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Journal of Pharmaceutical and Biomedical Analysis
30 (2002) 161–170

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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Short communication

Analysis of proprietary Chinese medicines for the presence of toxic ingredients by LC/MS/MS

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Received 19 December 2001; received in revised form 7 March 2002; accepted 30 March 2002

Abstract

This paper presents an LC/MS/MS approach for simultaneous qualitative and quantitative analysis of proprietary Chinese medicine products for the presence of toxic ingredient compounds. The target compounds include three C_{19}-diterpenoid alkaloids, two quinolizidine alkaloids, two indole alkaloids and four bufadienolide steroids. They were recognized as active compounds of several toxic/potent herbal materials commonly used in the preparation of some proprietary medicine products. These toxic/potent herbal materials include Radix Aconiti Lateralis, Radix Sophorae Tonkinensis, Semen Strychni and Venenum Bufonis. In the analysis, the LC/MS/MS system was set to record the MS spectra and respective multiple reaction monitoring (MRM) signals for different target compounds after they were separated and eluted from the column. The MS spectra were used for the qualitative analysis whereas the MRM signals for the quantitative analysis. In this study, totally 12 proprietary medicine products were tested and found to contain some of the target compounds at different levels.

Keywords: Proprietary Chinese medicines; LC/MS/MS; Toxic ingredient compounds

1. Introduction

Increasingly, Chinese medicine is receiving attention for its role as a kind of alternative medicine and health supplements. In response, health professionals and analysts have to develop effective means to assess the safety and efficacy of Chinese medicines. According to the practice of the traditional Chinese medicine, toxic/potent herbal materials might be used for specific therapeutic applications. For instance, the toxic/potent herbal materials Radix Aconiti Lateralis, Radix Sophorae Tonkinensis, Semen Strychni and Venenum Bufonis are commonly used in the preparations of some proprietary Chinese medicines (PCM) products. Their major toxic ingredients were identified as some kinds of alkaloids and steroids with details and chemical structures shown in Table 1 and Fig. 1 respectively. However, as concluded from clinical studies, these toxic ingredient compounds also act as active ingredients of the herbal materials concerned. History of medicinal use shows that unless these types of herbal materials were administered in

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0731-7085/02/$ - see front matter
PII: S0731-7085(02)00172-3

excessive doses, or with improper preparation, or when they were substituted erroneously, the PCM products containing them were generally safe. However, in the interests of the consumers, such PCM products should be monitored for the levels of the respective toxic ingredient compounds.

As one of the most sensitive and specific methods for molecular analysis, mass spectrometry has become the state-of-the-art spectrometric technique. Combining chromatography with mass spectrometry offers the advantages of both chromatography as a separation method and mass spectrometry as an identification method. Such kind of hyphenated technique is particularly useful for analysis involving complicated sample matrices such as in the cases of PCM products. The objective of this study was to develop simple and specific LC/MS/MS methods to identity and quantify those target toxic compounds present in PCM products.

2. Experimental

2.1. Apparatus

All experiments were performed using a Perkin-Elmer SCIEX (Thornhill, ON, Canada) API-300 pneumatically assisted electrospray triple-quadruple mass spectrometer equipped with a microgradient syringe pump (Model 140C, Applied Biosystems) and an autosampler (Model 200 LC, Perkin-Elmer, USA). The flow rate of the mobile phase was 200 μl/min with split ratio set at 9:1. The ionspray voltage, orifice voltage, focusing ring voltage, deflector voltage and the channel electron multiplier voltage were set at 5000, 41, 220, −400 and 2400 V, respectively. The flow of the nebulizer gas, curtain gas and collision gas were set at 0.95, 1.25 and 0.44 l/min, respectively.

2.2. Materials

Reference standards of cinobufotalin, bufalin, cinobufagin, resibufogenin, aconitine, mesaconitine, hypaconitine, matrine, oxymatrine, strychnine and brucine were obtained from the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, PRC. PCM were purchased from a local Chinese product emporium store.

2.3. Chemicals

Acetonitrile and methanol were of HPLC grade from Lab-Scan, Ireland. Formic acid and acetic acid were of analytical grade from Merck, Germany. Ammonium acetate was of analytical grade from Riedel-de Haen, Germany. Ammoium formate and tetramethylammonium hydroxide (TMAH) solution were of analytical grade from BDH, England.

2.4. Preparation of the sample

About 0.5 g homogenized sample was accurately weighed into a 15-ml centrifuge tube and mixed thoroughly with 10 ml respective mobile phase solution. The mixture was ultra-sonicated to extract for 30 min and separated by centrifugation at 2500 rpm for 15 min. The supernatent clear extract was filtered through 0.45-μm membrane filter prior to LC/MS/MS determination.

2.5. HPLC procedures

2.5.1. Strychnine/brucine

The alkaloids were separated on a 250 × 2.1 mm ID. Five micrometer Amino column from Restek, USA. The mobile phase used was acetonitrile: 0.4% v/v acetic acid with 10 mM ammonium acetate (85:15) at a flow rate of 200 μl/min.

Table 1
Toxic ingredient compounds identified in the herbal materials under study

Toxic/potent herbal material	Reported toxic ingredient compounds
Radix Aconiti Lateralis	Aconitine, Mesaconitine, Hypaconitine
Radix Sophorae Tonkinensis	Matrine, Oxymatrine
Semen Strychni	Strychnine, Brucine
Venenum Bufonis	Bufalin, Cinobufotalin, Cinobufagin, Resibufogenin

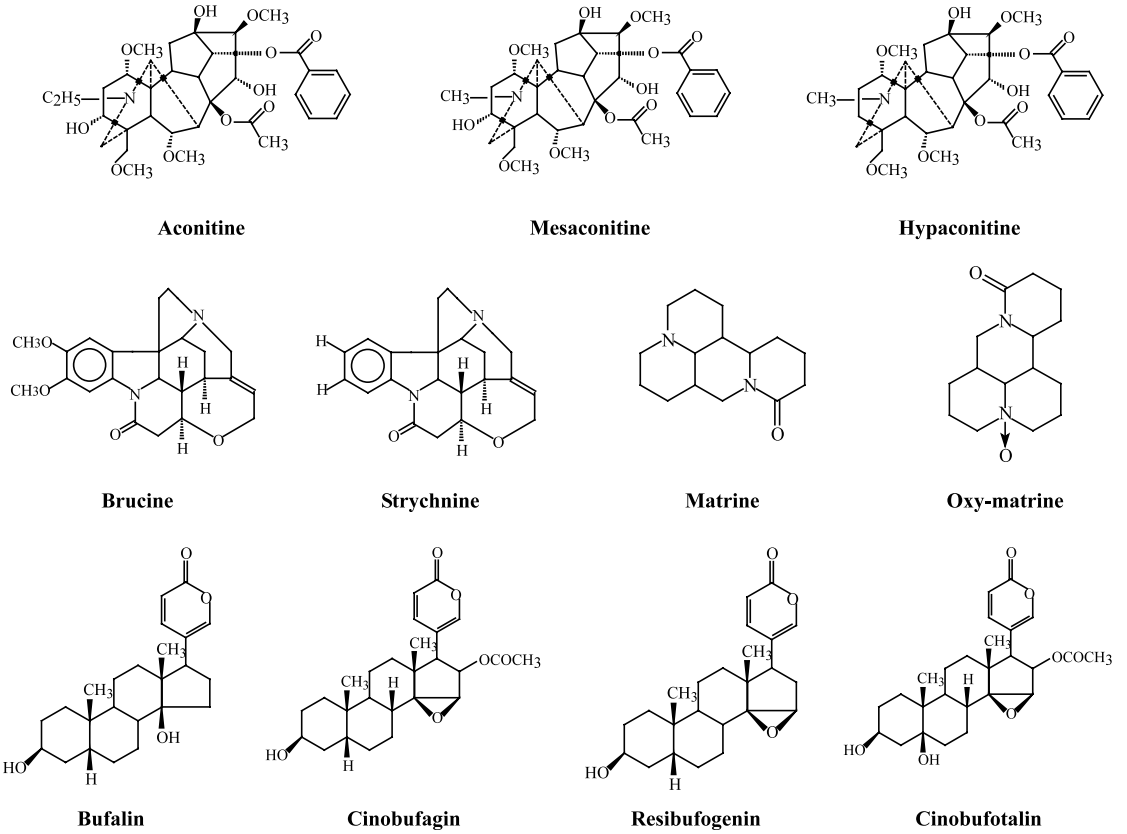

Fig. 1. Chemical structure of the 11 target toxic compounds.

2.5.2. Aconitine/mesaconitine/hypaconitine

The aconitine alkaloids were separated on a 250 × 2.1 mm ID. Five micrometer Hypersil C18 column from Alltech, USA. The mobile phase used was acetonitrile: 0.4% v/v acetic acid with 0.01% v/v TMAH (50:50) at a flow rate of 200 μl/min.

2.5.3. Matrine/oxymatrine

The alkaloids were separated on a 250 × 2.1 mm ID. Five micrometer Alltima C18 column from Alltech The mobile phase used was acetonitrile: formate buffer which contained 20 mM ammonium formate and 0.5% v/v formic acid (10:90) at a flow rate of 200 μl/min.

2.5.4. Bufalin/cinobufotalin/cinobufagin/resibufogenin

The bufadienolides were separated on a 250 × 2.1 mm ID. Five micrometer Hypersil C18 column from Alltech The mobile phase used was methanol containing 0.1% v/v acetic acid: 0.1% v/v acetic acid (55:45) at a flow rate of 200 μl/min.

3. Results

3.1. Detection and quantification of target toxic compounds

It was noticed that the three aconitine alkaloids might decompose gradually when stored in pure methanol. Hence it was recommended that all the reference standard solutions should be prepared and stored in respective mobile phase solutions and kept in refrigerator. The quantitative analysis was conducted using the tandem mass spectrome-

try technique provided by the triple quadrupole LC/MS system used in this study. As the system allowed the simultaneous monitoring of more than one pair of parent/daughter fragment ions, the technique was also named multiple reaction monitoring (MRM). The MRM responses were found to be linear over the concentration ranges examined for both samples and standards. Also, the quantification method was validated through the determination of the method performance parameters such as reproducibility, spiking recovery, linearity and detection limit. Table 3 shows the method validation data for the four different groups of target compounds.

Totally 12 PCM products were analyzed for the presence of target compounds expected on the basis of their claimed herbal ingredients. The results are summarized in Table 4 and were found to be consistent with the herbal ingredients claimed.

3.2. Confirmation of target toxic compounds

In the MRM scanning process, the triple quadrupole MS system also allowed the mass spectrum of the particular target compound to be recorded simultaneously. As such, the presence of the target compounds could be confirmed with its characteristic mass spectrum. This was particularly useful for minimizing the possibility of misidentification where sample matrices were complicated such as in the case of PCM products. Such system capability enables the target compounds to be qualitatively and quantitatively determined in a single LC run. In this connection, reference standard solutions of the target compounds had been analyzed and their characteristic MS fragment ions were identified as per Table 2. Typical print-outs of the MS analysis are also shown in Fig. 2.

4. Discussion

4.1. Optimization of LC conditions

Experienced LC/MS workers would all agree that most practical applications of LC/MS rely heavily on the chromatographic resolution power of the LC system. Otherwise, ion suppression induced by matrix components co-eluting with the target compounds would interfere with the ionization response [1]. In this respect, an appropriate mobile phase has to be selected to ensure that the peaks of the target compounds could be separated and well resolved within a reasonable analysis time. In order to achieve the optimal separation for each group of target compounds, the mobile phase systems were optimized separately for respective groups of target compounds according to the following consideration and observation:

1. Because of its lower viscosity, acetonitrile was usually preferred as the organic modifier of a mobile phase from the standpoint of pressure. However, in the determination of the bufadienolides, methanol was preferred because the polar interaction between methanol and the steroids offered better peak shapes in the analysis.
2. To enhance the ionization efficiency, acetic acid was usually required to be added to the mobile phase. However, in the determination of matrine and oxymatrine, it was observed that the $[M+H]^+$ ions could be enhanced when formic acid was used instead of acetic acid.
3. To tackle the peak-tailing problem in the determination of the aconitine alkaloids, TMAH was preferred as the mobile phase modifier because non-volatile salts would cause a dramatic decrease in the ionization efficiency [2].
4. In the determination of matrine, oxymatrine, strychnine and brucine, it was noticed that a buffered mobile phase could ensure reproducible results with improved peak symmetry. This was attributed to the fact that these alkaloids were all readily ionizable in solution due to their high basicity.
5. It was observed that amino column exhibited improved peak shape for brucine and strychnine compared with C18 column.
6. The separation and resolution of peaks were optimized by adjusting the ratio of the organic modifier in the mobile phase without

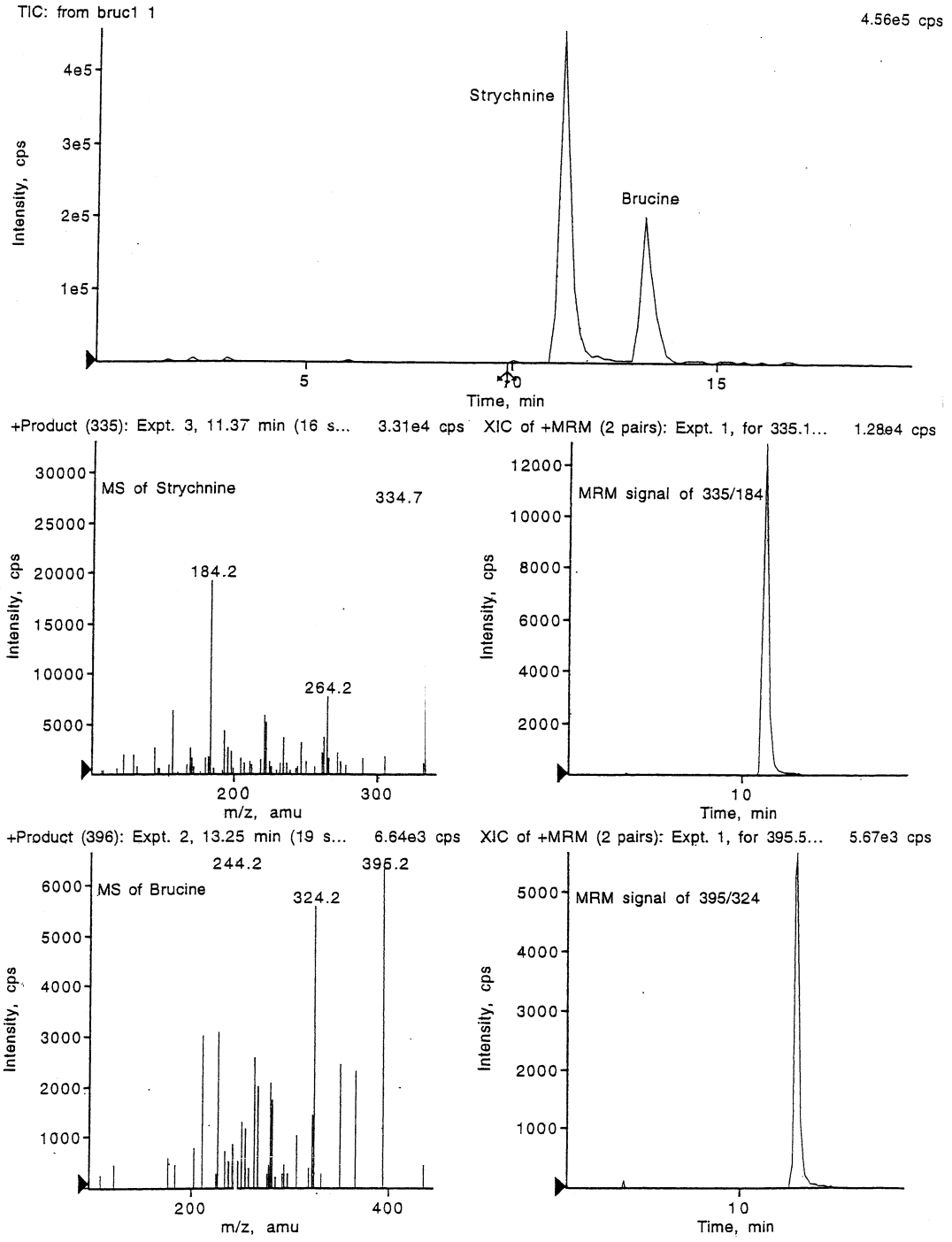

Fig. 2. Print-out of the MS analysis of a PCM products containing Semen Strychni as an ingredient herb.

the need of gradient flow. This would be an advantage if a stable baseline signal were required to enhance the sensitivity of the analysis.

The optimized LC conditions for the four different groups of target compounds are detailed in Section 2.

4.2. Fragmentation pathways of the target compounds

In contrast to GC/MS, there is not any universal LC/MS spectra library for identity matching. Hence, it would be necessary to identify the major characteristic fragment ions present in the mass

Table 2
Major fragment ions observed in the MS/MS spectra of the target compounds

Marker Compound	$[M+H]^+$	Fragment ions, m/z	MRM ion pair for quantification
Mesaconitine	**632**	572 → 540 572 → 354	632/572
Aconitine	**646**	586 → 554 586 → 368	646/586
Hypaconitine	**616**	556 → 524 556 → 338	616/556
Brucine	**395**	395 → 324 395 → 244	395/324
Strychnine	**335**	335 → 264 335 → 184	335/184
Matrine	**249**	249 → 247 → 148	249/148
Oxymatrine	**265**	265 → 247 → 148 265 → 205	265/247
Cinobufotalin	**459**	459 → 363 459 → 381 → 363	459/363
Cinobufagin	**443**	443 → 347 443 → 365	443/365
Resibufogenin	**385**	385 → 367 385 → 349 → 253	385/367
Bufalin	**387**	387 → 369 387 → 351 → 255	387/351

Table 3
Method validation data for the target compounds

	Mesaconitine	Aconitine	Hypaconitine	Brucine	Strychnine	Matrine	Oxymatrine	Cinobufotalin	Cinobufagin	Resibufogenin	Bufalin
Linearity											
Linearity range (mg/l)	0.5–20	0.5–20	0.5–20	0.1–5	0.1–5	0.5–20	0.5–20	0.5–20	0.5–20	0.5–20	0.5–20
Intercept	−2092	−3332	0	−517	427	1144	3413	−2481	3139	7310	4047
Slope	30065	110093	75675	30324	41632	8048	12405	129812	38230	32790	31413
Corr. coefficient	1.000	1.000	1.000	0.999	1.000	1.000	1.000	0.999	0.998	0.997	0.999
Limit of detection (ng)	0.6	0.4	0.5	1.5	1.8	1.4	1.0	0.6	1.5	1.6	0.7
Recovery average ($n = 5$) (%)	90.3	87.3	87.0	88.9	96.6	97.3	89.6	101.1	99.7	100.4	100.8
Method repeatability %RSD ($n = 5$)	5.4	5.4	4.3	3.6	4.1	2.7	1.3	4.5	3.5	2.4	3.9

Table 4
Results of the analysis of the 12 PCM products

Indole alkaloids	*Strychnine*	*Brucine*		
Xiao Jin Dan	0.17	0.17		
Shen Jin Dan	0.18	0.19		
Quinolizidine alkaloids	Matrine	Oxymatrine		
E Hou San	0.12	<0.01		
Bi Yab Jin Dan	0.52	0.01		
Diterpenoid alkaloids	Mesaconitine	Aconitine	Hypaconitine	
Aconitun compound Pills	1.4	0.5	27	
Chi Shung Chen Qi Pills	0.5	0.4	5.7	
Gui Fu Di Huang Pills	0.2	0.1	0.5	
Yao Kwei Pills	0.8	0.2	4.3	
Guan Jie Tong	<0.1	0.5	3.0	
Bufadienolides	Bufalin	Cinobufotalin	Cinobufagin	Resibufogenin
Liu Shen Wan (1)	0.24	0.13	0.57	0.25
Liu Shen Wan (2)	0.31	0.32	0.62	0.68
Liu Shen Wan (3)	0.48	<0.01	0.71	1.4

Note: The above concentration were in %w/w except that for diterpenoid alkaloids the units were mg/kg.

spectra if they were to be used for qualitative analysis. In this connection, reference MS spectra of the target compounds were studied and the fragmentation pathways leading to the formation of their major characteristic fragment ions are discussed as follows.

For the three C_{19}-diterpenoid alkaloids (i.e. aconitine, mesaconitine and hypaconitine), the mass spectra show protonated molecules ($[M+H]^+$) and characteristic losses of an acetic acid group ($[M+H\text{-}60]^+$) then followed by the elimination of three methanol groups and one benzoic acid ($[M+H\text{-}278]^+$) or just a single methanol group ($[M+H\text{-}92]^+$). For the indole alkaloids, strychnine and brucine, the mass spectra show protonated molecules ($[M+H]^+$) and fragment ions $[M+H\text{-}71]^+$ and $[M+H\text{-}151]^+$. The proposed fragmentation pathways are provided in Fig. 3.

For the quinolizidine alkaloids, matrine and oxymatrine, the mass spectra show protonated molecules ($[M+H]^+$) and fragment ions of m/z 247 and 148 with the proposed fragmentation pathways shown in Fig. 4. For the bufadienolides, the mass spectra of cinobufotalin and cinobufagin show protonated molecules ($[M+H]^+$) and characteristic losses of a 1,2-pyrone group ($[M+H\text{-}96]^+$) and an acetic acid plus a water molecule ($[M+H\text{-}78]^+$). It was noticed that, for cinobufotalin, the $[M+H\text{-}96]^+$ could also be due to the loss of an acetic acid plus two water molecules. For bufalin and resibufogenin, the mass spectra show protonated molecules ($[M+H]^+$) and characteristic losses of one water molecule ($[M+H\text{-}18]^+$), two water molecules ($[M+H\text{-}36]^+$) then followed by the elimination of a 1,2-pyrone group ($[M+H\text{-}132]^+$).

In the analysis of the PCM samples, the above major characteristic fragment ions were also observed in the MS spectra of the LC peaks suspected to be the respective target compounds. These characteristic MS spectra together with the LC retention times result in critical enhancement of the selectivity and specificity of the method for the present application.

4.3. Method comparison

According to the literatures, LC–UV was the most commonly used technique for the determination of the target compounds concerned [3–6]. However, lack of structural information rendered this technique inadequate for reliable qualitative identification. Recently, there was an increase in papers reporting the use of LC/MS for the analysis of Chinese medicines. Guo et al. [7] and Zhou

and Hamburger [8] both reported LC/MS conditions for the identification of brucine and strychnine in herbal extracts. Also, Wada et al. reported LC/MS conditions for the identification of aconitine alkaloids [9]. However, in these papers, only the MS spectra were provided without giving the details of the fragmentation ions and the respective fragmentation pathways. Also, validated procedure for quantitative analysis was lacking.

4.4. Prospective

The application of the above methodology was now being extended to cover other toxic/potent herbs that are also commonly used in the preparation of PCM products. The new target compounds included homogentistic acid from the herbs Rhizoma Pinelliae and Rhizoma Arisaematis, bullatin B and bullatin G from the herbs Radix Aconiti Brachypodi and Radix Aconiti Szechenyiani, atropine and scopolamine from the herbs Semen Hyoscyami and Flos Daturae Metelis. The LC/MS conditions for all these new target compounds had already been optimized and were being applied to the analysis of PCM products.

5. Conclusions

A new approach for simultaneous qualitative and quantitative analysis of PCM products for the presence of toxic ingredient compounds has been developed. With the use of a triple quadrupole LC/MS/MS system, concentrations of the target compounds present in the PCM products were determined by MRM signal whereas the presence of these compounds was confirmed by the MS spectra obtained. To facilitate the MS/MS determination, LC conditions for the four different groups of target compounds under study were also optimized. Totally 12 PCM products were tested and the results were found to be consistent with the herbal ingredients claimed.

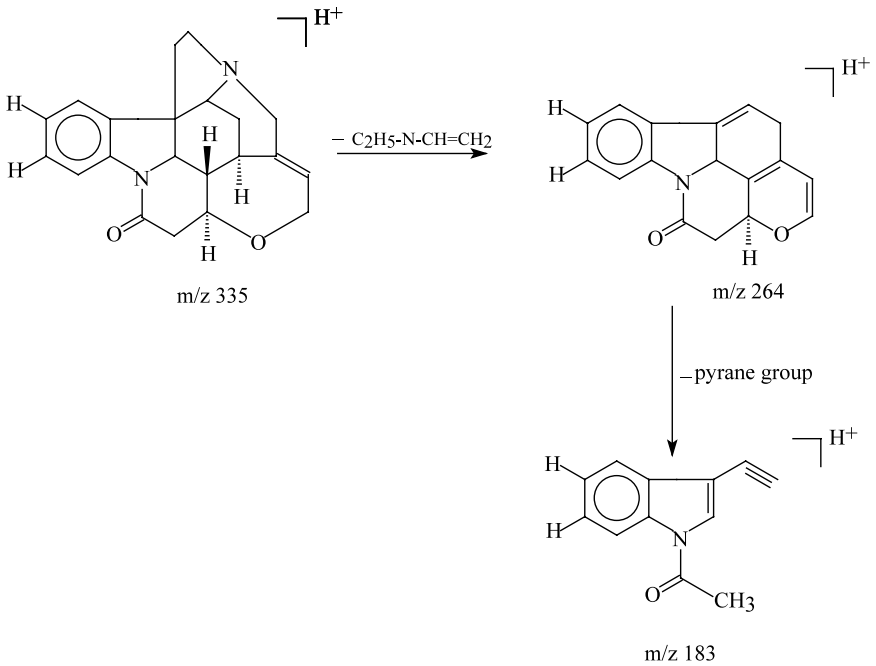

Fig. 3. Proposed fragmentation pathways for strychnine.

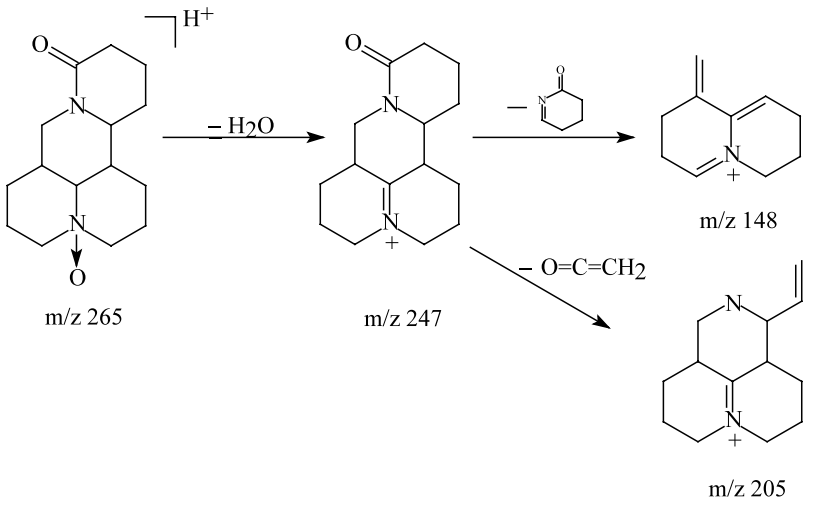

Fig. 4. Proposed fragmentation pathways for oxymatrine.

Acknowledgements

The authors wish to thank the Government Chemist of the Hong Kong SAR for his encouragement and permission to publish the results of the study.

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