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Validated liquid chromatography-tandem mass spectrometric method for the quantitative determination of daidzein and its main metabolite daidzein glucuronide in rat plasma

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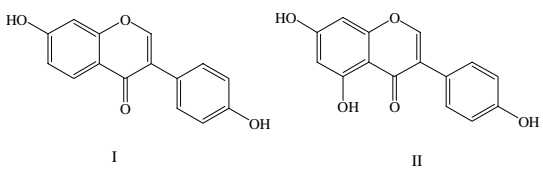
A highly selective and sensitive liquid chromatographic-tandem mass spectrometric (LC-MS-MS) method was developed and validated to determine daidzein and its main metabolite daidzein glucuronide in rat plasma. The analytes and internal standard genistein were extracted from plasma samples by *n*-hexane-diethyl ether (1 : 4, v/v), and separated on a C_{18} column. The mobile phase consisted of acetonitrile-water-formic acid (80 : 20 : 1, v/v/v). Detection was performed on a triple quadrupole tandem mass spectrometer by selected reaction monitoring (SRM) mode via atmospheric pressure chemical ionization (APCI) source. The method has a limit of quantification of 0.24 ng/ml. The linear calibration curves were obtained in the concentration range of 0.24–1000 ng/ml. The intra- and inter-day precisions were lower than 13.2% in terms of % RSD. The accuracy ranged from −0.5% to 2.4% in terms of % RE (relative error). This method was successfully applied to the determination of plasma concentration of daidzein and its main metabolite daidzein glucuronide in rats after an oral administration of 20 mg/kg daidzein.

1. Introduction

Daidzein (7-hydroxy-3-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one) is a naturally occurring isoflavone mainly present in leguminous plants, especially in soybeans, soy foods and *Pueraria lobata* Ohwi (Leguminosae). Several epidemiological studies in humans have suggested that daidzein intake was inversely associated with the incidence of hormone-dependent diseases, especially breast and prostate cancer (Pereira et al. 1994). In addition to its putative anticarcinogenic effects, daidzein has also been investigated as an antihyperlipidemic agent and a therapeutic substance to combat osteoporosis (Cassidy et al. 1994; Anderson et al. 1999). Daidzein tablets and capsules recommended dose 300 mg) have now been produced as drugs in China for the therapy of hypertension, coronary heart disease and cerebro-thrombus diseases.



I II

daidzein (I) and genistein (II, internal standard)

After oral administration, daidzein is subject to glucuronidation at the 7- or 4′-position, and daidzein glucuronide is its main metabolite (Brian et al. 2001). Daidzein glucuronide has usually been determined by an indirect method as follows: firstly, free daidzein (unconjugated) in biological samples was determined, subsequently total daidzein (unconjugated plus conjugated) was determined after enzymatic hydrolysis with β-glucuronidase, then total minus free daidzein yielded the amount of daidzein glucuronide (Holder et al. 1999; Daniel et al. 1999).

A wide variety of analytical techniques have been applied to the quantification of daidzein in biological fluids, including high-performance liquid chromatography-UV detection (HPLC-UV) (Brian et al. 2001), liquid chromatography-mass spectrometry (LC-MS) (Holder et al. 1999; Daniel et al. 1999; Nathan et al. 2002; Mary et al. 2001; Adrian et al. 2002), gas chromatography-mass spectrometry (GC-MS) (Adlercreutz et al. 1994; Tekel et al. 1999), enzyme-linked immunosorbent assay (ELISA) (Creeke et al. 1998) and time-resolved fluoroimmunoassay (TR-FIA) (Uehara et al. 2000; Wang et al. 2000). The HPLC-UV method had relative poor sensitivity and was time-consuming in chromatographic separation. GC-MS, ELISA and TR-FIA methods require complicated preparations of chemical or biological derivatives. Most of the existing LC-MS methods were applied to determine traces of daidzein in biological samples after ingestion of foods or other mixtures containing daidzein, but not daidzein as a therapeutic drug. Furthermore they were not fully validated methods. Therefore, the methodological improvements for the quantitative analyses of daidzein and its main metabolite daidzein glucuronide in biological fluids are still needed for preclinical trials aimed at defining single and multi-dose safety, efficacy and pharmacokinetic profiles.

In the present paper, a highly selective and sensitive LC-MS-MS method was developed and validated for the determination of daidzein and its main metabolite daidzein glucuronide in rats after an oral administration of 20 mg/kg daidzein.

2. Investigations and results

2.1. Mass spectrometry

By positive APCI mode, the analyte and internal standard formed predominantly protonated molecules $[M + H]^+$ in full scan spectra. To determine daidzein in the SRM mode, full scan and product ion spectra of daidzein and internal standard were investigated under the present HPLC conditions. Fig. 1 shows product ion spectra of $[M + H]^+$ ions of daidzein and genistein. Several fragment ions were observed in the product ion spectra. The major fragment ions at m/z 199 and 153 were chosen in the SRM acquisition for daidzein and genistein, respectively.

2.2. Method validation

Selectivity was assessed by comparing the chromatograms of six different batches of blank rat plasma with the corresponding spiked plasma. Fig. 2 shows typical chromatograms of a blank plasma sample, a blank plasma sample spiked with daidzein at the LLOQ and genistein, and a plasma sample from a Wistar rat 30 min after an oral administration. No significant interferences from endogenous substances with analyte or genistein were detected. Typical retention times for daidzein and genistein were 2.7 and 2.9 min, respectively.

Calibration standards were prepared by spiking 50 µl of the appropriate standard solutions of daidzein to 50 µl of blank rat plasma. Plasma concentrations were 0.24, 0.50, 1.50, 20.0, 100, 500, 1000 ng/ml for daidzein. The linear regression of the peak area ratios versus concentrations was fitted over the concentration range of 0.24–1000 ng/ml in rat plasma. A typical equation of the calibration curve was as follows: $y = 3.888 \times 10^{-4} + 6.030 \times 10^{-4}$ x ($r = 0.9981$), where y is the peak area ratio of daidzein to genistein, and x is the concentration of daidzein.

The present assay method had an LLOQ of 0.24 ng/ml with an accuracy of 14.3% and a precision of 12.7% ($n = 5$), which was sufficient for monitoring daidzein plasma levels over a period of 48 h after a single oral administration.

Table 1 summarizes the intra- and inter-day precision and accuracy for daidzein from QC samples. In this assay, the intra- and inter-day precisions ranged from 3.4% to 7.1% and from 6.3% to 13.2% for each QC level, respectively. The accuracy ranged from −0.5% to 2.4%. The results, calculated with a one-way ANOVA, indicated that the values were within the acceptable range; thus the method is accurate and precise (Shah et al. 2000).

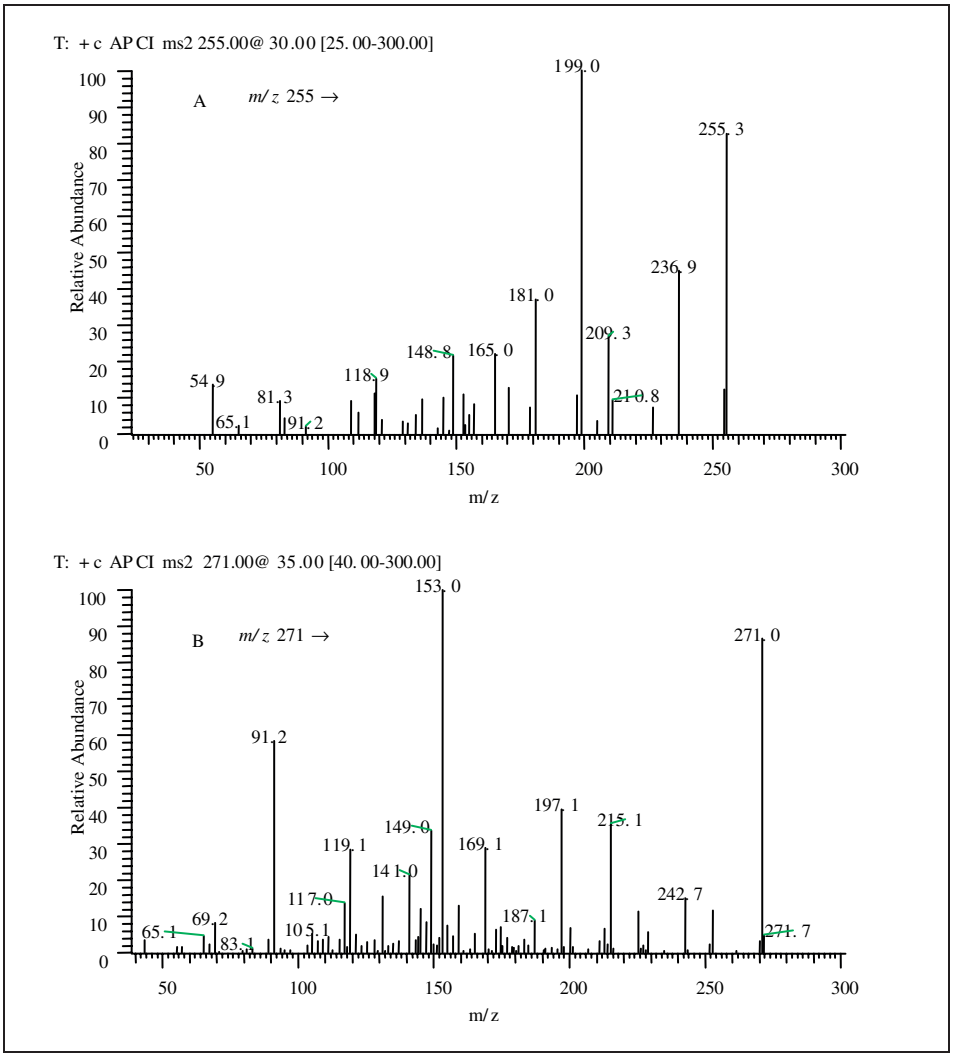


Fig. 1:
Full-scan product ion spectra of $[M + H]^+$ of (A) daidzein and (B) genistein

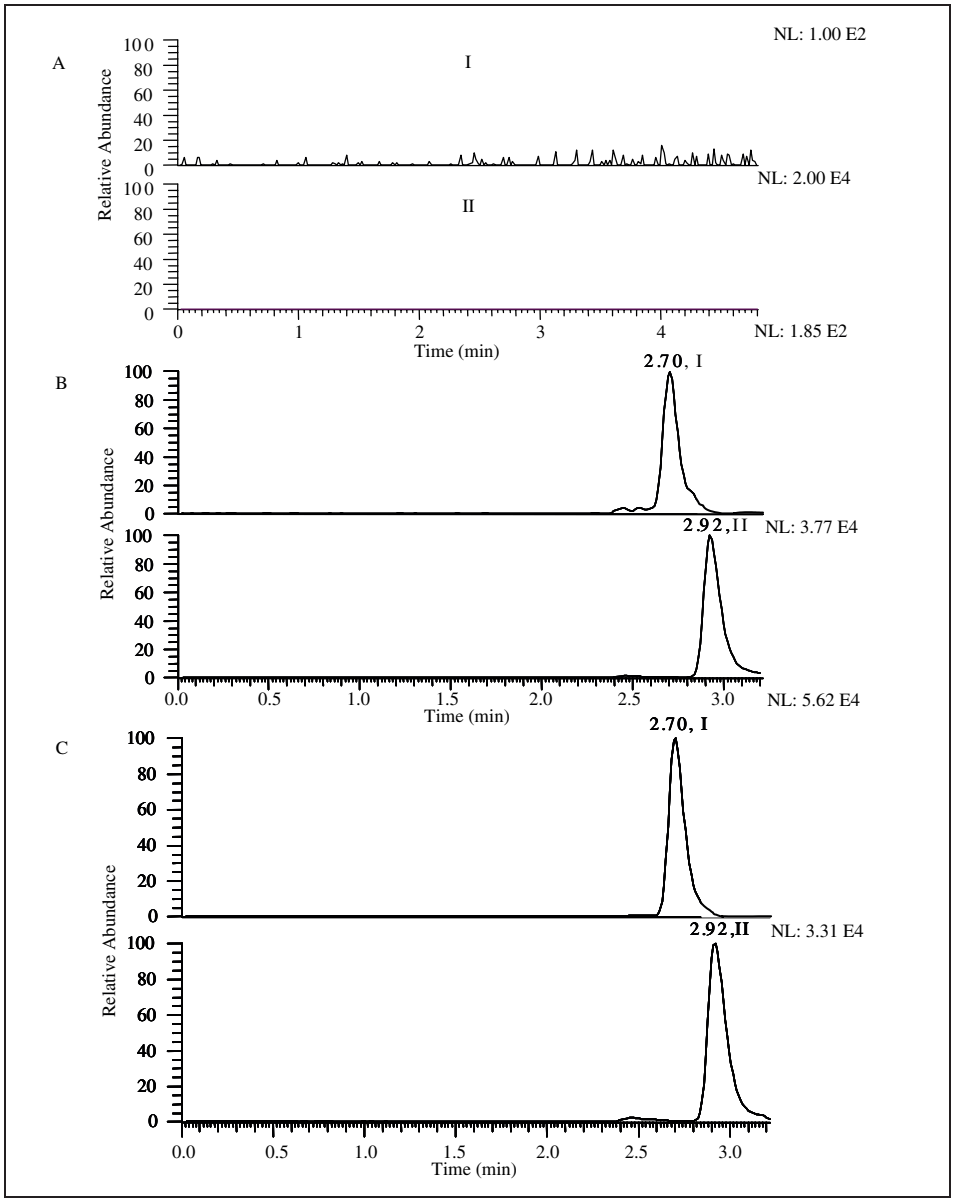


Fig. 2:
Representative SRM chromatograms of daidzein plasma samples determined by LC-MS-MS method. (A) A blank plasma sample; (B) A blank plasma sample spiked with daidzein at the LLOQ of 0.24 ng/ml and genistein (I.S., 50 µg/ml); (C) rat plasma sample collected at 30 min after oral administration of daidzein (20 mg/kg) to a Wistar rat. Peak I, daidzein; peak II, genistein

Table 1: Precision and accuracy of the LC-MS-MS method at determining daidzein in rat plasma

Concentration (ng/ml)		RSD (%)		RE (%)
Added	Found	Intra-day	Inter-day	
0.50	0.51	7.1	10.7	2.0
50.0	51.2	6.5	13.2	2.4
800.0	796.0	3.4	6.3	0.5

Table 2: Stability of daidzein in rat plasma (n = 6)

	Daidzein concentration (ng/ml)		
	0.50	50.0	800.0
Storage stability ($\leq$ 20 °C, relative error, %)			
0 day	2.6	−1.1	2.9
30 days	−4.7	−5.8	3.5
Freeze-thaw stability (relative error, %)			
0 cycle	−0.4	2.4	−2.1
3 cycles	−2.7	−5.6	−6.7
Processed plasma samples at room temperature (relative error, %)			
0 h	0.7	−3.2	0.3
24 h	−2.6	1.9	−3.8

The extraction recovery of daidzein, determined at three concentrations (0.50, 50, 800 ng/ml), were 73.8%, 75.1% and 76.3% (n = 6), respectively. The extraction recovery of genistein was also investigated as 64.2% (n = 6).
Daidzein in the plasma was shown to be stable for at least 30 days stored at −20 °C. The relative error (RE%) of daidzein between the initial concentrations and the concentrations of the following three freeze-thaw cycles ranged from −6.7% to 2.4%, which indicated the stability of daidzein during the three freeze-thaw cycles. Processed samples was also found to be stable in the reconstituted solution of acetonitrile-water-formic acid (80 : 20 : 1, v/v/v) for at least 24 h at room temperature. These data are summarized in Table 2.

2.3. Application of the method to the determination of plasma concentration in rats

The method was successfully applied to determine the plasma concentration of daidzein and its glucuronide following a single oral administration (20 mg/kg) to six rats. Mean plasma concentration-time profiles of daidzein and its glucuronide are presented in Fig. 3.

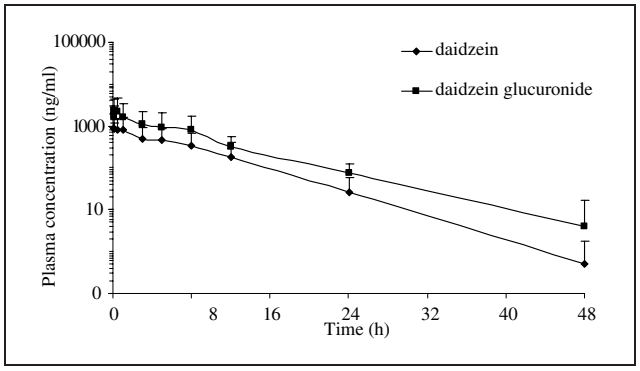


Fig. 3: Mean plasma concentration-time profiles of daidzein and its glucuronide after an oral administration of 20 mg/kg daidzein to 6 Wistar rats. Each point represents the mean + SD (n = 6)

3. Discussion

The possibility of using electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) sources under positive ion detection mode was evaluated. It was found that APCI can offer higher sensitivity and better linearity than ESI. The LC-MS-MS method had high selectivity because a pair of ions (precursor ion→product ion) derived from analytes of interest were monitored. The stable response of APCI source provided reproducibility of the measurement. The present LC-MS-MS method offers an LLOQ of 0.24 ng/ml in plasma sample. Under present LLOQ, the unconjugated daidzein concentration could be determined in plasma samples upto 48 h following a single oral dose of 20 mg/kg daidzein, which was sensitive enough to investigate pharmacokinetic behaviors of daidzein and its glucuronide, to establish the relationship between dose and pharmacological effect and identify doses that produce toxic responses in humans.

In analysis of biological samples, internal standards are usually needed to rectify the probable error in sample processing and determination. Genistein, an analogue of daidzein, was used as the internal standard in present study. The retention times of daidzein and genistein were 2.7 and 2.9 min, respectively.

In the assay β-glucuronidase was used to hydrolyze the conjugates for the determination of total daidzein in plasma. After investigation the enzymatic reaction conditions, 0.05 mol/L $NH_4H_2PO_4$ buffer (pH 5.0) was chosen as the hydrolysis medium, with a reaction time of 16 h. Under these conditions, complete enzymatic hydrolysis was obtained, allowing for the determination of total daidzein concentration.

In conclusion, a highly selective and sensitive LC-MS-MS method with a simple liquid-liquid extraction was successfully developed and validated to determine the plasma concentration of daidzein after oral administration of 20 mg/kg by rats.

4. Experimental

4.1. Chemicals and reagents

Daidzein and genistein (internal standard) were purchased from Huike Botanical (Xi'an, Shaanxi, China). β-Glucuronidase (EC 3.2.2.21., 542200 units/g of solid) was purchased from Sigma (St. Louis, MO, USA). Acetonitrile and methanol (Yuwang Chemical, Shandong, China) were of HPLC grade. Other chemicals were of analytical grade. Distilled water, prepared from demineralized water, was used throughout the study.

4.2. Instrumentation

A Finnigan TSQ API II tandem mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) source (San Jose, CA, USA), an Agilent 1100 autosampler (Agilent, Wilminton, DE, USA) and a Shimadzu LC-10AD pump (Kyoto, Japan) were used for LC-MS-MS analyses. Analytical data were acquired using Xcalibur 1.1 software (Finnigan) and quantitative processing was performed using LCQuan software (Finnigan).

4.3. LC-MS-MS conditions

The LC separation was performed using a Diamonsil C_{18} column (200 × 4.6 mm I.D., 5 μm, Dikma, Beijing, China) and a SecurityGuard C_{18} guard column (4 × 3.0 mm I.D., Phenomenex, Torrance, CA, USA). The isocratic mobile phase consisted of acetonitrile-water-formic acid (80 : 20 : 1, v/v/v) at a flow-rate of 0.75 ml/min. The column temperature was maintained at 20 °C.

The mass spectrometer was operated in the positive ion detection mode with the corona discharge current set at 4.00 μA. Nitrogen was used as the sheath gas (0.6 MPa) and auxiliary gas (3 L/min) for nebulization. The heated capillary and vaporizer temperatures were set to 280 and 450 °C, respectively. For collision-induced dissociation (CID), argon was used as the collision gas at a pressure of 0.19 Pa. Quantification was performed using selected reaction monitoring (SRM) of the transitions m/z 255→199 for daidzein and m/z 271→153 for genistein, respectively, with a scan time of 0.3 s per transition. The optimized collision energy of 30 eV and 35 eV were chosen for daidzein and genistein, respectively.

4.4. Sample preparation

To determine unconjugated daidzein, 50 μl internal standard solution (genistein, 50 μg/ml in methanol) and 50 μl water were added to 50 μl of rat plasma samples, respectively. Then 200 μl of 0.05 mol/L $NH_4H_2PO_4$ buffer (pH 5.0) was added. The mixture was vortexed for 10 s and extracted with 2 ml of *n*-hexane-diethyl ether (1 : 4, v/v) by shaking for 10 min. After centrifugation at 2000 g for 10 min, the organic phase was transferred into another tube and evaporated to dryness at 40 °C under a stream of nitrogen. The residue was reconstituted in 100 μl of the mobile phase and vortexed for 1 min. A 20-μl aliquot of the solution was injected onto the LC-MS-MS system.

To determine total daidzein, 100 μl of β-glucuronidase enzyme solution (1084.2 units/ml in 0.05 mol/L $NH_4H_2PO_4$ buffer, pH 5.0) was added to 50 μl aliquot of rat plasma. The mixture was incubated in a water bath at 37 °C for 16 h. After enzymatic hydrolysis, 50 μl internal standard and 50 μl water were added. The mixture was treated as described above.

Those plasma samples whose concentrations were higher than the highest calibration point were diluted appropriately with blank rat plasma in order to make the concentration within the range of standard curve before sample preparation.

4.5. Method validation

Plasma samples were quantified using the ratio of the peak area of daidzein to that of genistein as the assay response. Peak area ratios were plotted against concentrations and daidzein concentrations were calculated by a weighted ($1/x^2$) least squares linear regression.

To evaluate linearity, plasma calibration curves were prepared and assayed in triplicate on 3 consecutive days. Accuracy and precision were also assessed by determining QC samples at three concentration levels on the 3 different validation days. The accuracy was expressed by (mean observed concentration-spiked concentration)/(spiked concentration) × 100% and the precision by relative standard deviation (RSD).

The extraction recoveries of daidzein at three QC levels were determined by comparing the peak area ratios of analyte to internal standard in sample that had been spiked with analyte prior to extraction with samples to which the analyte had been added post-extraction. The internal standards were added to both sets of samples post-extraction.

The stability of daidzein in rat plasma was investigated under a variety of storage and process conditions. The storage stability under −20 ºC freezer conditions was evaluated for at least 30 days. The freeze-thaw stability of daidzein was assessed by analyzing QC samples at three concentrations undergoing three freeze (−20 ºC) thaw (room temperature) cycles. The stability in the reconstituted solution was investigated by placing QC samples at three concentrations under ambient conditions for 24 h.

4.6. Application of the method to determine plasma concentration in rats

Six Wistar rats (three males and three females, Laboratory Animal Center of Shenyang Pharmaceutical University, Shenyang, China) weighing 200 to 250 g were used in the studies. The rats were housed under standard conditions and had *ad libitum* access to water and a standard laboratory diet (isoflavones free). All rats were dosed following an overnight fast; food was returned 0.5 h after dosing. Water was available *ad libitum* throughout the experiments.

Polyethylene cannulas were implanted in the femoral vein 2 days before the experiment while the rats were anesthetized with pentobarbitone (50 mg/kg, i.p.). The cannulas were externalized at the back of the neck and filled with heparinized saline (20 units/ml). The rats were orally dosed with daidzein at 20 mg/kg (10 ml/kg, 2 mg/ml) in 0.9% NaCl solution

(pH 7.0). Serial blood samples (0.3 ml) were collected at 0, 0.083, 0.167, 0.50, 1.0, 3.0, 5.0, 8.0, 12, 24 and 48 h post dose. Plasma was separated by centrifugation at 2000 g for 10 min and stored frozen at −20 °C until analysis.

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