible, sensitive and accurate. It has been successfully applied to a stereoselective urinary excretion study of eriodictyol in humans and rats, and to the stereospecific quantification and determination of eriocitrin and eriodictyol in fruits and fruit juices.

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References

[1] A. Gil-Izquierdo, M.T. Riquelme, I. Porras, F. Ferreres, J. Agric. Food Chem. 52 (2004) 324–331.
[2] V. Exarchou, M. Godejohann, T.A. Van Beek, I.P. Gerothanassis, J. Vervoort, Anal. Chem. 75 (2003) 6288–6294.
[3] Y. Sudjaroen, R. Haubner, G. Wurtele, W.E. Hull, G. Erben, B. Spiegelhalder, S. Changbumrung, H. Bartsch, R.W. Owen, Food Chem. Toxicol. 43 (2005) 1673–1682.
[4] M. Kosar, H.J. Dorman, K.H.C. Baser, R. Hiltunen, J. Agric. Food Chem. 52 (2004) 5004–5010.
[5] I. Parejo, F. Viladomat, J. Bastida, G. Schmeda-Hirschmann, J. Burillo, C. Codina, J. Agric. Food Chem. 52 (2004) 1890–1897.
[6] A. Dapkevicius, T.A. van Beek, G.P. Lelyveld, A. van Veldhuizen, A. de Groot, J.P. Linssen, R. Venskutonis, J. Nat. Prod. 65 (2002) 892–896.
[7] E. Hvattum, Rapid Commun. Mass Spectrom. 16 (2002) 655–662.
[8] S.Y. Mo, Y.C. Yang, J.G. Shi, Zhongguo Zhong Yao Za Zhi 28 (2003) 339–341.
[9] S. Caccamese, C. Caruso, N. Parrinello, A. Savarino, J. Chromatogr. A 1076 (2005) 155–162.
[10] N.R. Srinivas, Biomed. Chromatogr. 18 (2004) 207–233.
[11] C.O. Miles, L.J. Main, J. Chem. Soc., Perkin Trans. II (1988) 195–198.
[12] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman, S. Spector, Eur. J. Drug Metab. Pharmacokinet. 16 (1991) 249–255.
[13] I. Erlund, E. Meririnne, G. Alfthan, A. Aro, J. Nutr. 131 (2001) 235–241.
[14] K. Minato, Y. Miyake, S. Fukumoto, K. Yamamoto, Y. Kato, Y. Shimomura, T. Osawa, Life Sci. 72 (2003) 1609–1616.
[15] V. Crespy, C. Morand, C. Besson, N. Cotelle, H. Vezin, C. Demigne, C. Remesy, Am. J. Physiol. Gastrointest. Liver Physiol. 284 (2003) G980–G988.
[16] C.Y. Yang, S.Y. Tsai, P.D.L. Chao, H.F. Yen, T.M. Chien, S.L. Hsiu, J. Food Drug. Anal. 10 (2002) 143–148.
[17] M. Krause, R. Galensa, Chromatographia 32 (1991) 69–72.
[18] P. Ficarra, R. Ficarra, C. Bertucci, S. Tommasini, M.L. Calabro, D. Costantino, M. Carulli, Planta Med. 61 (1995) 171–176.
[19] J.A. Yáñez, X.W. Teng, K.A. Roupe, N.M. Davies, J. Pharm. Biomed. Anal. 37 (2005) 591–595.
[20] J.A. Yáñez, N.M. Davies, J. Pharm. Biomed. Anal. 39 (2005) 164–169.
[21] C.A. Torres, N.M. Davies, J.A. Yáñez, P.K. Andrews, J. Agric. Food Chem. 53 (2005) 9536–9543.
[22] Z. Aturki, V. Brandi, M. Sinibaldi, J. Agric. Food Chem. 52 (2004) 5303–5308.
[23] USDA Database for the Flavonoid Content of Selected Foods. Updated on March 25, 2003 [http://www.nal.usda.gov/fnic/foodcomp/Data/Flav/flav.html]. Accessed on June 14, 2006.