

Study on Plasma Concentration and Bioavailability of Wogonin in Beagle's Dogs

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Abstract: **Objective** To develop an LC-MS/MS method for determining the concentration of wogonin in dog plasma and investigate the pharmacokinetics and bioavailability by different administrations of wogonin in Beagle's dogs. **Methods** LC-MS/MS was employed in determining the concentration of wogonin with the selected ion monitoring model after liquid-liquid extraction with ethyl acetate of dog plasma samples. The lower limit of quantification was 0.105 µg/L. Target ions were at m/z 285.0→270.0 for wogonin and 373.3→305.3 for finasteride. In a randomized, self-control, and cross-over study, six male Beagle's dogs were treated with different administration methods in three test periods. Pharmacokinetic parameters were calculated with DAS software (Ver. 2.0). **Results** The calibration curve was linear in the range of 0.105–107.36 µg/L for wogonin in dog plasma samples. The main pharmacokinetic parameters of ig administration (native drug of 15 mg/kg and solution preparation of 5 mg/kg) and iv route were as follows: C_{max} (2.5 ± 1.1), (7.9 ± 3.3), and (6838.7 ± 1322.1) µg/L, t_{max} (0.7 ± 0.3) and (0.3 ± 0.2) h for the both former, AUC_{0-t} (7.1 ± 2.0), (21.0 ± 3.2), and (629.7 ± 111.8) µg·h/L. The absolute bioavailability of native and solution of wogonin were (0.59 ± 0.35)% and (3.65 ± 2.00)%, respectively. **Conclusion** The validated method is convenient, sensitive, and specific, and the improvement of wogonin solubility could remarkably increase the absolute bioavailability.

Key words: Beagle's dogs; bioavailability; LC-MS/MS; pharmacokinetics; solubility; wogonin

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Introduction

Wogonin, 5,7-dihydroxy-8-methoxyflavone (Fig. 1), is one of the major bioactive flavonoid aglycones isolated from the roots of *Scutellaria baicalensis* Georgi (Labiateae), which has been commonly used in traditional Chinese medicines (TCM) as a remedy for the treatment of fever, cough, inflammation, and hypotension. In recent years, wogonin has attracted substantial interest as it possesses antihepatitis B virus (Guo *et al*, 2007), cancer-preventive (Lee *et al*, 2008; Zhang *et al*, 2008; Lu *et al*, 2008), and anticonvulsant effects (Park *et al*, 2007; Hui *et al*, 2002).

Up to now only limited data are available on the pharmacokinetics of wogonin in rats. The HPLC-UV method (Tsai *et al*, 1996) with the lower limit of quantitation (LLOQ) of 50 µg/L could not offer the

sensitivity necessary for the pharmacokinetic study of wogonin following ig administration. In contrast to the LC-MS/MS method (Chen *et al*, 2002; Kim *et al*, 2006), we describe a validated LC-ESI-MS method with relatively high-improved sensitivity, economy, and reliability, which was successfully applied to the investigation of the bioavailability of wogonin in dogs.

As the solubility of wogonin in water is very poor, DMSO-PEG 200-water (5:35:60) was once used as solvent for ig administration to rats in the study (Du *et al*, 2002). But DMSO used as the described dosage (10 mL/kg) may have some toxicity to animals and have some influence on the results. So we prepared wogonin to wogonin arginine for the study of the bioavailability of wogonin in dogs.

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Materials and methods

Chemicals, reagents, and animals

Both the native drug (purity > 99.7%) and the solution preparation (10 g/L, wogonin arginine) of wogonin were kindly provided by Hefei Con-source Medicine Technology Co., Ltd., China. The internal standard (IS) finasteride (Fig. 1) was purchased from National Institutes for Food and Drug Control (Beijing, China). Distilled water, prepared from demineralized water, was used throughout the study. Methanol (Merck, Germany) and formic acid (DIMA, USA) were of HPLC grade, acetic ether was commercially available and of analytical grade. Beagle's dogs were obtained from the Jia'an Animal Experimentation Breeding Center (Zhejiang, China) [SCXK (Zhejiang) 2003-0007], and the studies were approved by the Animal Ethics Committee of Anhui Medical University. The animals were cared according to the regulations of the Animal Committee at a constant temperature of $(22 \pm 1)^\circ\text{C}$, 12 h/12 h light-dark cycle and 10–15 air changes per hour for the feeding room.

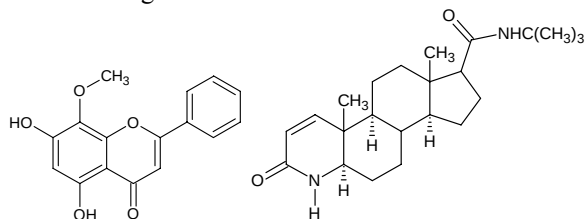


Fig. 1 Chemical structures of wogonin (A) and finasteride (B)

Standards and controls

A stock standard solution containing wogonin was prepared in methanol at a concentration of 1.0736 g/L. Appropriate serial dilutions of the stock solution were made in methanol for spiking blank dog plasma. Aliquots (10 μL) of the appropriately diluted stock solution of wogonin were added to 100 μL of blank dog plasma to yield working standards of the desired plasma concentrations. A stock standard solution of finasteride was prepared in methanol at a concentration of 1 g/L, and diluted to 250 $\mu\text{g/L}$ with methanol to yield the finasteride working solution. Calibration curves were obtained with wogonin-spiked dog plasma samples in the range of 0.105–107.36 $\mu\text{g/L}$. Additionally, spiked plasma samples were processed within each run as a means of assay quality control (QC).

Apparatus and chromatographic conditions

A Finnigan TSQ Quantum Discovery MAX system

with ESI interface and Xcalibur workstation software (Ver 1.4) for the data processing were utilized to perform the analytical procedures. Separation was carried out on a Phenomenex[®] Luna C₁₈(2) column (150 mm $\times$ 2.0 mm, 5 μm , Code No. 490902-2) with a C₁₈ guard column (Phenomenex, USA). Isocratic elution employed a mobile phase of methanol-water containing 0.1% formic acid (70:30) at a flow rate of 0.2 mL/min. A flow-switching technique was used by commanding the flow channel selection valve. The mobile phase was only loaded to MS around the times when the target peaks emerged, in order to prevent redundant co-elutes and interferences from polluting the analyzer and thus to increase the targets' MS signals. The optimized MS parameters were selected as follows: spray voltage 4.5 kV; capillary temperature 320°C ; source CID -4V ; sheath gas pressure 35 Arb; aux gas pressure 5 Arb. Multiple-reaction monitoring (MRM) used the transitions of the protonated molecules at m/z 285.0 $\rightarrow$ 270.0 for wogonin and 373.3 $\rightarrow$ 305.3 for finasteride with scan time of 0.5 s per transition.

Sample preparation

Plasma (100 μL) and finasteride (10 μL) working solution were added to a 2 mL plastic conical extraction tube. After vortex-mixing for 30 s, 1 mL of ethyl acetate was added; The tube was stoppered well and shaken vigorously for 3 min. Following centrifugation at $14\,000 \times g$ for 10 min, the upper organic layer was transferred into another plastic tube and evaporated to dryness at 45°C under a gentle stream of nitrogen. The residue was reconstituted in 100 μL mobile phase, and centrifuged at $14\,000 \times g$ for 10 min. The supernatant was transferred to an autosampler vial, and 10 μL was injected into the column for analysis.

Method validation

Validation was performed for samples of plasma according to *FDA Guidance for Industry, Bioanalytical Method Validation* (CDER, 2001). Specificity was assessed by analysis of six different samples of blank matrix with and without spiking with finasteride and wogonin. Linearity was assessed by weighted ($1/\chi^2$) least squares linear regression of calibration curves generated in triplicate on consecutive 3 d using analyte: finasteride peak area ratios. Intra- and inter-day precision (RSD) and accuracy (RE) were determined by analysis of six replicated QC samples on three different

days and inverse prediction of the concentrations from the calibration curve. The acceptance criteria for each calculated concentration were $RSD < 15\%$ and $RE < 15\%$ of the nominal value. The LLOQ was defined as the lowest concentration that could be determined with acceptable precision ($\pm 20\%$). Recoveries were estimated by comparing the peak areas of wogonin in three replicates of QC samples with those of post-extraction blank matrix extracts at the corresponding concentrations. Matrix effects of wogonin were evaluated by comparing the peak areas of post-extraction blank plasma spiked at concentrations of QC samples with the areas obtained by direct injection of corresponding standard solutions. Stability of wogonin in the plasma samples was established by analysis of three replicates of QC samples (low and high dose groups) under the following conditions: long-term stability at $-20\text{ }^{\circ}\text{C}$ for a whole month; short-term stability at $25\text{ }^{\circ}\text{C}$ for 6 h; in processed samples in autosampler vials for 10 h; and after three freeze-thaw cycles (-20 to $25\text{ }^{\circ}\text{C}$). The effect of dilution was evaluated for the analysis of plasma samples containing wogonin at concentrations higher than the upper limit of the standard curve by analysing six replicates of dog plasmas spiked with wogonin at 8.588 g/L and diluting with blank dog plasma to $1/100$.

Pharmacokinetic application

Six male Beagle's dogs, with the average weight of $(8.2 \pm 0.4)\text{ kg}$, were used in a randomized, self-control, and cross-over study, which involved three test periods with three types of treatments (iv route, ig administration of native drug and oral solution preparation) for each dog. One week was arranged as a washout phase among test periods in order to eliminate the influence by last administration. During each period, the dogs were fasted for 12 h before dosing and 3 h afterward, with free access to water. The dosages of wogonin were 5 mg/kg (0.5 mL/kg) for iv route and 15 mg/kg (5 mL/kg) for ig administration of native drug and 5 mg/kg (5 mL/kg) for ig administration of solution preparation. The dosing solution for iv route, prepared by dissolving the solution preparation in isotonic saline, was delivered using a 10 mL syringe via ear vein. The dosage preparation for ig approach was made of either native drug or solution preparation, in water containing 0.5% croscarmellose sodium (CMC-Na), and mixing well. All preparations were accomplished immediately

before drug administration.

A 2.0 mL volume of whole blood samples was collected from the forelimb vein prior to and at 0.083 , 0.167 , 0.5 , 0.75 , 1.0 , 1.5 , 2 , 3 , 4 , 5 , 6 , 8 , 12 , 24 , and 36 h for ig groups and 0.0 , 0.083 , 0.167 , 0.333 , 0.5 , 0.75 , 1.0 , 1.5 , 2 , 3 , 4 , 5 , 6 , 8 , and 12 h for iv group after drug administration into heparinized centrifuge tubes. Plasma was transferred out and stored at $-20\text{ }^{\circ}\text{C}$ till analysis after the centrifugation.

Results

Assay development

Finasteride was selected as the IS due to its similar chromatographic behavior and better extraction efficiency to wogonin as compared with diazepam, daidzein, and bergenin, etc. In the first quadrupole (Q1) full scan MS, wogonin and finasteride predominantly formed protonated molecular ions $[M + H]^+$ at m/z 285.0 and 373.3 , respectively. In full scan product ion MS, wogonin and finasteride produced only one fragment at m/z 270.0 and 305.3 which can be explained by the loss of a methyl group and tertiary butyl group from the corresponding precursor ion, respectively. The major fragment ions were chosen in the selected reaction monitoring (SRM) acquisition (Fig. 2).

A number of commercially available reversed phase HPLC columns (Zorbax extend C_{18} , Hypersil C_{18} , and Phenomenex Luna C_{18}) were evaluated and Phenomenex Luna C_{18} ($150\text{ mm} \times 2.0\text{ mm}$, $5\text{ }\mu\text{m}$) was chosen on the basis of superior chromatography at a flow rate of 0.2 mL/min . In terms of the mobile phase composition, methanol gives a similar response and less expensive than acetonitrile and water containing 0.1% formic acid provided good resolution with excellent peak shapes and satisfactory MS responses.

Protein precipitation as sample preparation always reduces the MS sensitivity with an increased number of subsequent injections. Moreover, if proteinization is not thorough, the impurity in sample liquid might block the LC-MS pipelines. In addition, for avoiding the high cost of solid phase extraction, different liquid-liquid extraction (LLE) conditions were evaluated including different pH values and organic extraction solvents. Wogonin could be well extracted with diethyl ether, ethyl acetate, dichloromethane, and ether-*n*-hexane ($4:1$). Among them, extraction efficiency with ethyl acetate was

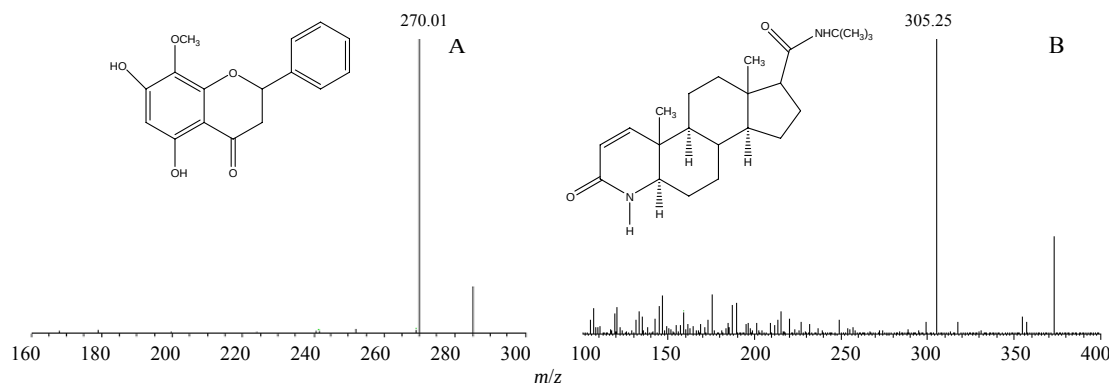


Fig. 2 Positive ion electrospray mass spectra obtained in scan mode from authentic samples of wogonin (A) and finasteride (B) with abundance of $[M + H]^+$

acceptable likewise, and similar to ether-*n*-hexane (4:1) (Chen *et al.*, 2002). Acidification of the plasma sample using 0.1 mol/L hydrochloric acid followed by extraction with ethyl acetate gave better recovery, while the result of neutralized condition with the same extraction solvent was also satisfactory. Considering the stability of metabolic and the convenience of following evaporation,

acidification was abandoned. Moreover, finasteride also results in good recovery under current condition.

Assay validation

Fig. 3 shows representative MRM chromatograms of wogonin and finasteride in dog blank plasma. The data showed there was no significant interference at the retention times of wogonin (5.73 min) or finasteride (6.37 min).

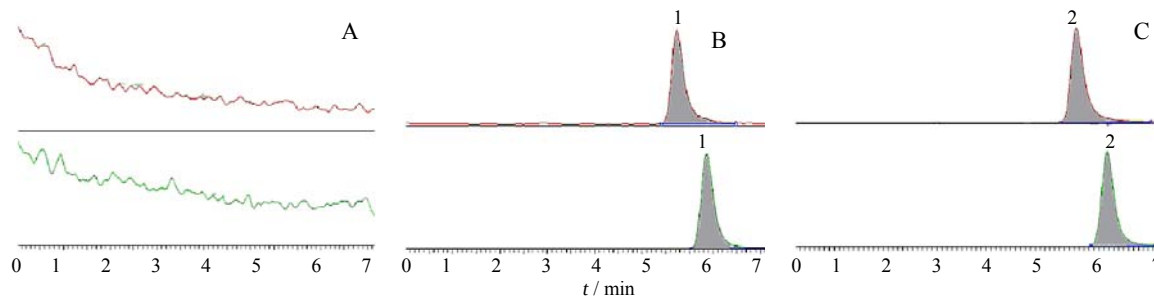


Fig. 3 Typical SIM chromatograms of blank Beagle's dog plasma (A), blank dog plasma spiked with wogonin and finasteride (B), and sample from a dog 0.5 h after iv injection of 5 mg·kg⁻¹ wogonin solution (C)

1: wogonin; 2: finasteride

Calibration curves were linear over the concentration in the range of 0.105–107.36 µg/L in dog plasma samples with correlation coefficients (r) > 0.997 and LLOQ of 0.105 µg/L. The sensitivity of this method for determining plasma concentrations of wogonin is higher than any other one ever reported. Besides, plasma diluted by 1 to 10 and 10 to 100 fold with blank matrix did not show any effect on the assay values, which allowed analysis after dilution for the samples which showed values greater than the quantifiable limits.

Analytical accuracy and precision data are shown in Table 1. Intra- and inter-day precision values, expressed as RSD, were less than 7% at all concentrations within the standard. Recovery results were all more than 84.0%, indicating the LLE efficiency was well-acceptable. Value of precision at

the LLOQ was 3.56%, with accuracy of –3.3% to –8.57%. The extraction recoveries of wogonin at 0.209, 8.588, and 85.88 ng/mL in the plasma were in the range of 84.7%–92.1%; The extraction recoveries of finasteride at 25 µg/L were in the range of 76.1%–96.2%.

Studies on matrix effects of wogonin at concentrations of 0.209, 8.588, and 85.88 µg/L gave concentrations under ±5% of nominal values. The finasteride at 25 ng/mL gave concentrations in the range of 81.2%–100.3% of the nominal value.

Wogonin was found to be stable after three freeze-thaw cycles, at ambient temperature for 6 h in plasma, in processed samples in autosampler vials for 10 h and kept in –20 °C for a whole month. All decompositions under this condition are low (< 10%) as compared to the samples being analyzed immediately.

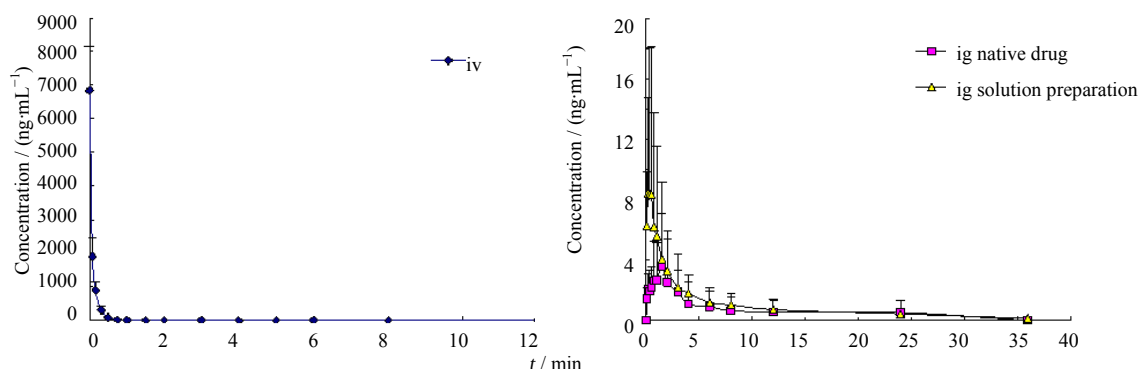
Table 1 Recovery, accuracy, and precision of analysis on wogonin in dog plasma

Spiked concentration / ($\mu\text{g}\cdot\text{L}^{-1}$)	Recovery		Intra-day			Inter-day		
	($\bar{X} \pm s$) / %	RSD / %	Measured concentration / ($\mu\text{g}\cdot\text{L}^{-1}$)	RE / %	RSD / %	Measured concentration / ($\mu\text{g}\cdot\text{L}^{-1}$)	RE / %	RSD / %
0.209	92.10 $\pm$ 6.50	7.00	0.21 $\pm$ 0.01	-1.36	6.67	0.22 $\pm$ 0.02	3.16	9.22
8.588	91.70 $\pm$ 1.20	1.30	8.96 $\pm$ 0.53	4.34	5.91	8.74 $\pm$ 0.51	1.77	5.85
85.89	84.70 $\pm$ 1.10	1.30	82.48 $\pm$ 6.30	-3.97	7.64	82.63 $\pm$ 4.37	-3.79	5.29

Pharmacokinetic and biopharmaceutical study in Beagle's dogs

Plasma concentrations of wogonin in dog after ig administration of native drug, solution preparation, and iv injection of solution were determined respectively based on the validated LC-ESI-MS method. A threesession crossover study was involved to evaluate pharmacokinetic parameters. Fig. 4 shows the mean plasma concentration-time profile in six Beagle's dogs following the three types of treatments. The pharmacokinetic parameters of pivotal moment

statistics were calculated using DAS software (Ver 2.0, Medical College of Wannan, China) and results for three treatments are delineated in Table 2. Absolute bioavailability was determined by dividing the dose-normalized area under the plasma concentration-time curve (AUC) resulting from ig administration by that from iv injection, which was expressed as $(\text{AUC}_{\text{ig}}) \times (\text{Dose}_{\text{iv}}) / (\text{AUC}_{\text{iv}}) \times (\text{Dose}_{\text{ig}}) \times 100\%$. The absolute bioavailabilities of native drug and solution preparation were $(0.59 \pm 0.35)\%$ and $(3.65 \pm 2.00)\%$, respectively.

**Fig. 4** Mean wogonin plasma concentration-time profile in six Beagle's dogs after three types of treatments**Table 2** Mean pharmacokinetic parameters for wogonin in dogs after three types of treatments ($\bar{X} \pm s$, $n = 6$)

Parameters	Unit	iv ($5 \text{ mg}\cdot\text{kg}^{-1}$)	ig native drug ($15 \text{ mg}\cdot\text{kg}^{-1}$)	ig solution preparation ($5 \text{ mg}\cdot\text{kg}^{-1}$)
t_{max}	h	—	0.7 ± 0.3	0.3 ± 0.2
C_{max}	$\mu\text{g}\cdot\text{L}^{-1}$	6838.7 ± 1322.1	2.5 ± 1.1	7.9 ± 3.3
$\text{MRT}_{(0-t)}$	h	0.12 ± 0.3	3.6 ± 0.2	5.3 ± 3.0
$t_{1/2z}$	h	5.9 ± 6.9	10.1 ± 8.9	5.1 ± 2.6
AUC_{0-t}	$\mu\text{g}\cdot\text{h}\cdot\text{L}^{-1}$	629.7 ± 111.8	7.1 ± 2.0	21.0 ± 3.2
$\text{AUC}_{0-\infty}$	$\mu\text{g}\cdot\text{h}\cdot\text{L}^{-1}$	631.9 ± 112.8	10.8 ± 5.0	21.9 ± 4.6

The concentration of wogonin in plasma by iv route declined sharply (over 1/6000) in post-dose 2 h. The parent drug can not be detected after 12 h. t_{max} and C_{max} of native drug and solution preparation ig administered were as follows: (0.7 ± 0.3) and (0.3 ± 0.2) h and (2.5 ± 1.1) and (7.9 ± 3.3) $\mu\text{g}/\text{L}$, respectively, and mean residence time (MRT) of ig administration was

obviously prolonged against iv route.

Result differences between native drug and solution preparation were rather amazing. C_{max} and AUC of solution preparation were six times over those of native drug, which led to an approximate sextuple absolute bioavailability. Elimination half life of the two preparations showed no significant

difference ($P > 0.05$). However, $t_{\max}$ of solution preparation was shortened.

Discussion

Previously most of the work on quantification of flavonoids compounds was operated in SIM mode of negative-ions of $[M - H]^-$. As far as wogonin is concerned, in our laboratory which supplied by methanol-water containing 0.1% formic acid (70:30) as the mobile phase with protonated molecular ions $[M + H]^+$ at m/z 285.0, it was found to exhibit more sensitive and well-reproducible results. Besides, the responses of wogonin to ESI were evaluated by recording the mass spectra scanned at m/z 100–400 in both positive and negative ionization modes with different mobile phase conditions. The positive mode yielded strong signals of $[M + H]^+$ of wogonin; On the contrary, no satisfied signals were obtained in negative mode under the same signal intensity. So protonated adducts of molecular ions were chosen for detection in the SIM mode at m/z 285.0→270.0 for wogonin and 373.3→305.3 for finasteride. The sensitivity (LLOQ 0.105 $\mu\text{g/L}$) of this method for determining plasma concentrations of wogonin is higher than any other one ever reported. Furthermore, the determination method was also successfully used in the assay of the bioactive metabolites (wogonin- β -glucuronide) in the plasma of the dogs (data not shown).

Wogonin has very poor solubility in water, thus solubility extent in intestinal tract may be most important to its absorption. Compared with iv injection, the bioavailability for the ig administration of wogonin is lower. To improve the dissolution rate of wogonin and enhance its absorption rate from ig dosage form, we have explored the feasibility of employing salification technology (wogonin arginine). As a result, solution preparation improved bioavailability of wogonin evidently. Wogonin in the solution formulation (wogonin arginine) seems to be better dissolved in the intestinal juice and well transported across the intestinal epithelial cells.

Conclusion

In this paper, a simple and rapid LC-ESI-MS method is validated to be accurate, precise, and robust for determination of wogonin in dog plasma. The bioavailability of wogonin is markedly improved, after which wogonin is made into wogonin arginine.

Acknowledgements

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References

- Chen XY, Wang HY, Du Y, Zhong DF, 2002. Quantitation of the flavonoid wogonin and its major metabolite wogonin-7 β -D-glucuronide in rat plasma by liquid chromatography tandem mass spectrometry. *J Chromatogr B* 775(2): 169-178.
- Du Y, Chen XY, Yang HY, Zhong DF, 2002. Determination of wogonin in rat plasma by liquid chromatography-tandem mass spectrometry. *Acta Pharm Sin* 37(5): 362-366.
- Guo Q, Zhao L, You Q, Yang Y, Gu H, Song G, Lu N, Xin J, 2007. Anti-hepatitis B virus activity of wogonin *in vitro* and *in vivo*. *Antiviral Res* 74(1): 16-24.
- Hui KM, Huen MS, Wang HY, Zheng H, Sigel E, Baur R, Ren H, Li ZW, Wong JT, Xue H, 2002. Anxiolytic effect of wogonin, a benzodiazepine receptor ligand isolated from *Scutellaria baicalensis* Georgi. *Biochem Pharmacol* 64(9): 1415-1424.
- Kim YH, Jeong DW, Paek IB, Ji HY, Kim YC, Sohn DH, Lee HS, 2006. Liquid chromatography with tandem mass spectrometry for the simultaneous determination of baicalein, baicalin, oroxylin A and wogonin in rat plasma. *J Chromatogr B* 844(2): 261-267.
- Lee DH, Kim C, Zhang L, Lee YJ, 2008. Role of p53, PUMA, and Bax in wogonin-induced apoptosis in human cancer cells. *Biochem Pharmacol* 75(10): 2020-2033.
- Lu N, Gao Y, Ling Y, Chen Y, Yang Y, Gu HY, Qi Q, Liu W, Wang XT, You QD, Guo QL, 2008. Wogonin suppresses tumor growth *in vivo* and VEGF-induced angiogenesis through inhibiting tyrosine phosphorylation of VEGFR2. *Life Sci* 82(17-18): 956-963.
- Park HG, Yoon SY, Choi JY, Lee GS, Choi JH, Shin CY, Son KH, Lee YS, Kim WK, Ryu JH, Ko KH, Cheong JH, 2007. Anticonvulsant effect of wogonin isolated from *Scutellaria baicalensis*. *Eur J Pharmacol* 574(2-3): 112-119.
- Tsai TH, Chou CJ, Tsai TR, Chen CF, 1996. Determination of wogonin in rat plasma by HPLC and its pharmacokinetics application. *Planta Med* 62(3): 263-266.
- Zhang HW, Yang Y, Zhang K, Qiang L, Yang L, Yang L, Hu Y, Wang XT, You QD, Guo QL, 2008. Wogonin induced differentiation and G1 phase arrest of human U-937 leukemia cells via PKC δ phosphorylation. *Eur J Pharmacol* 591(1-3): 7-12.