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Development of a method for comprehensive and quantitative analysis of armillarisin succinate ester in its medicinal preparations by liquid chromatography-ion trap mass spectrometry

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A rapid and sensitive method for the identification and quantification of armillarisin succinate ester (ASE), which is a patent drug, is described. The method used liquid chromatography - ion trap mass spectrometry (LC-IT-MS/MS). The ASE standard solution was directly infused into IT-MS for collecting MS^n spectra. The major fragment ions of ASE were confirmed by MS^n at *m/z* 333, 233, 202, 189 and 163 in negative ion mode, and *m/z* 335, 217, 189,175 and 161 in positive mode, respectively. The possible main fragment ions cleavage pathways were studied between in positive-ion mode and in negative-ion mode. Quantification was performed using the transitions *m/z* 333→233 in negative mode. The method is reliable and reproducible, and the detection limit is 2 ng/mL. The method was validated in the concentration range of 1.0 - 50 μg/mL, intra- and inter-day precision ranged from 1.98% to 4.06%, and the accuracy was 97.5−106.2%. The mean recovery of ASE was 97.2−105.3% with RSD less than 4.35%. A LC-IT-MS/MS has successfully applied to determine the ASE in its medicinal preparations.

1. Introduction

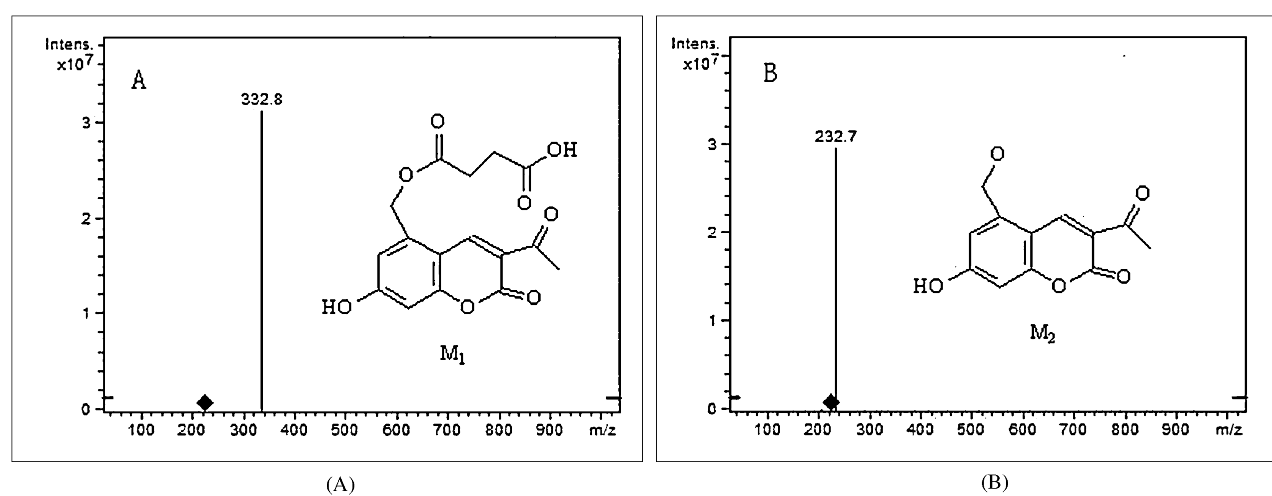
Armillarisin succinate ester (ASE) is a new prodrug originated in armillarisin A (3-acetyl-5-hydroxymethyl-7-hydroxycoumarin) (Wang et al. 2007). Armillarisin A was extracted from the fungus *Armillariella tabescens* (Scop. ex Fr.) Sing. It is used as a choleretic to improve bile secretion and regulate the pressure of the bile duct to ease inflammation and adjust liver function. It is an important component of traditional Chinese medicine for the treatment of infection of the biliary system, gastritis and hepatitis (Li et al. 2009; Shen et al. 2009; Sun et al. 2010). However, the armillarisin A injection is no longer used because of adverse effects of the solubilizer. In order to raise the solubility of armillarisin A, we prepared armillarisin A succinate ester (ASE) (Hermansky 1995; Li et al. 2009; Luo 2008). ASE has a good solubility and can take the place of armillarisin A in the treatment of infection of the biliary system, gastritis and hepatitis, which is a patent drug (Number: CN 1935802A).
Liquid chromatography–mass spectrometry (LC-MS) technology has demonstrated its value in analyzing complex mixtures. LC-MS has also become a powerful tool in the online structural characterization of various natural compounds due to its high sensitivity, great separation efficiency and unique ability of structure analysis (Li et al. 2008; Simirgiotis and Schmeda-Hirschmann 2010; Xu et al. 2006).
Determination of armillarisin A by HPLC coupled to UV (Hu et al. 2005) or MS (Wang et al. 2007; Li et al. 2009) detections have been reported. In prior, we had developed a HPLC to determine the content of ASE in the armillarisin succinate ester Injection (Li et al. 2009). HPLC in combination with tandem mass spectrometry (MS/MS) appears to be a suitable technique for the screening of ASE in terms of sensitivity and selectivity. The application of ion trap (IT) mass spectrometers in the structure elucidation, fragmentation behaviors and pathways of compound is well established (Klausen et al. 2010; Ferreres et al. 2010). Moreover, several reports show that IT mass spectrometers can also be very useful for quantitative analysis (Izumi et al. 2009; Wang et al. 2010). By IT-MS, ASE can be studied in both positive-ion and negative-ion modes. More importantly, MS^n (n = 2, 3) can afford much more confidence for compound identification and much lower noise level for quantification. The fragmentation map of a target analyte is useful in performing MS^n for either qualitative or quantitative analysis. Therefore, it is significant to investigate the fragmentation pathways of the compounds of interest.
To date, however, there is no comprehensive electrospray ionization (ESI)-MS fragmentation study of ASE. In this work, we describe a new method of structure elucidation based on MS^n. Direct infusion was used to collect MS^1 profile and targeted MS^{2-4} data of selected ions (ASE) in both positive-ion and negative-ion modes. Meanwhile, we developed a first simple and sensitive HPLC-MS/MS method for determination of ASE in its injection in negative-ion mode with *m/z* 333→233.

2. Investigation, results and discussion

2.1. *Identification and MS^n behavior of the reference compounds*

2.1.1. *Analysis of ASE by ESI-MS^n in negative ion mode*

ASE reference was dissolved in methanol and analyzed by ESI-MS^n in negative ion mode. The deprotonated molecule ion



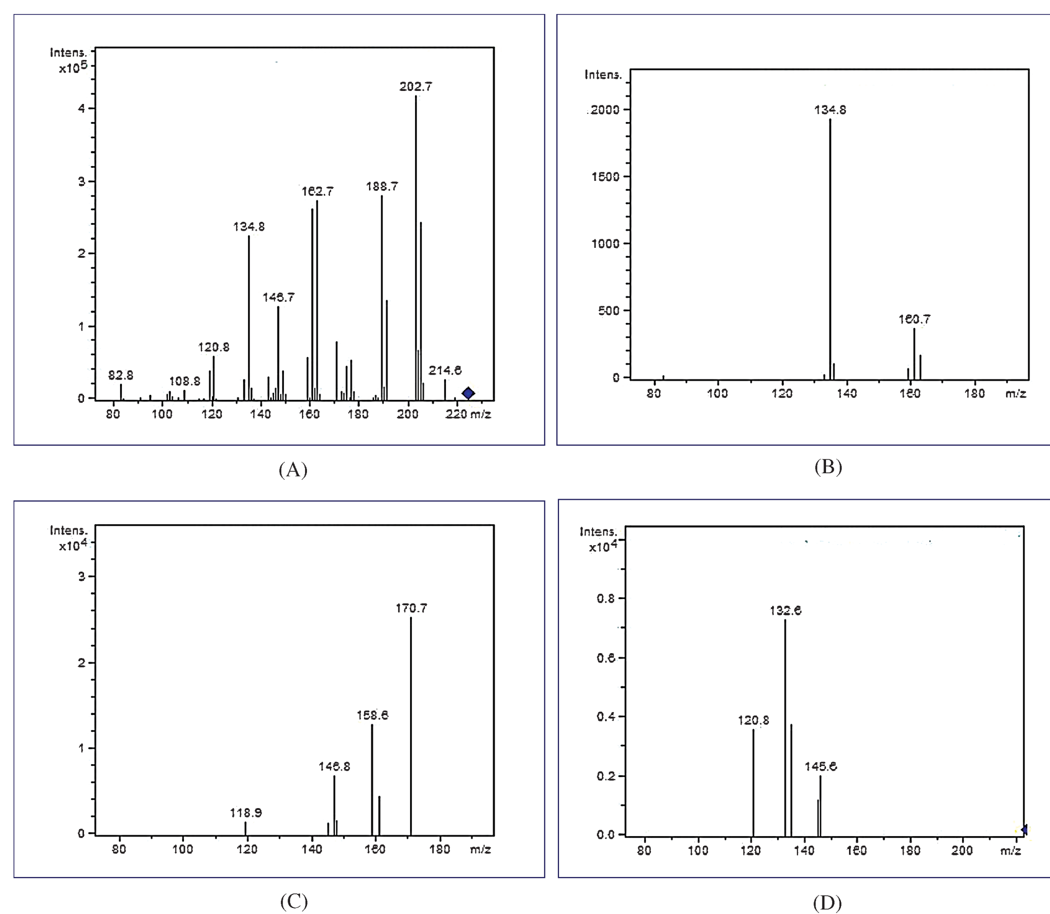
(A) (B)

Fig. 1: Chemical structure and deprotonated molecular ion by full–scan ESI-MS spectra (A) and chemical structure and major fragment ion (B) by full–scan ESI-MS^2 spectra of ASE in negative ion mode

of $[M-H]^-$ and major fragments ions were observed in the full scan MS and MS^n spectra in negative ion mode. Fig. 1 shows the deprotonated molecular ion, major fragment ion spectra and chemical structure by the ESI-MS and ESI-MS^2. The MS spectra from $[M-H]^-$ (M_1) of ASE contains the molecular ion and exhibited the highest intensity. The obtained fragment ion at *m/z* 233 was loss of $C_4H_5O_3$ from the molecule $[M-H]^-$ by ESI-MS^2 and the intensity was very high, too. The MS^3 spectra from *m/z* 333 > 233 ($[M-C_4H_5O_3]^-$, M_2) contains ions at *m/z* 215, 203, 189, 177, 171, 163, 147 and 135, was shown in Fig. 2A. Loss of H_2O forms the major ion at *m/z* 215 from M_2 and loss of CH_2O form the major ion at *m/z* 203, and then split to *m/z* 161 and 135. The product ion at *m/z* 233 can split to *m/z* 189 and 163, too. Figures 2B, C, D show the major fragment ion spectra at *m/z* 203,189 and 163 in ESI-MS^4. The fragment ion at *m/z* 171, 159, 147 comes from the *m/z* 189, *m/z* 161, 135 come from the *m/z* 203, and the *m/z* 145 come from the *m/z* 163, respectively. The possible main fragment ions cleavage pathway is shown in Fig. 3 .

2.1.2. *Identification and MS^n behavior of ASE by ESI-MS^n in positive ion mode*

ASE reference was dissolved in methanol and analyzed by ESI-MS^n in positive ion mode. The protonated molecule ion of $[M+H]^+$ and major fragments ions were observed in the full



(A) (B)

(C) (D)

Fig. 2: Full–scan ESI-MS^3 spectra at *m/z* 233 (A), full–scan ESI-MS^4 spectra at *m/z* 203 (B), *m/z* 189(C) and *m/z* 163(D) of ASE in negative ion mode

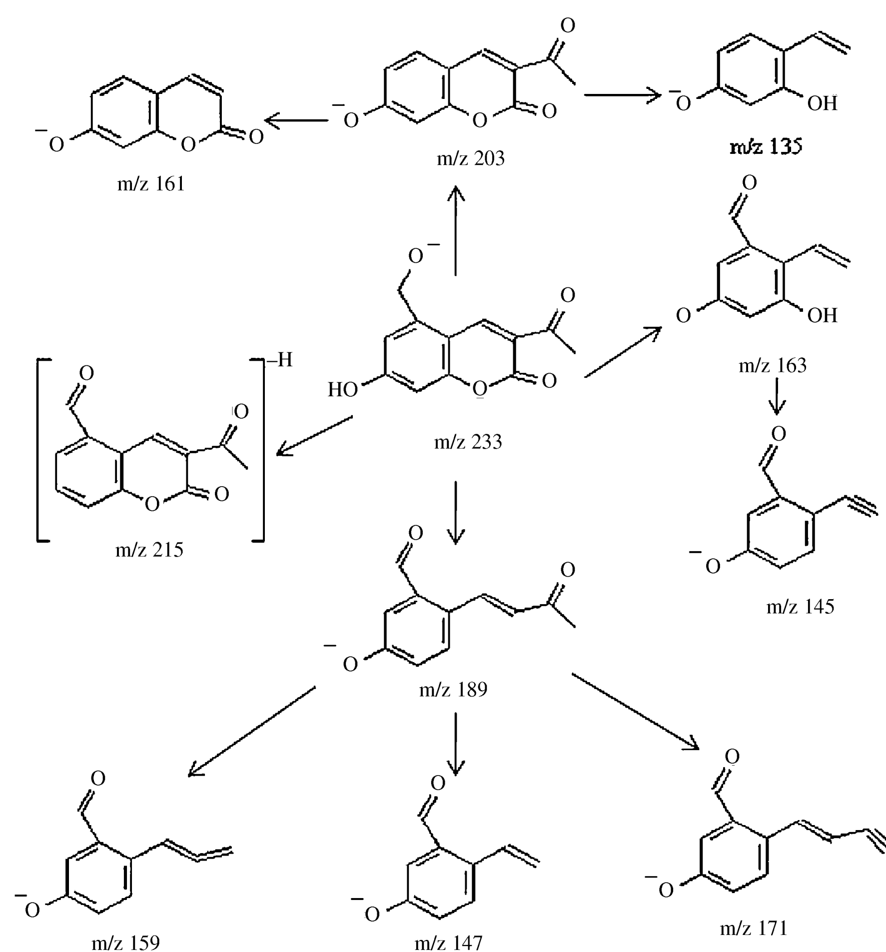


Fig. 3: Proposed fragmentation pathways of main fragment ions for ASE in negative ion mode

scan MS and MS^n spectra in positive ion mode. Figure 4 shows the protonated molecular ion, major fragment ion spectra and chemical structure by the ESI-MS and ESI-MS^2. The MS spectra from $[M+H]^+$ (M_3) of ASE contains the molecular ion and exhibited the highest intensity. The obtained fragment ion at *m/z* 217 was loss of succinic from the molecule $[M+H]^+$ by ESI-MS^2 and the intensity was very high, too. The MS^3 spectra from *m/z* 335, 217 ($[M\text{-}C_4H_5O_4]^+$, M_4) contains ions at *m/z* 189, 175, 161, 147, 133, 115, and 105 is shown in Fig. 5A. Loss of CO forms the major ion at *m/z* 189 from M_4, loss of C_2H_2O form the major ion at *m/z* 175, and loss CO_2 and CH_2 form the major ion *m/z* 161. Figures 5B, C, D show the major fragment ion spectra at *m/z* 189,175 and 161 in ESI-MS^4. The fragment ion at *m/z* 79 comes from the *m/z* 189, *m/z* 147, 119 come from the *m/z* 175, and the *m/z* 133 comes from the *m/z* 161, respectively. The possible main fragment ions cleavage pathway is shown in Fig. 6.

2.2. Quantification of ASE by HPLC/MS

Because ASE have a carboxyl group in its structure, they are acidic compounds, thus the weakly acidic isomers can

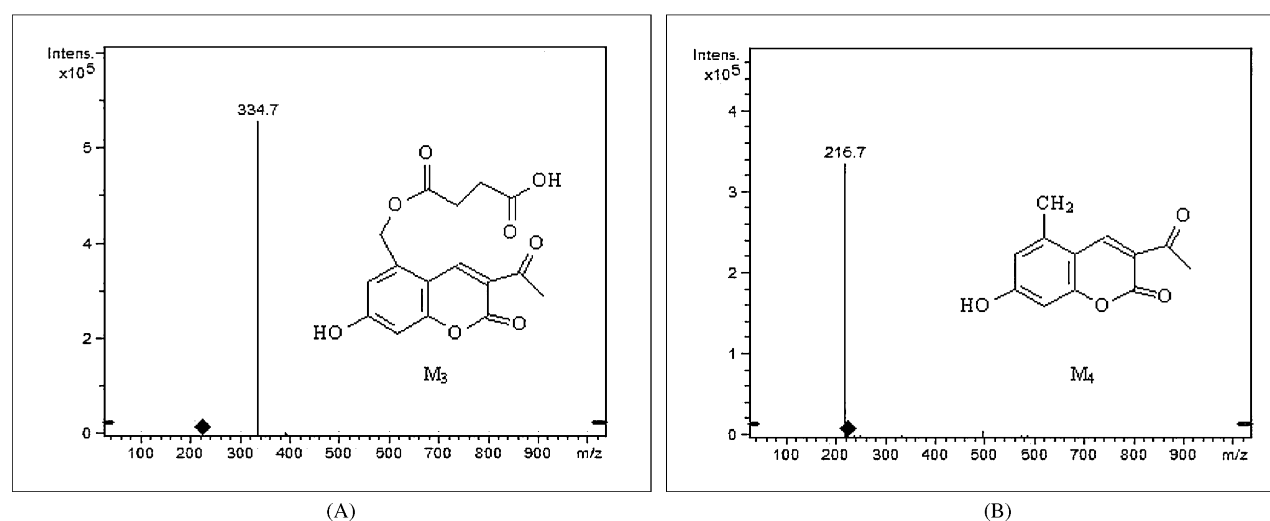


Fig. 4: Chemical structure and protonated molecular ion by full–scan ESI-MS spectra (A) and chemical structure and major fragment ion (B) by full–scan ESI-MS^2 spectra of ASE in positive ion mode

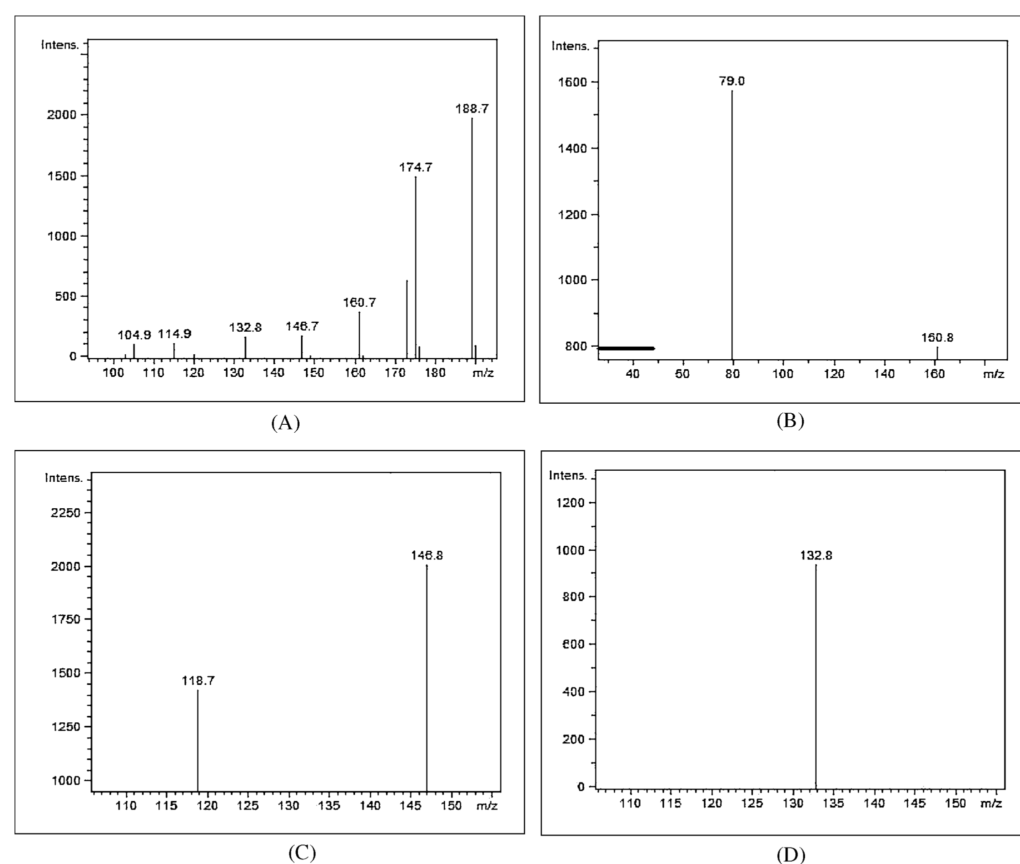


Fig. 5: Full–scan ESI-MS^3 spectra at *m/z* 217 (A), full–scan ESI-MS^4 spectra at *m/z* 189 (B), *m/z* 175(C) and *m/z* 161(D) of ASE in positive ion mode

be ionized readily to give anions. Those are expected for the negative ESI responses due to the carried negative charge in a basic solution with pHs higher above the pK_as. So the negative ion mode and ESI source were more adopted for the analysis of ASE. The standard solutions of the ASE (50 μg/ml) were directly injected into IT-MS in both the positive and negative ion modes, respectively. The MS spectra were detected. The value of signal-to-noise (S/N) ratio in the negative mode was higher than the value of S/N in the positive mode, because the noise of the negative mode was lower than that of the positive mode. So determination of ASE was performed in selected ion monitoring (SIM) mode with *m/z*: 333→233.

An HPLC/MS method was developed by optimizing the LC separation and ionization and ion transport conditions. A mobile phase composition must be selected to meet the requirement of mass spectrometric detection. The selection of mobile phase components is a critical factor to obtain good chromatographic peak shape and resolution. In order to enhance the ESI response,

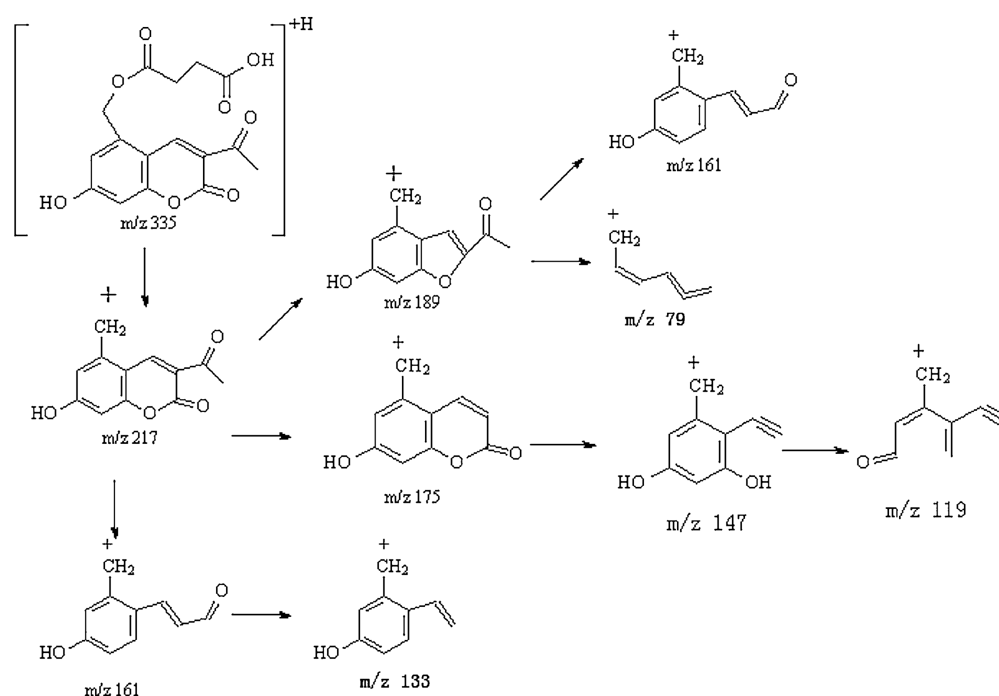


Fig. 6: Proposed fragmentation pathways of main fragment ions for ASE in positive ion mode

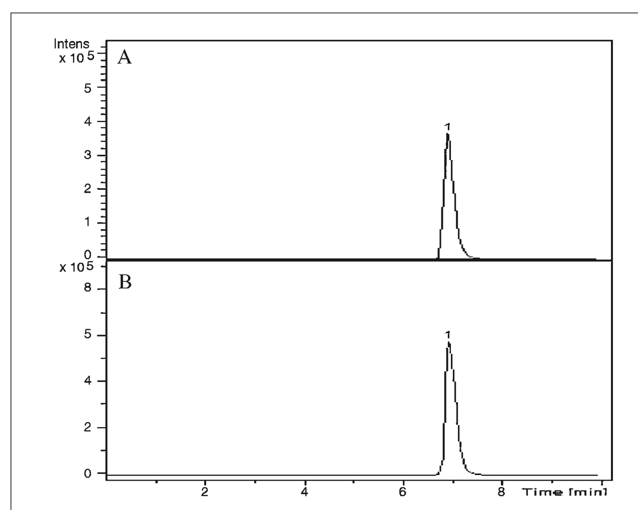


Fig. 7: Typical HPLC-MS profiles of ASE standard (A) and sample (B) by SIM

ammonium acetate is usually adopted in the mobile phase of the HPLC. The effect of the ammonium acetate concentration on separation was studied in the range from 5 to 30 mM. The acceptable retention and separation of analytes was obtained by using a solution of methanol-water containing of 20 mM ammonium acetate as the mobile phase. Figure 7 shows typical HPLC-MS/MS profiles of standard and sample. There were no foreign matter peaks that could interfere with the analyte and a stable baseline was maintained throughout.

2.3. Method validation

In order to increase the sensitivity of the developed method, quantitative determination was performed by SIM of ion at *m/z* 333→233 for ASE. The calibration curve was established by injecting six concentration levels of ASE (each in triplicate) in the concentration range 1.0−50 µg/mL. Linear regression was calculated by the peak areas of ASE as a function of concentration. The regression equation and its coefficient is $Y = (8 \times 10^6 \pm 10004)\ X + (2 \times 10^7 \pm 125009)$ ($R^2 = 0.992$) for ASE by LC-MS/MS. As can be seen, the proposed method presented excellent correlation coefficients and sensitivity.
The limit of detection (LOD) and limit of quantification (LOQ) for ASE standard were determined on the basis of the ASE/baseline signal-to-noise (S/N) ratio. A standard stock solution of ASE was initially prepared. Dilutions and injections of this standard were then made until an HPLC-MS/MS showed that the ASE peak height reached an S/N of approximately 10:1 and 3:1 for the LOQ and LOD solutions, respectively. Five injections of the LOQ and three injections of the LOD solutions were made and the relative standard deviation (RSD) for the LOQ solution was determined. The LOD and LOQ were determined to the same levels. The LOD of ASE was determined to be 2.0 ng/mL. The LOQ of ASE were determined to be 10.0 ng/mL $^{-1}$ with 4.8% of RSD for five consecutive injections.
The reproducibility of the method was proved by analyzing samples of ASE at three concentration levels, 1.0, 10 and 50 µg/mL. RSDs (n = 5) were 2.7–5.1%.
The precision of injection was evaluated by repeated injection of the sample solution six times. The RSD values of the peak areas for ASE were better than 3.06%. The acceptable intra- and inter-day precisions (% RSD) and accuracy (relative error, % RD) were set as < 5% and between -5% and 5%, respectively. Intra- and inter-day variabilities were determined by analysis of average amount of standards in quality control samples prepared by standard solutions at low, medium and high concentrations on three different days. The quality control samples were prepared as a single batch on the same day at each concentration, and then divided into aliquots that were stored at 4 °C until required for analysis. The calculated RSD from repeated measurements are summarized in Table 1. The assay precision ranged from 1.98% to 4.06%, and the accuracy was 97.5–106.2%.
Method recovery was calculated by spiking five samples with standard solution at low, medium and high concentrations of analyte. Two injections of each preparation were made and the theoretical amount of analyte in the sample preparations and the average percentage analyte recovered in the spiked solutions were calculated. The mean recovery for ASE was 97.2−105.3% with RSD less than 4.35%. Results are shown in Table 2.

Table 1: Intra- and inter-day precision and accuracy of ASE

Actual ASE concentration (µg/mL)	Intra-day RSD (%)	Intra-day Accuracy	Inter-day RSD (%)	Inter-day Accuracy
1.0	2.58	102.1	4.06	106.2
10	3.02	98.8	2.36	98.5
50	1.98	97.5	3.16	97.7

2.4. Applications

Analysis of real samples was performed on ASE injection (1.0 mL) diluted with methanol (100 mL). The quantification of ASE was performed by HPLC-MS and the contents of analyte in ASE injection were 97.2%, 98.6% and 99.1%, respectively. The results obtained by HPLC-IT-MS/MS were compared with HPLC-UV as the Student's t-test at 95% confidence level indicated no significant difference between the methods (Li et al. 2009).

3. Experimental

3.1. Chemicals and materials

ASE was synthesized by an organic chemistry group of Shenyang Pharmacy colleage (China) and was characterized using spectroscopic techniques (^{1}H NMR, ^{13}C NMR, IR, melting point and combustion microanalysis); HPLC grade methanol and ammonium acetate were obtained from Wuhan Analytical reagent company (Wuhan, China); Deionized water was purified using a Milli-Q system (Millipore, Bedford, MA, USA); Helium (purity, 99.999%) and liquid nitrogen were obtained from Wuhan Analytical Instrument Factory (Wuhan, China); Other reagents used in the experiment were of analytical grade and from commercial sources. Armillarisin succinate ester injection was supply by our laboratory (Shiyan, China).

3.2. Standard and sample preparation

The stock solution was prepared by dissolving 10.0 mg of ASE in 10 mL methanol. The stock solution was diluted with methanol to obtain 100 µg/mL as working standard solution. The solutions were kept at 4 °C before use. Standard solutions of ASE at various concentrations were prepared by diluting the working solution with methanol.
Aliquots of 1.0 mL of the injection of ASE were diluted in a volumetric flask with 100.0 mL of methanol. The solution was then filtered through a 0.22 µm membrane before injection.

Table 2: Recoveries of ASE in ASE injection

ASE content of sample (mg)	Spiked amount (mg)	Found amount (mg)	Recoveries Mean(%)	RSD (%)
1.875	1.0	2.974 ± 0.129	105.3	4.35
1.875	2.0	3.823 ± 0.066	97.2	1.73
1.875	3.0	4.839 ± 0.088	98.1	1.82

3.3. MS^n analysis of the reference compounds solution

A LC/MSD Trap SL Plus spectrometer (Agilent Technologies, Waldbronn, USA) equipped with electrospray ionization (ESI) interface and an ion trap mass analyzer was applied to the MS and multistage mass spectrometry (MS^n) analysis. System control and data analysis was provided by the Agilent LC Chemstation and by Bruker Daltonics Trap Control and QuantAnalysis. A syringe pump was used for the direct loop injections of reference compound working solutions, and the flow rate was set at 0.3 mL/h. The ESI source was used and operated in both positive and negative ion mode. Typical operating conditions are described as follows: drying gas (N_2) temperature of 350 °C, 5 L/min drying gas flow, 15 psi nebulizer gas (N_2) pressure, and 4,500 V of capillary voltage. Data were acquired with a smart target of 30 000 and a max accumulation time of 200 ms. First, full-scan MS spectra were obtained by scanning from 50–500 *m/z*. MS^2 acquisition of the most abundant ions in the full-scan MS mode was carried out. Finally, MS^{3-4} acquisition was used to confirm the identity of the analytes.

3.4. HPLC-MS/MS conditions and instrumentation

HPLC analyses were performed on an 1100 HPLC instrument (Agilent Technologies, Waldbronn, USA) coupled to a binary pump, a UV detection system, an autosampler, and a column thermostat. The sample was separated on DL- C_{18} column (5.0 μm, 150 × 4.6 mm; Japan). The mobile phase consisted of methanol and 20 mM ammonium acetate in water (80:20). The flow rate was 0.5 mL/min and the column temperature was set at 30 °C. The injection volume was 10 μL.

The Agilent 1100 HPLC system was coupled on-line to a LC/MSD Trap SL Plus spectrometer (Agilent Technologies, Waldbronn, Germany) equipped with electrospray ionization (ESI) source. The AutoMS operation parameters are described as follows: negative-ion mode (ESI^-); nitrogen drying gas, 10 L min^{-1}; nebulizer, 40 psi; gas temperature, 350 °C; Compound stability, 80%; mass range, 50–500 *m/z*. Detection of ASE was performed in selected ion monitoring (SIM) mode with *m/z*: 333→233.

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