A Validated HPLC Method with Dual Wavelength Detection on Chlorogenic Acid and Internal Standard in Plasma and Its Application in Pharmacokinetic Study in Rats

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Shortened title: Dual Wavelength Method for Chlorogenic Acid in Plasma

Abstract:

A validated high-performance liquid chromatographic (HPLC) method with dual wavelength detection was developed and applied to the determination of chlorogenic acid in rat plasma. Elution was performed on C_{18} column at 27°C with acetronitrile–water (0.2% phosphoric acid, pH=2.0) at different proportion according to a time-scheduled programme and pumped at a flow rate of 1.0 mL min^{-1}. The eluent was detected at 325 nm for chlorogenic acid and 370 nm for the internal standard, kaempferol. The intra-day and inter-day precisions were better than 3.58% and 3.66%, respectively. The standard curve for chlorogenic acid was linear (r^2 = 0.9993) in the concentration range of 0.1 – 15 μg mL^{-1}. Accuracy in the measurement of samples ranged from 91.97 to 101.81%. The limit of detection and the limit of quantification for chlorogenic acid in plasma were 30 and 90 ng mL^{-1}, respectively. This assay has been successfully applied in the pharmacokinetic study of chlorogenic acid through oral and intravenous administration in rats. It showed that the low bioavailability of chlorogenic acid and intravenous administration route is a better dosage regimen. It is a simple and sensitive analytical method with good accuracy and reproducibility. The current method also demonstrated that internal standard with different optimal UV absorbance from that of the analyte can also be used in an analytical method.

Keyword: HPLC, Rat, plasma, pharmokinetic, chlorogenic acid, internal standard

INTRODUCTION

Chlorogenic acid (3-*O*-ceffeoyl-D-quinic acid) is an ester of caffeic acid and quinic acid as depicted in Fig. 1. This polyphenolic compound can be found in coffee and numerous plant species including Chinese herbs. [1] Chlorogenic acid is an antioxidant with two phenolic groups for radical scavenging and its cation chelation properties. [2,3] Many studies have reported chlorogenic acid can protect lower density lipoproteins from oxidation.[4] Also, it was effective in preventing oxidative damage to human epithelial cells.[5] Moreover it can inhibit hepatic glucose 6-phosphatase involved in glucose metabolism, which may be a significant factor in the abnormal diabetic state.[6,7] Therefore, chlorogenic acid might contribute to the prevention of cardiovascular disease and Type 2 Diabetes.[8,9] Due to its biological importance, several papers have described its absorption pattern and bioavaliablility in human body.[10,11] HPLC methods have been employed in the pharmacokinetic studies of chlorogenic acid.[12,13] HPLC-UV method is most frequently used for the determination of chlorogenic acid in biological sample due to presence of chrmophore.[14] However, the accuracy of the method is limited by the extraction efficiency of chlorogenic acid from the biological sample. In some HPLC analytical method, calibration curves with an internal standard have been usually employed in the study of herbal materials in order to give good accuracy and reproducibility.[15] However, the selection of good internal standard was often difficult because there are only a few compounds with similar chemical behaviour (such as chemical structure and polarity) and comparable analytical response (such as optimal UV absorbance) to a certain target analyte available commercially. In order to demonstrate compounds with different optimal UV absorbance from that of the analyte

can also be a good internal standard, we tested kaempferol (a compound with optimal UV absorbance difference from chlorogenic acid) (Fig. 1) as an internal standard for developing an analytical method using dual wavelengths detection to quantify chlorogenic acid in plasma sample. Therefore, the purposes of the current work are to sort out the limitations of internal standards and develop a precise and accurate HPLC method for detecting chlorogenic acid that could be routinely applied to the quantification of chlorogenic acid in plasma sample. The application of the method reported in this study was also demonstrated by measuring the pharmacokinetics of chlorogenic acid through oral and intravenous administrations in rats.

EXPERIMENTAL

Reagents and chemicals

Chlorogenic acid, kaempferol (an internal standard), methanol (HPLC grade) and 85% phosphoric acid (HPLC grade) were purchased from Sigma Chemicals (St. Louis, MO, USA). All other organic solvents for chromatography (HPLC grade) were purchased from Merck, Germany. All other reagents are analytically pure and used without further purification. Distilled, de-ionized water was obtained in a PURELAB Option-R system (ELGA, UK).

Instrumentation

The chromatographic analysis were performed on Agilent 1100 Series system (Agilent Technologies, Waldbronn, Germany) conjunction with a quaternary pump, an autosampler, an autoelectronic degasser, a thermostated column compartment, a diode array-multiple wavelength detector and a computer with a ChemStation LC 3D software program for HPLC signal analysis. The separation was carried out on an Allsphere ODS-2 HPLC column (4.6 mm × 250 mm i.d., 5.0 μm particle size) (Alltech Associates, Inc).

Chromatographic condition

A gradient mobile phase consisting of A (acetonitrile) and B (0.2% phosphoric acid, pH = 2.0) were used to run the separation. A simple gradient elution was carried out as follows: 90% (v/v) solvent B was linearly decreased to 11% for the whole elution period (0–20 min). The total run time was 35 min including 15 min equilibration of the system with A: B = 1: 9 (v/v). All solvents are filtered with a 0.45 μm cellulose ester membrane prior to use. The mobile phase was degassed automatically using the electronic degasser system. The flow-rate was 1 mL min^{-1} and the column temperature at 27°C. The eluent was monitored by a UV detector. The detection wavelength was set at 325 nm for chlorogenic acid and 370 nm for the internal standard, kaempferol. An auto-sampler was utilized for sample injection with injection volume of 10 μL.

Calibration curves

The stock solutions of chlorogenic acid (10 mg mL^{-1}) and kaempferol (10 mg mL^{-1}) were prepared in methanol: 0.5% acetic acid (100:5, v/v). They were stored at 4°C away from light and were found to be stable for at less a month. Working solutions were

prepared daily in their corresponding solvents by appropriate dilutions of the stock solutions. Standard samples contained 0.1, 0.5, 1, 5, 10, 15 μg mL^{-1} chlorogenic acid. Kaempferol, an internal standard, was added to individual standard sample to give the final concentration of kaempferol which equals to 0.01 μg mL^{-1} for normalization. Each sample was analyzed in triplicate. Standard curves were established by determining the peak-area ratio (chlorogenic acid to kaempferol) of the HPLC chromatograms.

Sensitivity

The limit of detection (LOD) and limit of quantitation (LOQ) were defined as the lowest concentration of analyte in a sample that can be detected and quantified. These limits were determined on the basis of the signal-to-noise ratios of 3:1 and 10:1, respectively. The standard solutions of chlorogenic acid for LOD and LOQ were prepared by diluting them sequentially.

Repeatability

The precision of the chromatographic determination for the developed method was determined followed the method described before.[16, 17] It was expressed as a relative standard deviation (R.S.D.) and was calculated by triplicate injections of chlorogenic acid (6 and 10 μg mL^{-1} for intra-day and 5 and 10 μg mL^{-1} for inter-day). The standard sample was prepared and analyzed within 24 hrs for intra-assay precision. The inter-assay precision was determined using three independent experiments in different days. For the calibration curve, the calibration concentrations were back-calculated from their related peak area ratio (analyte to internal standard). The deviation from the nominal concentration was defined as accuracy.

Recovery

Recovery was calculated by comparing the peak areas of the extracted quality control samples with that of the un-extracted standard solutions containing the equivalent amount of analytes. The assay was carried out by adding standard solution of chlorogenic acid and the internal standard to plasma samples taken from normal rats. The final concentration of the sample was made up to 10 μg mL^{-1} of chlorogenic acid. The recovery percentage was calculated using the ratio of contents detected (actual) to those added (theoretical). The assay was repeated three times during the same day to obtain mean recovery data.

Blood collection

Male Sprague–Dawley rats (2-month-old, 280–330 g) supplied by the Centralized Animal Facility, the Hong Kong Polytechnic University, were fed on a standard laboratory diet with free access to water under the controlled temperature at 20–22°C and relative humidity of 50% with 12-h light/dark cycles prior to the study. Animals were surgically cannulated with polyethylene catheters (0.5 mm ID; 1.0 mm OD) on the right jugular veins under anesthesia with diethyl ether vapor.[18] The animals recovered in individual metabolic cages and fasted but were allowed to have free access to water overnight. Two groups of conscious and unconstrained cannulated rats (6 per group) were dosed with the chlorogenic acid solution intravenously (10 mg kg^{-1}) and orally (100 mg kg^{-1}), respectively. Serial venous blood samples (0.5 mL) were collected from the right jugular vein via the cannulated catheter into heparinized tubes at 15, 30, 60 120, 240 and 480 min after administration of drugs after dose. After each blood sampling, an

equivalent volume of heparinized normal saline (25% v/v, 0.5 mL) was injected into the animals to maintain a constant blood volume. The experimental protocol was conducted under the licence provided by the Government of the Hong Kong SAR and the Animal Subjects Ethics Sub-committee, The Hong Kong Polytechnic University and was consistent with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health and the principles outlined in the Declaration of Helsinki. Every effort was made to limit animal suffering and number of animals used.

Plasma sample preparation

The blood samples (0.5 mL) were centrifuged at 5000 rpm for 10 min to harvest the plasma. The plasma obtained was aliquots to a new micro-centrifuge tube and stored at -20ºC until analysis. To the aliquots of 200 μL plasma, 200 μL of internal standard containing 0.02 μg mL^{-1} kaempferol in methanol: 0.5% acetic acid (100:5, *v/v*) at pH = 2.0 was added for protein precipitation. The mixture was then vortex for 30 s and centrifuged at 130,000 rpm for 10 min. The supernatant was collected and filtered with a 0.20 μm nylon filter with diameter of 4 mm (Millipore, USA), and then 10 μL of filtrate was injected into HPLC.

Statistics

All data are expressed as means ± standard deviation (S.D.) and n denotes the number of replications for each data point. Where applicable, analysis of variance was used for statistical evaluation of significant differences among the multiple groups. All statistical comparison tests were performed by GraphPad Prism 4.02 for Windows (GraphPad Software, San Diego, California, U.S.A.). Differences were considered

significant when $p < 0.05$. Correlation of determination (r^2) was determined by Spearman's rank correlation analysis. For Pharmacokinetic parameters, including area under the plasma concentration–time profile from time zero to time infinity ($AUC_{0-\infty}$), maximum plasma concentration and time (C_{max} and T_{max}), were calculated by a PK Solutions 2.0 software (Summit Research Services, Ashland, U.S.A.) with a model-independent approach. Bioavailability was calculated as the ratio of the dose-normalized $AUC_{0-\infty}$ after oral administration (100 mg kg^{-1}) to that after bolus intravenous injection (10 mg kg^{-1}) of chlorogenic acid.

RESULTS AND DISCUSSION

Optimization of the HPLC chromatographic conditions

The HPLC solvent system applied in the experiment was optimized from different mobile phases, at various proportions, in order to obtain a good separation and resolution of target compounds in the chromatograms. Gradient elution is routinely applied to the separation of flavonoids in complicated biological samples, such as plasma, during a short-period run time. In the current study, a gradient solvent system composed of acetonitrile and 0.2% phosphoric acid (pH = 2.0) was selected as a mobile phase, since it gave a good separation and resolution of target peaks from other constituents in the plasma sample. For the acidic character of chlorogenic acid, an acidic mobile phase was employed to facilitate retention in the established solvent system of reverse phase chromatography and to separate the analyte with the polar component in plasma. Phosphoric acid was used to adjust the pH value and reduce the peak tailing;

therefore the produced peak shapes are symmetrical. The wavelengths selected for detection of chlorogenic acid and internal standard were 325 nm and 370 nm, respectively. They showed maximum absorption and good sensitivity of the target peaks. The retention times of chlorogenic acid and internal standard were 5.78 and 12.34 min, respectively. The typical chromatographic profiles of a mixed standard solution and sample solution are shown in Fig. 2. No interference was observed for target compounds in the chromatograms of the samples.

Calibration curves

The calibration curves were constructed based on the levels of chlorogenic acid present in pharmaceutical products. The linearity of the current analytical method for determination of chlorogenic acid was evaluated by analyzing a series of different concentrations. In this study, six concentrations were chosen, ranging from 0.1 μg mL^{-1} to 15 μg mL^{-1}. Each concentration was measured three times. The linearity and sensitivity parameters are shown in Table 1. This approach provides information on the variation in peak area between samples of the same concentration. The linearity of the calibration graphs was validated by the high value of the coefficient of determination (r^2) and the slope is statistically ($p < 0.001$) deviated from zero (Fig. 3). The standard curve of chlorogenic acid is $y = 0.1163x - 0.0005$, $r^2 = 0.9993$; where y is the peak-area ratio, x is the concentration in μgmL^{-1}. The results showed that the good linearity was achieved over the ranges for the analysis. The LOD was 30 ng mL^{-1} and LOQ was 90 ng mL^{-1}.

Precision and accuracy

The reproducibility of the method was examined by analyzing three standard solutions in three consecutive days and calculating the R.S.D and accuracy. The intra-day precision was lower than 3.58%, and the accuracy varied from 99.02% to 101.81%. The inter-day precision was lower than 3.66%, and the accuracy ranged from 91.97% to 93.69% (Table 2). In addition, the data for each concentration level were evaluated by one-way analysis of variance. There is no statistically significant difference between the mean results obtained from one level of day to another at the 95% confidence level. It indicated that the method is reliable and accurate.

Recovery

The extraction recovery of chlorogenic acid (n=3) from spiked rat plasma was acceptable. It was 77.15 ± 1.606 % with R.S.D. of 2.08% (n=3). Since the recovery of chlorogenic acid was lower than 80%, an internal standard was used to enhance the reliability and consistency of the method. For the current method, kaempferol was used. It recovery was 80.06 ± 1.606 % with R.S.D. of 2.32% (n=3). In fact, recovery of the analyte need not be 100% but the extent of recovery of an analyte and an internal standard should be consistent, precise and reproducible. Thus, kaempferol is a suitable internal standard.

Application of the method

The validated HPLC–UV method has been successfully used to determine chlorogenic acid in plasma samples obtained after intravenous (10 mg kg^{-1}) or oral (100 mg kg^{-1}) administration to rats. The plasma concentration-time profiles of chlorogenic acid with two different administrations are shown in Fig. 4. After the intravenous

administration, the concentration of chlorogenic acid was about 13.33 ± 1.226 μgmL^{-1} at the first sampling point (15 min). In the case of oral route, the results demonstrated that the absorption peak for chlorogenic acid was at the first 30 min with C_{max} = 2.03 ± 1.042 μg mL^{-1} and T_{max} = 30 min. The oral bioavailability of 1.2% was determined by comparison of the dose-normalized $AUC_{0-\infty}$ (45.45 mg mL^{-1}min^{-1}, 100 mg kg^{-1}) of oral dose to the $AUC_{0-\infty}$ (378.3 mgmL^{-1}min^{-1}, 10 mgkg^{-1}) of intravenous injection of chlorogenic acid. The low bioavailability is consistent with other studies which shown that chlorogenic acid is absorbed to a lesser extent than other dietary polyphenols, such as caffeic acid, in rats and human volunteer.[19-23] The difference in absorption of chlorogenic acid and other polyphenols has been shown to relate to microbial metabolism of chlorogenic acid by colonic microflora in gastrointestinal tract.[22, 24] In addition, the first-pass effect in liver may play a significant role on lower the bioavailability of chlorogenic acid.[25] Therefore, intravenous administration route is more suitable for chlorogenic acid among these the two studied dosage regimens.

Dual wavelengths detection on chlorgenic acid and internal standard is successfully used in HPLC method with UV detection. The resolution and separation of analytes from mixture is good and with high sensitivity. The result may implicate that analysis of several analytes in mixture with chromophores can be detected with single internal standard with different wavelengths.

CONCLUSIONS

A gradient HPLC-UV method for the quantification of the plasma concentrations of

chlorogenic acid has been developed. Kaempferol was used as an internal standard. Good separation and high resolution on chromatograms was obtained between the analytes and the internal standard at two different wavelengths. The utility of the method has been demonstrated by applying onto the pharmacokinetic study of chlorogenic acid through oral and intravenous administration in rats. The results showed that the low bioavailability of chlorogenic acid and intravenous administration route is better dosage regimen. The method is simple and efficient with excellent accuracy, precision, reproducibility and low detection limit. The current developed method is, undoubtedly, a good starting point for sorting out the limitation of internal standards in HPLC method.

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Table 1. Linearity and sensitivity parameters of the HPLC detection method for quantitation of chlorogenic acid in rat plasma

Parameters	**Results**
Linearity Range (μg mL^{-1})	0.1 – 15.0
Regression equation	y = 0.1163x - 0.0005
r^2	0.9993
LOD (μg mL^{-1})	0.03
LOQ (μg mL^{-1})	0.09
Recovery (%)[a]	77.15 ± 1.606

[a]Average of three determinations, S.D. means standard deviation.

Table 2. Intra- and inter-day precision and accuracy of chlorogenic acid in rat plasma

Concentration added ($\mu g\ mL^{-1}$)	Concentration measured ($\mu g\ mL^{-1}$) mean ± S.D.[a]	R.S.D. (%)[b]	Accuracy (%)[c]
Intra-day precision			
6	6.11 ± 0.218	3.58	101.81
10	9.55 ± 0.217	2.27	99.02
Inter-day precision			
5	4.598 ± 0.168	3.66	91.97
10	9.37 ± 0.191	2.04	93.69

[a]Average of three determinations, S.D. means standard deviation.

[b]RSD (%) (relative standard deviation) = (SD/mean) × 100.

[c]Accuracy (%) = [1 – (|mean concentration measured – concentration spiked|) / concentration spiked] × 100.

FIGURE CAPTIONS

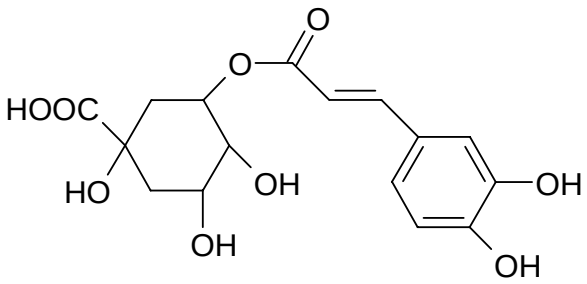
Figure. 1. Chemical structures of chlorogenic acid and kaempferol.

Figure. 2. Calibration curves of chlorogenic acid.

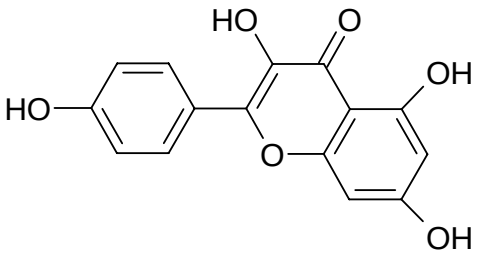
Figure. 3. Typical HPLC chromatograms of blank rat plasma at 325 nm (A) and 370 nm (B); rat plasma spiked with chlorogenic acid (CA) and internal standard, kaempferol, (IS) at 325 nm (C) and 370 nm (D); and rat plasma sample from rat intravenously administrated with 10 mgkg^{-1} chlorogenic acid at 325 nm (E) and 370 nm (F).

Figure. 4. Plasma concentration-time profile of chlorogenic acid in rats after a single intravenous (10 mg kg^{-1}) and oral (100 mg kg^{-1}) administration of chlorogenic acid.

FIGURES



Chlorogenic acid



Kaempferol

Figure 1

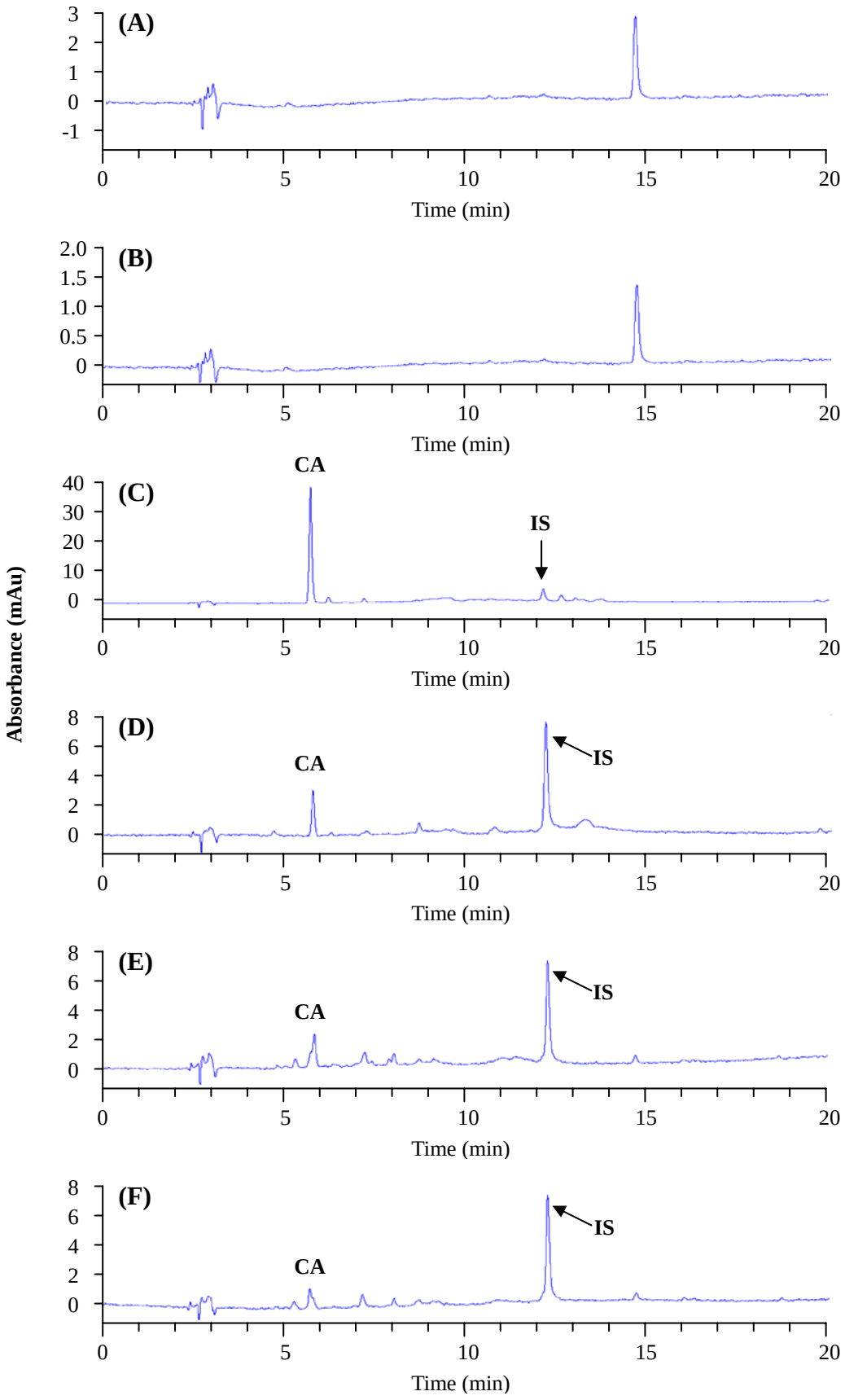


Figure 2

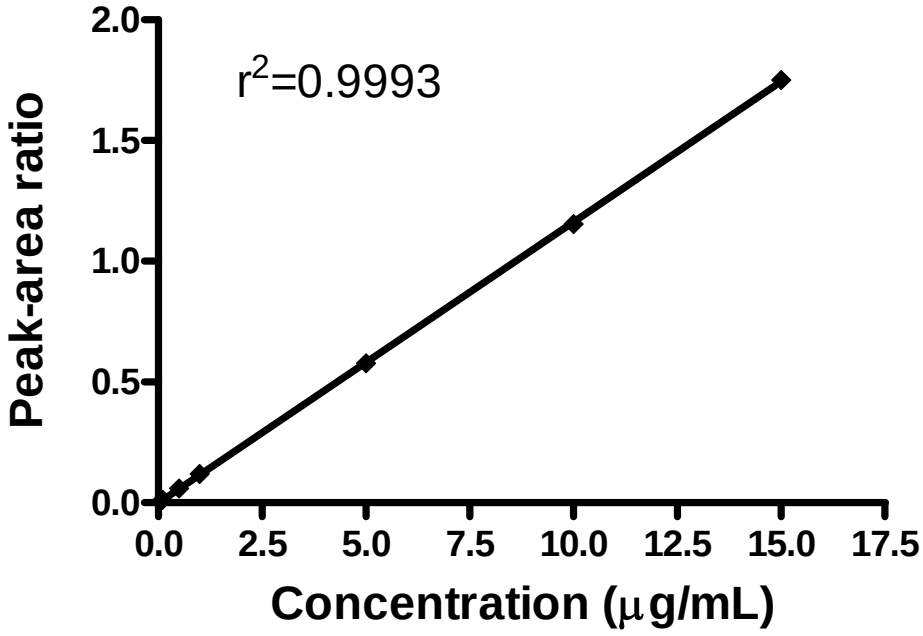

2.0
1.5
1.0
0.5
0.0
Peak-area ratio
r2=0.9993
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
Concentration (μg/mL)

Figure 3

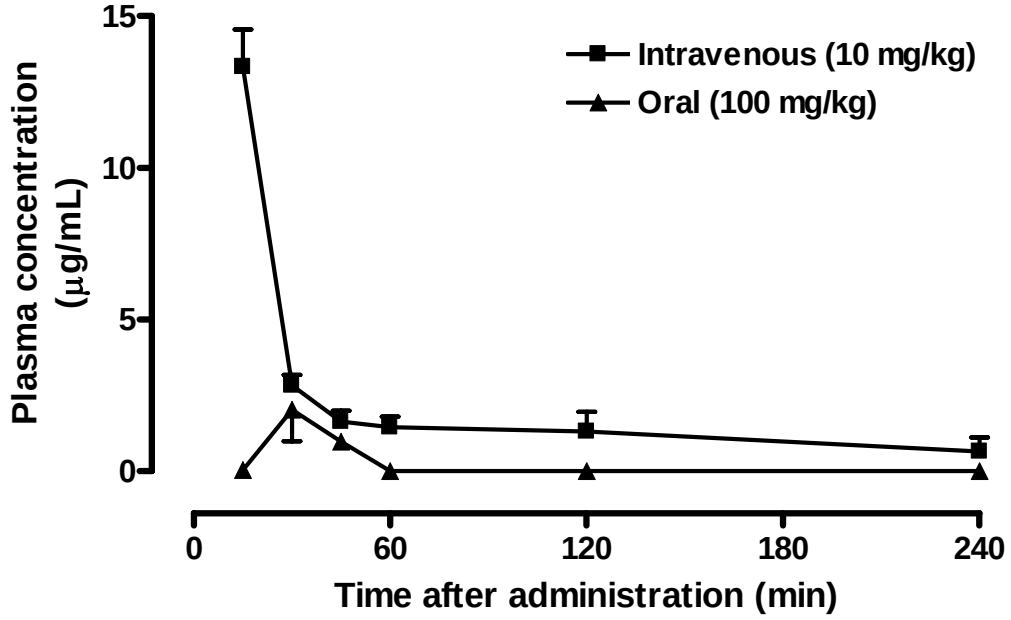

15
10
5
0
Plasma concentration
(μg/mL)
Intravenous (10 mg/kg)
Oral (100 mg/kg)
0
60
120
180
240
Time after administration (min)

Figure 4