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Determination of phloroglucinol in human plasma by high-performance liquid chromatography–mass spectrometry

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Abstract

A sensitive and selective liquid chromatographic method coupled with mass spectrometry (LC–MS) was developed for the quantification of phloroglucinol in human plasma. Resorcinol was used as internal standard, with plasma samples extracted using ethyl acetate. A centrifuged upper layer was then evaporated and reconstituted with mobile phase. The reconstituted samples were injected into a C_{18} XTerra MS column (2.1×100 mm) with 3.5-μm particle size. The analytical column lasted for at least 500 injections. The mobile phase was 15% acetonitrile (pH 3.0), with flow-rate at 200 μl/min. The mass spectrometer was operated in negative ion mode with selective ion monitoring (SIM). Phloroglucinol was detected without severe interferences from plasma matrix when used negative ion mode. Phloroglucinol produced a parent molecule ($[M–H]^-$) at m/z 125 in negative ion mode. Detection of phloroglucinol in human plasma was accurate and precise, with quantification limit at 5 ng/ml. This method has been successfully applied to a study of phloroglucinol in human specimens.

Keywords: Phloroglucinol

1. Introduction

Phloroglucinol (benzene-1,3,5-triol), is used as a smooth muscle relaxant. It has no anticholinergic potency and appears to be less toxic than most other antispasmodic agents.

Previous studies have reported two different methods for the qualitative and quantitative detection of phloroglucinol in plasma samples and pharmaceutical formulations, e.g., high-performance liquid chromatography with fluorimetric detection (HPLC-FLD) [1], colorimetry and gas chromatography (GC) [2] and gas chromatography with mass spectrometry detection (GC–MS) [3]. Pesez and Bartos [1] have shown that, although this compound does not fluoresce naturally, it can be converted into a fluorescent derivative by condensation with ethyl iodoacetate to form a coumarin derivative. Also, published GC–MS method has derivatization step using *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA). How-

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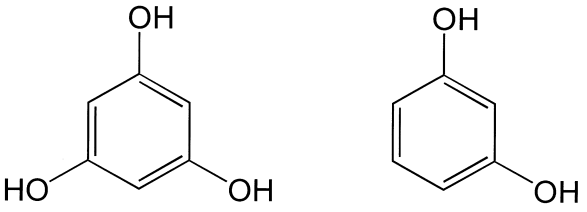
ever, these published methods are not ideal for pharmacokinetics work, because they are time-consuming, i.e., derivatization step, arduous sample preparation, and long chromatographic run times. Likewise, they need a relatively large amount of sample to reach a low quantification limit. In addition, detection of phloroglucinol using liquid chromatography–mass spectrometry (LC–MS) has yet to be reported.

Therefore, this study established a novel quantification method for detecting phloroglucinol in human plasma using liquid chromatography–negative electrospray ionization mass spectrometry (LC–ESI-MS) without derivatization step for pharmacokinetics study. This method is not only selective and reliable; it is also faster and simpler compared to other recently reported methods. Likewise, this method has been successfully applied to pharmacokinetic studies to determine the concentration of phloroglucinol in human plasma.



(A) Phloroglucinol (B) Resorcinol

Fig. 1. Structures of (A) phloroglucinol and (B) resorcinol (internal standard).

2. Experimental

2.1. Chemicals and solutions

All reagents and solvents were of analytical reagent grade. Phloroglucinol, resorcinol (internal standard), ascorbic acid, formic acid and phosphate buffer solution were purchased from Sigma–Aldrich (St. Louis, MO, USA), while acetonitrile, water and ethyl acetate were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Fig. 1 shows the structures of phloroglucinol and resorcinol.

To prepare standard stock solutions, 50 mg phloroglucinol and 50 mg resorcinol were weighed accurately and dissolved separately in 50 ml mobile phase. From these stock solutions, working standard solutions of phloroglucinol and other analytes were prepared through sequential dilutions with mobile phase to produce varying concentrations.

2.2. LC–MS–MS conditions

Tandem mass spectrometry (MS–MS) was performed on Quattro micro triple quadruple mass spectrometry equipped with electrospray ion source (Micromass, Manchester, UK). Twenty microliters of sample were delivered into the ESI source using the Waters HT system (high-performance liquid chromatography and autosampler, Model 2795, Waters, Milford, MA, USA) with C_{18} XTerra MS column (2.1×100 mm, 3.5-μm particle size). The mobile phase used after degassing was water–acetonitrile (85:15, pH 3.0 using 98% formic acid (pH 3.0 = water–acetonitrile–formic acid, 850:150:0.28 ml), with total running time of 10 min and flow-rate of 200 μl/min.

The following instrument settings were used: the electrospray interface was maintained at 300 °C with a nitrogen nebulization. The gas flow was 1100 l/h. For the negative ion detection with SIM scan mode, the following parameter settings were used: ion spray voltage at 3.48 kV, cone voltage at −29 V; extractor at −2 V; RF lens at −0.4 V; source temperature at 120 °C; collision cell entrance potential at 67 V; collision energy at 2 eV, collision cell exit potential at 67 V; and multiplier at −650 V. The peak widths of precursor ions were maintained at ~0.7 u half-height in the SIM mode.

2.3. Plasma samples collection and storage

Blood samples (5 ml) for the pharmacokinetic study were collected in heparin–lithium tubes, then immediately centrifuged for 10 min at 200 *g* and 10 °C. Plasma was separated in 1-ml fractions in the shade. An aqueous solution of ascorbic acid (0.4 ml at 0.2 g/l) was added to each fraction to prevent

oxidation, as reported for apomophine [4]. Fractions were then kept frozen at −75 °C until analysis.

2.4. Sample preparation

Plasma specimens (0.5 ml) were pipetted into conical glass tubes and spiked with 0.1 ml of 10 μg/ml internal standard solution. After adding 1.5 ml of 0.1 *M* phosphate buffer solution (pH 7.0), the plasma was vortex-mixed for 10 s and added to 6 ml ethyl acetate. The sample was then shaken for 10 min. The two phases were separated through centrifugation at 2000 *g* for 5 min. The upper organic layer (ethyl acetate layer) was transferred into another conical glass tube and completely evaporated at 40 °C under a stream of nitrogen. The dry residue was then reconstituted with 150 μl mobile phase and 20 μl injected into the LC–MS.

2.5. Validation procedures

Validation was performed based on ICH guidelines [5,6]. To assess the intra-day precision and accuracy of the method, five replicates of plasma standards at four concentrations (10, 50, 200, and 1000 ng/ml) were analyzed. Similarly, five replicates of the quality control samples at varying concentrations of 10, 50, 200, and 1000 ng/ml were analyzed to determine the initial inter-day precision and accuracy. The accuracy was expressed as (mean observed concentration)/(spiked concentration)×100%, with the precision expressed using the relative standard deviation (RSD).

2.6. Preliminary pharmacokinetic assay

A single 160-mg dose of phloroglucinol was administered orally to five volunteers who were advised about the nature and purpose of the study. The volunteers possessed good health and had not taken any medication for at least 2 weeks prior to the study. The group consisted of healthy males with a mean age of 24.7±2.8, mean weight of 67.6±6.0 kg, and mean height of 173.5±3.6 cm. Blood samples were taken 0, 0.17, 0.33, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, and 8 h after ingestion using heparin vacutainer collection tubes.

3. Results and discussion

Under electrospray ionization condition, phloroglucinol and resorcinol (internal standard) exhibited favorable sensitivity in negative ion detection mode. Fig. 2A shows the full scan Q1 negative ion spectra of phloroglucinol, while Fig. 2B shows the full scan Q1 negative ion spectra of internal standard. Both formed parent molecules $[M-H]^-$ as major ion peaks. Phloroglucinol produced a parent molecule ($[M-H]^-$) at m/z 125. On the other hand, resorcinol produced a parent molecule ($[M-H]^-$) at m/z 109.

For the chromatographic analysis and electrospray ionization of phloroglucinol, we initially attempted to develop a reversed-phase chromatographic system with methanol and water or acetonitrile and water. Such a system yielded an assay where phloroglucinol and internal standard were co-eluted. Moreover, formation of precipitates was observed in the mobile phase in cases where the amount of organic solvent was decreased. The amount of acetonitrile in mobile phase was then optimized at 15%. Likewise, the pH of the mobile phase was optimized at 3.0 using formic acid. However, the chromatogram of phloroglucinol showed high sensitivity and selectivity under these conditions. Fig. 3 shows the LC–MS chromatograms of phloroglucinol and internal standard in negative ion detection mode. Also, Fig. 4 shows LC–MS chromatogram of phloroglucinol in human blank plasma.

Fig. 3A shows the total ion chromatogram in human plasma by negative ion detection with selective ion monitoring, Fig. 3B shows the chromatogram of phloroglucinol and Fig. 3C shows the chromatogram of resorcinol (internal standard). In Fig. 3, using negative ion detection with SIM mode, phloroglucinol and resorcinol were detected without severe interferences from plasma matrix. Under these conditions, phloroglucinol and resorcinol exhibited favorable selectivity in negative ion detection mode. When we used positive ion detection mode, it was found that much higher ion intensities were achieved in the chromatographic conditions than negative ion detection mode. But positive ion detection mode has

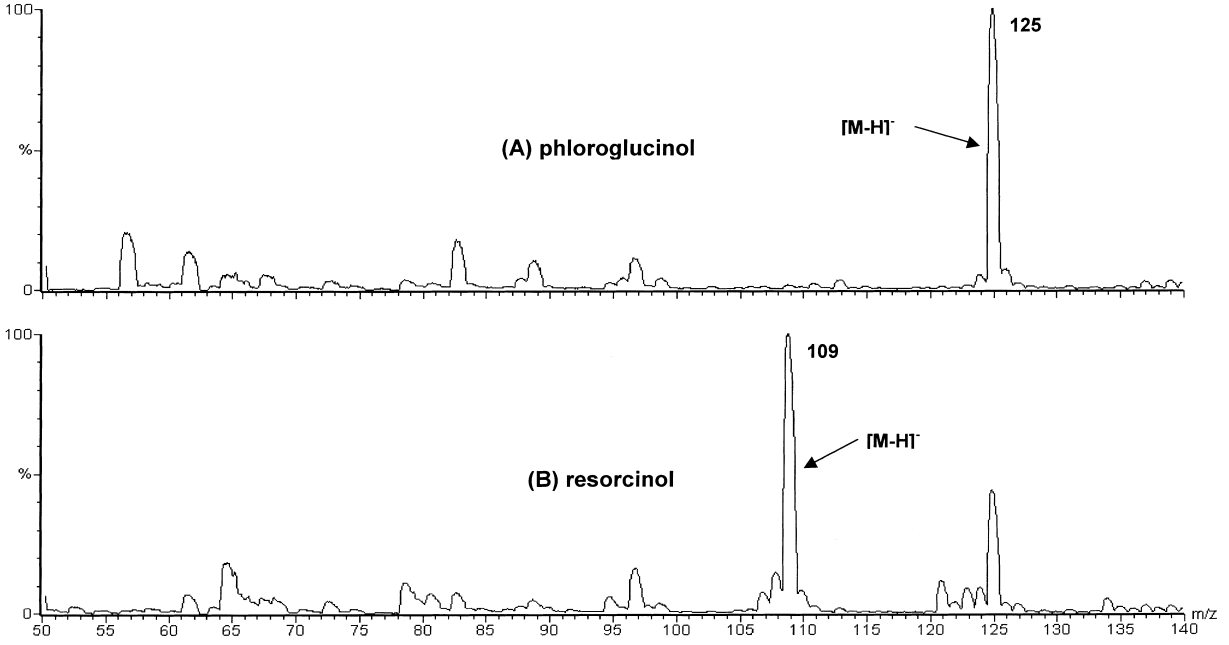


Fig. 2. Full scan Q1 negative spectra of (A) phloroglucinol and (B) resorcinol (internal standard).

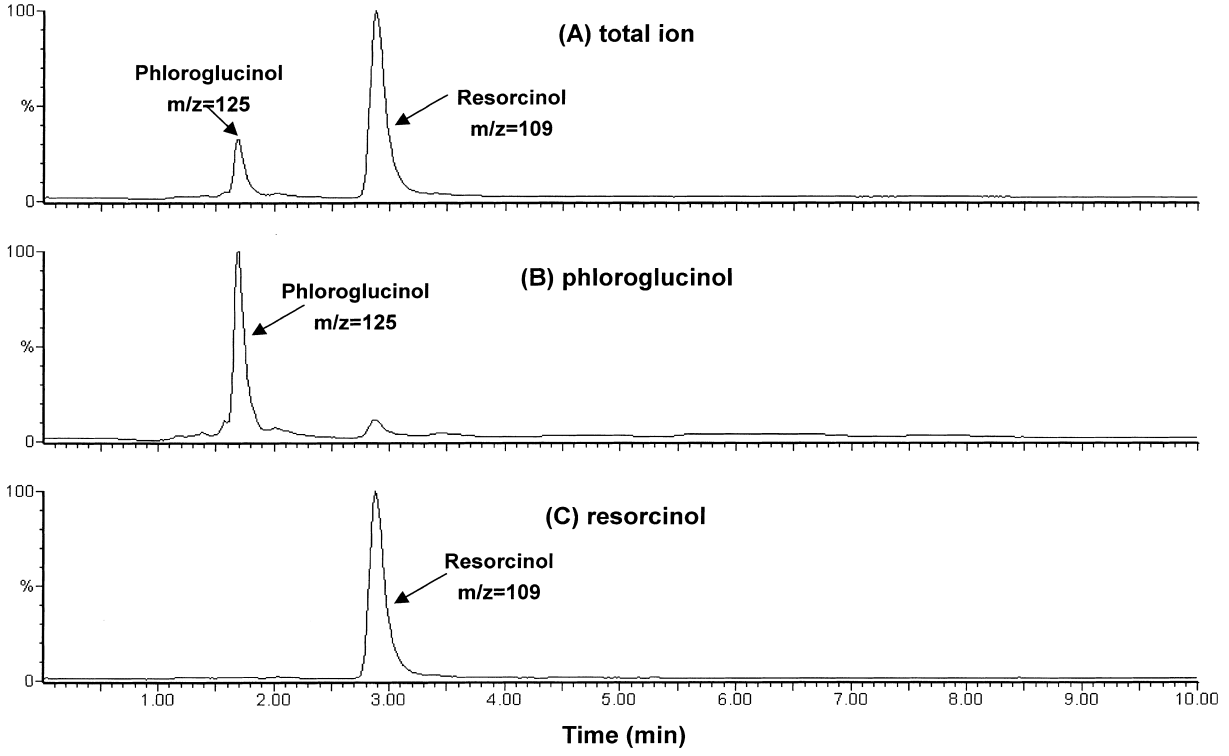


Fig. 3. LC–MS chromatograms of (A) total ion, (B) phloroglucinol and (C) resorcinol (internal standard) in human plasma by negative ion detection with selective ion monitoring.

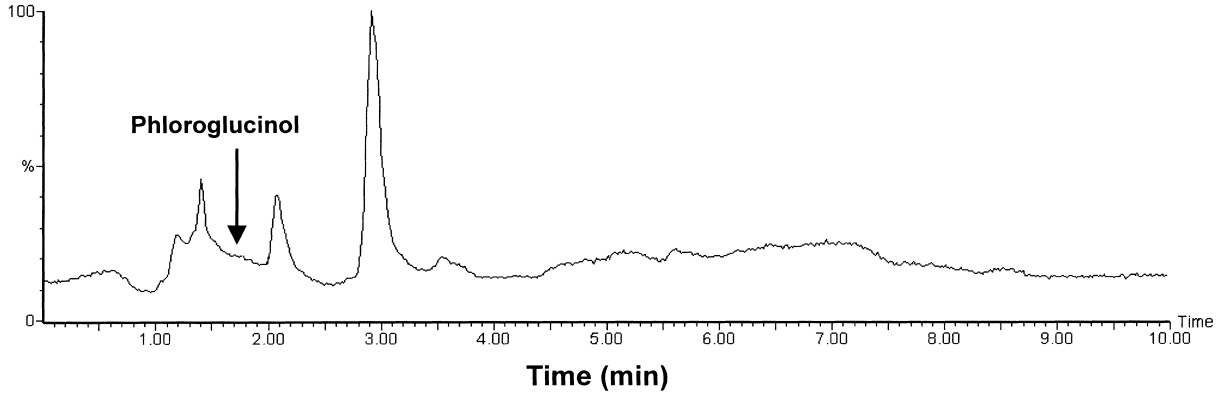


Fig. 4. LC–MS chromatograms of phloroglucinol in human blank plasma.

poor selectivity and high background than negative ion detection method. Consequently, negative ion detection mode was better than positive ion detection mode because quantification was easy in the low concentration range.

In the negative ion detection method, the intra-day precision expressed as % RSD was measured as 0.62–3.68% for 10, 50, 200, and 1000 ng/ml standard concentrations, with five replicates at each concentration level. The intra-day accuracy expressed as a percentage of nominal values was measured as 99.1–106.0% for four standard concentrations, with five replicates at each concentration level. Table 1 shows the intra-day precision and accuracy of measurement of phloroglucinol. The inter-day precision was measured as 0.82–3.33% for four standard concentrations, with five replicates at each concentration level. Conversely, the inter-day accuracy was measured as 100.4–102.2% for four standard concentrations, with five replicates at each concentration level. Table 2 shows the inter-day precision and accuracy for the measurement of phloroglucinol.

Standard calibration curves were constructed on different working days using the same human plasma. The response was linear throughout the concentration range of the study, with the coefficient of determination (r^2) greater than 0.9995 for all cases. And the standard calibration equations was $y = 0.015x - 0.0283$ (± 0.0005, standard deviation of three intercepts: -0.0283, -0.0288, -0.0278). Unit of y was area of analyte/internal standard and x was concentration of analyte/internal standard. Based on a signal-to-noise level (S/N) of 9–10, the quantification limit for phloroglucinol was found to be 5 ng/ml upon injection of 20 μl of the sample and the limit of detection was 2 ng/ml in negative ion detection mode.

Table 1
Intraday precision and accuracy of measurement of phloroglucinol when used negative ion detection with selective ion monitoring

Phloroglucinol nominal concentration (ng/ml)	Phloroglucinol (Mean±SD)[a] calculated concentration (ng/ml)	Accuracy (%)	Precision (% RSD)
10.0	10.6±0.2	106.0	1.89
50.0	50.2±1.8	100.4	3.58
200.0	198.2±7.3	99.1	3.68
1000.0	1003.3±6.2	100.3	0.62

[a] Five replicates at each concentration level ($n = 5$).

Table 2
Interday precision and accuracy of measurement of phloroglucinol when used negative ion detection with selective ion monitoring

Phloroglucinol nominal concentration (ng/ml)	Phloroglucinol (Mean±SD)[a] calculated concentration (ng/ml)	Accuracy (%)	Precision (% RSD)
10.0	10.2±0.3	102.0	2.94
50.0	51.1±1.7	102.2	3.33
200.0	200.7±5.3	100.4	2.64
1000.0	1001.8±8.2	100.2	0.82

[a] Five replicates at each concentration level ($n = 5$).

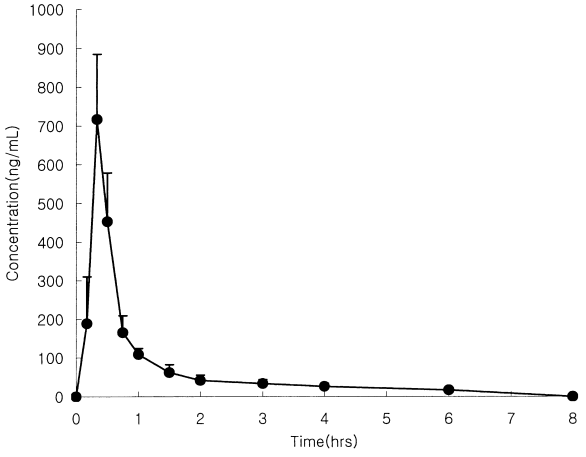


Fig. 5. Plasma concentration of phloroglucinol in human plasma–time curve after administration of a single dose of phloroglucinol.

Finally, determining the concentration of phloroglucinol in human plasma by negative ion detection was applied to pharmacokinetic studies. Fig. 5 shows the concentration of phloroglucinol in human plasma (five volunteers)–time curve after administration of a single dose of phloroglucinol. Fig. 5 indicates that the proposed method has been successfully applied to pharmacokinetic studies to determine the concentration of phloroglucinol in human plasma.

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