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Determination of soyasaponins Ba and Bb in human serum by high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry

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Abstract

A rapid, sensitive and selective high-performance liquid chromatography–tandem mass spectrometric method (HPLC–MS–MS) has been developed and validated for the determination of soyasaponins Ba and Bb in human serum using glycyrrhizin as internal standard (I.S.). Soyasaponins Ba and Bb were extracted from human serum by liquid–liquid extraction and cleaned up by C_{18} solid-phase extraction (SPE), followed by separation on a C_{18} reversed-phase column using acetonitrile/water containing 0.025% acetic acid as a mobile phase for gradient elution. Soyasaponins Ba and Bb, and I.S. were ionized by negative ion pneumatically assisted electrospray and detected by HPLC–MS–MS in the multiple-reaction monitoring (MRM) mode using precursor → product ion combinations at *m/z* 958 → 940, 942 → 924 and 822 → 351, respectively. The calibration curves were linear ($r^2 > 0.991$) in the concentration range of 0.5–100.0 ng/mL, with lower limits of quantification of 0.5 and 0.3 ng/mL for soyasaponins Ba and Bb, respectively, in human serum. Intra-day and inter-day relative standard deviations (R.S.D.) were less than 7.9 and 11.3%, respectively. The mean recoveries of soyasaponins Ba and Bb ranged from 92 to 101% and from 85 to 94%, respectively.

Keywords: Soyasaponin; Human serum; HPLC–MS–MS; MRM; ESI-MS

1. Introduction

Soyasaponins are triterpenoid glycosides with one or two polysaccharide chains which are mainly present in legumes [1]. Significant amounts of soyasaponins are found in soybeans and soy-based products between 0.2 and 114 μmol/g [2]. The potential relationship of soyasaponins with health effects, particularly cardiovascular health, cancer preventive effects, hepatoprotective effect and antiviral activities, and as a natural alternative to hormone replacement therapy [3–11] has recently generated a great deal of interest. Therefore, it is important to understand absorption and metabolic process of soyasaponins in the body since human beings obtain soyasaponins from the diet. However, only a few studies [12] reported the metabolism of soyasaponins. This maybe attributed to difficulties in the isolation of the authentic standards and in the establishment of more sensitive methods to detect soyasaponins.

Various analytical methods have been proposed for the identification and quantification of soyasaponins including reversed-phase high-performance liquid chromatography (HPLC) with UV detection [2–14], evaporative light scattering detection (ELSD) [15–17] and electrospray ionization (ESI) mass spectrometry (MS) detection [18–21]. These methods were mainly developed to determine relatively high concentrations of soyasaponins in hypocotyls and soybean-based products with comparatively simpler matrix compared with the human serum samples. Therefore, these methods, especially with UV and ELSD, probably suffered from a number of disadvantages including low sensitivity, extensive sample preparation and, in some cases, serious interference when they were used in the determination of soyasaponins in human serum with less than 10.0 ng/mL concentration.

High-performance liquid chromatography with tandem mass spectrometric detection (HPLC–MS–MS) has been widely used

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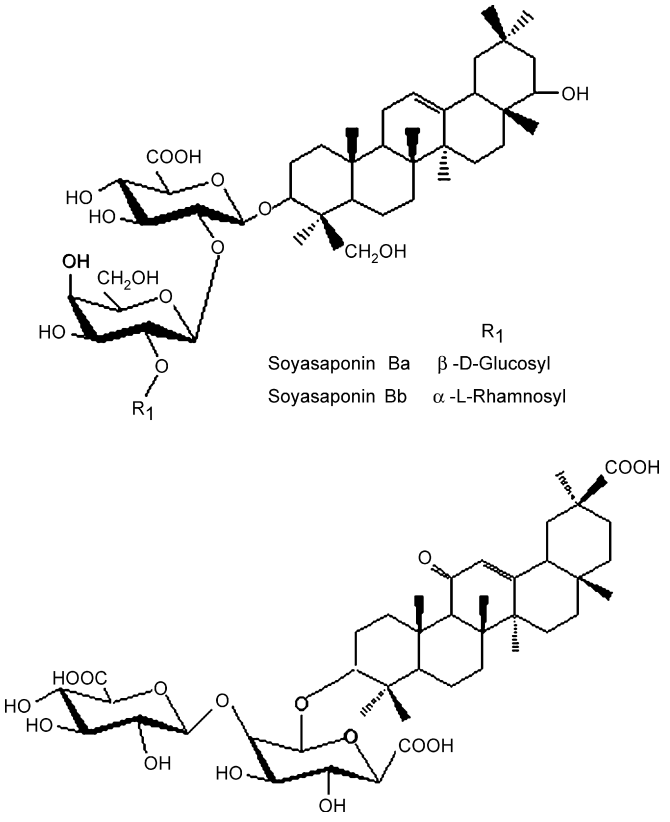

Fig. 1. Chemical structures of soyasaponins Ba, Bb (top) and glycyrrhizin (bottom).

for the analyses of drugs in biological fluids because of its excellent specificity, speed and sensitivity [22,23]. To the best of our knowledge, a HPLC–MS–MS method for the determination of soyasaponins in body fluids has not been reported. This paper describes the development and validation of a rapid, sensitive and specific HPLC–MS–MS method for the determination of soyasaponins Ba and Bb (Fig. 1), as representative soyasaponins of group B soyasaponins [13,24], in human serum using glycyrrhizin (Fig. 1) as internal standard (I.S.). This method is suitable for the study of soyasaponins Ba and Bb in human serum.

2. Experimental

2.1. Chemicals and solvents

Acetonitrile (MeCN), acetic acid (AcOH), ethanol (EtOH) and methanol (MeOH) (Merck, Darmstadt, Germany) were HPLC grade. Pure water was prepared by a Milli-Q water purification system from Millipore (Molsheim, France) and used throughout in all aqueous solutions. The soyasaponins Ba and Bb standards were obtained from ChromaDEX (>95% purity, Santa Ana, CA, USA). Glycyrrhizin was obtained from Fluka Chemie GmbH (>95% purity, Steinheim, Germany).

2.2. Human serum collection

The blank human serums were collected from three healthy volunteers who were recruited from Ningbo University (Ningbo, Zhejiang, China), 18–22 years old, and had not used any medication for 3 months prior to and during the study, and were instructed to avoid foods containing soyasaponins for 5 days prior to serum collection. The whole blood sample (3 mL) of each subject was collected into a 10 mL sterile centrifugal polypropylene tube by venipuncture. Following centrifugation (7800 rpm for 10 min), serums were stored in polypropylene tubes at −20 °C until analysis.

The serum Samples A, B, C, D and E were collected from five different subjects who were recruited from Ningbo University (Ningbo, Zhejiang, China), 18–22 years old, and had not used any medication for 3 months prior to and during the study, and were instructed to take some soybeans and soybean products, e.g. tofu, oily bean curd, for 3 days prior to serum collection. The sampling procedure was the same as that of the blank human serum.

2.3. Preparation of standard solutions

Authentic standards of the two available soyasaponins Ba and Bb, and I.S. were accurately weighed, transferred to volumetric flasks and dissolved in MeOH to make individual stock solutions of 1.0 mg/mL each. These solutions were thoroughly mixed and stored at 4 °C in tightly closed bottles until use and were stable for at least a month. Interim diluted solutions of soyasaponins Ba and Bb, and I.S. that were prepared with MeCN at the concentration of 10.0 μg/mL each were used for spiking human serum.

2.4. Preparation of spiked human serum samples

Appropriate amounts of the interim diluted standard solutions (10.0 μg/mL) of soyasaponins Ba and Bb, and 5.0 μL of the I.S. interim solution (10.0 μg/mL) were taken in a 10 mL polypropylene centrifuge tube and evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted with 0.5 mL of blank human serum, which was thawed to room temperature in advance. Final concentrations of soyasaponins Ba and Bb in the spiked human serum solutions were 0.1, 0.5, 1.0, 2.0, 5.0, 10.0, 50.0 and 100.0 ng/mL, respectively, whereas the I.S. concentration was 100.0 ng/mL. These spiked human serum solutions were used as the calibration standards, and three solutions in the concentration levels of 1.0, 10.0 and 100.0 ng/mL of soyasaponins Ba and Bb were considered as the quality control (QC) samples.

2.5. Sample preparation

The serum samples were thawed to room temperature before soyasaponins analysis. 0.5 mL human serum, 5.0 μL of I.S. interim solution (10.0 μg/mL), and 4.0 mL methanol were added to a 10 mL polypropylene centrifuge tube and vortex-mixed at room temperature for 5 min. The suspension was centrifuged at 7800 rpm for 10 min. The supernatant was removed, and the precipitate was resuspended in 4.0 mL of methanol. After the mixture was vortex-mixed for 5 min, the sample suspension was centrifuged again. After the second centrifugation, the two

Table 1
The MRM parameters for soyasaponins Ba and Bb, and I.S.

	Soyasaponin Ba	Soyasaponin Bb	I.S.
Precursor ion (*m/z*)	958	942	822
Product ion for detection and quantification (*m/z*)	940	924	351
Additional ions for confirmation (*m/z*)	526, 796, 598	526, 734, 880	645
Width (*m/z*)	1.0	1.0	1.0
Cutoff mass	300	300	300
Collision induced dissociation (CID) (V)	1.50	1.40	1.30
Retention time (min)	5.32	6.11	3.73

supernatants were combined and evaporated to dryness under a gentle stream of nitrogen at room temperature.

The residues were resuspended in 5.0 mL of 20% methanol and loaded onto a preconditioned C_{18} cartridge (3 mL/60 mg, Waters, Milford, MA, USA). The cartridge was washed with 5.0 mL of 5% methanol. Soyasaponins were eluted with 2.0 mL of HPLC-grade methanol. The elution was evaporated to dryness under a gentle stream of nitrogen, and the residues were reconstituted with 0.5 mL of 40% MeCN and filtered through a 0.45 μm nylon syringe filter (Agilent Technologies, Germany). Finally, an aliquot of 20 μL of the resultant solution was injected into the HPLC–MS system. Each analytical sequence included a blank serum, calibration standards, QC samples and a serum sample.

2.6. Chromatographic and mass-spectrometric conditions

The HPLC–MS system for method development and validation was an Agilent 1100 series LC/MSD Trap SL (Agilent Technologies, Germany) consisting of a quaternary gradient pump (G1311A), a column thermostat (G1316A), a degasser unit (G1379A), an autosampler (G1313A) and an ion trap mass spectrometer with an ESI interface (G2445D). The HPLC–MS system was controlled and data were analyzed on a computer equipped with LC/MSD Trap Software 4.2 (Bruker).

The separation was performed on a Waters AccQ.Tag column (150 mm × 3.9 mm i.d., 5 μm particle size, Waters, Milford, MA, USA) preceded by an Eclipse XDB-C_{18} column (12.5 mm × 4.6 mm i.d., 5 μm particle size, Agilent Technologies, USA), using 0.025% (v/v) AcOH in water (A) and 0.025% (v/v) AcOH in MeCN (B) as mobile phases in gradient mode at a constant column temperature of 35 °C. The elution program was as follows: 0 → 5.0 min, 40% B (isocratic); 5.0 → 8.0 min, 40 → 95% B (linear gradient). The column was washed at 95% B for 2.0 min and equilibrated for 2.0 min between runs at 40% B. Total sample to sample time was 12 min. The flow-rate was 0.5 mL/min and injection volume was 20 μL. Detection was carried out on an ESI-MS in the multiple-reaction monitoring (MRM) mode.

The ion trap mass spectrometer was used in the negative mode with a capillary voltage of 4.4 kV, a capillary exit voltage of 250 V, a dry temperature of 350 °C, a high-purity nitrogen (99.999%) dry gas of 9.0 L/min, a nitrogen nebulizer pressure of 35.0 psi, a cycle time of 1 s and a dwell time of 200 ms. The ESI interface and mass spectrometer parameters were optimized by direct infusion of soyasaponins Ba and Bb, and I.S. (1.0 mg/L) at a flow-rate of 0.5 mL/h to obtain maximum sensitivity. To determine the product ions of soyasaponins Ba and Bb, the deprotonated precursor ions $[M-H]^-$ at *m/z* 958 and 942, respectively, were isolated; helium gas was introduced into the trap to induce collision with the precursor ions. Throughout all the measurements, soyasaponins Ba and Bb, and I.S. were detected by MRM with a transition of *m/z* 958 → 940, 942 → 924 and 822 → 351, respectively. Table 1 outlines the values set for each of the studied soyasaponins and I.S.

2.7. Repeatability of the method

To investigate the accuracy of the method, five replicates of three spiked human serum samples with different concentrations were extracted and analyzed independently within 24 h to evaluate intra-day variation. The sample was extracted and analyzed five replicates on each of three separate days within a 7-day period to evaluate the inter-day variation.

3. Results and discussion

3.1. Method development

In order to detect soyasaponins Ba and Bb in human serum using MRM mode, precursor and product ions must be selected for soyasaponins Ba and Bb, and I.S., which should be as strong as possible and should be avoided of the interference from the actual samples. The negative and positive ion mass spectra of soyasaponins Ba and Bb, obtained from a direct infusion of 1.0 mg/L standard solution, are shown in Fig. 2. It can be seen that soyasaponins Ba and Bb, and I.S. in the negative mode were less fragmented than in the positive mode. Therefore, their abundances in the negative mode were stronger than those in the positive mode. It can also be seen that the most abundant peak is due to the loss of glucosides or water. The fragmentation of soyasaponins in the positive mode was extensive and characteristic, providing the information of the monosaccharide sequence of the oligosaccharide chain attached to the soyasapogenols [13]. No or only a few fragmentations of soyasaponins were observed in negative mode over a large range of capillary voltages (3500–4500 V) and a capillary exit voltage of 150–300 V. The molecular ions $[M-H]^-$ were observed as the primary signals. Therefore, it was better to use the negative ion mode over the positive ion mode for the detection of soyasaponins Ba and

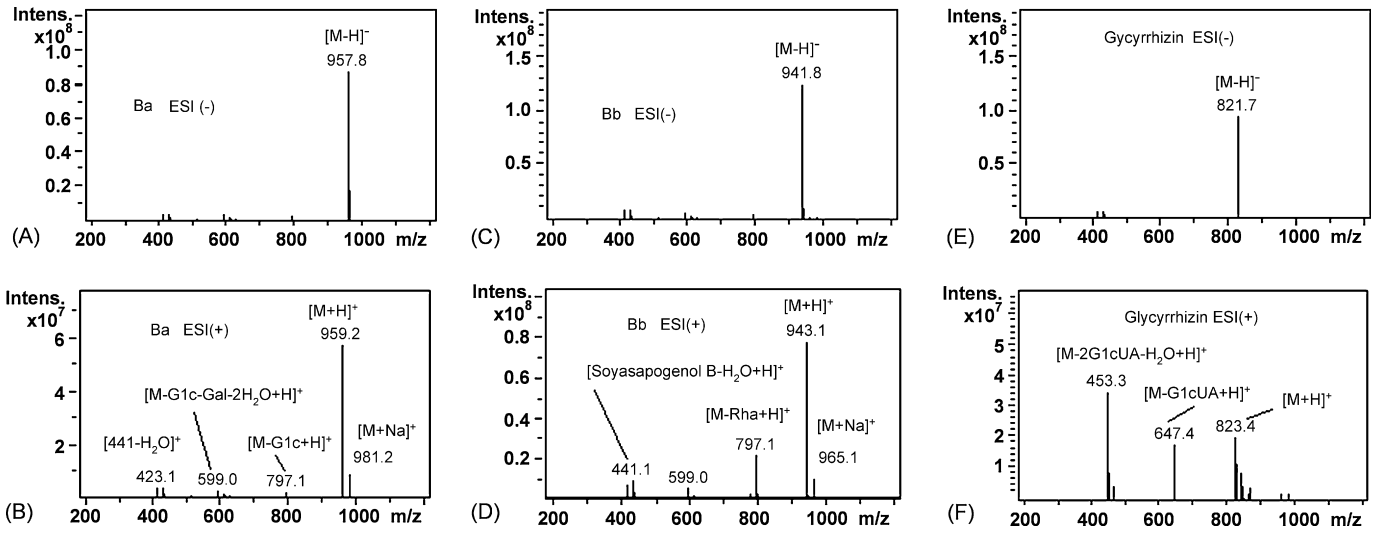

Fig. 2. Mass spectra of soyasaponins Ba, Bb and glycyrrhizin in both ESI (+) and ESI (−) modes by the direct infusion at a flow-rate of 0.5 mL/h with a concentration of 1.0 mg/L each. The abbreviations in the labels: Glc, β-D-glucopyranosyl; Gal, β-D-galactopyranosyl; GlcUA, β-D-glucoronopyranosyl; Rha, α-L-rhamnopyranosyl.

Bb, and I.S. to obtain the more intensive precursor. As a result, $[M-H]^-$ ions, *m/z* 958, 942 and 822 from soyasaponins Ba and Bb, and I.S., respectively, were selected as precursor ions and subsequently fragmented in MS–MS mode to obtain the product ion spectra, as shown in Fig. 3. The diamond in Fig. 3 marked the precursor ion.

The fragment ion at *m/z* 940 (loss of H_2O from $[M_{Ba}-H]^-$) was produced as the prominent product ion for soyasaponin Ba.

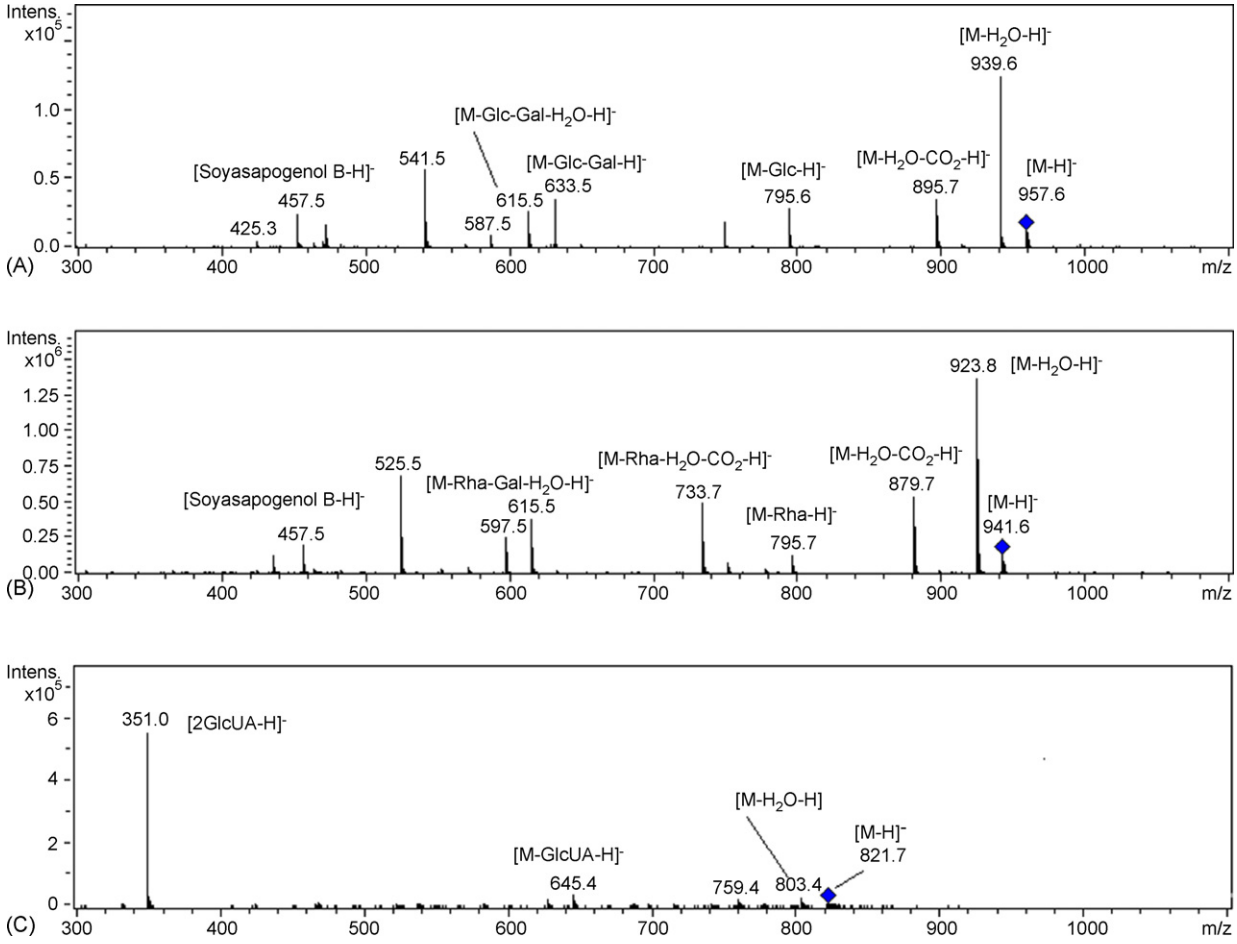

Fig. 3. Full-scan product ion spectra of $[M-H]^-$ for soyasaponins Ba (A), Bb (B) and glycyrrhizin (C) in ESI (−) mode by the direct infusion at a flow-rate of 0.5 mL/h with a concentration of 1.0 mg/L each. The abbreviations in the labels are the same as in Fig. 2. The diamond marked the precursor ion.

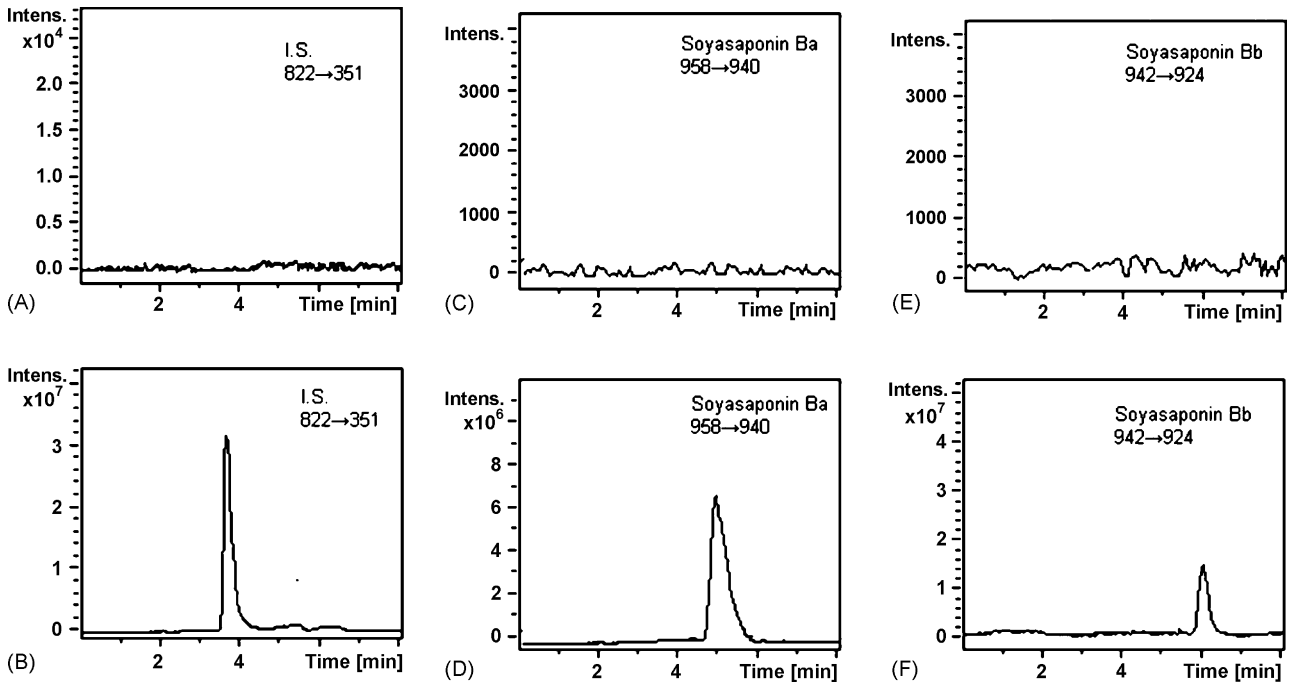

Fig. 4. Typical MRM chromatograms of soyasaponins Ba and Bb, and I.S.: (A) blank human serum of a transition (*m/z* 822 → 351) for I.S.; (B) blank human serum spiked with 100.0 ng/mL I.S. of a transition (*m/z* 822 → 351); (C) blank human serum of a transition (*m/z* 958 → 940) for soyasaponins Ba; (D) blank human serum spiked with 100.0 ng/mL soyasaponins Ba of a transition (*m/z* 958 → 940); (E) blank human serum of a transition (*m/z* 942 → 924) for soyasaponins Bb; (F) blank human serum spiked with 100.0 ng/mL soyasaponins Bb of a transition (*m/z* 942 → 924).

The major fragment ion for soyasaponin Bb was *m/z* 924 (loss of H_2O from $[M_{Bb} - H]^-$). The major fragment ion for I.S. was *m/z* 351 ($[2M_{GlcUA} - H]^-$). MRM mode was used for quantification, and achieved very high selectivity and sensitivity. Three pairs of MRM transitions were selected: *m/z* 958 → 940 for soyasaponin Ba, *m/z* 942 → 924 for soyasaponin Bb and *m/z* 822 → 351 for I.S.

Fig. 4 shows typical MRM chromatograms of soyasaponins Ba and Bb, and I.S. obtained from analyses of blank human serum sample, and blank human serum samples spiked with 100.0 ng/mL of soyasaponins Ba and Bb, respectively. Analyses of blank serum samples from 3 subjects did not show any interference at the retention times of soyasaponin Ba and Bb, and I.S. eluted at 5.32, 6.11, and 3.73 min, respectively, confirming the specificity of the present method.

3.2. Solid-phase extraction

The first attempt at serum extraction was only based on homogenization in methanol for protein precipitation. Although the procedure was quick, the extracts were fairly dirty. Column performance degraded after several hundred injections, evidenced by broad asymmetrical peaks and higher column pressure. This problem appeared to affect the whole LC column, as frequent guard column changes did not prevent deterioration of the analytical column and caused gradual loss of electrospray response. As a result, solid-phase extraction (SPE) was necessary for serum pretreatment. Solid-phase cartridge based on either C_{18} bonded silica or hydrophilic polymer was found to give acceptable results. The pretreatment method was validated using a Waters C_{18} SPE cartridge. It was assumed that matrix effects could be overcome by using a structurally similar internal standard which gave similar absolute recovery to soyasaponins, and by using calibration standards prepared in a similar matrix to the serum samples.

3.3. Validation of the HPLC–ESI–MS–MS method

Calibration curves for soyasaponins Ba and Bb were obtained using spiked human serum at the concentrations of 0.1, 0.5, 1.0, 2.0, 5.0, 10.0, 50.0 and 100.0 ng/mL, respectively, wherein the I.S. concentrations were 100.0 ng/mL. Three replicate injections of standards at each concentration were performed. Calibration curve for soyasaponins Ba was linear in the concentration range of 0.5–100.0 ng/mL, and that for soyasaponins Bb in the concentration range of 0.3–100.0 ng/mL. The regression equations were $C = 74.1Y - 0.159$ and $C = 105.3Y - 0.676$ for soyasaponins Ba and Bb, respectively, with coefficients of determination $r^2 > 0.991$. The peak area ratio (Y, soyasaponin/I.S.) was then used in conjunction with the calibration curve to derive the concentration (C, ng/mL) in human serum.

The limits of quantification for soyasaponins Ba and Bb were determined using a blank human serum sample spiked at lower concentration of 1.0 ng/mL standard solution, detected in MRM mode using glycyrrhizin as I.S. and evaluated by the criterion that the signal to noise ratio (S/N) should be >10, for quantification purposes. The limits of quantification were 0.5 and 0.3 ng/mL for soyasaponins Ba and Bb in human serum, respectively.

Table 2
The recoveries and the precisions of the method for spiked blank human serum samples

Analyte	Added (ng/mL)	Found (ng/mL)[a]	Recovery (%)	R.S.D. (%)	
				Intra-day[b]	Inter-day[c]
Soyasaponin Ba	1.0	0.94 ± 0.07	94	7.4	9.3
	10.0	9.2 ± 0.7	92	7.6	7.7
	100.0	101 ± 8	101	7.9	9.6
Soyasaponin Bb	1.0	0.85 ± 0.06	85	7.1	10.5
	10.0	9.4 ± 0.5	94	5.3	8.9
	100.0	91 ± 7	91	7.7	11.3

[a] Determined in 1 day, and data are presented as mean ± S.D. ($n = 5$).
[b] $n = 5$.
[c] $n = 5$ replicates × 3 days within a 7-day period.

Table 3
Concentrations of soyasaponins Ba and Bb in human serum samples (ng/mL)[a]

Analyte	Sample				
	A	B	C	D	E
Soyasaponin Ba	11.2 ± 0.9	3.2 ± 0.2	5.6 ± 0.4	2.2 ± 0.2	1.2 ± 0.1
Soyasaponin Bb	33.2 ± 2.0	6.5 ± 0.5	9.7 ± 1.0	3.7 ± 0.3	1.9 ± 0.1

[a] Data are presented as mean ± S.D. ($n = 3$).

The precision and accuracy were evaluated by calculating the intra-day and inter-day variation of three QC samples (1.0, 10.0 and 100.0 ng/mL). The results are shown in Table 2. The intra-day precision (R.S.D.) on the basis of peak area was less than 7.9 and 7.7% for soyasaponins Ba and Bb, respectively, whereas the inter-day precision (R.S.D.) on the basis of peak area was less than 9.6% and 11.3% for soyasaponins Ba and Bb, respectively.

The extraction recoveries of the analytes were obtained by comparison of the peak area of the analytes spiked in human serum before and after extraction. The mean recoveries of soyasaponins Ba and Bb from human serum at three concentrations of 1.0, 10.0 and 100.0 ng/mL were 94%, 92%, 101% and 85%, 94%, 91%, respectively, as shown in Table 2.

3.4. Application to the real serum samples

The suitability of this analytical protocol was demonstrated by the analyses of soyasaponins Ba and Bb in human serum for Samples A, B, C, D and E. The final concentrations of soyasaponins Ba and Bb are shown in Table 3. The results indicate that the proposed analytical method is suitable for the measurement of soyasaponins Ba and Bb in human serum at the concentrations expected to be present in human beings in pharmacokinetics studies.

4. Conclusions

A rapid, sensitive and specific HPLC–MS–MS method for the determination of soysaponins Ba and Bb in human serum using glycyrrhizin as I.S. was established and validated. The method was sensitive due to MRM detection mode and specific due to the inherent selectivity of tandem mass spectrometry. The method was successfully applied to the study of soyasaponins Ba and Bb in human serum samples.

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