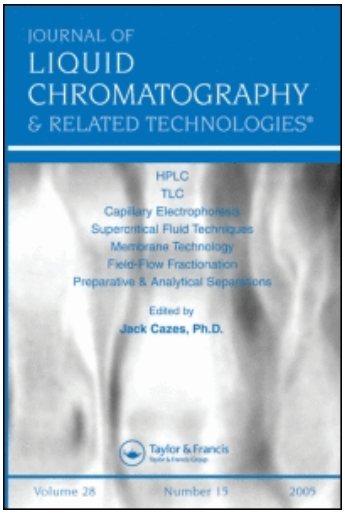

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A Valid Assay for the Pharmacokinetic Study of Gallic Acid from *Choerospondiatis Fructus* in Rabbit Plasma by LC/MS/MS

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A Valid Assay for the Pharmacokinetic Study of Gallic Acid from *Choerospondiatis Fructus* in Rabbit Plasma by LC/MS/MS

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Abstract: Gallic acid (GA), one of the bioactive compounds in *Choerospondiatis fructus*, was determined in rabbit plasma. The method involved a protein precipitation, a high performance liquid chromatographic separation, and an electrospray ionization ion trap mass spectrometric determination. Plasma samples were extracted by 0.5 mL 1.5% phosphoric acid/acetonitrile(v/v). Chromatographic separation was achieved on a C_{18} column using a mobile phase of 5.0% acetonitrile/water with 0.2% formic acid (pH 2.2). The calibration curve was linear in the range of 0.5–400 $ng \cdot mL^{-1}$ for plasma samples. The limit of detection was 200 $pg \cdot mL^{-1}$. Intra-and inter-day coefficients of variation were less than 8.0% and intra- and inter-day accuracies were within $\pm 5.0\%$ of the known concentrations. Finally, this assay was used to study the pharmacokinetics of GA in rabbit plasma following ingestion of *Choerospondiatis fructus* extracts. The statistical results

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indicated that the plasma drug concentration time course in rabbit was a 2-compartment open model.

Keywords: *Choerospondiatis fructus*, Gallic acid, Herb medicine, Pharmacokinetics, HPLC/MS/MS, ESI

INTRODUCTION

Choerospondiatis fructus is a traditional Mongolian herb medicine used in the treatment of many diseases, especially angina pectoris and myocardial infarction. Gallic acid (GA) (Figure 1), one of the abundant phenolic compounds in *Choerospondiatis fructus*, exhibits a variety of biological activities, including cardiovascular protection and anti-cancer, anti-inflammatory, and anti-oxidant activities.[1,2] Therefore, GA should be one of the active constituents responsible for the therapeutic effects of this medicinal plant.

Several analytical methods have been reported for the determination of GA concentrations in plant, wine, or other simple matrix, such as HPLC with UV or DAD detectors,[2–6] HPLC with mass spectrometry,[7,8] HPLC with microwave assisted isolation and solid-phase purification,[9] ion chromatographic method combined with electrochemical detection,[10] oscillating chemical reaction using the analyte pulse perturbation technique,[11] diffuse reflectance spectrometry,[12] and the biamperometric method.[13] However, less literature was spent on the determination of GA in biological samples. Much less information about the bioavailability and pharmacokinetic characteristics of GA in *Choerospondiatis fructus* were found.

We developed a sensitive and specific method to measure GA in rabbit plasma using high performance liquid chromatography/trap mass (LC/MS/MS) after administration of *Choerospondiatis fructus* extracts. This analytical method allows us to analyze low concentrations of parent GA and to elucidate the possible metabolic pathway in plasma with good reproducibility.

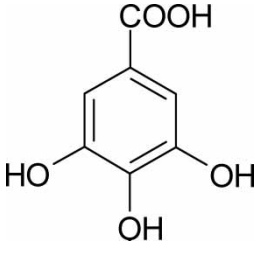

Figure 1. Structure of GA.

EXPERIMENTAL

Materials

Gallic acid standard (No: 110831-200502) was purchased from National Institute for the Control of Pharmaceutical and Biological Products in China. Formic acid, acetic acid, trifluoroacetic acid, phosphoric acid, and ρ-hydroxybenzcic acid (internal standard) were all obtained from Sigma-Aldrich (St. Louis, MO). Methanol and acetonitrile were all HPLC grade and purchased from Fisher Scientific (Springfield, NJ). *Choerospondiatis fructus* was purchased from Xi'an Medical Company in China and was identified by Professor Wang Junxian, Xi'an Jiaotong University.

Methods

Preparation of Standard Solutions for Plasma

A stock solution of 400.0 ng $\cdot$ mL^{-1} GA was prepared in 5.0% acetonitrile/water (v/v). Dilution of the stock solution with acetonitrile yielded working stock solutions at concentrations of 0.5, 5.0, 50.0, 100.0, 200.0, and 400.0 ng $\cdot$ mL^{-1}. The stock solution of I.S., ρ-hydroxybenzcic acid, was prepared at concentration of 96.0 ng $\cdot$ mL^{-1}. Dilution of this solution with acetonitrile yielded I.S. working solution at concentration of 0.096 ng $\cdot$ mL^{-1}. Samples were vortex mixed prior to protein precipitation and liquid-liquid extraction along with blank rabbit plasma, which contained neither GA nor internal standard. For precision and accuracy determinations, samples were prepared at GA concentrations of 1.0, 50.0, and 200.0 ng $\cdot$ mL^{-1} by the same step.

Sample Extraction

An aliquot of 0.50 mL rabbit plasma was transferred into a 5.0 mL Eppendorf tube in the presence of 10.0 μL I.S. Following addition of 1.5 mL 1.5% phosphoric acid/acetonitrile (v/v), the solution was vortexed for 60 s and centrifuged at 5,000 g for 10.0 min. An aliquot of 1.5 mL supernatant was aspirated into a 5.0 mL glass tube. Then, the resulting supernatant was dried under a N_2 stream and reconstituted with 0.5 mL 5.0% acetonitrile/water by vortex mixing. The reconstituted sample was filtered with a Φ 0.22 μm polytetrafluoroethylene membrane prior to analysis by LC/MS/MS.

Lc/Ms/Ms

A SL Agilent 1100 trap mass spectrometer (Walldboral, Germany) equipped with an electrospray ionization (ESI) interface, a series Agilent 1100 binary

pump, a series Agilent 1100 column oven, a series Agilent 1100 diode array detector (Walldboral, Germany), and 5.2 Chemistation software was used for data acquisition and processing. Chromatographic separation of the analytes of interest was achieved on a C_{18} column (particle size 5.0 μm, 250 mm × 4.6 mm, Littleforts, Philadelphia, USA). The mobile phase was 5.0% acetonitrile/water. The flow rate was $1.0\,mL \cdot min^{-1}$. The post column splitting ratio was set at 3:1 before the elution was transferred to ESI. The injection volume was 20.0 μL for all analytes.

The mass spectroscopy was performed in a negative mode using multiple reaction monitoring. Optimal operating parameters of ESI and multiple reaction monitoring transitions were obtained with respect to maximum signal intensity of molecular ions and fragment ions, by consecutively infusing standard solutions of GA ($100.0\,ng \cdot mL^{-1}$) and I.S. ($96.0\,ng \cdot mL^{-1}$), using a model 22 syringe pump (Harvard Apparatus, MA, USA) at a flow-rate of $500\,\mu L \cdot h^{-1}$. The optimum conditions of the interface were as follows: ion spray voltage of −4500 V, pressure of collision gas (He) of 1.3 m Torr and 40 p.s.i, flow rate of the nebulizer gas (N_2) and dry gas (N_2) of 40 p.s.i and $8.0\,L \cdot min^{-1}$, respectively. The dry temperature was set at 350°C. Analytes were detected by trap mass spectrometry using MRM with a 100 ms dwell time. The optimal transitions were m/z 168.5 (parent ion) to m/z 124.6 (product ion) for GA and m/z1 37.2 (parent ion) to m/z 93.4 (product ion) for I.S.

Calibration and Validation

The standard curve was prepared over a concentration range of 0.5–$400.0\,ng \cdot mL^{-1}$ with six different concentration levels as follows: 0.5, 5.0 50.0, 100.0, 200.0, and $400.0\,ng \cdot mL^{-1}$. Standard curves were run on each analysis day and the coefficient of determination r^2 was used to judge linearity. Calibration was performed by an internal standard method. The integration was processed on Chemistation software (Littleforts, Philadelphia, USA). The calibration curve was prepared by weighted linear regression analysis ($1/y^2$) of the peak area ratio (GA/internal standard) against concentration of calibration samples, where peak area was obtained from extract ion current spectrum. GA concentrations were calculated using the regression linear parameters. Samples that had concentrations exceeding the highest calibration value were diluted with control plasma prior to analysis.

Intra- and inter-day precision and accuracy for plasma samples were assessed through triplicate analyses of the same samples containing known amounts of GA with three samples per concentration level. Precision was evaluated as CV% of the mean of all the determinations at each concentration level. Accuracy was determined by comparing the calculated concentration with the known concentrations. The limit of detection (LOD) was assessed as the GA concentration at a signal-to-noise ratio of 3:1.

Stability

The effects of three freeze–thaw cycles (storage at $-20^{\circ}C$) on the compound stability in meconium were evaluated by repeated analysis ($n = 3$) of quality control samples (1.0, 50, and 200.0 $ng \cdot mL^{-1}$). The stability was expressed as a percentage of the initial concentration of the analytes spiked in blank rabbit plasma.

Pharmacokinetics Study

Twelve New Zealand rabbits (weight 1.8–2.2 kg) were provided by Animal Center of Xi'an Jiaotong University. After absolute dieting for 12.0 h, blank plasma was collected from the vein of their ears in clean heparinized glass tubes. Then, the water-extracted solution of *Choerospondiatis fructus* was administrated to the rabbits at the dosage of 15.0 mg/kg body weight. Plasma samples were collected at 0.33, 0.67, 1.0, 1.5, 2.0, 3.0, 5.0, 8.0, 12.0, 16.0, and 24.0 h, respectively. All the samples were prepared by the method described in Sample Extraction section.

RESULTS AND DISCUSSION

In this study, liquid chromatography-trap mass spectrometry with an ESI interface in negative mode with MRM was developed for the determination of GA in rabbit plasma samples. A previous method by Siranoush et al.[1] measured GA and conjuged GA by HPLC, using UV detection and isocratic elution. This assay was successfully used to measure galloyl glucuronide conjugates following a single dose of 50.0 mg GA (two acidum gallicum tablets) administration, but was unable to detect any parent GA before hydrolysis. Zhu et al.[3] described an HPLC method for the determination of tannic acid and its phenolic metabolites (GA and some other phenolic compounds) in biological fluids collected from sheep, following abomasal dosing of tannic acid at 1.0 g/kg body weight. However, the detection limit of his method was not low enough for the pharmacokinetic study.

LC/MS/MS Conditions

For the LC separation, a C_{18} column was used in our assay because it gave less peaks broadening and tailing and shorter run times, compared with a C_8 column. An acidic mobile phase (pH 2.2) with addition of formic acid was found to provide optimal separation and quantification of GA. Trifluoroacetic acid (TFA), acetic acid, and formic acid were all evaluated for suitability

under the mass spectrometric assay conditions. All of them are volatile, but the background noise using acetic acid was much higher than that using formic acid. TFA greatly suppressed the ionizing effect in negative ion electrospray. Therefore, formic acid was chosen to provide a high signal-to-noise ratio and increase the sensitivity of the assay.

It was found that 1.5% phosphoric acid/acetonitrile could effectively precipitate proteins and extract GA without any further preparation, which is different from previous reports.[4,5] The reason may be that phosphoric acid was useful for changing conjuged GA into free ones. We also compared the ESI-MS behavior of GA in positive mode with that in negative ones, and found that the negative ion mass spectrum was superior for the quantitation of GA under our conditions, due to better assay sensitivity.

The full scan and product ion scan mass spectra of gallic standards are presented in Figures 2a, b, respectively. Molecular ion m/z 168.5 was the most abundant ions for GA, and the greatest product ion from the parent ion m/z 168.5 was m/z 124.6. Therefore, the analyses were performed using MRM pairs of m/z 168.5 $\rightarrow$ m/z 124.6 for GA, and m/z 137.2 $\rightarrow$ 93.4 for I.S attributed to the same reason. The total ion current chromatogram of GA in standard solution and plasma samples after ingestion of *Choerospondiatis fructus* was showen in Figure 3a, b. The retention time of GA was 7.8 min. The noise level was lower than 80.0 cnt (intensity unit of the instrument). Due to the very high specificity of MS/MS, GA could be unambiguously identified by its MRM pairs (m/z 168.5 to m/z 124.6), even if it coelutes with other endogenous compounds.

Validation of the Assay

Validation was performed with regard to LOD, linearity, intra-, and inter-day precisions and accuracies in rabbit plasma. For the assay of six replicates on three different occasions, the LOD was 200 pg $\cdot$ mL^{-1} for the plasma samples. Compared with previous reports in the literature with LOQ values of 0.025 mg $\cdot$ mL^{-1},[1] the present method provides better sensitivity. The calibration curve of GA was linear over the concentration range of 0.5 $\sim$ 400.0 ng $\cdot$ mL^{-1} for plasma samples. Correlation coefficient of 0.999 was obtained for the relationship between peak area ratio and the corresponding calibration concentration. With regards to precision, CV% values for GA were less than 8.0% for inter-day and intra-day analysis (Table 1). The accuracies for intra- and inter-day analysis were within $\pm$5.0% of known concentrations (Table 1).

With reference to freeze–thaw stability assays for quality control samples, no relevant degradation was observed after any of the three freeze–thaw cycles, with differences in the initial concentration being less than 10%.

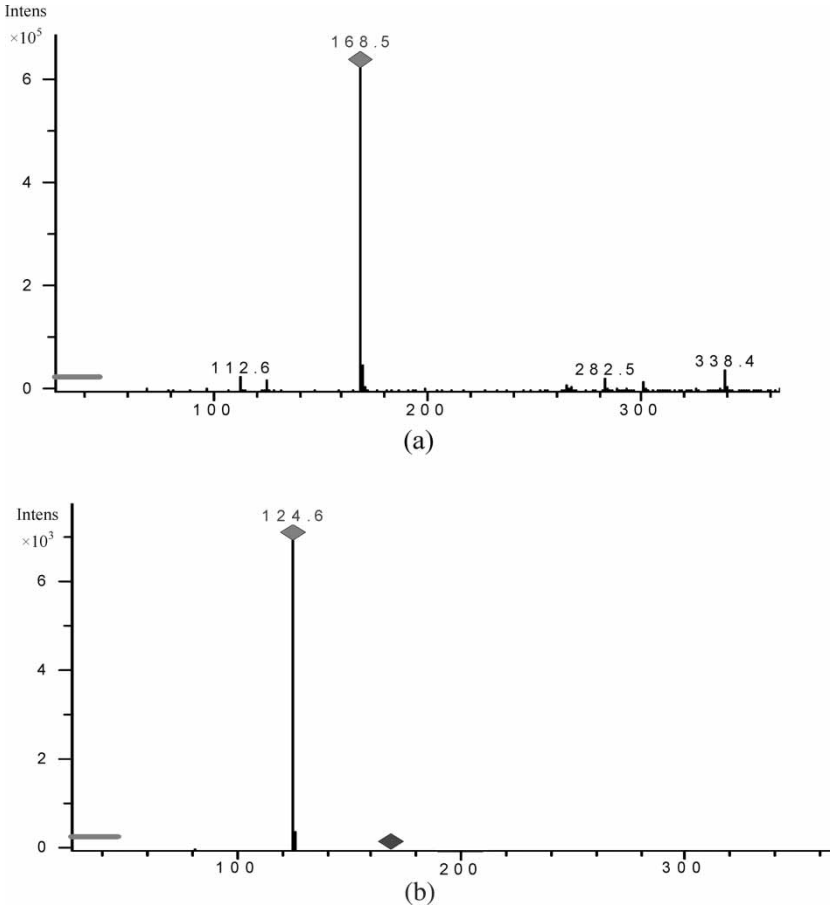

Figure 2. The parent- and product-ion scans of GA. a) The parent-ion scan spectrum of GA; b) The product-ion scan spectrum of GA. All the spectras were obtained with respect to maximum signal intensity, by consecutive infusing standard solutions of GA ($100.0\ ng \cdot mL^{-1}$), using a model 22 syringe pump.

Pharmacokinetics Study

After oral administration with the dose volume of 15.0 mg/kg weight to the rabbits, the plasma concentration of GA was determined by the described LC/MS/MS method. Figure 4 shows the plasma concentration time curve of GA following ingestion of *Choerospondiatis fructus* ($n = 12$). The statistical results by DAS version 2.0 (Drug and statistics software supported by Shanghai University of T.C.M) indicated that the plasma drug concentration time course in rabbit was confirmed to be a 2-compartment open model. The $t1/2\alpha$ was 0.073 h, C_{max} was $0.1083\ mg \cdot L^{-1}$, and t_{max} was 1.0 h.

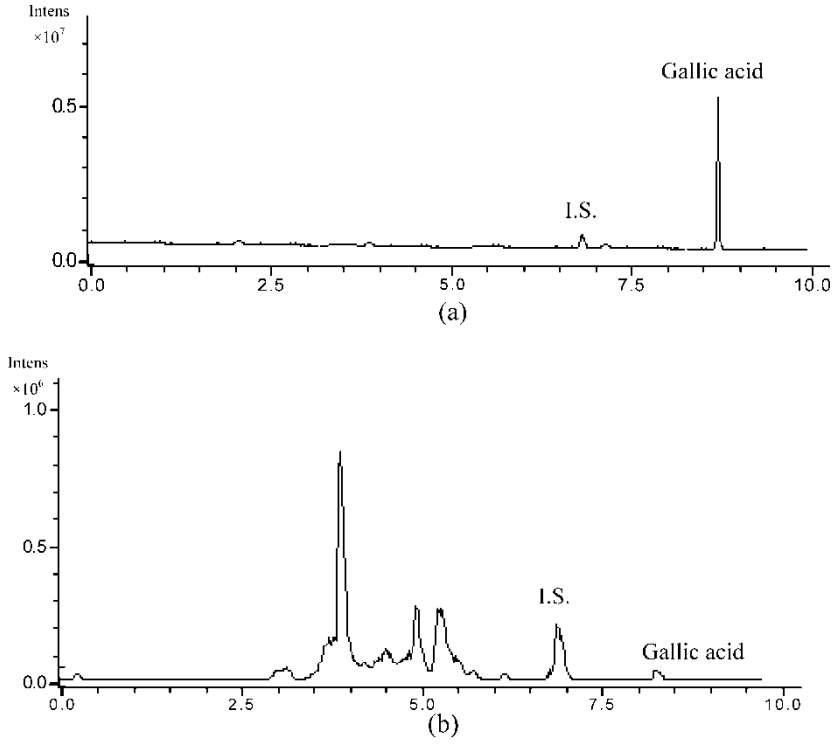

Figure 3. The total ion current chromatograms of GA standard solutions and plasma samples after oral admination. a) The total ion current chromatogram of GA satandard solution at the concentration of 50.0 ng · mL^{-1}; b) The total ion current chromatogram of the plasma sample collected at 0.67 h after ingestion of 15.0 mg/kg *Choerospondiatis fructus.* The concentration of I.S. is 0.096 ng · mL^{-1}.

Table 1. Intra-day and inter-day accuracy and precision for GA in rabbit plasma

	Added concentration (ng · mL^{-1})	Mean assayed concentration (ng · mL^{-1})	SD	Precision (CV %)	Accuracy (%)
Inter-day	4.0	4.1	0.1	2.4	102.5
	80.0	83.0	1.3	1.6	103.8
	400.0	396.7	8.4	2.1	99.2
Intra-day	4.0	3.8	0.3	7.8	95.0
	80.0	82.6	1.4	1.7	103.2
	400.0	407.6	13.2	3.2	101.9

Each individual value was the mean of triplicate determinations. The study was conducted over 4 days. Parameters were calculated as described in Section 2. Accuracy was Expressed as [(mean observed concentrations)/(nominal concentration) × 100].

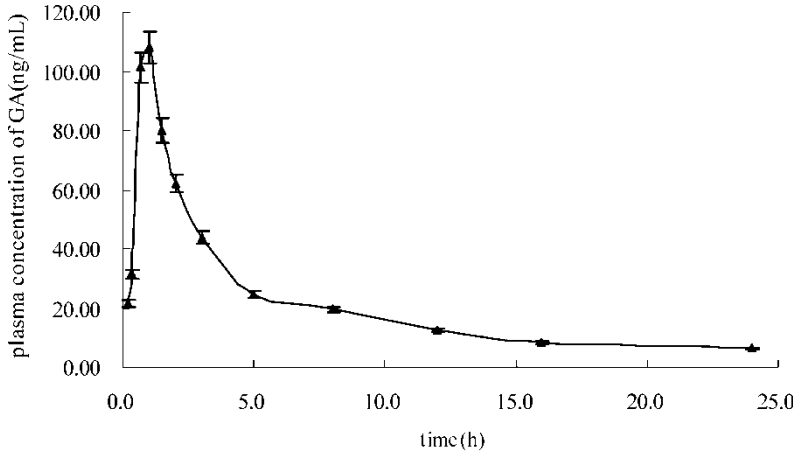

Figure 4. The plasma drug curve of GA following ingestion of 15.0 mg/kg *Choerospondiatis fructus.*

CONCLUSION

The method described in this work represents a highly sensitive and specific assay for the determination of GA in rabbit plasma samples. The assay demonstrated a LOD of 200 $pg \cdot mL^{-1}$ and intra- and inter-day coefficients of variation were 8.0% or less. The assay was successfully used to study the pharmacokinetics in rabbit plasma following the oral administration of 15.0 mg *Choerospondiatis fructus* per kilogram rabbit body weight, and suggested that this method could be used in the determination and pharmacokinetics study of GA in humans.

ACKNOWLEDGMENTS

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