Analysis of Synthetic Drugs in Chinese Medicine by High Performance Liquid Chromatography/Mass Spectrometry with In-Source Collision Induced Dissociation

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Sixteen chemical drugs, often found in adulterated Chinese medicine, were studied by high performance liquid chromatography/atmospheric ionization mass spectrometry. Under optimal conditions, three pairs of compounds were either coeluted or unresolved. The lack of chromatographic resolution and the lack of specificity in UV detection were overcome by a method based on high performance liquid chromatography/electrospray mass spectrometry. This method was capable of detecting the adulterants based on their retention times, molecular ions, and characteristic fragments resulting from in-source collision induced dissociation.

INTRODUCTION

The use of Chinese medicine to maintain human health and cure disease has a long and rich history. Because Chinese medicine is a nature based therapy, people believe that it is mild and less toxic than chemical drugs. However, to ameliorate symptoms quickly, traditional Chinese medicines are sometimes illegally adulterated with synthetic chemical drugs. This practice can be harmful to health and may result in side effects or drug allergy. Therefore, it is important to provide the public with reliable information about whether or not chemical drugs are present in Chinese medicine.

Conventional analytical method involves the separation of the adulterants by Thin-Layer Chromatography (TLC) following identification by retarding factor and/or ultraviolet spectroscopy. These methods are in general time consuming, labor intensive and more importantly lack the specificity for a high confidence in identification. Recently, methods based on high performance liquid chromatography (HPLC)[1-3] and capillary electrophoresis (CE)[4-6] have been developed for the analysis of synthetic drugs in traditional Chinese medicine. A variety of detection techniques, such as UV, fluorescence, and electrochemical methods have been developed for HPLC and CE. Although they have been shown to be superior to conventional TLC methods, these methods generally lack the specificity for high confidence identification and have only limited capabilities for structure elucidation. Moreover, analytes co-eluted or closely eluted can be identified solely based on their retention times.

Mass spectrometry is known for its high sensitivity and high specificity. In general, very high specificity can be achieved with minimal quantity of samples. The combination of mass spectrometry and chromatography proved to be a powerful method to identify the unknown compounds in a complicated mixture because absolute identification is often obtained with retention time, molecular ion and many structural characteristic fragment ions present in the mass spectra. Since its development in the 60s, gas chromatography/mass spectrometry (GC/MS) has been proved to be a very powerful analytical technique. Unfortunately, without chemical derivatization, this technique is limited to volatile and thermally stable compounds. In recent years, HPLC/MS methods based on atmospheric pressure ionization (API) have received considerable attention for the analysis of nonvolatile and thermally labile compounds. Therefore LC/MS is widely used in the fields of biology, biochemistry, environmental science, pharmaceutical chemistry and analytical chemistry.

We have evaluated HPLC/MS for the analysis of synthetic drugs in Chinese medicine. Preliminary results indicated that this method was capable of detecting adulterants in Chinese medicine.[7] The method has been explored further to the analysis of sixteen popular chemical drugs. The potential and the detailed analytical conditions of this method are discussed in this report.

EXPERIMENTAL

Chemicals

All chemical drugs, acetaminophen, bucetin, caffeine, diazepam, ethoxybenzamide, fenbufen, flufenamic acid, indomethacine, ketoprofen, mefenamic acid, niflumic acid, oxyphenbutazone, phenylbutazone, prednisolone, salicylamide and sulindac were obtained from Laboratories of Foods

and Drugs, Dep. of Health, Taipei, Taiwan. Water was purified with a Mill-Q system (Millipore Inc., Bedford, MA, U.S.A.). Ethanol, methanol and acetonitrile were of HPLC/Spectro grade, purchased from Merck (Darmstadt, Germany), LAB-SCAN (Labscan Ltd. Dublin, Ireland) and TEDIA (Fairfield, Ohio, U.S.A.), respectively. Acetic acid of analytical grade was purchased from Baker (Deventer, Netherlands). Chemical drugs were dissolved in ethanol and the concentration for each was 100 ppm.

Sample Preparation

Chinese medicine pills were obtained from National Laboratories of Foods and Drugs, Dep. of Health, Taipei, Taiwan. Each sample was pounded into powder. One hundred milligrams of sample was dissolved in 10 mL ethanol, extracted for 15 min by ultrasound and centrifuged for 1min to remove the undissolved sample from the solution.

HPLC

All HPLC separations were performed on a Microtech Prodigix4 HPLC system (Microtech Scientific Inc., U.S.A.). A custom packed GSK ODS-2 microbore column (5 μm, 1mm × 15cm) with Nucleosil 100-5 C_{18} (Macherey-Nagel, Düren, Germany) and a Nucleosil 120-5 C_{18} column (5 μm, 2mm × 25cm; Macherey-Nagel, Düren, Germany) were used throughout the study. A Rheodyne 7125 sampling valve (Rheodyne, U.S.A.) with a 5-μl loop was used as the injector. A linear gradient was employed for LC separations beginning with 85% of 0.1% acetic acid in acetonitrile followed by ramping the acetonitrile content to 100 % in a period of 30 min. UV detection was performed by using a Dynamax UV-C UV/Visible detector (Rainin MA, U.S.A.) with a flow cell (1.2 μl) at 235 nm.

HPLC/MS

Mass spectra were acquired using a Fisons Platform mass spectrometer (VG BioTech, Altrincham, United Kingdom) equipped with either an electrospray ion source or an atmospheric pressure chemical ionization source. The HPLC was coupled with an ultraviolet (UV) detector before connecting to the MS system. The 2 mm column was operated at a flow rate of 200 μl/min. The flow entering the ESI source was reduced to 15 μl/min using a post-column splitter. The 1 mm microbore column was operated at a flow rate of 50 μl/min and connected directly into the ion source. In ESI, the flow rates of nitrogen gas were set at 30 l/hr for nebulization and 400 l/hr for drying. In APCI operartion, the sheath gas was set at 150 l/hr and drying gas was set at 350 l/hr. Vaporizer temperature was 400 °C and the corona discharge pin was maintained at 3.5 kV. For in-source CID (collision induced dissociation) experiments, extraction lens voltage was set between 40 and 70 eV.

RESULTS AND DISCUSSION

Analysis of Chemical Drugs by HPLC/UV

Both narrow bore (2 mm i.d.) and microbore (1 mm i.d.) columns[8,9] were used as separation columns in this study. For narrow bore columns with higher flow rate (about 200 μl/min), it is easier to do the gradient elution without pre-column splitting. However, post column splitting is often needed to preserve the optimum ESI performance. Special equipment is often needed to do gradient elution with a microbore column. The advantage[10] of using microbore columns is that the lower flow rate (about 50 μl/min) makes it possible to connect HPLC directly with the mass spectrometry without splitting.

Sixteen chemical drugs (Fig. 1), often found in adulterated Chinese medicine, were separated with a Nucleosil 2mm C18 column and a GSK 1mm ODS-2 microbore column. The best chromatogram obtained from the 2 mm column is shown in Fig. 2a. Fourteen instead of sixteen peaks were observed (Fig. 2). Coinjection with authentic standards suggested that ketoprofen coeluted with oxyphenbutazone and indomethacine coeluted with niflumic acid. In addition, flufenamic acid was unresolved from mefenamic acid. The chromatogram obtained with a GSK 1mm ODS-2 microbore column is shown in Fig. 2b. The two chromatograms were very similar except for the elution order of diazepam. Diazepam was the 10th peak in Fig. 2a and was the last peak in Fig. 2b.

Analysis of Chemical Drugs by HPLC/MS

It is illegal to add synthetic drugs into Chinese medicine.[11,12] People committing the crime may face a maximum penalty of seven years in prison. To avoid any possibility of mistrial, analytical data with the highest quality should be pursued. The HPLC/UV data, as shown in Fig. 2, may not have the specificity needed to avoid a false positive error. Mass spectrometry is known for its sensitivity and specificity. Furthermore, by using mass spectrometry as the chromatography detector, co-eluting peaks or peaks with very similar retention time in HPLC/UV can often be deconvoluted by the resolving power of mass spectrometry.

Two of the most popular ionization methods in the coupling of HPLC with MS are atmospheric pressure chemical ionization (APCI) and ESI. The study of the 16 drugs by APCI and ESI suggested that ESI was superior to APCI because the sensitivity and the abundance of molecular ion in ESI were generally higher than those in APCI. The TIC and

mass chromatograms of the 16 compounds studied by ESI are shown in Fig. 3. The two co-eluting peaks were deconvoluted based on their difference in molecular weight. As can be seen in Fig. 3, the ketoprofen elutes a little earlier than oxyphenbutazone. A similar condition was observed on indomethacine and niflumic acid. Having the same molecular weight (M.W. 254), ketoprofen and fenbufen result in the appearance of two peaks at the trace of *m/z* 255 (19.80 and 20.40 min).

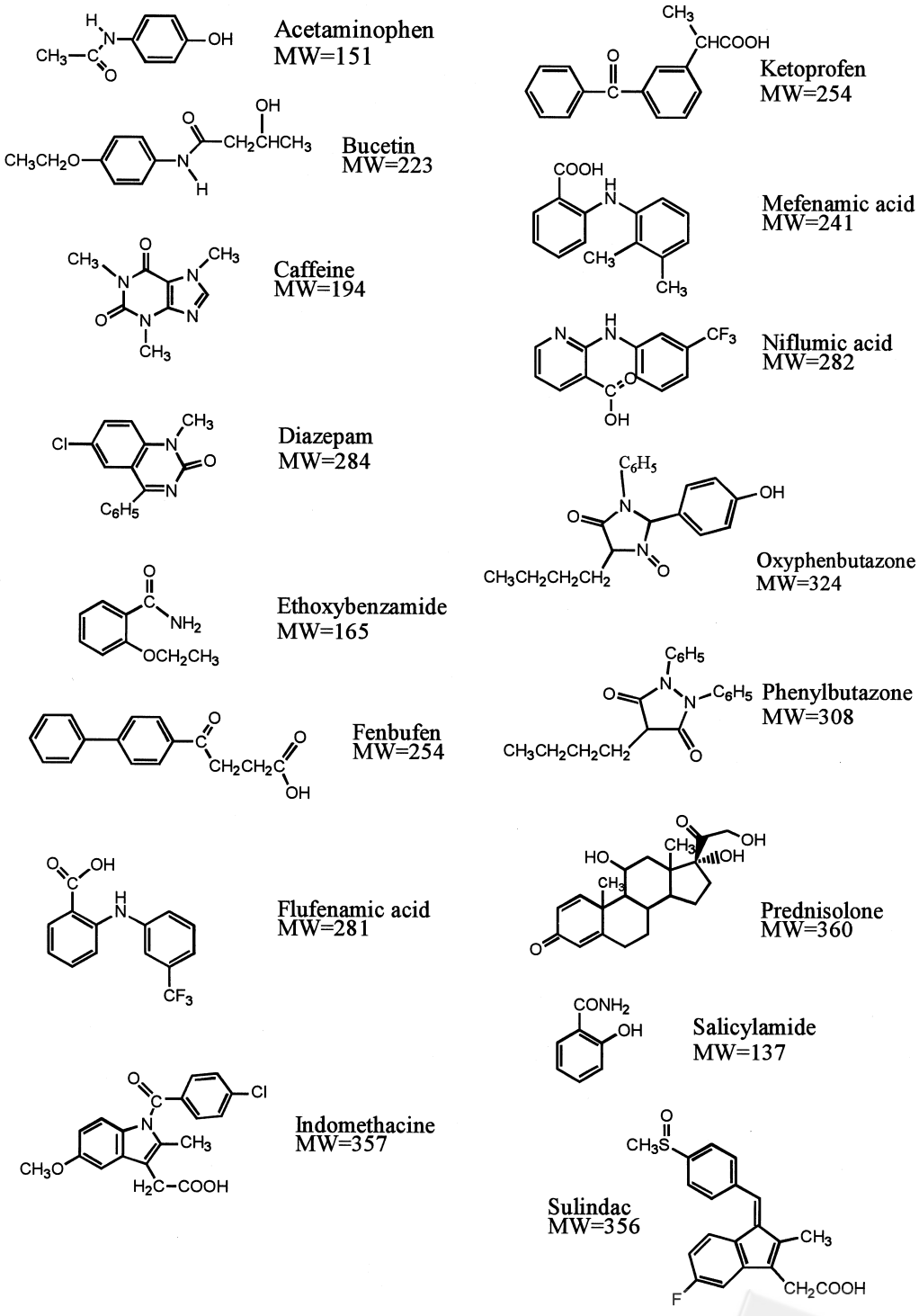

Fig. 1. Structures of the sixteen chemical drugs.

In-Source CID

For electrospray ionization, the analyte molecules are presented as preformed ions in solution. ESI is just a process of ejecting the preformed ion from liquid phase to gas phase.[13-16] Therefore, in comparison with other ionization methods, ESI produces ions with a minimum of internal energy and thus little or no fragment ions are observed. Based on retention time as well as molecular ion, HPLC/MS is more specific than HPLC/UV. However, specificity can be further improved if fragment ions are also included.

In general, it takes tandem mass spectrometry to produce fragment ions. Fortunately, with the development of in-source CID, fragment ions can be produced with a single quadrupole mass spectrometer. In the in-source CID, a potential difference is applied between the sample cone and the skimmer. When the molecular ions pass through the sample cone, they are accelerated by the potential difference. These fast moving ions are then collided with gas molecules and many structurally specific fragment ions are produced. In-source CID was used to produce fragment ions because the instrument used in this study is a single quadrupole mass spectrometer. The major fragment ions resulting from in-source CID were listed in Table 1. Except for diazepam, fragment ions were observed for the other fifteen drugs.

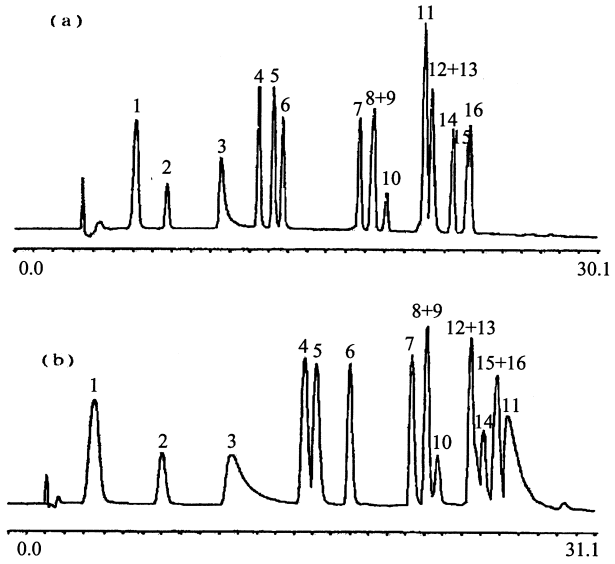

Fig. 2. Gradient elution of a mixture of sixteen chemical drugs, UV detection at 235 nm. A linear gradient of 85-100% acetonitrile containing 0.1% acetic acid over 30 min. (a) column, Nucleosil 120-5 C_{18} column (5 μm, 2mm × 25cm). (b) column, GSK ODS-2 C_{18} (5 μm, 1mm × 15cm). Peaks: (1) acetaminophen; (2) caffeine; (3) salicylamide; (4) bucetin; (5) ethoxybenzamide; (6) prednisolone; (7) sulindac; (8) ketoprofen; (9) oxyphenbutazone; (10) fenbufen; (11) diazepam; (12) indomethacine; (13) niflumic acid; (14) phenylbutazone; (15) flufenamic acid; (16) Mefenamic acid.

Analysis of Adulterants in Chinese Medicine

Several samples were analyzed by HPLC/ESIMS; the result of one sample is presented. The TIC and mass chromatograms (Fig. 4) suggested that four synthetic drugs were illegally added into the preparation. The *m/z* 195 and *m/z* 138 ions at 9.07 minutes correspond to the protonated molecular ion and the loss of $CONCH_3$ from the molecular ion of caffeine (MW 194). The *m/z* 166 and *m/z* 149 ions at 14.45 minutes correspond to the protonated molecular ion and the loss of NH_2 from the molecular ion of ethoxybenzamide

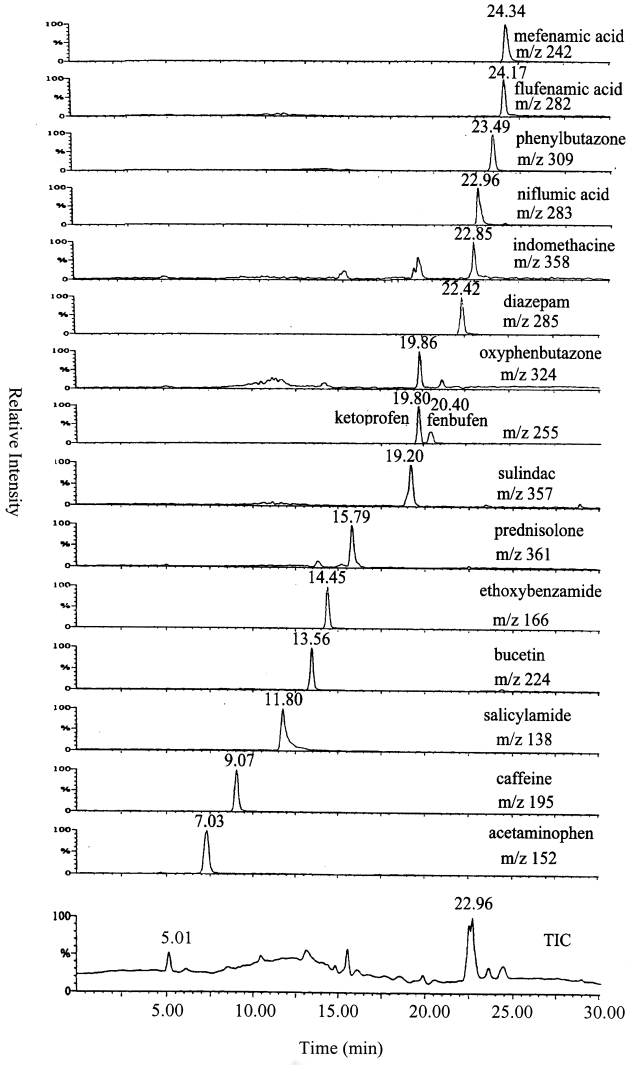

Fig. 3. The TIC and mass chromatograms of the sixteen chemical drugs studied by LC/ESI/MS.

Table 1. Molecular Ions and the Major in Source CID Fragment Ions of the Sixteen Chemical Drugs

chemical drug	molecular ion (*m/z*)	major fragment ions
acetaminophen	152	110
bucetin	224	206 164 136
caffeine	195	138
diazepam	285	none
ethoxybenzamide	166	149 121
fenbufen	255	237 181 209
flufenamic acid	282	264
indomethacine	358	139
ketoprofen	255	209
mefenamic acid	242	224
niflumic acid	283	265
oxyphenbutazone	325	204
phenylbutazone	309	211 120
prednisolone	361	343
salicylamide	138	121
sulindac	357	340 294 248 233

(MW 165). The *m/z* 285 ion at 22.42 minutes corresponds to the protonated molecular ion of diazepam (MW 284). The *m/z* 358 and *m/z* 139 ions at 22.85 minutes correspond to the protonated molecular ion and the loss of $CH_3O(C_6H_3)NC(CH_2CO_2H)C(CH_3)$ from the molecular ion of indomethacine (MW 357).

As can be seen in Fig. 4, unlike caffeine, ethoxybenzamide and indomethacine, diazepam was identified by retention time and molecular ion but not fragment ions. To further enhance the specificity of diazepam, a "two columns" approach was used. It is generally believed that specificity can be improved if the target compound displays different elution behavior in two different columns. As mentioned earlier, the elution order of diazepam is quite different between the GSK and the Nucleosil column. The data of the same sample analyzed by the custom packed GSK ODS-2 microbore column is shown in Fig. 5. The chromatogram is similar to the result from the Nucleosil column (Fig. 4) except the elution order of the tentatively assigned diazepam. This behavior is consistent with the data obtained with an authentic diazepam standard as shown in Fig. 2. Consequently, specificity of diazepam was improved.

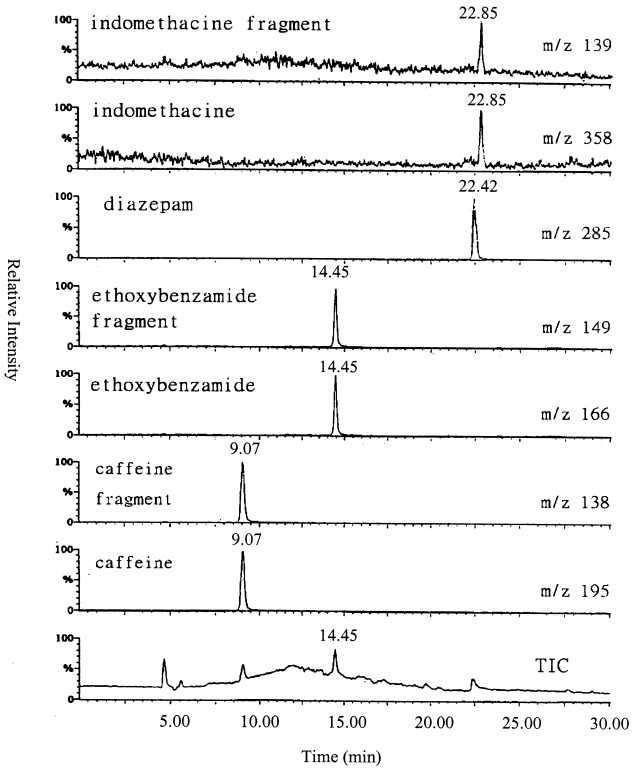

Fig. 4. The TIC and mass chromatograms of a real sample. The LC separation was performed on a Nucleosil 120-5 C_{18} column. The HPLC conditions were the same as in Fig. 2.

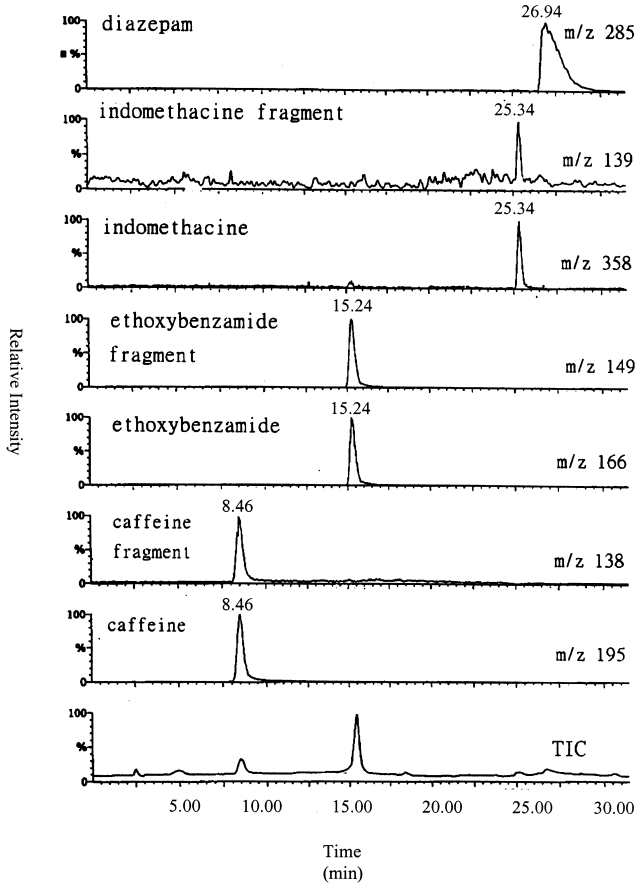

Fig. 5. The TIC and mass chromatograms of the same sample as in Fig. 4. The LC separation was performed on a GSK ODS-2 C_{18} column. The HPLC conditions were the same as in Fig. 2.

CONCLUSIONS

Whereas many methods can be used in the analysis of chemical drugs in adulterated Chinese medicine, these methods require excessive time and effort, and lack the specificity for high confidence identification. HPLC/ESIMS in combination with in-source CID proved to be a useful method for the analysis of synthetic drugs in Chinese medicine. One disadvantage of this single quadrupole mass spectrometric approach is that, unlike the CID in tandem mass spectrometry, there is no mass selectivity before the in-source CID. Therefore, the specificity would be inadequate if the coeluting compounds produce the same fragment ions. Another disadvantage of this approach is that the in-source CID may produce fewer fragment ions than the conventional CID. These disadvantages may, however, well be offset by the much cheaper price of a single quadrupole mass spectrometer.

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Key Words

Synthetic drugs in Chinese medicine; HPLC; ESI-MS; In-source CID.

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