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Simultaneous Determination of Caffeic Acid, Ferulic Acid and Isoferulic Acid in Rabbit Plasma by High Performance Liquid Chromatography

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ABSTRACT

A simple and sensitive high performance liquid chromatographic method with reverse-phase column for the simultaneous quantification of caffeic acid, ferulic acid, and isoferulic acid in rabbit plasma was developed. An ODS column (150 mm × 4.6 mm I.D., 5 μm) was used as the stationary phase and the mobile phase consisted of acetonitrile/glacial acetic acid/water (15:0.5:85, v/v, pH adjusted to 4.5). Flow-rate was 1.0 mL/min and UV absorbance was set at 321 nm. One hundred microliter of plasma was used to simultaneously measure the concentrations of caffeic acid, ferulic acid, and isoferulic acid. After a direct clean-up procedure with 10% of trifluoroacetic acid, the lower limits of quantification were 0.1 μg/mL and the standard curves were found to be linear over the concentration ranges of 0.1-100 μg/mL for caffeic acid, ferulic acid, and isoferulic acid. The average recoveries for caffeic acid, ferulic acid, and isoferulic acid were 96.2%, 98.0%, and 98.3%, respectively. The results for all compounds also showed good stability after three freeze/thaw cycles and storage at ambient temperature and 4°C for 24 hr. The assay method was successfully applied to the pharmacokinetic study of caffeic acid, ferulic acid, and isoferulic acid after an intravenous administration of caffeic acid to rabbits.

Key words: caffeic acid, ferulic acid, isoferulic acid, HPLC

INTRODUCTION

Caffeic acid (3,4-dihydroxycinnamic acid, CA) is found in a wide variety of foods including vegetables, fruits, tea, coffee, and wine[1-2]. Caffeic acid elicits several interesting and various biological responses, such as antibacterial[3-4], anti-fungal[5], anti-inflammatory[6], antiviral[7-8], anticancer[9-10], antioxidant[11-14], antimutagenic[15], and anti-diabetic activities[16]. Due to these biological activities, caffeic acid could be a good lead compound for new drug development. However, pharmacokinetic studies of caffeic acid and its related methylated metabolites, ferulic acid (FA) and isoferulic acid (IFA), have not been completely identified and studied.

Various analytical methods have been used for the determination of CA, FA, and IFA in natural products including high performance liquid chromatography (HPLC) and gas chromatography (GC) after derivatization. For analysis of the biological samples, most methods were used to analyze both caffeic acid and ferulic acid[17-19] or ferulic acid and isoferulic acid in plasma or urine[20-21]. However, reports on the simultaneous analysis of CA, FA, and IFA in biological samples are scarce. A recent study to determine CA, FA, and IFA in human plasma as metabolites was derived from artichoke leaf extract[21]. This method involved sample preparation followed analysis using high performance liquid chromatography on reversed-phase material with a polar endcapping (Aqua-C_{18}). The target compounds were monitored by electrochemical array detection (CoulArray). Calibration curves of caffeic acid, ferulic acid, and isoferulic acid were conducted in the ranges of 2.1-51.7 ng/mL, 2.2-53.7 ng/mL, and 1.1-52.6 ng/mL, respectively. Although this assay method shows high sensitivity; it needs about 500 μL of plasma and a complicated preparation procedure. It is also time consuming with one sample for total analytical time about 97.5 min including HPLC analysis taken over 87.5 min and column flushing about 10 min. In an animal model of our studies, the volume of biological sample is sparse. Thus, in order to develop a suitable method for the determination of metabolites in a metabolic study of caffeic acid after intravenous administration to rabbits and in accordance with the international

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guidelines for validation of bioanalytical methods[22], a simple and sensitive analytical method for simultaneously determining CA, FA, and IFA in biological samples was conducted.

MATERIALS AND METHODS

I. *Chemicals and Reagents*

Caffeic acid (CA) and ferulic acid (FA) were obtained from Sigma (St. Louis, MO, USA). Isoferulic acid (IFA) was purchased from Lancaster Synthesis (Lancashire, UK). Acebutolol used as the internal standard was supplied by the Coprima Sociedad Anónima (Barcelona, Spain). HPLC grade of acetonitrile, trifluoroacetic acid (TFA), phosphoric acid (85%), glacial acetic acid, and 2-aminoethanol were obtained from E. Merck (Darmstadt, Germany). All other chemicals were of analytical grade and used without further purification.

II. *Apparatus and Chromatographic Conditions*

The HPLC system consisted of a Shimadzu LC-$10AD_{VP}$ pump, SIL-HT_A/HT_C autosampler, SPD-$10A_{VP}$/$10AV_{VP}$ UV detector, and CLASS-VP Ver.6.1 system manager as data processor (Shimadzu Co., Kyoto, Japan). Separation was achieved by a Biosil Pro-ODS-U column (150 mm × 4.6 mm I.D., 5 μm, Biotic Chemical Co., Ltd., Taipei, Taiwan). A mobile phase composed of acetonitrile/glacial acetic acid/water (15:0.5:85, v/v, pH 4.5) was used throughout the analysis. Flow rate was 1.0 mL/min and UV absorbance was set at 321 nm. The mobile phase was filtered and degassed before use.

III. *Preparation of Solution*

Stock solutions of CA (1 mg/mL) and FA (1 mg/mL) were separately prepared by weighing 10 mg of CA or FA into a calibrated 10-mL volumetric flask and making volume to 10 mL with acetonitrile/85% phosphoric acid/water (10:0.01:90, v/v). Stock solution of IFA (1 mg/mL) was prepared by weighing 10 mg of IFA into a calibrated 10-mL volumetric flask and making volume to 10 mL with acetonitrile/85% phosphoric acid/water (25:0.01:90, v/v). Working solutions of CA, FA, and IFA were prepared by further dilution of each standard stock solution with acetonitrile/water (10:90, v/v) to 0.01, 0.1, and 1 μg/mL. Solutions were stored at 4°C until analysis.

IV. *Sample Preparation*

One hundred microliter of plasma were spiked into a clean culture tube and subsequently 15 μL of internal standard solution [10 ng/μL of acebutolol in 10% (v/v) of TFA] were added and mixed well. One hundred microliter of 10% (v/v) of trifluoroacetic acid were then added and vortex mixed for 1 min. Samples were centrifuged at 1,945 ×g for 10 min. An aliquot (25 μL) of supernatant was injected into the HPLC system.

V. *Standard Curve*

A standard curve was prepared by the addition of known quantities of CA-FA-IFA mixture to aliquots of plasma and prepared as described above for sample preparation. The concentration used for each sample was 0.1, 0.2, 0.5, 2, 5, 20, 50, and 100 μg/mL. The peak-height ratios of each compound to internal standard were plotted against the concentration of each compound prepared. Linearity was determined for all concentrations (0.1-100 μg/mL). The standard curve of each compound was determined by weighted least-square linear regression analysis corresponding to 1 over concentration square ($1/\text{concentration}^2$). The concentrations of CA, FA or IFA in the test samples were calculated using the regression parameters of CA, FA or IFA itself obtained from the standard curve.

VI. *Accuracy and Precision*

Four different concentrations (0.1, 0.3, 4 and 80 μg/mL) of CA, FA, and IFA were prepared with drug-free plasma and the concentrations were determined using the corresponding standard curves. Accuracy of the method was shown in relative errors (RE) which was calculated based on the difference between the mean calculated and added concentrations, while precision was evaluated by within- and between-run coefficient of variation (CV).

VII. *Recovery*

Recovery was calculated by comparing the peak-height of each compound in the plasma to that of the equivalent amounts in the aqueous solution.

VIII. *Freeze/thaw Stabilities and Post-Preparation Stability*

Freeze and thaw stability was tested by spiking samples at the concentrations of 0.15 and 80 μg/mL through three freeze/thaw cycles. Post-preparation stability was tested by plasma samples at the concentrations of 0.3 and 80 μg/mL, which were kept on bench at room temperature (25°C) and in a freezer at 4°C for 24 hr after preparation, respectively.

IX. *Rabbits Experiment*

Male New Zealand white rabbits which weighed 1.8-2.0 kg were used in pharmacokinetic studies. Before single intravenous administration of 10 mg/kg of caffeic acid, the rabbits were fasted overnight. Blood samples (1.0 mL) were collected at the following times: 0 (pre-drug), 5, 10, 20, 30, 60, 90, 120, 180, 240, 360, and 600 min from the marginal vein of the ear after intravenous administration, and placed into an ice bath immediately. Plasma was sepa-

rated after centrifugation at 1,945 ×g for 5 min and acidified by addition of 20 μL of 20% (v/v) of phosphoric acid and stored in a freezer at -80°C until analysis.

RESULTS AND DISCUSSION

I. *Sample Preparation*

The chemical structures of caffeic acid, ferulic acid and isoferulic acid are shown in Figure 1. As previously reported[23], CA has a fast degradation rate in higher pH solution and it is stable in the buffer solution with pH range of 2-5. Therefore, FA and IFA could also have the same properties. When the sample solutions were not of sufficient acidity, these hydroxycinnamates were present as protonated and nonprotonated species and subsequently degraded easily[21]. During the development of analytic method for simultaneous determination of CA, FA and IFA in rabbit plasma, we found that most commonly used solid-phase extraction was time-consuming and resulted in lower recovery and higher LOD. On the other hand, using acetonitrile as a protein-precipitating reagent would produce endogenous interference and/or low recovery. However, using TFA as a sample clean-up reagent could maintain post preparative sample in acidic condition and subsequently keep the stabilities of CA, FA, and IFA. Therefore, it could save concentration time of post sample preparation and simplify sample preparation procedure. After optimization, a pH value of the mobile phase at 4.5 was used. The baseline separation of CA, FA, IFA, a clear and sharp peak, good analytical sensitivity and no interference peak were obtained within a run-time of 20 min. Typical chromatograms are shown in Figure 2. No significant endogenous peak co-eluted with the target compounds as shown in the corresponding chromatogram of drug-free plasma. The retention times for CA, FA, IFA, and internal standard were 5.05, 10.94, 12.63 and 14.55 min, respectively.

II. *Calibration Curve and Linearity*

The method was validated for the concentration range of 0.1-100 μg/mL. A linear regression was performed using the equation $y = a + bx$ by weighted ($1/x^2$) over the standard curve range. The coefficients of determination obtained from the standard curve used in within-run and between-run validations, respectively, were greater than the value of 0.99. A statistical linear regression was performed for each compound using concentration against peak height ratio of target compound to internal standard. Three linear regression equations ($y = a + bx$; y = peak height ratio, x = concentration) were $b = 0.2792 \pm 0.0041$, $a = -0.0010$, $r^2 = 0.9962$ for CA, $b = 0.1392 \pm 0.0004$, $a = 0.0039$, $r^2 = 0.9983$ for FA, and $b = 0.1174 \pm 0.0010$, $a = 0.0000$, $r^2 = 0.9983$ for IFA. The results indicated linear relationship between peak height ratio and concentration over the concentration range of 0.1-100 μg/mL.

III. *Precision and Accuracy*

The detailed precision and accuracy data of CA, FA

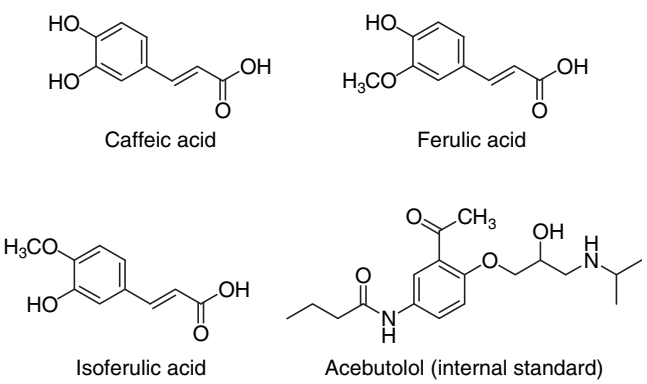

Figure 1. Chemical structures of caffeic acid, ferulic acid, isoferulic acid and acebutolol (internal standard).

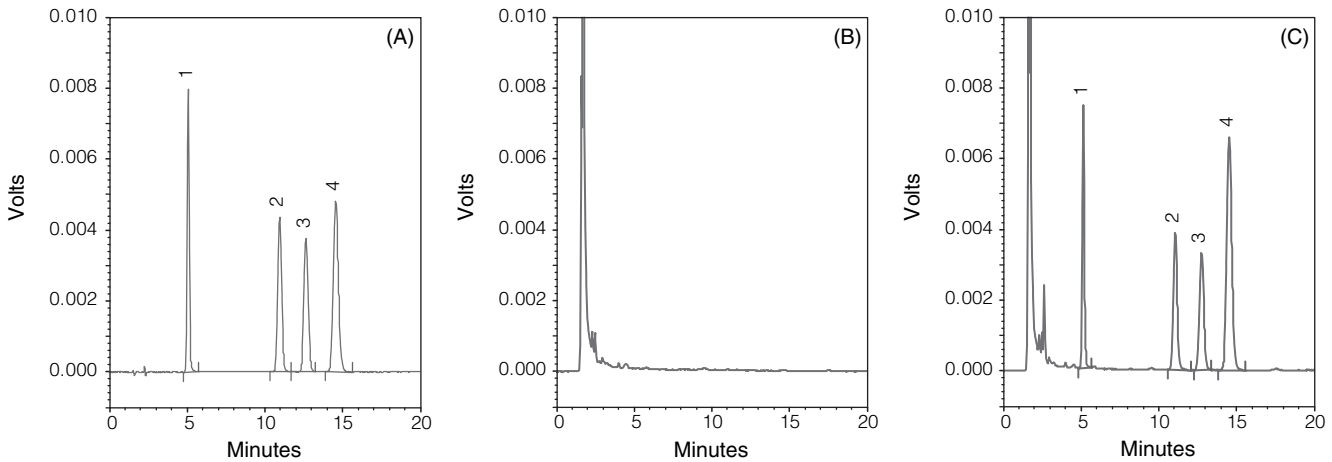

Figure 2. Typical chromatograms of (A) authentic compound, (B) drug-free plasma, (C) caffeic acid, ferulic acid, and isoferulic acid plasma, concentration at 4 μg/mL with internal standard. 1. caffeic acid; 2. ferulic acid; 3. isoferulic acid; 4. internal standard.

and IFA for within- and between-run are shown in Tables 1 and 2. In higher concentrations, both within- and between-run CV values were 1.4-3.4% for CA, 1.2-3.9% for FA, and 1.2-4.4% for IFA. The within- and between-run RE values were -3.9 to 7.3% for CA, -6.1 to 3.6% for FA, and -5.5 to 3.6% for IFA at three different concentration levels. These results indicated that the present method yields the acceptable precision and accuracy.

IV. *Lower Limitation of Quantification (LLOQ)*

The lower limitation of quantification (LLOQ) was defined as the lowest concentration on the standard curve that could be determined with acceptable precision and accuracy. Thus, the LLOQs all were 0.1 μg/mL for CA, FA, and IFA. At LLOQ level, CVs were 2.50–12.6% and RE values–11.7 to 19.4%. Precision and accuracy of within- and between-run at the LLOQ for CA, FA, and IFA met the requirements (both CV and RE ≤ 20%) for bioanalytical analysis[22].

V. *Recovery*

As shown in Table 3, after the direct de-protein procedure with 10% (v/v) of TFA solution, recoveries of CA, FA, and IFA at three different concentrations were 93.9-98.2%, 95.6-101.2%, 96.5-100.9%, respectively. The average recoveries of CA, FA, and IFA were 96.2%, 98.0%, and 98.3%, respectively. These results indicated that the direct de-protein procedure using 10% (v/v) of TFA solution is a good preparation method.

Table 1. Data for within-run precision and accuracy

		Nominal concentration (μg/mL)											
		Caffeic acid				Ferulic acid				Isoferulic acid			
Run		0.1	0.3	4	80	0.1	0.3	4	80	0.1	0.3	4	80
I	Mean	0.100	0.288	3.88	85.9	0.108	0.294	3.89	82.9	0.095	0.284	3.95	82.9
	S.D.	0.004	0.004	0.05	1.8	0.003	0.008	0.07	1.0	0.007	0.009	0.06	1.1
	CV (%)	4.4	1.5	1.4	2.1	3.0	2.6	1.8	1.2	7.5	3.2	1.5	1.4
	RE (%)	0.3	-3.9	-3.0	7.3	8.1	-2.1	-2.8	3.6	-5.3	-5.5	-1.2	3.6
II	Mean	0.102	0.298	3.95	82.2	0.088	0.282	4.00	81.2	0.116	0.310	4.01	79.8
	S.D.	0.004	0.006	0.08	2.0	0.007	0.010	0.05	1.4	0.010	0.006	0.05	1.1
	CV (%)	3.7	2.1	2.1	2.4	8.2	3.5	1.3	1.7	8.2	1.8	1.2	1.4
	RE (%)	2.5	-0.6	-1.1	2.7	-11.7	-6.1	0.0	1.5	16.2	3.4	0.2	-0.2
III	Mean	0.119	0.307	3.88	80.9	0.104	0.292	3.94	81.6	0.104	0.293	3.94	80.8
	S.D.	0.003	0.007	0.06	1.5	0.013	0.011	0.07	1.5	0.006	0.006	0.05	1.5
	CV (%)	2.5	2.3	1.4	1.8	12.6	3.9	1.8	1.8	5.4	2.0	1.2	1.9
	RE (%)	19.4	2.3	-3.1	1.1	3.7	-2.6	-1.5	2.0	4.4	-2.5	-1.5	1.0

n = 6 for each concentration and run.

Table 2. Data for between-run precision and accuracy

	Caffeic acid			Ferulic acid			Isoferulic acid		
Nominal concentration (μg/mL)	Calculated concentration (mean ± S.D.) (μg/mL)	C.V.[a] (%)	Relative error[b] (%)	Calculated concentration (mean ± S.D.) μg/mL)	C.V. [a] (%)	Relative error[b] (%)	Calculated concentration (mean ± S.D.) (μg/mL)	C.V. [a] (%)	Relative error[b] (%)
0.1	0.107 ± 0.009	8.4	7.0	0.104 ± 0.013	12.5	4.0	0.105 ± 0.012	11.4	5.0
0.3	0.298 ± 0.010	3.4	-0.7	0.292 ± 0.011	3.8	-2.7	0.295 ± 0.013	4.4	-1.7
4	3.90 ± 0.07	1.8	-2.4	3.94 ± 0.07	1.8	-1.5	3.97 ± 0.06	1.4	-0.9
80	83.0 ± 2.7	3.3	3.7	81.6 ± 1.5	1.8	2.0	81.2 ± 1.8	2.2	1.5

[a]CV = 100% × (S.D./mean).
[b]Relative error = 100% × (concentration calculated – spiking plasma concentration)/spiking plasma concentration.
[c]n = 18 for each concentration.

Table 3. Recoveries for caffeic acid, ferulic acid, and isoferulic acid

Nominal concentration (μg/mL)	Caffeic acid			Ferulic acid			Isoferulic acid		
	Water sample Peak height	Plasma sample Peak height	Recovery (%)	Water sample Peak height	Plasma sample Peak height	Recovery (%)	Water sample Peak height	Plasma sample Peak height	Recovery (%)
0.3	495 ± 3	486 ± 5	98.2	261 ± 5	264 ± 13	101.2	214 ± 3	216 ± 9	100.9
4	6761 ± 235	6349 ± 90	93.9	3356 ± 98	3262 ± 33	97.2	2866 ± 103	2791 ± 43	97.4
80	139947 ± 2025	135001 ± 4116	96.5	69824 ± 925	66783 ± 2139	95.6	59172 ± 628	57092 ± 1781	96.5

n = 3 for each concentration.

Table 4. Freeze and thaw stabilities of target compounds

	Nominal concentration (μg/mL)					
	Caffeic acid		Ferulic acid		Isoferulic acid	
Cycle	0.15	80	0.15	80	0.15	80
Initial	0.145 ± 0.010	80.5 ± 2.9	0.131 ± 0.007	78.0 ± 1.6	0.161 ± 0.013	75.4 ± 1.5
1	0.137 ± 0.005	83.1 ± 2.3	0.125 ± 0.020	79.6 ± 3.1	0.160 ± 0.007	76.4 ± 2.5
RE[a] (%)	-5.39%	3.27%	-4.54%	2.09%	-0.14%	1.29%
2	0.148 ± 0.005	81.9 ± 0.9	0.126 ± 0.015	79.8 ± 2.2	0.161 ± 0.011	77.1 ± 1.7
RE (%)	1.77%	1.80%	-3.96%	2.36%	0.11%	2.23%
3	0.139 ± 0.009	84.2 ± 1.5	0.136 ± 0.020	81.2 ± 2.6	0.158 ± 0.007	78.0 ± 3.0
RE (%)	-4.43%	4.68%	3.56%	4.18%	-1.83%	3.41%

[a]RE (%) = 100% × (concentration calculated – initial plasma concentration)/initial plasma concentration.
[b]n = 3 for each concentration and cycle.

Table 5. Post-preparation stability after storage at room temperature and 4°C for 24 hr

	Nominal concentration (μg/mL)					
	Caffeic acid		Ferulic acid		Isoferulic acid	
Cycle	0.3	80	0.3	80	0.3	80
Initial	0.287 ± 0.006	81.2 ± 1.4	0.280 ± 0.008	83.4 ± 0.5	0.285 ± 0.002	81.3 ± 0.3
Room temperature	0.302 ± 0.006	86.4 ± 1.1	0.300 ± 0.008	86.4 ± 0.6	0.304 ± 0.003	86.5 ± 0.5
RE[a] (%)	5.22%	6.39%	7.14%	3.64%	6.67%	6.45%
4°C	0.299 ± 0.005	85.5 ± 0.5	0.301 ± 0.020	85.8 ± 0.5	0.292 ± 0.006	85.5 ± 0.5
RE (%)	4.18%	5.21%	7.50%	2.90%	2.46%	5.16%

[a]RE (%) = 100% × (concentration calculated – initial plasma concentration)/initial plasma concentration.
[b]n = 3 for each concentration and cycle.

VI. *Freeze and Thaw Stability*

The freeze and thaw stability study indicated a short-term stability of the target compounds in plasma samples in the thaw procedure. Furthermore, some plasma protein can coagulate and precipitate after freezing and thawing, respectively. Because the target compounds can bind to the plasma protein, the phenomena of plasma protein coagulating in the frozen condition and precipitating after thawing to room temperature can result in concentration loss. As shown in Table 4, the differences of the measured concentrations for the target compounds between the initial and each cycle were less than 10% for two concentrations. This indicated that these compounds have good stability in plasma samples after three freeze and thaw cycles.

VII. *Post-Preparation Stability*

Post-preparation stability was used to evaluate the plasma sample stability after preparation. Plasma samples were prepared at concentrations of 0.3 μg/mL and 80 μg/

mL for each target compound and stored at 4°C and 25°C. Stability was monitored after 24 hr. Plasma samples stored at 4°C and room temperature for 24 hr showed good stabilities. The RE (%) ranged from 2.46 to 7.50%, showing that processed QC samples kept at 4°C and room temperature (25°C) were stable for at least 24 hr.

VIII. *Test Results*

The procedure was applied in pharmacokinetic studies by IV administration of CA to rabbits. Typical plasma concentration-time profiles of CA, FA, and IFA are shown in Figure 3. The plasma concentrations of CA, FA, and IFA were in the standard curve range. The plasma concentrations remained above the 0.1 μg/mL lower limit of quantification (LLOQ) for the entire sampling period.

The method developed by Wittemer *et al.*[21] required 500 μL of plasma and a complicated preparation procedure. In an animal model, the volume of biological sample was sparse. Therefore, this method could not apply to the animal studies. On the contrary, our developed method provides many advantages such as simple and fast sample preparation procedure. It also avoids the sample lost and decreases the analytic variance.

The results demonstrated that this method is simple, sensitive, precise and accurate and reproducible. From the data obtained after IV administration of CA to rabbits, it is concluded that the method described herein offers the opportunity to derive pharmacokinetic parameters with an acceptable accuracy and precision.

CONCLUSIONS

In the present study, the developed method was validated in accordance with international guidelines for the validation of bioanalytical methods[22], and was used for the determination of metabolites in a metabolic study of caffeic acid after intravenous administration to rabbits. The standard curves for each target compound showed a linear relationship over the concentration range of 0.1-100 μg/mL. Thus, this method could provide a good precision, accuracy, and reproducible assay for the simultaneous quantification of caffeic acid, ferulic acid, and isoferulic acid in rabbit plasma after intravenous administration of caffeic acid.

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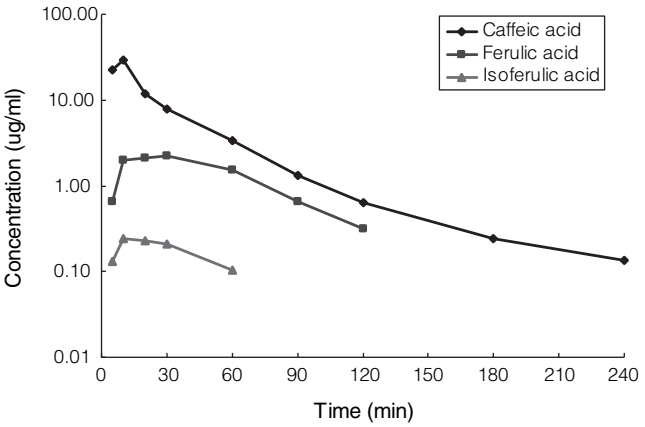

Figure 3. Plasma concentration-time profiles of caffeic acid, ferulic acid, and isoferulic acid, respectively, after a single intravenous administration of 10 mg/kg of caffeic acid to one rabbit.

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