Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Pharmaceutical and Biomedical Analysis 40 (2006) 305–311
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Rapid and reliable determination of illegal adulterant in herbal medicines and dietary supplements by LC/MS/MS

Qionglin Liang, Jun Qu, Guoan Luo*, Yiming Wang

Analysis Center, Tsinghua University, Beijing 100084, PR China

Received 3 March 2005; received in revised form 23 July 2005; accepted 28 July 2005
Available online 19 September 2005

Abstract

In recent years, dietary supplements and herbal medicines are increasing in popularity all over the world. However, it is problematic that some manufacturers illegally included synthetic drugs in their products. Due to the extremely complex matrices of those products, most existing methods for screening illegal adulterations are time-consuming and liable to false positive. In this paper, a robust LC/MS/MS method for the high-throughput, sensitive and reliable determination of illegal adulterations from herbal medicines and dietary supplements was established. Minimal LC separation was employed and MRM was used to simultaneously monitor the three transitions under their respective optimal collision energy for each compound. Positive results were determined only if well-defined peaks appeared at all of the three transitions and the ratios among the peak areas were within given threshold. In this study, the method had been applied for the screening of nine most commonly adulterated therapeutic substances, such as sildenafil (Viagra) and famotidine, and the lower limits of detection of these compounds ranged from 0.05 to 1.5 ng/ml. Little sample preparation was needed for this method and the analysis time was less than 5 min/sample. The reliability has been demonstrated by the test with blank matrix. Over 200 products that were under suspicion by SDA of China had been assayed and till now no false negative or positive result was found. This method is rapid, simple, reliable and capable of screening multiple adulterants in one run.

Keywords: Illegal adulterant; Herbal medicine; Dietary supplement; LC/MS/MS

1. Introduction

Nowadays herbal medicines and dietary supplements are taking an active part in people's health care, therapy and prevention of disease throughout the world [1,2]. These products are regarded by many as being harmless because of their natural origin and helpful to the treatment of some chronic diseases and the maintenance of physical fitness. Their share in the worldwide medicine market is remarkable and increases each year [3,4]. Nevertheless some manufacturers included synthetic drugs in the formula of their products marketed as 'herbal medicine' or 'dietary supplement', in order to improve the effect of their products [5]. In some categories of those products, such adulterations are indeed quite serious. For example [6], sildenafil (Viagra) was recently found being included in many herbal medicines and dietary supplements which were claimed having the effect of improving sexual ability and vitality. Other most common adulterants were listed in Table 1 [6]. Those manufacturers usually claimed that the effect of their products came from "purely natural substances" (which means totally harmless, declared by some manufacturers). This has violated relevant regulations and laws of most countries; in addition, the safety of those adulterated products has not been clinically tested and unpredictable effect on the health of users may be caused. In order to ban the production and marketing of those adulterated products, drug administrations urgently need a general and effective method to screen out those adulterated products from numerous brands of herbal medicines and dietary supplements appearing on the market.

The most widely applied methodologies for pharmaceutical analysis included HPLC, LC/MS and spectroscopic methods [7–11]. In recent studies, HPLC, GC/MS and LC/MS

* Corresponding author. Tel.: +86 10 62781688; fax: +86 10 62781688.
E-mail address: luoga@mail.tsinghua.edu.cn (G. Luo).

0731-7085/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2005.07.035

Table 1
Nine most common adulterants in herbal medicine and food supplements found in the market of China

Adulterant	Molecular weight	Number of samples examined	Number of positive results	Occurrence
Sildenafil (Viagra)	474.3	81	28	Products declared having the effect of improving sexual ability
Famotidine	337.4	47	18	Products declared being capable of keeping the fitness of stomach and helping the cure for gastrosis
Ibuprofen	206.3	14	3	Products declared being beneficial to the therapy for arthritis
Promethazine	284.4	19	2	Products declared having the effect of tranquilization or improving 'health status' of old people
Diazepam	284.7	11	3	Products declared being helpful to the remission of dysphoria and insomnia
Nifedipine	346.3	16	6	Products declared being capable of preventing hypertension and angina pectoris, and maintaining cardiovascular health
Captopril	217.3	19	8	Products declared being capable of preventing and healing hypertension
Amoxicillin	365.4	22[a]	2	Products declared being capable of healing tracheitis
Dextromethorphan	271.3		4	Products declared being effective for relieving cough and resolving phlegm

[a] Amoxicillin and dextromethorphan were simultaneously screened in one run for these samples since both of them had been suspicious to be potential adulterants in products declared being effective for a cold or respiratory disease. But no coexisting of these two drugs was found in the present study of 22 samples.

methods for screening of adulterants in herbal products were reported [12,13]. However, none of the existing methods is adequate to the reliable and high-throughput screening of adulterants from bulk herbal medicine and dietary supplement samples: first, these products often contain a mixture of herbs and other natural products, and the formulas of those products are considerably variant among brands, therefore the matrices of those samples will interfere both spectroscopic and chromatographic assays; for instance, in some cases the retention time (RT) of a compound from the matrix coincides with that of the analyte (false positive), and sometimes the RT of analytes migrated because of the interference from matrices (false negative). Second, the analysis times of existing methods are relatively long; for example, a typical HPLC analysis time for the assay of drugs from complex matrices is often more than 15 min, leaving alone the time needed for balancing the HPLC system. This made those methods not capable of handling large amount of samples, thus not suitable for the examination of numerous brands of such products on today's market.

This paper proposed a LC/MS/MS method for the rapid and reliable detection of synthetic drug adulterants in very complex matrices. Multiple reactions monitoring (MRM) was employed to monitor LC effluent by simultaneously using three selected transitions for each compound to improve the reliability based on the fact that the relative peak areas of the main product ions/transitions maintain a good stability [14] when each transition was monitored under the necessary condition of the Optimal Collision Energy (OCE) [15]. The applicability and accuracy of this method has been illustrated with over 200 on sale products, which were provided by the State Drug Administration (SDA) of China.

2. Experimental

2.1. Chemicals

Authenticated standards of sildenafil citrate, famotidine, nifedipine, captopril, diazepam, promethazine chloride, ibuprofen, amoxicillin·$3H_2O$ and dextromethorphan hydrobromide were from SDA (purities ≥99%, Beijing, China). HPLC grade methanol was from Tedia Inc. (Fairfield, OH). Trifluoroacetic acid (TFA, 98%) was obtained from Fluka (Buchs, Switzerland). Stock solutions of the analytical standards were prepared separately in mobile phase at the concentration of 5 μg/ml and stored at 4 °C.

2.2. Samples

All samples that were under suspicion of adulteration with undeclared synthetic drugs were taken from drug shops and markets in Beijing by SDA. These products were mainly from Japan, South Korea, China and some Southeast Asia countries. Most of them had been assayed with existing HPLC methods by the labs of SDA, and afterward a remarkable number of false positive or negative results had been found.

2.3. Sample preparation

These samples were mainly in three forms of preparations: capsules, tablets and oral solutions. For the sample preparation of capsules, the husks were removed and the powder was transferred to a 5 ml capped vial; then 3 ml mobile phase was added. A batch of samples was extracted in an ultrasonic washer for 10 min and centrifuged for another 10 min at 8000 rpm. Supernatants were 1000-fold diluted then ready

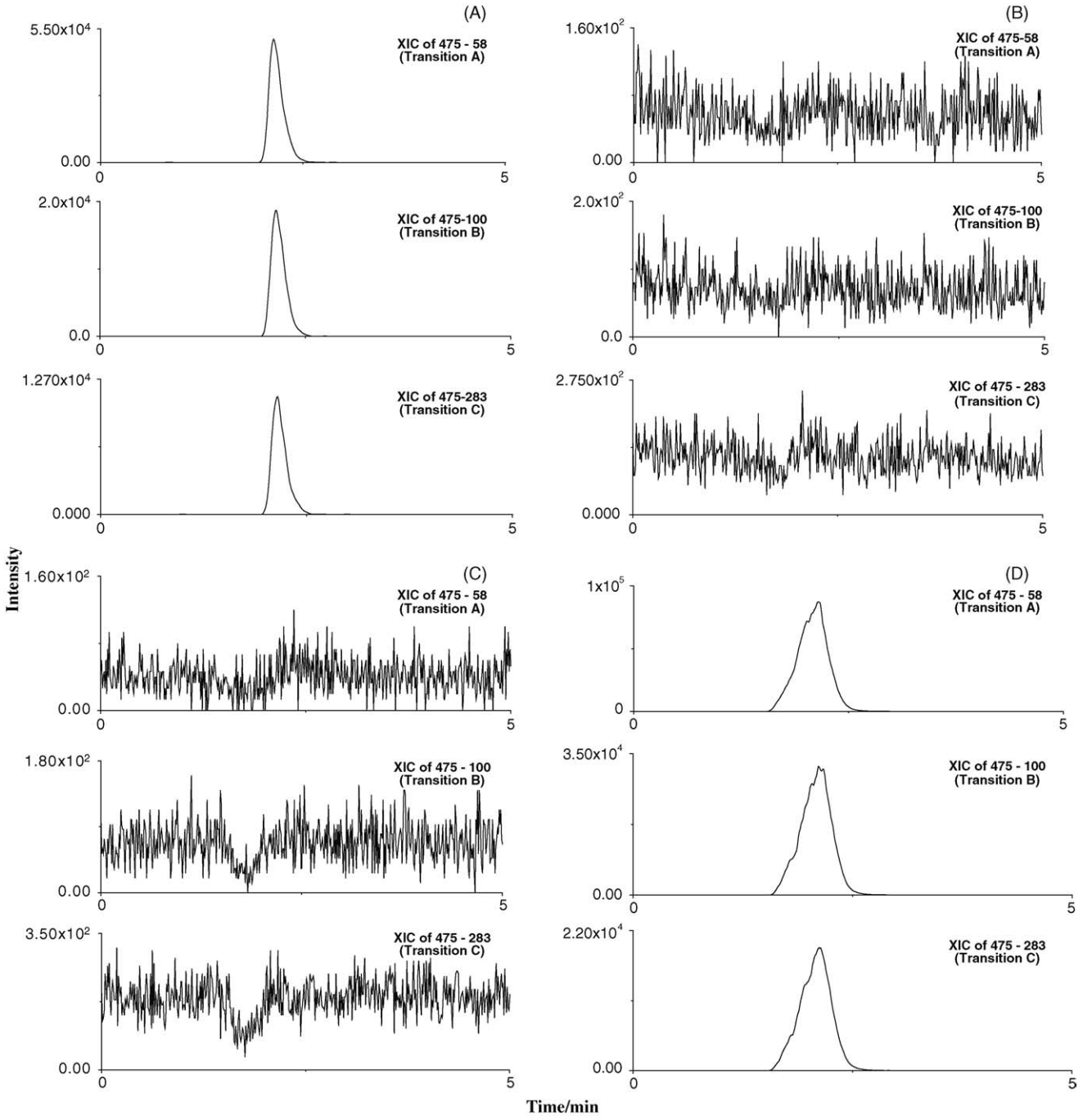

Fig. 1. Representive extracting ion current (XIC) chromatograms for the determination of sildenafil (Viagra) in herbal medicines and dietary supplements (A) XIC of 5 μg/ml standard solution, (B) XIC of blank, (C) XIC of a capsule made by a Hong Kong company (negative result) and (D) XIC of an oral solution made by a Japanese company (positive result).

for injection. For the tablets, they were smashed into small pieces and transferred to a 5 ml capped vial. Three milliliter of mobile phase was added and the samples were disintegrated and extracted in an ultrasonic washer for 15 min. After 10 min of centrifugation at 8000 rpm, supernatants were 1000-fold diluted. As to the oral solutions, 100 μl of the solution was mixed with 1 ml mobile phase in a 2 ml vial, and the following procedures were the same as those for the capsules, except that the supernatants were 100-fold diluted.

3. LC/MS/MS

A PE SCIEX API 3000 triple–quadrupole tandem mass spectrometer equipped with a Turbo Ion Spray Interface, an online degasser and a Perkin-Elmer binary pump (model 250) was used for LC/MS/MS analysis. For the investigation of the product spectra of analytes, standard solutions were directly infused to the interface by a syringe pump at a rate of 0.3 ml/h. For the LC/MS/MS analysis, the analytical column was a

Megachem C_{18} column (50 mm × 2.1 mm i.d., 5-μm particle size) and the injection volume was 20 μl. Corresponding mobile phase (see Table 2 for details) were used with a flow rate of 0.25 ml/min. MRM of MS/MS in positive ion mode was used for the specific detection of the analytes and three selected transitions were monitored simultaneously for each compound. The dwell time for each transition was 75 ms and the pause time for the changes of scan parameter was 8 ms. The pressure of collision gas (N_2) was 4 p.s.i. and corresponding collision energies were applied, respectively, for each transition. The flow rates of nebulizer gas (air), curtain gas (N_2) and drying gas (N_2) were, respectively, 10, 12 and 1.2 l/min. The ion spray voltage, orifice potential and ring focus voltage were set at 4800, 40 and 160 V, respectively. The interface temperature was set at 180 °C. Calculations of peak areas and peak area ratios were done by Macquan (PE Sciex) software.

4. Results and discussion

4.1. LC/MS/MS optimization

In order to speed the analysis without compromising the reliability of this method, we adopted mobile phases in which the analytes have small capacity factors, and meanwhile a high selective MS/MS strategy was employed to ensure the specificity of detection. With the mobile phase specified in Table 2, all of the analytes eluted within 5 min. The necessity of the use of a short C_{18} column before interface was justified by the results of some control experiments, which indicated that the use of such a column was capable of improving peak shapes, stabilizing signal intensities and narrowing peak widths of some analytes comparing with flow injection analysis (FIA). To select specific transitions for MS/MS detection, collisionally activated dissociation (CAD) fragments of each compound were investigated by direct infusion of standard solutions to MS/MS (spectra not shown). Three transitions were selected for each compound among its most intensive products, and were nominated as transition A, B and C, in the order of their relative abundance; to ensure the selectivity of those transitions, non-specific fragments such as the product of $[M + H–H_2O]^+$ and $[M + H–CO]^+$ were not selected, although in some cases they were quite intensive. The transitions selected for MRM of the analytes and their corresponding collision energies were listed in Table 2. In order to reduce the influence of matrix on ionization, a relative high source temperature was adopted [16].

4.2. The process for judgement

In our last study [14], we found that the peak area ratios (PAR) among the three transitions of each compound were quite stable in a wide signal intensity range (10^2–10^6), and independent of matrix when each transition was monitored under their respective optimal collision energy (OCE) [15]. Therefore, a strategy based on PARs among multiple transitions was proposed here to enhance the confidence of identification of analytes in complex matrix. Two PAR values were measured for each compound: the PAR, respectively, of transition B to A and C to A. The deviation% of a measured was defined as following:

$$\text{Deviation\%} \equiv \frac{\text{Measured PAR value} - \text{Stanard PAR value}}{\text{Stanard PAR value}} \times 100\%$$

To obtain the standard PAR values, we spiked the analytes to the blank matrix specified in the test of interference section at three levels (0.02, 0.5 and 40 μg/ml) and assayed aliquots of those spiked samples in triplicate, both inter-assay and intra-assay. The average PAR values for each compound by those experiments were taken as standard values. The threshold of ±10% for the deviation% was subjected to the fact that in those experiments the deviation% of all transitions were within ±5% (data not shown) which was comparable with the last study [15].

We established a 3-step process for the decision of experimental results. (1) Negative results were determined if no well-defined peak was found in the extracting ion currents (XIC). (2) A positive result was determined when not only peaks with the similar retention time appears in all of the

Table 2
The transitions, collision energies, mobile phases and lower limits of detection for the nine adulterants

Adulterant	Transitions (collision energy, eV)[a]	Mobile phase[b]	Lower limits of detection (ng/ml)[c]
Sildenafil (Viagra)	475 → 58(63), 475 → 100(41), 475 → 283(51)	Solution A	1.5
Famotidine	338 → 189(31), 338 → 259(15), 338 → 155(43)	Solution A	0.5
Ibuprofen	207 → 161(13), 207 → 119(29), 207 → 105(31)	Solution A	0.2
Promethazine	285 → 86(25), 285 → 198(27), 285 → 240(19)	Solution A	0.1
Diazepam	285 → 154(35), 285 → 193(41), 285 → 222(37)	Solution A	0.1
Nifedipine	347 → 315(11), 347 → 254(23), 347 → 271(31)	Solution A	0.5
Captopril	218 → 116(17), 218 → 172(15), 218 → 70(35)	Solution A	0.2
Amoxicillin	366 → 208(19), 366 → 134(41), 366 → 114(29)	Solution B	1
Dextromethorphan	271 → 215(31), 271 → 171(47), 271 → 159(39)	Solution A	0.05

[a] The three transitions of each compound were sorted by their intensities, and named, respectively, transitions A, B and C, in that order.
[b] Solution A is consisted of methanol: water: TFA = 78:22:0.1 (v/v), and solution B is consisted of methanol: water: TFA = 15:85:0.1 (v/v).
[c] The lower limit of detection of each compound was determined to a signal-to-noise ratio of 10:1 for the least intensive transition of each analyte by stepwise dilution of standard solution.

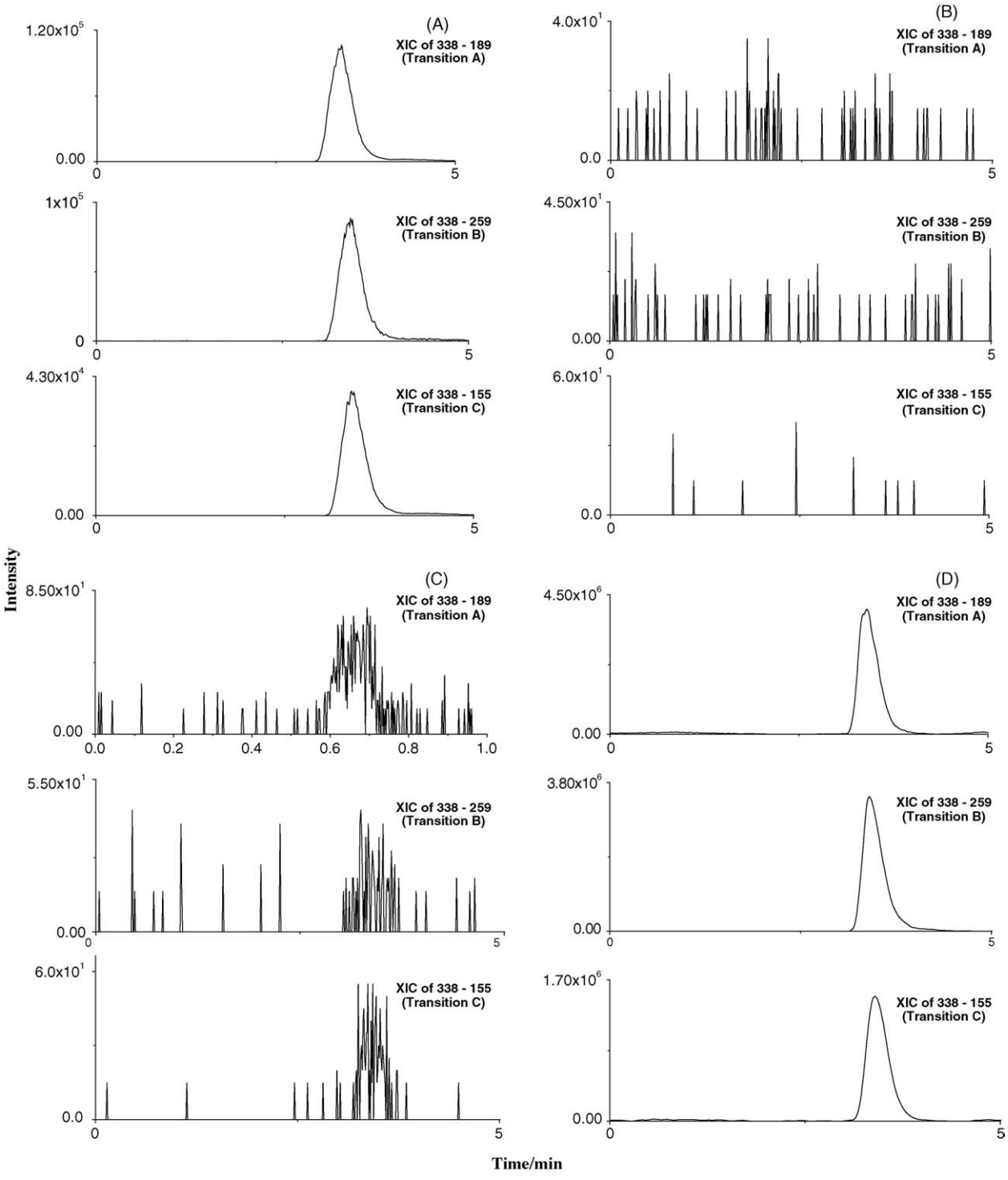

Fig. 2. Representive extracting ion current (XIC) chromatograms for the determination of famotidine in herbal medicines and dietary supplements (A) XIC of 5 μg/ml standard solution, (B) XIC of blank, (C) XIC of a capsule made by a Chinese company in 2002 (negative result) and (D) XIC of the same brand of capsule but produced in 2003 (positive result).

three XIC but also the deviation% of both measured PARs were within ±10% of the standard values. (3) If any peak appeared in some of the three XIC while the chromatograms and PARs data were not qualified for a positive result, a prolonged chromatographic separation before the interface of MS/MS should be used for further analysis.

4.3. Test of interference

In order to investigate interferences by matrices, we had concocted and analyzed a matrix-blank sample: eight brands of herbal medicines and dietary supplements (among which there were 3 capsules, 1 tablet and 4 oral solutions) that were

Table 3
Summary of test results of products for enhancing sexual potency

Dosage form	Number of samples examined	Number of positive results by this method	Number of positive results firstly provided by SDA's lab[a]	Number of positive results finally provided by SDA's lab[b]
Capsule	36	15	17	15
Tablet	17	8	9	8
Oral liquid	16	3	4 (plus 1 uncertain)	3
Medicated wine	9	2	1 (plus 1 uncertain)	2
honeyed pill	3	0	1	0
Sum up	81	28	34	28

[a] Screened using HPLC/UV method.
[b] Corroborated using a prolonged HPLC separation coupling with MS/MS detection.

declared free of the nine adulterants by SDA were extracted according to the procedure prescribed in this study. Aliquots of extracts were mixed and the mixture was experimentally assayed. No well-defined peak was observed with this blank matrix when using this method to detect the nine adulterants (chromatograms not shown).

4.4. Determination of samples

Over 200 samples have been assayed with this method. Among them were 74 positive results confirmed by this method. Most of the samples were determined either positive or negative in the first step of the decision process. There were only two occasions that the further analysis procedure of prolonged separation was needed, and those samples were afterward demonstrated negative. Till now no false positive or false negative result with this method was found, in contrary to 27 false positive and 6 false negative results with established HPLC methods by the labs of SDA. It is astonishing that the signal intensities of some samples were much higher than that of standard solutions, and it is estimated that the content of adulterants in those products greatly outstripped the normal prescribed dosage of those therapeutic compounds.

Some examples for the determination of sildenafil (Viagra) and famotidine, which were the two most common adulterants found in the market of China, were given as the following.

4.4.1. Determination of sildenafil (Viagra)

Presently the adulteration with undeclared sildenafil is critical in worldwide market of herbal medicine and dietary supplements. Those adulterated products were claimed being capable of improving sexual function with their natural ingredients and even were advertised with such words as 'liquid Viagra' or 'Viagra from the nature'. What is ridiculous is some of those products were even claimed being capable of improving both male and female 'sexual function'. Over 80 samples under suspicion of adulteration with sildenafil were investigated. Among them there were 28 positive by this method, versus 34 positive (6 of them were afterward proved false positive) by SDA labs using a classical HPLC method for the analysis of sildenafil, as shown in Table 3. The XIC of standard solution, blank and samples with and without adulteration of sildenafil were shown in Fig. 1.

4.4.2. Determination of famotidine

As an adulterant, famotidine is normally included in some products claimed to be capable of keeping the fitness of stomach and helping the cure of gastrosis. More than 40 samples were analyzed in this study and 18 positive results were found (there were 20 positive results by SDA labs using an established HPLC method). The XIC of standard solution, blank and samples with and without adulteration of famotidine were shown in Fig. 2. It is interesting that some manufacturers included this compound in their recent products, but not in earlier products; for example, the two products of the same brand illustrated in Fig. 2C and D.

The authors believed that this method possesses other applicable potential for the rapid and reliable detection of interested compounds in complex matrices because of its versatile characteristic. It is worthy of noticing that when this work had been just completed two publications reported the determination of vardenafil and tadalafil in addition to sildenafil in dietary supplements by HPLC coupling with ion trap MS [17,18]. It indicated that methods with the potential to detect mulitiple analogs of adulterants in one run of analysis, which can be easily achieved just by setting corresponding MRM transitions according to the presented method. Most importantly, for the enhanced confidernce of identification, it is not required for prolonged separation to get the reliable and rapid screening for target analytes, with a run of 2–5 min for this method while 15–30 min was usually required as reported in the literatures.

Acknowledgement

We gratefully acknowledge the support from the Key Project of Science and Technology Ministry (No. 2002BA906A29-3) and SDA of China.

References

[1] A.S. Bouldin, M.C. Smith, D.D. Graner, S.L. Szeinbach, D.A. Frate, E.M. Croom, Soc. Sci. Med. 49 (1999) 279–289.
[2] R. Yuan, Y. Lin, Pharm. Ther. 86 (2000) 191–198.
[3] D.J. Brown, S. Foster, Am. Pharmacist 119 (1997) 31–46.
[4] T.Y. Chan, J.C. Chan, B. Tomlinson, J.A. Critchley, Lancet 342 (1993) 1532–1534.

[5] T.Y. Chan, J.A. Critchley, Hum. Exp. Toxicol. 15 (1996) 5–12.
[6] B. Che, Internal communication from SDA.
[7] A.C. Mehta, Analyst 122 (1997) R83–R88.
[8] L. Tang, W.L. Fitch, M.S. Alexander, J.W. Dolan, Anal. Chem. 72 (2000) 5211–5218.
[9] O. Shakoor, R.B. Taylor, R.R. Moody, Analyst 120 (1995) 2191–2194.
[10] J.A. Ocana, F.J. Barragan, M. Callejon, Analyst 125 (2000) 2322–2325.
[11] M.S. Lee, E.H. Kerns, Mass Spec. Rev. 18 (1999) 187–279.
[12] S.Y. Liu, S.O. Woo, H.L. Koh, J. Pharm. Biomed. Anal. 24 (2001) 983–992.
[13] J.C. Reepmeyer, L.K. Revelle, I. Vidavsky, J. Chromatogr. A 828 (1998) 239–246.
[14] P. Sun, G.A. Luo, J. Qu, Chem. J. Chinese. Univ. 24 (2003) 2169–2172.
[15] J. Qu, Q.L. Liang, G. Luo, Y.M. Wang, Anal. Chem. 76 (2004) 2239–2247.
[16] J. Qu, Y.M. Wang, G.A. Luo, J. Chromatogr. A 919 (2001) 437–441.
[17] S.R. Gratz, C.L. Flurer, K.A. Wolnik, J. Pharm. Biomed. Anal. 36 (2004) 525–533.
[18] X.L. Zhu, S. Xiao, B. Chen, F. Zhang, S.Z. Yao, Z.T. Wan, D.J. Yang, H.W. Han, J. Chromatogr. A 1066 (2005) 89–95.