



## Stereospecific high-performance liquid chromatographic assay of isosakuranetin in rat urine<sup>☆</sup>

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### ABSTRACT

A stereospecific method of analysis of racemic isosakuranetin (5,7-dihydroxy-4'-methoxyflavanone) in biological fluids is necessary to study pharmacokinetics. A simple high-performance liquid chromatographic method was developed for the determination of isosakuranetin enantiomers. Separation was achieved on a Chiralpak<sup>®</sup> AD<sup>TM</sup>-RH column with ultraviolet (UV)-detection at 286 nm. The standard curves in urine 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 isosakuranetin enantiomers in rat urine.

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### 1. Introduction

Isosakuranetin, a flavanone flavonoid, has a chiral carbon center and thus exists in two enantiomeric forms (Fig. 1A). The stereochemistry of flavanones has been widely studied; carbon-2 acts as the chiral center that can occur in either the *S* or *R* configurations [1,2]; with the 2*S* configuration being predominant in nature [3] (Fig. 1B and C). Isosakuranetin has been identified as an important component of propolis [4–6], *Baccharis dracunculifolia* [7,8], *Terminalia fagifolia* [9], *Chromolaena odorata* [10], *Eupatorium odoratum* [11], and *Citrus sinensis* [12]. Didymine (isosakuranetin 7-rutinoside, Fig. 1B) and poncirin (isosakuranetin 7-neohesperidoside, Fig. 1C), glycosides of isosakuranetin, have been described in *Citrus bergamia* [13], *C. sinensis* [12,14], *Citrus paradisi* [12,15], and *Poncirus trifoliata* [16–21]. Flavanones in nature are found mostly as glycosides, attached to β-neohesperidose or β-rutinoside through the *c*-7 hydroxy group [15,22]. The flavanone neohesperidosides and rutinosides are mainly distinguished by their taste properties: the neohesperidosides are bitter, whereas the rutinosides are tasteless [22]. Hot alkali on 7-β-neohesperidosides splits off the B-ring and carbon-2 to yield phloracetophenone 4'-β-neohesperidoside;

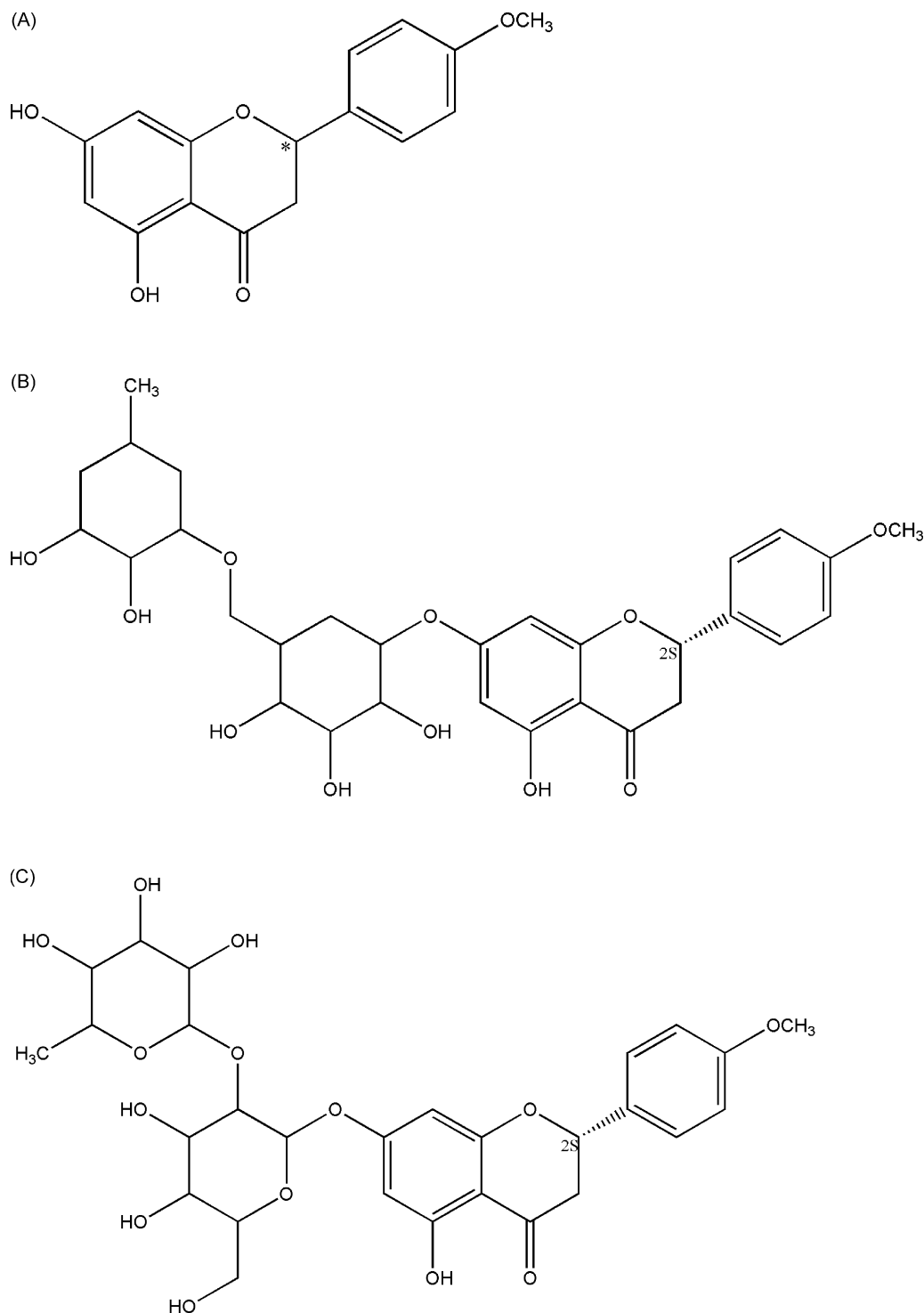
however, 7-β-rutinosides do not display phloracetophenone 4'-β-rutinoside formation when exposed to hot alkali but instead generate a sugar–aglycone bond split [22].

Methods used to identify isosakuranetin or its glycosides include paper chromatography [2], mass spectrometry [10], micellar electrokinetic chromatography (MECK) [23–25], capillary electrophoresis (CE) [25,26], high-performance liquid chromatography (HPLC) [1,4,5,7,12–16,18,27,28], HPLC–diode array detection–electrospray ionization mass spectrometry (DAD–ESI–MS) [14,29,30], and nuclear magnetic resonance (NMR) spectrometry [10,17].

Nevertheless, enantiomeric separation of isosakuranetin has only been attempted by a few groups including Asztemborska et al. [23], Park and Jung [24], Wistuba et al. [25], Kwon et al. [26], and Krause and Galensa [28] by means of MECK [23–25], CE [25,26], and HPLC [28]. Using sodium cholate (SC) and γ-cyclodextrin (γ-CD) as chiral modifiers with MECK, Asztemborska et al. described enantioseparation of isosakuranetin; however, resolution was poor (*R*<sub>s</sub> = 0.84) and complete separation was not accomplished. Wistuba et al. instead used sodium dodecyl sulphate (SDS) and γ-CD with MECK, obtaining higher resolution (*R*<sub>s</sub> = 1.78). Park and Jung applied highly sulphated cyclosophoraoses to MECK and obtained high-resolution (*R*<sub>s</sub> = 1.483) but like the other methods described for MECK the lack of commercial availability of this chiral selector limits the utility of this method. Enantioseparation of isosakuranetin was reported by Kwon et al. using cyclic β-(1→3), (1→6)-glucans from *Bradyrhizobium japonicum* with CE, high resolution was

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**Fig. 1.** Chemical structure of (A) isosakuranetin, (B) didymin, and (C) poncirin. The asterisk (\*) denotes the chiral center.

achieved ( $R_s = 1.41$ ), but the lack of commercial sources for this chiral selector also restricts the utility of this method. Likewise, Wistuba et al. developed a method using CE and anionic cyclodextrin derivatives as buffer additives. High resolution ( $R_s = 3.43$ ) was obtained with this method using sulfato- $\beta$ -CD at pH 7; however, this chiral selector is not commercially available. Finally, Krause and Galensa reported enantiomeric separation of isosakuranetin using cellulose triacetate as stationary phase in HPLC; baseline resolution is stated but no chromatographic data or  $R_s$  value is provided and

validation in biological fluids was not accomplished. Nevertheless, the authors concluded that the selectivity between enantiomers is not sufficient since enantiomers appear in a relatively narrow retention zone [28].

Isosakuranetin has been previously described to have antimycobacterial [10], antifungal [31], antioxidant [5,32], antibacterial [19], neuroprotective [33], anticancer [9,19,34], and antiallergic [21] properties. Poncirin and didymin were found to have numerous biological activities such as anti-inflammatory [16,17,20],

antioxidant [32], anticancer [34], anti-platelet [34], immunomodulatory [35], and anti-atherogenic [30] properties. However, there is a lack of information regarding the stereospecific activity or disposition of isosakuranetin enantiomers in biological matrices like urine and serum. Achiral analysis of isosakuranetin may be misleading in that absorption, distribution, metabolism and elimination may be stereoselective. Measuring enantiomers may facilitate establishment of more meaningful concentration effect relationships. Separation of enantiomers in urine is thus important to comprehensively understand the stereospecificity of action and elimination of isosakuranetin. The method described in the present study is the first validated method for the separation of isosakuranetin enantiomers under isocratic HPLC conditions using a polar organic mode applied to biological matrices with an internal standard and also employs a commercially available chiral column. Complete baseline separation of enantiomers with a 13-min retention time difference is of great advantage since it will facilitate the isolation of each enantiomer.

## 2. Experimental

### 2.1. Chemicals and reagents

Racemic isosakuranetin was purchased from Indofine Chemical Company (NJ, USA). Didymine (2*S*-isosakuranetin 7-rutinoside) was purchased from Extrasynthèse (Genay, France). 7-Ethoxycoumarin,  $\beta$ -glucuronidase from *Escherichia coli* Type IX A were purchased from Sigma Chemicals (MO, USA). HPLC-grade methanol was purchased from J. T. Baker (NJ, 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-10AT-VP pump, a SIL-10AF auto-injector, a SPD-M10A VP spectrophotometric diode array 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 Chiralpak® AD™-RH column (150 mm  $\times$  4.6 mm i.d., 5- $\mu$ m particle size, Chiral Technologies Inc., PA, USA). The mobile phase consisted of 100% HPLC-grade methanol filtered and degassed. Separation was carried out isocratically at  $25 \pm 1^\circ\text{C}$ , a flow rate of 0.40 ml/min, with ultraviolet (UV)-detection at 286 nm.

### 2.3. Stock and working standard solutions

Racemic isosakuranetin and 7-ethoxycoumarin (internal standard) solutions of 100.0  $\mu\text{g/ml}$  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  $\mu\text{g/ml}$  of each isosakuranetin enantiomer.

### 2.4. Sample preparation

To the working standards or urine samples (0.1 ml), 100  $\mu\text{l}$  of 7-ethoxycoumarin (internal standard) was added into 2.0 ml Eppendorf tubes. The mixture was vortexed (Vortex Genie-2, VWR Scientific, West Chester, PA, USA) for 1 min and centrifuged at 5000 rpm for 5 min (Beckman Microfuge centrifuge, Beckman Coulter Inc., Fullerton, CA, USA). The supernatant was collected and evaporated to dryness under compressed nitrogen gas using an N-Evap analytical evaporator (Organomation Associates, Inc., Berlin, MA, USA). The residue was reconstituted

with 200  $\mu\text{l}$  of mobile phase, vortexed for 1 min and centrifuged at 5000 rpm for 5 min, the supernatant was then transferred to HPLC vials and 150  $\mu\text{l}$  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 at 0.5, 1.0, 5.0, 10.0, 50.0 and 100.0  $\mu\text{g/ml}$  on the same day and on six different days within 1 week, respectively. The precision was evaluated by the relative standard deviation (RSD). The accuracy was estimated based on the mean percentage error of measured concentration to the actual concentration [36].

### 2.6. Recovery

The relative and absolute recovery for isosakuranetin enantiomers from biological fluids were assessed at 0.5, 1.0, 5.0, 10.0, 50.0 and 100.0  $\mu\text{g/ml}$  and the recovery of the internal standard was evaluated at the concentration used in sample analysis (100  $\mu\text{g/ml}$ ). The samples were prepared as described in Section 2.4. Urine was used as the matrix for the relative recovery studies, whereas water was used for the absolute recovery studies. A known amount of racemic isosakuranetin or 7-ethoxycoumarin was spiked into 0.1 ml blank rat urine or water to give the above concentrations. The extraction efficiency was determined by comparing the peak area ratio (PAR) of enantiomeric isosakuranetin and 7-ethoxycoumarin to the PAR of corresponding concentration injected directly in the HPLC without extraction.

### 2.7. Freeze–thaw and bench-top stability of isosakuranetin samples

The freeze–thaw stability of isosakuranetin enantiomers was evaluated in triplicate at 0.5 and 100.0  $\mu\text{g/ml}$  using quality control (QC) samples [37]. These samples were analyzed without being frozen at first, and then stored at  $-70^\circ\text{C}$  and thawed at room temperature ( $25 \pm 1^\circ\text{C}$ ) for three cycles.

The stability of isosakuranetin in reconstituted extracts during run-time in the HPLC auto-injector was investigated using pooled extracts from QC samples of two concentration levels 0.5 and 100.0  $\mu\text{g/ml}$  [37]. 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 the auto-injector ( $25 \pm 1^\circ\text{C}$ ).

### 2.8. Pharmacokinetic disposition of isosakuranetin in rats

Male Sprague–Dawley rats ( $n=3$ , average weight  $\sim 250$  g) were placed in a metabolic cage, and fasted for 12 h before dosing. The rats were cannulated [38] and dosed intravenously with 10 mg/kg racemic isosakuranetin in polyethylene glycol 400 and 1% dimethyl sulfoxide (DMSO) via the jugular vein. Urine samples (0.30 ml) were collected at 0–4, 4–8, 8–24, 24–48, 48–72, and 72–96 h and stored at  $-20^\circ\text{C}$  until analysis. Urine samples (0.1 ml) were assayed in duplicate with or without the addition of 40  $\mu\text{l}$  of 500 U/ml  $\beta$ -glucuronidase IX-A and incubated in a shaking water bath at  $37^\circ\text{C}$  for 2 h to liberate glucuronide conjugates [39]. The samples were evaporated under compressed nitrogen gas using an analytical evaporator after the addition of 100  $\mu\text{l}$  of internal standard. The residue was reconstituted with 200  $\mu\text{l}$  of mobile phase, vortexed for 30 s and centrifuged at 5000 rpm for 5 min, the supernatant was transferred to HPLC vials and 150  $\mu\text{l}$  of it was injected into the HPLC system.

## 2.9. Data analysis

Quantification was based on calibration curves constructed using PAR of isosakuranetin enantiomers to internal standard 7-ethoxycoumarin, against isosakuranetin concentrations using unweighted least squares linear regression.

## 2.10. Pharmacokinetic statistical analysis

Data were presented as mean and standard error of the mean (mean  $\pm$  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  $p < 0.05$ .

# 3. Results and discussion

## 3.1. Chromatography

Separation of isosakuranetin enantiomers and the internal standard in biological fluids was successfully achieved (Fig. 2). No interfering peaks co-elute with the compounds of interest (Fig. 2A). Didymine, the 7-rutinoside of isosakuranetin is described to be in the 2*S* configurational state and is sold as *S* configuration by Extrasynthèse [1,22,40]. When didymine was cleaved to its aglycone using  $\beta$ -glucuronidase and analyzed under our chromatographic conditions, the 2*S* configuration eluted at 38 min. The retention times of 2*S*- and 2*R*-isosakuranetin were approximately 38 and 51 min, respectively. The internal standard eluted at approximately 10 min (Fig. 2B). Optimal separation was achieved with 100% HPLC-grade methanol and a flow rate of 0.40 ml/min.

## 3.2. Linearity and LOQ

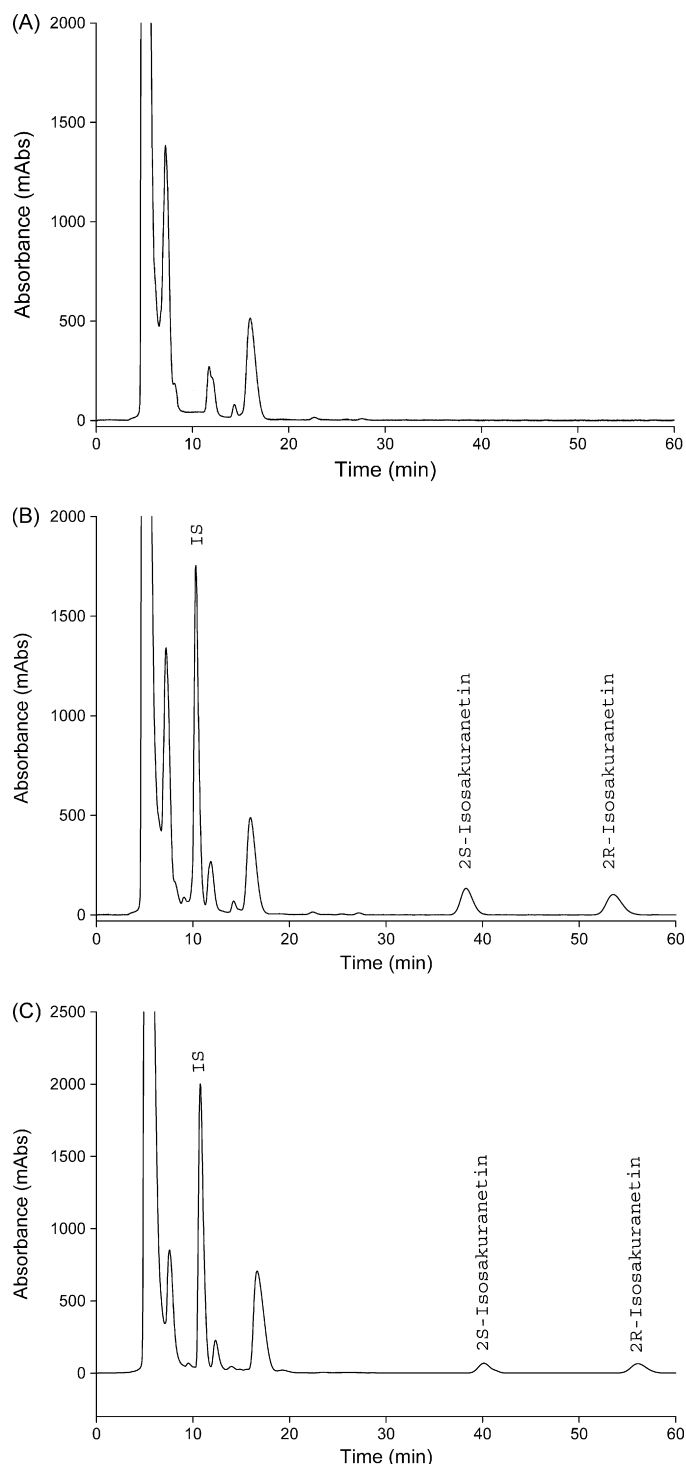
Excellent linear relationships ( $r^2 = 0.999$ ) were demonstrated between PAR of isosakuranetin enantiomers to the internal standard and the corresponding urine concentrations of isosakuranetin enantiomers over a range of 0.5–100  $\mu\text{g/ml}$ . Mean regression lines from the validation runs were described by 2*S*-isosakuranetin ( $\mu\text{g/ml}$ ) =  $0.0304x - 0.0081$  and 2*R*-isosakuranetin ( $\mu\text{g/ml}$ ) =  $0.0302x - 0.0068$ . LOQ of this assay was 0.5  $\mu\text{g/ml}$  with the corresponding between-day RSD of 7.97 and 10.90% for 2*S*- and 2*R*-isosakuranetin, respectively and bias of 3.26 and 2.11% for 2*S*- and 2*R*-isosakuranetin, 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 (RSD) calculated during replicate assays ( $n = 6$ ) of isosakuranetin enantiomers was <15% over a wide range of concentrations (Table 1). The intra- and inter-run bias assessed during the replicate assays for isosakuranetin enantiomers varied between  $-0.72$  and  $13.82\%$  (Table 1). These data indicated that the developed HPLC method is reproducible and accurate. The mean extraction efficiency for isosakuranetin enantiomers from biological fluids varied from 86.93 to 104.05% (Table 2). Absolute recovery, in comparison, varied from 93.39 to 109.97%.

## 3.4. Stability of isosakuranetin samples

No significant degradation was detected after the samples of racemic isosakuranetin in biological fluids following three freeze–thaw cycles. Recoveries of 2*S*- and 2*R*-isosakuranetin were respectively from 91.36 to 104.05% and 86.93 to 101.72% following three freeze–thaw cycles for isosakuranetin QC samples



**Fig. 2.** Representative chromatograms, of (A) drug-free rat urine demonstrating no interfering peaks co-eluted with the compounds of interest; (B) rat urine containing isosakuranetin enantiomers each with concentration of 10  $\mu\text{g/ml}$  and the internal standard, 7-ethoxycoumarin; and (C) rat urine containing isosakuranetin at 6 h after IV administration of racemic isosakuranetin. Detection of isosakuranetin enantiomers was attained at 286 nm, using flow rate of 0.40 ml/min of 100% HPLC-grade methanol on a Chiralpak<sup>®</sup> AD<sup>TM</sup>-RH column.

of isosakuranetin or 7-ethoxycoumarin. There was no significant decomposition observed after the reconstituted extract of racemic isosakuranetin was stored in the auto-injector at room temperature for 24 h; the measurements were from 90.06 to 98.52% of the initial value for extracts of racemic isosakuranetin in biolog-

**Table 1**Within- and between-day precision and accuracy of the assay for isosakuranetin (ISK) enantiomers in rat urine ( $n=6$ , mean, RSD, and Bias)

Added	Isosakuranetin concentration ( $\mu\text{g/ml}$ )									
	Observed				RSD%				Bias%	
	With in-day		Between-day		With in-day		Between-day		With in-day	Between-day
	2S-ISK	2R-ISK	2S-ISK	2R-ISK	2S-ISK	2R-ISK	2S-ISK	2R-ISK	2S-ISK	2R-ISK
0.5	0.52	0.51	0.52	0.52	8.58	14.49	7.97	10.90	3.26	2.11
1	0.97	0.97	1.01	1.14	8.05	0.22	11.77	5.55	-2.53	-3.29
5	5.36	5.30	4.90	4.77	3.24	4.23	10.67	9.60	7.20	5.96
10	10.90	10.96	10.43	10.17	1.34	2.08	10.32	10.54	8.97	9.57
50	51.85	52.02	51.95	52.51	1.18	1.77	5.62	3.41	3.71	4.04
100	99.89	99.85	99.27	99.28	0.36	0.14	1.54	0.97	-0.11	-0.15

ical fluids of 0.5 and 100.0  $\mu\text{g/ml}$ . The use of  $\beta$ -glucuronidase has previously been shown to have no effect on the stability and selectivity of enantioseparation assays of similar chiral flavanones [41].

### 3.5. Stereospecific pharmacokinetics of isosakuranetin in rats

The assay method was applied to the stereospecific determination of isosakuranetin in the urinary excretion study in rats ( $n=3$ , Fig. 2C). Following IV administration of racemic isosakuranetin, isosakuranetin enantiomers were detected in urine primarily as glucuro-conjugates. In Fig. 3 we report a cumulative total amount excreted in urine versus time plot in which the predominance of isosakuranetin glucuronides are demonstrated for both enan-

tiomeric forms over the aglycone. In addition, the cumulative excretion is stereoselective in favor of the 2S configuration.

## 4. Conclusions

In summary, the developed stereospecific HPLC method for isosakuranetin is sensitive, reproducible, and accurate. It has been applied for the first time to pharmacokinetic studies in rats. Further studies are ongoing in our laboratory to further characterize isosakuranetin as well as other flavonoid enantiomers activity and disposition.

## Acknowledgements

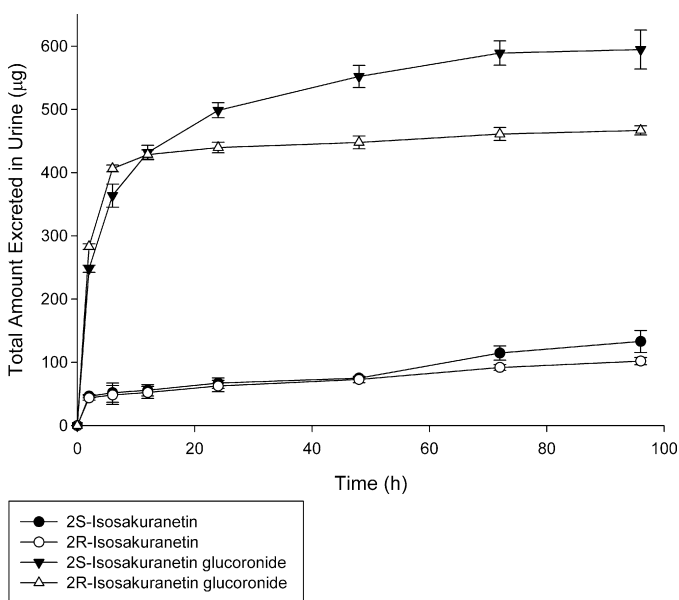
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**Table 2**Recovery of isosakuranetin enantiomers from rat urine ( $n=6$ )

Concentration ( $\mu\text{g/ml}$ )	Recovery (%) (mean $\pm$ SD)	
	2S-Isosakuranetin	2R-Isosakuranetin
0.5	100.25 $\pm$ 7.99	101.72 $\pm$ 11.08
1	104.05 $\pm$ 12.25	96.97 $\pm$ 12.54
5	91.36 $\pm$ 9.75	86.93 $\pm$ 8.71
10	95.69 $\pm$ 9.87	94.03 $\pm$ 8.49
50	102.22 $\pm$ 3.87	98.98 $\pm$ 5.35
100	99.46 $\pm$ 1.05	99.78 $\pm$ 1.23



**Fig. 3.** Total amount excreted in urine of enantiomeric isosakuranetin and its glucuro-conjugates following administration of racemic isosakuranetin (10 mg/kg) to rats ( $n=3$ , mean  $\pm$  S.E.M.).

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