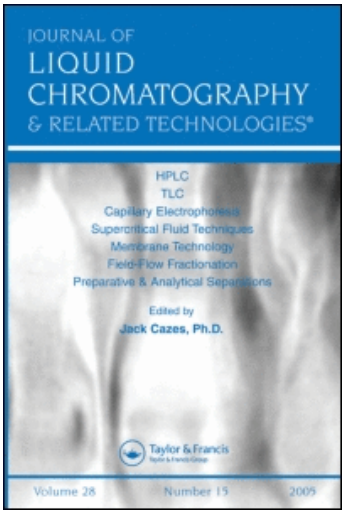

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Tang, Dao-quan , Yin, Xiao-xing , Zhang, Zun-jian , Gao, Yuan-yuan , Wei, Ya-qin , Chen, Yong-gang and Han, Lin(2009) 'Gradient HPLC-DAD for the Simultaneous Determination of Five Flavonoids in Plasma After Intravenously Administrated *Ginkgo biloba* Extract and its Application in the Study of Pharmacokinetics in Rats', Journal of Liquid Chromatography & Related Technologies, 32: 14, 2065 — 2079
To link to this Article: DOI: 10.1080/10826070903126948
URL: http://dx.doi.org/10.1080/10826070903126948

Journal of Liquid Chromatography & Related Technologies®, 32: 2065–2079, 2009

ISSN: 1082-6076 print/1520-572X online
DOI: 10.1080/10826070903126948

Gradient HPLC-DAD for the Simultaneous Determination of Five Flavonoids in Plasma After Intravenously Administrated *Ginkgo biloba* Extract and its Application in the Study of Pharmacokinetics in Rats

Dao-quan Tang,[1,2,3] Xiao-xing Yin,[3] Zun-jian Zhang,[1,2] Yuan-yuan Gao,[3] Ya-qin Wei,[3] Yong-gang Chen,[3] and Lin Han[3]

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Abstract: A HPLC-DAD method was used and validated for the simultaneous determination of five flavonoids (rutin, quercitrin, quercetin, kaempferol, and isorahamnetin) in rat plasma. Chromatographic separation was performed using a Kromasil C_{18} column (250×4.6 mm, 5 μm) maintained at 35°C. The mobile phase was a mixture of methanol and 0.1% formic acid with a step linear gradient. At 1.0 mL/min flow rate, the eluent of five flavonoids were detected simultaneously at 350 nm with good separation. Under optimum conditions, good linear relationship between the peak area and the concentrations were obtained in the ranges of $0.2525 \sim 20.2$, $0.1208 \sim 9.66$, $0.1008 \sim 20.16$, $0.031 \sim 2.46$, and $0.098 \sim 7.84$ μg/mL for rutin, quercitrin, quercetin, kaempferol, and isorhamnetin, respectively. The correlation coefficient for each analyte was above 0.999. The intra-day and inter-day precisions were better than 7% and 10%. The detection limit ($S/N = 3$) for rutin, quercitrin, quercetin, kaempferol, and isorhamnetin were 0.01, 0.02,

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0.006, 0.02 and 0.02 μg/mL, respectively. The method was validated for accuracy and precision, and it has been successfully applied to determine drug concentrations in rat plasma samples from rat that had been intravenously administrated *Ginkgo biloba* extract.

Keywords: Ginkgo biloba extract, High performance liquid chromatography, Isorhamnetin, Kaempferol, Pharmacokinetic, Quercetin, Quercitrin, Rutin

INTRODUCTION

Ginkgo biloba is an ancient Chinese phytomedicine which was used to treat various ailments including circulatory and demential disorders. Standardized leaf extracts have been proven to be clinically effective in the treatment of Alzheimer's disease, depression, diabetic neuropathy, impotency, memory impairment, peripheral vascular disease, intermittent claudication, vertigo and tinnitus.[1] The positive effects of *Ginkgo bibola* extracts (GBE) are thought to result from the synergistic action of two distinct groups of compounds, the flavonoids and triterpene lactones.[2] The flavonoids are responsible for the free radical scavenging effects of *Ginkgo biloba*[3–5], while the ginkgolides are potent anti-platelet factor (PAF) antagonists.[6] Various analytical techniques have been employed to standardize *Ginkgo biloba* products using the flavonoids and/or ginkgolides as marker compounds.

The flavonoids are a large group of polyphenols which occur naturally in the plant kingdom and include flavone and flavonol glycosides, acylated flavonol glycosides, biflavonoids, flavane-3-ols and proanthocyanidins.[7–9] Of these, special attention has been paid to the flavonols due to their abundance in GBE. Numerous flavonol glycosides have been identified as derivatives of the flavonol aglycones, quercetin, kaempferol, and isorhamnetin.[10] The current standardization approach of flavonols in *Ginkgo biloba* extracts is by calculating the total flavonol glycoside content from the aglycone concentration in extracts after acid hydrolysis.

Reversed-phase high-performance liquid chromatography (HPLC) with UV detection has been the most common method for the analysis of flavonols because they have strong chromophores.[11] On the other hand, liquid chromatography (LC) coupled to mass spectrometry (MS) has been demonstrated to be a powerful tool for the identification of natural products in crude plant extracts owing to their soft ionization which favours the analysis of flavonols, in addition to its high sensitivity and specificity.[12–13]

Compared to the extensive literature on phytochemical, quality control and clinical investigations, there was little data concerning the pharmacokinetics of the flavonoids in plasma after administrated GBE.

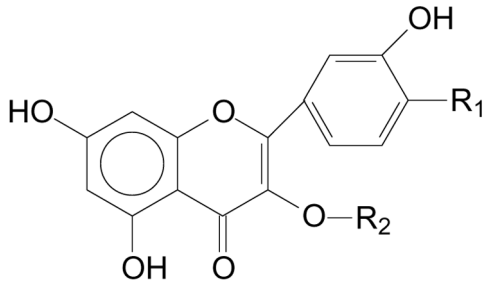
Yang et al. succeeded in this task using liquid chromatography/ultraviolet spectrometry to determinate six flavonoids and troxerutin in rat urine and chick plasma.[14] Wang et al. estimated quercetin and kaempferol concentrations in human urine using HPLC-UV.[15] Kazuo Ishii et al. used SPE-HPLC-UV to study concentration of rutin in human volunteers' plasma and quercetin in human plasma and urine.[16–17] Recently, Zhao et al. used liquid chromatography/electrospray ionization/mass spectrometry (LC/ESI/MS) to determine quercetin, kaempferol, isorhamnetin, bilobalide, ginkgolides A, B, C, and J in plasma.[18]

Many scholars have studied flavonoids in biological specimens, but there was little attention paid to the simultaneous determination of the concentrations of selected flavonols (rutin, quercitrin, quercetin, kaempferol, and isorahamnetin) in plasma and its pharmacokinetics after intravenously administrated GBE. There are literatures published the pharmacokinetic of GBE in rabbits by calculating the total flavonol glycoside content from the aglycone concentration after acid hydrolysis or total peak area of GBE in plasma,[19,20] but rutin, quercitrin, and other flavonol glycosides might be effective components in GBE and simultaneous determination of several effective components in vivo through optimizing the extraction method and chromatographic conditions might be suitable for the study of pharmacokinetic properties of GBE. So, this paper describes a gradient LC-DAD method to quantify five useful flavonoids in rat plasma after intravenously administrated GBE and was successfully applied to the pharmacokinetic study of GBE.

EXPERIMENTAL

Chemicals and Reagents

HPLC grade-methanol was obtained from Fisher Scientific (Fisher Scientific, USA). Rutin, quercitrin, quercetin, kaempferol, and isorhamnetin (see Fig. 1[7,9,10,11,14–16]) reference standard were purchased from National Institute for the Control of Pharmaceutical and Biological Products (NICPP, Beijing, China). The purity (99.5%) of these reference standards was assumed as provided by the suppliers. Water was prepared by automatic double purified water distilling apparatus (Shanghai Yarong Biochemical Factory, China).The mobile phase was degassed by Ultrasonic Generator (Wuxi Ultrasonic Generator Electronic Equipment Company, China) and filtered by 0.45 um filter (Autoscience Instrument Co. Ltd, China). GBE used in this paper was obtained from Xuzhou Enhua Pharmaceutical Factory (Xuzhou, China) respectively. GBE injection (20 mg/mL, dissolved with 30% PEG400 water solution) was prepared by our laboratory. All other chemicals were of analytical grade.



Flavonol	R_1	R_2
Rutin	OH	Rutinose
Quercitrin	OH	Rhamnose
Quercetin	OH	H
Kaempferol	H	H
Isorhamnetin	OCH_3	H

Figure 1. Chemical structures of five flavonoids.

Method Development

Different mobile phases were tried, such as water-acetonitrile-isopropanol-citric acid, acetonitrile-0.01% potassium dihydrogen phosphate (Phosphoric acid was used to regulate pH 2.0), methanol-0.1% formic acid, acetonitrile-0.1% formic acid, acetonitrile-0.3% formic acid, etc. Finally, methanol and 0.1% formic acid was selected as an appropriate mobile phase with a step linear gradient, which gave good resolution and acceptable peak parameters for rutin, quercitrin, quercetin, kaempferol, and isorhamnetin.

Chromatographic System

The HPLC (Shimadzu, Kyoto, Japan) instrument was equipped with a model series LC-10 ADVP pump, DGU-20A degasser, FCV-10ALVP, SCL-10AVP system controller, Rheodyne 7725 injector with a 20 μL loop and a SPD-20AVP Diode array detector. System control and data analyses were carried out using LCsolution software (Shimadzu). Separation have been done on a kromasil C18 column (5 μm particle size, 250 mm × 4.6 mm i.d. Dalian Elite Company, China) using a one step linear gradient. Mobile phase A (methanol) and B (0.1% formic acid) ratios

changed as follows: $0 \sim 5$ min, $35 \sim 40\%$A; $5 \sim 40$ min, $40 \sim 50\%$A; $40 \sim 50$ min, $50 \sim 60\%$A; $50 \sim 55$ min, $60 \sim 65\%$A. The total run time was 55 minutes at a flow rate of 1 mL/min. The eluent was monitored by a diode array detector and the detection wavelength was set at 350 nm. The sample injection volume was 20 μL and the column temperature was 35°C.

Preparation of Standard Stock Solutions

Individual standard stock solutions of the reference compounds were prepared by weighing accurately 5.05 mg rutin, 4.83 mg quercitrin, 5.05 mg quercetin, 1.23 mg kaempferol, and 0.98 mg isorhamnetin into 25 mL volumetric flasks filling them to volume using methanol. These solutions were stored at 4°C away from light and were found to be stable for at least 1 month. Working standard solutions for spiking plasma were freshly prepared by diluting the stock solution with methanol-0.1% formic acid (70:30, v/v) to appropriate concentrations.

The samples for plasma standard calibration curves were prepared by spiking the blank rat plasma (100 μL) with 100 μL of the appropriate working solution to yield the following concentrations: rutin $0.2525 \sim 20.2$ μg/mL, quercitrin at $0.1208 \sim 9.66$ μg/mL, quercetin at $0.1008 \sim 20.16$ μg/mL, kaempferol at $0.031 \sim 2.46$ μg/mL, isorhamnetin at $0.098 \sim 7.84$ μg/mL. Quality control samples used for the study of intra-day and inter-day accuracy and precision, extraction recovery and stability were prepared in the same way as the calibration samples.

Plasma Sample Preparation

Two hundred microliters of plasma was aliquoted in a centrifuge tube, and then spiked with acetone-diethyl ether (1:14, v/v) 1.5 mL by vortex mixing for 1 min. The mixture was centrifuged at 14,000 rpm for 15 min to separate water phase from the organic phase (I). The water phase was spiked with 1 mL methanol by vortex mixing for 1 min and centrifuged at 14,000 rpm for 15 min to separate protein from the organic phase (II). The organic phase (I) and the organic phase (II) were put together and transferred to a clean centrifuge tube. This mixture was evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted with 200 μL methanol-0.1% formic acid (70:30, v/v) and filtrated with 0.45 μm cellulose acetate membrane. 20 μL of the solution was injected into the chromatographic system.

Validation of the Assay Method

Calibration

The plasma samples for standard cure were prepared as described in plasma sample preparation. An aliquot of 20 μL of the resulting solution was injected into HPLC system, and each concentration was analyzed for five times. Based on the peak area, calibration lines of peak area versus analyte concentrations were plotted.

Detection Wavelength and Specificity

The rat plasma samples were detected at 254 nm and 350 nm by diode array detector under the chromatography conditions as described in chromatography system. Interference of endogenous or other substance in rat plasma was investigated. The specificity of the method was investigated by comparing the chromatograms of blank plasma samples from different rats with that of blank plasma spiked with standard solution and the samples collected after intravenous administration of GBE injection.

Recovery

Recovery was calculated by comparing the peak areas of the extracted quality control samples with that of the unextracted standard solutions containing the equivalent amount of analytes.

Precision

Spiked rat plasma samples with different concentrations of rutin, quercitrin, quercetin, kaempferol, and isorhamnetin were used for precision studies. The intra-day precision was calculated by analyzing five replicates of control samples within 1 day. The inter-day variability was estimated by analyzing samples on 5 separate days. The relative standard deviation (RSD) was used to estimate the precision.

Sensitivity

The limit of detection (LOD) was defined as the lowest concentration of the drug resulting in a signal-to-noise ratio of 3:1. The limit of quantification (LOQ) was defined as the lowest concentration of spiked plasma samples that can be determined with sufficient precision and accuracy, i.e., RSD less than 15%.

Stability

Stability of five flavonoids in rat plasma was examined in two ways: (1) The stability of analytes in the plasma stored at −20°C was determined in 0, 1, 2, 3, 7 days. (2) The stability of the analytes after extraction from plasma and dissolving in methanol-0.1% formic acid (70:30, v/v) at 4°C away from light was determined at 0, 24 and 48 h.

Application of the Analytical Method

Healthy male Wistar rats with an average weight of 200 g were provided by Xuzhou Medical College (Xuzhou, China). Animals were housed in a room with controlled temperature and humidity, and allowed free access to food and water. The rats were intravenously administrated GBE injection. Approximately 0.5 mL blood samples were collected from each rat after drug administration at 1, 2, 3, 5, 10, 15, 30, 60, 90, 120, 180 and 240 min, respectively. Venous blood samples from rats were collected into heparinized plastic tubes and were centrifuged immediately. The plasma samples were frozen at −20°C until required for LC analysis.

RESULTS AND DISCUSSION

In addition to the determination of effective components in traditional preparation medicines, it is more critical to clarify the destiny of effective components in vivo-the pharmacokinetic profiles. Because there are various effective components in a single preparation, simultaneous determination of several effective components in vivo through optimizing the extraction method and chromatographic conditions should be a quick and simple way to study the pharmacokinetic properties of several effective components in one preparation. Due to the significant difference in polarity and concentration between rutin, quercitrin, quercetin, kaempferol, and isorhamnetin, simultaneous determination of the five flavonoids in plasma becomes difficult in a single HPLC run. Although there are literatures concerning the determination of rutin, quercitrin, quercetin, kaempferol, and isorhamnetin simultaneously in *Ginkgo bibola* extract solid oral dosage or independently in plasma using HPLC with relatively fine sensitivity,[10,11,13] the methods may not be appropriate for the study of the traditional Chinese preparations in vivo for low drug concentration and interference of endogenous substance in plasma. The major contribution of the present HPLC method is to develop a simple extraction method and a gradient elution program to reduce the running time and reach satisfactory specificity and sensitivity for rutin, quercitrin,

quercetin, kaempferol, and isorhamnetin at the same time after intravenously administrated GBE.

Detection Wavelength and Specificity

In order to obtain the optimal detection wavelengths for the chromatography separation, the separated result of rutin, quercitrin, quercetin, kaempferol, and isorhamnetin was obtained at 254 nm and 350 nm by diode array detector. The results of different detection wavelength showed that the absorbance peaks of five flavonoids were different. Although they all had the best or better absorbance peaks at 254 nm, interference ingredients in the preparation have the same chromatographic behavior as rutin (see Figs. 2c and 2d). Therefore, the diode array detector was set at the wavelength of 350 nm in this method. The representative HPLC profiles of a blank plasma sample (Fig. 2a), a blank plasma sample spiked with standard solution (Fig. 2b) and plasma sample at 10 min after intravenous administration of GBE injection (Fig. 2c) were shown in Fig. 2. No interference was observed under the assay conditions. The peaks of the analytes in the plasma were identified by comparing their retention time with that of the standard and further confirmed by their on-line UV spectra.

Sample Preparation

In this study, several solvent systems, such as 10% trichloroacetic acid, methanol, acetonitrile, acetone, acetoacetate and acetone-diethyl ether with different composition ratios were tested for extraction of rutin, quercitrin, quercetin, kaempferol, and isorhamnetin from rat plasma. 10% trichloroacetic acid and methanol have better extraction recovery and precision for rutin and quercitrin than other solvent systems, but 10% trichloroacetic acid can split the peak of rutin. Among them best extraction and precision of quercetin, kaempferol, and isorhamnetin were observed when a mixture of acetone-diethyl ether (1:14, v/v) was chosen. Therefore, the rat plasma was extracted with methanol and acetone-diethyl ether (1:14, v/v) respectively and the two extracted solution were put together for simultaneous determination of rutin, quercitrin, quercetin, kaempferol, and isorhamnetin.

Linearity and Sensitivity

The calibration curve of each compound was established by injecting eight different concentrations consisting of five flavonoids, a good

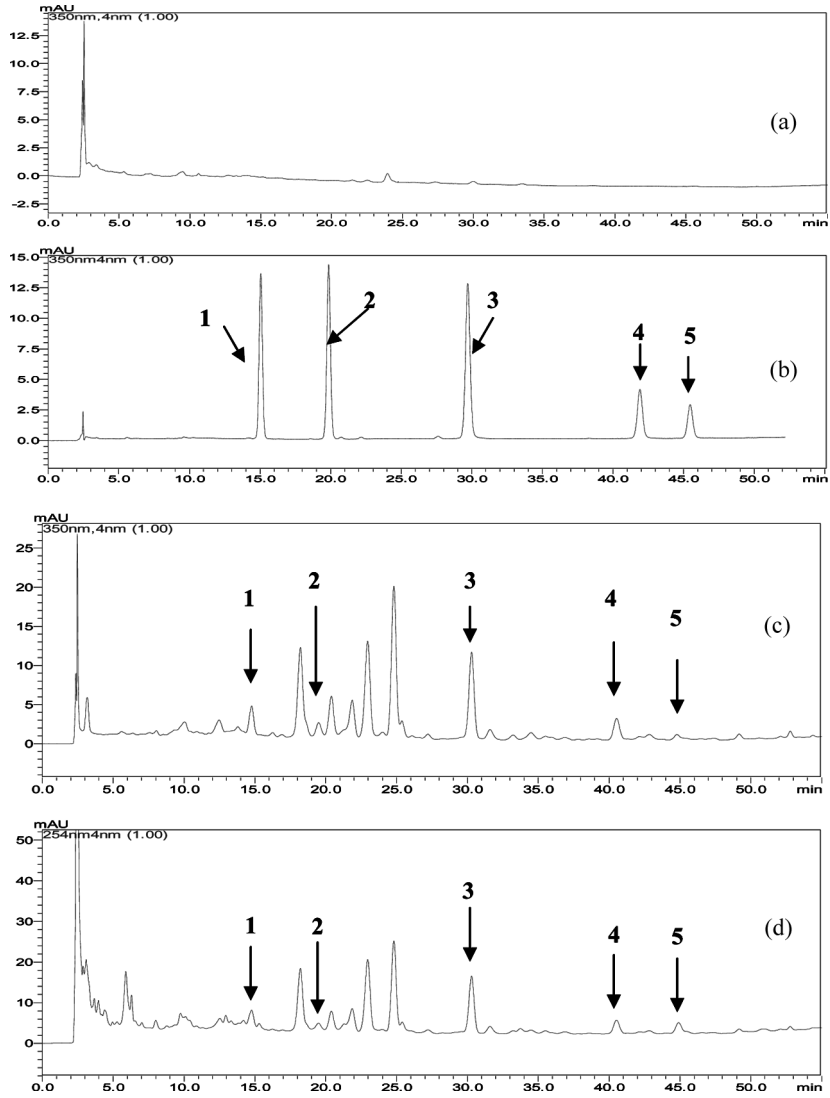


Figure 2. Chromatograms of rat plasma samples. (a) Blank plasma detected at 350 nm; (b) plasma spiked with Rutin, Quercitrin, Quercetin, Kaempferol and Isorhamnetin detected at 350 nm; (c) plasma sample at 10 min after intravenous administration of *Ginkgo biloba* extract injection detected at 350 nm; (d) plasma sample at 10 min after intravenous administration of *Ginkgo biloba* extract injection detected at 254 nm. (1. Rutin; 2. Quercitrin; 3. Quercetin; 4. Kaempferol; 5. Isorhamnetin).

correlation was found between the peak area (y) and the concentrations (x). The results of each compound, such as linearity, linear range, limit of detection (LOD), limit of quantification (LOQ) and correlation coefficient (R), were summarized in Table 1. The experimental results showed that the LODs of each flavonoid were very low (0.006–0.02 μg/mL), which indicated that this method was high sensitive.

Precision

Analytical precision data were shown in Table 2 and were expressed as relative standard deviation (RSD). The precisions of rutin, quercitrin, quercetin, kaempferol, and isorhmnetin calculated as the relative standard deviation (RSD) at low to high concentrations were better than 7% and 10% for intra-day and inter-day assays, respectively.

Recovery

The accuracy of the method, in terms of recovery efficiency, is a measure of the response of the analytical method to the entire quantity of the analyte contained in a sample. The recoveries of the five flavonoids in rat plasma samples were calculated and the results were shown in Table 2 with the mean values of five replicate injections. The recoveries for the five flavonoids were between 72.2% and 99.2% in rat plasma (see Table 2). From the results of recoveries, it can be concluded that the present method for the analysis of five flavonoids in plasma has a good accuracy.

Table 1. Linearity and sensitivity of detection for five flavonoids in rats plasma by RP-HPLC ($n^c = 5$)

Analyte	Linear range (μg/mL)	Regression equation	Correlation coefficient	LOD[a] (μg/mL)	LOQ[b] (μg/mL)
Rutin	0.126 ~ 20.2	Y = 9181 × −23.51	0.9995	0.01	0.04
Quercitrin	0.121 ~ 9.66	Y = 11276 × − 440.88	0.9999	0.02	0.07
Quercetin	0.101 ~ 20.2	Y = 41571 × − 3742.1	0.9977	0.006	0.02
Kaemferol	0.031 ~ 2.46	Y = 39793 × − 592.53	0.9990	0.02	0.07
Isorhamnetin	0.098 ~ 7.84	Y = 25563 × +35.203	0.9995	0.02	0.08

[a]LOD: Limit of detection.
[b]LOQ: Limit of quantitation.
[c]n: Number of determination.

Table 2. Extraction recoveries and intra-day and inter-day (5 separate days) precision for the determination of five flavonoids in rat plasma

		Recovery (n = 5)		Precision RSD[a] (%) (n = 5)	
Analyte	Nominal concentration (μg/mL)	Mean (%)	RSD (%)	Intra-day	Inter-day
Rutin	10.1	89.6	5.3	1.6	4.7
	2.53	80.5	7.7	2.3	4.1
	0.253	73.2	7.5	2.5	9.1
Quercitrin	4.83	87.2	4.5	2.9	9.3
	1.21	77.9	5.1	1.2	7.9
	0.121	76.3	6.3	3.9	8.5
Quercetin	10.1	83.1	4.1	2.4	9.2
	2.52	84.9	6.2	5.1	7.4
	0.252	72.2	5.2	6.1	8.2
Kaemferol	1.23	99.2	4.2	1.1	4.9
	0.308	84.3	5.3	2.6	6.8
	0.031	72.5	8.4	2.8	8.3
Isorhamnetin	3.92	86.3	3.9	3.3	6.6
	0.98	85.7	5.1	3.4	4.4
	0.098	72.3	7.9	2.9	8.6

[a]RSD: Relative standard deviation.

Storage Stability

The stability experiments were aimed to testing all possible conditions that the samples might experience after collection and prior to the analysis. So the stability of five flavonoids in rat plasma samples was investigated. The results of storage stability were summarized in Table 3. The stability test indicated that all five flavonoids were stable for at least 7 days in plasma at −20°C (see Table 3). In addition, the five flavonoids were also stable in prepared samples for 48 h (see Table 3) demonstrating the stability of the analytes in 2 days when placed at 4°C away from light before analysis.

Application to Pharmacokinetic Study

GBE was given to ten Wistar rats by intravenous administration at the dosage of 20 mg/kg. Blood samples (0.5 mL) were collected by vena caudalis at 1, 2, 3, 5, 10, 15, 30, 60, 90, 120, 180 and 240 min post-intravenous administration and were immediately centrifuged

Table 3. The stability of five flavonoids in plasma stored at −20°C was determined during 7 days as well as in prepared samples stored at 4°C away from light during 48 h (mean, n = 3)

		In plasma		In prepared samples	
Analyte	Nominal concentration (μg/mL)	Mean detected concentration (μg/mL)	RSD (%)	Mean detected concentration (μg/mL)	RSD (%)
Rutin	10.1	10.08	2.3	9.98	1.9
	2.53	2.41	6.5	2.49	3.5
	0.253	0.227	7.1	0.226	4.8
Quercitrin	4.83	4.72	4.2	4.59	3.8
	1.21	1.09	7.1	1.16	2.8
	0.121	0.123	5.2	0.123	2.2
Quercetin	10.1	9.93	1.9	9.92	2.7
	2.52	2.30	3.5	2.24	2.4
	0.252	0.254	3.5	0.261	2.1
Kaempferol	1.23	1.17	2.5	1.26	2.6
	0.308	0.273	3.6	0.303	4.9
	0.031	0.038	4.3	0.030	3.2
Isorhamnetin	3.92	3.88	4.3	3.90	1.5
	0.98	0.832	7.1	0.908	3.8
	0.098	0.092	5.1	0.086	5.6

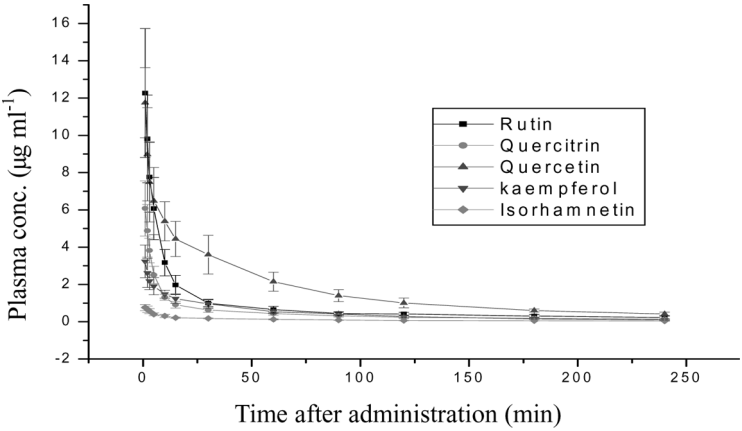


Figure 3. Plasma concentration versus time profiles of five flavonoids in rats in rats (n = 10) following intravenous administration of *Ginkgo biloba* extract injection at a single dose of 20 mg kg^{-1}.

Table 4. Pharmacokinetic parameters of five flavonoids in rats plasma (mean, n = 10)

Parameters	Units	Rutin	Quercitrin	Quercetin	Kaemperol	Isorhamnetin
AUC(0−*t*)	mg h/L	3.294	1.798	6.922	1.878	0.426
AUC(0−∞)	mg h/L	3.931	2.196	7.642	2.043	0.589
MRT(0−*t*)	h	0.911	1.023	1.056	1.03	1.188
*t*1/2	h	2.046	2.066	1.319	1.222	3.121
Cl[a]	L/h/kg	5.179	9.303	2.69	9.99	35.308
V[b]	L/kg	15.003	27.493	5.094	19.669	134.49

Cl[a]: clearance. V[b]: apparent volume of distribution.

(14000 rpm for 10 min) to separate the plasma fractions. The plasma samples were treated according to the procedure described in plasma sample preparation. And then the volume of 20 μL of the above solution was injected under the optimum conditions mentioned earlier. The chromatograms generated from the above rat plasma were shown in Fig. 2, respectively. It showed that there was no obvious interference on the analysis of five flavonoids in rat plasma matrixes. The concentration of five flavonoids was calculated according to the previously established linear regression equations in Table 1, and the mean plasma concentration-time profiles of the five flavonoids were shown in Fig. 3. The pharmacokinetic parameters from two-compartment model analysis were summarized by the DAS Ver2.0 (Drug and Statistics for Windows) program in Table 4.

CONCLUSION

Although there were many literatures about determination of rutin, quercetin, kaempferol, and isorhamnetin in biological specimen,[7,14–20] there was not simultaneously analysis of rutin, quercitrin, quercetin, kaempferol, and isorhamnetin in rat plasma. In summary, the RP-HPLC method mentioned here represented an excellent technique for firstly simultaneous separation and determination of five flavonoids in rat plasma after the intravenous administration with good sensitivity, precision and reproducibility. And the method employed simple sample preparation for rat plasma samples, and it is easily adaptable in many laboratories using commonly available HPLC equipment. Especially, it was successfully applied to determine drug concentrations in rat plasma samples that had been intravenously administered with GBE, and it will play a reference role on the determination of flavonoids in other medicinal plants, pharmaceutical preparations or clinic analysis.

ACKNOWLEDGMENT

The authors acknowledge support for this work by the Jiangsu University Natural Science Foundation of China (No. 07KJD360225).

REFERENCES

1. Chavez, M.L.; Chavez, P.I. Ginkgo (part 2) clinical efficacy, dosage, and adverse effects. Hosp. Pharm. **1998**, *33*, 1076–1095.
2. Ndjoko, K.; Wolfender, J.L.; Hostettmann, K. Determination of trace amounts of ginkgolic acids in *Ginkgo biloba* L. leaf extracts and phytopharmaceuticals by liquid chromatography–electrospray mass spectrometry. J. Chromatogr. B **2000**, *744*, 249–255.
3. Stackman, R.W.; Eckenstein, F.; Frei, B.; Kulhanek, D.; Nowlin, J.; Quinn, J.F. Prevention of age-related spacial memory deficits in a transgenic mouse model of Alzheimer's disease by chronic *Ginkgo biloba* treatment. Exp. Neurol. **2003**, *184*, 510–520.
4. Ellnain-Wojtaszek, M.; Kruczynski, Z.; Krasprzak, J. Investigation of the free radical scavenging activity of *Ginkgo biloba* leaves. Fitoterapia. **2003**, *74*, 1–6.
5. Goh, L.M.; Barlow, P.J.; Yong, C.S. Examination of antioxidant activity of *Ginkgo biloba* leaf infusions. Food Chem. **2003**, *82*, 275–282.
6. Chavez, M.L.; Chavez, P.I. Ginkgo (part 1): history, use, and pharmacologic properties. Hosp. Pharm. **1998**, *33*, 658–672.
7. Watson, D.G.; Oliveira, E.J. Solid-phase extraction and gas chromatography-mass spectrometry determination of kaempferol and quercetin in human urine after consumption of *Ginkgo biloba* tablets. J. Chromatogr. B. **1999**, *723*, 203–210.
8. Boue, S.M.; Carter-Weintjies, C.H.; Shih, B.Y.; Cleveland, T.E. Identification of flavone aglycones and glycosides in soybean pods by liquid chromatography–tandem mass spectrometry. J. Chromatogr. A. **2003**, *991*, 61–68.
9. Van Beek, T.A. Chemical analysis of *Ginkgo biloba* leaves and extracts. J. Chromatogr. A **2002**, *967*, 21–55.
10. Hasler, A.; Sticher, O.; Meier, B. Identification and determination of the flavonoids from *Ginkgo biloba* by high-performance liquid chromatography. J. Chromatogr. A **1992**, *605*, 41–48.
11. Dubber, M.J.; Kanfer, I. High-performance liquid chromatographic determination of selected flavonols in *Ginkgo biloba* solid oral dosage forms. J. Pharmaceut. Sci. **2004**, *7*, 303–309.
12. Mauri, P.; Pietta, P. Electrospray characterization of selected medicinal plant extracts. J. Pharm. Biomed. Anal. **2000**, *23*, 61–68.
13. Carbone, V.; Montoro, P.; Tommasi, N.; Pizza, C. Analysis of flavonoids from Cyclanthera pedata fruits by liquid chromatography/electrospray mass spectrometry. J. Pharm. Biomed. Anal. **2004**, *34*, 295–304.
14. Yang, G.J.; Liu, P.; Qu, X.L.; Xu, M.J.; Qu, Q.S.; Wang, C.Y.; Hu, X.Y.; Wang, Z.Y. The simultaneous separation and determination of six flavonoids

and troxerutin in rat urine and chicken plasma by reversed-phase high-performance liquid chromatography with ultraviolet–visible detection. J. Chromatogr. B. **2007**, *856*, 222–228.
15. Wang, F.M.; Yao, T.W.; Zeng, S. Determination of quercetin and kaempferol in human urine after orally administrated tablet of *Ginkgo biloba* extract by HPLC. J. Pharm. Biomed. Anal. **2003**, *33*, 317–321.
16. Kazuo, I.; Takashi, F.; Yasuji, K. Determination of rutin in human plasma by high-performance liquid chromatography utilizing solid-phase extraction and ultraviolet detection. J. Chromatogr. B. **2001**, *759*, 161–168.
17. Kazuo, I.; Takashi, F.; Yasuji, K. High-performance liquid chromatographic determination of quercetin in human plasma and urine utilizing solid-phase extraction and ultraviolet detection. J. Chromatogr. B **2003**, *794*, 49–56.
18. Zhao, Y.; Sun, Y.; Li, C. Simultaneous determination of ginkgo flavonoids and terpenoids in plasma: ammonium formate in LC mobile phase enhancing electrospray ionization efficiency and capacity. J. Am. Soc. Mass Spectrom. **2008**, *19*, 445–449.
19. Wang, F.M.; Wu, M.Z.; Zhou, L.; Yao, T.W.; Yu, Y.H. Study of assay method on the ginkgetin in plasma. China J. Chinese Materia Medica. **2003**, *28* (3), 279–281.
20. Yang, Q.J.; Wu, G.Z.; Xue, W.J.; Tang, Y.N.; Wu, X.Q. Determination of extract ginkgo biloba in blood plasma of rabbits. Herald of Medicine **2002**, *21* (11), 687–689.

Received December 20, 2008
Accepted March 4, 2009
Manuscript 6467