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Development of an LC-MS/MS method for quantitative analysis of Chlorogenic acid in human plasma and its application to a pharmacokinetic study in Chinese patients with advanced solid tumor

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ABSTRACT

A simple and specific, rapid resolution liquid chromatography-tandem mass spectrometry (LC–MS/MS) method was developed and validated for determination of chlorogenic acid in human plasma using neochlorogenic acid as the internal standard. Plasma samples were precipitated with methanol and separated on a Zorbax C18 column (50 × 2.1 mm, i.d. 1.8 μm) at a flow rate of 0.4 mL/min using a gradient mobile phase of methanol-water containing 0.1% formic acid (v/v). The detection was performed on a triple quadrupole tandem mass spectrometer by multiple reaction monitoring in negative ESI mode. The method was fully validated over the concentration range of 10–2000 ng/mL. The indicators of inter- and intra-day precision (RSD%) were all within 10.7%, and the accuracy (RE%) was ranged from -3.0% to 10.6%. Moreover, we evaluated this bioanalytical method by re-analysis of incurred samples as an additional measure of assay reproducibility. This method was successfully applied to pharmacokinetic study of CGA in Chinese subjects with advanced solid tumor after intramuscular injection administration of Chlorogenic acid for injection (CAFI).

1. Introdution

Chlorogenic acid (CGA) is a kind of depside formed by caffeic acid and quinic acid, which is commonly found in some food or traditional Chinese medicine herb, such as coffee, green tea, Lonicerae Japonicae Flos, Cortex Eucommiae, etc. CGA is one of the major ingredients in many traditional Chinese medicine injections or tablets including Mailuoning injection [1], Reduning injection [2], Shuanghua Baihe tablets [3]. It has protective effect against the myocarditis, cerebral ischemia, hypertension and liver injury through anti-inflammatory, antioxidative and free radical-scavenging activities [4–7]. In recent years, the effective anticancer activities were constantly confirmed and many antitumor mechanisms had been reported [8–17]. So it is essential to develop a quantitation method for CGA in order to investigate the pharmacokinetic profile for its clinic application, especially in patient with tumor.

Up to now, except one study [18] developing a LC–MS/MS method for the quantitaion of CGA itself in rat plasma, there have been several reports [1–3,18–24] concerning the simultaneous determination of CGA with other compounds in human or rat plasma samples using the LC–MS/MS method. The above methods have several shortcomings such as a large volume of plasma required [1,3,18,22], complicated extraction system and procedure [1,2,19,20,22–24] and long analysis time [2,21–23].

In the present study, a simple, rapid and specific LC–MS/MS method for the quantitation of CGA was developed in order to investigate the pharmacokinetics of CGA in human plasma. In brief, this method has advantages over the previous methods that include

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the small plasma volume required, a short running time and high reproducibility with incurred samples re-analysis (ISR) being completed. Furthermore, this method was validated according to FDA and EMA guidelines, and was successfully applied to pharmacokinetic (PK) study of CGA in Chinese subjects with advanced solid tumor after intramuscular administration of Chlorogenic acid for injection (CAFI) for the first time.

2. Experiment

2.1. Chemicals, materials and reagents

Chlorogenic acid and neochlorogenic acid (IS) (Fig. 1) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products. Chlorogenic acid injection (lyophilized powder) was supplied by Sichuan Jiu zhang Biotechnological Co., Ltd. (China). Methanol (HPLC grade) was purchased from Honeywell Burdick & Jackson (Muskegon, ML, USA). Formic acid was supplied by Sigma-Aldrich (St. Louis, MO, USA). HPLC grade water was obtained using a Milli Q system (Millipore, Bedford, MA, USA). Drug-free human plasma from healthy volunteers was supplied by Peking University Cancer Hospital & Institute blood bank and dipotassium EDTA was used as the anticoagulant.

2.2. Chromatographic conditions

The chromatographic separation was carried out on a series 1290 Infinity LC system (Agilent Technologies, USA) containing a binary pump, an autosampler and an online degasser. Mobile phase A was methanol and mobile phase B was water containing 0.1% formic acid. Gradient elution was applied at a flow rate of 0.4 mL/min through an Agilent Zorbax C18 column (50 x 2.1 mm i.d., 1.8 μm) thermostatted at 35 °C. A linear gradient from 15 to 95% A over 2 min and returned to the initial conditions within 0.1 min was applied. The column was equilibrated for 2 min before the next injection, leading to a total run time of 4 min. Sample injection volume was 5 μL and the auto-sampler temperature was set at 10 °C.

2.3. MS conditions

Mass spectrometric analysis was performed using a G6460A triple-quadrupole mass spectrometer from Aglient equipped with an ESI source in the negative mode. The mass spectrometer was operated in the MRM mode. The MRM transitions of CGA and the IS were both *m/z* 353.2 → 191.2. The ionization source conditions were capillary voltage 3.5 kV, cell acceletator 3 V, gas temperature 350 °C and shealth gas temperature 400 °C. The optimized collision energy was 12 V and dwell time was 200 ms for both CGA and IS. The gas flow rate was 11 L/min. Data acquisition and processing were performed using Agilent MassHunter Workstation Software (Version B.06.00).

2.4. Preparation of standard and quality control solutions

Stock solutions of CGA were prepared in duplicate in methanol at concentration of 1 mg/mL, one for calibration curve samples and the other for quality control (QC) samples. The stock solution of internal standard (IS) was prepared as above at the concentration of 1 mg/mL. Two serious of working solutions were obtained by mixing and diluting the stock solutions with methanol so as to prepare the plasma standard points of the calibration curve and the plasma QC samples with concentrations of 0.05, 0.1, 0.2, 0.5, 1, 2.5, 5, 10 μg/mL and 0.1, 1, 8 μg/mL, respectively. The IS working solution was prepared at 2 μg/mL by diluting the stock solution with methanol. The working solutions for the calibration curve were prepared freshly every day and other solutions were stored at −30 °C.

2.5. Preparation of standard and quality control plasma samples

An eight-point plasma calibration curve was prepared freshly every day. Each calibration sample was prepared by adding 20 μL of the respective standard solution to 100 μL of pooled blank human plasma to obtain the final concentrations of 10, 20, 50, 100, 200, 500, 1000, 2000 ng/mL. Each calibration curve included a blank sample (plasma processed without IS) and a zero blank sample (plasma processed with the IS). To prepare QC samples, 25 μL aliquots of working QC solutions were diluted with control human plasma in 5 mL volumetric flasks obtaining the QC plasma concentration of 20, 200, 1600 ng/mL. The proportion of methanol in the plasma was equal to 0.5% for all the QC samples. The three QCs were stored at −30 °C to check the analytes stabilities and as controls for future assays.

2.6. Processing samples

A volume of 100 μL of the actual sample, QC sample or 120 μL standard samples were transferred to a 1.5 mL Eppendorf polypropylene tube followed by addition 20 μL of the IS working solution. Furthermore, 20 μL of methanol were added to the actual sample and QC sample in order to keep the organic solvent proportion consistent with that of the mixture standard samples. After that, the mixture was vortexed and 300 μL of methanol (0.1% formic acid) were added. Each tube was thoroughly vortexed for 30 s and centrifuged at 12,000 *g* for 5 min at nominally 4 °C. Then 300 μL of the obtained supernatant were diluted with H_2O (0.1% formic acid) and filtered before they were transferred to an autosampler glass vial. Finally, 5 μL of the sample was injected for LC–MS/MS analysis.

2.7. Method validation

This study was conducted in compliance with the EMA and the FDA guidance on bio-analytical method validation [25–27]. The method was validated by examining the following parameters: selectivity, calibration curve performance, accuracy and precision, dilution integrity, sensitivity, carryover, stability of the analyte at various test conditions, recovery and matrix effect

2.7.1. Selectivity

In order to investigate potential interference from endogenous compounds that could coelute with the analyte and the internal standard, 100 μL of blank human plasma from six different sources with or without standard solutions of CGA and neochlorogenic acid was tested after the administration.

2.7.2. Calibration curve performance

The calibration curves, prepared using mixed plasma from 6 different lots, were constructed using 8 non-zero standards ranging from 10 to 2000 ng/mL for CGA. The linearity of the relationship between peak area ratio and concentration was determined by the correlation coefficient (R) obtained for the linear regression.

2.7.3. Accuracy, precision, dilution integrity and sensitivity

The intra- and inter-day reproducibility of the method was evaluated by assessing 5 replicate QC samples at low (LQC), medium (MQC) and high (HQC) concentration. Relative error (RE%) and relative standard deviation (RSD%) were calculated to evaluate the accuracy and precision, respectively.

In addition, sample concentrations above the upper limit of the standard curve should be diluted and dilution of samples should not affect the accuracy and precision. The dilution integrity was

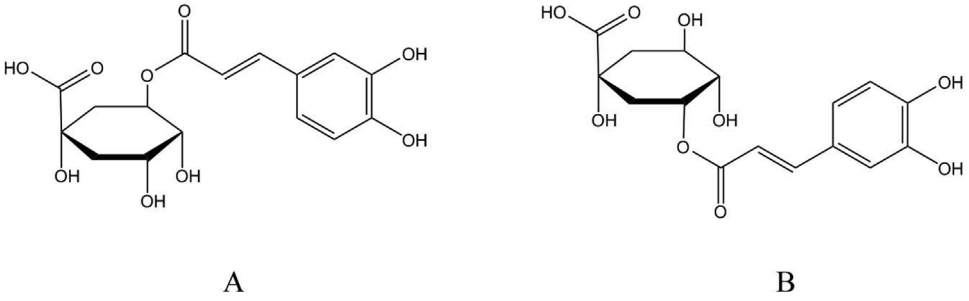

Fig. 1. The structures of the chlorogenic acid (A) and neochlorogenic acid (IS, B).

carried out by diluting an ultra-high QC samples at the two times of the highest calibration level. Five replicates of the ultra-high QC samples were diluted 10-fold with blank human plasma and analyzed.

The lowest limit of quantification (LLOQ) is the lowest analyte concentration of the bioanalytical method. The sensitivity was proved by calculating the accuracy and precision of the concentration from five LLOQ samples.

2.7.4. Carry over

Carryover was tested by injecting processed blank plasma samples after injecting an upper limit quantitation (ULOQ) sample. The response in the blank plasma at the retention times of the analyte or IS should be less than 20% of the mean response of a LLOQ sample for the analyte and less than 5% of the mean response for the IS, respectively.

2.7.5. Stability

The stability of the CGA stock solution was determined by comparing the peak area of a stock solution stored for a period of time with that of a freshly prepared solution of the same concentration. Both of the stock solutions were dissolved in methanol and diluted to 100 ng/mL using methanol:water (3:7,v/v).

Stabilities of CGA in plasma (freeze/thaw cycles, auto-sampler storage, short-term and long-term storage) were established. Freeze-thaw stability was determined by comparing the value of QC samples after 3 cycles of freeze/thaw (from −30 °C to 25 °C) with the nominal concentration. The auto-sampler stability was done by comparing the concentration of extracted QC samples after being kept in the auto-sampler at 10 °C for 24 h with the nominal value. In order to evaluate the short term stability, three levels of QC samples were extracted and determined after being placed at room temperature (25 °C) for 2 h. Then the acquired concentrations were compared to the nominal value. Long-term storage stability was acquired by assessing QC samples stored at −30 °C for 60 days.

2.7.6. Recovery and matrix effect

The extraction recovery of CGA was calculated by direct comparison of the peak area of analyte extracted from QCs at the three concentrations with that of the analyte spiked to the post-extraction blank samples at the same concentration. The average peak areas in spiked QCs post-extraction were then compared to those of corresponding working solutions at the same concentration. The ratio was defined as the matrix effect which refers to matrix ionization suppression or enhancement in MS-based methods [28]. The intersubject variability of the analyte responses from six different lots at each concentration level should be less than 15% [29]. The recovery of IS was evaluated in the same way at a plasma concentration of 400 ng/mL.

2.7.7. Incurred samples analysis (ISR)

The use of calibration standards and QC samples during validation may not mimic the actual study samples. Differences for instance in protein binding, back-conversion of known and unknown metabolites, sample inhomogeneity or concomitant medications, may affect the accuracy and precision of the analyte in such samples during processing and storage. It is therefore recommended to evaluate accuracy of incurred samples by reanalysis of study samples in separate runs at different days according to the EMA and FDA Draft for Guidance [25,26]. In the present study, the 10% of the samples had been reanalyzed including the samples around C_{max} and in the elimination phase in order to verify the reliability of the reported subject sample analyte concentrations. The analyses can be considered equivalent if two-thirds (67%) of the percentage difference [(repeat-original)*100/mean] of the results is within 20%.

2.8. Clinical application

This LC–MS/MS method was successfully applied to determine the concentrations of CGA in human plasma after administration of CAFI, which was approved for treatment of tumor by China State Food and Drug Administration (CFDA) in 2013. This was a preliminary experiment of a randomized, open-label, Phase I study to assess the pharmacokinetics and safety of CAFI in Chinese patients with advanced solid tumor. Eleven subjects were enrolled and received once-daily dose of 1, 2 or 3 mg/kg delivered by intramuscular infusion on day 1 and 28 concessive days from day 3 to day 30 for a 30-day treatment cycle, respectively. Serial blood samples from seven subjects were collected into tubes containing K_2-EDTA (as the anticoagulant) at the following time-points: before drug administration, and at 5, 10, 20, 30, 60, 90, 120, 180, 240 min after injection on days 1 and 30 of the first cycle of treatment. Plasma was obtained immediately by centrifugation of the blood samples at 3000 rpm for 10 min at 4 °C. Then the plasma was separated, split into 2 polypropylene tubes and stored as two independent aliquots at −30 °C pending analysis. All blood samples were collected under the full ethical approval of the ethics committee of the participating centers and only after the signature of informed consent from all the enrolled patients. The study was approved by the Ethics Committee of Beijing Cancer hospital (China). The phase I study was conducted according to the principles expressed in the Declaration of Helsinki.

3. Results and discussions

3.1. Extraction method optimization

The aim of sample preparation was to remove interferences and extract the analyte from the plasma samples. In this study, a simple and economical protein precipitation method was developed. Methanol and acetonitrile were investigated to identify the

more efficient precipitating agent for the sample extraction and less endogenous interference, and the result showed that methanol offered a clean chromatogram for a blank plasma sample. Chlorogenic acid is susceptible to oxidation under the light conditions and all the procedures should be protected from light. Furthermore, the 0.1% formic acid was added in the sample preparation because CGA was found to be stable in acidic conditions.

3.2. Internal standard selection

A good internal standard should mimic the analyte in the entire sample extraction, chromatographic elution and mass spectrometric detection. Stable isotopically labeled internal standards are ideal candidates for meeting the above criteria. However, isotopes are not always easily accessible due to the high cost or the technical difficulty in synthesizing them. Several compounds were investigated and neochlorogenic acid finally was found to be a suitable IS which has similar structure to CGA. Clean chromatograms were obtained and no significant interferences in the MRM channels at the relevant retention times were observed.

3.3. LC–MS/MS method optimization

To develop a sensitive and specific LC–MS/MS method for compound quantification, analytes were investigated in the positive and negative mode, and the negative mode was selected as the electrospray ionization condition for the current study because it provided stronger intensity. MS/MS parameters were optimized through an Agilent Automatic Optimizer to obtain the best response, and quantification analysis was performed in the dynamic MRM mode, because of its high selectivity and sensitivity.

To obtain the appropriate retention time and response, methanol, acetonitrile, water and formic acid were tested as mobile phases. Chlorogenic acid eluted from the column rapidly with an acetonitrile-water system, so the suitable retention time and resolution were difficult to obtain. Besides, 0.1% formic acid in water was found to enhance efficiency of ionization and obtain a better intensity than pure water. Thus, a methanol-water (0.1% formic acid) system was selected for the mobile phase.

3.4. Validation

3.4.1. Selectivity

Representative MRM chromatograms of blank plasma samples (Fig. 2A), plasma sample spiked with drug and IS (Fig. 2B) and a real human plasma sample (Fig. 2C) were obtained under the selected analytical conditions. The results showed the absence of any interference at the retention time of the analyte and IS.

3.4.2. Calibration curve performance

The calibration curves were validated over the concentration range 10–2000 ng/mL for CGA in human plasma. Correlation coefficients of all the curves were greater than 0.99 and the deviations of the back-calculated concentrations from their nominal values were within ±15%. Results were fitted to linear regression analysis using $1/x^2$ as the weighting factor.

3.4.3. Accuracy, precision, dilution integrity and sensitivity

The intra- and inter-day precision and accuracy of the method were determined from the analysis of 5 QC samples at three different concentrations and the results are summarized in Table 1. It was demonstrated that the method was reliable and reproducible since RSD% was below 15% and RE% was within ±15% for all the investigated concentrations.

Dilution integrity was examined by ten-fold dilution of ultra high QC samples. The results had displayed that the accuracy and precision of the diluted samples were within the acceptance range (data are shown in Table 1). Therefore, the plasma samples exceeding the ULOQ could be diluted with blank plasma using the tested dilution factors before analysis.

The LLOQ sample was used to evaluate the sensitivity of the method. The LLOQ was set at 10 ng/mL and a typical chromatography of plasma sample containing 10 ng/mL of CGA is shown in Fig. 2B. Five replicates of plasma samples at LLOQ level were investigated and the results met the acceptance criteria with accuracy of 5.4% (RE%) and precision of 1.7% (RSD%).

3.4.4. Carry over

A carryover test was executed and no response was detected at the expected retention times under a processed blank plasma sample injected after an ULOQ sample. There was no apparent carryover in the conditions of the present method.

3.4.5. Stability

CGA stock solution was stable at −30 °C for 4 months. The stability CGA in human plasma was investigated under a variety of storage and processing conditions and it was found to be stable under conditions as follows: at room temperature (25 °C) for 2 h; at 10 °C for 20 h kept at auto-sampler; after 3 freeze/thaw cycles (from −30 °C to 25 °C); at −30 °C for 60 days. The stability results are shown in Table 2.

3.4.6. Recovery and matrix effect

The protein perception method was employed to extract CGA from plasma. The extraction recoveries were 80.2%, 75.5%, 72.1% at 3 levels of QC samples (n = 5) with RSD% less than 15%, showing good consistency.

Matrix effects were 109.4%, 108.9%, 108.8% at 3 levels concentrations of QCs (n = 6), respectively. At the same time, matrix effects observed for IS was 103.3%. Furthermore, the intersubject variability from 6 different lots at each concentration level was less than 6.1%, indicating that no apparent matrix effect was found to affect the determination.

3.4.7. ISR

The accuracy of the method was further demonstrated by reanalysis of incurred plasma samples. The concentrations of CGA determined on the two analytical runs were very similar in all samples, being the percentage difference of the results within 20% for more than 70% of the total amount of samples re-analyzed. The results revealed that the present method was reproducible and reliable.

3.5. Clinical application

The pharmacokinetic analysis was processed with WinNonlin 6.3 software with noncompartmental model. Mean plasma concentration-time profiles of CGA after single dose administration of 1, 2 or 3 mg/kg CAFI and multiple-dose administration of 3 mg/kg CAFI are presented in Fig. 3. Main information were acquired below according to the pharmacokinetic parameter (shown in Table 3). Firstly, CGA was moderately absorbed with an average time to peak plasma concentration (T_{max}) of about 1 h and rapidly eliminated with short $t_{1/2}$ of 1 h. Secondly, there is no obvious accumulation of CGA after multiple doses and no additional adverse events were observed, showing the good safety of CAFI. Thirdly, C_{max} and $AUC_{0\rightarrow inf}$ of CGA had positive correlation with the dose from 57 to 282 mg obtained from the Dose-C_{max} and Dose - $AUC_{0\rightarrow inf}$ scatter diagram (shown in Fig. 4).

In addition, some pharmacokinetic parameters for CGA from different reports are shown in Table 4. It revealed that different

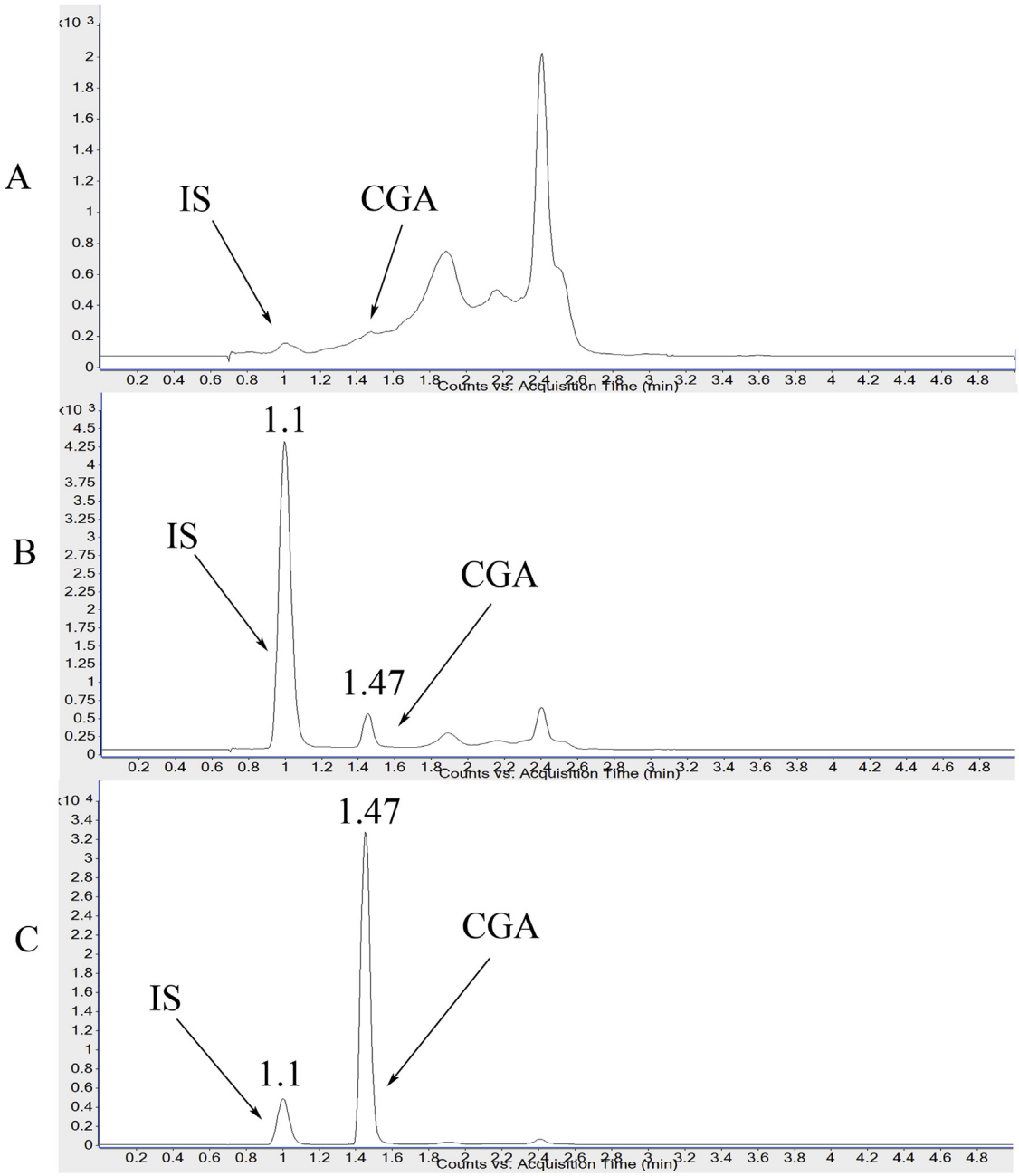

Fig. 2. Chromatogram of CGA and IS in plasma samples: (A) Blank plasma samples; (B) Blank plasma spiked with CGA (10 ng/mL) and IS; (C) Plasma samples obtained from a patient at 30 min after administration of CAFI containing 57 mg of CGA.

Table 1
Intra- and inter-day accuracy and precision of quality control samples.

	Intra-day (ng/mL)			Intra-day(ng/mL)			Diluted by 10-fold (ng/mL)
	Low (20.00)	Medium (200.0)	High (1600)	Low (20.00)	Medium (200.0)	High (1600)	Ultra-high (400)
Mean	21.04	211.7	1744	22.11	194.0	1628	391.6
SD	2.194	7.321	66.85	2.377	13.90	127.1	10.96
Precision(RSD%)	10.4	3.5	3.8	10.7	7.2	7.8	2.8
Accuracy(RE%)	5.2	5.9	9.0	10.6	−3.0	1.7	−2.1
n	5	5	5	15	15	15	5

mode of administration including oral administration, intramuscular injection and intravenous drip does not affect T_{max} and elimination rate ($t_{1/2}$). The bioavailability of CGA after intramuscular injection is much higher than that after oral administration. Although relatively higher C_{max} and $AUC_{0\rightarrow inf}$ were acquired using intravenous administration acquired, the good clinical compliance will be obtained with intramuscular injection administration especially for the patients with tumor.

Table 2
Stability results of QC samples at different conditions (n = 5).

Stability tests	Theoretical conc.(ng/mL)	Found conc. (ng/mL)	Precision (RSD%)	Accuracy (RE%)
Stock solution (−30 °C for 4 months)	1.12E + 05[a]	1.06E + 05[b]		
Freeze thaw (from −30 °C to 25 °C, 3 cycles)	20.00	21.21	8.3	6.0
	200.0	174.3	2.5	−12.8
	1600	1677	1.3	4.8
Auto-sampler (10 °C for 20 h)	20.00	20.81	9.4	4.1
	200.0	216.9	4.0	8.4
	1600	1770	6.2	10.6
Short term (25 °C for 12 h)	20.00	19.61	5.9	−1.9
	200.0	176.3	6.9	−11.8
	1600	1444	3.9	−9.7
Long term (−30 °C for 2 months)	20.00	19.21	10.7	−3.9
	200.0	177.5	7.4	−11.2
	1600	1630	7.3	1.9

[a] peak area of fresh stock solution.
[b] peak area of original stock solution.

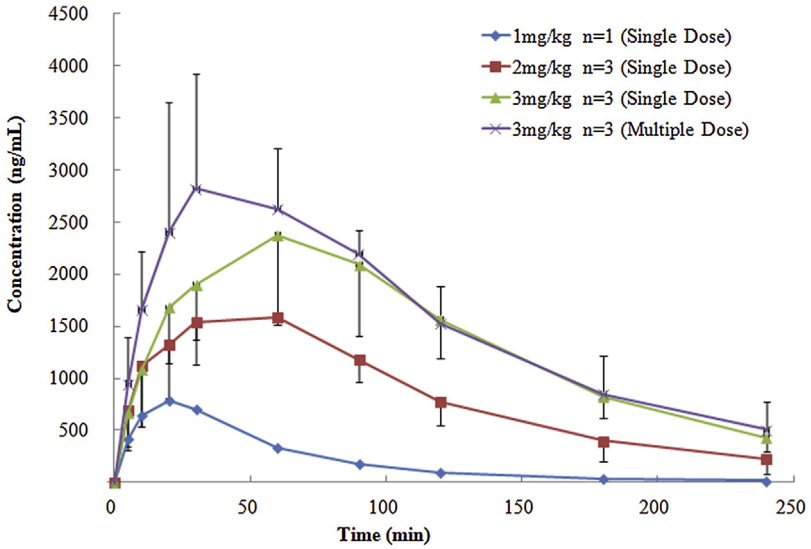

Fig. 3. Mean concentration-time curves of CGA in Chinese patients with solid tumor after administration of CGA at different dose level.

Table 3
Pharmacokinetic parameters of CGA after single dose 1, 2 or 3 mg/kg and multiple doses of 3 mg/kg CAFI in Chinese patients with advanced solid tumor.(The data were shown as average (SD) and T_{max} (min) was shown as median (minimum, maxmum)).

PK parameter	Single Dose 1 mg/kg(n = 1)	Single Dose 2 mg/ kg (n = 3)	Single Dose 3 mg/ kg (n = 3)	Multiple Dose 3 mg/ kg (n = 3)
$AUC_{0\rightarrow t}$ (ng·h mL^{-1})	821	3365 (460)	5477 (1273)	6214 (280)
$AUC_{0\rightarrow inf}$ (ng·h mL^{-1})	837	3754 (734)	6124 (1425)	7199 (547)
C_{max} (ng mL^{-1})	786	1727 (151)	2376 (683)	2914 (1043)
T_{max} (min)	20	30 (20, 60)	60 (30, 60)	30 (30, 60)
$t_{1/2}$ (h)	0.73	1.1 (0.4)	1.0 (0.1)	1.2 (0.5)
Vd/F (L/kg)	1.2	0.8 (0.1)	0.8 (0.1)	0.7 (0.2)
CL/F(L/H/kg)	1.2	0.5 (0.1)	0.5 (0.1)	0.4 (0)

Table 4
Pharmacokinetic parameters for CGA from different reports.

Administration mode	Dose (mg)	C_{max} (ng/mL)	T_{max} (h)	$t_{1/2}$ (h)	AUC (ng·h/ml)	Subjects
Oral administration [3]	3.64	0.8(0.5)	1.0(0.5)	1.3(0.6)	$1.1*10^{-3}(0.45*10^{-3})$	12 healthy voluteers
Intravenous drip [1]	6.6	255(66)	1.0(0.3)	1.4(0.5)	403(109)	10 healthy voluteers
Intramuscular injection	204	2615	1.0	1.2	6112	1 patient with advanced solid tumor from this study

4. Conclusion

A simple, specific, rapid analytical method had been developed and validated for the determination of CGA in human plasma. Simple protein precipitation extraction procedure and short run time can increase sample throughput that is important for large sample batches. This method was firstly applied to determine the CGA concentration in human plasma from Chinese patient with advanced solid tumor after intramuscular injection of CAFI and the corresponding PK parameters were also reported. The above information

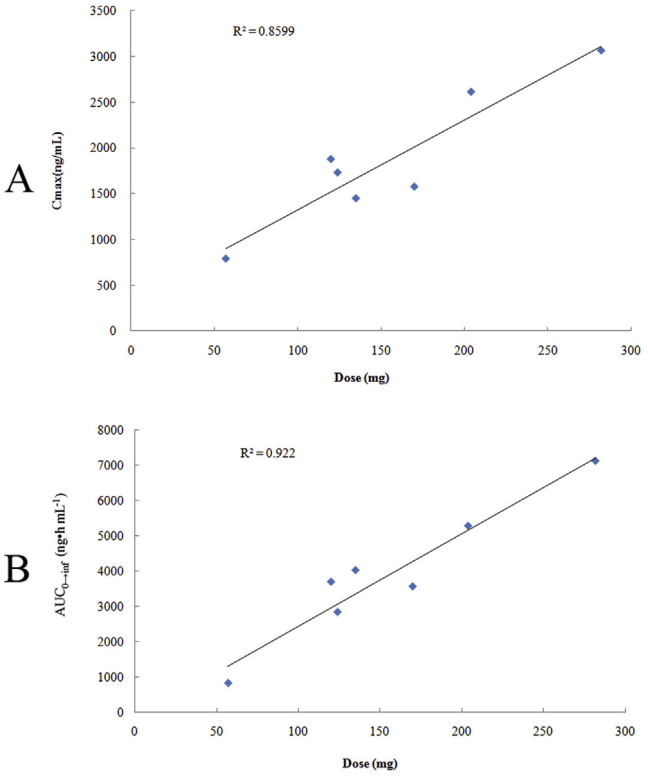

Fig. 4. The relationship between Dose and C_{max} (A), and between Dose and $AUC_{0\rightarrow inf}$ (B).

will be instructive for dose adjustment and investigating the pharmacokinetics and safety of CAFI in patient with advanced solid tumor in further study.

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Declaration of Competing Interest

All authors involved in this experiment claim that there are no conflicts of interest.

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