

# HPLC-UV Analysis of Phloretin in Biological Fluids and Application to Pre-Clinical Pharmacokinetic Studies

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## Abstract

A method of analysis of phloretin [3-(4-hydroxyphenyl)-1-(2,4,6-trihydroxyphenyl)propan-1-one] in biological fluids is necessary to study the kinetics of *in vitro* and *in vivo* metabolism and its concentration in natural products. A high-performance liquid chromatographic (HPLC) method was developed for the determination of phloretin in rat serum. Separation was achieved on a Chiralcel® OD-RH column with UV detection at 288 nm. The calibration curves were linear ranging from 0.5 to 100 µg/ml. The mean extraction efficiency was >95%. Precision of the assay was <14%, and was within 10.9% at the limit of quantitation (0.5 µg/ml). Bias of the assay was lower than 14%, and was within 9.22% at the limit of quantitation. The HPLC method was successfully applied to the pharmacokinetic study of phloretin in rats.

**Keywords:** Reversed-phase HPLC; UV-detection; Phloretin; Pharmacokinetics

**Abbreviations:** HPLC: High Performance Liquid Chromatograph; HPLC-MS: High Performance Liquid Chromatography-Mass Spectrometry; UV: Ultraviolet

## Introduction

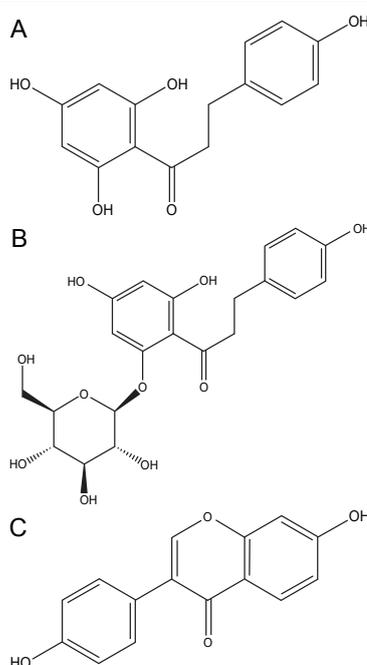
Phloretin [3-(4-hydroxyphenyl)-1-(2,4,6-trihydroxyphenyl)propan-1-one] is a hydrophobic, dihydrochalcone, polyphenolic compound (MW 274.3 g/mol, XlogP 2.6) that has been identified in apples and other natural sources including *Pieris japonica*, *Kalmia latifolia*, *Hoveniae Lignum*, and *Loiseleuria procumbens* [1-6] (Figure 1). Phloridzin is the glycoside of phloretin (phloretin-2'-glucose) which has been identified in apples, strawberries, and several other plants

including *Pieris japonica* and *Lithocarpus pachyphyllus* [5,7-9]. After consumption, it has been suggested that the glucose sugar moiety of phloridzin is rapidly cleaved off the parent compound in the gastrointestinal tract and liver to leave the aglycone, phloretin [7].

Phloretin is suspected to possess a range of pharmacological activities including potent antioxidant activity that has been attributed to its dihydrochalcone structure [10]. In specific studies, phloretin has also been shown to possess antithrombotic properties [11], hepatoprotective properties [6], ability to modulate cytochrome P450 1A1 expression [12] and ability to inhibit MRP1-mediated drug transport [13]. Most importantly, apples, which contain large amounts of phloretin and phloridzin, have been correlated with numerous health benefits including reduced risk of cardiovascular disease, asthma, some cancers, and diabetes [14].

Phloretin has been previously quantified utilizing a variety of methods in both natural products and biological samples, but to our knowledge, no validated methods have been reported in the literature. Kahle et al. assessed phloretin content in apple juices through high performance liquid chromatography (HPLC) using photo diode array detection and gradient elution [15]. Plasma samples have been assessed for phloretin content through HPLC with gradient elution but analysis used multielectrode coulometric detection [16]. Phloretin has been quantified in plasma, urine, and ileal fluid samples using high performance liquid chromatography-mass spectrometry (HPLC-MS); however, this method required a nearly hour long gradient program [17]. Finally, phloretin in urine has been assessed by using LC-MS-MS with a gradient elution [18].

Although these methods have been applied to phloretin



**Figure 1:** Structures of A) phloretin, B) phloridzin, and C) the internal standard, daidzein.

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quantification, none of these methods are ideal as they either have a long run time or use difficult to obtain analytical equipment. An isocratic reverse-phase HPLC assay utilizing ultraviolet detection would provide a method that would be applicable in many laboratory settings. Moreover, a method of analysis is needed to evaluate the pharmacokinetics and biotransformation of phloretin. The present study describes an isocratic, reversed-phase HPLC method using ultraviolet detection for the determination of phloretin in rat serum. We further describe the application of this assay to pharmacokinetic studies in rats including quantification in serum and urine.

## Experimental

### Chemicals and reagents

Phloridzin, phloretin, and daidzein were purchased from Sigma Chemicals (St. Louis, MO, USA). HPLC grade acetonitrile and water were purchased from J. T. Baker (Phillipsburg, NJ, USA). Phosphoric acid was purchased from Aldrich Chemical Co. Inc. (Milwaukee, WI, USA). Silastic<sup>®</sup> laboratory tubing was purchased from Dow Corning Corporation, (Midland, MI, USA). Intramedic<sup>®</sup> polyethylene tubing was purchased from Becton Dickinson Primary Care Diagnostics, Becton Dickinson and Company (Sparks, MD, USA). Monoject<sup>®</sup> 23 gauge (0.6 mm × 25 mm) polypropylene hub hypodermic needles were purchased from Sherwood Medical (St. Louis, MO, USA). Synthetic absorbable surgical sutures were purchased from Wilburn Medical US (Kernersville, NC, USA). Rats were obtained from Charles River Laboratories. Ethics approval for animal experiments was obtained from Washington State University.

### Chromatographic system and conditions

The HPLC system used was a Shimadzu HPLC (Kyoto, Japan), consisting of an LC-10AT VP pump, a SIL-10AF auto injector, a SPD-10A UV-VIS detector, and a SCL-10A VP system controller. Data collection and integration were accomplished using Shimadzu EZ Start 7.1.1 SP1 software (Kyoto, Japan). The analytical column used was Chiralcel<sup>®</sup> OD-RH column (150 mm × 4.6 mm i.d., 5- $\mu$ m particle size, Chiral Technologies Inc. Exton, PA, USA). The mobile phase consisted of acetonitrile, water and phosphoric acid (30:70:0.08, v/v/v), filtered and degassed under reduced pressure, prior to use. Separation was carried out isocratically at ambient temperature (25 ± 1°C) and a flow rate of 0.4 ml/min, with ultraviolet (UV) detection at 288 nm. To determine the optimal wavelength, a Shimadzu UV2100U UV-Vis Recording spectrometer was employed to measure the absorbencies over the range of 190 to 500 nm. The peak absorbance was 288 nm.

Peak purity was assessed using a Shimadzu SPD-M10AVP photodiode array detector over the absorbance range of 190-600 nm. No co-eluting peaks were seen over this absorbance range. Furthermore, the peak purity has been assessed using a Shimadzu LC-ESI-MS 2010EV in the positive and negative scan modes over the molecular weight range of 50 to 500 g/mol. No co-eluting peaks of a different *m/z* ratio than phloretin (*m/z* 273 in negative, *m/z* 275 in positive mode) were detected.

### Stock and working standard solutions

Methanolic stock solutions of phloretin (100  $\mu$ g/ml) and the internal standard, daidzein (100  $\mu$ g/ml), were prepared, protected from light, and stored at -20°C between uses, for no longer than 3 months. Calibration standards in serum were prepared daily from the stock solution of phloretin by sequential dilution with blank rat serum, yielding a series of concentrations namely, 0.5, 1, 5, 10, 50 and 100  $\mu$ g/ml.

### Sample preparation

To the working standards or samples (0.1 ml), 10  $\mu$ l of internal standard solution (daidzein at 100  $\mu$ g/ml) was added. The mixture was precipitated with 1 mL cold acetonitrile, vortexed for 1 min (Vortex Genie-2, VWR Scientific, West Chester, PA, USA), and centrifuged at 5,000 rpm for 5 min (Beckman Microfuge centrifuge, Beckman Coulter Inc., Fullerton, CA, USA). The supernatant was transferred and evaporated to dryness by a stream of nitrogen gas at room temperature. The residue was reconstituted with 400  $\mu$ l of mobile phase, vortexed for 1 min, and centrifuged at 5,000 rpm for 5 minutes. The supernatant was transferred to HPLC vials and 150  $\mu$ l was injected into the HPLC system.

### Precision and accuracy

The within-run precision and accuracy of replicate assays were tested by using six different concentrations of phloretin from 0.5-100  $\mu$ g/ml. The between-run precision and accuracy of the HPLC method was estimated from the results of five replicate calibration curves on five different days within one week with three repeat injections per concentration. The within-run precision and accuracy was examined from five calibration curves run within the same day. The precision was evaluated by the relative standard deviation (RSD). The accuracy was estimated based on the mean percentage error of measured concentration to the actual concentration [19].

### Relative recovery

Recovery of phloretin from biological fluids was assessed (n=5) at 0.5, 5 and 50  $\mu$ g/ml. A known amount of phloretin was spiked into 0.1 mL rat serum to give the above concentrations. 1 mL of cold acetonitrile was added to precipitate the proteins in the serum which was followed by centrifugation at 5,000 rpm for 5 min. The supernatant was transferred to new vials and injected for HPLC analysis. The extraction efficiency was determined by comparing the peak area ratio (PAR) of phloretin and IS to the PAR of corresponding concentration injected directly in the HPLC system without extraction.

### Freeze-thaw stability of phloretin samples

The freeze-thaw stability of phloretin was evaluated at three concentrations 0.5, 5 and 50  $\mu$ g/ml. These samples were analyzed via HPLC in triplicate without being frozen at first, and then stored at -20°C and thawed at room temperature (25 ± 1°C) for three cycles.

### Pharmacokinetic disposition of phloretin in rats

A male Sprague Dawley rat (n=1, 250 g) was anaesthetized using halothane and a silastic catheter was cannulated into the right jugular vein. The animal was allowed to recover in metabolic cages and fasted overnight. On the day of the experiment, the animal was dosed intravenously with phloretin (10 mg/kg) dissolved in 1% DMSO in polyethylene glycol 600. Blood samples (0.5 mL) were collected at 1, 30 min, 1, 2, 4, 6, 12 and 24 h post-dose. Following centrifugation of the blood samples, serum was collected and stored at -20°C until further preparation for HPLC analysis. Urine samples were also collected at 0, 2, 6, 12 and 24 h following phloretin administration.

### Pharmacokinetic sample preparation

Serum samples (0.1 mL) were run in duplicate with or without the addition of 40  $\mu$ l of 500 U/mL  $\beta$ -glucuronidase from *Escherichia coli* type IX-A and incubated in a shaking water bath at 37°C for 2 h to liberate any glucuronide conjugates [20, 21]. To 0.1 ml of serum, 10  $\mu$ l of IS was added. Proteins in the serum samples were precipitated



by the addition of 1 mL of ice-cold HPLC-grade acetonitrile followed by 1 min of vortexing and centrifugation at 5,000 rpm for 5 min. The supernatant was transferred to new centrifuge tubes and evaporated to dryness under compressed nitrogen gas at room temperature. The residue was reconstituted with 400  $\mu$ L of mobile phase, vortexed for 1 min, and centrifuged at 5,000 rpm for 5 min. The supernatant was transferred to vials and 150  $\mu$ L of it was injected into the HPLC system.

Urine samples (0.1 ml) were run in duplicate with or without the addition of 40  $\mu$ L of 500 U/mL  $\beta$ -glucuronidase from *Escherichia coli* type IX-A and incubated in a shaking water bath at 37°C for 2 h to liberate any glucuronide conjugates [20, 21]. To both duplicates, 10  $\mu$ L of IS was added. Samples were centrifuged at 5,000 rpm for 5 min, the supernatant transferred to new centrifuge tubes, and evaporated to dryness under compressed nitrogen gas. The residue was reconstituted and injected as indicated above. Standard curves in urine over the concentration range of 0.5 to 100  $\mu$ g/ml were prepared by spiking phloretin in blank rat urine and processed as above.

$\beta$ -glucuronidase from *Escherichia coli* type IX-A cleaves the glucuronidated metabolite back to phloretin. Therefore, by subtracting the concentrations of the samples without enzymatic hydrolysis from the samples with enzymatic hydrolysis, the concentration of the glucuronide can be calculated.

### Data analysis

Quantification was based on calibration curves constructed using peak area ratio (PAR) of phloretin to internal standard, against phloretin concentrations using unweighed least squares linear regression.

## Results and Discussion

### Chromatography

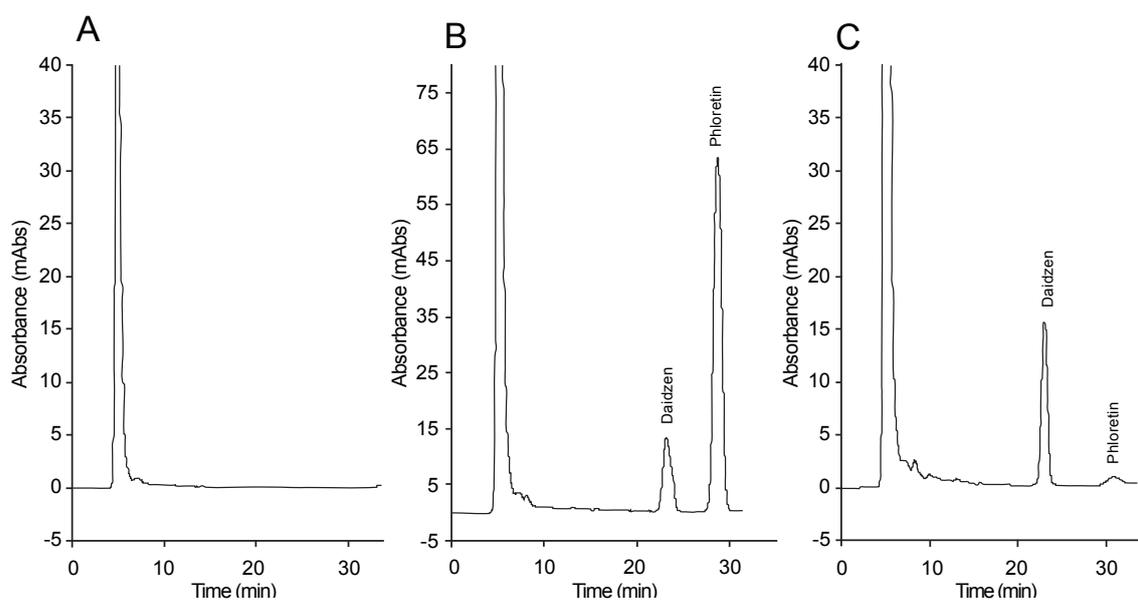
Separation of phloretin and the internal standard in biological fluids was achieved successfully. There were no interfering peaks

co-eluted with the compounds of interest in biological fluids (Figure 2). The retention times of internal standard, daidzein, and phloretin were approximately 24 and 30 min, respectively.

The performance of the HPLC assay was assessed according to criteria from the International Conference on Harmonization [19, 22]. Our acceptance criteria included: 1) no interference of the analytes from endogenous compounds; 2) peak shape based on symmetry, tailing factor and peak width; 3) linearity over concentration range; 4) between-day and within-day biases of <15% over all concentration points indicating accuracy; 5) between-day and within-day R.S.D. of <15% over all concentration points indicating precision; 6) relative recovery of 80-110%, 7) freeze-thaw stability over three cycles with recovery of 80-110%; and 8) limit of quantitation (LOQ).

In developing this assay, several other columns were attempted but poor separation and long run times resulted. The columns that we examined in the separation of phloretin included a Beckman Ultrasphere<sup>®</sup> Octyl column (150 mm  $\times$  4.6 mm, 5- $\mu$ m particle size), a Phenomenex<sup>®</sup> Prodigy 5-ODS-2 column (150 mm  $\times$  4.6 mm, 5- $\mu$ m particle size), a Dupont Golden Series Zorbax C<sub>8</sub> column, and Phenomenex<sup>®</sup> Luna C<sub>18</sub> (2) column (260 mm  $\times$  4.6 mm, 5- $\mu$ m particle size). A range of mobile phases were also employed when examining the above columns. The use of the Chiralcel<sup>®</sup> OD-RH column resulted in the best separation and retention times (~30 minutes) out of all the columns examined. Various compositions of mobile phase were tested to achieve the best resolution of phloretin. The optimal chromatogram was achieved when the combination of acetonitrile, water and phosphoric acid was 30:70:0.08 (v/v/v) and the flow rate was 0.4 mL/min.

The Chiralcel<sup>®</sup> OD-RH column used is packed with cellulose tris (3,5-dimethylphenylcarbamate) coated on 5  $\mu$ m silica-gel. In this column packing material, a helical groove exists with polar carbamate groups inside and hydrophobic aromatic groups outside the groove. Daidzein and phloretin are polar and may be inserted in the groove to and interact with the carbamate residues via hydrogen bond



**Figure 2:** Representative chromatogram of: A) blank serum demonstrating no interfering peaks co-eluted with the compounds of interest; B) rat serum containing IS and phloretin at a concentration of 50  $\mu$ g/mL; C) rat serum containing IS and phloretin with concentration of 0.5  $\mu$ g/mL (LOQ).



Phloretin concentration (µg/ml)			R.S.D (%)		Bias (%)	
Added	Observed		Within-day	Between-day	Within-day	Between-day
	Within-day	Between-day	Within-day	Between-day	Within-day	Between-day
0.5	0.546	0.546	9.62	10.9	9.22	9.18
1.0	1.02	0.96	3.94	9.77	2.11	-3.58
5.0	4.32	4.61	5.47	13.6	-13.5	-7.75
10	9.52	9.21	4.20	7.99	-4.79	-7.95
50	52.1	52.3	1.09	4.94	4.25	4.60
100	99.0	99.0	0.31	1.16	-1.01	-0.98

**Table 1:** Within- and between-day precision and accuracy of the assay for phloretin in rat serum (n = 5, mean, R.S.D., and Bias).

formation. In addition,  $\pi$ - $\pi$  interactions between the phenyl group of the cellulose carbamate and the aromatic groups of phloretin and daidzein occur.

### Linearity and LOQ

Excellent linear relationships for the HPLC method ( $r^2 = 0.998$ ) were demonstrated between PAR of phloretin to the internal standard and the corresponding serum concentrations of phloretin over a range of 0.5 to 100 µg/ml. The mean regression lines were described by phloretin (µg/ml) =  $0.0417x - 0.0227$ . The LOQ of this HPLC assay was determined based on a signal to noise ratio of 10:1. The LOQ was 0.5 µg/ml in biological fluids with the corresponding between day relative standard deviation of 10.9% and bias of 9.18% for phloretin. The back-calculated concentrations of calibration curve samples were within the acceptance criteria.

### Precision, accuracy and relative recovery

The within- and between-run precision (RSD) calculated during replicate assays of phloretin in rat serum was < 14% over a wide range of concentrations (Table 1). The intra- and inter-run bias assessed during the replicate assays for phloretin varied between -13.5 and 9.22% (Table 1). These data indicated that the developed HPLC method is reproducible and accurate. The relative recovery for phloretin relative to the internal standard from biological fluids varied from 95.2 to 108.3% (Table 2).

### Stability of phloretin samples

No significant degradation was detected by HPLC in the samples of phloretin in serum following three freeze-thaw cycles. The recoveries of phloretin were from 94.1 to 103% following three freeze-thaw cycles of phloretin at 0.5, 1, 5, 10, 50 and 100 µg/ml.

### Pharmacokinetics of phloretin in a rat model

The HPLC method was applied to the determination of phloretin in a pharmacokinetic study in a rat model (n=1). Following administration of phloretin intravenously (10 mg/kg), phloretin could be detected out to 6 h post-dose (Figure 3). The presence a phloretin glucuronide was also detected. The serum concentration vs. time profile demonstrated a rapid decline in concentration of phloretin within the first 30 min which was followed by a steady elimination phase (Figure 3).

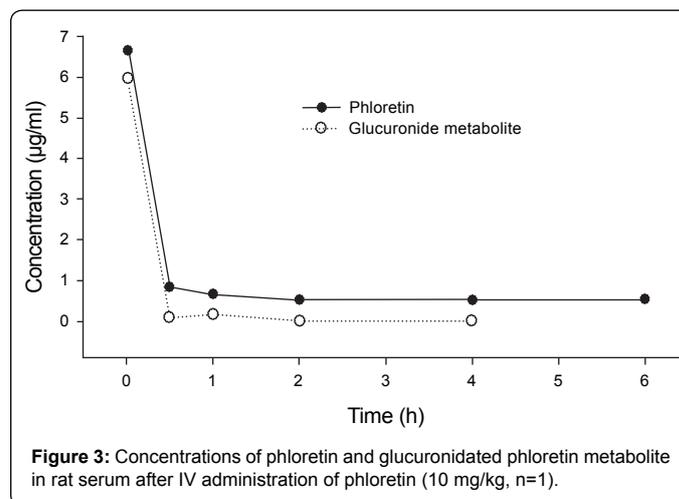
Phloretin Concentration (µg/ml)	Recovery (%) (mean ± SD)
0.5	95.2 ± 8.5
5.0	108.3 ± 1.3
50	107.5 ± 2.8

**Table 2:** Relative recovery of phloretin from rat serum (n = 3).

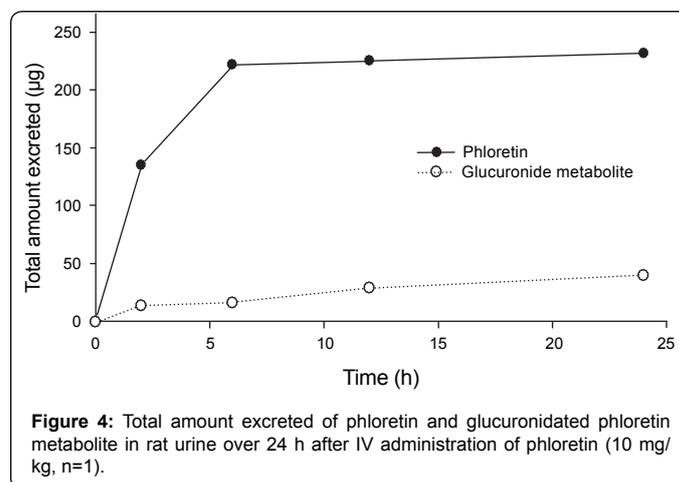
The HPLC method was also applied to the determination of phloretin in rat urine. Following IV administration (10 mg/kg) and analysis, excretion of the parent compound phloretin and a glucuronidated metabolite was observed (Figure 4). The total cumulative urinary excretion plot (Figure 4) indicates that phloretin is excreted predominantly as the aglycone.

### Conclusions

In summary, the developed HPLC method for phloretin is sensitive, reproducible, and accurate. It has been applied successfully in the study of the phloretin distribution and urinary pharmacokinetics. Further studies are ongoing in our laboratory to further characterize



**Figure 3:** Concentrations of phloretin and glucuronidated phloretin metabolite in rat serum after IV administration of phloretin (10 mg/kg, n=1).



**Figure 4:** Total amount excreted of phloretin and glucuronidated phloretin metabolite in rat urine over 24 h after IV administration of phloretin (10 mg/kg, n=1).



the pharmacological and toxicological activities of phloretin as well as other polyphenol phytonutrients.

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