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Short communication

Stereospecific high-performance liquid chromatographic validation of homoeriodictyol in serum and Yerba Santa (*Eriodictyon glutinosum*)

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Abstract

A stereospecific method of analysis of racemic homoeriodictyol (eriodictyol 3′-methyl ether) in biological fluids is necessary to study pharmacokinetics and disposition in fruits and herbs. A simple high-performance liquid chromatographic method was developed for the determination of homoeriodictyol enantiomers. Separation was achieved in a Chiralcel® OJ-RH column with UV-detection at 288 nm. The standard curves in serum were linear ranging from 0.5 to 100.0 μg/ml for each enantiomer. The mean extraction efficiency was >88.0%. 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 <15%, and was within 6% at the limit of quantitation. The assay was applied successfully to stereospecific disposition of homoeriodictyol enantiomers in rats and to the quantification of homoeriodictyol enantiomers in Yerba Santa (*Eriodictyon glutinosum*).

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

1. Introduction

Homoeriodictyol is a chiral flavanone consumed in citrus fruits, and herbal products [1]. Chiral flavanones may racemize depending on the substituted groups around the chiral center. The racemization process is significantly facilitated with temperature, moisture, solvent, and pH, among other factors [2]. In addition, it has been suggested that flavanones with a free hydroxyl group (OH^-) in the C4′ position (i.e. eriodictyol) racemize easier than flavanones with a methoxy group ($CH_3{}^-$) in that position (i.e. homoeriodictyol) [3]. Currently, few studies have been published on pharmacokinetics of homoeriodictyol [4,5]. These studies identified homoeriodictyol or its glucuro- and/or sulfo-conjugates as metabolites after ingestion of quercetin, eriodictyol, and eriocitrin. However, these studies did not assess the stereospecific homoeriodictyol disposition since the methods employed were achiral. Therefore, chiral methods to assess the metabolic pathways of homoeriodictyol enantiomers in biological fluids and their disposition in fruits, vegetables, and herbs are warranted.

Homoeriodictyol exists in two enantiomeric forms: *R*(+)- and *S*(−)-homoeriodictyol. *S*(−)-homoeriodictyol was reported as the predominant enantiomer in Yerba Santa [6]; although its actual concentration was not reported, and lack of stereospecific baseline separation and resolution was observed. The method described in the present study is the only validated method for the separation of homoeriodictyol enantiomers under reversed-phase high-performance liquid chromatography (HPLC) in biological matrices.

2. Experimental

2.1. Chemicals and reagents

Racemic homoeriodictyol was purchased from Indofine Chemical Company (NJ, USA). Racemic indoprofen, β-glucuronidase from *Escherichia coli* Type IX A, and β-glucuronidase from *Helix pomatia* Type-HP-2 were purchased from Sigma Chemicals (MO, USA). HPLC-grade acetonitrile and water were purchased from J.T. Baker (NJ, USA). Phos-

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phoric acid was purchased from Aldrich Chemical Co. Inc. (WI, USA). 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. The analytical column used was Chiralcel® OJ-RH column (150 mm × 4.6 mm i.d., 5-μm particle size, Chiral Technologies Inc., PA, USA) protected by a Chiralcel OJ-RH guard column (0.4 cm × 1 cm, 5-μm particle size). The mobile phase consisted of acetonitrile, water and phosphoric acid (22:78:0.1, v/v/v), filtered and degassed. Separation was carried out isocratically at 25 ± 1 °C, a flow rate of 1.0 ml/min, with ultraviolet (UV) detection at 288 nm.

2.3. Stock and working standard solutions

Racemic homoeriodictyol and racemic indoprofen (internal standard) solutions of 100.0 μg/ml were dissolved in methanol. Calibration standard curves were prepared yielding concentrations of 0.5, 1.0, 5.0, 10.0, 50.0, and 100.0 μg/ml of each homoeriodictyol enantiomer.

2.4. Sample preparation

To the working standards or samples (0.1 ml), 25 μl of racemic indoprofen (internal standard) was added into 2.0 ml Eppendorf tubes. The mixture was vortexed for 1 min and 1 ml of cold acetonitrile was added to precipitate proteins. The samples were centrifuged at 5000 rpm for 5 min. The supernatant was collected and evaporated to dryness under compressed nitrogen gas. The residue was reconstituted with 200 μl of mobile phase, vortexed and centrifuged, the supernatant was transferred to HPLC vials and 150 μl of it was injected into the HPLC system.

2.5. Precision and accuracy

The within-run and between-run precision and accuracy of the replicate assays ($n = 6$) were tested over a range of 0.5–100.0 μg/ml on the same day and 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 [7].

2.6. Recovery

Recovery of homoeriodictyol enantiomers was analyzed in the same concentration range (0.5–100.0 μg/ml). The samples were prepared as described in the sample preparation section. The extraction efficiency was determined by comparing the peak area ratio (PAR) of enantiomeric homoeriodictyol and *R*(−)-indoprofen to the PAR of corresponding concentration injected directly in the HPLC without extraction.

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

The freeze-thaw stability of homoeriodictyol enantiomers (0.5–100.0 μg/ml) was evaluated 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 homoeriodictyol in reconstituted extracts was investigated using pooled extracts from QC samples of one concentration level 10.0 μg/ml. The sample was kept in the sample rack of the auto-injector and injected into HPLC system every 4 h, from 0 to 24 h.

2.8. Pharmacokinetic disposition of homoeriodictyol in rats

Male Sprague–Dawley rats ($n = 6$, average weight ∼200 g) were cannulated [8] and dosed intravenously 10 mg/kg racemic homoeriodictyol in polyethylene glycol 400. Blood samples (0.30 ml) were collected at 0, 1, 30 min, 1, 2, 4, 6, 24, 48, 72, 96, and 120 h and serum obtained and stored at −20 °C. Serum samples (0.1 ml) were assayed in duplicate with or without the addition of 40 μl of 500 U/ml β-glucuronidase and incubated at 37 °C for 2 h to liberate glucuronide conjugates [9].

2.9. Quantification and racemization of Yerba Santa

One gram of Yerba Santa powder (American Health and Herbs, OR, USA) was extracted with 20 ml HPLC-grade methanol [6]. The extracts were evaporated to dryness using a rotary evaporator, and the dried samples were dissolved in 1 ml of mobile phase, vortexed and centrifuged. The supernatant was filtered through a 13 mm syringe filter. The solution was further diluted 10-fold, and 150 μl injected into the HPLC system. For racemization, the extracts were evaporated to dryness using a rotary evaporator and later reconstituted in 25% methanol in water [6]. The sample was heated for 1 h at 70 °C and filtered through a 13 mm syringe filter.

2.10. Data analysis

Quantification was based on calibration curves constructed using PAR of homoeriodictyol enantiomers to internal standard *R*(−)-indoprofen, against homoeriodictyol concentrations using unweighted least squares linear regression.

2.11. Pharmacokinetic and statistical analysis

Pharmacokinetic parameters [8] were calculated using WinNonlin® software (Ver. 5.1). Data were presented as mean and standard error of the mean (mean ± S.E.M.) and were analyzed for statistical significance using NCSS Statistical and Power Analysis software (NCSS, UT). Student's *t*-test was employed for unpaired samples with a value of $p < 0.05$.

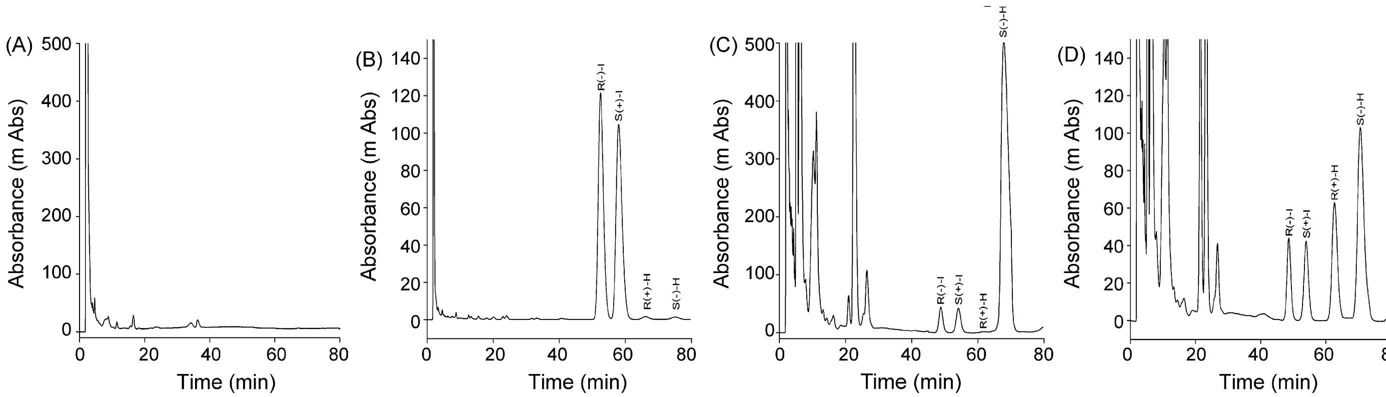

Fig. 1. Representative chromatograms of (A) drug-free serum demonstrating no interfering peaks co-eluted with the compounds of interest; (B) serum containing homoeriodictyol (H) enantiomers (*R*(+)-H, *S*(−)-H) each with concentration of 10 μg/ml and the internal standard, *R*(−)-indoprofen (*R*(−)-I); (C) Yerba Santa (*Eriodictyon glutinosum*) sample containing homoeriodictyol (H) enantiomers predominantly in the *S*(−)-form (*S*(−)-H) and the internal standard, *R*(−)-indoprofen (*R*(−)-I); and (D) Yerba Santa (*E. glutinosum*) after racemization (1 h heat treatment at 70 °C in 25% methanol). With this method, separation of racemic indoprofen is also achieved (*R*(−)-I, *S*(+)-I).

3. Results and discussion

3.1. Chromatography

Separation of homoeriodictyol enantiomers and the internal standard in biological fluids was achieved successfully (Fig. 1). No interfering peaks co-eluting with the compounds of interest (Fig. 1A and B). Under reverse-phase conditions *R*(+)-homoeriodictyol eluted first [6]. As shown in Fig. 1C, Yerba Santa contains mostly *S*(−)-homoeriodictyol. The retention times of *R*(+)- and *S*(−)-homoeriodictyol were approximately 69 and 78 min, respectively. The internal standard (*R*(−)-indoprofen) eluted at approximately 55 min (Fig. 1B). Optimal separation was achieved when the combination of acetonitrile, water, and phosphoric acid was 22:78:0.1 (v/v/v) and the flow rate of 1.0 ml/min.

3.2. Linearity and LOQ

Excellent linear relationships ($r^2 = 0.995$) were demonstrated between PAR of *R*(+)- and *S*(−)-homoeriodictyol to the internal standard and the corresponding serum concentrations of homoeriodictyol enantiomers over a range of 0.5–100 μg/ml. Mean regression lines from the validation runs were described by *R*(+)-homoeriodictyol (μg/ml) = $0.01880x - 0.0012$ and *S*(−)-homoeriodictyol (μg/ml) = $0.0178x - 0.0006$. LOQ of this assay was 0.5 μg/ml with the corresponding between-day R.S.D. of 6.55% and 5.94% for *R*(+)- and *S*(−)-homoeriodictyol, respectively, and bias of −10.80% and −14.97% for *R*(+)- and *S*(−)-homoeriodictyol, respectively. The back-calculated concentration of QC samples was within the acceptance criteria (Table 1).

3.3. Precision, accuracy and recovery

Within- and between-run precision (R.S.D.) calculated during replicate assays ($n = 6$) of homoeriodictyol enantiomers was <15% over a wide range of concentrations (Table 1). The intra- and inter-run bias assessed during the replicate assays for homoeriodictyol enantiomers varied between −14.97% and 14.53% (Table 1). These data indicated that the developed HPLC method is reproducible and accurate. The mean extraction efficiency for homoeriodictyol enantiomers from biological fluids varied from 88.54% to 112.17% (Table 2).

3.4. Stability of homoeriodictyol samples

No significant degradation was detected after the samples of racemic homoeriodictyol in biological fluids following

Table 1
Within- and between-day precision and accuracy of the assay for homoeriodictyol (H) enantiomers in rat serum ($n = 6$, mean, R.S.D., and bias)

Added	Homoeriodictyol concentration (μg/ml)											
	Observed				R.S.D. (%)				Bias (%)			
	Within-day		Between-day		Within-day		Between-day		Within-day		Between-day	
	R(+)-H	*S*(−)-H	*R*(+)-H	*S*(−)-H	*R*(+)-H	*S*(−)-H	*R*(+)-H	*S*(−)-H	*R*(+)-H	*S*(−)-H	*R*(+)-H	*S*(−)-H
0.5	0.57	0.57	0.45	0.43	14.84	14.94	6.55	5.94	13.52	14.53	−10.80	−14.97
1	1.04	1.03	1.11	0.86	5.46	4.71	10.63	9.37	4.02	3.45	10.62	−13.51
5	4.68	4.82	4.46	4.40	5.73	1.73	5.12	6.48	−6.34	−3.62	−10.79	−12.03
10	9.69	9.77	8.88	8.87	4.00	7.10	14.96	12.23	−3.11	−2.30	−11.23	−11.33
50	52.36	53.09	48.14	48.04	1.15	8.44	5.39	5.28	4.73	6.18	−3.72	−3.91
100	98.92	99.86	101.19	101.28	4.73	4.04	1.28	1.28	−1.08	−0.14	1.19	1.28

Table 2
Recovery of homoeriodictyol enantiomers from rat serum ($n=6$)

Concentration (μg/ml)	Recovery (%) (mean ± S.D.)	
	R(+)-Homoeriodictyol	*S*(−)-Homoeriodictyol
0.5	96.39 ± 5.66	103.98 ± 4.23
1	102.63 ± 10.91	112.17 ± 5.62
5	100.66 ± 5.32	97.81 ± 6.34
10	101.91 ± 5.40	96.66 ± 5.09
50	92.07 ± 4.96	88.54 ± 4.67
100	102.46 ± 1.32	103.40 ± 1.32

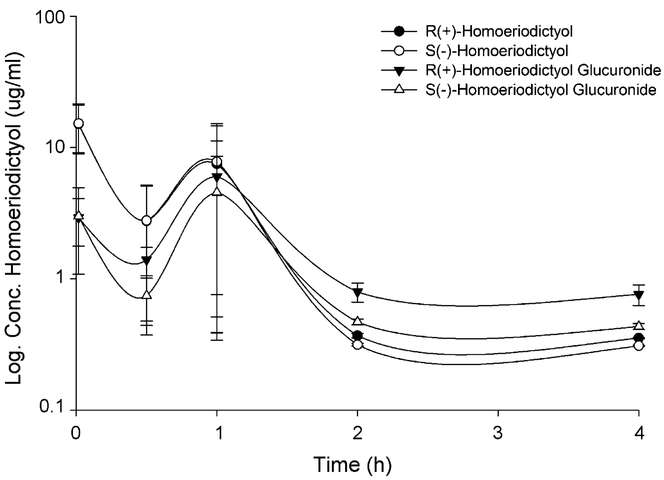

Fig. 2. Disposition in serum of enantiomeric homoeriodictyol and its glucuro-conjugates following administration of racemic homoeriodictyol (10 mg/kg) to rats ($n=6$, mean ± S.E.M.).

three freeze-thaw cycles. Recoveries of *R*(+)- and *S*(−)-homoeriodictyol were respectively from 85.35% to 107.37% and 85.53% to 103.86% following three freeze-thaw cycles for homoeriodictyol QC samples of homoeriodictyol or *R*(−)-indoprofen. There was no significant decomposition observed after the reconstituted extract of racemic homoeriodictyol was stored in the auto-injector at room temperature for 24 h; the measurements were from 97.87% to 98.90% of the initial value for extracts of racemic homoeriodictyol in biological fluids of 0.5–100.0 μg/ml.

3.5. *Stereospecific pharmacokinetics of homoeriodictyol in rats*

The assay method was applied to the study of homoeriodictyol disposition in rats ($n=6$). Following IV administration of homoeriodictyol the serum disposition was examined (Fig. 2). Homoeriodictyol enantiomers were detected in serum and urine primarily as glucuro-conjugates; *R*(+)-homoeriodictyol has a half-life of ∼11 h, whereas the *S*(−)-homoeriodictyol is ∼12 h.

3.6. *Quantification and racemization of homoeriodictyol in Yerba Santa (E. glutinosum)*

The HPLC method was applied to the quantification of homoeriodictyol enantiomers in Yerba Santa (Fig. 1). Yerba Santa has been reported to contain stereochemically enriched *S*(−)-homoeriodictyol [6]. As shown in Fig. 1C, Yerba Santa contains predominantly *S*(−)-homoeriodictyol (99.91%). The racemization process of homoeriodictyol under heated 25% methanol was examined. After racemization, the content of *S*(−)-homoeriodictyol decreased from 99.91% to 61.71% (Fig. 1D) and the content of *R*(+)-homoeriodictyol increased to 30.29%.

4. Conclusions

In summary, the developed stereospecific HPLC method for homoeriodictyol is sensitive, reproducible, and accurate. It has been applied for the first time to pharmacokinetic studies in rats and in examining the stereospecific concentrations of homoeriodictyol in organic and conventional lemon products (unpublished data). Further studies are ongoing in our laboratory to further characterize homoeriodictyol as well as other flavonoid enantiomers.

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