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Determination of caffeic acid in rabbit plasma by high-performance liquid chromatography

Yow-Shieng Uang, Fang-Lien Kang, Kuang-Yang Hsu*

Department of Pharmaceutics, School of Pharmacy, Taipei Medical College, 250, Wu-Hsing Street, Taipei 10502, Taiwan

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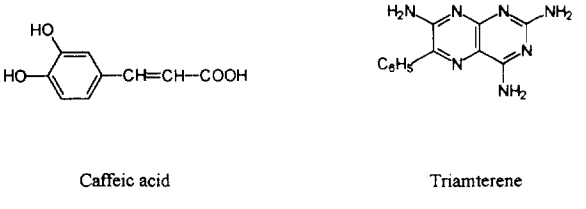
Abstract

A simple and sensitive high-performance liquid chromatographic method involving UV detection was developed for determination of caffeic acid in rabbit plasma. A Lichrosphere CN column (250 mm × 4 mm I.D., 5 μm) was used as the stationary phase and the mobile phase consisted of 2% acetic acid solution at a flow-rate of 1.0 ml/min. The UV absorbance was monitored at 320 nm. The plasma sample was acidified by the addition of 0.01 parts of concentrated phosphoric acid (85%) to maintain caffeic acid stability. After a simple clean-up procedure, the limit of quantitation achieved was 0.1 μg/ml, and the standard curve was found to be linear over the concentration ranges of 0.1–2.0 μg/ml and 0.1–40 μg/ml. The coefficient of variation for within- and between-run precision and accuracy was less than 10%, and the recovery was 82.3%.

1. Introduction

Caffeic acid (3,4-dihydroxycinnamic acid) as shown in Fig. 1, is a natural phenolic compound, which is widely distributed in vegetable and coffee products, where it is present as a degradation product of chlorogenic acid [1–3]. Some pharmacological properties of this substance have been described previously. For example, it has a strong and specific inhibitory activity towards 5-lipoxygenase, and can inhibit platelet aggregation and thromboxane biosynthesis [4–7]. As a result of its pharmacological activity, caffeic acid can be formulated to treat asthma and allergic-inflammatory disease [8].



Caffeic acid Triamterene

Fig. 1. Structures of caffeic acid and internal standard (triamterene).

There are several assay methods for determination of caffeic acid in natural products, including high-performance liquid chromatography (HPLC), and gas chromatography (GC) [9–14]. However, reports on the analysis of caffeic acid in biological samples are scarce. Only Camarasa et al. [15] developed a HPLC method with UV monitoring at 280 nm for analysis of caffeic acid in rat plasma after intravenous (i.v.) and oral (p.o.) administration of 40 mg/kg of caffeic acid to rats, and the limit of quantitation was 1.8 μg/ml of plasma.

* Corresponding author.

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For pharmacokinetic research, it is important both to maintain the stability and also to have a sufficient assay sensitivity for caffeic acid in biological samples. Due to the presence of a catechol group in the molecule, caffeic acid is unstable at both high and physiological pH, but is much more stable at low pH [9–11]. Until now, there have been no reports concerning the stability of caffeic acid in biological samples. Furthermore, the limit of quantitation should be improved to about 0.1 μg/ml for research involving low doses. Therefore, this previously reported method was considered to be insufficiently sensitive for caffeic acid analysis and unable to maintain good caffeic acid stability in plasma [15]. For these reasons, the purpose of this study was to improve the stability of caffeic acid in biological matrices and develop a simple, sensitive and reproducible HPLC method to assay caffeic acid in rabbit plasma.

2. Experimental

2.1. Chemicals and reagents

Caffeic acid and triamterene were obtained from Sigma (St. Louis, MO, USA). HPLC-grade acetonitrile and acetic acid were purchased from BDH Laboratory Supplies (Poole, UK). All other chemicals were analytical grade and used without further purification.

2.2. Apparatus and chromatographic conditions

The HPLC system consisted of an Alcott Model 760 pump (Alcott Chromatography, Norcross, GA, USA), a Shimadzu SIL-9A Automatic Sample Injector (Shimadzu, Kyoto, Japan), a Linear Model 200 Detector (Linear Instruments, Fremont, CA, USA), and a Shimadzu C-R7A Chromatopac Integrator. Separation was achieved on a Lichrosphere CN column (250 mm × 4 mm I.D., 5 μm, Merck, Darmstadt, Germany). The column eluent was monitored at UV 320 nm. The mobile phase was 2% acetic acid solution at a flow-rate of 1.0 ml/min which was filtered and degassed before use.

2.3. Solution preparation

A stock solution (2.5 mg/ml) of caffeic acid was prepared by dissolving 25 mg of caffeic acid in 10 ml of acetonitrile–mobile phase (2:8, v/v) and stored at 0°C. Working solutions were prepared in the mobile phase at concentrations of 5, 50 and 500 μg/ml.

2.4. Sample preparation

The sample preparation consisted of a clean-up procedure. Acetonitrile (0.5 ml), containing 4 μg/ml of triamterene as an internal standard, was added to 0.2 ml of rabbit plasma which was acidified by the addition of 0.01 parts of concentrated phosphoric acid (85%). The sample was vortex-mixed for 30 s, and then centrifuged for 10 min at 1945 *g*. The supernatant was transferred to a clean tube and evaporated to about 75–100 μl under a stream of nitrogen at ambient temperature. Then, 100 μl of distilled water was added and vortex-mixed for 30 s. Finally, 10 μl were injected onto the HPLC system.

2.5. Stability

The stability of caffeic acid in whole blood and plasma was studied. The concentration of caffeic acid for each study was 0.06 m*M*. The stability in whole blood at both 0°C and 25°C for 8 h was investigated. The stability in normal plasma and acidified plasma at 25°C for 24 h was compared. Acidified plasma was prepared by the addition of 0.01 parts of concentrated phosphoric acid (85%) to plasma.

2.6. Standard curve

A standard curve was prepared by the addition of known quantities of caffeic acid to aliquots of plasma and prepared according to the above sample preparation procedure. The concentrations used were 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, and 40 μg/ml. The peak-height ratio of caffeic acid to

triamterene was plotted against the concentration of caffeic acid added. Linearity was determined for all concentrations (0.1–40 μg/ml) and also for the lowest five concentrations (0.1–2 μg/ml) by simple linear regression analysis. The concentration of caffeic acid in the test samples was calculated using the regression parameters obtained from the standard curve.

2.7. Accuracy and precision

Four different concentrations of caffeic acid were added to drug-free plasma and the concentrations were determined using the corresponding standard curves. The accuracy of the method was shown in relative error and calculated based on the difference between the mean calculated and added concentrations, while precision was evaluated by calculating the within- and between-run coefficient of variation (C.V.).

2.8. Recovery

Caffeic acid recovery was calculated by comparing the peak-height ratios of caffeic acid in the plasma samples with those obtained from the analysis of equivalent amounts of aqueous solution.

2.9. Rabbit experiment

Six male New Zealand white rabbits which weighed about 2–3 kg were used in preliminary pharmacokinetic studies. Before single i.v. or p.o. administration of 10 mg/kg of caffeic acid, the rabbits were fasted overnight with water ad libitum. Blood samples (1.0 ml) were collected periodically at intervals from the marginal vein of the ear up to 140 min and 360 min after i.v. and p.o. administration, respectively, and placed into an ice bath immediately. Plasma was separated after centrifugation at 10 000 *g* for 5 min and acidified by the addition of 0.01 parts of concentrated phosphoric acid (85%), and stored at −20°C until analysis.

3. Results and discussion

Caffeic acid has a maximum UV absorbance at 320 nm, not at 280 nm. For analysis of biological samples, a previous report used a monitoring wavelength of UV 280 nm which would have more interference peaks than those at UV 320 nm. In addition, during sample preparation, the samples were not concentrated and therefore the sensitivity would be low [15]. In the development of our analytical method, the monitoring wavelength was set at UV 320 nm and the samples were concentrated after the clean-up procedure. Typical chromatograms are shown in Fig. 2. No significant endogenous peak coeluted with the caffeic acid as shown in the corresponding chromatogram of drug-free plasma. The retention times for caffeic acid and internal standard are 4.77 and 12.8 min, respectively.

There have been some studies using caffeic acid as a model compound to investigate the "brown reaction" of fruits [9–11]. Under a 100% oxygen condition, caffeic acid forms structural isomers by way of phenolate ion formation which reacts with oxygen. The higher the pH, the faster the degradation rate. In our study, we found that caffeic acid was stable in the pH range of 2–5 using buffer solutions incubated at 37°C for 72 h in normal atmosphere (unpublished data) which is the same as the results of previous reports [9–11]. Therefore, maintenance of the stability of caffeic acid in biological samples is very important. According to the above discussion, acidification of biological samples is a good method of stabilizing caffeic acid to prevent degradation. As shown in Fig. 3, caffeic acid is unstable in plasma, losing about 30% of its activity in 24 h at 25°C. However, caffeic acid shows good stability in acidified plasma for 24 h at 25°C. It could be inferred that caffeic acid is stable in acidic environments regardless of the sample type. In addition, the instability of caffeic acid in biological samples results from chemical degradation and not from enzymatic degradation. After the collection of blood samples, the stability of caffeic acid in whole blood was investigated. Because acidification of whole blood results in hemolysis, the stability of caffeic

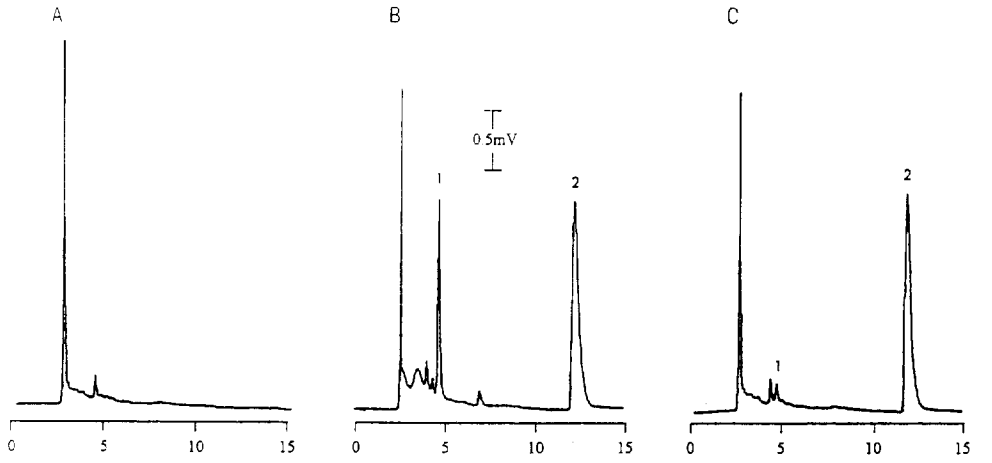

Fig. 2. Typical chromatograms of (A) drug-free plasma, (B) a plasma sample taken 45 min after p.o. administration of 10 mg/kg of caffeic acid to a rabbit, and (C) plasma spiked with a concentration of 0.1 μg/ml of caffeic acid. Peaks: 1 = caffeic acid; 2 = internal standard.

acid in whole blood was studied at 0°C and 25°C. The results show that caffeic acid was unstable at 25°C, but more stable at 0°C as shown in Fig. 4. At 0°C, caffeic acid in whole blood did not degrade during the first 2-h interval, whereas a loss of 30% was observed at 25°C, which indicates that a lowering of the temperature can temporarily maintain the stability of caffeic acid in whole blood. The blood samples were cooled in an ice-bath immediately after collection and then, after centrifugation, the plasma was separated and acidified to pH 2–4 by the addition of 0.01 parts of concentrated phosphoric acid (85%) to maintain the stability of caffeic acid in plasma. The acidified plasma samples were then stored at −20°C to minimize instability of the caffeic acid prior to analysis.

The standard curve for caffeic acid was made using nine spiked plasma samples over a concentration range of 0.1–40 μg/ml. In order to cover the concentration range and to increase the sensitivity without sacrificing precision and accuracy, it was necessary and advisable to use two standard curves. A linear relationship was obtained for both concentration ranges. Corresponding correlation coefficients (r^2) were over

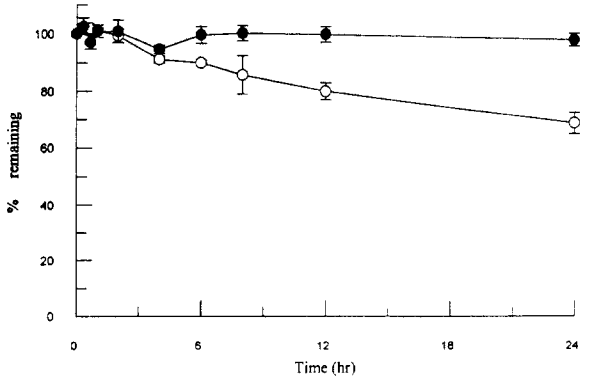

Fig. 3. Stability of caffeic acid in normal (○) and acidified (●) rabbit plasma at 25°C. Data are mean ± S.D. of three different samples.

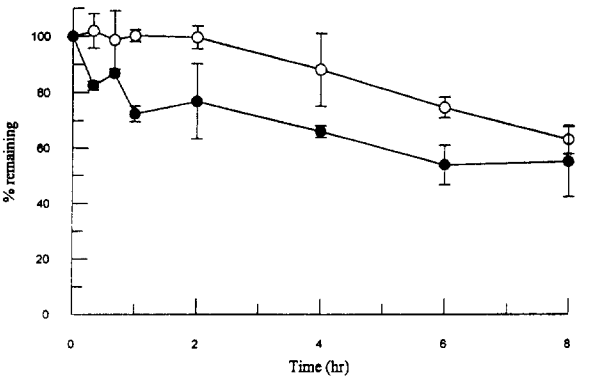

Fig. 4. Stability of caffeic acid in rabbit whole blood at 0°C (○) and 25°C (●). Data are mean ± S.D. of three different samples.

0.999 for all concentrations and also for the lowest five concentrations from each of six different standard curves. The regression parameters ($n = 6$) were $y = 0.84753x + 0.0077447$ for the lowest five concentrations and $y = 0.85560x + 0.0092562$ for all concentrations (y is peak height ratio; x is spiking concentration). The coefficient of variation of the slope for six different standard curves was 6.87% for the lowest five concentrations and 6.10% for all concentrations. As Table 1 shows, the precision and accuracy for each of the standard curve concentration points after back-calculation were 0.25% to 5.77% and −2.00% to 5.00%, respectively. These results indicate that the standard curve had a good linearity after simple linear regression analysis and was reproducible, and also shows that the method permits the determination of caffeic acid in plasma over a relatively wide range of concentrations.

The criteria for determination of the limit of quantitation in plasma is based on signal-to-noise, the reproducibility of the response and the variability of the back-calculated concentration [16]. Spiked plasma samples with a final concentration of 0.1 μg/ml were prepared ($n = 6$) and analyzed and were found to have a signal-to-noise ratio ≥5. The mean peak-height ratio and standard deviation were 0.0959 and 0.0100, respectively. The mean peak-height ratio was greater than 3 standard deviations and the coefficient of variation of the peak-height ratio was only 10.4%. The coefficient of variation and relative error of the back-calculated concentration were 5.77% and 4.00%, respectively. These results indicate that the limit of quantitation is 0.1 μg/ml and that the variability is less than 15%, which shows that the limit of quantitation has acceptable accuracy, precision and reproducibility. A typical chromatogram is shown in Fig. 2C.

Within-run and between-run accuracy and precision were examined by performing replicate analyses of plasma samples ($n = 6$) to which four different known concentrations of caffeic acid had been added. As Table 2 shows, the within-run precision was between 1.93% and 4.40% over the concentrations examined. In addition, between-run precision was between 3.56% and 5.66%. The accuracy for within-run and between-run was from 3.60% to 6.00% and from 1.07% to 6.00%, respectively, over the concentrations examined. These results show that the method has both good reproducibility and accuracy.

The absolute recovery for caffeic acid in plasma after the clean-up procedure was found to be 82.3%, as shown in Table 3. This result is similar to that of a previous report which drew a reasonable inference from protein binding [15].

The procedure was applied in pharmacokinetic

Table 1
Reproducibility of the standard curve

Spiking plasma concentration (μg/ml)	Calculated concentration (mean ± S.D.) (μg/ml)	C.V.[a] ($n = 6$) (%)	Relative[b] error (%)
0.100	0.104 ± 0.006	5.77	4.00
0.200	0.210 ± 0.007	3.33	5.00
0.500	0.491 ± 0.015	3.05	−1.80
1.00	1.00 ± 0.02	2.00	0.00
2.00	2.00 ± 0.01	0.50	0.00
5.00	4.90 ± 0.07	1.43	−2.00
10.0	10.1 ± 0.2	1.98	1.00
20.0	20.1 ± 0.1	0.50	0.50
40.0	40.0 ± 0.1	0.25	0.00

[a] C.V. = 100% × (S.D./mean).
[b] Relative error = 100% × (calculated concentration − spiking plasma concentration)/spiking plasma concentration.

Table 2
Precision and accuracy

Spiking plasma concentration (μg/ml)	Within-run			Between-run		
	Concentration measured (mean ± S.D.) (μg/ml)	C.V. (n = 6) (%)	Relative error (%)	Concentration measured (mean ± S.D.) (μg/ml)	C.V. (n = 16) (%)	Relative error (%)
0.150	0.159 ± 0.007	4.40	6.00	0.159 ± 0.009	5.66	6.00
0.750	0.778 ± 0.030	3.86	3.70	0.758 ± 0.033	4.35	1.07
2.50	2.59 ± 0.05	1.93	3.60	2.53 ± 0.09	3.56	1.20
15.0	15.9 ± 0.4	2.52	6.00	15.4 ± 0.6	3.90	2.67

studies by i.v. and p.o. administration of caffeic acid to rabbits. Typical plasma concentration–time profiles are shown in Fig. 5. The plasma concentrations of caffeic acid were in the standard curve range. The maximum concentration of caffeic acid was 1.16 ± 0.33 μg/ml and plasma concentrations remained above the 0.1 μg/ml quantitation limit for the entire sampling period after p.o. administration of 10 mg/kg of caffeic acid to rabbits, but below the limit of quantitation of a previous report (1.8 μg/ml) [15].

These results demonstrate that this method is simple, sensitive, reproducible and accurate and meets the requirement of the report of the conference on Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmaco-

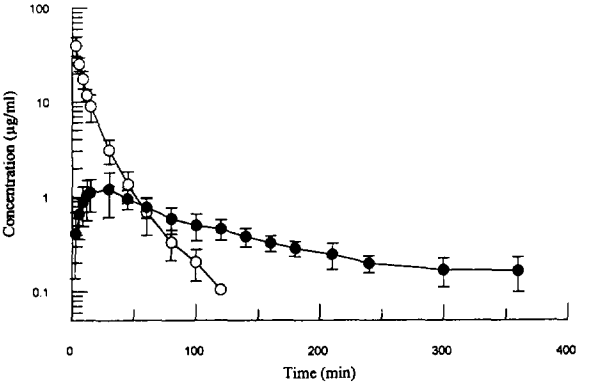

Fig. 5. Plasma concentration–time profiles of caffeic acid after i.v. (○) and p.o. (●) administration of 10 mg/kg of caffeic acid in crossover administration to six rabbits. Shown as mean ± S.D.

Table 3
Recovery data

Spiking concentration (μg/ml)	Peak-height ratio (mean ± S.D.)		Recovery[a] (%)
	Water sample (n = 3)	Plasma sample (n = 3)	
0.150	0.111 ± 0.001	0.095 ± 0.002	85.6
0.750	0.589 ± 0.012	0.466 ± 0.007	79.1
2.50	1.85 ± 0.10	1.58 ± 0.01	85.4
15.0	12.2 ± 0.1	9.62 ± 0.16	78.9

[a] Recovery = 100% × (peak-height ratio of plasma sample/peak-height ratio of water sample).

kinetic Studies [17]. From the data obtained after i.v. and p.o. administration of caffeic acid to rabbits, it is concluded that the method described here offers the opportunity to derive pharmacokinetic parameters with an acceptable accuracy.

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