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Analysis of Chinese medicine preparations by capillary electrophoresis–mass spectrometry

Hua-Tao Feng, Ling-Ling Yuan, Sam Fong Yau Li*
Department of Chemistry, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260, Singapore

Abstract

In Chinese medicines, herbs are usually prepared before use by patients. Since the preparation procedures convert the original component into one or more products, study of the procedures is usually complex and involves several compounds. On-line coupling of capillary electrophoresis (CE) to mass spectrometry (MS) allows both the efficient separation of CE and the specific and sensitive detection of MS to be achieved. In this study, CE–MS was applied to the determination of alkaloids in Maqianzi (the seed of *Strychnos pierrian*) and Wutou (aconite root, *Radix aconiti praeparata*) during the preparation procedure. With optimal CE–MS conditions, alkaloids in both prepared and unprepared Maqianzi were determined successfully in the total ion current (TIC) mode. However, single ion monitoring (SIM) had to be applied for the separation of aconitum alkaloids and their hydrolysis products. Quantification data indicated that MS detection under SIM mode is more sensitive than UV detection. Based on the CE–MS method developed, the hydrolysis of aconitum alkaloids in water and methanol was also studied.

Keywords: Pharmaceutical analysis; *Strychnos pierrian*; *Aconite* roots; Plant materials; Alkaloids

1. Introduction

Herbal medicines have a long history in health care. After thousands of years of practical experience, ~700–800 kinds of crude herbal drugs are commonly used in China [1]. The active components in some herbs can cause serious toxic effects to humans, for example the alkaloids in Wutou (aconite root, *Radix aconiti praeparata*) and Maqianzi (the seed of *Strychnos pierrian*) [2,3]. Obviously, efficient determination procedures for these active components are very important for the development of herbal medicine [4], especially for toxic components whose misuse can be fatal.

It is difficult to determine the active components in Wutou and Maqianzi because they are usually prepared before use by patients. The purpose of preparation is to reduce toxicity; Wutou usually is oven-roasted and neglected to convert aconitum alkaloids into their hydrolysis products [5], while Maqianzi is treated by heating in oil or sand at 240–250 °C for 3–5 min. The hydrolysis products of aconitum alkaloids are hard to determine by UV detector because of their weak UV absorbance, especially the final degradation products aconine, hypaconine and mesaconine, which lose both the acetyl and benzoyl functions during hydrolysis.

CE is promising for the separation and analysis of active ingredients in herbal medicine as it needs only

*Corresponding author. Tel.: +65-6874-2681; fax: +65-6779-1691.
E-mail address: chmlifys@nus.edu.sg (S.F.Y. Li).

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small amounts of standards and can analyze samples rapidly. Mass spectrometry (MS), a sophisticated analytical technique that can be used for both qualitative and quantitative determinations, can give information regarding the mass and the structure of unknown components present in a sample mixture. Moreover, the single ion monitoring (SIM) mode of MS also provides a powerful tool for identification of analytes when the efficiency of the separation technique is not good enough.

The invention of ''soft ionization'' methods, such as electrospray ionization (ESI), made the online coupling of CE to MS possible [6]. Online coupling provides both the efficient separation of CE and the specificity and sensitivity of MS detection. Since the beginning of the 1990s, CE–MS has developed significantly as demonstrated by a growing number of published applications in the analysis of proteins [7–10], drugs [11–13], carbohydrates [14], etc., and has been proven to be a very useful tool.

CE–MS had been applied to the analysis of alkaloids in several reports. Strum et al. studied the isoquinoline alkaloids by CE–MS [15] and Henion and colleagues studied the determination of trace impurities in peptide and alkaloid samples [16]. Unger et al. applied CE–MS to the separation of four different groups of alkaloids consisting of monoterpenoid indole alkaloids, protoberberines/benzophenanthridines, beta-carboline alkaloids and isoquinolines from poppy [17]. Stöckigt et al. applied CE–ESI-MS to the determination of Rauvolfia root alkaloids [18]. All these studies suggest that the CE–MS system is sufficiently sensitive and reliable to provide acceptable quantitative analysis of alkaloids. However, so far there are no reports on the application of CE–MS in the preparation of herbal medicines.

In this study, CE–MS was applied to the analysis of alkaloids in the two herbs mentioned above. The CE–MS interface and MS parameters were optimized; the influence of preparation procedures on concentrations of alkaloids was studied.

2. Experimental

2.1. Materials

Aconitum alkaloids (aconitine, hypaconitine, mesaconitine) and brucine were supplied by the National Institute for the Control of Pharmaceutical and Biological Products of China (NICPBP, Beijing, China). Strychnine was purchased from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade. Water ($\geq$18 MΩ) used throughout the experiments was generated by a NANOpure ultrapure water system (Barnstead, IA, USA). Fused silica capillary (50 μm I.D.) was the product of Polymicro Technologies (Phoenix, AZ, USA).

Unprepared Maqianzi and Wutou were purchased from a Chinese medicine store in Singapore. Prepared Wutou was purchased from the same store and used for methanol extraction directly. Unprepared Maqianzi was treated by heating in oil or sand at 240–250 °C for 4 min to provide the prepared herb.

2.2. Instruments

CE with UV detection was carried out using a CE-L1 capillary electrophoresis system (CE Resources, Singapore) and a SPD-10A UV–Vis detector from Shimadzu (Kyoto, Japan). Data acquisition and recording of electropherograms were accomplished with a CSW Chromatography Station (Data Apex, Czech Republic). The total length of the capillaries was 50.0 cm, and the effective length from the injection end to the detection window was 38.0 cm. The capillary was rinsed with 0.1 *M* NaOH for 15 min, followed by water and the appropriate electrolyte solution for 15 min each. UV detection was made at 200 nm and the separation voltage was set at 15 kV. The running electrolyte was refreshed after ten runs.

Mass spectrometry was performed with a Finnigan LCQ quadrupole ion trap MS and an electrospray ionization source (Finnigan MAT, San Jose, USA). Ions were detected in the positive mode and the spray voltage was set to 4.0 kV. The sheath liquid was a mixture of water–methanol (1:9, v/v) with 0.5% acetic acid and delivered at a flow-rate of 3 μl/min. The temperature of the heated capillary was 200 °C. The flow-rate of sheath gas was 10 A.U. and no auxiliary gas was used. The MS system was set with scan ranges of m/z 200–500 for Maqianzi and 200–700 for Wutou; the mass analyzer performed scans every 0.7 s. The SIM electropherograms of protonated molecular ions were acquired by selecting the ranges of m/z ±0.5 from the full scan

mass spectra. The CE capillary (75 cm) was set 0.05 mm outside the ESI needle. The voltage applied by CE was 30 kV. Sample loadings were made with the hydrodynamic mode (100 mbar for 12 s). The capillaries were conditioned as for the CE–UV system. The CE–MS system was tuned with 20 mg/l hypaconitine introduced through the CE capillary at 500-mbar pressure.

2.3. Buffer, alkaloid standards and sheath liquid solution

Stock standard solutions of strychnine and brucine were prepared in methanol, while stock solutions of aconitum alkaloids were dissolved in chloroform since they are unstable in both water and methanol. The solutions used in analyses were made by drying accurate amounts of stock solutions and dissolving in water–methanol (50:50, v/v) . The separation electrolyte for the analysis of aconitum alkaloids, containing 40 m*M* ammonium acetate and 0.1% acetic acid, was prepared daily in methanol. Ammonium acetate (80 m*M*) and 0.1% acetic acid were dissolved in water–methanol (40:60, v/v) for determining alkaloids in Maqianzi. Between runs, the capillary was flushed with buffer solution for 2 min.

2.4. Sample preparation

Pulverized herb (1 g) was immersed in 10 ml methanol at room temperature overnight. After 30-min ultrasonication, the solution was made up to volume with methanol and passed though 0.20-μm filters. Subsequently, the extracts were analyzed within 1 day.

3. Results and discussion

3.1. Set-up of CE system

The separation conditions of alkaloids in the seed of *Strychnos pierrian* and aconite root had been studied in a previous work [19]. Only non-aqueous buffers achieved the separation of aconitum alkaloids, and higher concentrations of methanol caused better separations. Ammonium acetate and acetic acid were used in both CE–UV and CE–MS experiments. For aconitum alkaloids, 40 m*M* ammonium acetate and 0.1% acetic acid were dissolved in pure methanol for best separation. For alkaloids in *S. pierrian*, buffer containing 80 m*M* ammonium acetate and 0.1% acetic acid was dissolved in a mixture of 40% water and 60% methanol because a higher methanol concentration caused too long a retention time.

A high voltage of 30 kV was applied by the CE system because a longer separation capillary was required for coupling the CE and MS systems and the positive spray voltage added to the end of the CE capillary can also decrease part of the total voltage in the capillary.

3.2. The position of CE capillary in the ESI needle

According to other reports, CE–MS can be performed with the CE capillary either inside or outside the ESI needle. Our study showed that it was hard to maintain a stable electric current in CE capillary when it was too far outside the ESI needle, but it would be too difficult to maintain a stable MS signal when the capillary was put too far inside the ESI needle. To keep both the electric current and MS signal stable, the CE capillary should be from 0.1 mm inside the ESI needle to 0.4 mm outside.

The signal intensity increased as the distance of the capillary outside the ESI needle increased. However, greater noise was caused at the same time; the signal-to-noise ratio was subsequently decreased. In contrast, though the signal intensity decreased when the CE capillary was put inside the ESI needle, a better signal-to-noise ratio, which directly determines the sensitivity of detection, resulted. Finally, 0.05 mm outside the needle was selected as the best position because there was a relatively large dead volume when the capillary was put inside the ESI needle.

To aid electric contact and prevent the peeling of polyimine coating immersed in non-aqueous solutions, 2 mm of polyimine coating on the capillary outlet end was removed. The coating was burned off in the report of Xia and colleagues [20]. However, when the melted coating cools irregular knots may form and block the sheath liquid flow and affect the original position of the ESI needle. In this study, the polyimine coating was caused to swell by immersing

the outlet end into acetonitrile overnight before peeling off.

3.3. Sheath liquid composition and flow rate

Two organic solvents, methanol and 2-propanol, were tested as the organic modifiers of sheath liquid. Methanol was chosen as a suitable modifier, since 2-propanol introduced greater noise but did not result in a more intense signal. In the solution containing 0.1% acetic acid, the signal intensity increased when the proportion of methanol increased from 50 to 100%. However, a pure organic solvent could make the electric current unstable and harm the repeatability of the MS signal. Therefore, the ratio of methanol/water was set to 9:1. In the range from 0.1 to 1%, the concentration of acetic acid did not cause notable changes of signal intensity, so 0.5% acetic acid was finally chosen. It was indicated that the flow rate of sheath liquid should be small but sufficient to give stable spray and electrical contact; 3 μl/min was found to be suitable.

3.4. Sheath gas flow rate and spray voltage

A low flow rate of sheath gas should be used for the electrospray since a high flow rate of sheath gas influences sensitivity; moreover, too high a sheath gas flow rate (for example, 40 A.U. for the Finnigan MS system) could result in a driving force making the separation buffer move far faster than the electroosmotic flow (EOF). The flow rate of sheath gas was set to 10 A.U. in this study.

When the spray voltage was changed from 3.0 to 5.0 kV, no obvious difference was found. Therefore, the voltage was set to 4.0 kV throughout the study. As a ''soft ionization'' method, the MS spectra obtained from ESI indicated alkaloids, mainly the protonated molecular ion modes ($[M+H]^+$). The spectra of strychnine and brucine are shown in Fig. 1.

3.5. Analysis of alkaloids in Maqianzi

Major alkaloids in Maqianzi are strychnine and brucine, but it also contains small amounts of other components, such as vomicine, novacine and icajine [2,21]. The preparation procedure for Maqianzi reduces the toxicity by converting the toxic alkaloids strychnine and brucine into isobrucine, isostrychnine, isobrucine *N*-oxide, isostrychnine *N*-oxide, etc.

Extraction procedure was applied to prepared and unprepared Maqianzi. Four main components were found in the unprepared herb: strychnine, brucine, novacine and icajine (Fig. 2). The separation of peaks could be achieved in the total ion current mode (TIC). Inaccuracy resulting from the different sample loading amounts and spray efficiencies of different

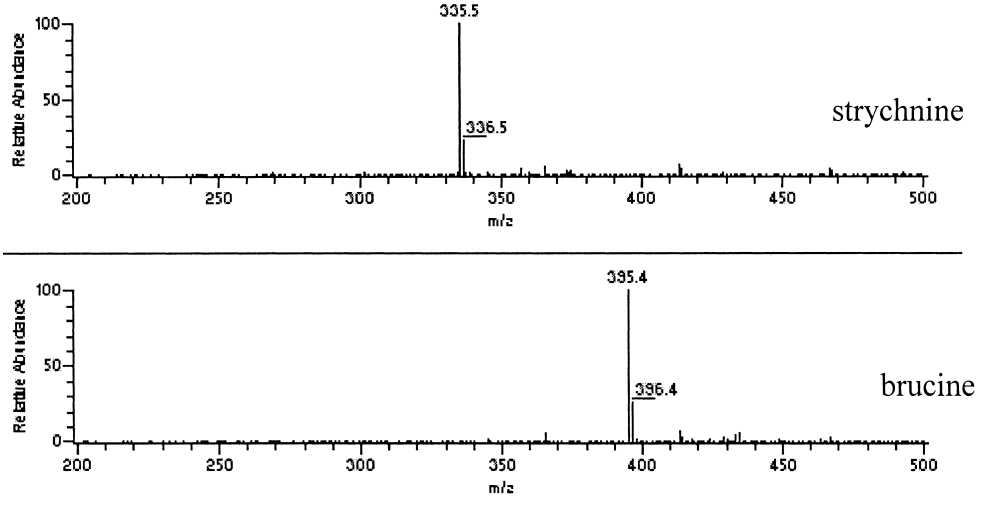


Fig. 1. MS spectra of strychnine and brucine using ESI.

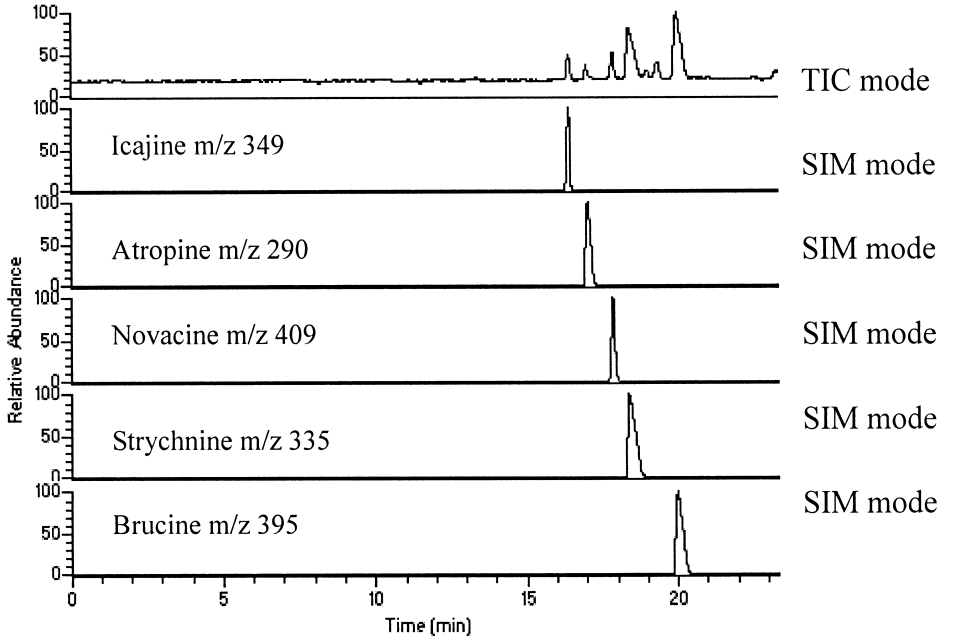


Fig. 2. Electropherogram of unprepared Maqianzi sample. Running electrolyte: 80 m*M* ammonium acetate and 0.1% acetic acid dissolved in a mixture of water–methanol (40:60, v/v). Sample introduction: hydrodynamic mode, 100 mbar for 12 s. Separation voltage: 30 kV. MS: spray voltage, 4.0 KV; flow rate of sheath gas, 10 U; flow rate of sheath liquid, 3 μl/min; temperature of heated capillary, 200 °C. The sheath liquid was mixture of water–methanol (1:9, v/v) with 0.5% acetic acid.

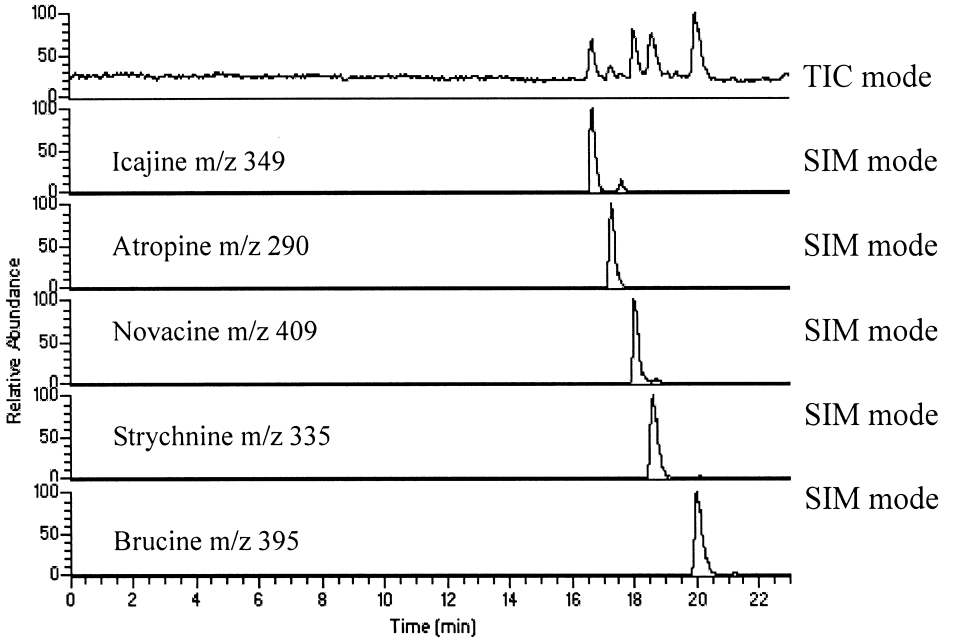


Fig. 3. Electropherograms of prepared Maqianzi sample. Other conditions as in Fig. 2.

runs, can be minimized by adding an internal standard. Therefore, 40 m*M* atropine was added into the following standard and sample solutions to act as the internal standard.

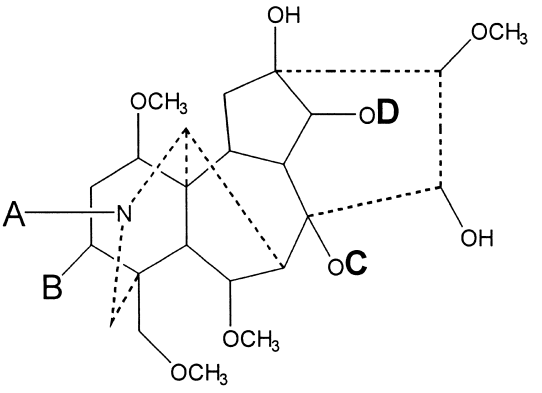
Strychnine, brucine, novacine and icajine also were the main components found in the herb prepared in hot sand for 4 min (Fig. 3). Taking the peak area of atropine as reference, the peak areas of strychnine and brucine after preparation were only ~75.3 and ~78.9% of the values before preparation. The result confirmed the decrease in strychnine and brucine concentrations during the preparation procedure.

To test the quantification of the system, five mixtures of strychnine and brucine with concentration levels from 0.2 to 50 mg/l were measured. The peak areas were adjusted by comparing the peak areas of strychnine and brucine with the peak area of atropine. The calibration curves of strychnine and brucine exhibited linear dynamic ranges with correlation coefficients (R^2) of 0.9920 for strychnine and 0.9906 for brucine. Taking atropine as internal standard, the repeatabilities of peak areas (RSD, 5.51% for strychnine and 2.85% for brucine) were measured by five injections of a solution containing 40 mg/l of each alkaloid. The detection limits ($S/N=3$) of strychnine and brucine were 0.12 and 0.13 mg/l, respectively, indicating that the sensitivity of MS detection using the SIM mode was better than that of UV detection, which gave a detection limit of ~1 mg/l for these two alkaloids in previous work [19].

3.6. Analysis of alkaloids in Wutou

The structures of aconitum alkaloids and their hydrolysis products are listed in Fig. 4; nine alkaloids were involved in the hydrolysis. Taking aconitine as an example, it is first converted to benzoylaconine, and then to aconine during the preparation procedure.

The rates of conversion of aconitum alkaloids to their hydrolysis products in different solvents was studied first. Aconitum alkaloids were very unstable in water. More than 90% of alkaloids were converted to their hydrolysis products after 3 days. A solution of hypaconitine refluxed in water for 2 h indicated



Alkaloids	A	B	C	D	MW
Aconitine	Et	OH	Acetyl	Benzoyl	645
Hypaconitine	Me	H	Acetyl	Benzoyl	615
Mesaconitine	Me	OH	Acetyl	Benzoyl	631
Benzoylaconine	Et	OH	H	Benzoyl	603
Benzoylhypaconine	Me	H	H	Benzoyl	573
Benzoylmesaconine	Me	OH	H	Benzoyl	589
Aconine	Et	OH	H	H	499
Hypaconine	Me	H	H	H	469
Mesaconine	Me	OH	H	H	485

Fig. 4. The structures of aconitum alkaloids and their hydrolysis products.

that the alkaloid existed mainly as hypaconine and aconine (Fig. 5).

Aconitum alkaloids dissolved in methanol were also unstable and existed mainly as their intermediate hydrolysis products (benzoylaconine, benzoylhypaconine and benzoylmesaconine) after 2 months. However, it is worth noting that hypaconitine dissolved in methanol for 1 year might be converted to benzoyldeoxyaconine (M_r 587) and deoxyaconine (M_r 483), instead of benzoylhypaconine and hypaconine (Fig. 6).

Though the concentrations of hydrolysis products after refluxing in water were not known precisely, the solution could be used to test the separation of aconitum alkaloids and their hydrolysis products. A sample containing nine alkaloids was prepared by mixing a standard solution of aconitum alkaloids with the same solution after refluxing.

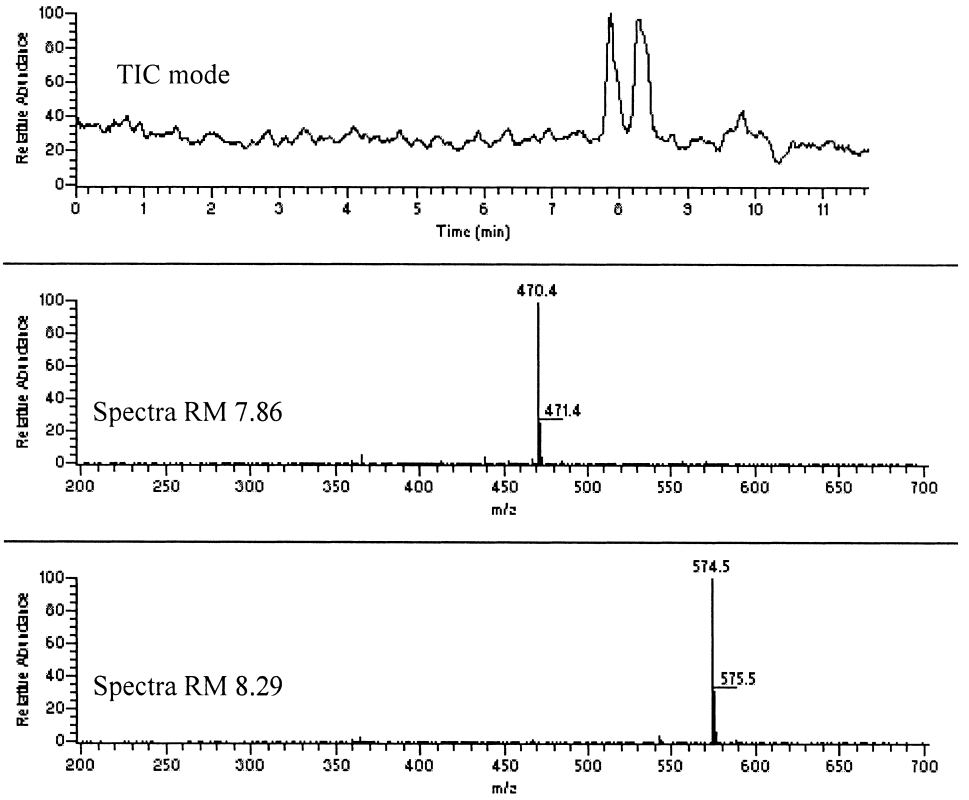


Fig. 5. Electropherograms of hypaconitine dissolved in water after refluxing in water for 2 h. Running electrolyte: 40 m*M* ammonium acetate and 0.1% acetic acid dissolved in methanol directly. RM: retention time. Other conditions as in Fig. 2.

A normal CE system was used with UV detection set at 200 nm and a separation voltage of 15 kV. The peaks of aconitine, hypaconitine and mesaconitine were shown clearly. The peaks of benzoylaconine, benzoylhypaconine and benzoylmesaconine could be also identified, but baseline separation could not be achieved. The resolution could not be improved by changing the buffer pH value or the water–methanol ratio. No peak for the final degradation products aconine, hypaconine and mesaconine was found due to the weak UV absorbance of those components. CE–MS was applied subsequently. Unfortunately, the separation of nine alkaloids was still unsatisfactory, and SIM mode had to be applied to identify the compounds (Fig. 7).

Methanol extracts of prepared and unprepared Wutou were tested by CE–MS. The peaks of both aconitum alkaloids and hydrolysis products were found from the unprepared herb, showing that hydrolysis might begin in the live plant. However, the peaks of several hydrolysis products could not be found from the extract of the prepared herb. This finding may indicate that toxicity was reduced by alkaloids released also into the decoction solution.

4. Conclusions

The coupling of CE with MS combines two generally accepted powerful analytical techniques. In this study, the CE–MS method was successfully applied to the analysis of alkaloids in herbs before and after preparation. Alkaloids in Maqianzi were separated successfully with the TIC mode; comparison of the detection limits of a previous study showed that the sensitivity of MS detection was better than that of UV detection. The rates of conversion of aconitum alkaloids to their hydrolysis

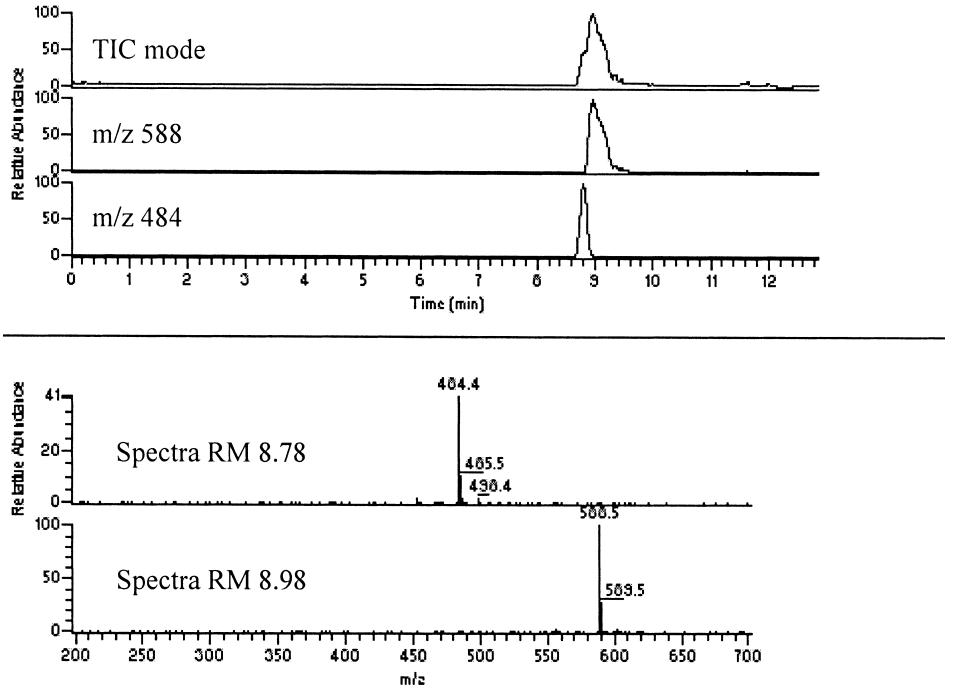


Fig. 6. Electropherograms of hypaconitine dissolved in methanol for 1 year. Other conditions as in Fig. 5.

products in water and methanol were studied. The CE–MS method was also applied to the analysis of prepared and unprepared Wutou. However, the separation of nine alkaloids was not satisfactory and identification could only be achieved under SIM mode.

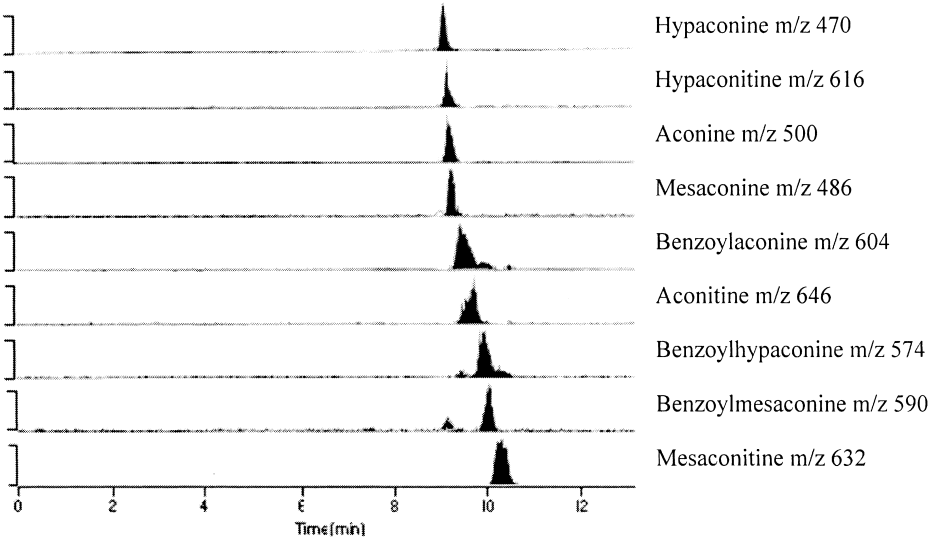


Fig. 7. Separation of aconitum alkaloids and their hydrolysis products under SIM mode. Other conditions as in Fig. 5.

Application of the ESI technique ensured the generation of mass spectra with only one dominating signal corresponding to the protonated ion $[M+H]^+$. The separated components can potentially be further characterized by MS–MS. To our knowledge, this study is the first report of CE–MS in the field of Chinese medicine preparation and is valuable for future application of CE–MS in controlling the preparation of Chinese medicine.

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