Simultaneous analysis of isomers of escin saponins in human plasma by liquid chromatography–tandem mass spectrometry: Application to a pharmacokinetic study after oral administration

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ARTICLE INFO

ABSTRACT

A rapid and sensitive bioassay based on liquid chromatography–tandem mass spectrometry (LC–MS/MS) for the simultaneous determination of four isomeric escin saponins (escin Ia, escin Ib, isoescin Ia and isoescin Ib) in human plasma has been developed and validated. Sample preparation of plasma after addition of telmisartan as internal standard (I.S.) involved solid-phase extraction (SPE) on C18 cartridges. Separation was based on reversed phase chromatography using gradient elution with methanol–acetonitrile (50:50, v/v) and 10 mM ammonium acetate solution (pH 6.8). MS/MS detection in the positive ion mode used multiple reaction monitoring of the transition at m/z 1113.8 → 807.6. Stability issues with the four saponins required the addition of formic acid to plasma samples prior to storage at $-80\,^{\circ}C$ and analysis within 30 days. The method was linear at concentrations up to 10 ng/mL with correlation coefficients > 0.996 for all analytes. The lower limit of quantitation (LLOQ) for all four saponins was 33 pg/mL. Intra- and inter-day precisions (as relative standard deviation) were all <15% and accuracies (as relative error) in the range −5.3% to 6.1%. The method was successfully applied to a pharmacokinetic study of escins in healthy volunteers after oral administration of sodium aescinate tablets containing 60 mg escin saponins.

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1. Introduction

Escin is a mixture of triterpenic saponins obtained from the seeds of the horse chestnut tree (*Aesculus hippocastanum*) [1]. It exhibits anti-inflammatory [2], anti-edematous and vasoprotective properties [3–5] and has remarkable efficacy in treating chronic venous insufficiency (CVI), haemorrhoids and post-operative edema [6]. Recently, the isolation and structural characterization of the saponin constituents in escin has been carried out revealing the presence of at least 10 individual compounds including escin Ia, Ib, IIa, IIb, IIIa, IIIb, IV, V and VI as well as isoescin Ia and Ib. Of these, the isomeric escin Ia and Ib (β-escin) together with isoescin Ia and Ib (α-escin) (Fig. 1) constitute the major bioactive constituents [7–11].

Numerous analytical methods have been reported for the determination of escin including colorimetry [12], thin-layer chromatography [13], enzyme immunoassay [14], high-performance liquid chromatography (HPLC) [15], radioimmunoassay (RIA) [16,17] and LC–MS [8]. However, in the main, these methods have only been applied to herbal medicinal products, extracts of the seeds or *in vitro* studies of transport through the skin. The RIA method has been applied to human plasma after administration of single or multiple doses but only to the determination of total β-escin [16,17]. In addition, it suffers from a tedious and time-consuming sample preparation involving a two-step incubation lasting 30 h, potential interference from endogenous and analogous compounds and can only quantitate the total amount of β-escin. In order to assay the individual α- and β-saponins in biological samples, we have developed an LC–MS/MS method suitable for application to pharmacokinetic studies of escin. The assay is characterized by simple sample preparation and much greater sensitivity than the RIA method [17] having a lower limit of quantitation (LLOQ) of 33 pg/mL for each saponin compared to 0.5 ng/mL for total β-escin.

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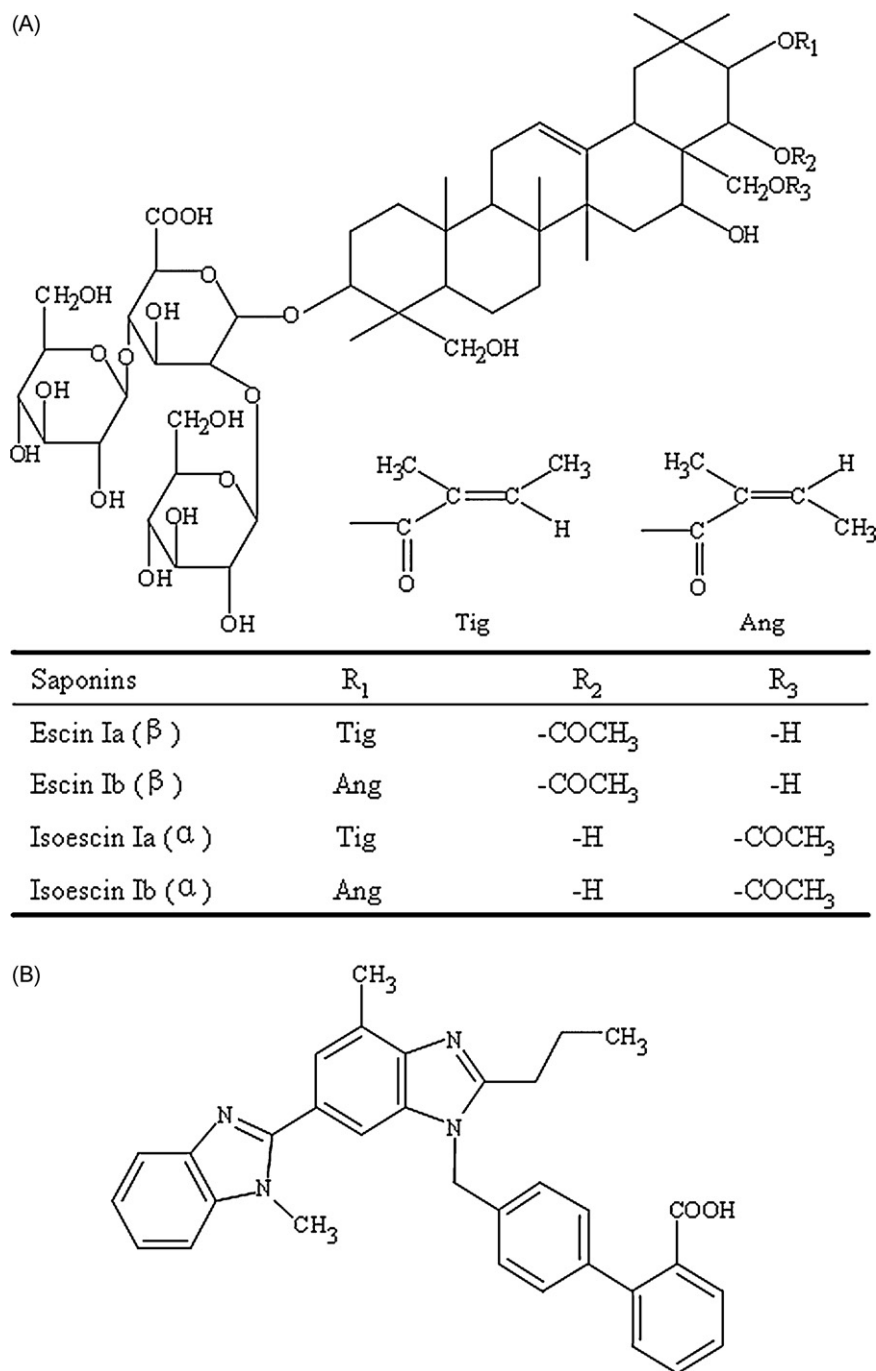
(A)

Saponins	R₁	R₂	R₃
Escin Ia (β)	Tig	-COCH₃	-H
Escin Ib (β)	Ang	-COCH₃	-H
Isoescin Ia (α)	Tig	-H	-COCH₃
Isoescin Ib (α)	Ang	-H	-COCH₃

(B)

Fig. 1. Chemical structures of (A) escin Ia, escin Ib, isoescin Ia, isoescin Ib and (B) telmisartan (I.S.) (Tig = tiglic acid; Ang = angelic acid).

2. Experimental

2.1. Chemicals and reagents

Escin Ia, escin Ib, isoescin Ia and isoescin Ib (>98.0% purity based on assay by HPLC with UV detection) were isolated from seeds of the horse chestnut tree according to published methods [7,8]. Structural identity was confirmed by comparison of ¹H NMR spectra with those reported by Yoshikawa et al. [7,8]. Telmisartan (I.S., >99.5% purity) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Sodium aescinate tablets containing 30 mg escin saponins were supplied by Luye Pharmaceutical Co. Ltd. (Yantai, China); each tablet contained 9.3, 5.7, 8.4 and 3.6 mg of escin Ia, escin Ib, isoescin Ia and isoescin Ib,

respectively. HPLC grade methanol and acetonitrile were purchased from Fisher Scientific (Fair Lawn, NJ, USA). All other chemicals were analytical grade and used without further purification. Doubly distilled water was used throughout the study. Blank human plasma (drug free) was obtained from the Changchun Blood Donor Service (Changchun, China).

2.2. Preparation of calibration standards and quality control samples

Individual stock solutions of escin Ia, escin Ib, isoescin Ia, isoescin Ib and telmisartan (1 mg/mL) were prepared in methanol–water (40:30, v/v) and stored at 4 °C when not in use. Mixed standard solutions containing 0.17, 0.50, 1.7, 5.0, 17 and
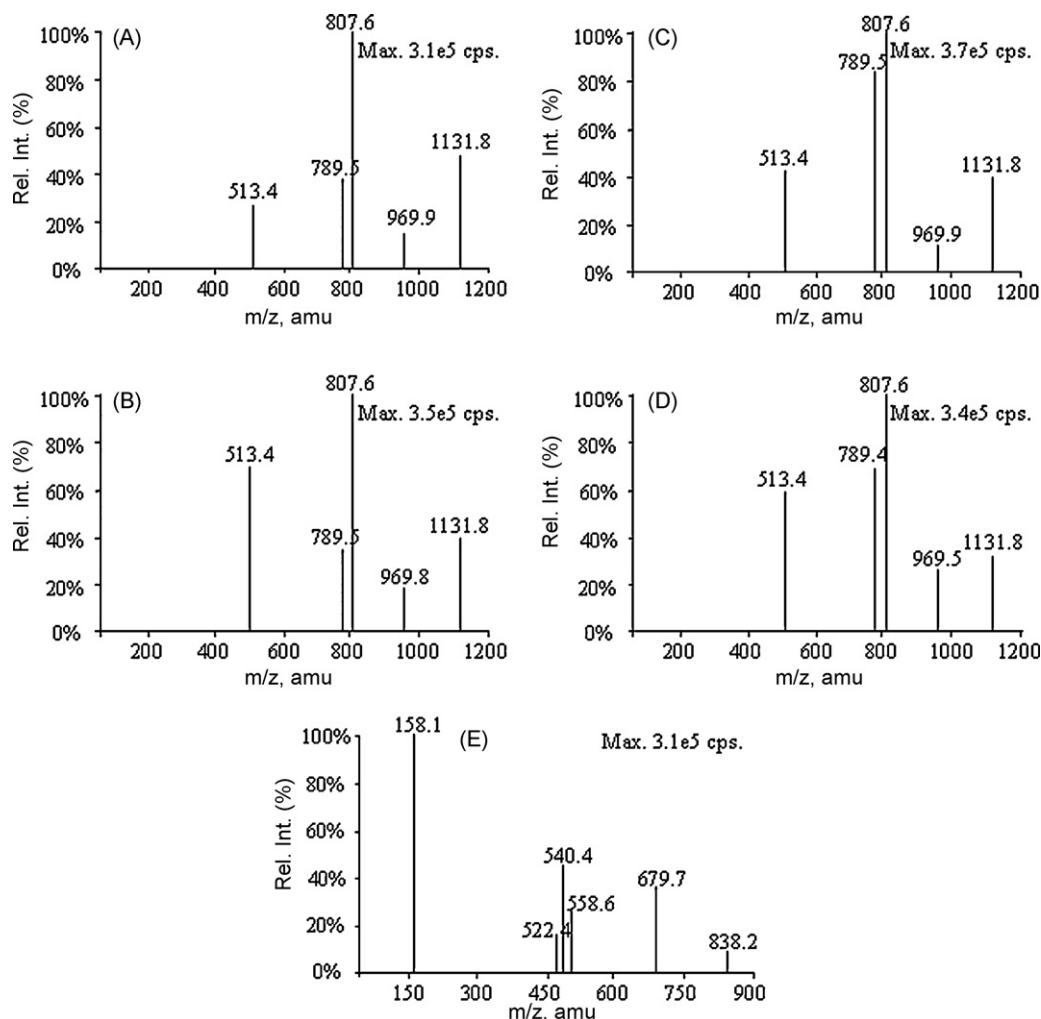
Fig. 2. Full-scan product ion spectra of [M+H]⁺ for escin Ia, escin Ib, isoescin Ia, isoescin Ib (A) and telmisartan (B).

50 ng/mL of each saponin were prepared by dilution of mixtures of the stock solutions using methanol–water (10:40, v/v). Quality control (QC) solutions containing 0.50, 5.0 and 40 ng/mL of each saponin and a working I.S. solution (0.50 ng/mL) were prepared by the same procedure. Calibration standards and QC samples were prepared by adding 50 μL 1% formic acid and 50 μL standard or QC solution to 250 μL blank human plasma.

2.3. LC–MS/MS conditions

The LC–MS system consisted of an Agilent 1100 series HPLC (Agilent Technologies, Palo Alto, CA, USA) and Applied Biosystems Sciex API 4000 mass spectrometer (Applied Biosystems/MDS Sciex, Concord, ON, Canada) using electrospray ionization (ESI). Chromatography was performed on an HC-C18 column (5 μm, 150 mm × 4.6 mm i.d., Agilent Technologies) maintained at 25 °C using gradient elution with 10 mM ammonium acetate in purified water (pH 6.8) as solvent A and methanol–acetonitrile (50:50, v/v) as solvent B. Before starting a run, the HPLC system was equilibrated for 10 min with a mixture of 45% A, 55% B. The gradient program was then as follows: 0.00–0.50 min 55% B, 0.50–7.00 min 55% B to 70% B, 7.00–7.50 min 70% B to 55% B, 7.50–8.00 min 55% B. The flow rate was set at 1.0 mL/min and an approximately 1:1 split of the column eluant was included prior to entry into the mass spectrometer.

ESI was performed in the positive ion mode with nebulizer, heater and curtain gas (all N_2) at 55, 45 and 15 units, respec-

tively. The IonSpray Voltage and heater gas temperature were set at 5500 V and 400 °C, respectively. Instrument response was optimized by syringe pump infusion (10 μL/min) of a solution in mobile phase containing individual saponins and telmisartan into the stream of mobile phase eluting from the column. The pause time was set at 5 ms and the dwell time at 200 ms.

The detector was operated at low mass resolution (to maximize sensitivity) in the multiple reaction monitoring (MRM) mode using the transitions of the protonated molecular ions of the four saponins at m/z 1131.8 → 807.6 and of telmisartan at m/z 515.2 → 276.1. Declustering potentials were 95 V for the saponins and 100 V for telmisartan. The collision gas (N_2) was set at 5 units and collision energies of 21 and 65 eV were used for the four saponins and telmisartan, respectively. The instrument was interfaced to a computer running Applied Biosystems Analyst version 1.3.2 software.

2.4. Sample preparation

To 300 μL acidified human plasma in a 1.5 mL Eppendorf tube were added 50 μL I.S. solution (0.50 ng/mL), 50 μL methanol–water (10:40, v/v) and 500 μL water. After vortexing for 30 s, the mixture was loaded onto an SPE cartridge (AccuBond II ODS-C18, 1 mL/100 mg, Agilent, USA) pre-conditioned with methanol and then water. The cartridge was washed with 1 mL water, sucked dry and eluted with 2 × 0.5 mL aliquots of methanol. The eluate was
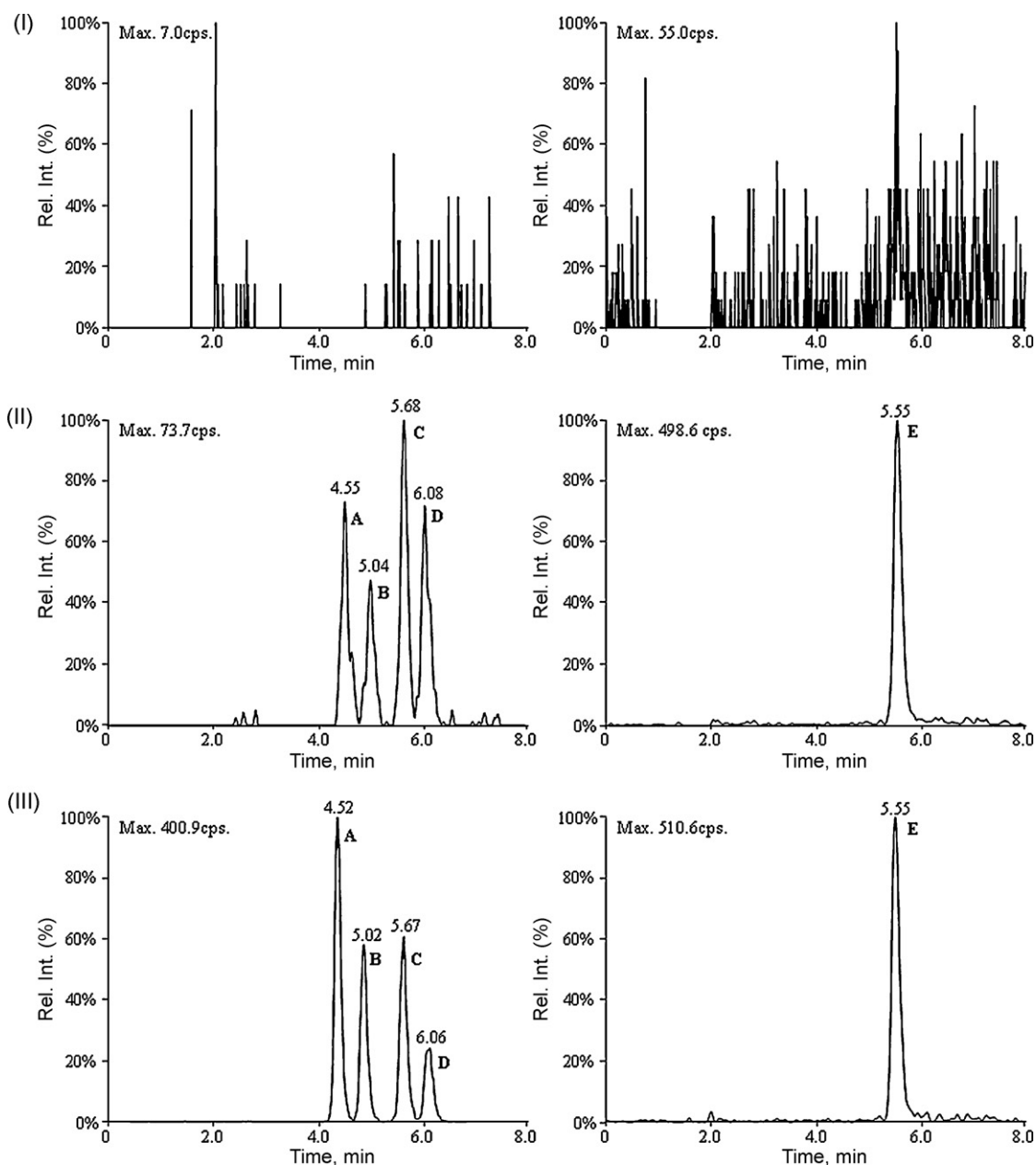
Fig. 3. Representative LC-MRM chromatograms for (A) escin Ia, (B) escin Ib, (C) isoescin Ia, (D) isoescin Ib and (E) I.S. (telmisartan) in human plasma samples: (I) A blank plasma; (II) blank plasma spiked with escin Ia, escin Ib, isoescin Ia, isoescin Ib (33 pg/mL for each saponin) and I.S.; and (III) from a volunteer 1.0 h after oral administration of sodium aescinate tablets containing 60 mg escin saponins.

evaporated to dryness under a gentle stream of N_2 at 30 °C using a Zymark TurboVap LV drying system (Zymark, USA) and reconstituted with 150 μL methanol–water (40:30, v/v) prior to LC–MS/MS analysis using an injection volume of 30 μL.

2.5. Method validation

The method was fully validated according to the FDA Guidance for Industry, Bioanalytical Method Validation [18].

Specificity was assessed by assay of pooled blank plasma from six healthy volunteers. Linearity was assessed by linear regression of calibration curves based on peak area ratios of analytes to I.S. weighted according to $1/x^2$ (x = concentration).

Intra- and inter-day precision (as relative standard deviation (R.S.D.)) and accuracy (as relative error (R.E.)) were determined by assay of six replicates of each QC sample on three separate days using independently prepared calibration curves. The acceptance

criteria for each back calculated concentration were precision < 15% and accuracy < 15% of the nominal value.

The LLOQ was defined as the lowest concentration that could be determined with signal/noise (S/N) > 5, precision of ±20% and accuracy of ±20%.

Recoveries of the four saponins were determined by comparing peak areas of extracted QC samples with those of post-extraction blank plasma spiked at corresponding concentrations. Matrix effects (ME) 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. Process efficiency (PE) was estimated by comparing the peak areas of QC samples with those of corresponding standard solutions.

Stability of the saponins in acidified human plasma was established by analysis of three replicates of QC samples under the following conditions: Long-term stability at −80 °C for 30 days;

Table 1
Accuracy and precision for the analysis of escin Ia, escin Ib, isoescin Ia and isoescin Ib in human plasma (data are for analysis of 6 replicates on 3 different days).

Analyte	Concentration (ng/mL)		Accuracy (R.E. %)	Precision (R.S.D. %)	
	Spiked	Mean calculated		Intra-day	Inter-day
Escin Ia	0.033 (LLOQ)	0.035	5.1	8.1	
	0.10	0.10	3.1	6.0	2.6
	1.0	0.99	−1.2	6.2	7.4
	8.0	7.9	−1.1	5.6	12.8
Escin Ib	0.033 (LLOQ)	0.032	−3.1	8.3	
	0.10	0.10	3.0	9.4	12.5
	1.0	0.97	−2.6	7.4	7.7
	8.0	7.8	−2.7	5.1	14.1
Isoescin Ia	0.033 (LLOQ)	0.031	−5.3	9.6	
	0.10	0.10	1.8	5.7	2.5
	1.0	0.96	−3.7	5.4	6.8
	8.0	8.0	−0.2	4.4	6.5
Isoescin Ib	0.033 (LLOQ)	0.035	6.1	11.1	
	0.10	0.098	−1.8	7.1	4.4
	1.0	0.98	−2.1	4.9	3.2
	8.0	7.7	−3.3	3.5	9.5

short-term stability at 25 °C for 2 h; after three freeze/thaw cycles (−80 °C to 25 °C). Stability of the saponins in processed samples in autosampler vials for 8 h and in stock solutions for one month at −80 °C and at room temperature for 6 h were also investigated.

2.6. Pharmacokinetic study

The study was carried out in 10 healthy male volunteers aged 20–30 years with body mass index (BMI) of 20–24. The study was approved by the Ethics Committee of the Affiliated Hospital of Bethune University of Medical Sciences, Changchun, China. Volunteers were selected after completing a thorough medical, biochemical and physical examination. All volunteers gave informed consent after the aims and risks of the study were fully explained.

After a 12 h fast, volunteers were given a single oral dose of two sodium aescinate tablets containing 60 mg escin saponins with 250 mL water. Blood samples (3.0 mL) were collected by venepuncture into heparinized tubes prior to and at 0.5, 0.67, 0.83, 1, 1.5, 2, 3, 4, 6, 8, 12, 24 and 36 h after dosing. Plasma was prepared by centrifugation at 15,000 × g for 10 min after which 200 μL of 1% formic acid was added to 1 mL plasma. Plasma samples were stored at

Table 2
Matrix effects, recovery and process efficiency of escin Ia, escin Ib, isoescin Ia and isoescin Ib (data are mean ± SD, n = 3).

Analyte concentration (ng/mL)	Matrix effects (%)	Recovery (%)	Process efficiency (%)
Escin Ia			
0.10	100.4 ± 2.0	84.7 ± 0.8	85.2 ± 0.9
1.0	98.5 ± 3.4	88.0 ± 4.0	86.6 ± 1.2
8.0	99.0 ± 6.5	89.2 ± 2.4	88.7 ± 4.1
Escin Ib			
0.10	97.5 ± 2.3	87.3 ± 1.4	85.0 ± 1.0
1.0	98.0 ± 2.5	86.2 ± 4.2	84.8 ± 1.9
8.0	100.6 ± 1.6	83.0 ± 2.4	84.5 ± 0.5
Isoescin Ia			
0.10	105.3 ± 5.2	89.6 ± 3.7	91.4 ± 4.7
1.0	101.7 ± 5.3	81.4 ± 4.8	85.4 ± 2.1
8.0	105.1 ± 3.8	80.5 ± 1.8	84.7 ± 4.2
Isoescin Ib			
0.10	102.7 ± 3.9	83.8 ± 5.0	86.0 ± 1.4
1.0	102.3 ± 6.5	88.8 ± 6.4	90.6 ± 4.3
8.0	105.2 ± 6.7	80.8 ± 0.7	85.1 ± 6.5
I.S.			
0.10	98.6 ± 1.8	86.4 ± 2.6	85.1 ± 6.6

−80 °C and analyzed within 30 days. Each analytical run included a plasma blank, a zero-level standard (blank plasma *plus* I.S.), and a set of calibration standards and QC samples in duplicate.

Pharmacokinetic parameters were analyzed by noncompartmental analysis using the TopFit 2.0 software package (Thomae GmbH, Germany). The maximum plasma concentration (C_{max}) and time to reach it (T_{max}) were determined directly from the data. The terminal phase rate constant (λ_z) was calculated as the negative of the slope of the log-linear terminal portion of the plasma concentration–time curve using linear regression of at least the four last concentration–time points. The terminal phase half-life ($t_{1/2}$) was calculated as $0.693/\lambda_z$. The area under the curve from zero time to the last observed time (AUC_{0-t}) was calculated by the linear trapezoidal rule for ascending data points and by the log-linear trapezoidal rule for descending data points. The total area under the curve ($AUC_{0-\infty}$) was calculated as $AUC_{0-t} + C_t/\lambda_z$, where C_t is the last measurable drug concentration. The apparent total body clearance (CL/F) and apparent volume of distribution associated with the terminal phase (V_z/F) were calculated as $CL/F = dose/AUC$ and $V_z/F = (CL/F)/\lambda_z$, respectively.

3. Results and discussion

3.1. Mass spectrometry

All four saponins and the I.S. responded best to positive ionization and protonated molecular ions $[M+H]^+$ were present as major peaks for all compounds. Small amounts of $[M+Na]^+$ were also detected for the four saponins. For all isomers, similar fragment ions could be observed (Fig. 2) with product ion spectra of $[M+H]^+$ showing fragment ions at m/z 969.9 ($[M–C_6H_{10}O_5+H]^+$), 807.6 ($[M–2C_6H_{10}O_5+H]^+$), 789.5 and 513.4. Since the ion at m/z 807.6 was the most abundant, the transition m/z 1131.8 → 807.6 was used as quantifier and the transition m/z 1131.8 → 789.5 as qualifier for all four saponins. The collision energy was optimised to produce the maximum response for the two product ions.

3.2. Liquid chromatography

Because the same MRM transition was used to analyse the four isomers, chromatographic separation was required. After evaluating different combinations of acetonitrile, methanol and ammonium acetate solution, it was found that methanol gave a higher response than acetonitrile but with poor resolution, while methanol–acetonitrile (50:50, v/v) gave excellent resolution and

Table 3
Stability of escin Ia, escin Ib, isoescin Ia and isoescin Ib under various storage conditions (data are mean $\pm$ SD, $n = 3$).

Analyst concentration (ng/mL)	Long-term ($-80\,^{\circ}$C)	Short-term	Freeze–thaw	Post-preparative
Escin Ia				
0.10	104.1 ± 0.4	103.9 ± 3.9	100.5 ± 2.4	102.3 ± 3.2
1.0	90.8 ± 4.8	100.2 ± 6.8	104.5 ± 1.2	102.1 ± 2.3
8.0	94.5 ± 7.2	100.2 ± 2.5	97.4 ± 4.6	98.6 ± 3.5
Escin Ib				
0.10	110.3 ± 0.7	98.6 ± 2.6	108.2 ± 4.0	108.1 ± 4.2
1.0	101.3 ± 3.2	98.3 ± 2.9	96.2 ± 4.2	97.3 ± 0.9
8.0	96.5 ± 1.9	97.5 ± 4.2	99.6 ± 3.9	95.7 ± 3.2
Isoescin Ia				
0.10	101.1 ± 2.0	106.1 ± 1.2	99.3 ± 2.7	103.2 ± 5.2
1.0	102.5 ± 1.4	98.5 ± 6.6	93.6 ± 2.3	97.6 ± 1.1
8.0	98.1 ± 8.3	97.4 ± 0.2	100.3 ± 6.9	95.7 ± 3.9
Isoescin Ib				
0.10	97.8 ± 0.2	94.9 ± 5.0	101.1 ± 2.7	97.6 ± 1.9
1.0	101.8 ± 1.4	96.8 ± 6.3	102.2 ± 1.8	101.7 ± 1.4
8.0	100.9 ± 5.6	98.9 ± 2.7	98.6 ± 5.9	97.8 ± 3.2

peak shapes with good signal-to-noise ratios. The inclusion of 10 mM ammonium acetate solution enhanced the intensities of the $[M+H]^{+}$ peaks, improved resolution and reduced the cycle time of the assay. Further enhancement in response was obtained using gradient instead of isocratic elution. In terms of I.S., a stable isotope or structural analogue was not available and telmisartan was eventually chosen on the basis of its similarity in retention, recovery and ionization to the four saponins. Of a number of commercially available C18 columns evaluated (Nova-Pak C18, Nucleosil C18, Hypersil C18, Zorbax extend C18 and HC-C18), HC-C18 was found to give the most satisfactory chromatography.

3.3. Sample preparation

In investigating sample preparation, simple protein precipitation gave rise to severe matrix suppression (approximately 30% of the signal of the saponins and I.S.). Liquid–liquid extraction (LLE) using ethyl acetate or dichloromethane gave low extraction recoveries while SPE using methanol as eluting solvent produced good recovery of all four isomers. To improve flow through the SPE cartridges, acidified plasma samples were diluted with water before loading, and the high recoveries of all four saponins from the SPE cartridge were confirmed by repeated elutions with methanol. Similar results were reported by Montenegro et al. [15] who used SPE for sample preparation in the determination of escin in skin permeation studies.

3.4. Method validation

3.4.1. Selectivity

Typical chromatograms of the four saponins are presented in Fig. 3. The assay was free of interference from endogenous substances in plasma. Retention times of escin Ia, escin Ib, isoescin Ia, isoescin Ib and I.S. were 5.62, 5.96, 6.43, 6.76 and 6.16 min, respectively.

3.4.2. Linearity, LLOQ, precision and accuracy

Calibration curves were linear over the concentration range 0.033–10 ng/mL with correlation coefficients (r) > 0.996; typical equations were as follows:

Escin Ia :	$y = 0.7478x + 0.0285,$	$r = 0.9969$
Escin Ib :	$y = 0.8757x + 0.0248,$	$r = 0.9986$
Isoescin Ia :	$y = 0.7071x + 0.0204,$	$r = 0.9977$
Isoescin Ib :	$y = 0.7891x + 0.0051,$	$r = 0.9991$

where y represents the peak area ratio and x represents the plasma concentration of the analyte. The LLOQ was determined to be 33 pg/mL for the four saponins.

Table 1 summarizes intra- and inter-day precisions and accuracies for the four isomers. Intra- and inter-day precisions were <15% with accuracies in the range -5.3 to 6.1%.

3.4.3. Matrix effects, recovery and process efficiency

Recovery, matrix effects and process efficiency results are presented in Table 2. Recoveries of the four saponins were >80.5% and the recovery of the I.S. was $86.4 \pm 2.6\%$. In terms of matrix effects, the peak areas of the four saponins in post-extraction spiked blank plasma samples were 97.5–105.3% of those in the corresponding standard solutions, and that of the I.S. was $97.3 \pm 3.2\%$. The process efficiency which represents a combination of matrix effects and recovery was >84% for all saponins and I.S. Overall, these results indicate that no endogenous substances significantly influence recovery, sensitivity or ionization of the I.S. and saponin isomers.

3.4.4. Stability

Previous research has shown that, in acidic aqueous solutions, β-escins undergo an intramolecular acylic migration involving the C_{21}, C_{22} and C_{28} hydroxyl groups to form the corresponding α-escins [8]. In this study, the effect of pH on the interconversion was studied by dissolving β-escin in phosphate buffers at pH 2, 3, 4, 5, 6, 7 and 8 and analyzing the solutions after various times. It was found that no isomerization occurred at pH 2–3 but did occur at pH ≥ 4. On this basis, 1.0% formic acid was added to plasma samples to adjust the pH to 2–3 prior to storage. The saponins and I.S. were then found to be stable under all the conditions evaluated. Detailed stability data are shown in Table 3.

3.5. Pharmacokinetic study

The method was able to detect the four saponins in human plasma for 36 h after the single oral dose of sodium aescinate containing 60 mg escin saponins. The concentration–time profiles are shown in Fig. 4 and pharmacokinetic parameters are summarized in Table 4. Peak concentrations of the four saponins were in the range 0.38–1.8 ng/mL at approximately 2 h after dosing. In some subjects, plasma concentration–time profiles exhibited multiple peaks suggesting some form of redistribution or enterohepatic recycling occurs in certain individuals. As evident in Fig. 4, β-escins are cleared more rapidly from human plasma than α-escins, which
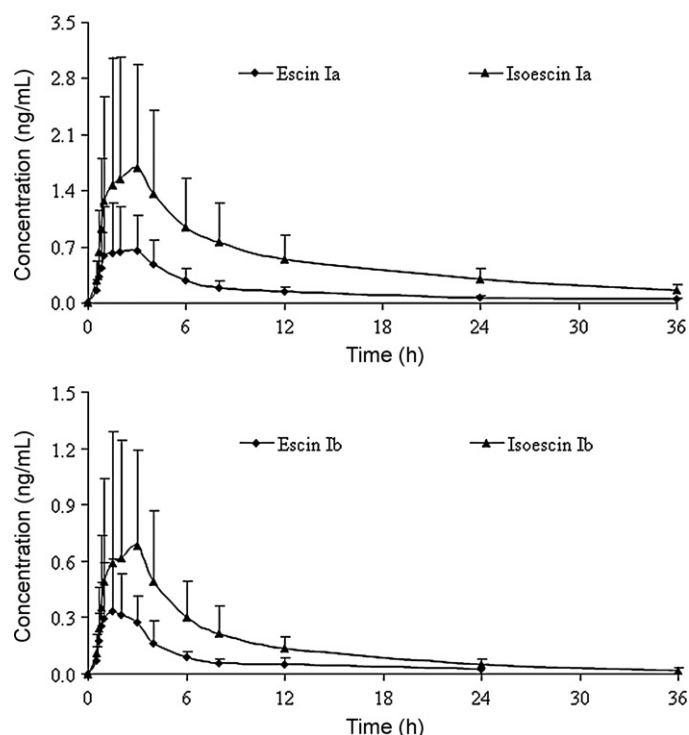
Fig. 4. Mean plasma concentration–time curves of escin Ia, escin Ib, isoescin Ia and isoescin Ib after oral administration of sodium aescinate tablets containing 60 mg escin saponins to healthy male volunteers (data are mean ± SD, $n = 10$).

Table 4
Pharmacokinetic parameters for escin Ia, escin Ib, isoescin Ia and isoescin Ib after oral administration of sodium aescinate tablets containing 60 mg escin saponins to healthy male volunteers (data are mean ± SD, $n = 10$).

Parameters	Escin Ia	Escin Ib	Isoescin Ia	Isoescin Ib
T_{max} (h)	2.6 ± 0.8	2.1 ± 0.7	2.4 ± 0.8	2.1 ± 0.1
C_{max} (ng/mL)	0.77 ± 0.64	0.38 ± 0.26	1.82 ± 1.60	0.74 ± 0.73
$t_{1/2}$ (h)	8.5 ± 3.2	4.7 ± 3.0	13.7 ± 3.8	8.4 ± 2.5
λ_z (1/h)	0.10 ± 0.05	0.21 ± 0.12	0.05 ± 0.01	0.09 ± 0.03
V_z/F (L)	1530 ± 680	1110 ± 680	1110 ± 500	2750 ± 1380
CL/F (mL/min)	1020 ± 410	2900 ± 1360	964 ± 426	3890 ± 1940
AUC_{0-t} (ng h/mL)	5.2 ± 3.6	1.6 ± 0.9	19.3 ± 12.4	5.6 ± 3.8
$AUC_{0-\infty}$ (ng h/mL)	5.7 ± 3.9	1.8 ± 0.9	22.4 ± 13.6	5.8 ± 3.8

indicates that elimination of escins is configuration specific. Further studies of the absorption, distribution, metabolism and excretion of the individual saponins in rat and dog are underway in our laboratory.

4. Conclusion

In the present study, a rapid and sensitive LC–MS/MS assay was developed for the simultaneous determination of four isomeric bioactive saponins, namely, escin Ia, escin Ib, isoescin Ia and isoescin Ib in human plasma. The method was applied to a clinical pharmacokinetic study following oral administration of sodium aescinate tablets. This is the first report of the use of LC–MS/MS to evaluate the pharmacokinetics of escin.

Acknowledgments

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