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High-performance liquid chromatographic determination of 3′-hydroxy-5′-(4-isobutyl-1-piperazinyl)benzoxazinorifamycin (KRM-1648) and its deacetyl metabolite in plasma, whole blood, urine and tissue samples in rats

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Abstract

A reversed-phase high-performance liquid chromatographic method was developed for the determination of 3′-hydroxy-5′-(4-isobutyl-1-piperazinyl)benzoxazinorifamycin (KRM-1648, I), a new rifamycin derivative, and its 25-deacetyl metabolite (KRM-1671, II) in plasma, whole blood, tissues and urine from rats. I and II were coextracted with an internal standard from each sample matrix by solid-phase extraction (Bond Elut). Plasma and urine were directly loaded onto Bond Elut, while whole blood and tissues were homogenized and extracted with methanol or dichloromethane–chloroform prior to Bond Elut extraction. The extracts were chromatographed on Shim-pack CLC-ODS(M) using acetonitrile–0.02 *M* citrate buffer containing 0.1 *M* sodium perchlorate (2:1, v/v), and peaks were detected at 643 nm. The validation data showed that the assays for I and II in plasma, whole blood, tissues and urine were selective, accurate and reproducible.

1. Introduction

KRM-1648, 3′-hydroxy-5′-(4-isobutyl-1-piperazinyl)benzoxazinorifamycin (I), is a new antibiotic with excellent antimicrobial activity, especially against *Mycobacterium tuberculosis* and *Mycobacterium avium* complex [1–5]. One of its major metabolites is 25-deacetyl-3′-hydroxy-5′-(4-isobutyl-1-piperazinyl)benzoxazinorifamycin, which also has antimicrobial activity [6]. A sensitive, selective, and reproducible method for determination of I and its metabolite is necessary to assess the pharmacokinetic properties of the parent compound. Many high-performance liquid chromatographic (HPLC) methods for related rifamycin derivatives, rifampicin, rifapentine and rifabutin have been developed to determine the plasma, biliary and urinary levels of parent drugs and their 25-deacetyl metabolites [7–10]. These methods relied on liquid–liquid extraction for sample clean-up, causing lengthy handling times and low sensitivity. Therefore, radiochemical determination methods were developed for tissue distribution studies of these compounds [11–13].

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The purpose of this study was to develop a sensitive, selective and reproducible HPLC method for the simultaneous determination of I and its 25-deacetyl metabolite (KRM-1671, II) not only in plasma and urine, but also in whole blood and tissues. Solid-phase extraction was employed as a sample clean-up procedure, resulting in a rapid and quantitative extraction of I and II from a complex sample matrix. These compounds were completely separated by reversed-phase chromatography and sensitively measured by Vis detection with a limit of quantitation of 0.02 to 0.08 μg/ml for the biological fluids and 0.2 μg/g for tissues for both analytes.

2. Experimental

2.1. Materials

The analytes, 3′-hydroxy-5′-(4-isobutyl-1-piperazinyl)benzoxazinorifamycin (I) and 25-deacetyl-3′-hydroxy-5′-(4-isobutyl-1-piperazinyl)benzoxazinorifamycin (II), as well as the internal standard 3′-hydroxy-5′-(4-isopentyl-1-piperazinyl)benzoxazinorifamycin (I.S.) were synthesized in our laboratory and their chemical structures are shown in Fig. 1. Acetonitrile and water used for the mobile phase were HPLC grade (Nacalai Tesque, Kyoto, Japan). All reagents and buffer solutions were prepared with analytical-reagent grade chemicals. The Bond Elut C_8 column (500 mg) and Bond Elut LRC SI column were supplied by Analytichem International (Harbor City, CA, USA). Biological fluids or tissues were obtained from male Crj-CD rats of eight to ten weeks old (Charles River Japan, Kanagawa, Japan).

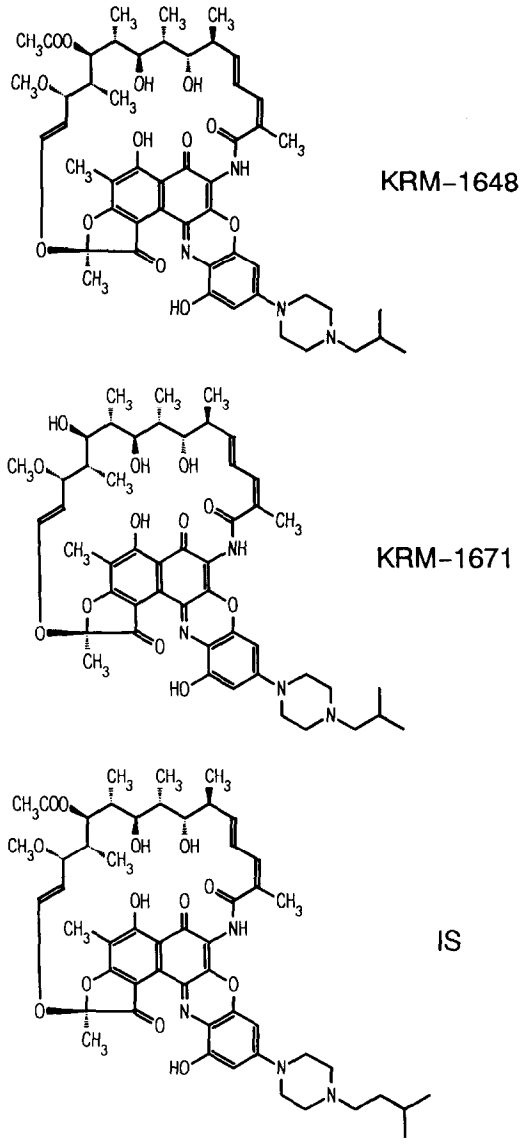

Fig. 1. Chemical structures of I (KRM-1648), II (KRM-1671) and internal standard (I.S.).

2.2. Extraction procedure

Plasma

The I.S. (20 μl of 15 μg/ml in methanol) was added to 1.0 ml of plasma and the mixture was loaded onto the Bond Elut C_8 column conditioned with 3 ml of methanol and 6 ml of 5% methanol. The column was washed with 3 ml of water and allowed to air dry. I and II retained on the column were then eluted with 2 ml of ethyl acetate–methanol (1:1, v/v). The eluate was evaporated to dryness with a centrifugal evaporator and the residue was reconstituted in 200 μl of methanol. The samples were transferred to glass autosampler vial inserts, and 50 μl were injected onto the chromatographic system.

Whole blood

A 0.4-ml sample was placed in a 10-ml glass-stoppered tube, and the I.S. (20 μl of 20 μg/ml in methanol) and 5 ml of methanol were added to 0.4 ml of whole blood. The mixture was homogenized by sonication for 10–20 s, and then shaken using a mechanical shaker (Yayoi, Tokyo, Japan) for 10 min at 240 vertical strokes/min followed by vacuum filtration through a filter paper. The filtrate was evaporated to dryness, and the residue was dissolved in 10 ml of chloroform. After the solution was loaded onto the Bond Elut LRC SI column conditioned with 10 ml of chloroform, the column was then washed with 6 ml of *n*-hexane and allowed to air dry. I and II retained on the column were then eluted with 2 ml of acetone. The elute was evaporated, and reconstituted in the same way as described in the plasma clean-up procedure.

Urine

Urine samples were treated using the same procedures as for the plasma samples, with the following exceptions: I.S. was added in a concentration of 25 μg/ml, elution was performed with 6 ml of ethyl acetate–methanol (1:1, v/v), and the sample was reconstituted in 400 μl of methanol and a 40-μl volume was injected.

Tissues

After the I.S. (20 μl of 20 μg/ml in methanol) and 10 ml of dichloromethane–chloroform (1:4, v/v) were added to 0.2 g of well-minced tissue samples, the tissue was homogenized using a Physcotron handy microhomogenizer (NITI-ON Medical Supply, Funabashi, Japan) for 30–60 s. The sample homogenates were centrifuged at 800 *g* for 5 min and filtered through filter paper. The filtrate was subjected to the same sample clean-up procedure using a Bond Elut LRC SI column employed in the whole blood treatment, except for reconstitution in 400 μl of methanol and a 20-μl injection volume.

2.3. Apparatus and HPLC conditions

The apparatus used for the analysis of plasma, whole blood, lung, and urine samples consisted of a Model LC-10AD pump (Shimadzu, Kyoto, Japan), a Model L-4200 UV-Vis detector (Hitachi, Tokyo, Japan), a Wisp 712 automatic sample processor (Waters, Associates, Milford, MA, USA) and a model C-R4A Chromatopac data processor (Shimadzu). Analyses of other tissue samples were performed with the following apparatus: a Model 880-PU pump (Japan Spectroscopic, Tokyo, Japan), a Model 870-UV detector (Japan Spectroscopic), a Wisp 712 automatic sample processor (Waters) and a Model C-R4A Chromatopac data processor (Shimadzu). Samples (20 to 50 μl) were injected onto a guard column of LiChroCART (LiChrospher 100, RP-18 endcapped, 5 μm, 20 mm × 4.6 mm I.D., E. Merck, Darmstadt, Germany) in series with a Shim-pack CLC-ODS(M) column (5 μm, 250 mm × 4.6 mm I.D., Shimadzu) maintained at 40°C. The mobile phase was acetonitrile–0.02 *M* citrate buffer containing 0.1 *M* sodium perchlorate, pH 4.3 (2:1, v/v) at a flow-rate of 1.0 ml/min. The eluate was monitored at a visible wavelength of 643 nm.

2.4. Calibration

A standard solution containing I and II, and an I.S. solution were separately prepared in methanol at concentrations of 1 mg/ml. Each of the standard solutions were diluted with methanol to obtain spiking solutions. Standard curve samples of various biological fluids or tissues were freshly prepared by spiking aliquots of the standard solutions into the blank samples and were cleaned up and analyzed as described above. Peak-area ratios of each analyte to the I.S. were measured and the calibration curves were constructed by an unweighed least-squares linear regression analysis.

2.5. Precision and accuracy

The precision and accuracy of the assay were assessed by the intra- and inter-assay coefficients of variation (C.V.) and relative errors (deviation between the concentration spiked and found) by determining each of two analytes at three concentrations (low, medium and high).

2.6. Absolute recoveries

Absolute recoveries of both analytes and the internal standard were determined as follows: the concentrations of each compound in spiked rat sample at one or two doses (low and high) was determined using the external standard method (single-point calibration, peak area) and compared with theoretical values.

3. Results and discussion

3.1. Selectivity

Typical chromatograms of blank rat samples (plasma, whole blood, lung, liver, kidney, spleen, abdominal adipose tissue and urine), spiked rat samples and rat samples obtained 8 h after an oral dose of 30 mg/kg of I are shown in

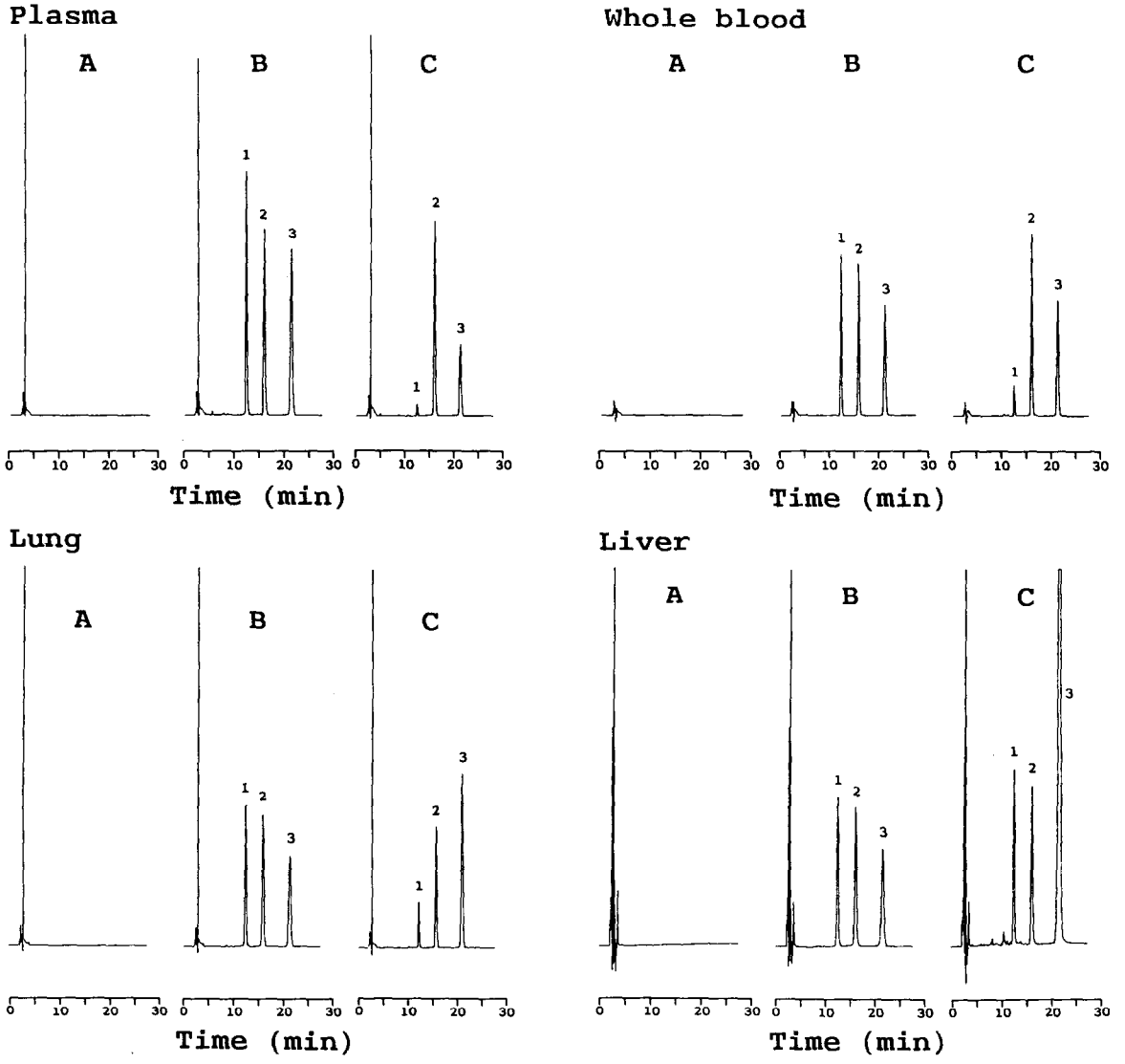

Fig. 2. Chromatograms of (A) blank rat samples, (B) rat samples spiked with I and II to give a final concentration of 0.3 μg/ml for plasma and urine, 0.75 μg/ml for whole blood and 1.5 μg/g for tissues, for each analyte, (C) rat samples obtained 8 h after an oral dose of 30 mg/kg of I. Peaks: 1 = II; 2 = I.S.; 3 = I.

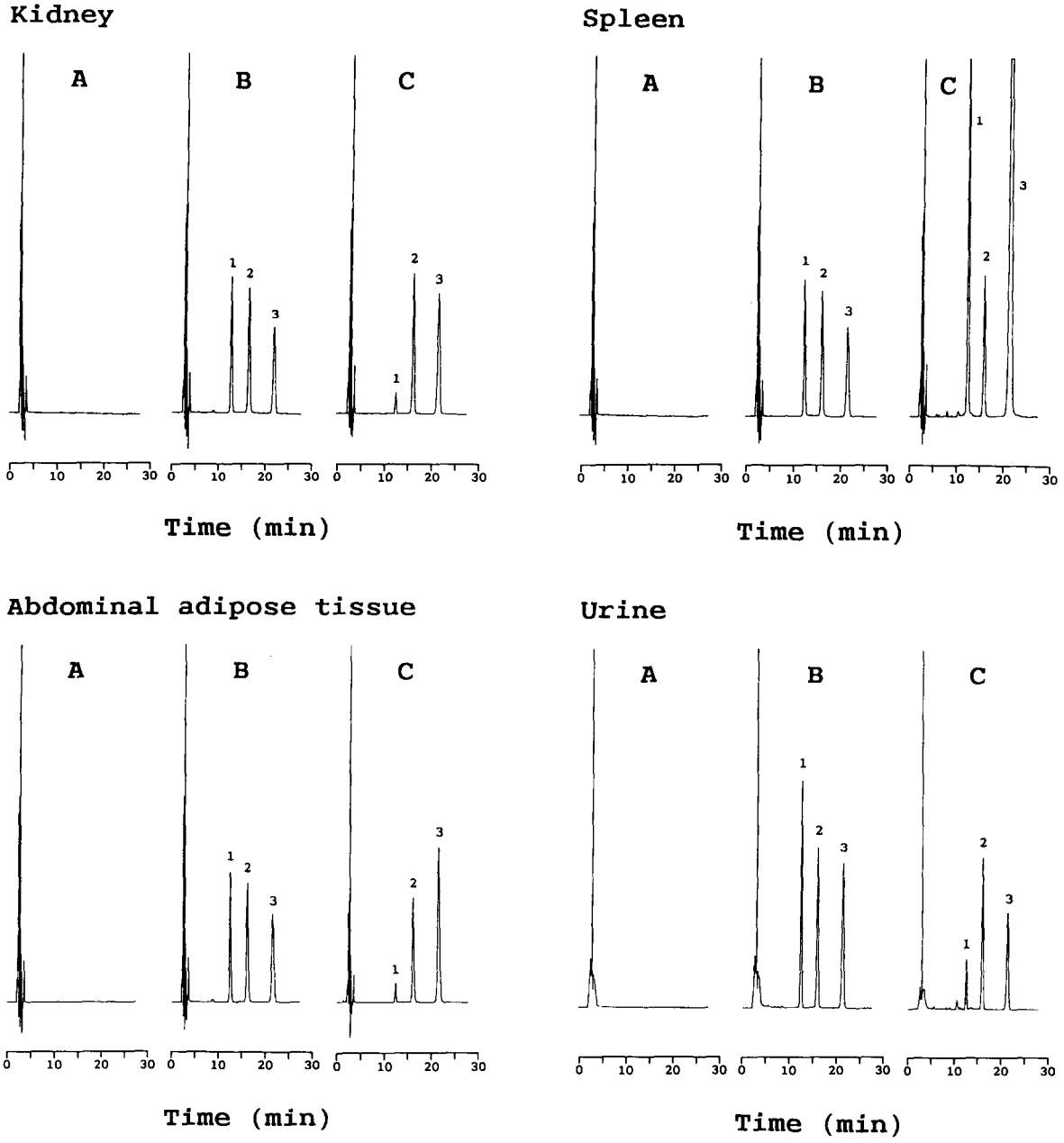

Fig. 2. Continued

Fig 2. No interference peaks at the retention times of I, II, and I.S. were observed in the chromatograms of blank rat samples. The peaks of the analytes and I.S. were sharp, symmetrical, and showed good resolution. Our strategy for the selectivity was employing solid-phase extraction and Vis detection. After screening of solid-phase extraction columns such as C_8 (octyl), C_{18} (octadecyl), PH (phenyl), CH (cyclohexyl) and SI (silica), we selected C_8 and SI as suitable solid phases for plasma and urine, and for whole blood and tissues, respectively. The SI solid

phase enabled to isolate and retain the analytes and I.S. from all tissues including adipose tissue, a very non-polar matrix. In addition, the use of a visible wavelength of 643 nm for detection eliminated the peaks from endogenous substances which could not be eliminated by UV detection. Conveniently, the Vis spectrum of I, II and I.S. showed a maximum absorption at 643, 642 and 639 nm with rather high molar absorptivity of $5.94 \cdot 10^4$, $4.97 \cdot 10^4$ and $4.96 \cdot 10^4$ $(\text{mol/l})^{-1}$ cm^{-1}, respectively when dissolved in mobile phase.

3.2. Standard curves

For both analytes, the standard curve range was 0.01 to 1 μg/ml for plasma, 0.02 to 10 μg/ml for whole blood, 0.01 to 10 μg/ml for urine, and 0.1 to 10 or 30 μg/g for tissues. Four or six points were analyzed with 5 determinations per point. The parameters of the resulting standard curves are given in Table 1.

Standard curves for all sample types were linear over the concentration range studied for both compounds. The correlation coefficients for both analytes were 0.9999 or 1.0000. The intercept values of the standard curves for all sample types ranged from −0.0329 to 0.0255 for I and from −0.0415 to 0.0141 for II, and were not significantly different from zero by Student's t test ($p > 0.05$).

3.3. Precision and accuracy

The intra- and inter-assay C.V.s for I and II varied from 1.0 to 10.2% for plasma, from 1.0 to 6.1% for whole blood, from 1.2 to 5.5% for urine and from 0.5 to 10.0% for tissues (Tables 2 and 3). The relative errors for both analytes ranged from −15.0 to 5.0% for plasma, from

Table 1
Linear regression parameters for plasma, whole blood, urine and tissues for I and II

Tissue	Slope	Intercept	Correlation coefficient	Concentration range (μg/ml or μg/g)
Compound I				
Plasma	3.8707	−0.0080	0.9999	0.01– 1.00
Whole blood	1.2048	0.0086	1.0000	0.02–10.00
Urine	2.3431	0.0255	1.0000	0.01–10.00
Lung	0.5983	0.0152	1.0000	0.10–30.00
Liver	0.6131	−0.0126	1.0000	0.10–30.00
Kidney	0.5814	0.0052	1.0000	0.10–10.00
Spleen	0.5815	−0.0329	1.0000	0.10–30.00
Abdominal adipose tissue	0.5838	−0.0053	1.0000	0.10–30.00
Compound II				
Plasma	3.8626	−0.0121	1.0000	0.01– 1.00
Whole blood	1.1719	−0.0112	1.0000	0.02–10.00
Urine	2.3605	0.0141	1.0000	0.01–10.00
Lung	0.5926	−0.0057	1.0000	0.10–30.00
Liver	0.6045	−0.0348	1.0000	0.10–30.00
Kidney	0.5849	−0.0075	1.0000	0.10–10.00
Spleen	0.5796	−0.0415	1.0000	0.10–30.00
Abdominal adipose tissue	0.5808	−0.0117	1.0000	0.10–30.00

Table 2
Precision and accuracy of intra- and inter-assay of I in plasma, whole blood and tissues in rats

Tissue (μg/ml or μg/g)	Concentration	Intra-assay ($n = 5$)		Inter-assay ($n = 5$)	
		C.V. (%)	Relative error (%)	C.V. (%)	Relative error (%)
Plasma	0.02	6.6	0.0	4.0	5.0
	0.1	2.1	0.0	1.3	1.0
	1.0	2.7	0.2	1.0	−0.5
Whole blood	0.06	3.9	−15.0	3.7	−16.7
	1.0	0.5	−4.5	1.7	−1.9
	10.0	0.9	−3.9	3.2	−0.5
Urine	0.08	1.7	−17.5	3.8	−20.0
	1.0	1.7	−2.3	1.7	−0.4
	10.0	2.5	12.6	1.7	10.4
Lung	0.2	3.8	5.0	5.3	3.0
	2.0	1.0	−6.0	5.4	−3.6
	50.0	2.5	−3.3	2.1	1.9
Liver	0.2	5.7	3.5	2.8	6.0
	2.0	0.9	−7.4	6.9	−3.1
	50.0	2.2	−2.4	4.2	1.0
Kidney	0.2	5.3	10.5	4.8	−5.5
	2.0	2.0	2.2	10.0	−1.9
	50.0	1.5	9.7	1.6	8.9
Spleen	0.2	3.4	25.5	3.8	28.0
	2.0	1.2	9.1	5.8	4.9
	50.0	4.0	1.2	4.4	5.8
Abdominal adipose tissue	0.2	1.4	8.0	6.2	4.5
	2.0	1.3	6.1	5.0	3.4
	50.0	1.7	7.3	2.7	8.9

−16.7 to −0.5% for whole blood, from −20.0 to 12.6% for urine, and from −11.0 to 18.5% for tissues except in spleen at low concentration.

It has been recommended to use an internal standard for HPLC assay to compensate for the potential variations at extraction, injection or other chromatographic manipulations [14,15]. Our results indicate that an I.S. structurally similar to the analytes can function as an internal standard with good precision and accuracy. Furthermore, the mean absolute recoveries of I, II, and I.S. from plasma, whole blood, urine and tissues were consistent and ranged from 76.6 to 95.6% (Table 4). This was also the basis for the high reproducibility of this assay method.

3.4. Sensitivity

The limit of detection (LOD) and the limit of quantitation (LOQ) were used to assess the sensitivity of the method. The LOD is defined as the amount of analyte giving a peak height three times the maximum noise peak of a blank biological sample observed at the retention time of each analyte. The LOQ is defined at a relative

Table 3
Precision and accuracy of intra- and inter-assay of II in plasma, whole blood and tissues in rats

Tissue	Concentration (μg/ml or μg/g)	Intra-assay ($n = 5$)		Inter-assay ($n = 5$)	
		C.V. (%)	Relative error (%)	C.V. (%)	Relative error (%)
Plasma	0.02	7.8	−15.0	10.2	−5.0
	0.1	1.8	−10.0	3.7	−6.0
	1.0	2.5	−4.0	1.6	−3.6
Whole blood	0.06	6.1	−3.3	5.6	−6.7
	1.0	0.6	−5.8	1.5	−6.7
	10.0	1.0	−4.2	1.6	−3.3
Urine	0.08	1.2	−16.3	5.5	−7.5
	1.0	1.5	−1.9	3.6	2.3
	10.0	2.8	11.3	2.0	8.9
Lung	0.2	1.3	15.5	1.5	16.0
	2.0	0.6	−4.9	1.3	−4.1
	50.0	2.4	−3.8	1.0	−1.4
Liver	0.2	2.1	14.0	3.0	18.5
	2.0	0.5	−5.6	1.8	−4.0
	50.0	1.5	−2.6	3.1	−2.1
Kidney	0.2	3.5	−11.0	3.9	−3.0
	2.0	1.2	−4.3	2.3	−2.2
	50.0	1.4	2.5	1.1	2.3
Spleen	0.2	2.1	24.0	1.3	27.0
	2.0	0.9	2.1	1.4	2.0
	50.0	3.4	−1.0	3.1	2.1
Abdominal adipose tissue	0.2	2.2	−3.0	3.5	0.0
	2.0	0.9	−1.0	1.6	−1.1
	50.0	1.5	2.4	1.6	4.4

error less than ±20% and a C.V. less than 10% in the intra-assay. The LODs for I and II were 0.8–1.1 ng/ml or g and 0.6–0.8 ng/ml or g, respectively for various biological fluids or tissues. The LOQ for both analytes was 0.02 μg/ml for plasma, 0.06 μg/ml for whole blood, 0.08 μg/ml for urine and 0.2 μg/g for all tissue samples. A lower LOQ could be achieved by increasing the HPLC injection volume or by increasing the amount of sample used for the extraction.

It should be possible to apply this method with minor modification to the determination of I and II in biological samples from other animals.

4. Conclusions

A sensitive and reproducible HPLC method was developed to determine the concentration of I and its metabolite simultaneously in plasma, whole blood, tissues, and urine in rats. This method employs a solid-phase clean-up procedure that permits the analysis of large numbers

Table 4
Absolute recovery of I, II and internal standard from plasma, whole blood, urine and tissues

Tissue (μg/ml or μg/g)	n	Concentration	Recovery (%) (mean ± S.D.)		
			I	II	I.S.
Plasma	5	0.02	94.5 ± 3.7	87.5 ± 7.7	
	5	1	95.6 ± 2.7	95.5 ± 2.6	
	10	0.3			93.4 ± 3.7
Whole blood	5	0.1	82.2 ± 2.6	76.6 ± 2.7	
	5	10	85.2 ± 1.6	82.8 ± 1. 7	
	10	1			82.5 ± 1.9
Urine	5	0.2	87.3 ± 2.5	89.9 ± 2.3	
	5	10	89.4 ± 1.5	91.8 ± 1.4	
	10	0.5			88.7 ± 2.4
Lung	5	0.5	78.6 ± 0.7	77.7 ± 0.9	
	5	50	87.8 ± 3.1	88.9 ± 3.0	
	10	2			81.3 ± 3.2
Liver	5	0.5	85.7 ± 1.0	83.4 ± 1.8	
	5	50	90.4 ± 1.9	92.0 ± 1.8	
	10	2			83.5 ± 1.5
Kidney	5	0.5	86.3 ± 0.5	84.6 ± 1.5	
	5	50	89.7 ± 1.4	92.1 ± 0.6	
	10	2			84.3 ± 0.8
Spleen	5	0.5	81.4 ± 2.4	80.4 ± 3.0	
	5	50	88.5 ± 3.2	90.4 ± 2.8	
	10	2			86.3 ± 3.0
Abdominal adipose tissue	5	0.5	79.7 ± 3.1	77.3 ± 1.6	
	5	50	88.8 ± 1.6	89.5 ± 1.7	
	10	2			83.1 ± 3.2

of samples which is required in pharmacokinetic studies. Pharmacokinetic studies using this method are now under way.

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