Oral Pharmacokinetic Comparison of Different Genistein Tablets in Beagle Dogs

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An accurate and sensitive analytical method has been developed for the quantification of genistein in dog plasma using high-performance liquid chromatography/tandem mass spectrometry. Genistein and diclofenac (internal standard) were extracted from the plasma sample using methyl tert-butyl ether and then separated on an Agilent Zorbax C18 column using a gradient mobile phase. The detector was a Q-trap mass spectrometer with an electrospray ionization interface operating in the multiple reaction monitoring mode. The assay was linear over the concentration range of 0.10–500 ng/mL with a lower limit of quantification of 0.10 ng/mL. The method was shown to be reproducible and reliable, with inter-day and intra-day accuracy and precision within ±15%. The method was successfully applied to a pharmacokinetic comparison of immediate and extended release tablets in beagle dogs after oral administration. Immediate release tablets showed rapid genistein absorption, with mean peak concentration of 726 ± 199 ng/mL reached at 0.2 ± 0.0 h. However, the absorption of genistein was considerably slower and more sustainable for extended release tablets. The relative bioavailability of the extended release tablet over the immediate release formulation was estimated to be 134 ± 47% based on the AUC\text{inf} values from non-compartmental analysis.

Introduction

Genistein (4',5,7-trihydroxyisoflavone), the predominant phytoestrogen found in soy-based products, is an isoflavone with structural features similar to those of 1β-estradiol (1). Numerous activities have been reported from in vitro studies, including inhibition of tyrosine protein kinase, inhibition of topoisomerase II, inhibition of cellular proliferation, stimulation of apoptosis and interactions with estrogen receptors (2). Recently, it has been established that genistein has anticancer effects, such as the prevention of breast and prostate cancer (3–7). A diet rich in genistein has been associated with lower incidences of both prostate and breast cancer, particularly for people in southeast Asia, where soy and its constituents, such as genistein, are consumed at high levels. Therefore, many formulations or health foods containing genistein have been developed for preventing or lowering the incidence of human cancers.

When administered orally, genistein may be rapidly absorbed and cleared in animals and human, just like other isoflavones (8). To confirm the preceding hypothesis and thus further improve the compliance and pharmacokinetic performance of genistein, formulations of one immediate and one extended release tablet were prepared in a laboratory, and the extended release tablet was designed to obtain the sustained release of genistein.

Several analytical methods have been reported for the analysis of genistein in various sample matrices, including liquid chromatography with ultraviolet detection (LC–UV) (9–12), radio-labeled assay (13) and gas chromatography–mass spectrometry (GC–MS) (14). The high-performance liquid chromatography (HPLC–UV) method is a general analytical method for the determination of many isoflavones in biological fluids, however, it shows lower sensitivity than LC–electron capture detection (ECD), GC–MS or LC–MS. GC–MS has been utilized for the determination of isoflavones. However, derivatization prior to GC–MS analysis is often required for the determination of genistein. In addition, LC–tandem mass spectrometry (LC–MS–MS) has been used to identify and quantify genistein in some biological fluids (15–18). Nevertheless, methodological improvements for the quantitative determination of genistein in biological fluids are still needed in pre-clinical trials for pharmacokinetic and efficacy research.

This paper describes the development and validation of analytical methods based on LC–MS–MS for determining plasma genistein in beagle dogs that had been administered immediate and extended release tablets. The sensitivity of LC–MS–MS detection in combination with methyl tert-butyl ether (MTBE) extraction serves to provide additional confidence over previous LC–MS methods regarding the accuracy and precision of the determinations by directly providing quality control and assurance information [e.g., retention times and recoveries of the internal standard (IS)] in every sample throughout large sample sets.

In addition, the sample preparation is much more efficient than that required for GC–MS analysis. The validated LC–MS–MS method was successfully applied for a pharmacokinetic comparison study of genistein after oral administration of immediate and extended release tablets to beagle dogs.

Experimental

Chemicals and reagents

The drug substance of genistein was supplied by Shenyang Pharmaceutical University (purity >99.3%; Shenyang, China). Reference standards of genistein and diclofenac (used as IS) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC-grade methanol was purchased from Yuwang Chemical Co. (Shandong, China). Distilled water, prepared by a Milli-Q water purification system from Millipore (Molsheim, France),
was used throughout the study. All other chemicals were of analytical grade. Blank dog plasma (drug-free and anticoagulated with heparin sodium) was prepared in the laboratory.

**Immediate and extended release tablets**

The immediate release (IR) tablet is a coated tablet with 50 mg genistein as the active ingredient. At least 80% of the dose is released within 30 min (conditions: water, 37°C, United States Pharmacopeia (USP) paddle, 50 rpm). The IR tablet shows fast disintegration within 30 s in water at 37°C. In contrast, the extended release (ER) tablet is a coated matrix formulation, which releases the 50 mg of genistein gradually over 12 h under the same conditions as described previously. The *in vitro* dissolution is specified with 20–40% release within 1 h, 45–65% within 4 h, and 80–100% within 12 h. Figure 1 shows the *in vitro* dissolution profiles of these two formulations.

**Stock solutions**

A stock solution of genistein was prepared in methanol at a concentration of 1.0 mg/mL. Standard solutions (1.00, 2.00, 5.00, 20.0, 100, 500, 2,000 and 5,000 ng/mL) and quality control (QC) solutions (2.00, 100 and 4,000 ng/mL) were prepared by serial dilution of the stock solution with methanol. A stock solution of diclofenac (1.0 mg/mL) was also prepared in methanol and then diluted with methanol to a final concentration of 1,000 ng/mL. All solutions were stored at 4°C and used within one month of preparation.

**LC–MS–MS analysis**

A Shimadzu LC-20AD series HPLC system (Shimadzu, Japan) coupled to an Applied Biosystems Sciex 4000 Q-trap mass spectrometer (Concord, Ontario, Canada) via an electrospray ionization (ESI) source was used for analysis. Applied Biosystems Analyst software package, version 1.5.1, was used to control the LC–MS–MS system and for data acquisition and processing. Genistein and IS were separated on an Agilent Zorbax C18 column (4.6 × 50 mm i.d., 3.5 μm particle size; Agilent Technologies, Palo Alto, CA), maintained at 20°C. The mobile phase consisted of water containing 0.1% formic acid (A) and methanol containing 0.1% formic acid (B) delivered at a flow rate of 0.40 mL/min. The gradient was as follows: 0 min 10% B, 0.6 min 10% B, 1.5 min 95% B, 3.5 min 95% B, 3.51 min 10% B and 5 min stop. A two-phase switching valve was used to divert the pre-eluent from entering the ion source.

The mass spectrometer was operated in the negative ESI mode with multiple reaction monitoring (MRM) at unit resolution. Nitrogen was used as the nebulizer, heater and curtain gas, as well as the collision activation dissociation (CAD) gas. The precursor-to-product ion transitions were monitored at \(m/z\) 268.9 → 158.8 for genistein and at \(m/z\) 293.9 → 249.8 for diclofenac. Mass spectrometer instrumental parameters were tuned to maximize the generation of precursor and fragment ions by infusion of a solution of genistein and IS into the ESI source at 25 μL/min. Optimum parameters were as follows: nebulizer (GS1), heater (GS2) and curtain gas flow rates, 45, 60 and 10 units, respectively; ionspray needle voltage, –4,500 V; heater gas temperature, 550°C; collision gas (N₂); medium; declustering potential, –95 V for genistein and –30 V for diclofenac; collision energies, –40 eV for genistein and –16 eV for diclofenac.

**Sample preparation**

After thawing at room temperature for approximately 30 min and vortexing for 30 s, aliquots of 50 μL plasma were mixed with 5 μL of methanol (or standard or QC solution) and 10 μL of IS solutions (1,000 ng/mL diclofenac in methanol) and 500 μL of MTBE. After vortexing for 1 min and then centrifugation at 5,000 g for 10 min, aliquots of 300 μL supernatants were removed and evaporated to dryness at 40°C under a gentle stream of nitrogen. The residues were dissolved in 100 μL of the mixture of methanol and water (50:50, v/v), and then transferred to HPLC vials. A volume of 10 μL of this solution was then injected onto the column. Samples with concentrations exceeding that of the upper limit of quantification (ULOQ, 500 ng/mL) were diluted with blank dog plasma before analysis.

**Validation**

Linear calibration curves in dog plasma were generated by plotting the peak area ratio of genistein to the IS versus the known plasma genistein concentrations over the range of 0.10–500 ng/mL. Slope, intercept and coefficient of determination values were estimated using least square regression analysis.

QC plasma samples containing low, medium and high genistein concentrations were used to evaluate the precision and accuracy of the assay method. The intra-day assay precision and accuracy were obtained by analyzing six replicates of the QC samples in duplicate using a calibration curve constructed on the same day. The inter-day assay precision and accuracy were obtained by analyzing six QC samples in duplicate using calibration curves constructed on three different days. Intra-day and inter-day precisions of the method were expressed by \([\text{standard deviation}]/(\text{mean concentration})\) × 100. Accuracy of the method was expressed by \([\text{mean measured concentration} – \text{nominal concentration}]/(\text{nominal concentration})\) × 100.
100. The mean values and relative standard deviation (RSD) for QC samples at three concentration levels were calculated using a one-way analysis of variance (ANOVA). The assay precision was reflected by the RSD (%) and the assay accuracy was reflected by the relative percentage error from the theoretical drug concentrations. The lower limit of quantification (LLOQ) was defined as the lowest concentration on the calibration curve with acceptable precision (RSD ≤ 20%) and accuracy [relative error (RE) within ±20%].

The extraction recoveries of genistein from dog plasma (expressed as a percentage) were calculated as the ratio of the slope of a calibration curve for genistein in spiked plasma to that in spiked mobile phase. Matrix effects from endogenous substances present in extracted dog plasma may cause ion suppression or enhancement of the signal. Matrix effects for genistein were evaluated by comparing peak areas of post-extraction blank plasma spiked at concentrations of QC samples with the areas obtained by direct injection of corresponding standard solutions.

The stability of genistein in dog plasma was investigated at three QC levels. Stability tests of the analyte were performed on six replicates of three QC concentrations after (i) three freeze (−20°C) and thaw cycles, (ii) storage of reconstituted extract at 20°C for 24 h and (iii) storage at −80°C for a month, respectively.

Pharmacokinetic study

Six beagle dogs (three males and three females; Laboratory Animal Center of Beijing Vital River, Beijing, China) weighing 10 to 12 kg were used in a randomized, two-period, crossover study, with a wash period of two weeks. The dogs were housed under standard conditions and had ad libitum access to water and a standard laboratory diet (free of genistein, identified using the same LC–MS-MS method). Food and water was available ad libitum throughout the experiments. The beagle dogs were orally administrated with genistein at 100 mg (two tablets for each dog, 50 mg/tablet, approximately 10 mg/kg). Serial blood samples (0.5 mL) were collected at predose, 0.17, 0.50, 1.0, 2.0, 4.0, 8.0, 12, 24, 36 and 48 h post-dose from the foreleg vein. Plasma was separated by centrifugation at 5,000 g for 5 min and stored frozen at −20°C until analysis.

Pharmacokinetic parameters, including half-life (t1/2), maximum plasma time (tmax) and concentration (Cmax), area under concentration–time curve (AUClast and AUCinf) and mean residence time (MRT) of genistein were analyzed by the non-compartmental method using WinNonlin Version 5.1 (Pharsight Corporation, Mountain View, CA). All results were expressed as arithmetic mean ± standard deviation (SD).

The pharmacokinetic parameters were analyzed by Student’s t-test. A probability level of P<0.05 was defined as statistically significant.

Results and Discussion

Method development

Regarding mass spectrometer detection, both genistein and diclofenac produced strong signals in the negative ion mode due to the presence of hydroxyl group and/or carboxyl group in their structures. The ion spray voltage was limited to -4,500V to reduce in-source dissociation and the source temperature was increased to 550°C to improve the response of genistein. Other parameters were adjusted appropriately to optimize ionization. Full-scan product ion spectra of [M-H]− ions and fragmentation pathways of genistein and diclofenac are shown in Figure 2. The transition m/z 268.9 → 158.8 was chosen for quantification of genistein and m/z 293.9 → 249.8 was used as the qualifier.

In general, matrix effects are a significant problem in LC–MS-MS analysis of biological samples, but in this assay, comparison was performed using genistein neat solution in methanol and post-extraction blank plasma sample spiked with genistein stock solution. No significant signal suppression or enhancement was found under the present conditions.

An IS is usually required in LC–MS-MS analysis with similar retention behaviors, recoveries and ionization efficiencies as those of the analyte. Daidzein and chrysin, structure analogues of genistein, were first considered to be IS, but there was some interference in blank dog plasma, which may have resulted from the standard diet given to the beagle dogs. Finally, diclofenac was selected as the IS because its chromatographic behavior and extraction efficiency were similar to those of genistein, and there was no interference from the standard diet.

Many commercially available reversed-phase HPLC columns and various mobile phases were evaluated for chromatographic behavior and the ionization responses of genistein and IS. A gradient delivery of a mixture of water (containing 0.1% formic acid) and methanol (containing 0.1% formic acid) gave the best response and retention. An Agilent XDB-C18 column was chosen for quantification of genistein and

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Full-scan product ion spectra of [M-H]− ions and fragmentation pathways: genistein (A); diclofenac (B).
(4.6 × 50 mm i.d., 5 μm) with gradient delivery offered satisfactory chromatography with minimal matrix effects.

In terms of sample preparation, genistein and diclofenac are sufficiently lipophilic to allow a one-step liquid–liquid extraction (LLE) procedure with satisfactory recovery (>70%).

**Method validation**

The detection of genistein and diclofenac by MRM mode was highly selective, and no co-eluting peaks were observed in the positions of genistein and diclofenac, even under several different separation conditions, which proved the peak homogeneity of the analyte and IS. Typical chromatograms are shown in Figure 3. Under current conditions, the analytes were free of interference from endogenous substances and tablet excipients, and yielded retention times of 2.91 and 3.09 min for genistein and IS (diclofenac), respectively. The run time was set at 5 min because full chromatographic separation was necessary to avoid matrix effects. The standard curve was linear in the range 0.10–500 ng/mL with an LLOQ of 0.10 ng/mL. A typical equation of the standard curve was \( y = 0.015x + 0.0003, \quad r^2 = 0.9963 \). Precision and accuracy were satisfactory at the three concentration levels studied (Table I) (19).

Several extraction solvents (dichloromethane, ethyl acetate and MTBE) were tested to optimize recovery. The best recovery for genistein was obtained with MTBE. Recoveries of

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**Figure 3.** Typical chromatograms: blank plasma (A); blank plasma spiked with genistein (0.10 ng/mL) and IS (200 ng/mL) (B); a dog plasma sample collected at 1 h after an oral administration of ≈10 mg/kg genistein extended release tablets (calculated genistein concentration: 12.6 ng/mL, 10-fold dilution using blank dog plasma) (C). Peak I, genistein; Peak II, diclofenac.
genistein spiked at 0.20, 10.0 and 400 ng/mL were 81.0, 82.1 and 82.4%, respectively, and recovery of the IS was approximately 71%. The precision for recoveries (in terms of RSD) was all within 15%. The stability of genistein in drug-free plasma was found to be satisfactory (RE within ±15%; Table II) under all conditions. Genistein was largely stable, both in stock solutions kept at 4°C and in the mobile phase under autosampler conditions for 12 h (>90% drug remaining).

Pharmacokinetic comparison
Mean plasma concentration-time profiles of genistein in dogs after oral administration of immediate and extended release tablets are shown in Figure 4. The plasma genistein pharmacokinetic parameters with arithmetic mean and standard deviation are summarized in Table III.

For oral administration of IR tablets, the primary pharmacokinetic parameters of \( T_{\text{max}} \), \( C_{\text{max}} \), \( t_{1/2} \), MRT, AUC\(_{\text{last}}\) and AUC\(_{\text{inf}}\) were estimated to be 0.2 ± 0.0 h, 7.26 ± 19 ng/mL, 4.3 ± 1.0 h, 3.7 ± 0.7 h, 1.245 ± 442 h/ng/mL and 1.264 ± 448 h/ng/mL, respectively. IR tablets showed rapid genistein absorption with mean peak concentration of 726 ± 19 ng/mL reached at 0.2 ± 0.0 h.

Two factors probably contributed to the rapid absorption of the IR preparation in dogs: a pharmaceutical formulation specifically designed for rapid disintegration of the tablets and good absorption of genistein from the gastrointestinal gut.

For oral administration of ER tablets, the primary pharmacokinetic parameters of \( T_{\text{max}} \), \( C_{\text{max}} \), \( t_{1/2} \), MRT, AUC\(_{\text{last}}\) and AUC\(_{\text{inf}}\) were estimated to be 1.7 ± 0.5 h, 1.47 ± 16 ng/mL, 4.6 ± 0.2 h, 7.9 ± 0.4 h, 1.518 ± 134 h/ng/mL and 1.519 ± 134 h/ng/mL, respectively. Significant differences in genistein pharmacokinetic parameters were observed in the values of \( T_{\text{max}} \), \( C_{\text{max}} \) and MRT \((P < 0.05)\) between the immediate and extended release tablets. The absorption of genistein was considerably slower \((T_{\text{max}} \text{ 1.7 h})\) and more sustainable for ER tablets; it was observed that the peak plasma concentration accounting for ~19% of maximal immediate absorption was obtained at 1.7 h post-dose and maintained for ~10 h. The relative bioavailability of the ER tablet over the immediate release formulation was estimated to be 134 ± 47% based on the AUC\(_{\text{inf}}\) values from non-compartmental analysis. The ER tablet produced a lower but more sustainable genistein exposure, allowing for an extended dosing interval that could improve compliance.

In summary, orally administered immediate and extended release genistein tablets exhibited significantly different plasma concentration-time profiles that were consistent with their corresponding in vitro dissolution profiles. The IR tablet produced rapid genistein absorption, whereas absorption rates were considerably lower and sustained for the ER tablets. The AUC in dogs did not significantly differ between the two release types of formulations, and the relative bioavailability of IR over ER was calculated to be 134 ± 47% by non-compartmental analysis.

Conclusion
An accurate and sensitive LC–MS–MS method has been developed for the determination of genistein in dog plasma and shown to be suitable to investigate genistein pharmacokinetics after oral route of administration. The method was successfully applied to a pharmacokinetic comparison of immediate and

![Figure 4. Mean plasma concentration-time profile of genistein determined by LC–MS–MS method after oral administration of ~10 mg/kg genistein immediate and extended release tablets to beagle dogs. Each point represents the mean ± SD \((n = 6)\).](image-url)
extended release tablets in beagle dogs after oral administration. The extended release tablet showed more sustainable absorption than the immediate release formulation, as indicated by the pharmacokinetic results of longer $T_{\text{max}}$ and MRT. Both types of formulations offered similar genistein exposure.

References