

# Determination of phloroglucinol by HPLC-MS/MS and its application to a bioequivalence study in healthy volunteers

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**Abstract.** – **OBJECTIVE:** The aim of this study was to compare the pharmacokinetic characteristics of phloroglucinol between an orally disintegrating tablet and an orally lyophilized tablet of phloroglucinol in healthy volunteers under fasting condition.

**PATIENTS AND METHODS:** A rapid and simple method based on high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) method has been developed and validated for the determination of phloroglucinol in human plasma. The plasma sample was prepared by liquid-liquid extraction, and paracetamol was chosen as the internal standard. Phloroglucinol and IS were separated on a C<sub>18</sub> column with a mobile phase consisted of methanol/water (80:20 v/v) with 0.02% formic acid. HPLC-MS/MS analyses were performed on a triple- quadruple tandem mass spectrometer by monitoring protonated parent→daughter ion pairs at m/z 125.0→56.9 for phloroglucinol, and m/z 150.2→107.0 for paracetamol (IS). The method was the high sensitivity with a lower limit of quantification (LLOQ) of 1.976 ng/mL.

**RESULTS:** Drug and IS were detected by HPLC/MS/MS with negative electrospray ionization (ESI). Accuracy and precision for the assay were determined by calculating the intra- and inter-batch variation of quality control (QC) samples at three concentration levels. The relative standard deviation (RSD) was less than 15.0%. The detection and quantitation of drug and IS within 4.5 min make this method suitable for high-throughput analyses. In this study, the C<sub>max</sub> of phloroglucinol were calculated to 515.6 ± 134.4 ng/mL and 536.0 ± 144.8 ng/mL for the test drug and the reference drug, respectively. The AUC<sub>0-t</sub> values were 459.5 ± 81.03 ng·mL<sup>-1</sup>·h and 491.8 ± 95.17 ng·mL<sup>-1</sup>·h for the test drug and the reference drug; 24 subjects completed the study, respectively. The geometric mean ratio (GMR) and the 90% confidence intervals (CIs) of C<sub>max</sub> and AUC<sub>0-t</sub> of phloroglucinol were 97.1 (90.2-103.9) and 93.8 (88.7-99.2), respectively.

**CONCLUSIONS:** The method was employed for the first time during pharmacokinetic studies of phloroglucinol in human plasma following a single dose of phloroglucinol 160 mg tablets. There was no significant difference in pharmacokinetic profiles between the two treatments.

Key Words

Phloroglucinol, HPLC-MS/MS, Bioequivalence, Chinese volunteers.

## List of abbreviations

HPLC-MS/MS = liquid chromatography-tandem mass spectrometry; IS = internal standard; LLOQ = Lower limit of quantification; ESI = Electrospray Ionization; GMR = Geometric mean ratio; CIs = confidence intervals; AUC = area under the concentration time curve; AUC<sub>0-t</sub> = AUC from 0 h to time t; AUC<sub>0-∞</sub> = AUC from time 0 extrapolated to infinity; C<sub>max</sub> = maximum plasma concentration; QC = Quality-control; RSD = relative standard deviation; DEV = Deviation; ROS = reactive oxygen species; DNA = deoxyribonucleic acid; GC = Gas chromatograph; GC-MS = Gas chromatograph- tandem mass spectrometry; CFDA = China Food and Drug Administration; AE = After-effect.

## Introduction

Phloroglucinol (1,3,5-trihydroxybenzene; 1,3,5-benzenetriol) is a bioactive compound that has been used as a smooth muscle relaxant. Phloroglucinol significantly decreased the level of radiation-induced intracellular ROS and damage to cellular components such as the lipid, DNA and protein. Phloroglucinol is a phenolic compound

which chemical structure includes an aromatic phenyl ring, consisting of a hydroxyl group. Similar to other phenolic compounds, phloroglucinol shows a variety of biological activity and it is widely used in medicine, cosmetics, pesticides, paints, cements and dyeing, implicating its safety as a drug<sup>1</sup>. Previous studies for the qualitative and quantitative detection of phloroglucinol in biological fluid, such as high-performance liquid<sup>2</sup>, gas chromatography (GC)<sup>3</sup>, gas chromatography with mass spectrometry antispasmodic agents, and detection (GC-MS)<sup>4</sup>, have been reported. These analyze methods are often involved complex sample preparation and long runtime. The lower limit of quantitation (LLOQ) of these methods was 5 ng/mL for phloroglucinol, which could not properly evaluate the elimination phase in human pharmacokinetic study. As a common analytical tool for various compounds, HPLC-MS/MS possesses several advantages over other methods in terms of speed, sensitivity, selectivity, etc. The current study described a rapid, sensitive and practical HPLC-MS/MS method for the determination of phloroglucinol in human plasma. To the best of our knowledge, the use of HPLC-MS/MS has not been demonstrated for determination bioequivalence studies of phloroglucinol. This work established a novel quantification method for detecting phloroglucinol in human plasma using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). The aim of our study was to assess the bioequivalence of 2 formulations of phloroglucinol in healthy volunteers. The pharmacokinetic study of oral phloroglucinol was conducted in 24 healthy volunteers. The pharmacokinetic profiles of orally administered phloroglucinol were obtained for the first time; the development and validation of the analytical methods used in this study have not been previously reported. The bioequivalence study describes a rapid and selective HPLC-MS/MS method using a Diamonsil C<sub>18(2)</sub> analytical column (4.6 mm×150 mm, 5 μm, Dikma Technologies Inc., Lake Forest, CA, USA), which enables the determination of phloroglucinol with good accuracy at low drug concentrations in plasma. The plasma samples were prepared by liquid-liquid extraction with ethylacetate, evaporation and reconstitution. We have demonstrated the applicability of this method for bioequivalence study in Chinese volunteers. The fully validated method was successfully applied to a pharmacokinetic study of phloroglucinol in human plasma.

## Patients and Methods

### Study Design

The study was approved by the Independent Ethics Committee of Xijing Hospital, (Xian, Shaanxi, China) and was conducted in accordance with the Declaration of Helsinki concerning medical research in human beings and the principles of the International Conference on Harmonization Guideline for Good Clinical Practice. Written informed consent was obtained from each subject before screening procedures. The clinical trial was conducted at the Xijing Hospital clinical trial center (Xian, Shaanxi, China) from December 2013 to August 2014 (Government identifier of clinical trial: 2011L01299). This was an open-label, randomized, single-dose, two-way crossover study in healthy male subjects.

### Chemicals and Reagents

The tests of phloroglucinol orally disintegrating tablets (80 mg/tablet, LOT: 111101) were provided by the Wuhan Zhonglian Pharmaceutical, Ltd. (Wuhan, China). The reference of phloroglucinol orally Lyophilized tablets (80 mg/tablet, LOT: N9280) were provided by French Cephalon Pharmaceutical, Co., Ltd., (La Defense, France). Standard phloroglucinol (the structures were showed in Figure 1, purity > 99.9%) and internal standard paracetamol (purity > 99.0%) was provided by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol and acetonitrile were of HPLC-grade and purchased from Thermo-Fisher Scientific (Waltham, MA, USA). The water was distilled and purified using a Milli-Q Water Purification System (Bedford, MA, USA). Other chemicals were all of HPLC-grade. Blank human plasma was obtained from the Blood Center of Xijing Hospital and was stored at -80°C<sup>5-7</sup>.

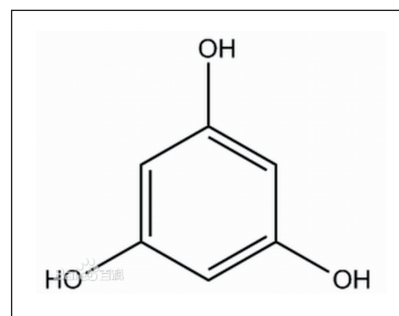


Figure 1. Chemical structure of phloroglucinol.

### Instruments and Analytical Conditions

The instrumentation employed for HPLC-MS/MS analysis was an AB-Sciex API4000 triple quadrupole mass spectrometer (Foster City, CA, USA) interfaced with a Nexera HPLC-30A (Shimadzu, Shimane, Kyoto, Japan). The components of the HPLC system were a binary solvent manager and a sample manager. Chromatographic separation was achieved using A Diamonsil C<sub>18(2)</sub> analytical column (4.6 mm × 150 mm, 5 μm, Dikma Technologies Inc., Lake Forest, CA, USA). The best separation was achieved using a mobile phase consisted of 80:20 (v/v), methanol (MeOH) (mobile phase A) and 0.02% formic acid in water (mobile phase B), at a total flow rate of 0.5 mL/min. The column temperature was kept at 35°C and injection volume was 5 μL. The HPLC system was coupled with an API 4000 tandem mass spectrometer (Concord, Ontario, Canada), equipped with an Electrospray Ionization (ESI) source. The mass axis of the mass spectrometer was properly calibrated, all data were collected and analyzed by Analyst software (Applied Biosystems/MDS SCIEX 1.6, Foster City, CA, USA). The quantification determination was performed using the multiple reaction monitoring (MRM) method with the transitions of *m/z* 125.0 → 56.9 for Phloroglucinol, *m/z* 150.2 → 107.0 for IS. The electrospray ionization voltage in negative mode was set 5500 and 4500 V, respectively. The turbo spray temperature was maintained at 550°C. Nebulizer gas (gas 1) and heater gas (gas 2) were set as 45 and 50, respectively. The curtain gas was kept at 10 and the interface heater was on. The compound dependent parameters for phloroglucinol and other compounds in MRM mode were summarized in Table I.

### Preparation of Standard Solutions

Standard 988.0 μg·mL<sup>-1</sup> stock solutions of phloroglucinol and the IS were prepared separately in methanol. Standard working solutions of phloroglucinol at concentrations of 1.976, 4.940,

9.880, 24.70, 98.80, 247.0, 494.0 and 988.0 ng/mL, and a 75 ng/mL solution of IS, were prepared by serial dilution of stock solutions.

### Preparation of Calibration and Quality Control Samples

Drug-free plasma was spiked with standard solutions to prepare calibration standards with final concentrations of 1.976, 4.940, 9.880, 24.70, 98.80, 247.0, 494.0 and 988.0 ng/mL of phloroglucinol. Quality-control (QC) samples containing phloroglucinol were prepared in a similar manner at four concentration levels: 1.976 ng/mL (lower limit of quantitation, LLOQ), 3.952 ng/mL (low, LQC), 49.40 ng/mL (middle, MQC), and 741.0 ng/mL (high, HQC). All standard stock solutions were kept at -80°C before analysis. Standards and quality controls were extracted daily before analysis using the procedure described below for plasma samples.

### Sample Preparation

The plasma sample (0.5 mL) was mixed with 4 mL ethyl acetate after addition of 20 μL IS solution (75 ng/mL) and 50 μL 1% hydrochloric acid. The mixture was vortex-mixed for 3 min and centrifuged for 10 min at 4000 rpm. The upper organic phase was transferred and evaporated to dryness under a gentle stream of nitrogen in a water bath of 45°C. The residue was reconstituted in 200 μL of the mobile phase by vortex-mix for 1 min. After being centrifuged for 10 min at 16,000 rpm, the sample was transferred to the glass auto sampler vial insert and 5 μL aliquot was injected into the chromatographic system.

### Method validation Specificity

The specificity of the method was validated by comparing chromatograms of phloroglucinol and internal standard (IS), blank plasma, blank plasma spiked with phloroglucinol and IS, and plasma from 6 healthy male volunteers after

**Table I.** Intra-day and inter-day precision and accuracy for analysis of phloroglucinol in plasma.

Nominal Conc.	Intra-day (n=5)			Inter-day (n=15)		
	Mean ± SD (ng·mL <sup>-1</sup> )	RSD (%)	RE (%)	Mean ± SD (ng·mL <sup>-1</sup> )	RSD (%)	RE (%)
3.952	4.306±0.52	3.54	3.37	4.093±0.36	5.62	2.81
49.40	49.72±0.67	5.41	-1.05	54.90±0.58	11.4	1.45
741.0	706.1±2.03	0.68	2.06	732.1±1.69	4.10	3.02

$$\text{DEV} (\%) = \left[ \frac{\text{Nominal concentration} - \text{Mean observed concentration}}{\text{Nominal concentration}} \right] \times 100$$

**Figure 2.** Computational formula 1: computational formula of DEV (%).

oral 160 mg phloroglucinol orally disintegrating tablets. Typical chromatograms were shown in Figure 4. Retention times of phloroglucinol and IS were 2.78 min and 2.94 min, respectively; the overall run time was within 5 min. No significant interfering peak was observed around phloroglucinol and IS during analysis.

#### **Lower Limit of Quantification**

The lower limit of quantification (LLOQ) was defined as the lowest concentration of the calibration curve, at which both precision and bias were less than 20%. The current assay offered an LLOQ of 1.976 ng/mL in plasma of phloroglucinol, which was sufficient for the study of pharmacokinetics following a single oral administration of phloroglucinol.

#### **Extraction Recovery**

The extraction recoveries of phloroglucinol were determined at 3 QC levels by comparing the peak areas of extracted plasma samples with those of extracted blank plasma spiked with corresponding concentrations. The extraction recovery of IS was also evaluated in the same way. The results showed the mean extraction recoveries of phloroglucinol at QC concentrations (3.952, 49.40 and 741.0 ng/mL) and IS were  $86.19 \pm 2.01\%$ ,  $90.24 \pm 2.05\%$ ,  $91.10 \pm 1.47\%$ , and  $90.41 \pm 1.02\%$ .

#### **Linearity of Calibration Curve**

The calibration curves of phloroglucinol were produced by daily analysis of the spiked calibration samples at 8 concentrations in the range of 1.976-988.0 ng/mL. The linearity of calibration curves was assessed by linear regression with a weighting factor of the reciprocal of the concentration squared ( $1/x^2$ ). Therefore, the calibration curves were expressed by the equation of  $y = 0.131x - 0.00521$  (y represents the plasma concentration of phloroglucinol and x represents the

ratio of phloroglucinol peak area to that of the IS) ( $R^2 = 0.9998$ ), in which y represents the peak-area ratio of phloroglucinol to IS, and x represents the concentration ratio of phloroglucinol.

#### **Precision and Accuracy**

Intra-day accuracy and precision were determined by replicate analyses (n=5) of each of the three QC samples (3.952, 49.40 and 741.0 ng/mL) performed on the same day. Inter-day accuracy and precision were determined by replicate analyses (n=5) of the same QC concentrations on three different days. Accuracy was calculated as the percent deviation (% DEV) of the mean observed concentration from the nominal concentration according to the below expression (Figure 2). The precision and accuracy of each analyte in the intra- and inter-day runs were within  $\pm 15\%$  at 3.952, 49.40 and 741.0 ng/mL within  $\pm 20\%$  at LLOQ (Figure 3).

#### **Stability**

The stabilities of phloroglucinol in plasma were evaluated as follows. The stabilities of phloroglucinol in plasma samples at QC levels were examined under different study conditions and stored at  $-80^\circ\text{C}$  for 50 days. Freeze/thaw stabilities were determined after freezing ( $-80^\circ\text{C}$ ) and thawing ( $25^\circ\text{C}$ ) QC samples for 3 cycles. Stability of post-extracted samples in the HPLC-MS/MS auto-sampler at room temperature for 24 h was also observed. There was no significant degradation during preparation and storage processes than contrast samples.

#### **Application to a Bioequivalence Study**

For the bioequivalence test, the geometric mean ratios of  $C_{\max}$  and  $AUC_{0-t}$  of phloroglucinol were calculated by WinNonlin software (version 6.2; Pharsight Corporation, Mountain View, CA, USA). If the 90% confidence intervals (CIs) of the geometric mean ratio of primary parameters were within the range 0.8 to 1.25, the new formulation was considered to meet the criteria for bioequivalence. A mixed-effects analysis of variance (ANOVA) model was performed on the log-transformed  $C_{\max}$  and AUC with random effects of sequence-nested subject, and fixed effects of sequence, period, and treatment. The pharmacokinetic parameters of

$$\text{RSD} (\%) = \left[ \frac{\text{SD}}{\text{M}} \right] \times 100.$$

**Figure 3.** Computational formula 2: computational formula of RSD (%).

phloroglucinol were estimated using a no compartmental method with WinNonlin software (version 6.2; Pharsight Corporation, Mountain View, CA, USA). The maximum concentration ( $C_{\max}$ ) and area under the concentration-time curve from zero (pre-dose) to time of last quantifiable concentration ( $AUC_{0-t}$ ) were the primary parameters, and time to reach  $C_{\max}$  ( $t_{\max}$ ), half-life ( $t_{1/2}$ ), and  $AUC_{0-\infty}$  were estimated as secondary parameters.  $C_{\max}$  and  $t_{\max}$  were obtained from the observed plasma concentration-time profile.  $AUC_{0-t}$  was calculated according to the linear trapezoidal rule.  $AUC_{0-\infty}$  was calculated with the following equation:  $AUC_{0-\infty} = AUC_{0-t} + Ct/\lambda_z$ , where  $Ct$  is the last observed concentration and  $\lambda_z$  is the elimination rate constant. The  $t_{1/2}$  was calculated as  $0.693/\lambda_z$ . Subjects who completed the study were included in the pharmacokinetic analysis.

### Statistical Analysis

Pharmacokinetic parameters were calculated using the no compartmental analysis method with WinNonlin version 6.2 (Pharsight Corporation, Mountain View, CA, USA).  $C_{\max}$  and  $t_{\max}$  were obtained directly from the concentration-time data of phloroglucinol.  $AUC_{0-t}$  was calculated according to the linear trapezoidal rule.  $AUC_{0-t}$  was calculated as the sum of  $AUC_{0-t}$  and  $Ct/\lambda$ .  $Ct$  was the last measured concentration and  $\lambda$  was the slope of linear regression of the log-transformed concentration-time curve. The value of  $t_{1/2}$  was calculated as the ratio of  $0.693/\lambda$ . Independent-samples  $t$ -tests or nonparametric tests were used to determine statistically significant differences between the pharmacokinetic parameters. A linear-regression model was used to determine the dose proportionality. The dose proportionality of phloroglucinol was estimated by fitting an appropriate model. The model assumed a linear relationship between natural log transformed pharmacokinetic parameter ( $AUC_{0-t}$ ,  $AUC_{0-\infty}$ , and  $C_{\max}$ ) and natural log transformed dose. The proportionality constant and its corresponding 90% confidence intervals (CIs) were compared with the modified acceptance range. For all the analyses,  $p < 0.05$  was considered statistically significant.

## Results

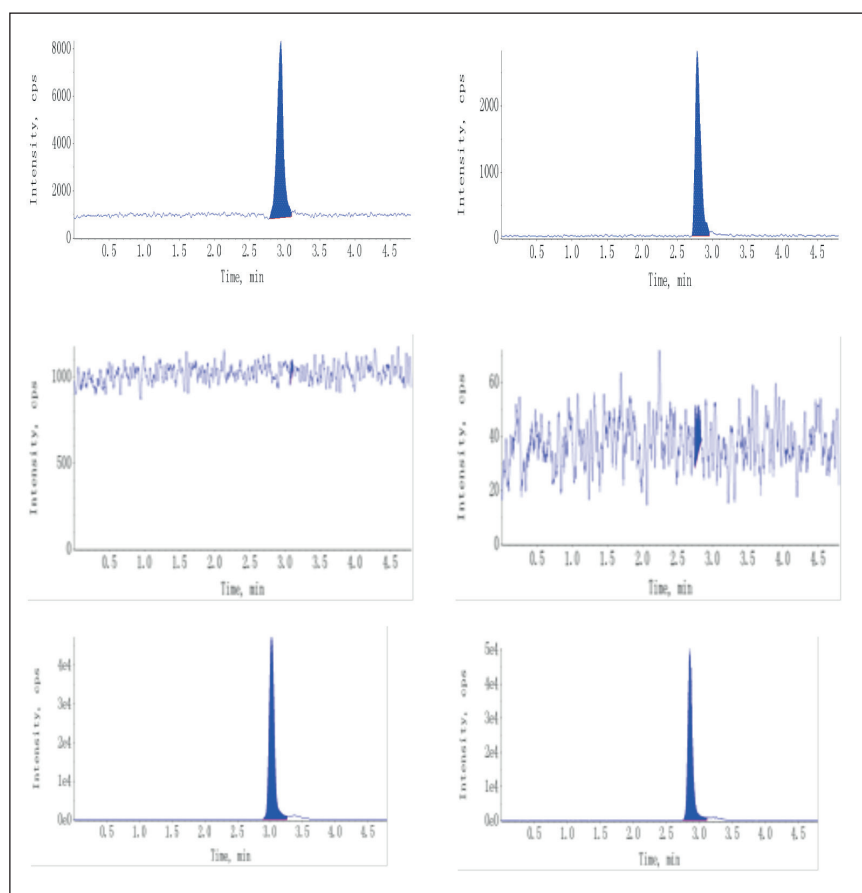
### Method Validation

Typical MRM chromatograms obtained from the drug-free plasma, plasma spiked with standard phloroglucinol (4.940 ng/mL) and IS (75 ng/

mL), and plasma samples obtained 1 h after oral administration of phloroglucinol and spiked with IS (75 ng/mL) were shown in Figure 4. Retention times of phloroglucinol and the IS were 2.8 and 2.9 min, respectively. No interference from endogenous peaks was observed at these retention times. The lower limit of quantitation was 1.976 ng/mL with a precision (% RSD) and accuracy (% DEV) of 11.2% and -3.0%, respectively. The linear regression of the ratio of the areas of the analyte and internal standard peaks vs. the concentration was weighted by  $1/x^2$ . Calibration curves for phloroglucinol in plasma were linear from 1.976 to 988.0 ng/mL, as shown by the mean correlation coefficient ( $r^2$ ) of 0.9998 ( $n=5$ ) obtained for intra-day analyses. The line equation for this calibration curve was  $y = 0.131x - 0.00521$ , where  $y$  is the peak area ratio of phloroglucinol to the IS and  $x$  is the concentration of phloroglucinol in 1.976-988.0 ng/mL. Intra-day and inter-day accuracy and precision were summarized in Table II. The intra-day and inter-day precision values (% RSD) for the various concentrations ranged from 3.22% to 11.0% and 3.74% to 10.2%, respectively. Intra-day and inter-day accuracy values ranged from -3.2% to 2.2% and from -5.0% to 1.5%, respectively. Both accuracy and precision were found to be acceptable for bio-analytical applications. The stabilities of phloroglucinol in plasma were evaluated as follows. The stabilities of phloroglucinol in plasma samples at QC levels were examined under different study conditions. Blood samples (5 ml) were collected at 5 min, 10 min, 15 min, 20 min, 30 min, 45 min, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0 h post-dose. Plasma was separated, decanted, frozen and stored at  $-80^\circ\text{C}$  for 50 days before analysis. Freeze/thaw stabilities were determined after freezing ( $-80^\circ\text{C}$ ) and thawing ( $25^\circ\text{C}$ ) QC samples for 3 cycles. Stability of post-extracted samples in the HPLC auto-sampler at room temperature for 24 h was also observed. There was no significant degradation during preparation and storage processes than contrast samples.

### Sample Extraction

In the present study, we optimized simple liquid-liquid extraction procedure, which is fast enough for high-throughput analysis. The mean percent recovery ( $n=3$ ) of phloroglucinol from plasma at QC concentrations (3.952, 49.40 and 741.0 ng/mL) ranged from 86.19%, 90.24, to 91.10% and the mean percent recovery of the IS at a concentration 75 ng/mL was 90.41% with an acceptable precision (% RSD < 15.0).



**Figure 4.** Typical chromatograms of phloroglucinol in plasma. (A) Human plasma spiked with phloroglucinol and I.S (B) blank human plasma (C) the plasma sample from a volunteer at the time points of 20 min following administration.

### Application to a Clinical Bioequivalence Study

The validated HPLC-MS/MS method was successfully applied to a bioequivalence study. Twenty-four male volunteers' pharmacokinetics data were obtained after 160 mg oral dose of phloroglucinol were administered. Phloroglucinol plasma concentrations appeared to be comparable between test and reference preparations (Figure 5). There were no significant differences between preparations and periods in all of the pharmacokinetic parameters (Table II). The 90% confidence interval (90% CI) for the test/reference ratio of  $C_{\max}$ ,  $AUC_{0-8}$  and  $AUC_{0-\infty}$  were shown

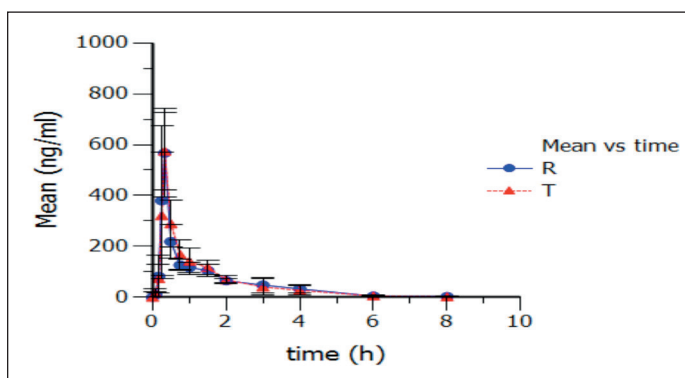
in (Table III). All 90% CIs were within the bioequivalent interval of 80.0-125.0%.

### Discussion

There was no report about the pharmacokinetic study of phloroglucinol in Chinese subjects, so it was important to characterize the pharmacokinetic parameters of phloroglucinol to study that the phloroglucinol performs in healthy Chinese volunteers. The 90% CIs for the geometric mean ratio for  $C_{\max}$  and  $AUC_{0-t}$  of phloroglucinol fell entirely within the regulatory criteria for bio-

**Table II.** The main pharmacokinetic parameters of phloroglucinol in Chinese volunteer (means  $\pm$  SD, n=24).

Parameters	Test	Reference	p-value
$t_{1/2}$ /h	1.384 $\pm$ 0.8217	1.249 $\pm$ 0.5595	0.835
$C_{\max}$ /ng·mL <sup>-1</sup>	515.6 $\pm$ 134.4	536.0 $\pm$ 144.8	0.796
$t_{\max}$ /h	0.2600 $\pm$ 0.02703	0.2667 $\pm$ 0.03319	0.855
$AUC_{(0-8)}$ /ng·mL <sup>-1</sup> ·h	459.5 $\pm$ 81.03	491.8 $\pm$ 95.17	0.874
$AUC_{(0-\infty)}$ /ng·mL <sup>-1</sup> ·h	466.6 $\pm$ 80.57	497.7 $\pm$ 96.00	0.764



**Figure 5.** The average concentration-time curves of test and reference products of phloroglucinol.

quivalence. The acceptable range is 80.0-125.0% according to the current Food and Drug Administration guidelines of CFDA.

We enrolled 24 subjects into the study, with the mean age (SD) of 27.5 (3.6%) years (range = 22-32 years), weight of 62.5 (6.0%) kg (range = 54.0-72.0 kg), and height of 171.8 (5.4%) cm (range=169.0-189.0 cm). All of the subjects completed the study.

The results demonstrated that phloroglucinol was bioequivalent when compared with the test drug and the reference drug. In the present work, the  $C_{max}$  of phloroglucinol were calculated to  $515.6 \pm 134.4$  ng/mL and  $536.0 \pm 144.8$  ng/mL for the test drug and the reference drug, respectively. The  $AUC_{0-t}$  values were  $459.5 \pm 81.03$  ng·mL<sup>-1</sup>·h and  $491.8 \pm 95.17$  ng·mL<sup>-1</sup>·h for the test drug and the reference drug, respectively. 24 subjects completed the study, respectively. The geometric mean ratio (GMR) and the 90% confidence intervals (CIs) of  $C_{max}$  and  $AUC_{0-t}$  of phloroglucinol were 97.1 (90.2-103.9) and 93.8 (88.7-99.2), respectively. These comparable results suggest that bioanalytical and pharmacokinetic analysis had been appropriately performed in this study<sup>8-16</sup>. A single 160 mg dose of phloroglucinol was well tolerated and safe in both test and reference formulations. The study was completed without clinically relevant AEs attributable to the drug. Physical examination, electro-cardiograms, and laboratory tests did not suggest any clinically significant abnormalities. MS/MS parameters were optimized to gain the

maximum response for Phloroglucinol and IS, simultaneous. Both the positive and negative ion modes were investigated. The response of negative ion was much more sensitive and selective than positive ion for phloroglucinol and IS. The response of phloroglucinol was improved by optimizing electronic parameters, such as ion spray voltage, capillary temperature, sheath gas, auxiliary gas and the collision energy. Phloroglucinol and IS were detected separately in negative ion mode, using a single quadrupole mass spectrometer. Using a triple quadrupole mass spectrometer of a higher selectivity, the analyte and IS could be determined simultaneously. The chromatographic conditions, especially the composition of mobile phase, were optimized to achieve a good resolution and symmetric peak shapes for the analyte and the IS, as well as a short analytical time. It was found that a mobile phase consisted of methanol/water (80:20 v/v) with 0.02% formic acid could achieve this purpose. After careful comparison of several columns, A Diamonsil C<sub>18(2)</sub> analytical column (4.6 mm×150 mm, 5 μm, Dikma Technologies Inc., Lake Forest, CA, USA) gave the best chromatogram at a flow rate of 0.5 mL/min. Using these chromatographic conditions, the analytical time was 4.5 min. Our method seemed much more convenient. In the present study, we optimized simple liquid-liquid extraction procedure, which is fast enough for high-throughput analysis. These results suggest that developed sample preparation procedure is acceptable for extraction of phloroglucinol and

**Table III.** Bioequivalence analysis of the test and reference formulations of phloroglucinol.

Parameters	F%	90% CI	p-value
$C_{max}$ /ng·mL <sup>-1</sup>	93.8	90.2%-103.9%	0.732
$AUC_{(0-8)}$ /ng·mL <sup>-1</sup> ·h	95.6	88.7%-99.2%	0.645
$AUC_{(0-24)}$ /ng·mL <sup>-1</sup> ·h	97.5	89.0%-99.6%	0.768

IS from plasma samples. In this study, the peak concentration and AUC of phloroglucinol were proportional to the dose after single dosing with 160 mg phloroglucinol in healthy Chinese volunteers, suggesting linear plasma pharmacokinetic properties. Our study revealed that phloroglucinol is rapidly absorbed after oral administration, reaching a peak concentration within approximately 0.26 h. The elimination half-life of phloroglucinol after single dosing in healthy Chinese volunteers was approximately 1.3 h (Table II). The pharmacokinetic findings from our study were in agreement with those from previously published studies in the literature<sup>2</sup>.

### Conclusions

A simple, rapid and accurate HPLC-MS/MS method was developed to quantify phloroglucinol in human plasma. Validation results have shown that the method is robust and meets the requirements of the bioequivalence study after the orally disintegrating tablet administration of therapeutic dose. To the best of our knowledge, this is the first HPLC-MS/MS method concerning the quantitation of phloroglucinol in human plasma. The assay showed a short analytical time (5 min per sample), a high sensitivity (an LLOQ of 1.976 ng/mL), sufficiently sensitive for this bioequivalence study, and the sample preparation procedure involved a one-step liquid-liquid extraction. The developed method was successfully applied to determine phloroglucinol in human plasma, and was proved to be suitable for use in phase I clinical bioequivalence study after administration of phloroglucinol (160 mg) in healthy Chinese volunteers. Based on the regulatory requirements and criteria for bioequivalence, the 2 formulations were considered bioequivalent.

### Ethical Approval

The study was approved by the Independent Ethics Committee of Xijing Hospital, and was conducted in accordance with the Declaration of Helsinki concerning medical research in human beings and the principles of the International Conference on Harmonization Guideline for Good Clinical Practice. Written informed consent was obtained from each subject before screening procedures.

### Conflict of interest

X.-Q. Li and R.-T. Wang are working for Xi'an Libang Zhaoxin Biological Technology Co., Ltd., Xi'an, China. All authors declare that they have no conflict of interests related to this study.

### Acknowledgments

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