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Quantitative analysis of isoflavone aglycones in human serum by solid phase extraction and liquid chromatography–tandem mass spectrometry

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Abstract

This paper describes a liquid chromatography–electrospray-tandem mass spectrometry (LC–ESI-MS/MS) for the qualitative and quantitative analysis of three isoflavone aglycones (glycitein, daidzein and genistein) in human serum. Positive ion mode was used for the detection of these compounds and selective reaction monitoring (SRM) was employed for quantitative measurement. The SRM transitions monitored were as 285.0 → 242.0, 270.0 for glycitein, 255.0 → 137.0, 153.0, 181.0, 199.0 for daidzein and 271.0 → 153.0, 215.0 for genistein. d_3-Daidzein was used as an internal standard for quantitative measurement. The linearity was good from 0.5 to 500 ng/ml. The detection limit based on a signal-to-noise ratio of three was 0.27, 0.38 and 0.29 ng/ml for glycitein, daidzein and genistein, respectively. A newly developed solid phase extraction (SPE) procedure was developed for sample pre-treatment. Good recovery, 92.3–103.2%, for three isoflavone aglycones were obtained. This newly developed method was successfully applied to evaluate isoflavone pharmacokinetic in human serum after oral administration.

Keywords: Isoflavone aglycones; Liquid chromatography–tandem mass spectrometry; Pharmacokinetic

1. Introduction

Phytoestrogens have a high potential for putative health benefits like chemoprevention of breast and prostate cancer, atherosclerosis, osteoporosis, hyperlipidemica, cardiovascular diseases and relief of post-menopausal complaints [1–6]. One important group of phytoestrogens are isoflavone aglycones like glycitein (GCT), daidzein (DDZ) and genistein (GEN). These compounds are found in soy and soy products and the structures of the analytical components are shown in Fig. 1. In general, three isoflavone aglycones are subjects to hepatic glucuronidation at the 7- or 4′-position and are also substrates for sulphotransferases [7]. Because of the extensive glucuronidation and sulphate conjugation, it is difficult to analyze the concentration of total isoflavone aglycones which lads to experimental difficulty in a relationship between dose and pharmacological effect.

Several analytical methods for the analysis of these compounds in various sample matrices have been reported, including liquid chromatography with ultraviolet/visible detection (LC-UV) [8–11], LC with electrochemical detection (LC-ECD) [12] and gas chromatography–mass spectrometry (GC–MS) [13–16]. GC–MS has been utilized for the determination of isoflavones in urine. However, derivatization prior to GC–MS analysis is often required for the determination of these compounds. In addition, LC–tandem mass spectrometry (LC–MS–MS) has been used to identify and quantify isoflavone aglycones in milk, tissue and biological fluids [17–19]. Nevertheless, methodological improvements for the quantitative determination of free and conjugated forms of isoflavones in biological fluids continue to be needed for clinical trials aimed at pharmacokinetic and efficacy.

In this study, we explored the utilization of solid phase extraction and LC–ESI-MS/MS for quantitative determination of GCT, DDZ and GEN in human serum. To our knowledge, these isoflavones in serum were measured simultane-

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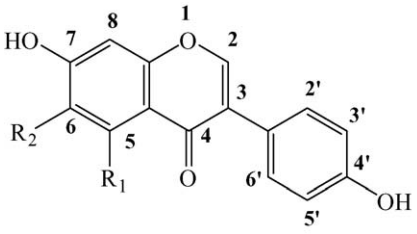

Compounds	M.W	R_1	R_2
Glycitein (GCT)	284	H	OCH_3
Daidzein (DDZ)	254	H	H
Genistein (GEN)	270	OH	H
3',5',8-d_3-Daidzein (internal standard)	257	H	H

Fig. 1. Structure of isoflavone aglycones and internal standard.

ously for the first time. Three isoflavone aglycones were extracted and pretreated by a newly developed solid phase extraction procedure after enzymatic hydrolysis and then analyzed by LC–ESI-MS/MS. In addition, the time profiles of total and unconjugated of GCT, DDZ and GEN in human serum were evaluated after a single dose of soy-food powder containing glycitein/glycitin, daidzein/daidzin and genistein/genistin.

2. Experiment

2.1. Chemicals and reagents

Purified water from a Milli-Q system from Millipore Corp. (Bedford, MA, USA) and HPLC grade acetonitrile and methanol (Mallinckrodt Baker, Paris, KY, USA) were used. Analytical grade acetic acid and methyl *tert*-butyl ether were from Aldrich (St. Louis, MO, USA). Daidzein and genistein were purchased from Sigma (St. Louis, MO, USA). Glycitein was obtained from Nakahara (Japan). 3′,5′,8′-d_3-Daidzein was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Stocks standard solutions of ∼100 μg/ml in methanol were prepared and used for further dilutions by water/methanol (50/50 v/v). d_3-Daidzein (10 ng/ml)was used as internal standard. Sodium acetate and β-glucuronidase/arylsulphatase (100,000 units ml^{-1} β-glucuronidase and 1000–5000 units ml^{-1} arylsulphatase from *Helix pomatia*) was from Sigma. Oasis HLB solid-phase extraction (SPE) cartridge (1 cm^3, 30 mg, Waters Corp., Milford, MA, USA) was used from sample pre-treatment.

2.2. Subjects

Three healthy women aged 70, 61and 53 were recruited upon contacting the Department of Obstetrics and Gynecology at the Taipei Veterans General Hospital for the purpose of post-menopausal complaints. Participants in the study were ingested a soy-food powder containing 6.8 mg glycitein/glycitin, 26.8 mg daidzein/daidzin and 18.2 mg genistein/genistin. Ethical approval was given by the Hospital Ethics Committee for clinical studies.

Table 1
Gradient elution program

Time (min)	*A* (%)	*B* (%)
0	65	35
20	25	75
25	10	90
30	65	35
40	65	35

A: water with 0.3% (v/v) acetic acid; *B*: acetonitrile with 0.3% (v/v) acetic acid.

2.3. Liquid chromatography

Separation of the isoflavone aglycones was done by HPLC with an Waters 616 HPLC system equipped with an Agilent Zorbax Bonus-RP C18 column (2.1 mm × 150 mm, 5 μm) and on-line filter (4 μm, Upchurch Scientific, Washington, USA). The mobile phase consists of acetonitrile/water gradient; the water and acetonitrile fractions each contain 0.3% (v/v) acetic acid. Gradient elution condition is summarized in Table 1. The flow rate was 0.2 ml/min and the injection volume was 20 μl.

2.4. Mass spectrometry

Analyses were performed on a Finnigan TSQ-7000 mass spectrometer (Thermoelectron Crop., San Jose, CA) equipped with the ESI positive mode. Xcalibur software (version 1.1) was utilized for mass spectrometer control, data acquisition and data processing. The heated capillary temperature was set to 225 °C. Nitrogen was used as sheath gas and auxiliary gas with a pressure set at 60 and 20 psi. The spray voltage of ESI-MS system was operating at 5.0 kV. MS/MS was employed for determination of each isoflavone aglycone and its corresponding internal standard, using argon as collision gas set a pressure of 2.2 mTorr.

2.5. Extraction of isoflavone from human serum

Whole blood was centrifuged at 3000 × *g* for 10 min and serum was frozen at −20 °C prior to analysis. One hundred and fifty microliters serum were added to 5 ml sodium acetate buffer (pH 5.0) and 30 μl β-Glucuronidase/arylsulphatase containing 150 μl ISTD (10 ng/ml). The mixture was vortex-mixed and incubated for 3 h at 37 °C. The reacted solution was passed through a mix mode HLB SPE cartridge (1 cm^3, 30 mg) that has been preconditioned with methanol (1 ml) and water (1 ml). This SPE cartridge packed with a macro-porous copolymer that has hydrophilic and lipophilic functions. The sample loaded SPE cartridge was further washed with water (2 ml) and isoflavone aglycones were eluted by 1 ml 0.1% formic acid in methyl *tert*-butyl ether (MTBE)–methanol mixture (80/20 v/v). The eluted solution was concentrated to dryness under nitrogen gas. The dried extracts were re-dissolved in 150 μl methanol–water (50/50 v/v).

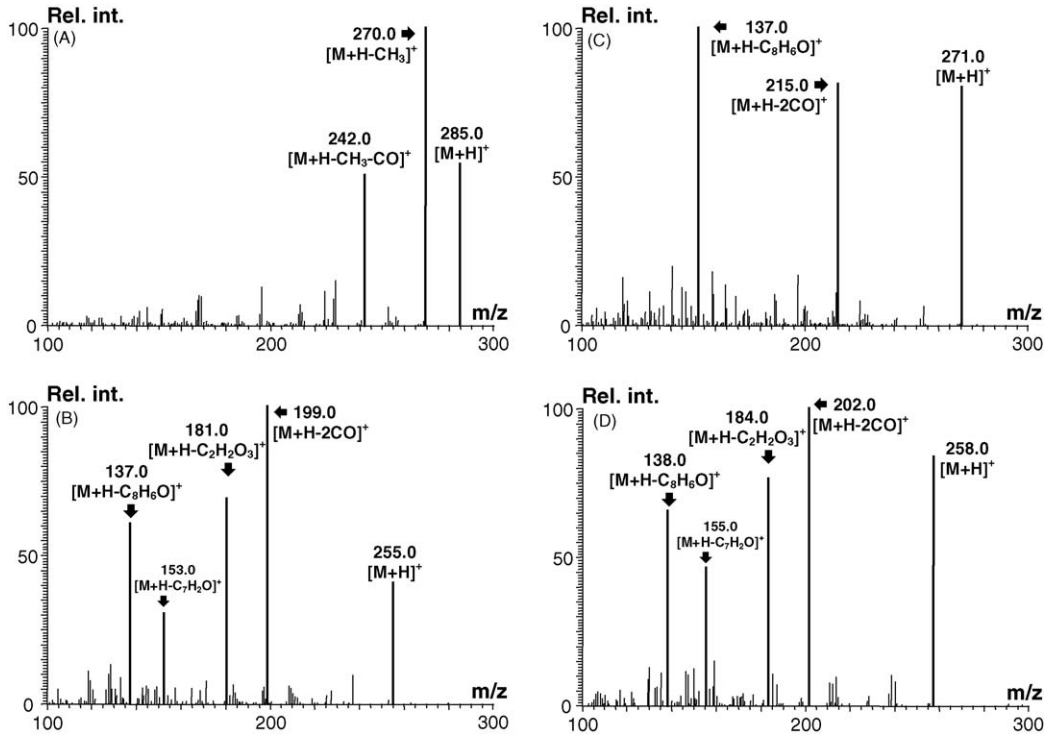

Fig. 2. Tandem mass spectrum of the $[M+H]^+$ ions of (A) GCT, (B) DDZ, (C) GEN and (D) ISTD. (Precursor ions at *m/z* 285.0, 255.0, 271.0 and 258.0, respectively.)

3. Results and discussion

3.1. ESI-MS and ESI-MS^2 of GCT, DDZ and GEN

In positive mode ESI-MS detection, all of isoflavone aglycones gave a major precursor ion $[M+H]^+$ and little fragmentation. The effect of $[M+H]^+$ abundance by the addition of acetic acid (0–1%) was examined. The abundance of $[M+H]^+$ intensified as the concentration of acetic acid increased from 0 to 0.4% and diminished when more than 0.5% of acetic acid was added. This is attributed to the enhancement of protonation of analytical molecules when small amount of acetic acid was added. As the concentration of acetic acid exceed 0.5%, the competition of acetic acid molecule for the positive charge on ESI droplet reduce the intensity of $[M+H]^+$. As the acetic acid increased to 0.3%, the intensity of $[M+H]^+$ of GCT, DDZ and GEN increased approximately 5, 10 and 20 times, respectively. In this study, 0.3% of acetic acid was added in the mobile phase for the rest of this study.

The ESI-MS/MS results and tentative assignment of fragment ions of these analytical components and ISTD are shown in Fig. 2. The characteristic MS/MS fragmentation of these compounds has been reported [19]. Similar results were observed in this study. For GCT, $[M+H]^+$, $[M+H\text{-}CH_3]^+$ and $[M+H\text{-}C_2H_3O]^+$ were the major MS^2 fragment ions. For DDZ, $[M+H]^+$, $[M+H\text{-}2CO]^+$, $[M+H\text{-}C_2H_2O_3]^+$, $[M+H\text{-}C_7H_2O]^+$ and $[M+H\text{-}C_8H_6O]^+$ were detected. For GEN, $[M+H]^+$, $[M+H\text{-}2CO]^+$ and $[M+H\text{-}C_8H_6O]^+$ were determined.

3.2. LC–ESI-MS/MS of GCT, DDZ and GEN

The separation of the isoflavone aglycones was performed using Zorbax Bonus-RP C18 column with water/acetonitril gradient elution. Acetic acid was added into mobile phase to improve the resolution of GCT, DDZ and GEN. With addition of acetic acid, a greatly improved on chromatographic separation with sharper peak shape and better peak symmetry of analytical compounds was observed. Similar result of the enhancement of LC separation by lowing pH of mobile phase has been reported [10]. A typical LC–ESI-MS–MS chromatogram of the standard solution is shown in Fig. 3. GCT, DDZ GEN and ISTD was eluted at 9.4, 11.9, 16.8 and 11.9 min, respectively.

3.3. Quantitative analysis, detection limit and precision

Selective reaction monitoring (SRM) was employed for the quantitative measurement. Table 2 is the summary of quantitative ions, linearity and detection limit (LOD) of each analytical

Table 2
Calibration data and detection limit of isoflavone aglycones by LC–ES-MS/MS

	Quantative ion	Calibration data[a]	Detection limit (ng/ml)
GCT	270.0, 242.0	$Y=0.2261X+0.1122$	0.27
DDZ	199.0, 181.0, 153.0, 137.0	$Y=0.1338X+0.1211$	0.38
GEN	215.0, 153.0	$Y=0.1248X+0.1190$	0.29

[a] X, concentration (ng/ml); Y, peak area ratio (area of each peak/area of ISTD); ISTD: 10.0 ng/ml.

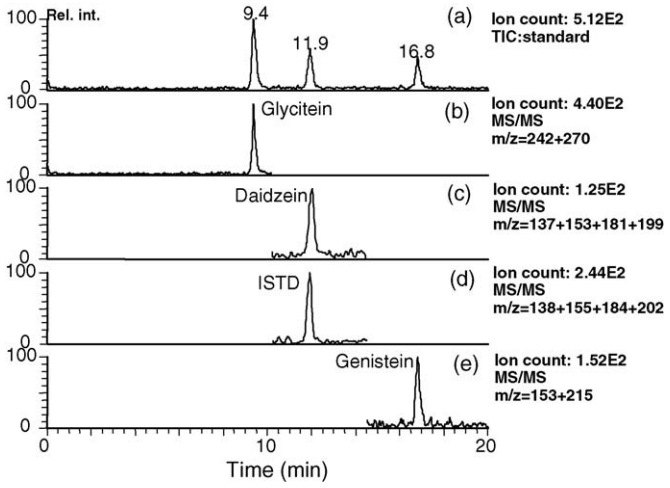

Fig. 3. LC–ESI-MS/MS chromatography of standard solution (isoflavone aglycones: 5 ng ml^{-1}, internal standard: 10 ng ml^{-1}): (a) total ion chromatogram; (b)–(e) select reaction monitoring.

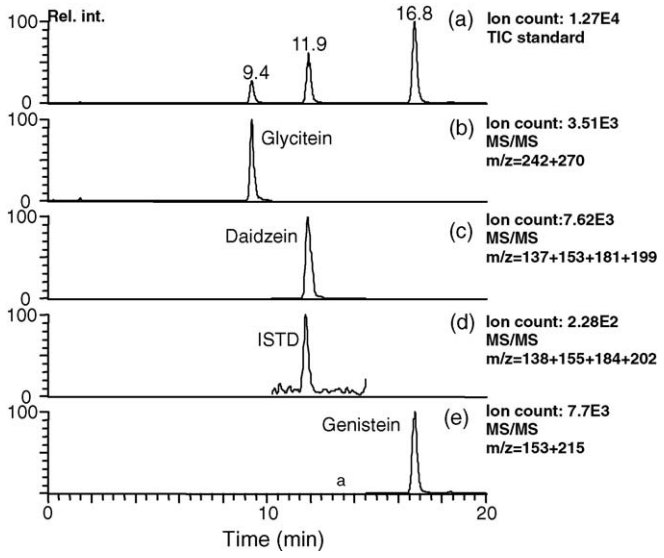

Fig. 4. LC–ESI-MS/MS chromatography of the extraction sample of human subject 2 at 6 h and preparation with enzymatic hydrolysis (ISTD: 10 ng ml^{-1}): (a) total ion chromatogram; (b)–(e) select reaction monitoring.

compound. The linearity of this assay was evaluated using a series of standard solutions; each standard was measured in triplicate. The calibration curve for each analytical component was constructed using least-squares linear regression of quantities of each analyte versus peak area ratio of analyte peak area to that of internal standard. The linearity was good from 0.5 to 500 ng/ml. The detection limit of the GCT, DDZ and GEN was ranged from 0.27 to 0.38 ng/ml in serum extracts based on a criterion of signal-to-noise ratio of 3. The LODs of this present method were superior or equal to those previously reported results [17–19].

The inter- and intra-day precisions of LC–ESI-MS–MS detection was examined by replicated analysis of standard solution (0.5, 1.0, 5.0, 10.0, 50.0, 100.0, 500.0 ng ml^{-1}). Standards were prepared and analyzed each day. A total of three series of standards were analyzed over a week-long period and each standard was measured in triplicate. The intra- and inter-day precisions showed a coefficient of variance (CV) ranging from 0.1 to 11.1 and 0.1 to 8.9%, respectively. In addition, the accuracy ranging from 96.1 to 116.1% was obtained.

3.4. Recovery of SPE

In order to examine the extraction recoveries of this newly developed SPE method, spiked serum samples and standard solutions were analyzed. In this study, GCT, DDZ and GEN were spiked to serum and then extracted by the said extraction method. A typical LC–ESI-MS/MS chromatogram of extracted human serum is shown in Fig. 4. There was no additional peak of endogenous component in serum and the added enzyme detected in the SPE treated serum sample. The recovery was evaluated by the response of a spiked serum as a fraction of that a cor-

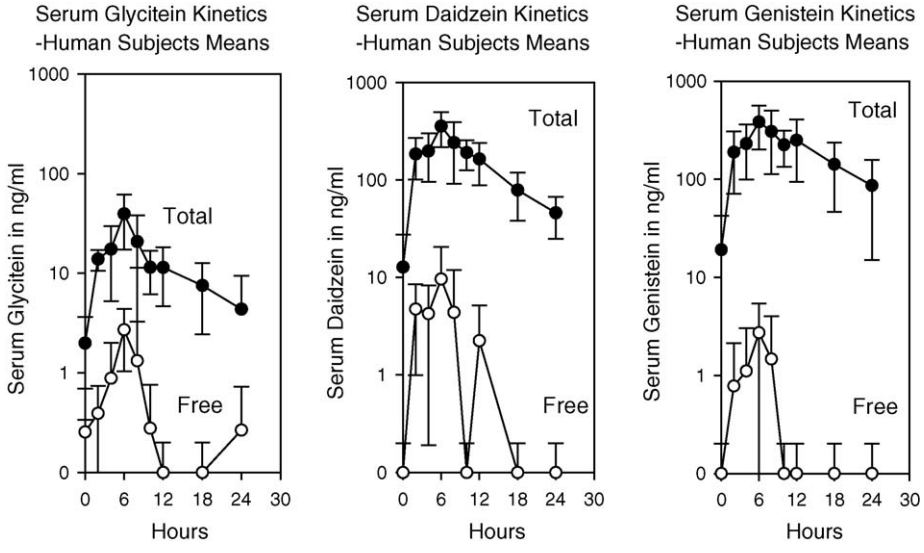

Fig. 5. The subjects means serum appearance and disappearance curves for total and free (unconjugated) isoflavone aglycones in three post-menopausal women.

Table 3
Recovery of isoflavone aglycones spiked serum samples[a]

	1.5 ng/ml	7.5 ng/ml	15.0 ng/ml	45.0 ng/ml
GCT	97 ± 4	92 ± 2	103 ± 2	101 ± 5
DDZ	93 ± 3	96 ± 5	102 ± 4	100 ± 3
GEN	103 ± 3	97 ± 3	96 ± 3	92 ± 3

[a] Recovery, % average ± S.D. ($n = 3$).

responding standard in methanol/water (50:50 v/v). The results of this study are summarized in Table 3. Good recoveries ranging from 92 to 103% were obtained. The results indicated that this newly developed assay was suitable for the determination of these compounds in serum samples.

3.5. Time course study of GCT, DDZ and GEN in human serum

To understand the relationship of total isoflavone aglycones effect and amount of soy-food, this newly developed method was employed to evaluate sample obtained in human serum after a single 40 g dose of soy-food powder was administered to three post-menopausal women. The concentration profiles of GCT, DDZ and GEN in serum are shown in Fig. 5. The results indicated the concentrations of the analytical components in serum reached the maximum at 6 h after administered and the C_{max} concentration of GCT, DDZ and GEN was 39.4, 356.7 and 383.8 ng/ml, respectively.

4. Conclusion

In summary, a LC–ESI-MS/MS method was developed to determine the isoflavone aglycones in human serum. In addition, a newly developed solid-phase extraction method effectively removed the endogenous components and enhanced the sensitivity of assay. Good recoveries and detection limits were obtained. This newly developed method has successfully applied to evaluate three isoflavone aglycones in human serum samples for 24 h period. It demonstrated that this method is suitable for pharmacokinetic studies.

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