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Determination of gambogic acid in human plasma by liquid chromatography-atmospheric pressure chemical ionization–mass spectrometry

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Abstract

A high-performance liquid chromatography-atmospheric pressure chemical ionization–mass spectrometry (HPLC-APCI–MS) method was established for the determination of gambogic acid (GA) in human plasma using ursolic acid as the internal standard (I.S.). Plasma samples were extracted with ethyl acetate and separated on a Hanbon Lichrospher 5-C18 column with a mobile phase of acetonitrile–tetrahydrofuran–water (70:23:7, v/v). Gambogic acid was determined by using atmospheric pressure chemical ionization (APCI) in a single quadrupole mass spectrometer. HPLC-APCI–MS was performed in the selected ion monitoring (SIM) mode using target ions at $[M-H]^-$ *m/z* 627.4 for gambogic acid and $[M-H]^-$ *m/z* 455.4 for the I.S. Calibration curve was linear over the range of 3.108–4144 μg/L. The lower limit of quantification was 3.108 μg/L. The intra- and inter-run precisions were less than 12.3 and 14.1%, respectively. The method has been successfully applied to study the pharmacokinetics of gambogic acid in patients with malignant tumour.

Keywords: Gambogic acid; HPLC-APCI–MS; Pharmacokinetics

1. Introduction

Gambogic acid (GA, Fig. 1) is a cytotoxic compound isolated from the gamboge resin of the *Garcinia hanburyi* tree found in Southeast Asia [1–3]. The resin is used as a traditional medicine in China for the treatment of cancers [4]. In recent years, GA has been discovered to be a novel apoptosis inducer [5–8]. Pre-clinical investigations indicate that GA may induce apoptosis in different cancer cell lines [5,7], inhibit the growth of SMMC-7721 [9,10] and the proliferation of human lung carcinoma SPC-A1 cells [11], and repress telomerase activity and telomerase reverse transcriptase mRNA expression in the cells [11]. Kasibhatla et al. [8] reported that transferrin receptor is a target for GA. Zhao et al. [6] concludes that GA does not affect normal cells, but it can induce apoptosis in tumour cells selectively.

Recently, a formulation of GA injection has been developed by Jiangsu Kanion Pharmaceutical Co., Ltd. (Lianyungang, China) and approved by State Food and Drug Administration of China to be put into phase I clinical trial as a new drug to treat cancer. So far, the pharmacokinetic profile of GA in humans has not been reported yet. As entrusted by Jiangsu Kanion Pharmaceutical Co., Ltd., the investigation of the pharmacokinetics of the GA injection was carried out. To evaluate the pharmacokinetics of GA injection in humans, a sensitive method is required. Several TLC scanning [12], high-performance liquid chromatography (HPLC)-UV [13,14], and HPLC-ELSD [15] methods for quantification of GA in gamboge resin and GA injection have been reported, in which the lower limit of quantification (LLOQ) was 4–25 mg/L. These methods offer the reliable analysis of GA in gamboge resin and GA injection. However, they are not suitable for the determination of GA in human

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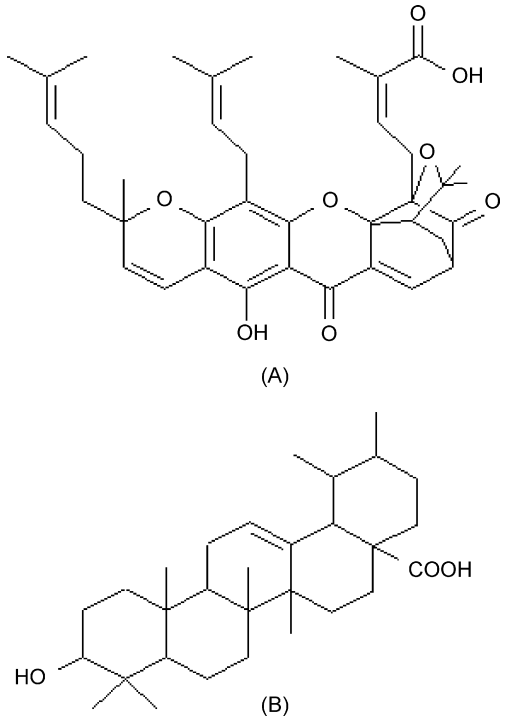

Fig. 1. Chemical structures of GA (A) and ursolic acid (B).

plasma. Hao et al. [16] developed an HPLC-UV method for the determination of GA in dog plasma, in which the LLOQ was 67 μg/L. This method is sensitive enough for the evaluation the pharmacokinetics of GA in dogs. But to evaluate the pharmacokinetics of GA in humans, a method with an LLOQ of 67 μg/L is not sensitive enough because the plasma concentration levels of GA on the terminal elimination phase were below this LLOQ. In this article, we report a rapid and sensitive high-performance liquid chromatography-atmospheric pressure chemical ionization–mass spectrometry (HPLC-APCI–MS) method with an LLOQ of 3.108 μg/L. The assay is suitable for the study of pharmacokinetics of GA in patients with malignant tumour.

2. Experimental

2.1. Materials and reagents

GA (99.6% purity) was obtained from Jiangsu Kanion Pharmaceutical Co., Ltd. (Lianyungang, China). Ursolic acid (I.S., 98.3% purity) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The test drug was GA injection containing 20 mg of GA per vial, which was provided by Jiangsu Kanion Pharmaceutical Co., Ltd. (Lianyungang, China). Acetonitrile and tetrahydrofuran were of gradient grade for liquid chromatography (Merck, Germany). Ethyl acetate was of analytical grade purity and was purchased from Nanjing Chemical Reagent Co., Ltd. (Nanjing, China).

2.2. Instrument and conditions

HPLC-APCI–MS analyses were performed using an Agilent Technologies Series 1100 LC/MSD SL system (Agilent Technologies, Palo Alto, CA) with a Hanbon Lichrospher 5-C18 column, 5 μm, 150 mm × 4.6 mm i.d. (Jiangsu Hanbon Science & Technology Co. Ltd., China). The mobile phase was acetonitrile–tetrahydrofuran–water (70:23:7, v/v) at a flow rate of 1.0 ml/min. The column temperature was maintained at 25 °C. A quadrupole mass spectrometer equipped with an atmospheric pressure chemical ionization source was set with a drying gas (N_2) flow of 4 L/min, nebulizer pressure of 60 psi, drying gas temperature of 350 °C, capillary voltage of 3 kV, and the negative ion mode. The fragmentor voltage was 80 V. HPLC-APCI–MS was performed in selected-ion monitoring mode using target ions at $[M-H]^-$ m/z 627.4 for GA and $[M-H]^-$ m/z 455.4 for I.S. The MS data acquisition was started at 2 min after sample injection, and the stream selection valve was set to waste until data acquisition was started.

2.3. Preparation of working solutions

The stock solution of GA was prepared at 1.036 g/L in acetonitrile and stored at −20 °C. A series of working solutions of GA was prepared at concentrations of 103.6 mg/L, 10.36 mg/L, 1.036 mg/L, and 103.6 μg/L by serially diluting the stock solution with acetonitrile in separate 10-mL volumetric flasks. The stock solution of I.S. was prepared at 1.010 g/L in acetonitrile and stored at −20 °C. A solution containing 4.040 mg/L I.S. was also prepared by further diluting the stock solution of I.S. with acetonitrile. All the solutions were stored at −20 °C.

2.4. Sample preparation

A 1-mL aliquot plasma sample was extracted with 5 mL ethyl acetate after addition of 50 μL I.S. solution (4.040 mg/L). Following centrifugation and separation, the organic phase was evaporated to dryness under a stream of nitrogen in a water bath of 30 °C. The residue was reconstituted in 500 μL of mobile phase, and a 20-μL aliquot was injected into the HPLC-APCI–MS system.

2.5. Calibration curves and lower limit of quantification

Calibration standards of GA were prepared by supplementing appropriate amount of the working solutions in blank plasma obtained from healthy volunteers, and the percentages of the acetonitrile working solution added to blank plasma were 1–3%. Standard curves were prepared in the range of 3.108–4144 μg/L for GA at concentrations of 3.108, 10.36, 31.08, 103.6, 207.2, 518.0, 1036, 2072, and 4144 μg/L. These calibration standards of GA were extracted and assayed. The GA calibration curve was constructed by plotting the peak area ratio of GA to the I.S. versus the concentration of GA, using weighted least squares linear regression (weighting factor was $1/C^2$) [17–19]. The LLOQ was

defined as the lowest concentration on the calibration curve at which precision was within 20% and accuracy was within ±20% [17], and it was established using five samples independent of standards.

2.6. Preparation of quality control samples

The quality control (QC) samples were prepared in blank plasma at concentrations of 3.0, 50.0, 500.0 and 4000 μg/L for GA. The QC samples were prepared independent of the calibration standards and analyzed with processed test samples at intervals in each run. The results of the QC samples provided the basis of accepting or rejecting the run.

3. Assay validation

3.1. Precision and accuracy

The QC samples were prepared and analyzed on three consecutive days (one run per day) to evaluate the accuracy and the intra- and inter-run precision of the analytical method. The accuracy as well as the intra- and inter-run precision of the method was determined by analyzing five replicates at 3.108, 51.80, 518.0 and 4144 μg/L of GA along with one standard curve on each of the 3 days. Assay precision was calculated using the relative standard deviation (RSD%). The accuracy is the degree of closeness of the determined value to the nominal or known true value under prescribed conditions [16]. Accuracy is defined as the relative deviation in the calculated value (E) of a standard from that of its true value (T) expressed as a percentage (RE%). It was calculated by using the formula $RE\% = (E - T)/T \times 100$.

The accuracy of the assay was checked by preparation of QC samples at the start of the clinical study. These QC samples were assayed along with clinical samples in each run to monitor the performance of the assay and to assess the integrity and validity of the result of the unknown clinical samples analyzed.

3.2. Assay selectivity

The selectivity of the method was assessed by comparing the chromatograms of the blank plasma samples from six healthy volunteers and twenty-eight malignant tumour patients with the corresponding spiked plasma. Each blank plasma sample was tested using the proposed extraction procedure and HPLC-APCI–MS conditions. No interference from the blank plasma samples at the expected retention time of GA or I.S. was observed.

Usually, we prefer choosing a compound that has similar chemical structure to the analyte or an isotope of the analyte as the internal standard. However, for some nature products, such as GA, it is difficult to obtain their isotopes or the internal standards that have chemical structures similar to them. In fact, the necessary requirements for a proper internal standard include that it should have similar retention to the analyte, be well resolved from the analyte and other peaks, and mimic the analyte in any sample preparation steps, but it does not have to have chemical structure similar to the analyte [20]. GA is a kind of nature acids that has complicated chemical structure, and it is difficult for us to obtain an internal standard that has similar chemical structure to it or is an isotope of it. After screening of some acids, ursolic acid was chosen as the internal standard in the assay of GA. Ursolic acid is a triterpenoid acid found in food, medicinal herbs, and various other plants, so the issue of potential interference from it should be considered. The main plant foods eaten by Chinese people are from monocotyledoneae plants (such as rice, barley, and corn) and cruciferae plants (such as radish, celery cabbage, cole, and greengrocery). Ursolic acid has not been found in these kinds of plants (grains and vegetables). May be ursolic acid exists in some other foods (such as some fruits). But the quantity of ursolic acid absorbed by human bodies from those foods is too less in the daily life, so it cannot be detected by our HPLC-APCI–MS method reported in this article. The test result of the blank plasma samples from six healthy volunteers and 28 malignant tumour patients mentioned above demonstrated this issue. The predose blank plasma samples of the volunteers (malignant tumour patients) were checked using the proposed extraction procedure and HPLC-APCI–MS conditions to ensure that no interferences (including the detectable endogenous ursolic acid) were encountered.

3.3. Extraction recovery

The extraction recovery of GA was evaluated by analyzing five replicates at 3.108, 51.80, 518.0, and 4144 μg/L of GA. Recovery was calculated by comparison of the peak areas of GA extracted from plasma samples with those of injected standards.

3.4. Stability

The stability of GA in plasma was studied under a variety of storage and handling conditions at low (3.108 μg/L) and high (4144 μg/L) concentration levels. The short-term temperature stability was assessed by analyzing three aliquots of each of the low- and high-concentration samples that were thawed at room temperature and kept at this temperature for 8 h. Freeze–thaw stability (−20 °C in plasma) was checked through three freeze–thaw cycles. Three aliquots of each of the low- and high-concentration levels were stored at −20 °C for 24 h and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 24 h under the same conditions. The freeze–thaw cycles were repeated three times and then analyzed on the third cycle. The long-term stability was determined by analyzing three aliquots of each of the low- and high-concentration levels stored at −20 °C for 2 months.

3.5. System suitability test

Prior to running each run of clinical plasma samples, the instrument performance (e.g., sensitivity, reproducibility of chromatographic retention and separation, plate number, and

tailing factor) was determined by the analysis of the reference standard of GA, I.S., blank plasma, and plasma spiked with GA and I.S.

3.6. Clinical study design and pharmacokinetic analysis

The clinical study protocol was reviewed and approved by the Ethics Committee of Cancer Hospital Affiliated to Chinese Academy of Medical Sciences. All volunteers were given written informed consent to participate in the study according to the principles of the Declaration of Helsinki. Six Chinese patients with malignant tumours participated in the study. Following an overnight fast, each volunteer received a single intravenous (IV) dose of 35 mg/m^2 of the GA injection. The intravenous infusion of the GA injection was designated to finish within 2 h. Blood was sampled pre-dose and at 0.167, 0.333, 0.667, 1.333, 2, 3, 4, 6, 9, 12, 24, 36, and 48 h following dosing for determination of plasma concentration of GA. Model-independent pharmacokinetic parameters were calculated for GA. The maximum plasma concentrations (C_{max}) and their time (t_{max}) were noted directly. The elimination rate constant (k_e) was calculated by linear regression of the terminal points of the semi-log plot of plasma concentration against time. Elimination half-life ($t_{1/2}$) was calculated using the formula $t_{1/2} = 0.693/k_e$. The area under the plasma concentration–time curve [$AUC_{(0-t)}$] to the last measurable plasma concentrations (C_t) was calculated by the linear trapezoidal rule. The area under the plasma concentration–time curve to time infinity ($AUC_{0-\infty}$) was calculated as follows: $AUC_{0-\infty} = AUC_{0-t} + C_t/k_e$.

4. Results and discussion

4.1. Conditions of chromatography

When selecting the mobile phase for HPLC-MS, attention should be paid to the influence of mobile phase on the chromatographic retention and the MS sensitivity. To assay acidic compounds by HPLC, the mobile phase is often adjusted to acidic pH with acids to improve the chromatographic peak shape and retention of the analytes. The results of the experiment showed that after adjusting the mobile phase to an acidic pH with formic acid or acetic acid, the symmetric chromatographic peaks of GA and I.S. were obtained, but the problems of much longer retention time and the decreased MS sensitivity also occurred. To achieve shorter run time and improve the chromatographic peak shape of GA and I.S., tetrahydrofuran was added in the mobile phase. The results of the experiment also showed that the MS sensitivity of GA and I.S. was increasing along with the increase in the ratio of acetonitrile in the mobile phase. But when the ratio of organic portion in the mobile phase exceeded 93%, the ionization efficiency decreased. Finally, high sensitivity, good separation, and short run time were obtained by

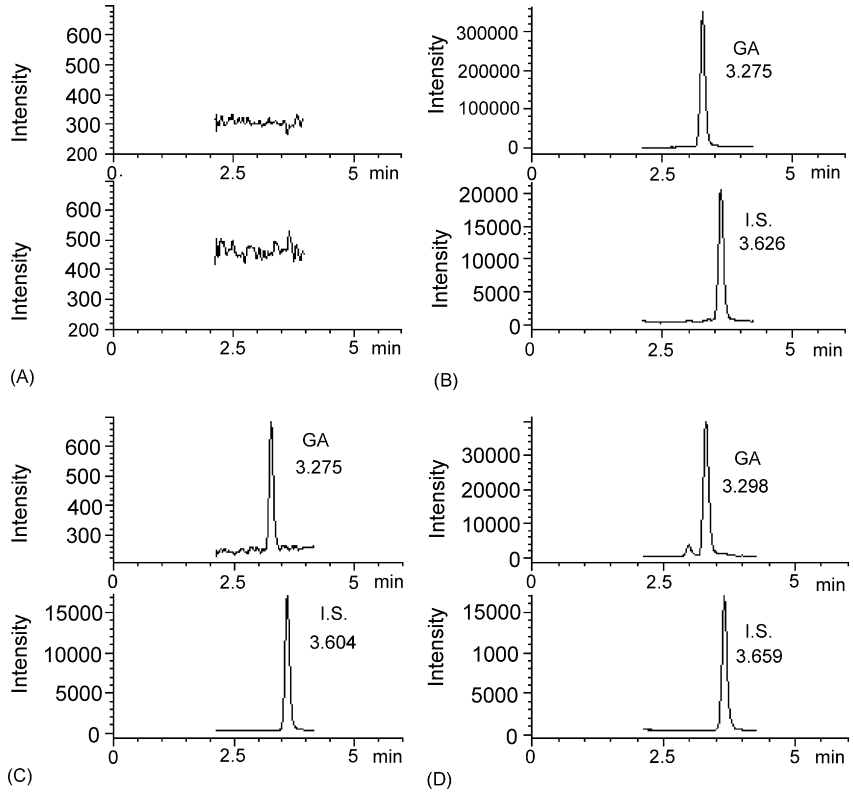

Fig. 2. Typical SIM chromatograms of blank plasma (A), plasma spiked with GA (3892 μg/L) and I.S. (B), LLOQ for GA in plasma (3.108 μg/L) and I.S. (C), plasma obtained from a patient at 6 h after a single 35 mg/m^2 IV dose of the GA injection, the plasma concentration of GA was estimated to be 416.0 μg/L (D).

using a mixture solution of acetonitrile–tetrahydrofuran–water (70:23:7, v/v) as the mobile phase. Representative selected-ion chromatograms are shown in Fig. 2 in which the retention time was 3.2 min for GA and 3.6 min for I.S.

4.2. Conditions for APCI-MS

Because GA is a weak acid, the negative ion mode was adopted in the LC–MS. Usually, electrospray ionization (ESI) is used for medium- to high-polarity analytes, and atmospheric pressure chemical ionization (APCI) is used for low- to medium-polarity analytes. In fact, sometimes we cannot easily decide which ionization technique is the best, and we need to investigate the relative merits of the compound under the two different ionizations. So, both APCI and ESI sources were evaluated for assay development in negative ion mode. APCI produced greater sensitivity and exhibited less interference than we were able to achieve with ESI. Thus, APCI in negative ion mode was adopted for the assay of GA.

Although MS/MS is not available on the instrument used in this experiment, there are still fragment ions [21], deprotonated molecule ions [22,23], and protonated molecule ions [24,25] that can be selected as the target ions of the analytes in the SIM. By adjusting the fragmentor voltage to different values, the different base peaks (the highest ion peak in the mass spectrum, which can be selected as the target ions of the analytes) were obtained. As the fragmentor voltage was set at a lower value, the base peak obtained in the mass spectrum of GA is the deprotonated ion $[M-H]^-$ at *m/z* 627.4. When the fragmentor voltage exceeded 150 V, the intensity of the fragment ion $[M-COOH]^-$ at *m/z* 583.5 increased obviously, and it became the base peak at 180 V. If the fragmentor voltage was set at the values less than 180 V, selecting the deprotonated ion $[M-H]^-$ *m/z* 627.4 as the target ion can achieve the higher assay sensitivity. When the fragmentor voltage exceeded 180 V, selecting the fragment ion $[M-COOH]^-$ *m/z* 583.5 as the target ion can achieve the higher sensitivity. When the fragment ion $[M-COOH]^-$ *m/z* 583.5 was selected as the target ion, the highest assay sensitivity was obtained with the fragmentor voltage of 200 V. However, this sensitivity was still less than the one achieved by selecting the deprotonated ion $[M-H]^-$ *m/z* 627.4 as the target ion with the fragmentor voltage less than 150 V. So, the deprotonated ion $[M-H]^-$ *m/z* 627.4 was finally selected as the target ion of GA in the assay.

In order to determine the optimal fragmentor voltage, the intensities of the deprotonated ion $[M-H]^-$ of GA at *m/z* 627.4 were compared at fragmentor voltages of 30, 60, 70, 80, 90, 100, 120, 150, 180, 200, and 250 V. The results showed that while selecting the deprotonated ion $[M-H]^-$ *m/z* 627.4 as the target ion of GA, the highest sensitivity of the assay could be achieved by using an 80 V fragmentor voltage. Therefore, a fragmentor voltage of 80 V was used to carry out the APCI-MS in the assay. Fig. 3(A) shows a full-scan APCI-negative mass spectrum of GA at an 80 V fragmentor voltage. At this fragmentor voltage, the base peak in the mass spectrum of I.S. was the negative molecular ion $[M-H]^-$ of I.S. at *m/z* 455.4, See Fig. 3(B). Therefore, the negative molecular ion

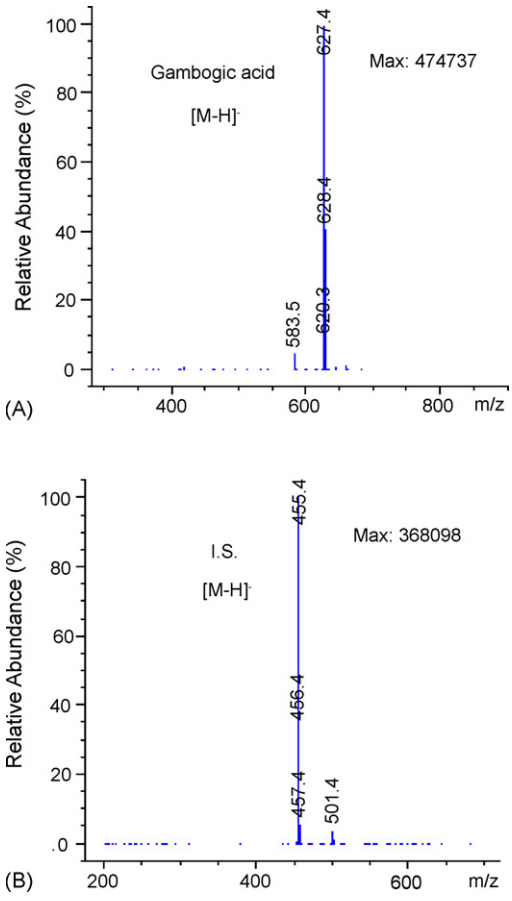

Fig. 3. Mass spectra of the negative ion of GA (A) and I.S. (B) at 80 V fragmentor voltage.

$[M-H]^-$ at *m/z* 455.4 was selected as the target ion of I.S. in the SIM.

4.3. Method validation

4.3.1. Calibration curve and sensitivity

The calibration curves, which related the concentrations of GA to the peak area ratio of GA to I.S., showed good linearity over the range of 3.108–4144 μg/L. The typical calibration curve for GA had a slope of 0.004468 ± 0.000151, an intercept of -0.000483 ± 0.002405 and $R = 0.9982$. Calibration curves were prepared and analyzed with each run of clinical samples and QC samples. The LLOQ for GA in plasma was 3.108 μg/L.

The HPLC-APCI–MS method reported in this article was developed for the GA pharmacokinetic study in the phase I clinical trial of the GA injection. In the pilot study of this phase I clinical trial, the pharmacokinetic profiles of six preliminary doses of 10, 25, 35, 45, 60, and 75 mg/m^2 GA were studied. The GA pharmacokinetic profile reported in this article is only the pharmacokinetic profile of GA at the dose of 35 mg/m^2. In fact, to evaluate the pharmacokinetics of GA at the dose of 35 mg/m^2, an LLOQ of 3.108 μg/L is not necessary. But, to evaluate the pharmacokinetics of GA at the dose of 10 mg/m^2, an LLOQ of 3.1 μg/L is required because at this dose level, many of the GA plasma concentration levels on the terminal elimination phase are about 3–10 μg/L.

Table 1
Matrix effect data for GA at 3.108, 51.80, 518.0, and 4144 μg/L in five batches of human plasma ($n = 5$)

Concentration of GA (μg/L)	ME (mean ± SD) (%)
3.108	106.1 ± 8.2
51.80	94.8 ± 5.3
518.0	95.6 ± 5.7
4144	101.3 ± 3.9

4.3.2. Matrix effect (ME)

The matrix effect was defined as the direct or indirect alteration or interference in response to the presence of unintended analytes (for analysis) or other interfering substances in the sample [17]. The matrix effect of the assay was determined by comparing the peak areas of analytes resolved in the reconstituted solution of the blank plasma sample (the final solution of blank plasma after extraction and reconstitution) (A) with those resolved in mobile phase (B). ME was calculated by using the formula ME (%) = $A/B \times 100$. The matrix effect of the assay was evaluated at four GA concentration levels of 3.108, 51.80, 518.0, and 4144 μg/L, and five samples at each level were analyzed. The blank plasma samples used in this study were five different batches of human blank plasma. If the ME values exceed the range of 85–115%, an exogenous matrix effect is implied. The ME data at four plasma concentration levels of GA in five different batches of human plasma are presented in Table 1. The results showed that there was no matrix effect of the analytes observed in this study.

4.3.3. Assay precision and accuracy

The intra- and inter-run precision and accuracy are summarized in Table 2. The precision was calculated by using one-way ANOVA. The results in Table 2 demonstrate that the precision and accuracy of this assay are acceptable [17].

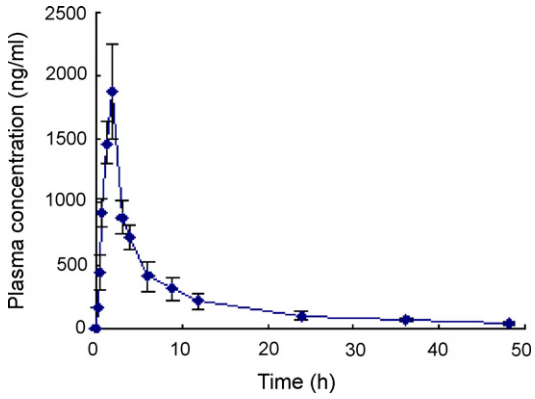

Fig. 4. Mean GA plasma concentration–time profile in six patients with malignant tumours after a single 35-mg/m^2 IV dose of the GA injection.

4.3.4. Extraction recovery

Because of its high hydrophobicity, GA can be easily extracted from plasma with ethyl acetate without adjustment of the pH value of the plasma. The results of the experiment showed that there was no significant difference between the recovery values obtained by extraction of the plasma samples with ethyl acetate with or without adjustment of the plasma pH to the acidic value. Ethyl acetate was chosen as the extraction solvent for its higher extraction efficiency with respect to GA and the I.S. The recovery values of GA from human plasma with ethyl acetate, determined at four concentrations of 3.108, 51.80, 518.0 and 4144 μg/L, were 70.2 ± 6.0%, 74.1 ± 5.9%, 74.0 ± 4.1% and 77.7 ± 4.0% ($n = 5$), respectively.

4.3.5. Stability

The stability of GA was studied under a variety of storage and handling conditions. The results in Table 3 showed that no significant degradation occurred after being kept at room temperature for 8 h and during the three freeze-thaw cycles for

Table 2
Accuracy and precision for the analysis of GA in human plasma (in pre-study validation, three runs, five replicates per run)

Added to plasma (μg/L)	Mean measured concentration (μg/L)	RE (%)	Intra-assay RSD%	Inter-assay RSD%
3.108	3.231	4.0	12.3	14.1
51.80	53.84	3.9	7.8	7.9
518.0	529.1	2.1	3.7	7.7
4144	3936	−5.0	3.1	5.6

Table 3
Stability data of GA in human plasma under various storage conditions ($n = 3$)

Storage conditions	Added C (μg/L)	Found C (μg/L)	Inter-run RSD (%)	RE (%)
Room temperature for 8 h	3.108	3.150	7.7	1.4
	4144	3861	5.9	−6.8
Three freeze-thaw cycles	3.108	3.561	11.6	14.6
	4144	4371	3.5	5.5
2 months at −20 °C	3.108	3.020	5.9	−2.8
	4144	3955	1.9	−4.6

the GA plasma samples. GA in plasma at −20 °C was stable for at least 2 months.

4.4. Application

The method described above was successfully applied to the pharmacokinetic study in which plasma concentrations of GA in six patients with malignant tumours were determined up to 48 h after receiving a single 35 mg/m^2 IV dose of the GA injection. The mean plasma concentration-time curve of GA is shown in Fig. 4. After administration of a single IV dose of 35 mg/m^2 GA, the C_{max} and k_e were 1877 ± 374.3 μg/L and $0.04486 \pm 0.00373\ h^{-1}$, respectively. Plasma concentrations declined with the $t_{1/2}$ of 15.54 ± 1.30 h. The AUC_{0-t} and $AUC_{0-\infty}$ values obtained were 11483 ± 1849 μg h/L and 12660 ± 2225 μg h/L, respectively.

5. Conclusions

The method had a good sensitivity and specificity for the determination of GA in human plasma. No significant interferences and matrix effect caused by endogenous compounds were observed. The assay is suitable for pharmacokinetic study of GA in human subjects.

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