Journal of Chromatography B, 877 (2009) 3595–3600

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Sensitive determination of kaempferol in rat plasma by high-performance liquid chromatography with chemiluminescence detection and application to a pharmacokinetic study

Qunlin Zhang*, Yunjing Zhang, Zhongtang Zhang, Zidong Lu

School of Pharmacy, Anhui Medical University, 81 Meishan Road, Hefei, Anhui 230032, PR China

ARTICLE INFO

Article history:
Received 5 May 2009
Accepted 30 August 2009
Available online 4 September 2009

Keywords:
Kaempferol
High-performance liquid chromatography
Chemiluminescence
Cerium(IV)
Rhodamine 6G
Rat plasma
Pharmacokinetics

ABSTRACT

A simple and sensitive method based on high-performance liquid chromatography (HPLC) coupled with chemiluminescence detection was developed for the quantitation of kaempferol (KA) in rat plasma. Isorhamnetin (IS) was used as an internal standard. Plasma samples were prepared only by acidification with 20% phosphoric acid and protein precipitation using methanol. Good separations of kaempferol and internal standard were achieved with an isocratic elution using a mobile phase consisting of methanol and aqueous 0.4% phosphoric acid (47:53, v/v) within 25 min. The detection limit for kaempferol was 1.0×10^{-9} g/ml. The mean accuracy was within 80.0–100.2%, and the intra- and inter-day precision had RSD (%) < 5.0. The sample preparation method was able to produce high recovery (≥80.0%). The proposed method with wide linear range has been successfully applied to a pharmacokinetic study in SD rats after oral administration at a dose of 2500 or 1250 mg/kg bodyweight (BW) kaempferol. Kaempferol concentration was detectable in plasma up to 24 h post-dosing, and the pharmacokinetic parameters of T_{max}, C_{max}, $AUC_{0-\infty}$, $MRT_{0-\infty}$, and $T_{1/2}$ of kaempferol were reported.

1. Introduction

Flavonoids are a large group of naturally occurring polyphenols with a wide range of physiological benefits. They are subdivided into six classes: flavones, flavanones, isoflavones, flavonols, flavanols, and anthocyanins varying in their structure characteristics around the heterocyclic oxygen ring [1]. Kaempferol (3,5,7,4′-tetrahydroxy flavone), a major representative of the flavonol subclass, has received much attention during the past few years because of its anticarcinogenic [2,3], antiatherogenic [4], antioxidant [5], antiphlogistic [6], and osteogenic properties [7]. In order to better understand the pharmacological effects of flavonols, researchers have sought to perform their pharmacokinetic studies [8]. However, to our best knowledge, the pharmacokinetic data of kaempferol is still extremely limited. Only a liquid chromatography method with diode-array detector (DAD) for the simultaneous determination of quercetin, kaempferol, and isorhamnetin in rabbit plasma was developed and applied in the pharmacokinetics after intragastric administration of the ethanol extract from *Pollen Typhae* to rabbit. It was found that the concentration–time profile of kaempferol was best fitted to the two-open compartment model [9].

Analytical methods employed for the quantitative determination of drugs and their metabolites in biological samples are the key determinants of pharmacokinetic study. Up to now, analysis of flavonoids has been accomplished by thin-layer chromatography (TLC) [10], gas chromatography (GC) [11,12], capillary electrophoresis (CE) [13,14], micellar electrokinetic chromatography (MEKC) [15], and high-performance liquid chromatography (HPLC) [16–25]. For the separation and determination of kaempferol in biological samples, high-performance liquid chromatography (HPLC) has been shown to be suitable, in which UV [24], electrochemical [19], fluorescent [17] and mass [22] detections were used. However, UV detection suffers from lack of sensitivity and limited linear range. Electrochemical detection has poor reproducibility, fluorimetric detection often required derivatization step, and MS detection needs special and expensive equipment. Altogether, these methods often require tedious sample preconcentration. For example, the sample preparation of kaempferol in rabbit plasma was carried out as following. After the deproteinization process, the supernatant was extracted for three times with n-butanol-acetoacetate, and then the organic layer was combined and dried by nitrogen gas [9]. This cannot allow high-throughput analysis required for a pharmacokinetic study. Moreover, recovery will

* Corresponding author. Tel.: +86 551 5161115; fax: +86 551 5167735.
E-mail address: qlzhang@ahmu.edu.cn (Q. Zhang).

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doi:10.1016/j.jchromb.2009.08.046

decrease after the long exposure time of sample preparation, because flavonols with polyphenolic hydroxyl group are not stable and readily oxidized. For the pharmacokinetic study, a matrix-based standard curve must cover the entire range of concentrations in the unknown biological samples. Instead, the standard curve should be redetermined or samples be reassayed after dilution with the matrix [26]. Sometimes, the potential ranges between minimum plasma concentration and maximum plasma concentration cover two or more orders of magnitude. Therefore, it is a challenge to develop a highly sensitive and high-throughput assay with wide linear range for the quantitation of kaempferol in biological samples in the pharmacokinetic study.

In recent years, due to its high sensitivity, wide linear range and simple instrumentation, HPLC coupled with chemiluminescence (CL) detection has become more and more attractive for the determination of compounds at trace levels in complex matrices [27] and has proved to be a suitable method for the separation and quantitation of some active flavonols in phytopharmaceuticals in our previous work [28]. However, as far as we know, there has been no report on HPLC-CL method used for the determination of flavonols occurring in biological samples such as plasma. In this paper, further study focuses on the minimal sample pretreatment and the selective and sensitive detection of trace levels of kaempferol aglycone in rat plasma by HPLC-CL method. The proposed method was successfully applied to the pharmacokinetic studies of rats, and the pharmacokinetic parameters were obtained.

2. Experimental

2.1. Chemicals and solutions

Methanol was of HPLC grade. All other chemicals were of analytical-reagent grade and redistilled water was used to prepare all solutions, with the exception of kaempferol fed to the animals, which was of 98.5% purity. Cerium(IV) sulphate tetrahydrate was purchased from Sinopharm Chemical Reagent Co., Ltd., (Shanghai, China). Rhodamine 6G was purchased from Merck (Darmstadt, Germany). Kaempferol and isorhamnetin reference standards were purchased from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

Stock solutions of 2.0×10^{-4} g/ml kaempferol and internal standard were prepared by dissolving appropriate amount of kaempferol and isorhamnetin in methanol, respectively. These stock solutions remained stable for three months if stored in a refrigerator at 4 °C. 1.0×10^{-3} mol/l rhodamine 6G was prepared with redistilled water and kept at ambient temperature in the dark. The working cerium(IV) solutions were prepared daily in sulfuric acid prior to use. The HPLC mobile phases were prepared fresh daily, filtered through a 0.45 μm membrane filter (Bandao, Shanghai).

2.2. Instrumentation

The HPLC-CL detection system consisted of an HPLC system and a CL detection system, as shown in Fig. 1. The HPLC system was Shimadzu LC-20A series (Shimadzu Corporation, Japan), consisting of a quaternary pump, a vacuum degasser, a thermostated column compartment, a diode-array detector (DAD) system, a manual sample valve injector with a 20 μl loop, and an analytical column (Hypersil ODS, 200 mm × 4.6 mm ID 5 μm; Dalian Elite Analytical Instruments Co., Ltd., China). The CL detection was performed with a flow injection CL system (Remax, China) consisted of a model IFFM-E peristaltic pump, a mixing tee, and a model IFFS-A CL detector equipped with a flat glass coil (used as reaction coil and detection cell) and a photomultiplier operated at −900 V.

2.3. Chromatography

The analysis was performed on a ODS column at 30 °C with isocratic elution at a flow rate of 1.5 ml/min. The mobile phase was composed of methanol and aqueous 0.4% phosphoric acid (47:53, v/v). Detection by DAD was performed at 365 nm. The UV spectra were recorded between 200 and 400 nm for the identification of the potential metabolites of kaempferol in plasma and for the test of peak purity. The column effluent from DAD was first mixed on-line with rhodamine 6G solution via a PEEK tube, then combined with cerium(IV) solution containing sulfuric acid in a mixing tee. Solutions of cerium(IV) and rhodamine 6G were delivered by a peristaltic pump at a flow rate of 1.5 ml/min, respectively.

2.4. Plasma sample preparation

100 μl of plasma sample of kaempferol was spiked with 5.0 μl isorhamnetin stock solution (internal standard, 2.0×10^{-4} g/ml methanol), and acidified with 10 μl 20% phosphoric acid solution to adjust the plasma for pH 2.5, followed by protein precipitation with 200 μl of methanol. The mixture was vortexed for 1.0 min and centrifuged for 10 min at 10,000 × *g*. 10 μl of the supernatant was directly injected into HPLC.

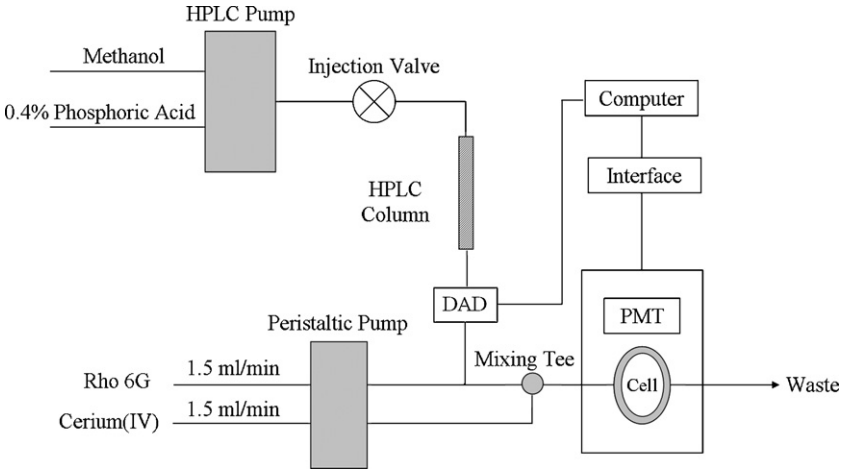

Fig. 1. Schematic diagram of the HPLC-CL system used for the determination of kaempferol.

2.5. Animals

Animal studies were performed according to the Guidelines for the Care and Use of Laboratory Animals approved by the Committee of Ethics of Animal Experimentation of Anhui Medical University (Hefei, China).

12 Sprague–Dawley rats (6 males and 6 females) aged 7 weeks (200 ± 20 g) were supplied by at the Laboratory Animal Center of Anhui Medical University, housed in an air-conditioned room (temperature, 22–25 °C; relative humidity, 55 ± 5%), and kept on a light/dark cycle of 12/12 h. Free access to food and mains drinking water was allowed throughout the study except for fasting 12 h before the experiment.

2.6. Method validation

2.6.1. Selectivity

Selectivity was investigated by examining peak interference from six independent sources of blank plasma.

2.6.2. Linearity and sensitivity

The linearity of the method was evaluated over the range of 1.0×10^{-9}–4.0×10^{-6} g/ml using blank plasma spiked with kaempferol. The tested concentrations of kaempferol were 1.0×10^{-9}, 2.0×10^{-9}, 3.0×10^{-9}, 2.0×10^{-8}, 8.0×10^{-8}, 5.0×10^{-7}, 1.0×10^{-6}, 2.0×10^{-6}, and 4.0×10^{-6} g/ml. The concentration range was selected according to the potential plasma concentration of kaempferol in the pharmacokinetic study. Calibration curve was constructed by plotting logarithm of kaempferol concentration against the logarithm of the CL intensity ratio (kaempferol/IS). The signal-to-noise ratio (S/N) of 3 was set as the threshold for calculating the detection limit.

2.6.3. Precision, accuracy and recovery

Precision was expressed as the relative standard deviation (RSD) of each calculated concentration while accuracy was calculated as the percentage of the found concentration to the added concentration. The intra-day precision was tested with five spiked samples at three concentration levels of 2.0×10^{-8}, 1.6×10^{-7} and 1.0×10^{-6} g/ml. The inter-day precision and accuracy were evaluated by injecting five times every day on five consecutive days.

The recovery was determined by comparing the CL intensity ratio (kaempferol/IS) obtained from the blood after pretreatment with those obtained by direct injection of the same amount of analyte in standard solution. Replicate analysis ($n = 5$) of plasma samples at three concentration levels was used for the determination of recovery.

2.7. Application to pharmacokinetic study

12 Sprague–Dawley rats (6 males and 6 females) were randomly allocated into two dosage groups of 6 animals each (3 males and 3 females) and administered orally at a dose of 2500 or 1250 mg/kg bodyweight (BW) kaempferol in 0.5% sodium carboxy methyl cellulose (CMC-Na). Blood samples were collected from the retro-orbital plexus into heparinized tubes prior to dosing and at 5.0, 15.0, 30.0, 45.0 min, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 12.0, and 24.0 h post-dosing. Plasma was isolated by centrifugation at 4 °C (4000 × *g*, 5 min) and stored at −20 °C until analysis.

Pharmacokinetic (PK) parameters including area under concentration–time curve (AUC_{0-t}), maximum plasma concentration (C_{max}), time to reach the maximum concentrations (T_{max}), mean residence time (MRT_{0-t}) were estimated by a non-compartmental analysis based on statistical moments using Drug And Statistics 2.0 (DAS 2.0) (Mathematical Pharmacology Professional Committee of China, Shanghai, China).

3. Results and discussion

3.1. Optimization of HPLC system

As for HPLC-CL detection, the mobile phase of HPLC is not only suitable for the separation of kaempferol and internal standard but is also compatible with the cerium(IV)–rhodamine 6G CL reaction. Several mobile phases have been reported for the separation of flavonols on RP-C18 column, such as methanol–tetrafluoracetic acid–water [16], methanol–acetic acid–water [21], and acetonitrile–acetic acid–water [24]. In this work, the mobile phase of methanol–phosphoric acid–water was found to be suitable for the separation of flavonols and compatible with the cerium(IV)–rhodamine 6G CL system in sulfuric acid medium. The mobile phase containing methanol and aqueous 0.4% phosphoric acid (47:53, v/v) was considered optimal for isocratic elution and for the CL intensities of kaempferol and internal standard. Isorhamnetin was selected as the IS due to its suitable CL intensity, retention time and good chromatographic separation.

3.2. Optimization of CL system

To obtain the maximal relative CL intensity, the effects of rhodamine 6G, cerium(IV), sulfuric acid concentration, and flow rate on relative CL intensity were investigated. The kaempferol concentration used for all the optimization experiments was 5.0×10^{-7} g/ml.

The effects of rhodamine 6G concentration on the relative CL intensities of kaempferol were tested over the range 1.0×10^{-6}–2.0×10^{-5} mol/l. The results in Fig. 2a showed that the optimum concentrations of rhodamine 6G for kaempferol was 3.0×10^{-6} mol/l. Lower concentrations of rhodamine 6G gave lower CL emission because of fewer energy receptors, and higher concentrations produced decreased CL emission due to self-absorption of radiation.

The effects of cerium(IV) concentration on the relative CL intensities of kaempferol were investigated in the range 1.0×10^{-4}–6.0×10^{-3} mol/l in 0.5 mol/l sulfuric acid medium, as shown in Fig. 2b. For kaempferol the maximum enhanced CL intensity was reached at a concentration of 2.0×10^{-4} mol/l. Above this concentration, the CL intensities decreased. Lower concentrations of oxidant produced lower CL emission, whereas CL emission decreased with higher cerium(IV) concentrations. Because of the increased collisional energy transfer between molecules caused by higher cerium(IV) concentration, some excited-state rhodamine 6G molecules return to the ground state by a non-radiative internal transfer process, which would decrease the chemiluminescent quantum yield. Therefore, 2.0×10^{-4} mol/l cerium(IV) was selected for kaempferol determination.

Cerium(IV) is not readily soluble in water, but becomes stable when dissolved in sulfuric acid. The effects of sulfuric acid concentration on the relative CL intensities of kaempferol were studied over the range 0.3–0.7 mol/l, as shown in Fig. 2c. The relative CL intensities for kaempferol increased with increasing sulfuric acid concentration up to 0.5 mol/l. Thus, 0.5 mol/l sulfuric acid was chosen for further studies.

The flow rates of solutions are very important to CL reactions and should be regulated. Under the above selected conditions, the effects of flow rate on the relative CL intensity of kaempferol were studied over the range 0.5–3.5 ml/min in each stream (Fig. 2d). The flow rates of cerium(IV) and rhodamine 6G solution are set at the same value for convenience. The CL intensities for kaempferol increased with increasing flow rate up to 1.5 ml/min. Above that

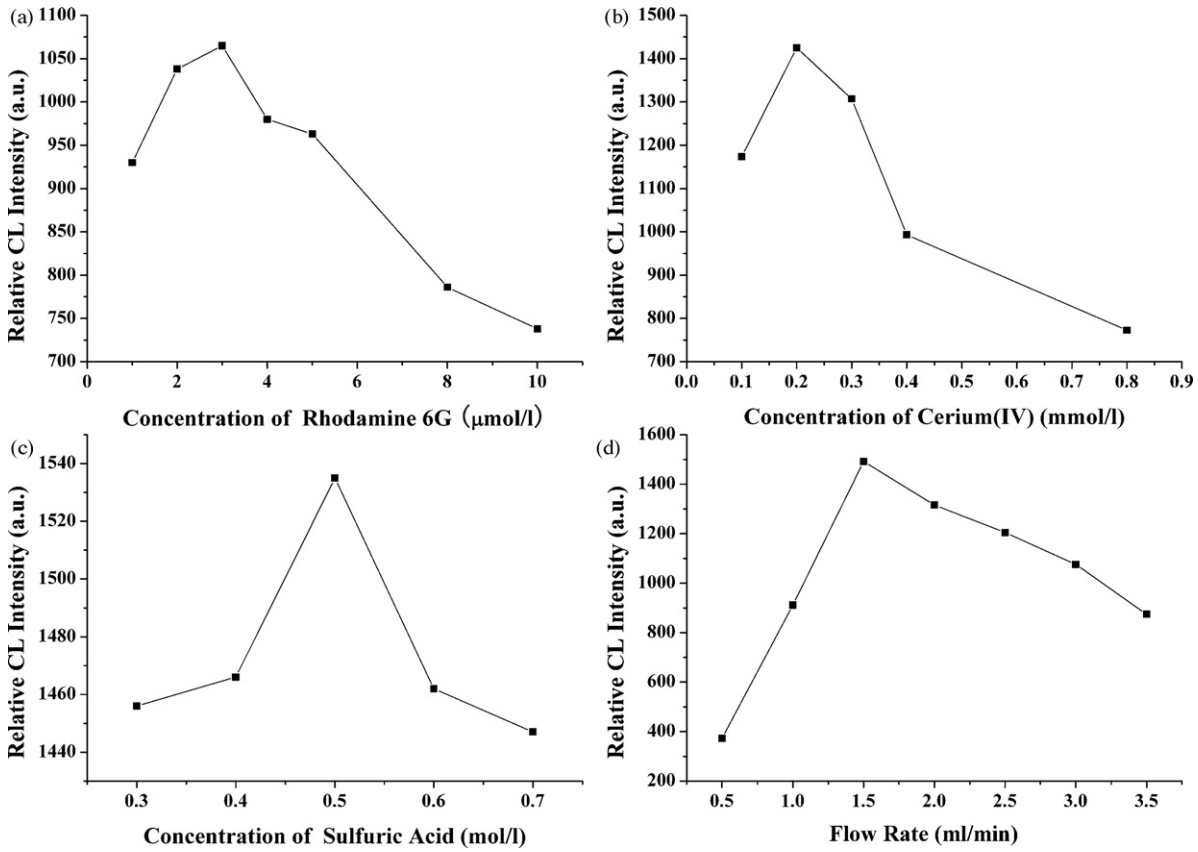

Fig. 2. Effects of the concentration of (a) rhodamine 6G: under the condition of 1.0×10^{-4} mol/l cerium(IV); (b) cerium(IV): under the condition of 3.0×10^{-6} mol/l rhodamine 6G; (c) sulfuric acid and (d) flow rate on the relative CL intensities. The kaempferol concentration used for all the optimization experiments was 5.0×10^{-7} g/ml.

value, the CL intensities decreased. The greatest emission occurred at a flow rate of 1.5 ml/min. Therefore, a flow rate of 1.5 ml/min was maintained throughout the current investigation.

3.3. Chromatography

An HPLC system equipped with a Hypersil ODS column was used to separate and analyse flavonols. Using an isocratic mobile phase composed of methanol and aqueous 0.4% phosphoric acid (47:53, v/v), good separation was achieved within 20 min. The retention times were 15.6 min for kaempferol and 18.3 min for isorhamnetin.

3.4. Method validation

3.4.1. Selectivity

Figs. 3 and 4 show the representative HPLC chromatograms of blank plasma, blank plasma spiked with kaempferol and IS, and plasma obtained at 30 min after oral administration of kaempferol (2500 mg/kg BW) detected with DAD at 365 nm and post-column CL detection, respectively. No endogenous peak interferences with kaempferol and IS. It is worth noting that kaempferol with lower concentration in plasma sample obtained at 30 min after oral administration of kaempferol (2500 mg/kg BW) cannot be detected using DAD detection, but was detectable by CL detection. The potential metabolites are marked as the peaks of 1–3 at the earlier retention time in the chromatograms detected with DAD as well as CL. Peak 1 probably is the coelution of a potential metabolite with endogenous substances which are not fully separated. The ultraviolet (UV) spectrum with the maximal absorption around 217 and 272 nm (Fig. 5) of peak 2 is essentially identical with those of hydroxyphenylacetic acids in blood samples of rats after oral administration of flavonoids extracted from Ginkgo biloba leaves [29]. It is reported that *p*-hydroxyphenylacetic acid was the metabolite of kaempferol [30] and that the CL intensity of cerium(IV)–rhodamine 6G system can be strongly enhanced by phenolic compounds [31]. Accordingly, the sharp peak 2 was observed at the retention time of 3.8 min in the CL chromatogram (Fig. 4). Thus, peak 2 was assumed to be *p*-hydroxyphenylacetic acid. The UV spectrum of peak 3 at 5.0 min with the maximal absorption around 263 and 348 nm exhibits the similar absorption pattern to those of flavonols. Studies show that kaempferol can

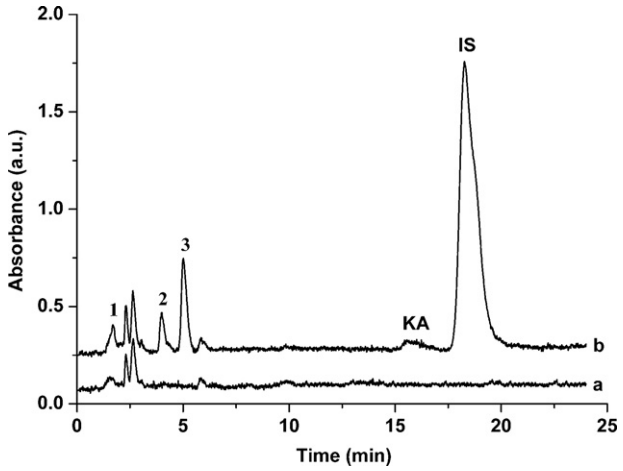

Fig. 3. Representative HPLC chromatograms of (a) blank plasma and (b) plasma sample taken at 30 min after oral administration of kaempferol (2500 mg/kg BW) detected with DAD at 365 nm. Peaks 1–3 are the potential metabolites of kaempferol. KA: kaempferol, IS: isorhamnetin.

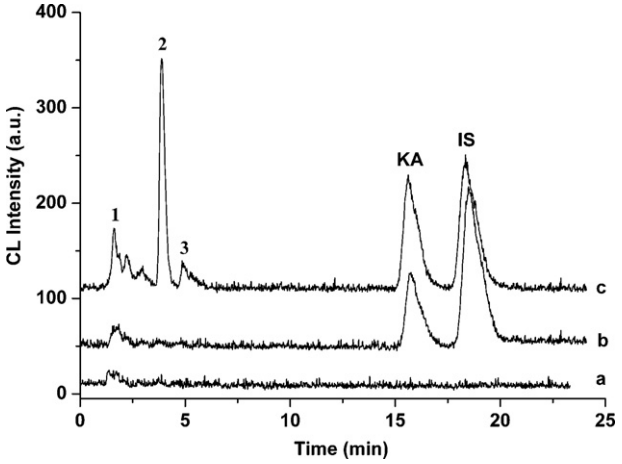

Fig. 4. Representative HPLC chromatograms of (a) blank plasma, (b) plasma spiked with 1.6×10^{-7} g/ml kaempferol and 1.0×10^{-5} g/ml isorhamnetin and (c) plasma sample taken at 30 min after oral administration of kaempferol (2500 mg/kg BW) with the post-column CL detection. Peaks 1–3 are the potential metabolites of kaempferol. KA: kaempferol, IS: isorhamnetin.

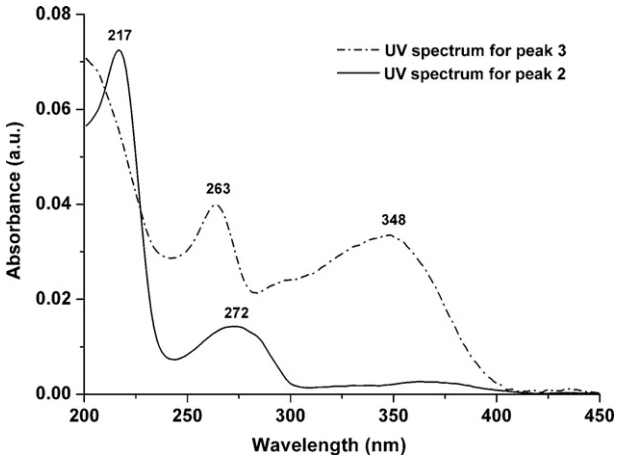

Fig. 5. UV spectra detected by DAD of peaks 2 and 3 in Fig. 3.

be metabolized into quercetin [32], and quercetin is transformed into its conjugates with only very small amounts of free quercetin in plasma [17,33,34]. All the above studies, together with the fact that the retention time of free quercetin (if present) was around 10 min in the proposed reversed phase chromatographic condition and that the conjugated quercetin will be eluted earlier due to its higher polarity, suggest that peak 3 is the conjugates of quercetin. However, the structures of metabolites need further confirmation by LC–MS.

3.4.2. Linearity and sensitivity

The calibration curve obtained in blank plasma showed good linear relationship over the range of 2.0×10^{-9}–2.0×10^{-6} g/ml. The regression equation was $y = 0.8626x + 5.553$ ($r = 0.9991$, $n = 7$),

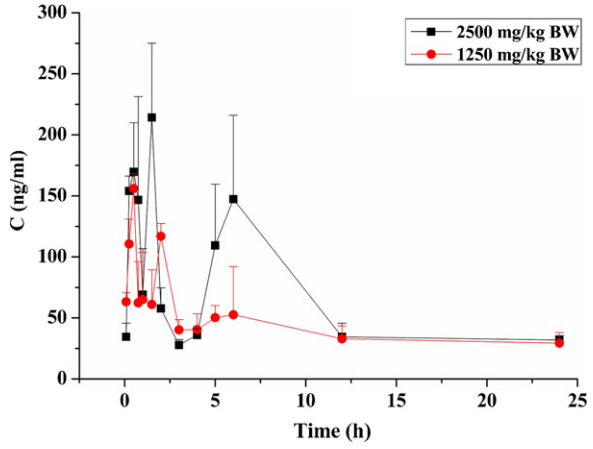

Fig. 6. Plasma concentration (±SD)–time profile after oral administration of kaempferol to SD rats ($n = 6$) [■ 2500 mg/kg BW, ● 1250 mg/kg BW].

where y is the logarithm of the CL intensity ratio of kaempferol to the internal standard and x is the concentration of kaempferol. Detection limit (S/N = 3) for blank plasma was 1.0×10^{-9} g/ml. The linear range and detection limit was compared with those obtained by HPLC with UV, FD, ECD, and MS detection in the literatures. Table 1 indicates that the proposed method has wider linear range and lower detection limit than that of the method using UV [18,21], FD [14], and ECD [17], and is almost as sensitive as that of HPLC–MS–MS method [19]. Therefore, the present HPLC-CL method does offer an alternative, sensitive and simple approach to the detection of kaempferol.

3.4.3. Precision, accuracy and recovery

The method was found to have good accuracies and reproducibilities for kaempferol at all three levels of concentrations. The mean accuracy for kaempferol is within 80.0–100.2%, and the intra- and inter-day precision had RSD (%) < 5.0. The sample preparation method was able to produce high recovery (≥80.0%). The results are summarized in Table 2.

3.5. Application to a pharmacokinetic study

The validated analytical method has been successfully applied to a pharmacokinetic study. The mean plasma concentration–time curve of kaempferol is shown in Fig. 6. The pharmacokinetic parameters of T_{max}, C_{max}, $AUC_{0-\infty}$, $MRT_{0-\infty}$, and $T_{1/2}$ of kaempferol in SD rats are given in Table 3. Kaempferol was detectable in plasma up to 24 h post-dosing. The kaempferol exhibited multiple peak concentrations after SD rats received single oral doses of kaempferol. These findings suggested a possible enterohepatic recirculation, which are consistent with the pharmacokinetic profiles of other flavonols [35]. However, this hypothesis needs further investigation.

Table 1
Comparison of kaempferol detection in plasma by HPLC with different detectors.

Sample	Method	Linear range	Detection limit	Reference
Rat plasma	HPLC-UV	5.0×10^{-8}–2.0×10^{-6} g/ml	2.0×10^{-8} g/ml	[21]
Chicken plasma	HPLC-UV	1.0×10^{-7}–7.0×10^{-5} g/ml	3.0×10^{-8} g/ml	[18]
Pig plasma	HPLC-FD	Not reported	3.0×10^{-9} g/ml	[14]
Human plasma	HPLC-ECD	4–6000 nmol/l	1.6 nmol/l	[17]
Rat plasma	HPLC–MS–MS	1.0×10^{-9}–2.0×10^{-7} g/ml	Not reported	[19]
Rat plasma	HPLC-CL	2.0×10^{-9}–2.0×10^{-6} g/ml	1.0×10^{-9} g/ml	This work

Table 2
Precision, accuracy and recovery of kaempferol in rat plasma ($n = 5$).

Added (ng/ml)	Found (mean ± SD) (ng/ml)	Precision (% RSD)	Accuracy (%)	Recovery (mean ± SD) (%)
Intra-day				
20	16.2 ± 0.6	3.7	80.8	89.2 ± 3.1
160	151.3 ± 3.0	2.0	94.6	97.1 ± 1.7
1000	1001.2 ± 32.8	3.3	100.1	95.3 ± 2.7
Inter-day				
20	16.0 ± 0.7	4.4	80.0	90.0 ± 4.3
160	150.7 ± 4.6	3.1	93.9	97.4 ± 2.4
1000	1002.4 ± 13.0	1.3	100.2	95.1 ± 2.4

Table 3
Mean pharmacokinetic parameters of kaempferol after oral administration at doses of 2500 mg/kg BW and 1250 mg/kg BW in SD rats.

Parameters	Doses	
	2500 mg/kg BW	1250 mg/kg BW
T_{max} (h)	1.21 ± 0.46	1.08 ± 0.43
C_{max} (ng/ml)	232.90 ± 5.14	165.67 ± 1.99
$AUC_{0-\infty}$ (ng h/ml)	1728.41 ± 57.31	729.01 ± 29.44
$MRT_{0-\infty}$ (h)	13.38 ± 3.87	4.77 ± 2.06
$T_{1/2}$ (h)	9.27	3.30

4. Conclusion

A new HPLC-CL method has been developed for the determination of kaempferol in plasma based on the chemiluminescent enhancement of the cerium(IV)–rhodamine 6G system in sulfuric acid medium. The proposed method allows for the detection of kaempferol in plasma without preconcentration step and also has high sensitivity which is comparable to HPLC–MS. The HPLC-CL method was successfully applied to a pharmacokinetic study of kaempferol. The application potential of the proposed HPLC–CL method to kaempferol in different matrices such as urine and tissues is under further investigation.

Acknowledgements

This study was supported by Natural Science Foundation of Anhui Province (070413113), Natural Science Foundation of Education of Anhui Province (2006kj306B), and Doctoral Foundation of Anhui Medical University (XJ2005008).

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