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Determination of a new isoquinolinedione derivative, 7-anilino-5,8-isoquinolinedione, in plasma, urine and tissue homogenates by high-performance liquid chromatography

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Abstract

A high-performance liquid chromatographic (HPLC) method was developed for the determination of a new isoquinolinedione derivative, 7-anilino-5,8-isoquinolinedione (IQO4), in rat plasma, urine, blood and tissue homogenates using diazepam as an internal standard. A 2 volume of acetonitrile was added to deproteinize the biological sample. A 50 μl aliquot of the supernatant was injected onto a C_{18} reversed-phase column. The mobile phase, 0.05 M acetate buffer (pH 3):acetonitrile:methanol (40:40:20, v/v/v), was run at a flow rate of 1.5 ml/min. The column effluent was monitored using an ultraviolet detector set at 298 nm. The retention times for IQO4 and the internal standard were approximately 5 and 7 min, respectively. The detection limits of IQO4 in rat plasma, urine and tissue homogenates (including blood) were 0.05, 0.1 and 0.1 μg/ml, respectively. The coefficients of variation of the assay were below 9.4% for rat plasma, urine and tissue homogenates. No interferences from endogenous substances were found.

Keywords: IQO4; HPLC; Pharmacokinetics

1. Introduction

Quinones such as 6-phenylamino-5,8-quinolinedione (LY83583) [1–3], and 9,10-phenanthraquinone and 1,4-naphthoquinones [4] inhibit nitric oxide synthase (NOS). LY83583 inhibits NOS activity [2,3], lowers intracellular cyclic guanosine-3′,5′-monophosphate (cGMP) in several tissues [1], and thus inhibits the release of endothelium-derived relaxing factor (EDRF) (= NO) [1]. Recently, a new isoquinolinedione derivative, 7-anilino-5,8-isoquinolinedione (IQO4, Fig. 1), was developed as an inhibitor of endothelium-dependent vasorelaxation and NOS. IQO4 strongly inhibited the acetylcholine-induced vaso-

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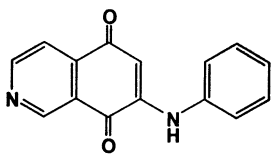
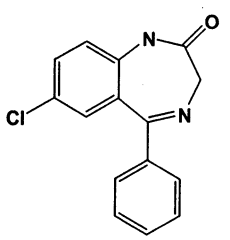
relaxation of phenylephrine-precontracted rat aorta with the intact endothelium [5]. Further pharmacological investigations of IQO4 as an inhibitor of endothelial and neuronal NO synthases are in progress. IQO4 is now being evaluated in preclinical trial as a vasorelaxation agent. Some derivatives of isoquinolinedione synthesized by our laboratories were unstable in blood and especially after intravenous administration to rats. Therefore, the high-performance liquid chromatographic (HPLC) analysis and especially pharmacokinetic studies of isoquinolinedione seemed not to be published yet.

This paper describes an HPLC method with deproteinization of the sample with acetonitrile for the determination of IQO4 in rat plasma, urine and tissue homogenates. The pharmacokinetics and tissue distributions of IQO4 after intravenous administration of IQO4 to a male Sprague-Dawley rat were also reported.



IQO4



Diazepam (the internal standard)

Fig. 1. Chemical structures of IQO4 and diazepam (the HPLC internal standard).

2. Experimental

2.1. Chemicals

IQO4 for the HPLC assay was supplied by College of Pharmacy, Ewha Womans University (Seoul, South Korea) and diazepam, the internal standard for the HPLC assay (Fig. 1), was donated by Hwan In Pharmaceutical Company (Seoul, South Korea). Various pH solutions ranging from 1 to 2 (HCl–KCl buffer), 3 ($KHC_8H_4O_4$–HCl buffer), 4 and 5 ($KHC_8H_4O_8$–NaOH buffer), 6 and 7 (KH_2PO_4–NaOH buffer), 8 and 9 (H_3BO_3–KCl–NaOH buffer), 10 and 11 ($NaHCO_3$–NaOH buffer), 12 (Na_2HPO_4–NaOH buffer), to 13 (KCl–NaOH buffer) were purchased from Shinyo Pure Chemicals (Osaka, Japan). Other chemicals were of reagent grade or HPLC grade and therefore were used without further purification.

2.2. Preparation of stock and standard solutions

A stock solution of IQO4 (1 mg/ml) was prepared in methanol:dimethylsulfoxide (1:1, v/v). Appropriate dilutions of stock solution were made with methanol. Standard solutions of IQO4 in rat plasma, urine and tissue homogenates (1 g of each tissue or organ was homogenized (Ultra-Turrax, T25, Janke and Kunkel, IKA-Labortechnik, Staufen, Germany) with 4 volumes of 0.9% NaCl injectable solution, centrifuged for 10 min at $9000 \times g$ and the supernatant was collected) were prepared by spiking with an appropriate volume (less than 10 μl/ml of biological sample) of the diluted stock solution, giving final concentrations of 0.05, 0.1, 0.2, 0.5, 1, 2, 10 and 20 μg/ml for plasma, 0.1, 0.5, 1, 2, 10 and 20 μg/ml for urine, and 0.1 and 1 μg/ml for supernatant of tissue homogenates. Only the HPLC assay results on two or three concentrations (0.05, 0.5 and 20 μg/ml for plasma, 0.1, 1 and 20 μg/ml for urine, and 0.1 and 1 μg/ml for tissue homogenates) are listed in Table 1. The internal standard solution was prepared by dissolving diazepam in acetonitrile to give a final concentration of 5 μg/ml.

Table 1
Response factors and accuracies of IQO4 at various concentrations in rat plasma, urine and tissue homogenates using the deproteinization method

Theoretical concentration (μg/ml)	Response factor[a]	Accuracy[b] (%)
Rat plasma		
20	0.444 (1.8)	102 (1.8)
0.5	0.427 (2.2)	98.4 (2.2)
0.05	0.444 (9.4)	102 (9.4)
Rat urine		
20	0.365 (8.4)	98.4 (8.4)
1	0.372 (6.0)	100 (6.0)
0.1	0.366 (1.4)	98.7 (1.4)

Values in parentheses are coefficients of variation (%); $n = 5$.
[a] [Drug peak height (cm) divided by its concentration (μg/ml)]/[internal standard peak height (cm) divided by its concentration (μg/ml)]; mean.
[b] (Mean observed concentration/theoretical concentration) × 100; mean.

2.3. Sample preparation

IQO4 (IQO4 was dissolved in dimethylsulfoxide), 5 mg/kg, was intravenously administered for 30 min (total injection volume was 0.3 ml) via the jugular vein of a male Sprague-Dawley rat (Charles River, Atsugi, Japan). Blood samples (0.12 ml) were collected at various times from the carotid artery. After centrifugation, plasma samples (0.05 ml) were stored in the −70 °C freezer (Revco ULT 1490 D-N-S, Western Mednics, Asheville, NC) prior to HPLC analysis of IQO4. Urine sample was collected for 24 h. Pretreatment of a rat, cannulation method and handling of plasma and urine samples were the same as reported previously [6]. A 100 μl volume of acetonitrile containing the internal standard (5 μg/ml of diazepam) was added to a 50 μl aliquot of rat plasma or urine samples [7,8]. After vortex-mixing and centrifugation at 9000 × *g* for 10 min, a 50 μl aliquot of the supernatant was injected directly onto the HPLC column. IQO4, 5 mg/kg, was also intravenously administered to another rat. At 10 min postinfusion of the drug, each rat tissue (or organ) was homogenized with 4 volumes of 0.9% NaCl injectable solution using a tissue homogenizer (Ultra-Turrax, T25, Janke and Kunkel) and immediately centrifuged at 9000 × *g* for 10 min. A 50 μl aliquot of the supernatant was collected and treated as described for rat plasma and urine samples. Two volumes of distilled water were added to rat blood sample to facilitate the hemolysis of blood cells and a consistent release of IQO4 from blood when deproteinize blood samples to increase the reproducibility of IQO4 [9,10]. After vortex-mixing and centrifugation at 9000 × *g* for 10 min, a 50 μl aliquot of the supernatant was collected and processed as described for rat plasma and urine samples.

2.4. HPLC apparatus

The HPLC system consisted of a model 7120 injector (Rheodyne, Cotati, CA), a model 307 pump (Gilson, Middleton, WI), a reversed-phase (C_{18}) column (Lichrosorb; RP-18; 25 cm, 1 × 4.6 mm i.d.; particle size, 10 μm; Merck, Darmstadt, Germany), a model 118 UV/Vis detector (Gilson) and a model 1200 recorder (Linear, Reno, NV). The mobile phase, 0.05 M acetate buffer (pH 3):acetonitrile:methanol (40:40:20, v/v/v), was run at a flow rate of 1.5 ml/min and the column effluent was monitored by an UV detector set at 298 nm.

2.5. Stability in various pH solutions and rat whole blood, plasma, urine and liver homogenates

IQO4 stock solution in methanol was spiked (less than 10 μl/ml) in each glass test tube containing 10 ml of various pH solutions ranging from 1 to 13 to make 10 μg/ml of IQO4. After vortex-mixing, each test tube was placed in a water-bath shaker kept at 37 °C and at a rate of 50 oscillations per minute. At 0, 1, 2, 3, 4, 6, 8, 12 and 24 h, a 50 μl aliquot was sampled from the each test tube and immediately injected (without deproteinization with acetonitrile) onto the HPLC column. A Sprague-Dawley rat (Charles River) liver was homogenized with 4 volumes of 0.9% NaCl injectable solution using a tissue homogenizer (Ultra-Turrax T25, Janke & Kunkel) at 4 °C. After centrifugation at 9000 × *g* for 10 min, 10 ml of the supernatant was collected and similar

experiment was again performed at 10 μg/ml of IQO4. Rat whole blood, plasma, and urine at 10 μg/ml of IQO4 were also incubated. At designated time intervals, an aliquot of sample was collected.

3. Results and discussion

The λ_{max} values of IQO4 in methanol were 280 and 298 nm based on the UV absorption spectra. The absorption at 298 nm was greater than that at

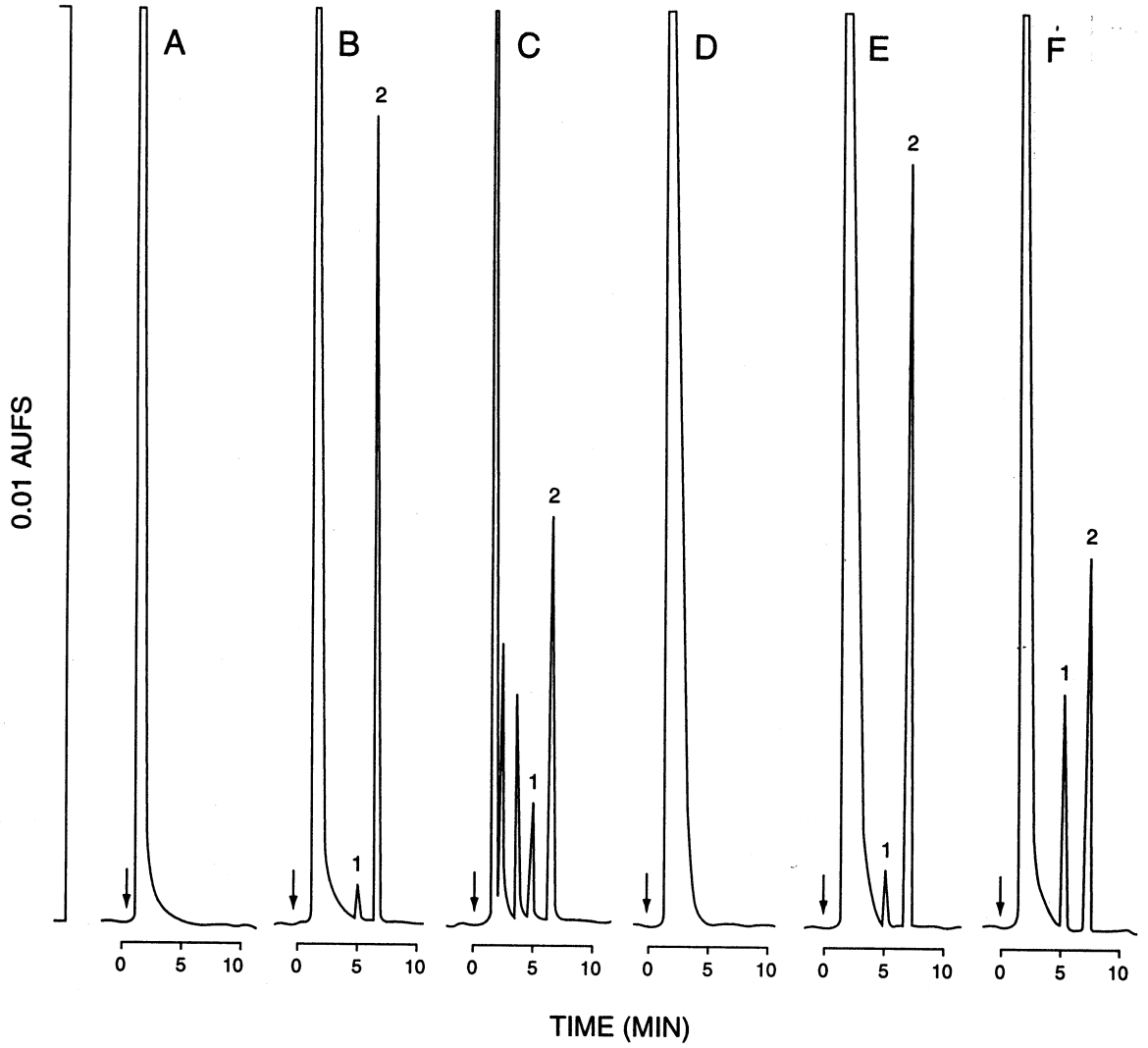


Fig. 2. Chromatograms after deproteinization of drug-free rat plasma (A), rat plasma spiked with 0.1 μg/ml of IQO4 and 5 μg/ml of internal standard (B), plasma collected from a male Sprague-Dawley rat 5 min (0.658 μg/ml) after 30 min intravenous infusion of 5 mg/kg of IQO4 (C), drug-free rat urine (D), rat urine spiked with 0.2 μg/ml of IQO4 and 5 μg/ml of internal standard (E) and urine collected from a male Sprague-Dawley rat between 0 and 24 h (1.67 μg/ml) after 30 min intravenous infusion of 5 mg/kg of IQO4 (F). Peaks: 1 = IQO4 (5 min); 2 = internal standard (7 min). The arrows mark the points of injection. The detector's sensitivity was set at 0.01 AUFS and the recorder's sensitivity was set at 10 mV (A, B, D and E) and 20 mV (C and F).

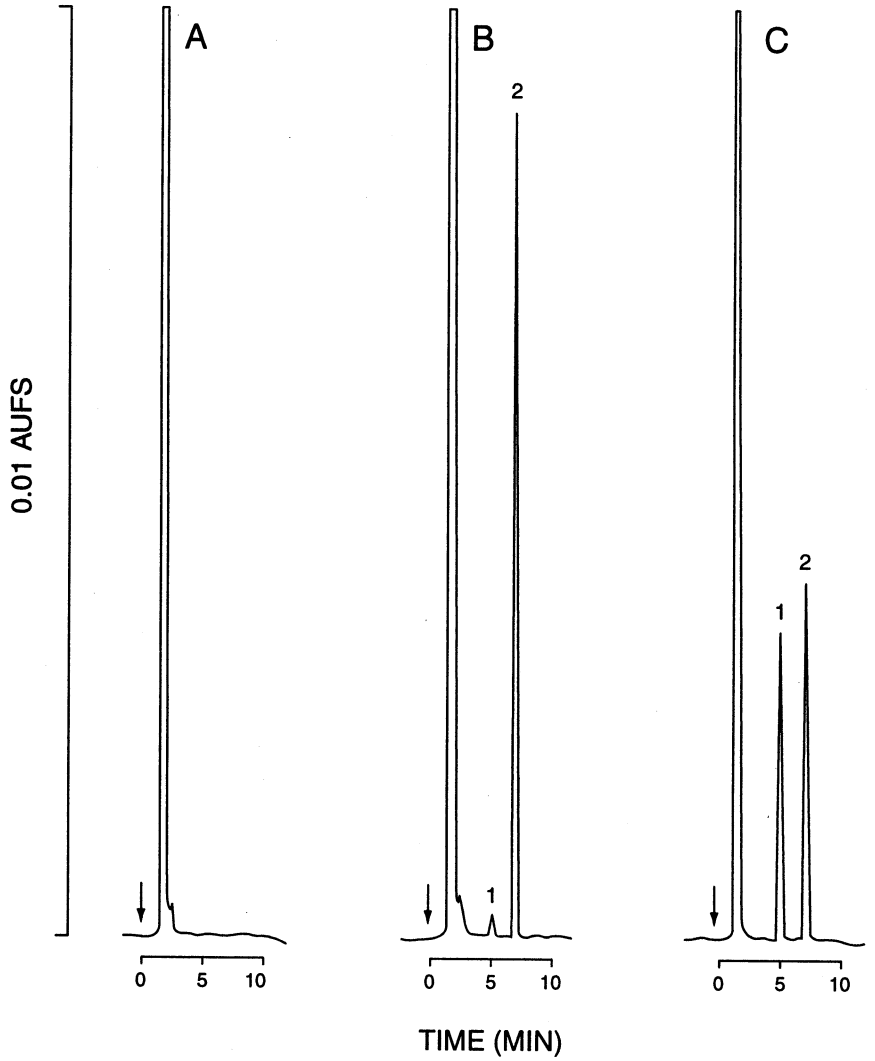


Fig. 3. Chromatograms after deproteinization of drug-free rat lung homogenates (A), rat lung homogenates spiked with 0.1 μg/ml of IQO4 and 5 μg/ml of internal standard (B) and lung homogenates collected from a male Sprague-Dawley rat at 10 min (3.44 μg/ml) after 30 min intravenous infusion of 5 mg/kg of IQO4 (C). Peaks: 1 = IQO4 (5 min); 2 = internal standard (7 min). The arrows mark the points of injection. The detector's sensitivity was set at 0.01 AUFS and the recorder's sensitivity was set at 10 mV (A and B) and 20 mV (C).

280 nm. Hence, 298 nm was chosen considering the background and assay sensitivity. Fig. 2 shows typical chromatograms of drug-free rat plasma, drug standards in rat plasma spiked with 0.1 μg/ml of IQO4, plasma collected 5 min after intravenous administration of 5 mg/kg of IQO4 to a male Sprague-Dawley rat, drug-free rat urine, drug standards in rat urine spiked with 0.2 μg/ml of IQO4, and urine collected between 0 and 24 h after intravenous administration of 5 mg/kg of IQO4 to a male Sprague-Dawley rat using the deproteinization method; the corresponding chromatograms for rat lung homogenates are shown in Fig. 3. No interferences from endogenous substances were

observed in any of the biological samples. Additional peaks were observed in rat plasma sample (Fig. 2C), this could be unknown metabolites of IQO4 since approximately 95% of the intravenously administered IQO4 were metabolized in rats as will be discussed in pharmacokinetic studies. The retention times for IQO4 and the internal standard were approximately 5 and 7 min, respectively. It is to be noted that the biological sample was deproteinized with two volumes of acetonitrile; therefore, the concentration of acetonitrile was approximately 67% (v/v). The concentration of acetonitrile in the mobile phase was 40% (v/v/v). The injection volume after deproteinization was 50 μl and the flow rate of the mobile phase was 1.5 ml/min. Although the elution strength of injected fluid after deproteinization was higher than that of the mobile phase, the retention time of IQO4 in all the biological fluids was fairly constant.

The detection limits for IQO4 in rat plasma, urine and tissue homogenates were 0.05, 0.1 (Table 1) and 0.1 μg/ml (Table 2), respectively, based on a signal-to-noise ratio of 3.0. The coefficients of variations (CVs) for response factor and accuracy were both lower than 9.4 and 8.4% for rat plasma and urine, respectively (Table 1). The ranges of CVs of IQO4 in rat plasma and urine were 0.21–

Table 2
Response factors and accuracies of IQO4 at various concentrations in rat blood and tissue homogenates using the deproteinization method, $n = 4$

Tissue[a]	Theoretical concentration (μg/ml)	Response factor[b]	CVs (%)	Accuracy[c] (%)	CVs (%)
Blood	1	0.525	1.9	96.1	1.9
	0.1	0.567	0.92	104	0.92
Brain	1	0.424	7.1	98.3	7.1
	0.1	0.437	3.2	101	3.2
Fat	1	0.400	3.2	95.9	3.2
	0.1	0.434	4.4	104	4.4
Heart	1	0.410	3.9	102	3.9
	0.1	0.398	4.9	98.5	4.9
Kidney	1	0.255	0.62	104	0.62
	0.1	0.235	9.5	95.9	9.5
Intestine	1	0.146	5.8	106	5.8
	0.1	0.129	5.4	93.8	5.4
Liver	1	0.341	4.3	103	4.3
	0.1	0.321	8.1	96.9	8.1
Lung	1	0.255	2.9	105	2.9
	0.1	0.228	1.5	94.2	1.5
Mesentery	1	0.416	2.9	103	2.9
	0.1	0.388	6.2	96.6	6.2
Muscle	1	0.393	6.2	98.7	6.2
	0.1	0.402	4.9	101	4.9
Spleen	1	0.439	3.7	103	3.7
	0.1	0.416	3.9	97.2	3.9
Stomach	1	0.381	5.5	101	5.5
	0.1	0.366	8.0	97.7	8.0

[a] Tissue samples were homogenized with 4 volumes of 0.9% NaCl injectable solution.
[b] [Drug peak height (cm) divided by its concentration (μg/ml)]/[internal standard peak height (cm) divided by its concentration (μg/ml)]; mean.
[c] (Mean observed concentration/theoretical concentration) × 100; mean.

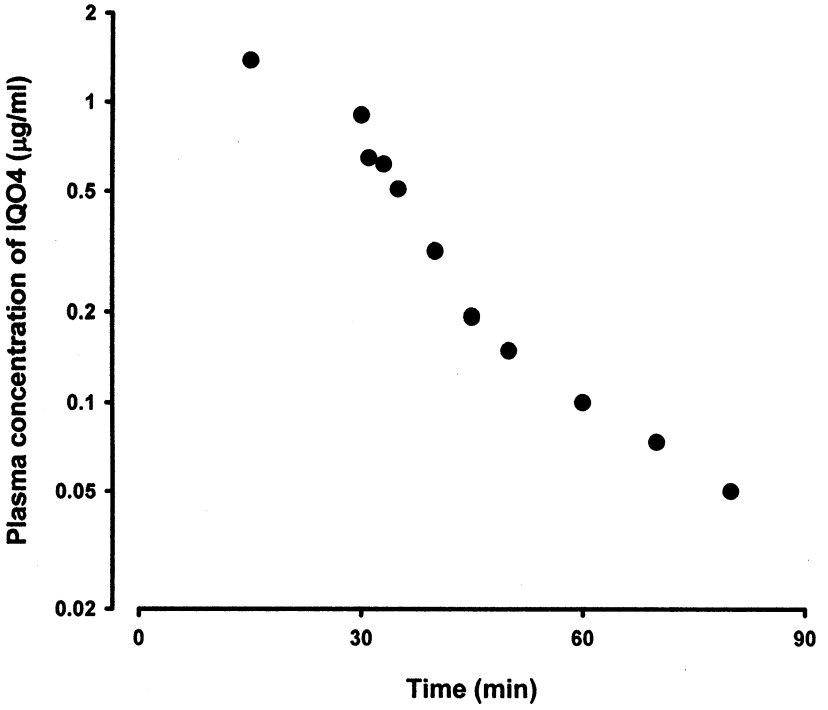


Fig. 4. Arterial plasma concentration–time profile of IQO4 after 30 min intravenous administration of 5 mg/kg of IQO4 to a male Sprague-Dawley rat.

9.4 and 1.2–8.4%, respectively, within the concentration ranges of 0.05–20 μg/ml (Table 1). The ranges of CVs for IQO4 in rat tissues were 0.62–9.5% within the concentration ranges of 0.1–1 μg/ml (Table 2). The accuracies [(mean observed concentration/theoretical concentration) × 100] of IQO4 were 94.9–106 and 94.7–104% for rat plasma and urine, respectively, within the concentration ranges of 0.05–20 μg/ml for rat plasma and urine (Table 1). The accuracy of IQO4 in rat tissues ranged from 93.8 to 106% within the concentration ranges of 0.1–1 μg/ml (Table 2). The peak height of the internal standard was comparable for the plasma and urine samples (Fig. 2). However, the peak height of IQO4 was less in the urine sample than that in the plasma sample. Therefore, the response factors of IQO4 in the urine samples were lower than those in the plasma samples (Table 1). This could be the result of binding and/or adsorption of IQO4 to the endogenous compounds in urine. Similar results were also found with azosemide [8], YH1885, a new proton pump inhibitor [11], and methotrexate [12]. It should be noted that because of the poor water solubility of IQO4, the relative recovery of IQO4 compared with water could not be measured in the present study.

This HPLC method was also successful for the pharmacokinetic studies in a rat. After 30 min intravenous infusion of IQO4, 5 mg/kg, to a rat, the arterial plasma concentration–time profile of IQO4 is shown in Fig. 4. The terminal half-life, total body clearance, apparent volume of distribution at steady state (V_{SS}) and mean residence time of IQO4 were 15.0 min, 120 ml/min/kg, 3013 ml/kg, and 25.2 min, respectively.

The tissue concentration of IQO4 was also measured 10 min after 30 min intravenous infusion of IQO4, 5 mg/kg, to a rat; the values were 1.10 μg/ml for plasma and 2.21, 1.60, 12.0, 1.20, and 3.19 μg/g tissue for the liver, heart, lung, small intestine, and kidney, respectively.

IQO4 was stable for up to 24 h incubation in various pH solutions ranging from 1 to 8 at 10 μg/

ml of IQO4. However, IQO4 was unstable in pH solution ranging from 9 to 13; the disappearance rate constants of IQO4 were 0.0164 h^{-1}, 0.0457 h^{-1}, 0.411 h^{-1}, 0.0341 h^{-1}, and 0.124 min^{-1} for pHs of 9, 10, 11, 12 and 13, respectively, and the corresponding disappearance half-lives were 42.1 h, 15.2 h, 1.69 h, 20.4 h and 5.60 min. IQO4 was also unstable for up to 24 h incubation in rat plasma, urine and liver homogenates at 10 μg/ml of IQO4. The disappearance rate constants of IQO4 were 0.0436, 0.0530 and 0.174 h^{-1} for rat plasma, urine and liver homogenates, respectively, and the corresponding disappearance half-lives were 15.9, 13.1 and 3.98 h.

Acknowledgements

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