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Simultaneous Determination of Baicalein, Baicalin, Wogonin, and Wogonoside in Rat Plasma by LC-MS/MS for Studying the Pharmacokinetics of the Standardized Extract of Scutellariae Radix

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A new composition of standardized Scutellariae Radix extract (HPO12) was developed for treatment of Alzheimer's disease. For the preclinical pharmacokinetic study of HPO12, a rapid, sensitive, and selective LC-MS/MS method was developed and validated for the simultaneous determination of 4 bioactive compounds, baicalein, baicalin, wogonin, and wogonoside. After extraction with ethylacetate, chromatographic analysis was performed on a Thermo C_{18} column (150 mm × 2.1 mm, 3 μm) with a mobile phase consisting of 0.1% formic acid (A) and 0.1% formic acid in 95% acetonitrile (B) by using gradient elution at a flow rate of 250 μL/min. Analytes introduced to a mass spectrometer were monitored by multiple reaction monitoring (MRM) in positive ion mode. Using 25 μL of plasma sample, the method was validated over the following concentration ranges: 25-5000 ng/mL for baicalein, 20-40000 ng/mL for baicalin, 1-1000 ng/mL for wogonin, and 5-10000 ng/mL for wogonoside. The intra- and inter-day precision and accuracy of the quality control samples at the 4 concentrations showed ≤ 13.7% relative standard deviation (RSD) and 86.6-105.5% accuracy. The method was successfully applied to determine the concentrations of baicalein, baicalin, wogonin, and wogonoside in rat plasma after intraperitoneal and oral administrations of HPO12.

Key Words : Scutellariae radix, HPO12, LC-MS/MS, Pharmacokinetics

Introduction

The dried root of *Scutellaria baicalensis* Georgi, known as Scutellariae Radix, is a key medicinal plant used in traditional Asian medicine, in countries including Korea, China, and Japan. Scutellariae Radix has long been used for treatment of dermatitis, diarrhea, fever, inflammatory disease, hepatic disease, and hypertension.[1,2] Moreover, it has been reported that Scutellariae Radix can protect neurons from cell death[3] and improve learning and memory functions following impairment caused by transient cerebral ischemia in mice.[4] Gao *et al*. demonstrated the neuroprotective effects of baicalein, baicalin, wogonin, and wogonoside from *S. baicalensis* in cultured human neuroblastoma cells.[5] Moreover, wogonin has shown to have a potent neuroprotective effect by inhibiting the inflammatory activation of microglia.[6]

Recently, a new composition of Scutellariae radix extract, named HPO12, was prepared from 70% ethanol extract of Scutellariae radix. The main bioactive constituents of this extract are baicalein, baicalin, wogonin, and wogonoside. HPO12 demonstrated a synergistic neuroprotective effect compared with the effect of the individual ingredients in our pharmacological assay; this extract is currently under development as a therapeutic agent for treatment of Alzheimer's disease. Therefore, a preclinical pharmacokinetic study was required for predicting the efficacy, safety, and toxicity of HPO12.

Various analytical methods using high-performance liquid chromatography (HPLC), liquid chromatography-mass spectroscopy (LC-MS), or liquid chromatography with tandem mass spectrometry (LC-MS/MS) detection have been developed for analysis of the principal ingredients of Scutellariae Radix, mainly flavonoids, in biological samples.[7-13] To our knowledge, two studies have reported the simultaneous determination of the bioactive flavonoids of Scutellariae Radix including all of baicalein, baicalin, wogonin, and wogonoside in biological samples with full validation data by using LC-MS/MS.[14,15] However, in those studies, the analytical method was validated in the restricted concentration ranges. In one of those reports,[14] the analytical method was validated in the concentration ranges of 0.1 to 20 μg/mL for all the flavonoids tested. Scutellariae flavonoids are reported to be extensively metabolized after being absorbed mainly in the form of aglycones from the gastrointestinal tract.[16] Accordingly, aglycones such as baicalein and wogonin are likely to exist in plasma at a lower concentration than glycones, possibly less than 0.1 μg/mL. This was mentioned as a possible reason why the aglycones could not be detected by the authors of the previous study. In

another report,[15] the calibration curve could not cover higher concentrations for the pharmacokinetic analysis of non-oral routes of administration, i.e. intravenous or intraperitoneal. Therefore, to more clearly and conveniently characterize the pharmacokinetic properties of Scutellariae flavonoids, it is necessary to develop an analytical method for the simultaneous determination of both glycones and aglycones present in a low and wide concentration range.

In the present study, we developed and validated the LC-MS/MS method for the simultaneous determination of baicalein, baicalin, wogonin, and wogonoside at lower and wider concentration ranges in rat plasma. Subsequently, our method was successfully applied for determining the plasma concentration of these 4 flavonoids after intraperitoneal and oral administration of HPO12 in rats.

Experimental

Chemicals and Reagents. Baicalein, baicalin, and wogonin were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan), and wogonoside was purchased from Delta Information Center for Natural Organic Compounds (Anhui, China). Quercetin (internal standard, IS) was obtained from Sigma Chemical Co. (St. Louis, MO), and HPO12 was provided by NeuMed Co. Ltd. (Seoul, Korea). HPO12 was standardized to contain 3.78% baicalein, 19.8% baicalin, 0.62% wogonin, and 3.82% wogonoside. Polyethylene glycol (PEG; average molecular weight, 400) and L(+) ascorbic acid were purchased from Sigma Chemical Co. (St. Louis, MO). HPLC grade ethyl acetate, acetonitrile, and methanol were purchased from Mallinckrodt Baker Inc. (Phillipsburg, NJ, USA).

Sample Preparation. A 10-μL aliquot of ascorbic acid (100 mg/mL), a 10-μL aliquot of methanol containing 120 μg/mL of quercetin (internal standard, IS), and 1 mL of ethyl acetate were added to each 25-μL aliquots of the biological samples. The samples were vortex-mixed and centrifuged, and the supernatant was collected and dried under nitrogen evaporation. The residue was dissolved in 100 μL of HPLC initial mobile phase, and a 10-μL sample was injected onto an LC-MS/MS system. The calibration standards and quality control (QC) samples were prepared by adding appropriate concentrations of the stock solutions of the 4 flavonoids (10 μL) to blank rat plasma (90 μL) and were analyzed in the same manner as described above.

LC-MS/MS Analysis. The LC-MS/MS system consisted of a LC-10ADvp binary pump system with an API 2000 triple-quadrupole mass spectrometer (Applied Biosystems-SCIEX, Concord, Canada) equipped with a TurboIonSpray source. The HPLC mobile phases consisted of 0.1% formic acid (A) and 0.1% formic acid in 95% acetonitrile (B). Chromatographic separation was achieved on a reversed-phase Thermo C_{18} column (150 mm × 2.1 mm, 3 μm; Thermo Scientific Inc., Waltham, MA) using gradient elution at a flow rate of 0.25 mL/min. The gradient began with 15% eluent B, increased linearly to 65% eluent B for 3 min, and then to 85% eluent B for 1 min; this percentage was maintained for 2 min. The gradient was then changed back to the initial condition over 0.3 min and was kept in this condition for 1.7 min. The total run time was 8 min. Electrospray ionization (ESI) was performed in the positive mode with nitrogen as the nebulizing turbo spray, and curtain gas with the optimum values set at 40, 75, and 40 (arbitrary units). Multiple reaction monitoring (MRM) detection was employed using nitrogen as the collision gas (4 arbitrary units), with a dwell time of 150 ms for each transition; the transitions monitored were *m/z* 271→123 for baicalein, *m/z* 447→271 for baicalin, *m/z* 285→270 for wogonin, *m/z* 461→285 for wogonoside, and *m/z* 309→69 for IS.

Analytical Method Validation. The analytical method was validated according to the "Guidance for Industry, Bioanalytical Method Validation" presented by the US Food and Drug Administration (2001).[17] The specificity of the method was determined by analyzing 5 different batches of blank rat plasma to examine the presence of chromatographic interference from endogenous matrix. Calibration curves were prepared by plotting peak area ratios of analyte/IS against analyte concentrations and were analyzed by linear least-squares regression analysis. The calibration curves for baicalein, baicalin, wogonin, and wogonoside were constructed over the concentration ranges of 25-5000 ng/mL, 20-40000 ng/mL, 1-1000 ng/mL, and 5-10000 ng/mL, respectively, in triplicate. The intra- and inter-day precision and accuracy were assessed on the same day and on 5 separate days by repeated analyses ($n = 5$) of the QC samples at 4 concentration levels (25, 100, 1000, and 5000 ng/mL for baicalein; 20, 200, 2000, and 40000 ng/mL for baicalin; 1, 50, 100, and 1000 for wogonin; and 5, 250, 5000 and 10000 ng/mL for wogonoside). The post-preparative stability was evaluated by analyzing the concentration of the extracted QC samples 24 h after being placed in the autosampler at 4 °C. Matrix effects were estimated by a post-extraction addition approach, as previously described.[18] The peak areas of the standards-spiked plasma extract samples and the corresponding standards dissolved in the HPLC initial mobile phase at a concentration of 100 ng/mL for all analytes were compared and represented as percentages.

Animal Experiments. Male Sprague-Dawley rats weighing 210-250 g were purchased from Taconic Farms Inc. (Samtako Bio Korea, O-San, Korea). The animals were housed in a temperature- and moisture-controlled (23 °C ± 2 °C and 55% ± 10%, respectively) clean room with a 12-h light/dark cycle, and were allowed free access to food and water. A day before HPO12 administration, the rats were anesthetized with pentobarbital, and 1 femoral artery of each rat was cannulated for blood sampling by using PE-50 tubing (Becton Dickinson, Lincoln Park, NJ). The cannulae were fixed to the head and neck. HPO12 was dissolved in 50% (v/v) PEG 400 solution in water. The rats were subjected to overnight fasting, with free access to water before HPO12 administration. The HPO12/PEG solution was administered intraperitoneally or orally via a feeding tube at a dose of 200 mg/kg HPO12 in a volume of 2 mL/kg. Aliquots of approximately 200 μL of heparinized blood samples were

collected via the femoral artery cannula at 0 (to serve as a control), 0.167, 0.333, 0.5, 1, 90, 2, 3, 4, 6, 8, 12, and 24 h after intraperitoneal administration, and 0, 0.25, 0.5, 1, 2, 3, 4, 5, 6, 8, 12, 24, 36, and 48 h after oral administration. Further, blood samples were centrifuged to separate the plasma, and two 25-μL aliquots of plasma from each sample were stored at −70 °C until analysis. The cannula was flushed with approximately 200 μL of heparinized 0.9% NaCl-injectable solution (10 units/mL) immediately after each blood sampling to prevent occlusion.

Pharmacokinetic Analysis. Pharmacokinetic parameters were determined by a non-compartmental analysis using the WinNonlin (Pharsight Corporation, Mountain View, CA) program. The total area under the plasma concentration-time curve from time zero to the last measured time, in plasma (AUC_{last}), was calculated using the trapezoidal rule method. The peak plasma concentration (C_{max}) and time to reach C_{max} (T_{max}) were determined directly from the experimental data.

Results and Discussion

Investigation of LC-MS/MS Condition. The LC-MS/MS analysis was performed in positive ion mode. The electrospray ionization of baicalin, baicalein, wogonin, and wogonoside produced protonated molecular ions at *m/z* 447.5, 271.4, 285.0, and 461.3, respectively. The product ion mass spectra were obtained for each protonated molecular ion (Figure 1). Based on the major product ions shown in the product ion mass spectra, MRM transition for each compound was selected: *m/z* 271.4→123.1 for baicalein, *m/z* 447.5→271.3 for baicalin, *m/z* 285.0→270.0 for wogonin, and *m/z* 461.3→285.1 for wogonoside.

Specificity and Selectivity. The specificity of the developed method was presented by comparing MRM chromatograms for blank rat plasma, spiked rat plasma and plasma collected 10 min after intraperitoneal administration of HPO12. As shown in Figure 2, any significant peaks interfering with baicalin, baicalein, wogonin, wogonoside, or IS were not observed in blank rat plasma.

Linearity and Lower Limit of Quantitation (LLOQ). The calibration curves for baicalein, baicalin, wogonin, and wogonoside were linear over the concentration ranges investigated with correlation coefficients (r) greater than 0.99 (Table 1). The limits of quantification of baicalein, baicalin, wogonin, and wogonoside were 25, 20, 1, and 5 ng/mL, respectively, at which precision was less than 20% and accuracy was within 80-120%. The developed method could achieve lower LOQ values and wider analytical range compared with the previously reported methods by optimizing the sample injection volume.[14,15]

Precision and Accuracy. Precision and accuracy of the assay were determined by replicate analyses (n = 5) of the QC samples at 4 concentrations, by performing the analytical runs on the same day for intra-day assays, and on 5 consecutive days for inter-day assays. The intra- and inter-day accuracy of the method were between 86.6% and 105.5%, and the intra- and inter-day precision were less than 9.03% and 13.7%, respectively, for all the compounds analyzed. The intra- and inter-day assay results are summarized in Table 2.

Stability. The long-term and short-term stabilities and freeze-thaw cycle stability for the 4 flavonoids in rat plasma have already been established in previous studies.[14,15] Therefore, the tests for these 3 stabilities were omitted and a post-preparative stability was evaluated in the present study. The test was conducted with 4 QC samples for each

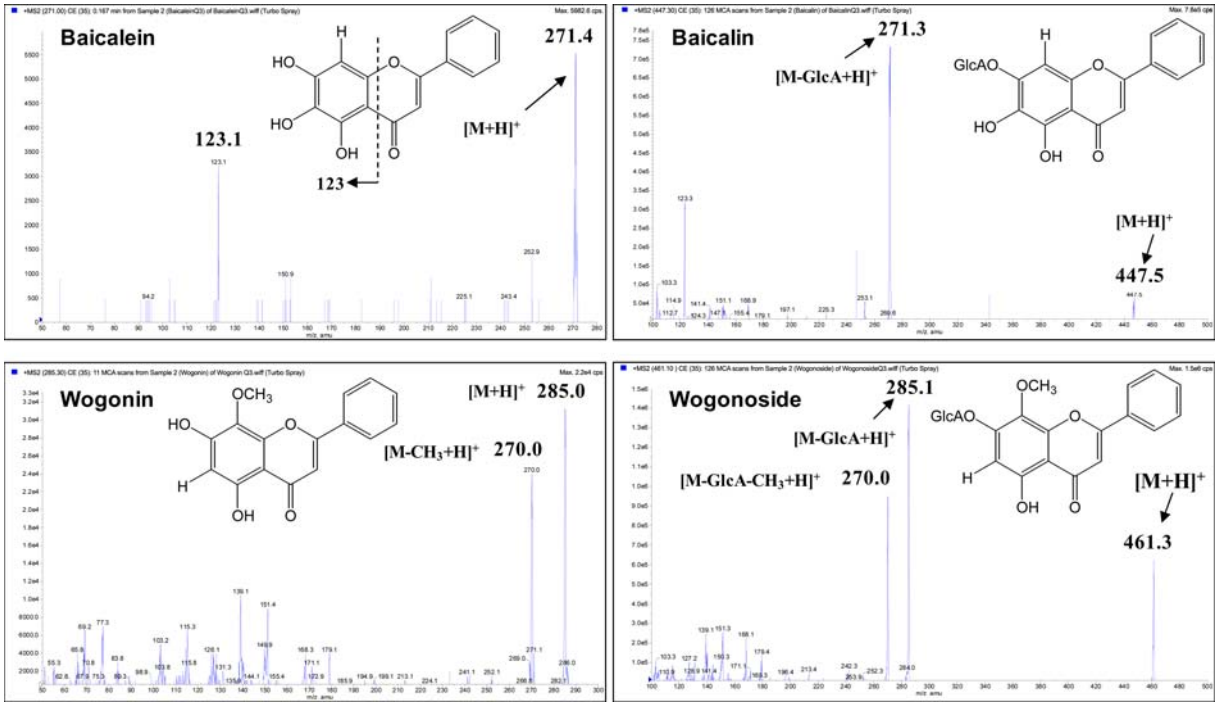

Figure 1. Product ion mass spectra of baicalein, baicalin, wogonin, and wogonoside.

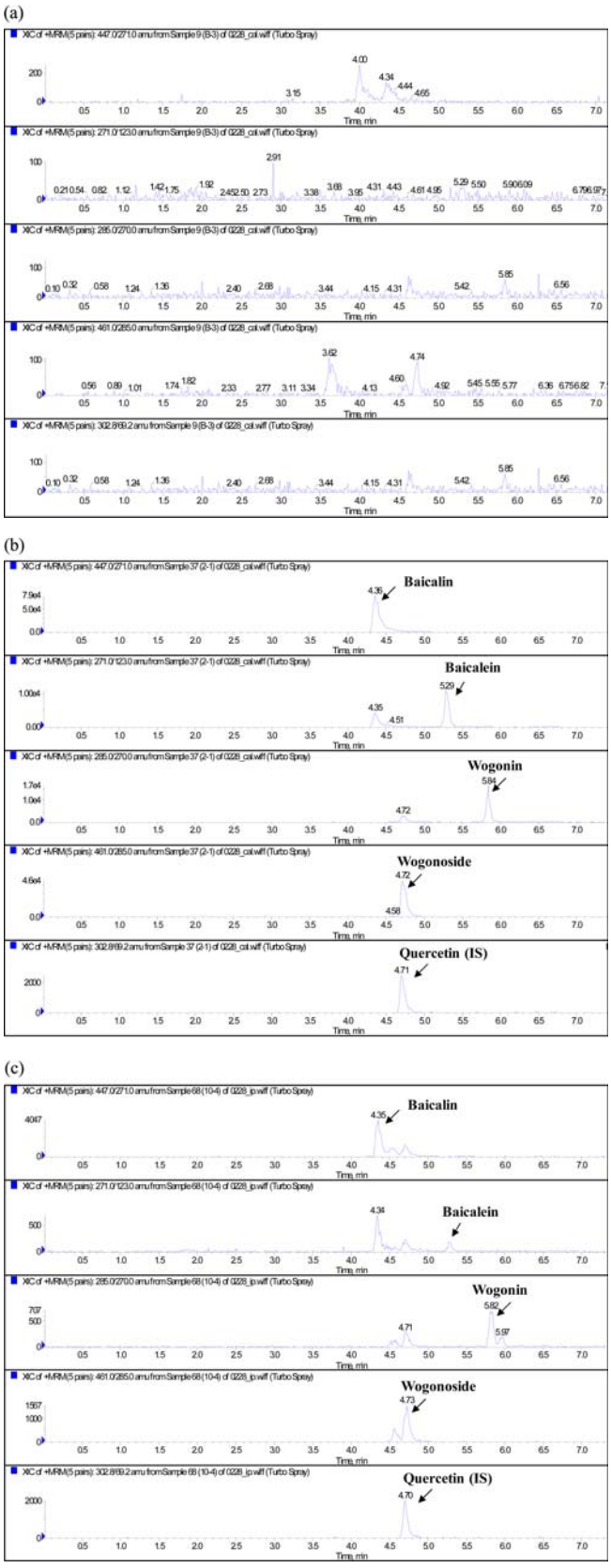

Figure 2. Representative selected reaction monitoring chromatograms of baicalein, baicalin, wogonin, and wogonoside. (a) Blank rat plasma, (b) blank rat plasma spiked with standards and IS, and (c) plasma sample 10 min after intraperitoneal administration of HPO12 (200 mg/kg) to rats.

Table 1. Calibration curves parameters of 4 flavonoids in rat plasma[a]

	Slope	Intercept	R	Range (ng/mL)	
				LLOQ[b]	ULOQ[c]
Baicalein	0.000682	-0.0064	0.9999	25	5000
Baicalin	0.00213	0.0373	0.9985	20	40000
Wogonin	0.00426	0.0125	0.9996	1	1000
Wogonoside	0.00422	0.0337	0.9992	5	10000

[a]Values are the mean of three calibration curves. [b]LLOQ, lower limit of quantification. [c]ULOQ, upper limit of quantification.

Table 2. Intra- and inter-day accuracy and precision for the determination of 4 flavonoids in rat plasma

Theoretical conc.	Intra-day (n=5)			Inter-day (n=5)		
	Conc. found (ng/mL)	Accuracy (%)	Precision (% RSD)	Conc. found (ng/mL)	Accuracy (%)	Precision (% RSD)
Baicalein						
10	9.5 ± 0.7	94.8	8.0	9.6 ± 0.4	95.6	4.2
25	21.7 ± 0.4	86.6	1.7	23.4 ± 1.5	93.7	6.2
100	87.6 ± 1.4	87.6	1.6	93.4 ± 5.6	93.4	6.0
1000	1013.5 ± 99.6	101.4	9.8	933.0 ± 104.7	93.3	11.2
Baicalin						
100	106.7 ± 4.7	106.7	4.4	93.0 ± 7.2	93.0	7.7
200	192.0 ± 7.0	96.0	3.6	193.5 ± 10.1	96.8	5.2
2000	2030.0 ± 70.7	101.5	3.5	2060.0 ± 220.2	103.0	10.7
40000	39800.0 ± 3140.1	99.5	7.9	37625.0 ± 5159.1	94.1	13.7
Wogonin						
1	0.96 ± 0.09	96.3	9.1	0.99 ± 0.06	98.5	6.6
2	2.07 ± 0.20	103.4	9.6	1.96 ± 0.16	97.8	8.0
10	8.72 ± 0.26	87.2	3.0	9.60 ± 1.12	95.9	11.7
50	45.08 ± 1.53	90.2	3.4	44.60 ± 2.06	89.2	4.6
Wogonoside						
5	4.7 ± 0.3	94.0	6.6	4.6 ± 0.3	91.9	5.9
10	9.3 ± 0.4	93.0	4.3	9.5 ± 1.0	94.8	11.0
250	232.3 ± 10.9	92.9	4.7	225.2 ± 5.7	90.5	2.5
5000	5197.5 ± 176.9	104.0	3.4	4410.0 ± 154.5	88.2	3.5

compound in the autosampler at 4 ºC. All compounds tested were shown to be stable with acceptable recoveries (89.1%-113.5%) and precisions (less than 15%) in the autosampler for 24 h after being extracted and processed.

Matrix Effect. The matrix effects were evaluated using a post-extraction addition approach. The recovery of baicalein, baicalin, wogonin, and wogonoside from rat plasma was 87.6-105.4% at 100 ng/mL for all analytes. These results

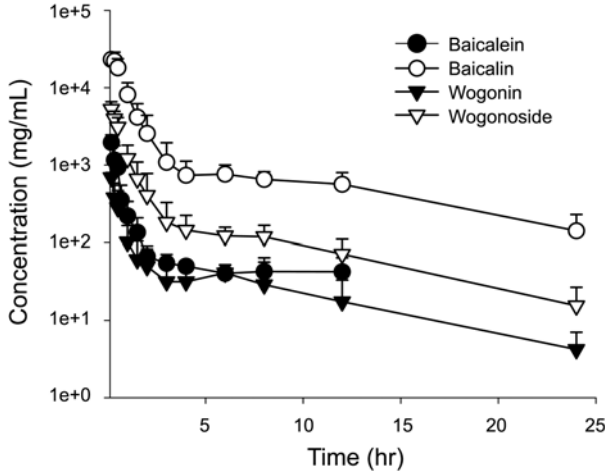

Figure 3. Mean arterial plasma concentration-time profiles of (a) baicalein, (b) baicalin, (c) wogonin, and (d) wogonoside after intraperitoneal administration of HPO12 to rats at a dose of 200 mg/kg (n = 4, mean ± SD).

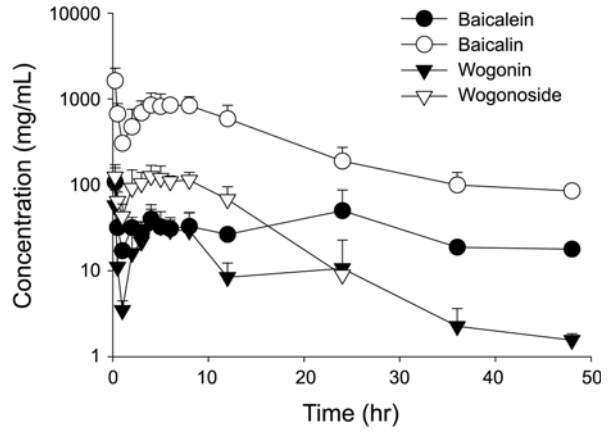

Figure 4. Mean arterial plasma concentration-time profiles of (a) baicalein, (b) baicalin, (c) wogonin, and (d) wogonoside after oral administration of HPO12 to rats at a dose of 200 mg/kg (n = 4, mean ± SD).

indicate that no significant ion suppression or enhancement was caused by matrix ions.

Pharmacokinetic Study of HPO12. The mean arterial plasma concentration-time profiles of 4 major flavonoids after intraperitoneal administration of HPO12 at doses of 200 mg/kg in rats are shown in Figure 3, and some relevant pharmacokinetic parameters are listed in Table 3. The plasma concentrations declined rapidly for up to 3 h, and then slightly increased; the second peak appeared at 6-12 h; this could be due to enterohepatic circulation.[19] The peaks found in the MRM channel for baicalin, wogonin, and wogonoside are supposed to be their isobaric forms, generated through the metabolism of Scutellariae flavonoids (Figure 2). The mean arterial plasma concentration-time profiles of the 4 major flavonoids after oral administration of HPO12 at doses of 200 mg/kg in rats are shown in Figure 4, and some relevant pharmacokinetic parameters are listed in Table 3. After oral administration, all flavonoids tested were absorbed rapidly from the rat GI tract; the drug was detected in the plasma from the first blood sampling time (15 min). The first peak occurred at 15-30 min, and the second at 6-12 h after administration, and then plasma concentrations fluctuated up to the last measured time. This could be due to enterohepatic circulation[19] and subsequent continuous absorption of the flavonoids.

Table 3. Pharmacokinetic parameters of baicalein, baicalin, wogonin, and wogonoside after intraperitoneal and oral administration of HPO12 at a dose of 200 mg/kg to rats

	Parameter	Intraperitoneal	Oral
Baicalein	AUC_{last} (μg·hr/mL)	2.18 ± 0.63	1.52 ± 0.49
	T_{max} (hr)	0.17 ± 0.00	6.19 ± 11.88
	C_{max} (μg/mL)	1.96 ± 0.49	0.122 ± 0.051
Baicalin	AUC_{last} (μg·hr/mL)	32.7 ± 6.2	17.9 ± 3.4
	T_{max} (hr)	0.25 ± 0.09	0.25 ± 0.00
	C_{max} (μg/mL)	24.9 ± 2.93	1.87 ± 0.45
Wogonin	AUC_{last} (μg·hr/mL)	0.802 ± 0.316	0.478 ± 0.276
	T_{max} (hr)	0.17 ± 0.00	0.688 ± 0.875
	C_{max} (μg/mL)	0.691 ± 0.109	0.0057 ± 0.0322
Wogonoside	AUC_{last} (μg·hr/mL)	5.47 ± 0.93	1.71 ± 0.35
	T_{max} (hr)	0.17 ± 0.00	1.88 ± 2.24
	C_{max} (μg/mL)	5.27 ± 1.31	0.17 1± 0.016

Conclusion

A sensitive, accurate and precise LC-MS/MS method has been developed and validated for the simultaneous determination of baicalein, baicalin, wogonin, and wogonoside in rat plasma. The developed method was successfully applied for determining the plasma concentration of these 4 flavonoids after intraperitoneal and oral administration of HPO12 in rats for the preclinical pharmacokinetic study.

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