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Screening and Identification of Multi Compounds in Yitongshu Injection using Combination of Liquid Chromatography/Time-of-Flight Tandem Mass Spectrometry

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An approach for screening and identification of multi components in complex traditional Chinese medicine systems using combination of liquid chromatography/time-of-flight tandem mass spectrometry technique was described in this paper. The chemical profile of Yitongshu injection, a well-known traditional Chinese formula in China, was studied using the established method as for an application. As a result, 22 components in Yitongshu injection were identified in this complex traditional Chinese medicine. The method was validated by imperatorin, ligustilide and osthol, three representative compounds in Yitongshu injection. The determination of three compounds was performed by an HPLC-UV method. The results indicated that the developed HPLC method is simple, sensitive and reliable for the determination of three representative compounds with a good linearity($r^2 > 0.9995$), precision(relative standard deviation (RSD) < 1.9) and the recovery range of 93.8-97.7 %. This study is expected to provide a rapid, sensitive, economical and systematical method for the identification and further quality evaluation of complex traditional Chinese medicines.

Key Words: Yitongshu injection, Identification, Mass spectrometry, Liquid chromatography.

INTRODUCTION

In recent years, the traditional Chinese medicine (TCM) has been given increasing popularity worldwide for their complementary therapeutic effects to the western drugs but with minimum side effects[1,2]. However, the application of TCM is often based on long-term empirical and traditionally clinical uses. This has highlighted the necessary need for up-to-date scientific information on TCM to assure their quality, safety and efficacy. The effects of TCM are, of course, brought about by their chemical constituents; thus, the chemical analysis of TCM is especially important because it helps to understand which chemical components exist inside and which ingredients are the real bioactive ones for certain therapeutic effects and then to establish scientific and rational quality control methods. Most of the TCM are mixtures of up to 2 or 15 herbal plants or extracts and each herb comprises hundreds of different constituents. Therefore, systematical and comprehensive analysis of TCM is a difficult, in some respect even more challenging task.

The classical chemical research method for constituents of herbal prescription is usually time intensive and expensive. In addition, the effectiveness of a prescription can not be evaluated by only a few compounds. High-performance liquid chromatography-electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) had been shown to be a useful analytical tool for the identification of the known compounds in TCM prescriptions[3-5]. However, because of the complexity of the TCMs and the lack of all those appropriate standards in one laboratory, uncertainties may still exist in the identification and elucidation of multi compounds. To solve this problem, a more powerful methodology which could offer higher quality structural information is therefore required for the characterization of the complex TCM systems. Liquid chromatography/time-of-flight mass spectrometry (LC/TOF-MS) has become a suitable technique for the precise and sensitive analysis[6,7]. Benefit from the increased resolving power, accurate mass measurement and high full-scan capability, LC/TOF-MS can provide the elemental compositions of the peaks which have no stand to identify with low limited accuracy (routinely within 5 ppm) in complex matrices. Currently, this strategy has been successfully developed and applied in the analysis of environmental contaminants including pharmaceuticals and pesticide degradates[8-11]. However, to the best of our knowledge, only a few researches on the complex TCM systems with this technique have been reported yet[12-14].

Yitongshu injection, a well-known TCM formula in China, has been widely used in clinical practice[15-17]. It is prepared

from seven medicinal materials, including Herba Asari, Radix Angelicae Sinensis, Rhizoma Chuanxiong, Rhizomza Seu Radix Notopterygii, Radix Angelicae Pubescentis, Raidix Saposhnikoviae and Radix Angelicae Dahuricae. Due to the extreme complexity of the multi components, the characterization and quality assessment of Yitongshu injection are rather difficult work. To our best of knowledge, there are few published literatures about major bioactive components of Yitongshu injection up to now. The aim of this work is to screen and identify the main constituents in Yitongshu injection using combination of LC/TOF-MS, then to establish a sensitive and accurate method for simultaneous quantitative bioactive components of them.

EXPERIMENTAL

Ligustilide (A0219) was purchased from Shanghai Winherb Medical Science Co., Ltd. Imperatorin (batch number: 110827-200407) and osthole (batch number: 0822-200204) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (China). HPLC grade acetonitrile was purchased from Caledon Laboratories Ltd. (Georgetown Ont., Canada). Ultrapure water was self-made in our laboratory. Acetic acid was of an HPLC grade. (YuWang, ShanDong, China).

Yitongshu injections were self-made with the help of Pharmaceutical Center of Shenyang Pharmaceutical University.

Sample preparation: The samples of Yitongshu injection for LC/TOF-MS analysis were filtered through a 0.45 μm membrane filter before the injection.

HPLC condition for identification: HPLC/DAD analysis was carried out on an Agilent 1200 series HPLC consisting of G1379B Degasser, G1376A Cap Pump, G1367B Hip-ALS, G1316A TCC and G1365B MDW. Separation was performed on a Diamonsil C_{18} analytical column (250 mm × 4.6 mm i.d., 5 μm, Dalian Zhonghuida, China) at 35 ºC. The mobile phase consisted of two solvents: 0.1 % aqueous formic acid (solvent A) and acetonitrile (solvent B). The whole screening procedure was as the following gradient: 0-15 min, 20-29 % B; 15-20 min, 29-33 %; 20-50 min, 33-40 %; 50-55 min, 40-43 %; 55-85 min, 43-65 %. The flow rate was 1 mL/min and 10 μL of sample solution was injected in each run.

Liquid chromatography/time-of-flight tandem mass spectrometry: The HPLC system was coupled to an Agilent 1200 LC/MSD TOF (Agilent Corp, Waldbronn, Germany) equipped with an electrospray interface. The electrospray source includes dual nebulizers-one nebulizer for the LC eluent and the other for the internal reference solution. The reference standards was sodium formate, introduced into the TOF-MS with a automated calibrant delivery system (CDS), which would be used as the internal standard for acute mass weight calibration. Accurate mass measurements of the components were obtained with this calibrant delivery system and thus achieved with this on-line prompt calibration.

The HPLC conditions for the LC/TOF-MS analysis were the same as the HPLC method, except for that one-second of the eluent was introduced into the TOF-MS system with a split valve. TOF-MS analysis was performed in both positive (ESI+) and negative (ESI-) ion mode under the following operation parameters: capillary voltage 3500 V; drying gas 4 L/min; nebulizer 0.8 psig; gas temp 230 ºC; fragmentor voltage 175V (ESI+) and 190V (ESI-); skimmer voltage 60 V; octopole dc1 37.5V (ESI+) and -38.0 V (ESI-); octopole RF 250V. The full-scan carried out by LC/MSD TOF was recorded across the mass range 50-2000 m/z.

HPLC-UV condition for determination of three representative compounds: Diamonsil C_{18} (250 mm × 4.6 mm i.d. 5 μm) colum, acetonitrile and H_2O (50:50) as mobile phase, flow rate is 1.0 mL min^{-1}, detection length of UV is 300 nm, injection volume is 20 μL.

RESULTS AND DISCUSSION

HPLC separation for identification: In order to screen and separate the multi components within a reasonable time, high-gradient slope and aqueous formic acid in the mobile phase were applied. The wavelength for the detection was selected with the use of a MDW detector. The representative HPLC chromatogram of Yitongshu injection is shown in Fig. 1. The proposed method is therefore acceptable as well as adequate for further MS analysis.

Procedure for the identification of multi components: The screening, identification and further confirmation of multi

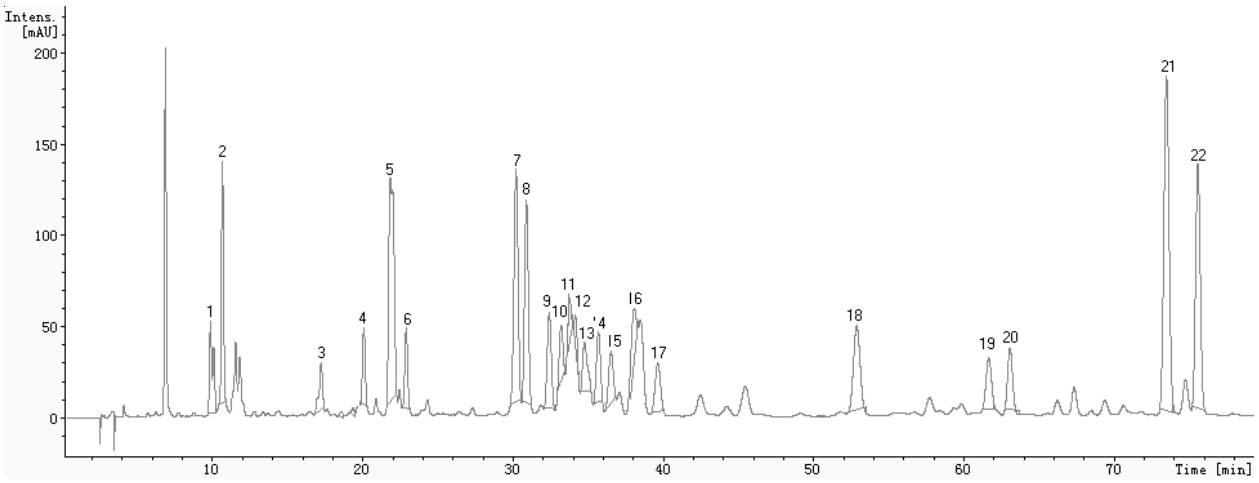

Fig. 1. HPLC-UV chromatogram (320 nm), most of the compounds in Yitongshu injection could appear in the chromatogram in the fixed wavelength of 320 nm. The number of peaks marked in Figs. 1 and 2 is corresponding to Table-1

components in Yitongshu injection were performed by LC/TOF-MS, which could provide the elemental compositions of both the molecular ions and characteristic fragment ions according to the acute mass weight. The elemental composition of every peak was calculated by time-of-flight software . Considering the possible elemental composition of potential components existing in Yitongshu, the number and types of expected atoms was set as follows: carbons ≤ 30, hydrogens ≤ 50, oxygens ≤ 20, nitrogens ≤ 5. The double bond equivalent (rdb) parameter was set from 0-20 and the option of electron state was selected as "even". The accuracy error threshold was fixed at 5 ppm for a strict criterion.

The total ion chromatograms (TIC) of Yitongshu injection obtained by LC/TOF-MS in positive ion mode were presented in Fig. 2. We didn't give the chromatogram of negative ion mode, because the negative ion mode was found to have few sensitive peaks in its chromatogram.

Identification of multi components in the complex system was then carried out by LC/TOFMS according to the following procedure. The accurate mass spectrum of each peak in the HPLC or MS chromatogram and the empirical formulae corresponding to the probable existent compounds in Yitongshu were obtained subsequently. After that, tentative identifications were performed by detailed studies of their MS/MS spectral data and by comparison with the published literatures.

As a result, 22 components in Yitongshu were identified with the relative data in Tables 1 and 2. The results listed in these tables exhibited excellent coherence with the mass spectrometries. The exact identification of each group of components was outlined below.

Exact identification of each group of components: Firstly we set up a database containing about 400 compounds which were mostly available in the Yitongshu by referencing the published literatures[18-23]. The structures of these compounds were obtained by SciFinder Scholar 2007. Because we have obtained the high resolution mass weights and possible molecular formulae of 22 chromatographic peaks in the Table-1, we can narrow the choices of each chromatographic peak by comparing its molecular formula with compounds in the database. If the molecular formula of one peak has only one compound in our database, we can definite that it is our target compound. If not, we must further analyze the relative MS/MS data for more detailed information for the components' elucidation.

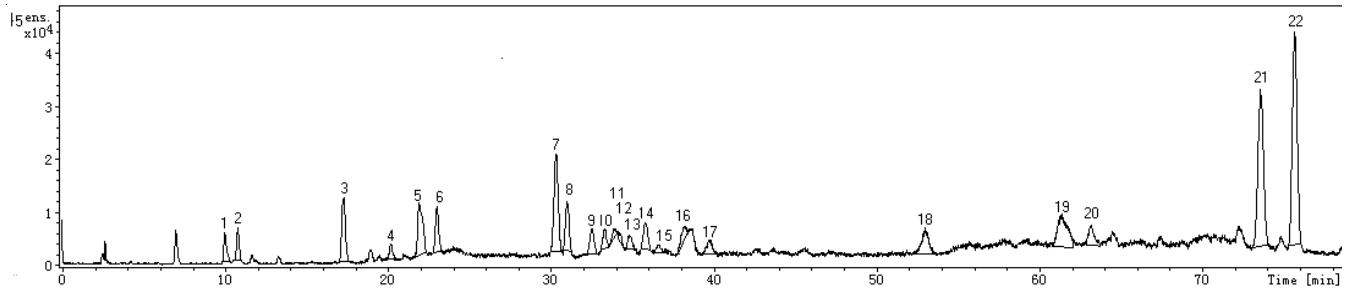

Fig. 2. LC/TOF-MS total ion chromatogram (TIC) of Yitongshu injection in positive ion mode

TABLE-1
COMPOUNDS WERE IDENTIFIED BY LC/TOF-MS

Peak No.	t_R (min)	Selected ion	Formula	Measured mass (m/z)	Calculated mass (m/z)	Error		rdb	Compounds
						mDa	ppm		
1	10.0	$[M + H]^+$	$C_{16}H_{18}O_6$	307.1171	307.1176	0.56	1.8	7.5	Cimifugin
2	10.8	$[M + H]^+$	$C_{15}H_{18}O_6$	295.1170	295.1176	0.58	2.0	6.5	Angelitriol
3	19.4	$[M + H]^+$	$C_{16}H_{18}O_5$	291.1221	291.1227	0.62	2.1	7.5	5-O-Methylvisamminol
4	20.2	$[M + H]^+$	$C_{14}H_{14}O_4$	247.0963	247.0965	0.19	0.8	7.5	Columbianetin
5	22.8	$[M + H]^+$	$C_{16}H_{16}O_6$	305.1015	305.1020	0.45	1.5	8.5	Oxypeucedanin hydrate
6	23.0	$[M + H]^+$	$C_{17}H_{18}O_7$	335.1149	335.1125	-2.35	-7.0	8.5	Byakangelicin
7	30.1	$[M + H]^+$	$C_{20}H_{24}O_7$	377.1580	377.1595	1.47	3.9	8.5	Angelol A
8	31.0	$[M + H]^+$	$C_{20}H_{24}O_7$	377.1593	377.1595	0.18	0.5	8.5	Angelol B
9	32.5	$[M + H]^+$	$C_{20}H_{24}O_7$	377.1608	377.1595	-1.35	-3.6	8.5	Angelol D
10	33.2	$[M + H]^+$	$C_{20}H_{24}O_7$	377.1609	377.1595	-1.47	-3.9	8.5	Angelol G
11	33.8	$[M + H]^+$	$C_{20}H_{26}O_7$	379.1769	379.1751	-1.74	-4.6	7.5	Angelol C
12	34.2	$[M + H]^+$	$C_{20}H_{26}O_7$	379.1773	379.1751	-2.12	-5.6	7.5	Angelol E
13	34.8	$[M + H]^+$	$C_{20}H_{26}O_7$	379.1769	379.1751	-1.75	-4.6	7.5	Angelol F
14	35.8	$[M + H]^+$	$C_{20}H_{24}O_7$	377.1581	377.1595	1.34	3.6	8.5	Angelol K
15	36.6	$[M + H]^+$	$C_{20}H_{24}O_7$	377.1588	377.1595	0.70	1.9	8.5	Angelol H
16	38.7	$[M + H]^+$	$C_{20}H_{26}O_7$	379.1727	379.1751	2.43	6.4	7.5	Angelol L
17	39.7	$[M + H]^+$	$C_{13}H_{10}O_5$	247.0579	247.0601	2.16	8.7	8.5	Pimpinellin
18	52.9	$[M + H]^+$	$C_{14}H_{12}O_3$	229.0843	229.0859	1.60	7.0	8.5	Angenomalin
19	61.3	$[M + H]^+$	$C_{12}H_{16}O_2$	193.1220	193.1223	0.33	1.7	4.5	Senkyunolide
20	63.1	$[M + H]^+$	$C_{16}H_{14}O_4$	271.0979	271.0965	-1.45	-5.3	9.5	Imperatorin
21	73.5	$[M + H]^+$	$C_{12}H_{14}O_2$	191.1055	191.1067	1.13	5.9	5.5	Ligustilide
22	75.6	$[M + H]^+$	$C_{15}H_{16}O_3$	245.1163	245.1172	0.96	3.9	3.9	Osthol

TABLE-2
MS AND MS/MS SPECTRA OF YITONGSHU INJECTION

Peak No.	t_R (min)	Compounds	M_w	MS (pos/neg)	
				MS (m/z)	MS/MS (m/z)
1	10.0	Cimifugin	306	307 $[M+H]^+$329, $[M+Na]^+$, 635$[2M+Na]^+$	259, 233, 221
2	10.8	Angelitriol	294	295 $[M+H]^+$, 317 $[M+Na]^+$, 611 $[2M+Na]^+$	191, 147, 131
3	19.4	5-O-Methylvisamminol	290	291 $[M+H]^+$, 313 $[M+Na]^+$	243, 217, 205, 189, 176
4	20.2	Columbianetin	246	247 $[M+H]^+$, 269 $[M+Na]^+$	175, 158, 147
5	22.8	Oxypeucedanin hydrate	304	305 $[M+H]^+$, 327 $[M+Na]^+$, 631 $[2M+Na]^+$	247, 147, 119, 91
8	23.0	Byakangelicin	334	335 $[M+H]^+$,357 $[M+Na]^+$	317, 299, 233, 205, 183
7	30.1	Angelol A	376	377 $[M+H]^+$, 399 $[M+Na]^+$, 775 $[2M+Na]^+$	205, 191, 175, 160, 147, 131, 121, 104
8	31.0	Angelol B	376	377 $[M+H]^+$, 399 $[M+Na]^+$, 775 $[2M+Na]^+$	219, 205, 191, 161
9	32.5	Angelol D	376	377 $[M+H]^+$, 399 $[M+Na]^+$, 775 $[2M+Na]^+$	259, 227, 203, 191, 175, 160
10	33.2	Angelol G	376	377 $[M+H]^+$, 399 $[M+Na]^+$, 775 $[2M+Na]^+$	205, 191, 160, 131
11	33.8	Angelol C	378	379 $[M+H]^+$, 401 $[M+Na]^+$	219, 205, 191, 175, 160, 147, 131
12	34.2	Angelol E	378	379 $[M+H]^+$, 401 $[M+Na]^+$	219, 205, 191, 176, 160, 147
13	34.8	Angelol F	378	379 $[M+H]^+$, 401 $[M+Na]^+$, 779 $[2M+Na]^+$	206, 205, 191, 160, 147, 107
14	35.8	Angelol K	376	377 $[M+H]^+$, 399 $[M+Na]^+$, 775 $[2M+Na]^+$	259, 205, 203, 191, 160
15	36.6	Angelol H	376	377 $[M+H]^+$, 399 $[M+Na]^+$	259, 227, 203, 191, 175, 160, 131
16	38.7	Angelol L	378	379 $[M+H]^+$, 401 $[M+Na]^+$	219, 191, 175, 160
17	39.7	Pimpinellin	246	247 $[M+H]^+$, 269 $[M+Na]^+$	217, 189, 171, 161, 143, 133
18	52.9	Angenomalin	228	229 $[M+H]^+$, 251 $[M+Na]^+$	213, 131
19	61.3	Senkyunolide	192	193 $[M+H]^+$, 215 $[M+Na]^+$	147, 137, 115
20	63.1	Imperatorin	270	271 $[M+H]^+$, 293 $[M+Na]^+$	185, 153, 145
21	73.5	Ligustilide	190	191 $[M+H]^+$, 213 $[M+Na]^+$	161, 133, 107
22	75.6	Osthol	244	245 $[M+H]^+$, 267 $[M+Na]^+$	189, 131, 103, 77

Characterization of coumarins: The major constituents of this herbal prescription could be classified into three types: coumarins, chromones and volatile oils. Fig. 3 showed the MS and MS/MS spectra and the proposed fragmentation pattern of peak 22, a simple coumarin (osthole). The MS spectra in the positive mode exhibited an abundant parent ion $[M + H]^+$ at m/z 245 and ion $[M + Na]^+$ at m/z 267, while a fragment ion at m/z 189 was also observed. The MS/MS spectra of 245

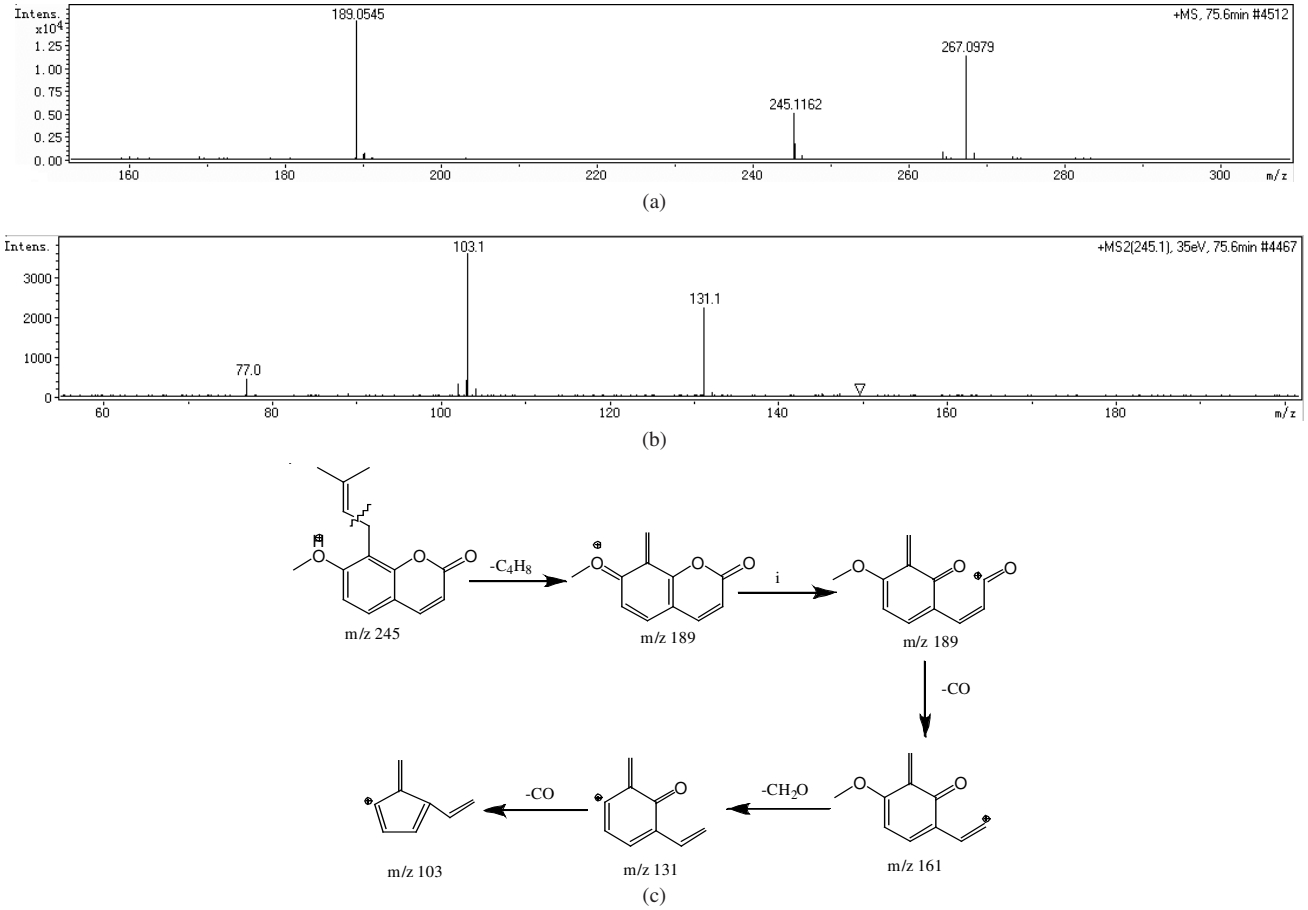

Fig. 3. MS (a) and MS/MS (b) spectra and the proposed fragmentation pattern (c) of osthole

m/z exhibited an abundant fragment ion at m/z 131and 103. The fragment ion at m/z 189 could be attributed to the loss of C_4H_8 from the parent ion. This was further fragmented to yield an ion with m/z 161, signaling the loss of CO. Subsequent loss of 30 mass units equating to the loss of CH_2O occurred with a peak observed at m/z 131. Finally the loss of 28 mass units equating to the loss of CO occurred with a peak observed at m/z 103.

Validation of this method: There are three standards in our laboratory. They are imperatorin, ligustilide and osthol. According to contrast of retention time and the fragment ions of standards, it is perfectly confirmed the deduction of the three compounds. So it is shows that this method is accurate and reliable.

Determination of three representative compounds

Optimization and validation of HPLC method: The chromatographic conditions were developed and optimizing both standards and Yitongshu injection samples. Reversed phase HPLC was used according the previous literatures. Varying the ratios of water and acetonitrile, all the peaks are symmetrical and well resolved from each other when the ratio of water and acetonitrile is 50:50. Fig. 4 compares the chromatograms of a mixed standard solution *versus* those of Yitongshu injection sample under the same HPLC conditions.

Linearity and detection limit: Results from the calibration study and the limits of detection for the imperatorin, ligustilide and osthole are summarized in Table-3. The linearity of the calibration curves have been verified by correlation study and the correlation coefficients are all better than 0.9995. The detection limits are in the range of 0.009-0.013 μg mL^{-1} for the three compounds.

Precision, reproducibility and stability: The precisions of both peak area and retention time measurements were found to be better than 1.9 % (RSD, n = 6) for all the target compounds. The reproducibility (RSD) of the proposed method, on the basis of peak areas for six replicate injections, was 1.83-1.93 %. The variation in the retention times of all the peaks was less than 0.52 % for six replicate injections. The stability (RSD) of the measurements for the three compounds is 1.92-2.03 % (n = 6) for the peak areas and 0.49-0.86 % (n = 6) for the retention times.

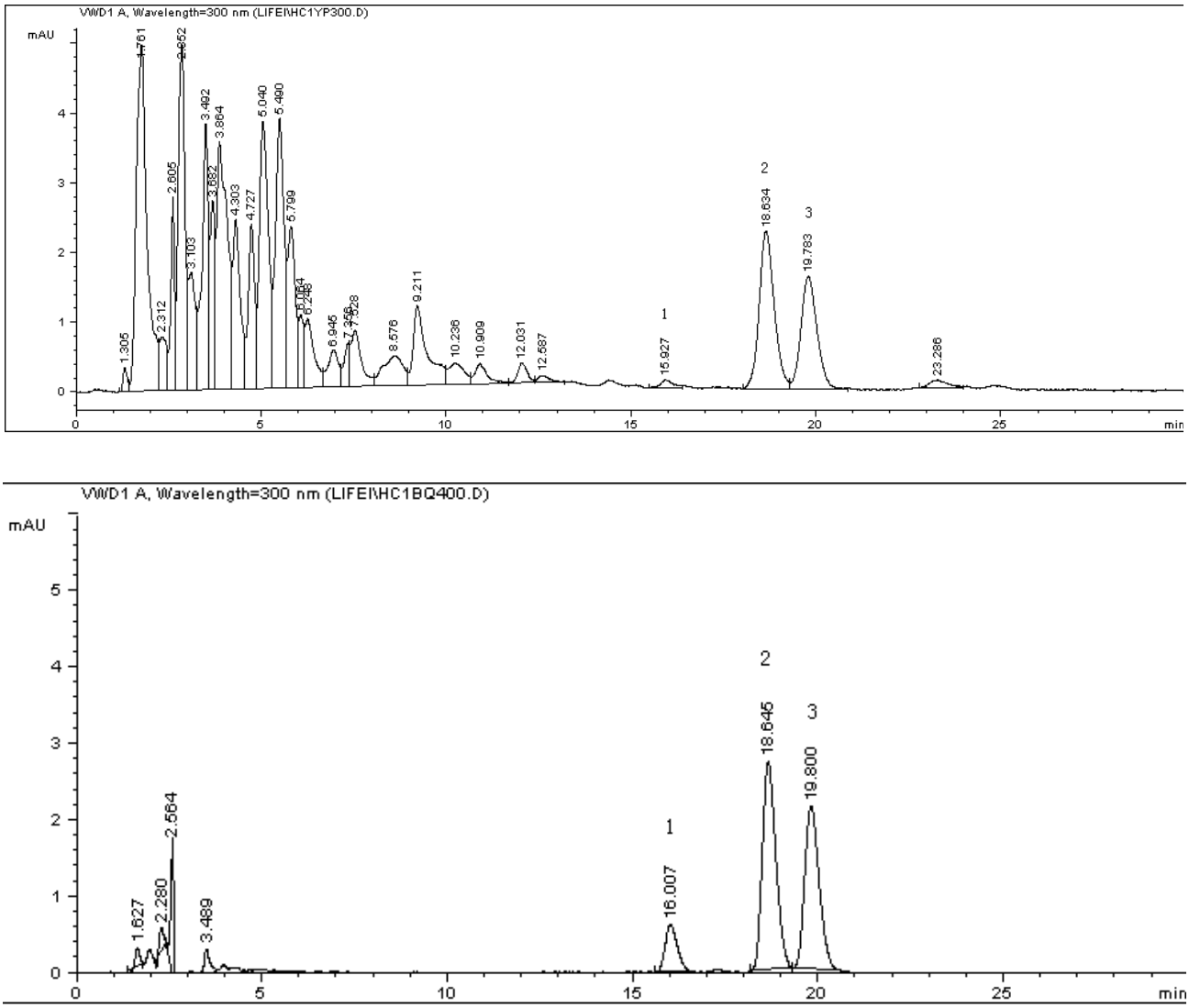

Fig. 4. HPLC of a Yiotngshu injection sample (A) in comparison with that of its three effective constituents standard (B). (1) Imperatorin (2) Ligustilide (3) osthole

TABLE-3
CALIBRATION CURVE, LINEAR RANGE AND DETECTION LIMITS OF IMPERATORIN, LIGUSTILIDE AND OSTHOLE

Compounds	Calibration curve	Linear range (μg)	Correlation coefficient	Detection limits (μg mL^{-1})
Imperatorin	y = 112.62x + 1.4115	0.0158-0.505	0.9995	0.010
Ligustilide	y = 16.839x + 2.3005	0.519-33.2	0.9999	0.013
Osthole	y = 22.802x + 2.435	0.328-20.99	0.9998	0.009

TABLE-4
RECOVERY OF THREE TARGET COMPOUNDS DETERMINED BY STANDARD ADDITION METHOD (n = 3)

Compounds	Original amount (mg)	Added amount (mg)	Detected amount (mg)	Recovery (%)	RSD (%)
Imperatorin	0.026	0.032	0.030	93.8	2.31
Ligustilide	3.462	4.15	4.03	97.1	3.21
Osthole	1.836	2.62	2.56	97.7	3.55

Recovery: Recoveries for the three target compounds were determined by standard addition method in which three repeated analyses of the spiked samples were run within the same day. The results are summarized in Table-4. The recoveries are within the range of 93.8-97.7 % and the RSD values of all the three target compounds from three replicate injections are better than 4.0 %, demonstrating the good recovery and precision of the method.

Conclusion

In this work, a reliable and powerful analytical method by using liquid chromatography/time-of-flight mass spectrometry for rapid screening and identification of multi components in a traditional Chinese medicine (TCM) formula, Yitongshu injection, was established. As a result, 22 components in the complex system were identified. According to the literature, most of the identified compounds in Yitongshu possess pharmacological activities related to the clinical application of this formula. So the identification of the 22 components equals to identify the main pharmacodynamic substances in this formula. Meanwhile, the application of the method to the commercial products of Yitongshu also provided the chemical support for the chromatographic fingerprint technology and facilitates to improve the quality control standard of this TCM formula. On the whole, the LC/TOF-MS has a powerful capability for screening and identification of multi components. This method can identify the components in the complex systems without long-time-consuming isolation and purification period, just relying on the abundant MS and MS/MS data acquired using LC/TPF-MS, the comprehensive investigation of the previous literatures and a much smaller amount of relative standards, which totally reduced those extremely tedious work. It would provide a rapid, sensitive, economical and systematical method for the improvement of quality control of traditional Chinese medicine in the future.

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