Qualitative and quantitative analysis of the major constituents in traditional Chinese medicine Danmu injection using LC-ESI-MS^n and LC-DAD

Fen-Xia Zhu, Jing-Jing Wang, Xiu-Feng Li[1], E Sun, Xiao-Bin Jia

Key Laboratory of New Drug Delivery System of Chinese Meteria Medica, Jiangsu Provincial Academy of Chinese Medicine, Nanjing 210028, [1]Engineering Centers for Li Nationality Medicine, Hainan Pharmaceutical Factory, Co., Ltd, Wuzhishan 572200, P.R. China

Submitted: 03-07-2013 Revised: 16-08-2013 Published: 24-07-2014

ABSTRACT

Background: Danmu injection, a traditional Chinese medicine (TCM) preparation made from *Nauclea officinalis*, has been commonly used for the treatment of cold, fever, swelling of throat in China. However, the chemical constituents in Danmu injection have not been clarified yet. **Objective:** a HPLC/DAD/ESI-MS^n method was developed for qualitative and quantitative analysis of the components in Danmu injection. **Materials and Methods:** The chromatographic separation was performed on a Welch Material XB-C18 (4.6mm × 250mm, 5μm) using gradient elution with acetonitrile (A) and water containing 0.1% formic acid (B) as mobile phase at a flow rate of 1.0 ml/min. **Results:** Twenty-five compounds, including phenolic acid and phenol glycoside, iridous glycoside and glycoalkaloid were identified or tentatively deduced on the base of their retention behaviors, UV absorption, MS and MS^n data with those elucidated references or literature. In addition, eleven compounds were simultaneously determined by HPLC–DAD, which was validated and successfully applied for determination of major components in Danmu injection. **Conclusion:** The results suggested that the established qualitative and quantitative method would be a powerful and reliable analytical tool for the characterization of multi-constituent in complex chemical system and quality control of Danmu injection.

Key words: Danmu injection, HPLC–DAD–ESI-MS^n; major constituents, *Nauclea officinalis,* quality control

Access this article online

Website: www.phcog.com

DOI: 10.4103/0973-1296.137365

Quick Response Code:

INTRODUCTION

Traditional Chinese medicines (TCMs) have been widely used in many oriental countries for thousands of years.[1] In the past decades, the TCM injection has been achieved great development and become a very important formulation. However, with the widespread usage of TCM injection, many serious adverse reactions are reported in recent years,[2-6] mainly due to the lack of a practicable and reliable quality control to monitor the properties changed in the procedure from preparation, transportation and storage to clinic usage.

Danmu injection is a modern formula prepared from *Nauclea officinalis* (commonly known as Danmu) which is a traditional Chinese medicine growing in the southern part of China and the only one species in genus *Nauclea* in China. *Nauclea officinalis* has been used for the treatment of cold, fever, swelling of throat, pink eyes and so on because of its anti-inflammatory effects.[7,8] Although the main constituents in *Nauclea officinalis* were found to be alkaloids and its glucoside,[9-12] the chemical ingredient identification method for Danmu injection was still lacking, which resulted in the difficulty in the quality control of Danmu injection. Xie *et al.*, determined five compounds in *Nauclea officinalis* leaves by HPLC.[13] As for the quantitative analysis of Danmu injection, the single alkaloid strictosamide has been determined,[14,15] and three alkaloids and one phenol acid had been determined by HPLC.[16] However, the above-mentioned methods could not provide a comprehensive chemical profile for Danmu injection.

In the last decades, hyphenated techniques, such as LC–ELSD,[17] LC–DAD,[18] LC–MS,[19] GC–MS,[20] and LC–NMR/MS,[21] have been widely applied for the

Address for correspondence:
Dr. Xiao-Bin Jia, Key Laboratory of New Drug Delivery System of Chinese Meteria Medica, Jiangsu Provincial Academy of Chinese Medicine, Nanjing 210028, P.R. China.
E-mail: jxiaobin2012@126.com

analysis of the constituents in the botanic extracts and TCMs. Among them, LC–MS becomes more popular for direct identification of multi-component and quality control, due to its wide suitability, sensitivity, and sufficient structural information.[22] In the present study, a sensitive HPLC/DAD/ESI-MS^n method was established for rapid separation, reliable identification and quantification of the multiple components in Danmu injection. Twenty-five constituents were identified by comparison of their retention times, UV absorption and MS spectra with those elucidated references or literature data; 11 of the identified ingredients with high content were simultaneously determined by the established HPLC–DAD approach. According to the results, phenolic acid, iridous glycoside and glycoalkaloid were the main constituents in Danmu injection; furthermore, it was found that protocatechuic acid was also the abundant compound except for glycoalkaloid. This study represents the first detailed investigation of the constituents of Danmu injection and provides an applicable method for its quality evaluation.

MATERIALS AND METHODS

Chemicals and materials

HPLC-grade acetonitrile and formic acid were purchased from Tedia (Fairfield, OH, USA). Ultrapure water was prepared by a Milli-Q System (Millipore, Bedford, MA, USA) for preparing samples and mobile solution. Other reagents were of analytical grade. All solvents and samples were filtered through 0.45 μm membrane filters before analysis.

Protocatechuic acid (**D1**) and chlorogenic acid (**D6**) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China); Neochlorogenic acid (**D3**) and cryptochlorogenic acid (**D9**) were obtained from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, China); Loganin (**D7**) and sweroside (**D13**) was purchased from Shanghai Yuanye Bio-Technology Co. Ltd (Shanghai, China); 3, 4-dimethoxyphenol β -D-apiofuranosyl (1 → 6)-β-D-glucopyranoside (**D5**), kelampayoside A (**D8**), naucleamide A-10-O-β-D-glucopyranoside (**D15**), naucleamide G (**D17**), pumiloside (**D18**), 3-epi-pumiloside (**D19**), 3α, 5α-tetrahydrodeoxycordifoline lactam (**D23**), strictosamide (**D24**) and vincosamide (**D25**) were isolated from *Nauclea officinalis* in our laboratory and their structures were confirmed based on spectroscopic analysis (MS, ^{1}H-NMR and ^{13}C-NMR).[23] The purities of all the standards were not less than 98%.

Five batches of Danmu Injection (110601, 110616, 110401, 110801 and 110925) were supplied by Hainan Pharmaceutical Factory Co., LTD. (Wuzhishan, China).

Standard and sample solutions preparation

The standard substances of D1 (20.16 mg), D3 (6.32 mg), D6 (7.96 mg), D9 (6.16 mg), D15 (17.12 mg), D17 (16.00 mg), D18 (16.20 mg), D19 (12.08 mg), D23 (8.48 mg), D24 (60.12 mg) and D25 (60.12 mg) were accurately weighed and dissolved respectively in 10 mL volumetric flask with methanol to give individual stock solutions. The mixed standard solution containing protocatechuic acid (**D1,** 161.28 μg/mL), neochlorogenic acid (**D3,** 31.60 μg/mL**)**, chlorogenic acid (**D6,** 39.80 μg/mL), cryptochlorogenic acid (**D9,** 30.80 μg/mL), naucleamide A-10-O-β-D-glucopyranoside (**D15,** 85.60 μg/mL), naucleamide G (**D17,** 80.00 μg/mL), pumiloside (**D18,** 81.00 μg/mL), 3-epi-pumiloside (**D19,**60.40 μg/mL), 3α,5α-tetrahydrodeoxycordifoline lactam (**D23,** 42.40 μg/mL), strictosamide (**D24,** 601.20 μg/mL) and vincosamide (**D25,** 41.00 μg/mL) in methanol was prepared and diluted with methanol to six different concentrations with the ranges listed in Table 1. The mixed standard solutions were filtered through a 0.45 μm membrane prior to injection. All solutions were stored at 4°C in refrigerator before analysis.

A sample of 1.0 mL injection was diluted to 10 mL with ultrapure water in a volumetric flask before being filtered through a 0.45 μm membrane filters. An aliquot of each filtrate was injected into the HPLC instrument for analysis.

HPLC-DAD-ESI- MS SYSTEM

Chromatographic analysis

An Agilent series 1200 HPLC instrument (Agilent, Waldbronn, Germany) equipped with a quaternary pump,

Table 1: Linear regression data, LOD and LOQ of the investigated compounds

Analyte	Calibration curve	Linear range (μg/mL)	R^2	LOQ (ng)	LOD (ng)
D1	*y*=31.629 *x*-11.25	3.22~161.28	1.0000	4.86	1.62
D3	*y*=13.359 *x*-5.1437	0.63~31.60	0.9998	6.40	1.28
D6	*y*=11.952 *x*+0.218	0.80~39.80	0.9999	5.71	1.43
D9	*y*=13.934 *x*-1.351	0.62~30.80	1.0000	8.86	2.21
D15	*y*=32.635 *x*+16.098	1.71~85.60	0.9999	9.56	4.28
D17	*y*=20.472 *x*+2.505	1.60~80.00	1.0000	8.00	2.67
D18	*y*=44.942 *x*-4.7128	1.62~81.00	1.0000	8.20	2.05
D19	*y*=43.316 *x*-5.4285	1.21~60.40	1.0000	6.00	1.50
D23	*y*=23.723 *x*-5.5732	0.85~42.40	0.9999	4.24	1.06
D24	*y*=31.835 *x*+84.135	12.02~601.20	0.9999	80.13	20.03
D25	*y*=34.35 *x*-8.4458	0.82~41.00	1.0000	3.42	1.37

LOD: Limits of detection; LOQ: Limits of quantification

a diode-array detector, an auto sampler and a column compartment was used. The samples were separated on a Welch Material XB-C18 (4.6 mm × 250 mm, 5 μm, Welch Material). The mobile phase consisted of acetonitrile (A) and water containing 0.1% formic acid (B). A gradient program was used as follows: 0~70 min, 1%~35% A, 70~75 min, 35%~95% A. Flow rate was 1.0 mL/min, Column temperature was 25°C. The injection volume was 20 μL. Detection wavelength was set at 260 nm for protocatechuic acid, 327 nm for neochlorogenic acid, chlorogenic acid and cryptochlorogenic acid, 245 nm for pumiloside and 3-epi-pumiloside, 226 nm for naucleamide A-10-O-β-D-glucopyranoside, naucleamide G, 3α, 5α-tetrahydrodeoxycordifoline lactam, strictosamide and vincosamide, respectively.

Mass spectrometry

Mass spectrometry was performed using Agilent 6310 mass spectrometers (Agilent, Waldbronn, Germany) equipped with ESI interface and ion trap analyzer. The ESI-MS spectra of samples and reference compounds were acquired in both positive and negative ionization modes. The parameters were as follows: the drying gas temperature was set at 350°C and the capillary voltage was set at 3.5 kV, skimmer voltage at 40 V, Flow rate of nebulizing gas (N_2) 12 L/min; and pressure of drying gas (N_2) 35 psi. Mass spectrometry was performed in the full-scan mode (MS1) and automatic multiple-stage fragmentation-scan modes (MS2–MS4) over an m/z scan range of 50-1000.

Method validation

The method validation was performed after the optimum conditions established. Solutions containing 11 standard substances at six different concentrations were injected in triplicate. Calibration curves were established by plotting the peak area versus concentration of each analyte. The limits of detection (LOD) and limits of quantification (LOQ) were measured with the signal-to-noise ratio of 3~4 and 10~12 as criteria, respectively.

Intra- and inter-day variations were utilized to assess the precision of the method. The intra-day variation was determined by analyzing six replicate samples within 1 day and the inter-day variation was examined in 3 consecutive days. Recovery was used to evaluate the accuracy of the method. A certain amount of Danmu injection sample was spiked with the mixed standard solution. The mixture was processed and analyzed using the method mentioned above, and three replicates were performed for the analysis. Variations were expressed by relative standard deviation (RSD) in all tests above.

RESULTS

Identification of chemical constituents in Danmu injection

The reference standards and Danmu injection sample were analyzed using the optimized LC–DAD-ESI-MS^n method, and 25 peaks were observed in Danmu injection sample [Figure 1]. The chemical structures of the 25 compounds were characterized based on their retention behavior and their UV spectra obtained on-line [Table 2]. Different types of compounds showed different UV absorption characteristics. For MS analysis, both negative and positive modes of ESI mass spectra were examined in this study. Generally, in the positive mode, $[M + H]^+$ ions of alkaloids of sufficient abundance could be subjected to MS^n analysis and provided more structural information, while in the negative mode, $[M - H]^-$ or $[M + 46–H]^-$ were observed. Due to the use of formic acid in mobile phase, there were adducted ions of $[M + 46–H]^-$ corresponding to $[M + HCOOH - H]^-$, which provided valuable information for composition of the constituents.

Twenty-five compounds were tentatively identified on the basis of their retention times, UV absorption, MS data with those of the reference standards and the literature data (chemical structures shown in Figure 2. Among the identified constituents, peaks D1, D3, D5, D6, D7, D8, D9, D13, D15, D17, D18, D19, D23, D24 and D25 were identified as protocatechuic acid, neochlorogenic acid, 3,4-dimethoxyphenol β -D-apiofuranosyl (1→6)-β-D-glucopyranoside, chlorogenic acid, loganin, kelampayoside A, cryptochlorogenic acid, sweroside, naucleamide A-10-O-β-D-glucopyranoside, naucleamide G, pumiloside, 3-epi-pumiloside, 3α-5α-tetrahydrodexycordifoline lactam, strictosamide and vincosamide by comparing the standard substance, respectively.

The identified compounds can be classified into three classes, namely phenolic acid and its phenol glycoside, iridous glycoside and glycoalkaloid. A total of 7 phenolic acids or phenol glycoside (peaks D1, D2, D3, D5, D6, D8 and D9) were identified, most of which produced $[M-H]^-$ ion. 4 iridoid glycoside (peaks D4, D7, D11 and D13) and 14 glycoalkaloid (D10, D12, D14, D15, D16, D17, D18, D19, D20, D21, D22, D23, D24 and D25) were characterized by MS^n analysis due to their fragmentation pathways, as well as by comparing with reference standards and previous studies. Alkaloids and glycoalkaloids are the main ingredients in *Nauclea officinalis*.[24-26] For MS analysis, glycoalkaloid gave $[M + H]^+$ ions and were observed to

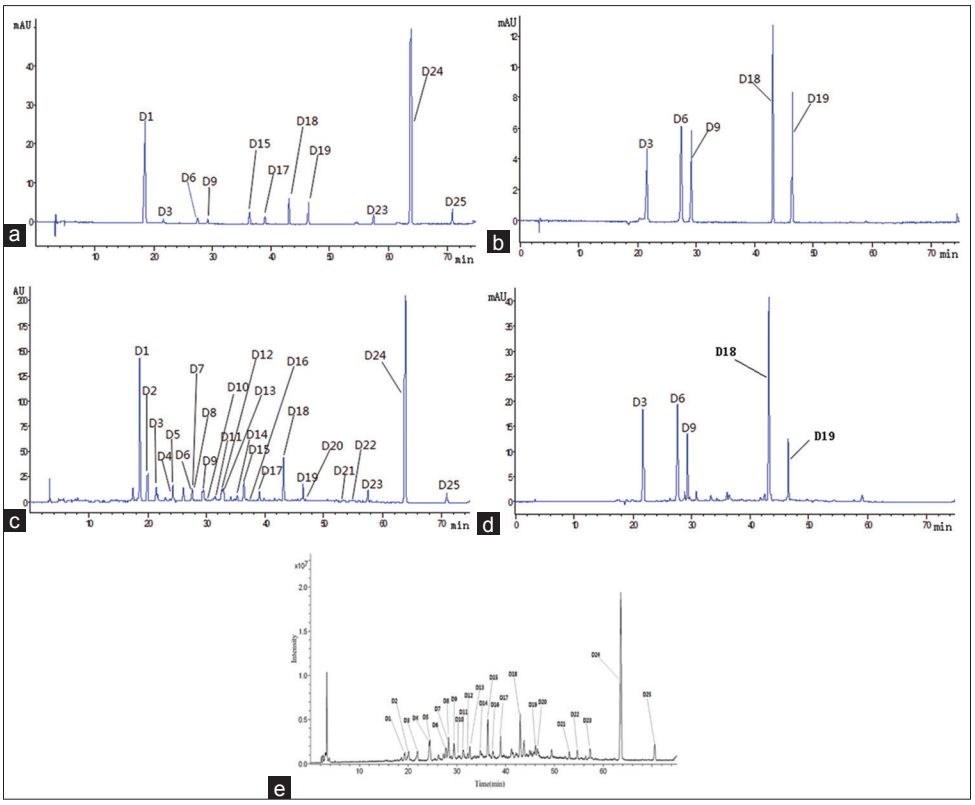

Figure 1: HPLC Chromatograms of standard substances (a and b) and sample (c and d) determined at 260 nm (a and c) and 325 nm (b and d); TIC chromatogram of DM injection in negative ionization modes (e)

undergo the Retro–Diels–Alder (RDA) fragmentation reaction [Figures 1 and 2 and Table 2].

The ESI-MS of protocatechuic acid, neochlorogenic acid, chlorogenic acid and cryptochlorogenic acid all produced $[M - H]^-$ ion in the negative mode. The fragment ion *m/z* 109 was found in the MS/MS spectrum of protocatechuic acid, which was followed by neutral loss of CO_2 (44 Da). The $[M - H]^-$, $[2M - H]^-$ ions and the characteristic ions at m/z 191 [quinicacid $- H]^-$, 173 [quinic acid $- H - H_2O]^-$ and 135 [caffeoyl $- H - COO]^-$ were observed in the MS^n spectra of chlorogenic acid.[27]

The ESI-MS^n of 3,4-dimethoxyphenol β-D-apiofuranosyl (1 → 6)-β-D-glucopyranoside and kelampayoside A

The 3,4-dimethoxyphenol β -D-apiofuranosyl (1 → 6)-β-D-glucopyranoside was found in the genus *Nauclea* for the first time and kelampayoside A was isolated from *Nauclea officinalis* for the first time in our lab, respectively.[23] The structures of the two compounds were very similar to each other except for the $–OCH_3$ at the C-5 position. The analogies of structures indicated the similarities of their MS^n fragmentation behaviors. In their ESI-MS spectra, the $[M + Na]^+$ ion were obtained in the positive mode, while in the negative mode, the $[M - H]^-$, $[M + HCOOH - H]^-$ and $[M + 2H_2O - H]^-$ ions were simultaneously observed. The MS/MS spectrum showed a significant fragment ion $[C_{11}H_{18}O_9 - H]^-$ ion at *m/z* 293 which was attributed to the loss of a C-1 unit, at the same time, other ions *m/z* 233, *m/z*149 and *m/z*131 were observed [Figure 3].

The ESI-MS^n of sweroside

The $[M - H]^-$, $[M + 2H_2O - H]^-$ and $[M + HCOOH - H]^-$ ions were shown in the negative mode. In the MS/MS spectrum analysis of $[M + HCOOH - H]^-$ ion, the ion caused by the losses of glucose and other units successively were observed [Figure 4].

In common with sweroside, loganin gave the $[M - H]^-$, $[M + 2H_2O - H]^-$ and $[M + HCOOH - H]^-$ ions in the negative mode. The MS/MS spectrum of *m/z* 435 revealed a radical aglycone ion $[M - glu - H]^-$ at *m/z* 227 as the base peak, resulting from the loss of glucose (162 Da). In the further MS^n analysis of $[M\text{-}glu - H]^-$ ion of loganin, the obvious $[M\text{-}glu\text{-}H_2O - H]^-$ ion at *m/z*209 was observed, meanwhile, other ions *m/z*127 and *m/z*101 were observed [Figure 5]. In the positive mode, the ESI-MS^n of loganin was consistent with literature.[28]

Table 2: HPLC-DAD-ESI/MS identification results of 25 chemical compositions in Danmu injection

Peak no.	Retention time (t_R, min)	UV λ_{max} (nm)	Characteristic ion fragments (m/z)	Identification
D1	19.2	259.6, 292.8	153[M-H]$^-$; MS^2:109	Protocatechuic acid
D2	20.1	252.5, 291.6	461[M-H]$^-$; MS^2:293,167	Vanillic acid-4-O-β-D-apiofuranosyl (1"→6')-β-D-glucopyranoside
D3	21.7	218.3, 327.3	353[M-H]$^-$; MS^2:191 (100),179; MS^3:173,135	Neochlorogenic acid
D4	24.3	234.8	375[M-H]$^-$; MS^2:213,169	Logaric acid
D5	24.6	228.1, 279.7	493[M+HCOOH-H]$^-$,483[M+2H_2O-H]$^-$,447[M-H]$^-$(100); MS^2:293 (100),233,149,131,125	3,4-dimethoxyphenol β-D-apiofuranosyl (1"→6')-β-D-glucopyranoside
D6	27.6	214.7, 329.7	353[M-H]$^-$; MS^2:191 (100),179; MS^3:173,135	Chlorogenic acid
D7	28.1	241.8	435[M+HCOOH-H]$^-$(100),425[M+2H_2O-H]$^-$; MS^2:388,227 (100); MS^3:209,177,127,101	Loganin
D8	28.3	204.2, 271.4	523[M+HCOOH-H]$^-$,513[M+2H_2O-H]$^-$(100),477[M-H]$^-$; MS^2:293 (100),233,149,131,125	Kelampayoside A
D9	29.3	218.3, 327.3	353[M-H]$^-$; MS^2:191 (100),179,173; MS^3:135	Cryptochlorogenic acid
D10	30.2	224.2, 273.3	519[M+H]$^+$; MS^2:357 (100),339; MS^3:321,309,160	Iso-naucleamide A-10-O-β-D-glucopyranoside
D11	31.3	241.5	431[M+HCOOH-H]$^-$(100),403[M+H_2O-H]$^-$; MS^2:393,231,213,167,101	7-dehydrated logaric ether
D12	32.3	225.2, 274.1	519[M+H]$^+$; MS^2:357 (100),339;MS^3:321,309,160	Iso-naucleamide A-10-O-β-D-glucopyranoside
D13	32.6	246.6	403[M+HCOOH-H]$^-$ (100),393[M+2H_2O-H]$^-$; MS^2:357 (100),195,179,125	Sweroside
D14	34.7	224.3, 273.4	681[M+H]$^+$; MS^2:519,357 (100);MS^3:339,321,309,160	Iso-naucleamide A-10-O-β-D-gluco pyranosyl(1→6)-β-D-glucopyranoside
D15	36.2	224.2, 272.6	519[M+H]$^+$; MS^2:501,357 (100),339; MS^3:321,309,160	Naucleamide A-10-O-β-D-glucopyranoside
D16	37.3	208.1,243.2	531[M+H]$^+$; MS^2:511,369,349,299	Naucleoxoside C
D17	38.9	224.2, 271.4	489[M+H]$^+$; MS^2:471,327 (100),309; MS^3:309,186,160	Naucleamide G
D18	43.0	241.8, 327.3	513[M+H]$^+$; MS^2:351,333,281; MS^3:263,185,158	Pumiloside
D19	46.0	243.0, 327.3	513[M+H]$^+$; MS^2:351,333,281; MS^3:263,185,158	3-epi-pumiloside
D20	46.4	226.2	514[M+H]$^+$; MS^2:353 (100),283,171,187	10-hydroxystrictosamide
D21	54.6	226.8	515[M+H]$^+$; MS^2:353 (100),335,317,283; MS^3:283,265,160,132	Naucleoxoside A or B
D22	57.2	226.3	515[M+H]$^+$, MS^2:353 (100),335,317,283; MS^3:283,265,160,132	Naucleoxoside A or B
D23	63.5	224.2	543[M+H]$^+$; MS^2:381 (100),363,335; MS^3:311,317,293,265,169	3α-5α-tetrahydrodeoxycordifoline lactam
D24	57.2	226.5	499[M+H]$^+$; MS^2:337 (100),267,171; MS^3:171 (100),154,144,130,118	Strictosamide
D25	70.5	226.5	499[M+H]$^+$; MS^2:337 (100),267,171; MS^3:171 (100),154,144,130,118	Vincosamide

HPLC-DAD-ESI/MS: High performance liquid chromatography-diode array detector- electrospray ionisation-mass spectrometry, MS: Mass spectrometr

The ESI-MS^n of naucleamide A-10-O-β-D-glucopyranoside

The ESI-MS^n of the naucleamide A-10- O-β-D-glucopyranoside has been well investigated.[29] The fragmentation pathway of $[M + H]^+$ ion m/z 519 was shown in the positive mode. Firstly, the $[M + H]^+$ ion produced a prominent ion m/z 357 in the MS/MS spectrum, which revealed a neutral loss of 10-*O*-glucose residue. And then the obtained ion produced the prominent $[M - glu - H_2O + H]^+$ ion at m/z 339. Then the $[M\text{-}glu - H_2O + H]^+$ ion of m/z 339 was selected for further MS^4 analysis to generate ions at m/z 321, m/z 309 and m/z 160 were also observed, which were attributed to the losses of H_2O, CH_4O and RDA cleavage of ring C, respectively.

The ESI-MS^n of naucleamide G

The fragmentation of naucleamide G exhibited a little difference from that of naucleamide A-10-O-β-D-glucopyranoside because of the CH_2OH at the C-16 position. In the MS/MS spectrum, the $[M - glu + H]^+$ ion at m/z 327 was found as the base peak. And then the obtained ion produced the prominent $[M - glu - H_2O + H]^+$ ion at m/z 309. Successively, in the MS^4 spectrum of m/z 309, two ions at m/z186 and 160 were observed, which were

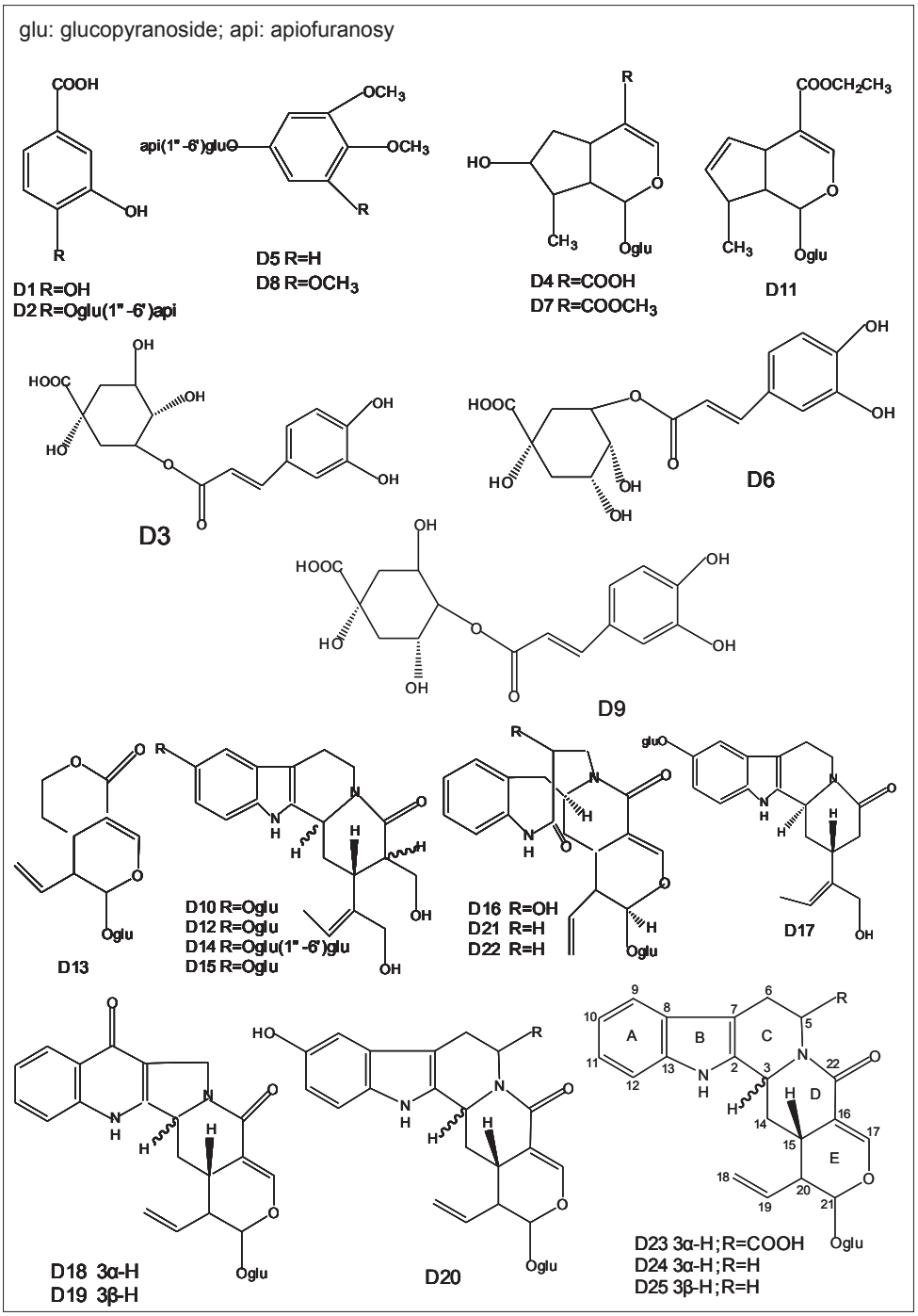

Figure 2: The structures of 25 compounds identified in Danmu injection

assigned as the RDA cleavage of ring D and C, respectively [Figure 6].

The ESI-MS^n of pumiloside and its isomer 3-epi-pumiloside

Pumiloside and 3-epi- pumiloside, with the same MS spectra, UV, molecular formulae, are isomers. Pumiloside and its isomer 3-epi- pumiloside are the only two quinoline alkaloids in *Nauclea officinalis,*[24-26] and pumiloside is higher content in Danmu injection. The ESI-MS^n of the pumiloside and 3-epi- pumiloside had been studied.[29,30] In their ESI-MS spectra, the $[M + H]^+$ ion was observed, while the significant $[M - glu + H]^+$ ion was found in the MS/MS spectrum. The $[M - glu + H]^+$ ion was triggered by the loss of H_2O and CO_2 in the MS^3 spectrum, at the same time, the ion at m/z 281 caused by the loss of neutral fragments $C_4H_6O_4$ of RDA cleavage of ring E could be observed in the MS^3 spectrum. Then the ion m/z 281 was selected for further MS^4 analysis to generate two ions at m/z 185and 158,

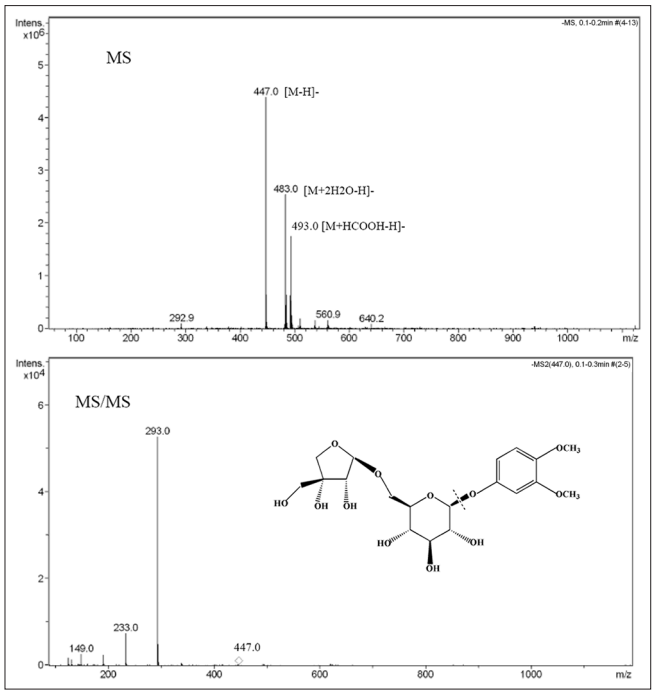

Figure 3: MS, MS/MS spectrum of 3,4-Dimethoxyphenol β-D-apiofuranosyl(1→6)-β-D-glucopyranoside in the negative mode

which were assigned as the RDA cleavage of ring D and C, respectively [Figure 7].

The ESI-MS^n of strictosamide and vincosamide

Strictosamide and vincosamide are isomers. Strictosamide is the main ingredient and the highest content in *Nauclea officinalis*[24-26] and Danmu injection. In the positive mode, the $[M + H]^+$ ion at *m/z* 499 was shown in full scan mass spectrum. $[M + H]^+$ ion generated the base peak at *m/z* 337, originating from the loss of glucose residue in the MS/MS spectrum. In the MS^3 spectrum of at *m/z* 337, $[M - glu + H]^+$ ion yielded fragment ion $[M - glu\text{-}C_4H_6O_4 + H]^+$ at *m/z* 267, which was assigned as the RDA cleavage of ring E of loss neutral fragments (70Da), at the same time, the ions at *m/z* 319, *m/z*171 and *m/z*141 were observed, which were attributed to the losses of H_2O, $C_9H_{10}O_3$ and RDA cleavage of ring C, respectively. Successively, in the MS^4 spectrum of *m/z* 267, the ions at *m/z*171 was observed, which were attributed to the losses of RDA cleavage of ring D.[29,30] In the MS^5 spectrum of *m/z*171, the ions at *m/z*118, 130 and 154 were observed. Vincosamide is the same fragmentation pathway with strictosamide [Figures 8 and 9].

The ESI-MS^n of 3α, 5α-tetrahydrodeoxycordifoline lactam

The structures of 3α, 5α-tetrahydrodeoxycordifoline lactam and strictosamide were very similar to each other except for "–COOH" groups at the C-5 position. In the positive mode, the CO_2 lost in the MS/MS spectrum,

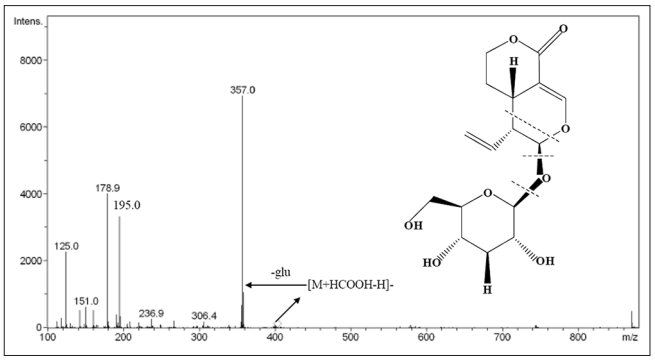

Figure 4: MS/MS spectrum of sweroside

and then $[M\text{-glu-}CO_2^- + H]^+$ ion *m/z* 335 was observed in the MS^3 spectrum, successively, in the MS^4 spectrum of *m/z* 265 and *m/z* 169 were observed, which were attributed to the losses of RDA cleavage of ring E and D, respectively [Figure 10].

On the chromatogram, D6, D7 and D8 are in one peak, but their molecular mass is different. We can extract their molecular mass and ion fragments in the TIC chromatogram, and deduced their structures. By the same token, D4, D5, D12 and D13 were identified.

Method validation of the quantitative analysis

The calibration curves, linear ranges, LOD and LOQ of 11 analyses were performed using the developed HPLC–DAD method described in section 2.4 [Table 1]. Reasonable correlation coefficient values ($r^2 > 0.9998$) indicated good correlations between investigated standards concentrations and their peak areas within the ranges tested. The ranges of LOD and LOQ for all the analytes were from 1.06 ng to 20.03 ng, and 3.42 ng to 80.13 ng, respectively. The intra- and inter-day variations (RSD) of the 11 analyses were in the range from 1.31 to 2.42 and 1.14 to 2.82% [Table 3], respectively. The developed method has good accuracy with the recoveries were between 95.97 and 109.30%. It was also found that the analyses in the sample solution were stable for 24 h with a RSD less than 3.1%. All these values fall within acceptable limits, which indicates this method, is reliable with significant repeatability, recovery and precision. The results proved that HPLC is appropriate for analyzing and assessing the quality of Danmu injection.

Quantitative determination of Danmu injection

Typical chromatograms for the quantitative determination of 11 main compounds in Danmu injection were shown in Figure 1. A total of five different batches of Danmu injection were tested using the developed HPLC–DAD method. The contents of 11 investigated compounds were determined and the results were listed in Table 4. It was found that strictosamide (**D24**) was the most abundant compound, protocatechuic acid (**D1**), naucleamide

A-10-O-β-D-glucopyranoside (**D15**), naucleamide G (**D17**) and pumiloside (**D18**) are higher content, while other 6 compounds were found to be lower in Danmu injection.

CONCLUSION

Chemical constituents of Danmu injection was systematically and thoroughly investigated by HPLC–DAD–ESI-MS^n, which provided full-scale qualitative and quantitative information for analysis of chemical constituents in Danmu injection. Twenty-five compounds

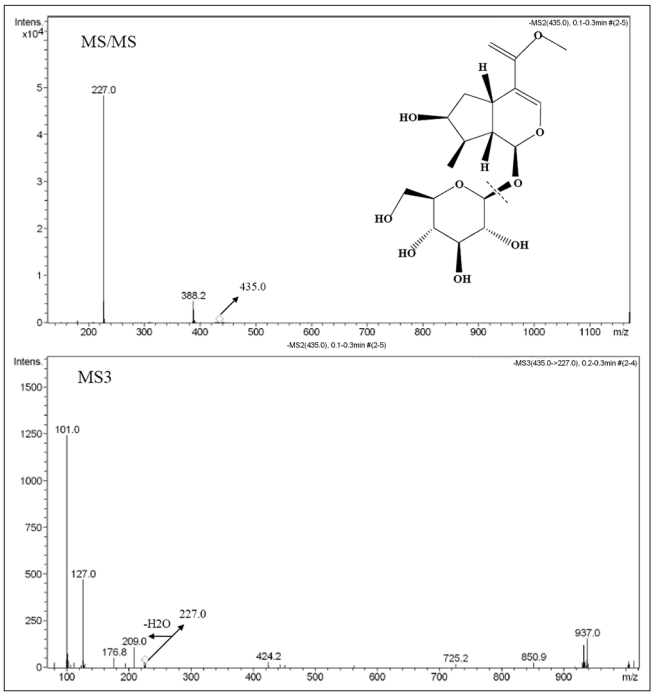

Figure 5: MS2, MS3 spectrum of loganin

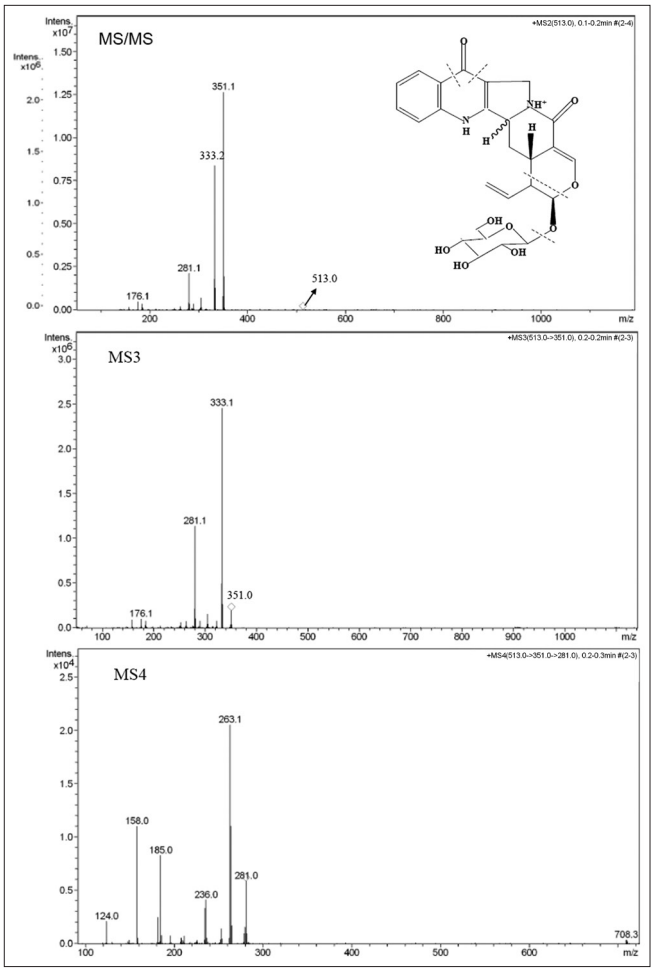

Figure 7: MS/MS, MS3and MS4 spectrum of pumiloside and 3-epipumiloside

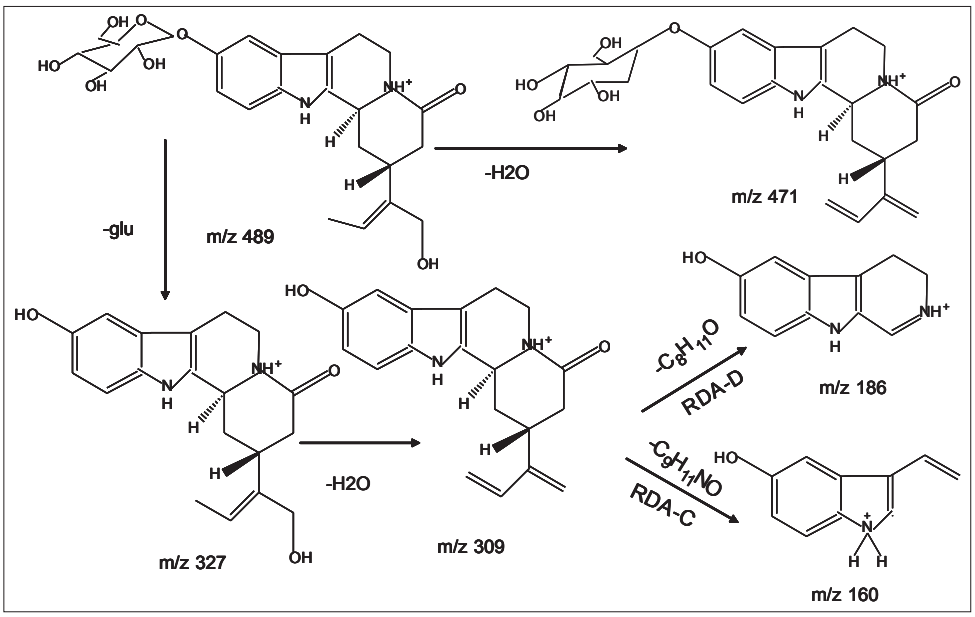

Figure 6: Fragmentations pathways of naucleamide G

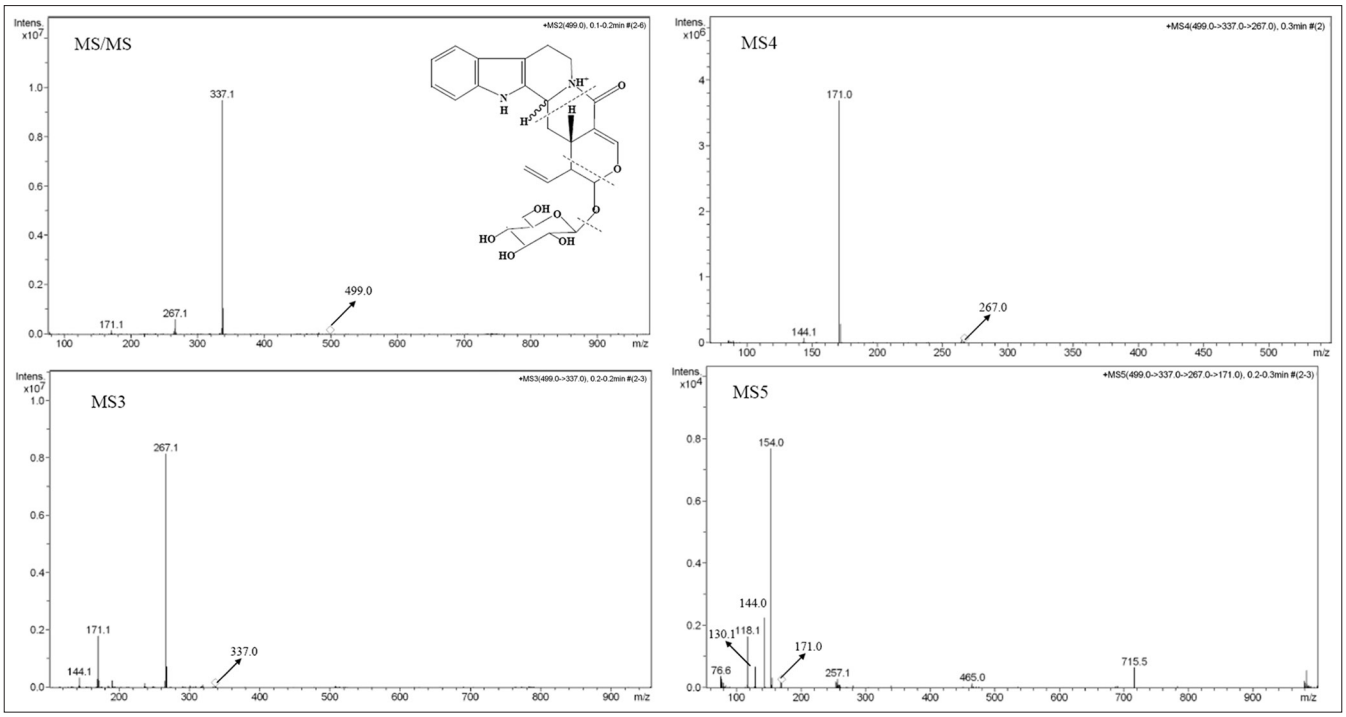

Figure 8: MS/MS, MS3,MS4,MS5 spectrum of strictosamide and vincosamide

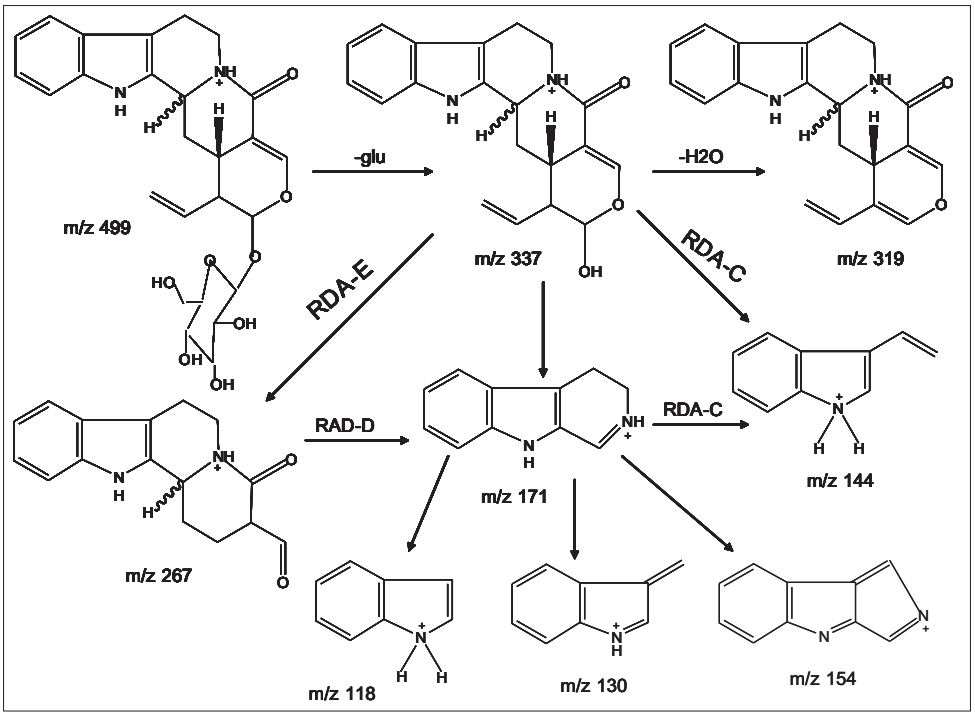

Figure 9: Fragmentations pathways of strictosamide and vincosamide

including phenolic acid, phenol glycoside, iridous glycoside and alkaloid glycoside were identified; eight (D3, D5, D6, D8, D9, D11, D17 and D23) of them were never reported previously in *Nauclea officinalis*.

The quantitative method was proved to have excellent linearity, good accuracy, sensitivity and repeatability. The results would provide the chemical support for the further pharmacokinetic studies and for effective quality assessment of Danmu

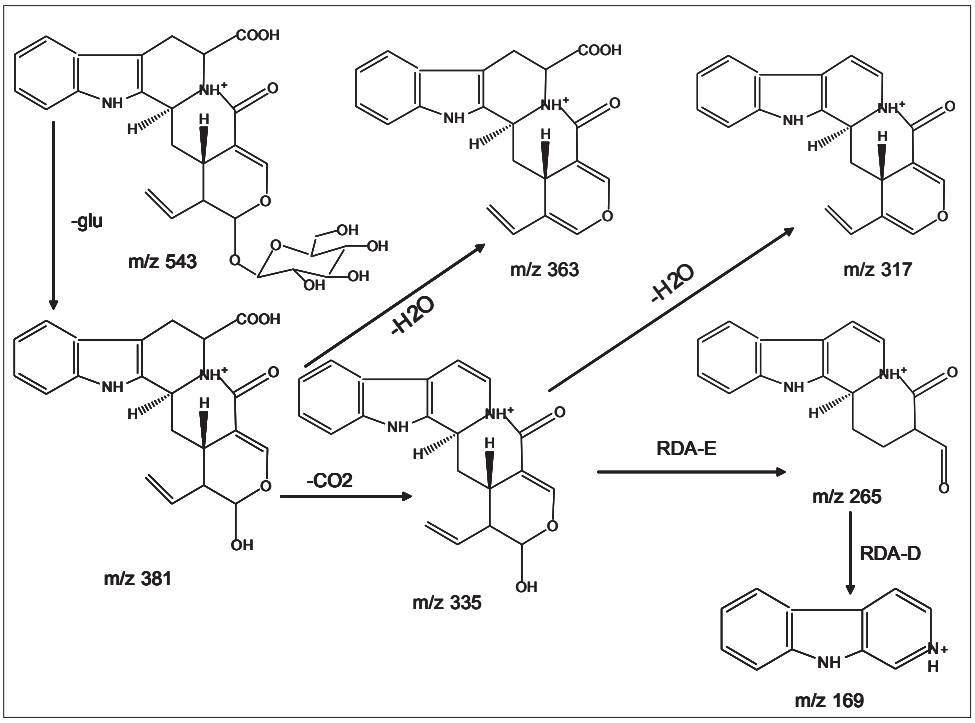

Figure 10: Fragmentations pathways of 3α,5α-tetrahydrodeoxycordifoline lactam

Table 3: Precisions and recoveries of 11 analyses (110801)

Analyte	Intra-day (*n*=6) (μg/mL)		Inter-day (*n*=3) (μg/mL)		Recoveries (*n*=6)				
	Means±S.D	RSD (%)	Means±S.D	RSD (%)	Initial (μg)	Added (μg)	Found (μg)	Recovery (%)	RSD (%)
D1	26.36±0.36	1.37	26.08±0.44	1.69	263.60	266.11	542.91	104.96	4.23
D3	11.42±0.17	1.49	11.09±0.30	2.71	114.20	113.76	223.39	95.97	2.53
D6	9.40±0.14	1.49	9.61±0.11	1.14	94.00	95.52	191.45	102.02	3.36
D9	7.55±0.12	1.59	7.45±0.21	2.82	75.50	73.92	148.32	98.61	4.07
D15	20.02±0.26	1.30	20.17±0.43	2.13	200.20	194.40	389.70	97.48	3.78
D17	16.81±0.24	1.43	16.26±0.37	2.28	168.10	169.12	352.95	109.30	2.27
D18	38.64±0.54	1.40	38.94±0.70	1.80	386.40	386.91	775.82	100.65	3.45
D19	19.11±0.42	2.20	20.83±0.43	2.06	191.10	192.00	391.34	104.29	1.79
D23	8.69±0.21	2.42	7.99±0.22	2.75	86.90	84.40	174.33	103.59	2.07
D24	123.89±1.96	1.58	125.53±2.25	1.79	1238.90	1202.40	2428.80	98.96	1.64
D25	3.96±0.052	1.31	4.05±0.062	1.53	39.60	39.36	80.44	103.77	1.47

RSD: Relative standard deviation; S.D: Standard deviation

Table 4: The content of 11 compounds in five batches of Danmu injection (*n*=3, μg/mL)

Analyte	110801	110601	110616	110401	110925
D1	259.65	254.62	239.64	353.91	423.87
D3	115.25	82.81	37.71	100.57	106.55
D6	96.97	20.39	21.04	83.62	84.57
D9	75.75	76.02	22.95	96.77	65.91
D15	199.28	289.51	157.18	237.55	168.34
D17	166.18	489.55	74.83	356.92	102.2
D18	390.41	177.47	142.84	193.94	126.85
D19	196.12	20.75	15.32	73.94	18.95
D23	83.25	120.91	47.04	62.55	26.35
D24	1240.91	800.54	1294.15	789.57	1080.43
D25	40.66	21.81	56.57	39.65	25.37

injection, which would be of significant importance for the safety use and modern research of Danmu injection.

ACKNOWLEDGEMENTS

The research was financially supported by the National Natural Science Foundation of China (No. 81203001).

REFERENCES

1. Normile D. The new face of traditional Chinese medicine. Science 2003;299:188-90.
2. Li YK, Zhang JY. Improvement and enhancement of traditional Chinese medicine injections. Chin J Chin Mater Med

2011;36:1905-9.
3. Hu CQ, Xu MZ, MA Y, Yu FP, Li J, Wang C, *et al*. Determination of the allergic impurities in the parenteral injection of Chinese traditional medicines containing Salviam iltiorrhiza. Acta Pharm Sin 2008;43:518-22.
4. Wu YP, Zhang HZ, Wang JG. Chuanhuning injection analysis of 300 cases of adverse reactions. Chin J Pharmacoepidemiol 2008;17:176-8.
5. Wang Y, Xu YJ, Liu D. Chuanxinlian injection analysis of 278 cases of adverse reactions. Chin J Hosp Pharm 2011;31:701-3.
6. Wu Y, Liang CG, Shen LZ, Sun L, Yan AZ, Lin RC, *et al*. Studies on the reason of severe adverse reaction (ADR) in clinics induced by Yuxingcao injections (I). Chin J Pharm Anal 2007;27:782-5.
7. Kang WY, Yang XS, Zhao C, Hao XJ. Advances in studies on indole alkaloids of genus Nauclea. Chin Tradit Herbal Drugs 2002;33:762-5.
8. Compiling Groups of Countrywide Herbal Medicine of China. Compilation of Countrywide Herbal Medicine of China. Beijing: People's Medical Publishing House; 1975. p. 624.
9. Lin M, Liu X, Yu DQ, Dou SQ, Zhang YJ, Li QM. The structure determ ination of a new alkaloid-nauclefiline in Nauclea officinalis. Acta Pharm Sin 1985;20:902-5.
10. Lin M, Li SZ, Liu X, Yu DQ. Studies on the structures of two new alkaloidal glucosides of Nauclea officinalis Pierre ex Pitard. Acta Pharm Sin 1989;24:32-6.
11. Kakuguchi Y, Ishiyama H, Kubota T, Kobayashi J, Naucleamide F. A new monoterpene indole alkaloid from Nauclea latifolia. Heterocycles 2009;79:765-71.
12. Xuan WD, Chen HS, Yuan ZX, Zhu P. Chemical constituents of Nauclea officinalis. Chin J Nat Med. 2005;3:181-3.
13. Xie DW, Li YH, Zhao L, Ding G, Yuan SW, Xu J, *et al*. Simultaneous quantification of five compounds from Nauclea officinalis leaves by High Performance Liquid Chromatography. J Food Drug Anal 2012;20**:**489-94.
14. Zhang JQ, Mao CN, Yang WL, Lai WY, Fu NG, Sheng L, *et al*. The qualitative research of the isovincoside lactone in different parts of Nauclea officinalis Pierrc ex Petard. Chin J Pharm Biomed Anal 2008;28:1654-7.
15. Li MH, Ding G, Shen M, Liu JJ. Identif ication and Content Determination of strictosamide in Lignum Naucleae and its injection. J Nat Med 2006;4:104-6.
16. Wang JJ, Jiang J, Zhu FX, Li XF, Jia XB. Simultaneous determination of phenolic acid and three alkaloids in Nauclea officinalis and its preparations by HPLC. Chin Tradit Pat Med 2012;34:2326-30.
17. Wan JB, Yang FQ, Li SP, Wang YT, Cui XM. Chemical characteristics for different parts of Panax notoginseng using pressurized liquid extraction and HPLC–ELSD. J Pharm Biomed Anal 2006;41:1596-601.
18. Guan J, Lai CM, Li SP. A rapid method for the simultaneous determination of 11 saponins in Panax notoginseng using ultra performance liquid chromatography. J Pharm Biomed Anal 2007;44:996-1000.
19. Zheng XT, Shi PY, Cheng YY, Qu HB. Rapid analysis of a Chinese herbal prescription by liquid chromatography-time-of-flight tandem mass spectrometry. J Chromatogr A 2008;1206:140-6.
20. Su S, Hua Y, Duan JA, Shang E, Tang Y, Bao X, *et al*. Hypothesis of active components in volatile oil from a Chinese herb formulation, 'Shao-Fu-Zhu-Yu decoction', using GC–MS and chemometrics. J Sep Sci 2008;31:1085-91.
21. Schmidt S, Piechotta C, Godejohann M, Win T, Nehls I, Mügge C. Characterisation of commercially available linear alkylbenzenesulfonates by LC-SPE-NMR/MS (liquid chromatography-solid phase extraction-nuclear magnetic resonance spectroscopy-mass spectroscopy). Talanta 2010;82:143-50.
22. Steinmann D, Ganzera M. Recent advances on HPLC/MS in medicinal plant analysis. J Pharm Biomed Anal. 2011;55:744-57.
23. Zhu FX, Wang JJ, Song J, Ding SM, Jia XB. Chemical constituents of Nauclea officinalis. Acta Pharm Sin 2013;48:276-80.
24. Xuan WD. Studies on bio-active constituents of Nauclea officinalis and Ervatamia. Yunnanensis. Second Military Medical University Doctoral Dissertation, 2005.
25. Hu X. Nauclea officinalis: Chemical components and pharmacological effects. Shengyang Pharmaceautical University Doctoral Dissertation, 2007.
26. Sun JY. The chemical constituents and their bioactivities of Nauclea officinalis Pierre ex petard and turpinia arguta seem. Shandong University Doctoral Dissertation, 2008.
27. Ruan M, Li Y, Li X, Luo JG, Kong LY. Qualitative and quantitative analysis of the major constituents in Chinese medicinal preparation Guan-Xin-Ning injection by HPLC–DAD–ESI-MS^n. J Pharm Biomed Anal 2012;59:184-9.
28. Zhou LL, Liu WQ, Yan CY, Song FD, Liu SY, Zhu JH. Study on the loganin by ESI-MS^n and FTICR-MS. J Chin Mass Spectrom Soc. 2008;229:332-5.
29. Li Q, Zhang YF, Wu B, Qu H. Identification of indole alkaloids in Nauclea officinalis using high-performance liquid chromatography coupled with ion trap and time of flight mass spectrometry. Eur J Mass Spectrom 2011;17:277-86.
30. Zhu FX, Jia XB, Li XF, Wang JJ, Lu TT. Study on the dissociation laws of two alkaloids by ESI-MS and determination of two alkaloids by HPLC in Danmu Injection. J Nanjing TCM Univ 2011;27:492-4.

Cite this article as: Zhu F, Wang J, Li X, Sun E, Jia X. Qualitative and quantitative analysis of the major constituents in traditional Chinese medicine Danmu injection using LC-ESI-MS^n and LC-DAD. Phcog Mag 2014;10:254-64.

Source of Support: Nil, **Conflict of Interest:** None declared.