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Stereospecific analysis of sakuranetin by high-performance liquid chromatography: Pharmacokinetic and botanical applications☆

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ABSTRACT

A stereospecific method for analysis of sakuranetin was developed. Separation was accomplished using a Chiralpak® AD-RH column with UV (ultraviolet) detection at 288 nm. The stereospecific linear calibration curves ranged from 0.5 to 100 μg/mL. The mean extraction efficiency was >98%. Precision of the assay was <12% (relative standard deviation (R.S.D.)%), and within 10% at the limit of quantitation (0.5 μg/mL). Bias of the assay was lower than 10%, and within 5% at the limit of quantitation. The assay was applied successfully to pharmacokinetic quantification in rats, and the stereospecific quantification in oranges, grapefruit juice, and matico (*Piper aduncum* L.).

1. Introduction

The importance of understanding the stereospecific disposition of flavonoids has increased in the past decade. Our laboratory has developed assays for the separation, characterization, and detection of chiral flavonoids in biological matrices, fruits, and plants [1]. Sakuranetin, [(+/−)7-*O*-methylnaringenin] [2], or [(+/−)5,4′-dihydroxy-7-*O*-methoxyflavanone] [3,4] is a chiral flavanone [5–8] phytoalexin [9,10] that has been detected in rice (*Oryza sativa* L.) [3,8,11–13], fingerroot (*Boesenbergia pandurata*) [4], yerba santa (*Eriodictyon californicum*) [5], spiked pepper (*Piper aduncum*) [6], *Piper crassinervium* [7], *Piper lhotzkyanum* [14], *Ongokea gore* [15], and *Populous davidiana* [16]. Stereospecific quantification has yet to be performed.

Sakuranetin has been synthesized, induced, and produced in variety of ways. Synthesis of sakuranetin derived from naringenin involves naringenin 7-*O*-methyltransferase [3,11,12]. Induction of sakuranetin was achieved by ultraviolet (UV) light [3,10,11,17–19], blast infection [3,11], copper chloride ($CuCl_2$) [3,11,17,20], amino acid conjugates of jasmonic acid (JA) [3,12,17], methionine [17,20], coronatine [21], and chitosan oligomers [17] in rice leaves. This induction of sakuranetin could be counteracted by tiron [20], kinetin [21], and zeatin [21]. Production of sakuranetin in rice cells [11,21] and rice leaves [11,20,21] was accomplished by endogenously applying JA, ethylene, and ethephon.

Sakuranetin has been analyzed spectroscopically through the use of nuclear magnetic resonance (NMR) [7,15,16,22], infrared spectroscopy (IR) [7,16], UV [7,16], mass spectrometry (MS) [7], liquid chromatography–mass spectrometry (LC–MS) [8], high-pressure liquid chromatography (HPLC) [8], and electron ionization mass spectroscopy (EI-MS) [22]. In a study where volatiles released from rice leaves (extracted in methanol) were quantified, the presence of sakuranetin was detected using electrospray mass spectrometry in the positive ion mode [8].

When administered orally to mice, racemic sakuranetin exhibited significant elastase release [23] and inhibition of platelet aggregation, which was accompanied by a decrease in blood flow [22]. Sakuranetin has also shown to be a cytotoxic compound to KB nasopharyngeal carcinoma cells with an ED_{50} of 10 μg/mL[6]. Sakuranetin exhibited cyclooxygenase-1 (COX-1) inhibition with an IC_{50} value of 196.1 μM [16]. It has been previously shown that sakuranetin can be metabolized by *Cunninghamella elegans* [2] and *Escherichia coli* (*E. coli*) containing *Streptomyces avermitilis* [18]. Sakuranetin has demonstrated various plant defense roles and it has been reported to have antibacterial [6], antifungal [7,10–12,17–19], antimicrobial [10,12], and anti-inflammatory [23] properties.

To date, there are no published reports on the stereospecific separation and quantification of sakuranetin enantiomers in biological fluids, fruits, or herbs. This study reports the first stereospecific

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HPLC analysis for sakuranetin and its application for the quantification of sakuranetin enantiomers in biological matrices and botanicals.

2. Experimental

2.1. Chemicals and reagents

Racemic sakuranetin was purchased from Extrasynthèse (Genay, France). β-Glucuronidase type IX A (β-glucuronidase) and *Helix pomatia* β-glucuronidase type HP-2 (*H. pomatia*) were purchased from Sigma Chemicals (St. Louis, MO, USA). HPLC grade acetonitrile, methanol, and water were purchased from Fisher Scientific (Federal Way, WA, USA). Oranges and grapefruit juice were obtained from a local grocery store. Matico (*P. aduncum* L.) leaves were collected from the Tingo Maria region of the Huallaga river valley in the Peruvian tropical rainforest. Rats were obtained from Charles River Laboratories. Animal ethics were approved by the Institutional Animal Care and Use Committee at Washington State University.

2.2. Chromatographic system and conditions

The Shimadzu HPLC (Kyoto, Japan) system, consisted of a 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 integrations were carried out using Shimadzu EZ Start 7.1.1 SP1 software. The analytical column was a Chiralpak® AD-RH column (4.6 mm × 150 mm i.d., 5 μm particle size, Chiral Technologies Inc., Exton, PA, USA). The mobile phase consisted of methanol and water (95:5, v/v); prior to use was filtered and degassed. Separation was carried out isocratically at an ambient temperature at a flow rate of 0.6 mL/min, with ultraviolet detection at 288 nm.

2.3. Stock and working solutions

Racemic sakuranetin and 7-ethoxycoumarin (internal standard) solutions were prepared using methanol to obtain a final concentration of 100.0 μg/mL, protected from light, and stored at −20 °C for no more than 3 months. Standard curves were prepared at determined concentrations of 0.5, 1.0, 5.0, 10.0, 50.0, and 100.0 μg/mL (working standards) for each sakuranetin enantiomer.

2.4. Serum and urine disposition of sakuranetin in rats

Male Sprague–Dawley rats ($n = 3$, average mass ∼250 g) were jugular-vein cannulated and dosed intravenously with 10 mg/kg of racemic sakuranetin in 98% polyethylene glycol 400 (PEG 400) and 2% dimethyl sulfoxide (DMSO). Whole blood (500 μL) was collected at 0 h, 1 min, 1, 2, 4, 6 h post-dose and centrifuged for 5 min at 5000 rpm, collected serum was stored at −70 °C until analysis. Urine was collected at 0, 2, 6, 12, 24, 48, 72, 96, and 120 h post-dose and stored at −70 °C until analysis.

2.5. Sample preparation

2.5.1. Standard curves

Working standard (100 μL), internal standard (IS) (100 μL), and blank male rat serum were combined into 2.0 mL Eppendorf tubes and vortexed for 30 s. Cold acetonitrile (1.0 mL) was added to precipitate proteins. Working standards were centrifuged for 5 min at 5000 rpm. The supernatant was collected and dried to completion under compressed nitrogen gas. The dried working standard was reconstituted in mobile phase (400 μL), centrifuged for 5 min at 5000 rpm, transferred to HPLC vials, and 100 μL was injected into the HPLC system.

2.5.2. Serum

Serum samples collected from a pharmacokinetic study were separated into two (total and free) 2.0 mL Eppendorf tubes of 200 μL in each. To the total samples, 40 μL of β-glucuronidase was added and incubated at 37 °C for 2 h to liberate any glucuronide conjugates without decomposition of the parent compound [24]. To both free and total samples, 100 μL of IS and 1.0 mL of cold acetonitrile was added, vortexed for 30 s, centrifuged for 5 min at 5000 rpm. Supernatants were removed and dried to completion under compressed nitrogen, reconstituted in mobile phase (400 μL), centrifuged for 5 min at 5000 rpm, transferred to HPLC vials, and 100 μL was injected into the HPLC system. β-Glucuronidase from *E. coli* type IX-A cleaves specifically any glucuronidated metabolites back to the corresponding aglycone. Therefore, the samples without enzymatic hydrolysis (free samples) were utilized to determine the concentration of the aglycones, whereas the samples with enzymatic hydrolysis (total samples) were utilized to determine the concentration of the aglycones originally present plus the concentration of the major glucuronidated metabolites converted to their respective aglycones by the cleavage action of the enzyme. By subtracting the free sample concentration from the total sample, the stereospecific concentration of the glucuronidated metabolites can be calculated [24].

2.5.3. Urine

Urine samples collected from this pharmacokinetic study were separated into two (total and free) 2.0 mL Eppendorf tubes, 200 μL in each. To the total samples, as with the serum samples, β-glucuronidase was added and incubated as indicated in Section 2.5.2. To the free and total samples 100 μL of IS was added, samples were vortexed for 30 s, centrifuged for 5 min at 5000 rpm. Supernatants were removed and dried to completion under compressed nitrogen, reconstituted in mobile phase (400 μL), centrifuged for 5 min at 5000 rpm, transferred to HPLC vials, and 100 μL was injected into the HPLC system. As with the serum samples, β-glucuronidase from *E. coli* type IX-A cleaves specifically any glucuronidated metabolites back to the corresponding aglycone. Therefore, the same calculations can be utilized to determine the concentration of the aglycones and the metabolites in urine.

2.5.4. Oranges

The oranges were cut and pressed to isolate the juice, peel, flavedo, and albedo samples. The peel, flavedo, and albedo samples were frozen with liquid nitrogen and ground to a powder using a mortar and pestle.

2.5.4.1. Juice. The juice that was squeezed from the oranges was separated into a set of total and free samples, with 500 μL in each 15.0 mL conical tube. Samples were dried to completion and stored at −70 °C until further processing. *H. pomatia* is a β-glucuronidase that cleaves specifically the glycosylated sugar moiety of flavanones as previously described [25–29]. Therefore, free samples were utilized to determine the concentration of the aglycones, whereas the total samples were utilized to determine the concentration of the aglycones originally present plus the concentration of the glycosides converted to aglycones by the cleavage action of the enzyme. By subtracting the free sample concentration from the total sample, the concentration of glycoside epimers can be calculated.

To the total samples with *H. pomatia*, dried samples were reconstituted in 1.0 mL of cold water, followed by the addition of 110 μL of

0.78 M sodium acetate–acetic acid (pH 4.8), 100 μL of 0.1 M ascorbic acid, and 200 μL crude preparation of *H. pomatia* was incubated at 37 °C for 24 h as previously described [30].

After incubation, 1.0 mL of ice-cold acetonitrile was added to stop the reaction. IS (100 μL) was added to both the total and the free samples, vortexed for 30 s and centrifuged for 5 min at 5000 rpm. Supernatant was collected and dried under compressed nitrogen gas to completion. This residue was reconstituted with 400 μL of mobile phase, centrifuged for 5 min at 5000 rpm and transferred to HPLC vials and injected 100 μL.

2.5.4.2. Peel, flavedo, and albedo. The peel, flavedo, and albedo samples were separated into total and free samples (~0.100 g) in each 15.0 mL conical tube. Extractions were carried out with 1.5 mL of 100% methanol at an ambient temperature with a homogenizer for 1 min. Samples were vortexed for 30 s and centrifuged for 5 min at 5000 rpm. The total samples were incubated with *H. pomatia* as described in Section 2.5.4.1.

2.5.5. Grapefruit juice

Ruby red grapefruit juice was obtained and separated into a set of total and free samples with 500 μL in each 15.0 mL conical tube. Samples were dried to completion and stored at −70 °C until further processing. The free and total samples were processed as described in Section 2.5.4.1.

2.5.6. Plant

Collected leaves were air dried, ground to a fine power and extracted with 95% ethanol at an ambient temperature with constant shaking for 2 h. These extracts were dried under compressed nitrogen gas. The total samples were incubated with *H. pomatia* as described in Section 2.5.4.1.

2.6. Precision and accuracy

Within-run and between-run precision and accuracy was tested using replicate assays ($n = 9$) over the concentration 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.

2.7. Recovery and stability

Recovery of the sakuranetin enantiomers was analyzed over the concentration range of 0.5–100.0 μg/mL. The samples were prepared as described in Section 2.4. The extraction efficiency was determined by comparing the peak area ratio (PAR) of the sakuranetin enantiomers and 7-ethoxycoumarin to the PAR of the corresponding concentration injected directly into the HPLC without extraction.

Bench top stability of sakuranetin was investigated by preparing standard curve samples as in Section 2.5.1. Samples were injected, left in the auto-injector rack for 24 h at an ambient temperature and injected the following day.

2.8. Data analysis

Quantification of concentrations were calculated from standard curves based on the PAR of the sakuranetin enantiomers to the IS against sakuranetin concentrations using unweighted least squares linear regression.

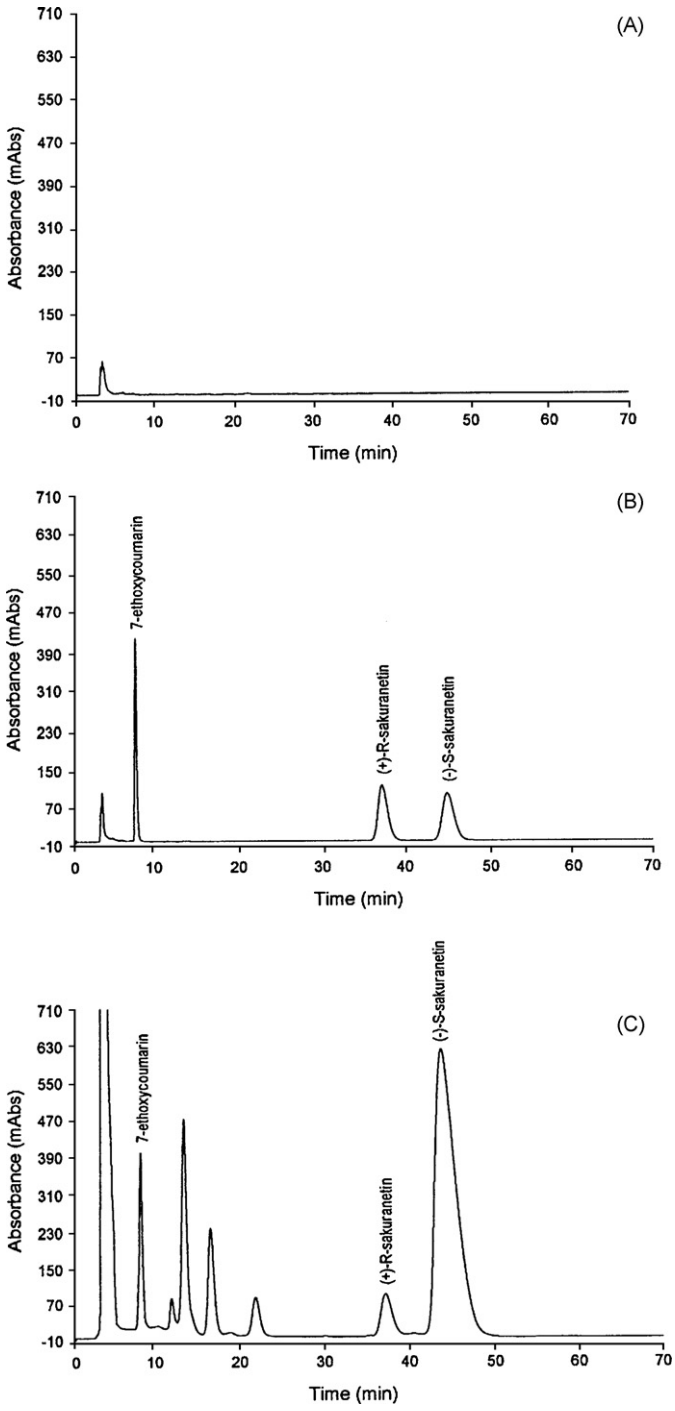

Fig. 1. Representative chromatograms of (A) sakuranetin-free serum (B) serum containing sakuranetin enantiomers (100 μg/mL), and (C) orange juice after incubation with *Helix pomatia* and 7-ethoxycoumarin was used as IS.

2.9. Pharmacokinetic analysis

WinNonlin® software (version 5.1) was used to calculate the pharmacokinetic parameters.

2.10. Circular dichroism

A stock solution of 500 μg/mL of racemic sakuranetin was injected into the HPLC. Fractions of each enantiomer peak were collected into separate 50 mL conical tubes, dried to completion under compressed nitrogen gas at an ambient temperature, and

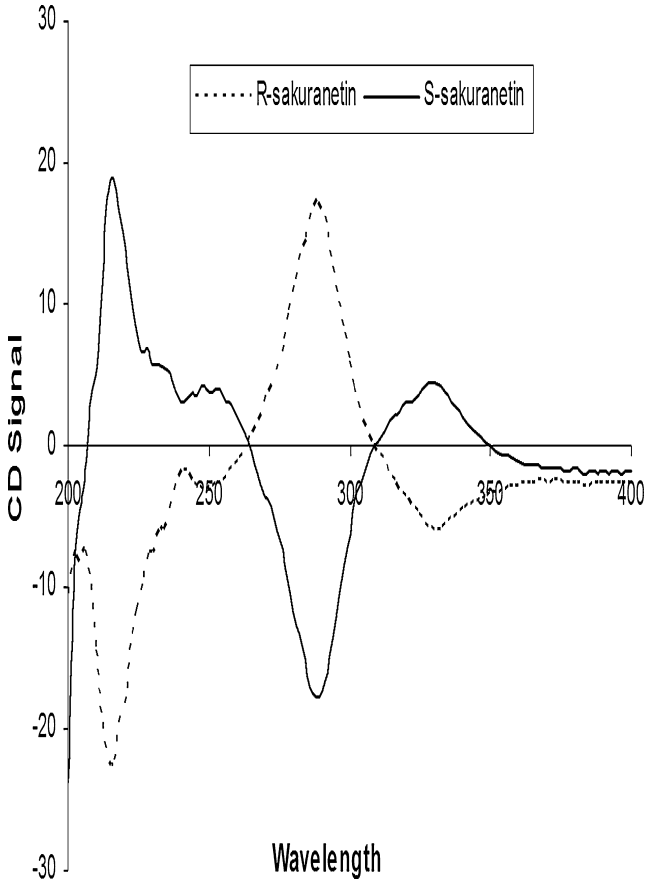

Fig. 2. Circular dichroism scans of individual sakuranetin enantiomers.

stored at −20 °C. These fractions were reconstituted in 2.0 mL of 100% HPLC grade methanol the day of the experiment. At an ambient temperature circular dichroism (CD) wavelength scans were collected using an AVIV Stopped Flow Circular Dichroism Spectrometer Model 202SF (AVIV Instruments Inc., Lakewood, NJ, USA) on the two collected enantiomer fractions.

3. Results and discussion

3.1. Chromatography

Separation of the sakuranetin enantiomers in serum was achieved (Fig. 1). Under reverse-phase conditions the IS eluted at

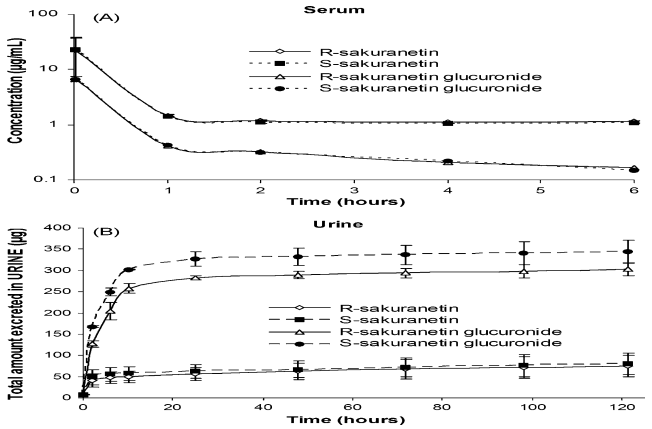

Fig. 3. Pharmacokinetic disposition the enantiomers of sakuranetin and sakuranetin glucuronide in (A) serum and (B) urine after intravenous administration of racemic sakuranetin (10 mg/kg) to rats ($n = 3$; mean ± S.D.).

Table 1
Within-run and between-run precision and accuracy of the assay for sakuranetin enantiomers ($n = 9$, mean, R.S.D. and bias)

Sakuranetin (µg/mL)	Observed		R.S.D. (%)				Bias (%)			
	Within-day		Between-day		Within-day		Between-day		Within-day		Between-day	
	R-Sakuranetin	*S*-Sakuranetin	*R*-Sakuranetin	*S*-Sakuranetin	*R*-Sakuranetin	*S*-Sakuranetin	*R*-Sakuranetin	*S*-Sakuranetin	*R*-Sakuranetin	*S*-Sakuranetin	*R*-Sakuranetin	*S*-Sakuranetin
0.5	0.49	0.48	0.49	0.48	6.95	9.41	6.95	9.41	−2.63	−4.81	−2.63	−4.81
1	0.97	0.90	0.97	0.96	3.24	11.42	3.24	3.26	−3.41	−9.69	−3.41	−3.88
5	4.82	4.83	5.04	5.01	4.14	4.24	0.95	6.31	−3.60	−3.31	0.82	0.19
10	10.09	10.18	10.47	10.03	8.52	9.50	9.93	3.92	0.89	1.84	4.72	0.28
50	50.27	50.41	49.54	49.52	3.69	3.84	1.61	3.41	0.56	0.81	−0.93	−0.96
100	99.74	99.82	100.60	100.22	0.88	0.68	1.97	0.80	−0.25	−0.18	0.60	0.22

Table 2
Recovery and stability of sakuranetin enantiomers ($n = 9$; mean ± S.D.)

Concentration (μg/mL)	Recovery (%)		Stability (%)	
	R-Sakuranetin	*S*-Sakuranetin	*R*-Sakuranetin	*S*-Sakuranetin
0.5	90.64 ± 0.034	87.53 ± 0.044	98.15 ± 0.019	101.02 ± 0.0097
1	96.58 ± 0.024	95.49 ± 0.031	98.01 ± 0.022	98.53 ± 0.016
5	93.78 ± 0.22	92.24 ± 0.28	100.83 ± 0.29	106.95 ± 0.23
10	104.82 ± 0.35	94.61 ± 0.39	99.25 ± 0.047	97.74 ± 0.15
50	99.84 ± 0.055	100.35 ± 0.12	99.65 ± 0.12	100.68 ± 0.24
100	100.08 ± 0.059	100.13 ± 0.094	99.67 ± 0.23	100.83 ± 0.54

8 min and the (+)-*R*- and (−)-*S*-sakuranetin enantiomers eluted at 39 and 46 min, respectively.

There were no interfering peaks co-eluting with the compounds of interest (Fig. 1A–C). The order of elution was verified by comparing collected circular dichroism data to circular dichroism data reported by Jerz et al. and Gaffield [15,31]. It was confirmed that the first eluted enantiomer peak is (+)-*R*-sakuranetin and the second eluted enantiomer peak is (−)-*S*-sakuranetin (Fig. 2).

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), stability of reconstituted extracts, precision, accuracy, and recovery. Various compositions of mobile phase were tested to achieve the best stereospecific elution of sakuranetin.

The selection of IS was determined after numerous attempts with other selected compounds. 7-Ethoxycoumarin was selected because of the large difference in retention time from the sakuranetin enantiomers which allows for baseline separation of IS and sakuranetin.

3.2. Linearity and limit of quantification

Linear relationships (average $R^2 = 0.9998$) were established for each sakuranetin enantiomer to the IS based on the PAR and working standard serum and urine concentrations. Mean regression lines from the precision and accuracy runs were described by (+)-*R*-sakuranetin (μg/mL) = 0.05 × −0.13 and (−)-*S*-sakuranetin (μg/mL) = 0.05 × −0.12. The limit of quantification of this assay in both serum and urine was 0.5 μg/mL which corresponded to an R.S.D. of 6.95 and 9.41% for (+)-*R*- and (−)-*S*-sakuranetin with a bias of −2.63 and −4.81%, respectively (Table 1). The calculated concentration of QC samples was within the accepted criteria.

3.3. Precision and accuracy

Within-run and between-run precision was calculated in replicate assays ($n = 9$) of the sakuranetin enantiomers was <12% over the standard concentration range. The intra- and inter-run bias assessed during the replicate assays for the sakuranetin enantiomers varied between −9.69 and 4.72%. These data indicates that the developed HPLC method is reproducible and accurate.

3.4. Recovery and stability

The mean extraction efficiency for the sakuranetin enantiomers from rat serum and urine varied from 87.53 to 90.64% (Table 2). No significant degradation of sakuranetin was detected in samples that investigated bench top stability over the 24-h period compared to initial values.

3.5. Stereospecific disposition of sakuranetin in rats

The HPLC method has been applied successfully to stereospecific determination of sakuranetin in rat serum and urine. Following intravenous (IV) administration of 10 mg/kg of sakuranetin, sakuranetin enantiomers were detected in rat serum as both the glucuronidated metabolite and the free aglycone (Fig. 3A). The serum half-life of the parent compound (sakuranetin) was determined to be ∼19 h for both enantiomers. The area under the curve (AUC_∞) is the total amount of exposure of the drug to the body over time is 225.68 ± 58.34 and 260.10 ± 50.23 μg h/mL for (+)-*R*- and (−)-*S*-sakuranetin, respectively. The volume of distribution (V_{ss}) values can be reported as 0.40 ± 0.057 and 0.64 ± 0.076 L/kg for (+)-*R*- and (−)-*S*-sakuranetin, respectively. Sakuranetin enantiomers in serum appeared to demonstrate comparable pharmacokinetic disposition patterns which parallels our findings with other stereogenic flavonoids [25–29,32].

In urine, the half-life in the (+)-*R*- and (−)-*S*-sakuranetin enantiomers is approximately 27 and 35 h, respectively. The fraction excreted unchanged in urine (F_e) for the (+)-*R*-sakuranetin is 6.97 ± 3.08% and (−)-*S*-sakuranetin is 9.86 ± 2.71%. For sakuranetin as well as its major metabolite, there appears to be enantioselective renal excretion (Fig. 3B).

3.6. Quantification sakuranetin in oranges, grapefruit juice, and matico (*P. aduncum L.*)

The HPLC method has been applied to the stereospecific quantification of sakuranetin in oranges, grapefruit juice, and matico. Application of this novel HPLC method allowed for the direct quantification of sakuranetin enantiomers and for the indirect quantification of sakuranetin glycoside epimers. (−)-*S*-Sakuranetin was the predominant form found in oranges, grapefruit juice, and matico. Both enantiomers were found predominantly in a glycosidic form. The highest concentration of (−)-*S*-sakuranetin glycoside of the samples examined was the orange juice, followed by the peel, albedo, flavedo, grapefruit juice, and matico (Table 3). Sakuranetin in oranges, grapefruit, and matico has yet to be reported in the United States Department of Agriculture (USDA) Database for the Flavonoid Content of Selected Foods [33].

Table 3
Stereospecific concentrations in oranges, grapefruit juice, and matico (*Piper aduncum* L.) cleaved by *H. pomatia*

	R-Sakuranetin (μg/mL)		*S*-Sakuranetin (μg/mL)	
	Aglycone	Glycoside	Aglycone	Glycoside
Orange juice	0.21[a]	55.63	2.42	737.08
Orange peel	0.45[a]	9.55	0.26[a]	559.87
Orange flavedo	0.21[a]	12.63	0.23[a]	265.97
Orange albedo	0.21[a]	32.63	0.26[a]	397.66
Grapefruit juice	1.97	58.86	1.107	63.44
Matico	3.40	4.15	10.11	ND

[a] Values are below the LOQ; ND, not detectable.

4. Conclusion

In summary, the developed stereospecific HPLC assay is reproducible, sensitive, and accurate. It has been successfully applied to the stereospecific quantification of sakuranetin and glucuronidated metabolite in serum and urine from rats and to the stereospecific quantification and determination of sakuranetin and its glycoside in oranges, grapefruit juice, and matico (*P. aduncum* L.). Detection of sakuranetin enantiomers has been reported for the first time in oranges, grapefruit juice, and matico. Further studies are being conducted in our laboratory to further characterize sakuranetin pharmacokinetics and pharmacological activity of each enantiomer.

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