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Biological fingerprinting analysis of the traditional Chinese prescription Longdan Xiegan Decoction by on/off-line comprehensive two-dimensional biochromatography

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Abstract

A comprehensive two-dimensional biochromatography method using a silica-bonded human serum albumin (HSA) column and a RP-HPLC column was developed for the biological fingerprinting analysis of bioactive components in a traditional Chinese medicine (TCM) prescription, Longdan Xiegan Decoction (LXD). The biochromatography with HSA-immobilized stationary phase was applied to study the interaction of multiple components in LXD with HSA in the first dimension, and fractions of HSA column were further separated by a silica monolithic ODS column (on-line)/an ODS column (off-line) coupled with a diode array detector and an atmospheric pressure chemical ionization mass spectrometer (APCI-MS). More than 100 compounds in LXD that interacted with the immobilized HSA were separated and analyzed. Among them 19 compounds were identified based on their retention values, UV spectra, molecular weights and mass spectra. The results show that the developed comprehensive two-dimensional biochromatography system reported here is capable of being used for biological fingerprinting analysis of natural products in complex matrices such as extracts of TCMs and their prescriptions.

Keywords: Comprehensive two-dimensional biochromatography; Longdan Xiegan Decoction; HSA column; Biological fingerprinting analysis; Traditional Chinese medicine

1. Introduction

Multidimensional liquid chromatography systems have been regarded as a powerful separation technique for its large peak capacity, effective separation and high resolution. Different types of multidimensional chromatography systems have been developed and widely used in the separation and characterization of various organic mixtures including amines [1], proteins [2], alcohol ethoxylates [3], peptides [4], polymers [5], and atmospheric aerosols [6].

Because of the complexity of traditional Chinese medicines (TCMs), the screening and analysis of multiple compounds with polyvalent effects in TCMs is a formidable challenge. In recent years, with the development of enzyme/receptor technology in drug discovery, more rapid and efficient methods have been established to obtain information on drug binding properties to proteins. However, it is rather feasible to apply such technology in the area of TCMs since most of the methods are designed for single drug to single target binding study. Biological fin-

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gerprinting analysis (BFA) method has been proposed to probe interaction of multiple components in TCMs with a tethered target and defined as an approach for comparing the fingerprints of an array of chemicals before and after their interaction with a biological system. Recently we have developed biochromatography methods to probe the interaction of the extracts of TCM with immobilized HSA, α_1-acid glycoprotein (α_1-AGP) and liposome [7–9]. And microdialysis-HPLC was also used for probing the interaction of extracts of TCMs with DNA, α_1-AGP and HSA in homogeneous phase [10–13]. BFA, especially using biochromatography, offers a convenient method for the study of interaction between multi-components in the extracts of TCMs and tethered targets. However, the low column efficiency and peak capacity of biochromatography limit its screening application. Therefore, the two-dimensional (2D) biochromatography may be useful for the identification and characterization of bioactive components in TCMs [7].

Longdan Xiegan Decoction (LXD) is one of the most popular traditional Chinese medicine prescriptions for treatment of jaundice, cystitis, conjunctival congestion earache, scrotum and extremitas inferior eczema as well in Chinese traditional medication [14]. The prescription consists of 10 medicinal materials including Gentianae Radix, Scutellarae Radix, Gardeniae Fructus, Rehmanniae Radix, Alismatis Rhizoma, Plantaginis Semen, Angelicae Sinensis Radix, Clematidis Armandii Caulis, Glycyrrizae Radix et Rhizoma and Bupleuri Radix. Although LXD preparation as a whole has not been elucidated for its bioactive constituents, some of the compounds contained in the ingredient herbs have been tested and have shown pharmacological properties. For instance, geniposide isolated from the fruits of *Gardenia* showed anti-angiodenic and anti-inflammatory activities [15,16], gentiopicroside isolated from *Gentiana* root indicated antibacterial and free radical scavenging activities [17], and the flavonoids baicalin and baicalein from *Scutellaria* root showed multi functional efficacies, including anti-bacterial, antivirus, anti-inflammation and liver protective activities [18,19].

The conventional procedure for discovery of bioactive components from a Chinese herb is laborious and time-consuming, especially from a complex prescription such as LXD. In this work, we extended a comprehensive 2D biochromatography system coupled with atmospheric pressure chemical ionization (APCI) mass spectrometry for biological fingerprinting analysis of LXD. In this 2D biochromatography system, the biochromatography with HSA immobilized stationary phase was used as the first dimension to probe the interaction of various components in LXD extract with HSA. An ODS column, the second-dimensional column, was chosen as a tool with relatively powerful separation and resolution for the analysis of fractions from the HSA column. The on-line 2D separation technique is used for its fast analysis and the off-line 2D separation technique is used for its better separation efficiency.

2. Experimental

2.1. Chemicals and reagents

Methanol and acetonitrile were of chromatographic grade, distilled water used in all experiments was purified by a MilliQ system (Milford, Bedford, MA, USA), and other chemicals were of analytical grade. LXD was prepared in our laboratory. The reference compounds of 6-epiferrtoside, ferrtoside, genipin-1-gentiobioside, geniposide, gentiopicroside, 6-arabinopyranosyl-8-glucopyranosylchrysin, liquiritin and baicalin were isolated from LXD and were identified by UV, MS and NMR spectral

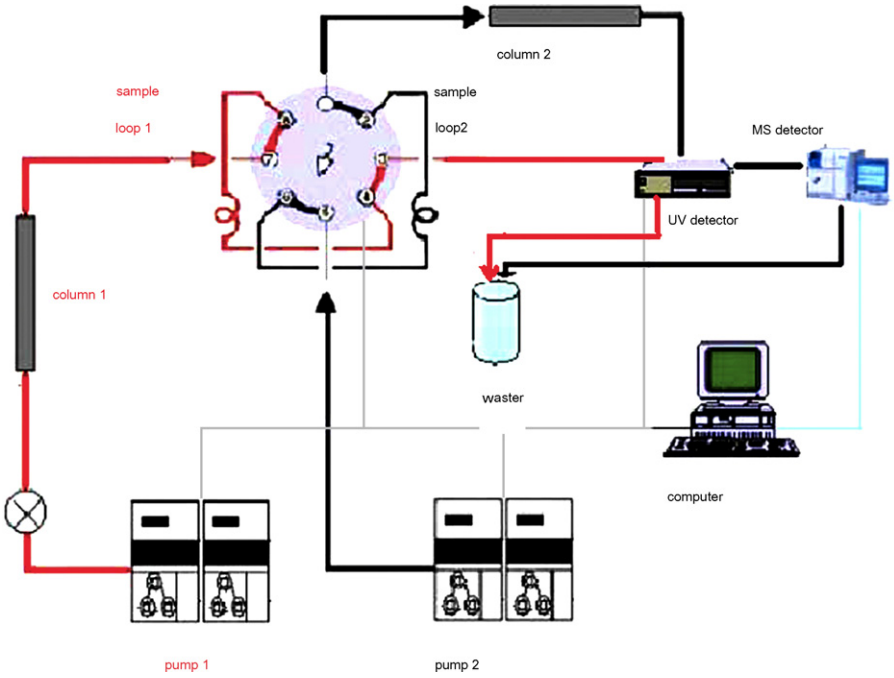

Fig. 1. Schematic representation of a comprehensive two-dimensional liquid chromatography system coupled to mass spectrometry.

data, and their purities were greater than 98% as determined by HPLC based on normalized peak areas.

2.2. Preparation of LXD

The powdered samples of the mixture of Gentianae Radix (2.4 g), Scutellarae Radix (1.2 g), Gardeniae Fructus (1.2 g), Rehmanniae Radix (2.4 g), Alismatis Rhizoma (2.4 g), Plantaginis Semen (1.2 g), Angelicae Sinensis Radix (1.2 g), Clematidis Armandii Caulis (1.2 g), Glycyrrizae Radix et Rhizoma (1.2 g) and Bupleuri Radix (2.4 g) were immersed in 168 mL water for 1 h and then boiled for 1 h. The extract was then filtered through a 0.45 μm membrane and stored at 4 °C in the absence of light for subsequent experiments.

2.3. Apparatus and instruments

A schematic representation of the two-dimensional liquid chromatography system appears in Fig. 1. This is a modification from a previously reported device [20]. The pump 1 delivers mobile phase through the injector into column 1. The outlet of column 1 is attached to an eight-port valve on which two loops are installed. The effluent filled in the loops can be pumped out by another pump to pass through column 2, and then the effluent of column 2 flows through a diode array detector and a mass spectrometer detector. The effluent of column 1 can also be directly collected by a connected fraction collector for off-line separation purpose.

The two-dimension separation was performed on a Shimadu HPLC system (Shimadzu, Kyoto, Japan) consisting of two LC-10ADvp pumps, a SPD-M10Avp diode array detector (DAD), a SCL-10Avp system controller and LCMS solution workstation.

The 2D LC was controlled by a computer running an in-house custom program with visual C++6.0 software (Microsoft Corp., Redmond, WA, USA) and the chromatographic data were collected with a WDL-95 chromatography workstation (National Chromatographic R&A Center, Dalian, China). The obtained chromatographic data with normalization of peak time was also controlled by an in-house written software. 2D chromatogram was graphically represented with the aid of software Fortner Transform (version 3.4, Fortner, Savoy, IL, USA). 3D landscape images were displayed using a homemade program with MATLAB 5.3 software (The Math Works, Sherborn, MA, USA).

2.3.1. On-line 2D separation

Separation in the first dimension was performed on the HSA column that was prepared using a previously described method [21]. The dimensions of the column were 150 mm × 4.6 mm with a particle size of 5 μ. The gradient elution program utilized two solvents: solvent A was 100% acetonitrile; solvent B was 10 mM ammonium acetate. A linear gradient elution starting from 1% A to 7% A in 25 min, linearly increased to 15% A at 35 min and finally maintained at this concentration was adopted. The flow rate was set at 0.1 mL/min, and the injection volume was 5 μL.

Chromonolith Speed ROD column with 50 mm × 4.6 mm, i.d. (Merck, Darmstadt, Germany) was used as the second dimension column. The mobile phase was composed of acetonitrile (A) and water (B) by linear gradient elution starting from 1% A to 60% A in 3.5 min, and then returned to the initial mobile phase of 1% A and held for 1.5 min for re-equilibration at a flow rate of 3.0 mL/min.

The effluent was first monitored at 254 nm by a UV detector, and then split by a microsplitter valve (Upchurch Scientific, Oak Harbor, WA, USA), resulting in a flow rate of 0.8 mL/min into the mass spectrometer. The APCI probe voltage was set at 1800 V, nebulizing gas flow was 2.5 L/min, the APCI, CDL and block temperature was set at 400, 250 and 200 °C, respectively. The mass range (*m*/*z*) was from 50 to 1000 and the scan speed was set at 0.5 s.

In the on-line separation mode, the two loops work alternatively for collecting from column 1 and injecting into column 2; as one loop fills with the effluent from column 1, the fraction of effluent in the second loop is pumped through the column 2. Once the contents of the second loop transfer to the column 2, the effluent in the first loop are just fulfilled and then pumped to column 2 while the second loop refills. Thus, one cycle comprises the filling of a loop with effluent of column 1 and separation on column 2.

2.3.2. Off-line 2D separation

The first dimensional chromatographic condition for the HSA immobilized column was the same as above, but the flow rate was set at 0.5 mL/min. Effluent fractions were collected every 8 min using an automated collector (Huxi, Shanghai, China) monitored by a UV detector. The effluent from the collector was evaporated to dryness and dissolved in 20 μL of methanol and 5 μL was injected by the manual injector into a Kromasl ODS column (150 mm × 4.6 mm, prepared in-house, National Chromatographic R&A Center, Dalian, China) for the secondary chromatography separation using a binary mobile phase of A (acetonitrile) and B (water) The linear gradient elution started from 5% A to 90% A in 65 min, and then returned to the initial mobile phase of 5% A and held for 15 min for re-equilibration at a flow rate of 0.8 mL/min. The effluent was monitored by the PDA set at 254 nm followed by mass spectral analysis.

3. Results and discussion

Human serum albumin (HSA) is one of the most important drug binding proteins in human plasma. This protein also maintains oncotic pressure as well as transporting and binding small molecules. The pharmacological activity of all drugs including those in mixtures prepared from Chinese herbs will depend on the interactions of these compounds with HSA.

Biochromatography of HSA-immobilised affinity chromatography was successfully and rapidly used for screening six principal anthraquinone components from *Rheum.palmatum* L. and two principal bioactive components ferulic acid and liguistilides from *Angelica sinensis* (Oliv.) Diels. [7,22] The chemical components were separated on a HSA column due to the varied interaction between analytes and the immobilized protein, so the retention values of the analytes correlate with the interaction

strength between analytes and HSA. Usually, the biochromatography column is used as the first dimensional column due to the ability of separation on the basis of interactions between the protein and the compounds and the second dimensional column adopts a reversed phase column because of its powerful resolution and facility for MS detection. In the on-line comprehensive two-dimensional liquid chromatography system, one cycle comprises the filling of a loop with effluent of the HSA column (1) and the separation on the reversed phase column (2). So it is most important that the timing for each fraction collected from the first column must match the timing for one run in the second dimensional separation. Once the contents of the first loop completely transfer to the column 2, the effluent from the column 1 just fulfills the second loop and pumps to column 2 again to start another run on the column 2. If the flow rate in the second dimension is not fast enough, it is necessary to reduce the flow rate in the first dimension, which will increase band diffusion on the HSA-immobilised column. A stationary phase with good permeability will increase the second dimension separation speed and decrease the peak band diffuse in the HSA column. This can further improve the 2D LC separation efficiency. Monolithic silica columns are well-suited for this purpose because of its high permeability and excellent mass-transfer properties of the monolithic skeleton. Therefore, the combination of a HSA-immobilised column and a silica monolithic ODS column not only meets the requirement of probing interaction of multiple compounds in the LXD with the immobilized proteins but also enables fast separation.

In order to obtain an optimised 2D biochromatographic separation condition, the separation behaviors of the components in LXD on the HSA column, silica monolithic ODS column and conventional ODS column were conducted separately using the one-dimensional mode, and their HPLC profiles were shown in Fig. 2a–c, respectively. It can be seen in Fig. 2a that the analysis time on the HSA column was about 70 min and the peak width was up to several minutes. Most components in the extract showing weaker interaction with protein were eluted in the first few minutes and the later eluted peaks had wider peak width, one of the reasons is that they had stronger affinity to the immobilized HSA [7]. The column efficiency seems fairly low due to the dynamic and kinetic properties of the interaction. The LXD extract was separated on the silica monolithic ODS column with fast speed and the analysis was completed in 5 min (Fig. 2b). It is obvious that the silica monolithic ODS column displayed more efficient separations than the HSA column. However, the resolution was poor for most of the peaks overlapped within 3 min elution time. Although the conventional ODS column demonstrated even more higher resolution capacity than the monolithic ODS column (Fig. 2c), it needs a longer eluting time for one cycle. In on-line comprehensive 2D liquid chromatography, the analysis time of the second dimension should be short enough to allow the analysis of a large number of cycles and hence the silica monolithic ODS column was chosen for the second dimension separation.

In order to obtain a higher resolution in the first dimension separation in the on-line mode, the flow rate of the first dimension separation was set at 0.1 mL/min, the loop volume was set

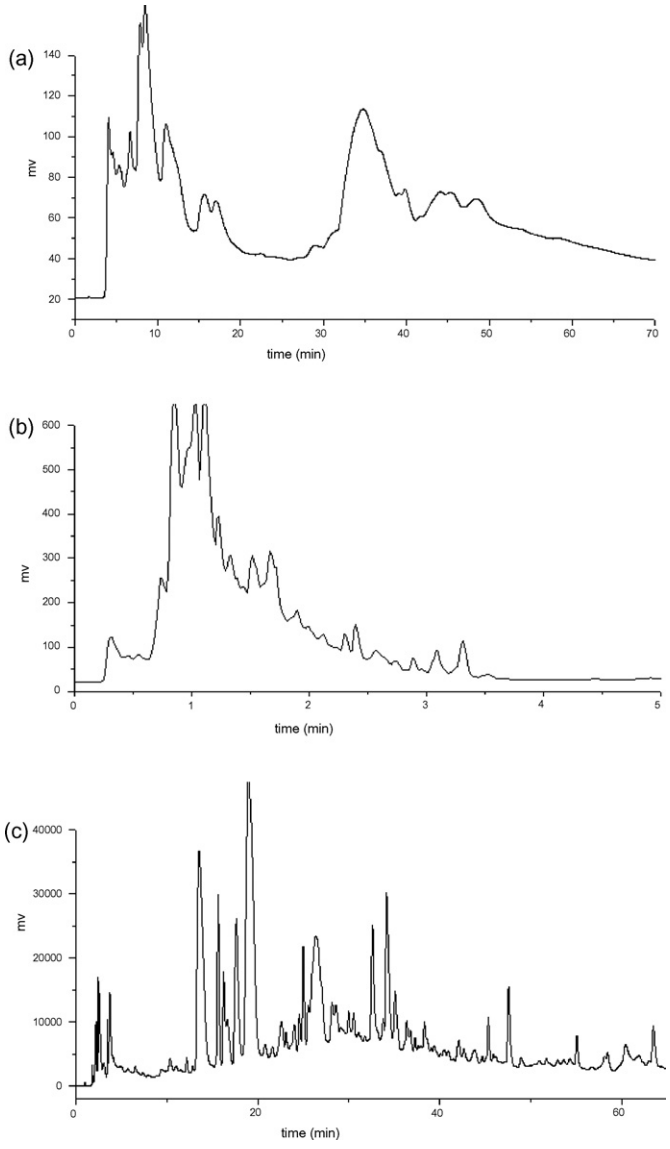

Fig. 2. Separation of Longdan Xiegan Decoction on (a) HSA column, (b) silica monolithic ODS column and (c) conventional ODS column. (a) Mobile phase, 1–15% ACN in 10 mM ammonium acetate buffer (pH 7.0); flow rate, 0.5 mL/min; injection volume, 5 μL; detection wavelength, 254 nm. (b) Liner gradient elution from 1% ACN to 60% ACN in 5 min; flow rate, 3.0 mL/min. (c) Liner gradient elution from 1% ACN to 90% ACN in 65 min; flow rate, 0.8 mL/min; other conditions are the same as (a).

at 0.5 mL. Each cycling was finished in 10 min, 5 min for the first dimensional separation and 5 min for the second dimension, and totally 70 cycles were conducted (see Table 1).

Fig. 3 shows two-dimensional contour plots of the components in LXD. The *x*- and *y*-axes of this contour plot represent the retention times of the primary and the secondary columns, respectively. It can be seen that the spots are thick and spread in the first 5 to 63 cycles, which indicated that many components have interacted with the HSA stationary phase. In Fig. 3a the response of high-abundance components and background were so high before 50 s in the ODS column, that it dwarfed the response of the low-abundance components and they could not be clearly seen in the 2D plot. These low abundance responses

Table 1
Cycling schedule of on-line comprehensive 2D chromatography

Cycle	Each loop filling or evacuating time (min)	Loop filling from column A (HSA), time (min)	Separation on column B (monolithic ODS), time (min)
1	5	0–5	5–10
2	5	5–10	10–15
3	5	10–15	15–20
...	...	...	...
70	5	345–350	350–355

were enhanced by an in-house written program (Fig. 3b). Each spot represents a peak of the two-dimensional separation and the degree of intensity represents the height of a chromatographic peak. In the area of 0–70 min on the *x*-axis and 50–300 s on the *y*-axis, over 50 components were separated by 2D biochromatography. However, peaks in the area of 0–70 min on the *x*-axis and 0–50 s on the *y*-axis are very vague. By comparing the original 3D chromatogram with the normalized 3D chromatogram, it can be seen clearly that more components can be detected directly and distinctly with the second-dimensional separation by the silica monolithic ODS column. From the above analysis, it can be found that more than 60 peaks can be seen in a three-dimensional chromatogram.

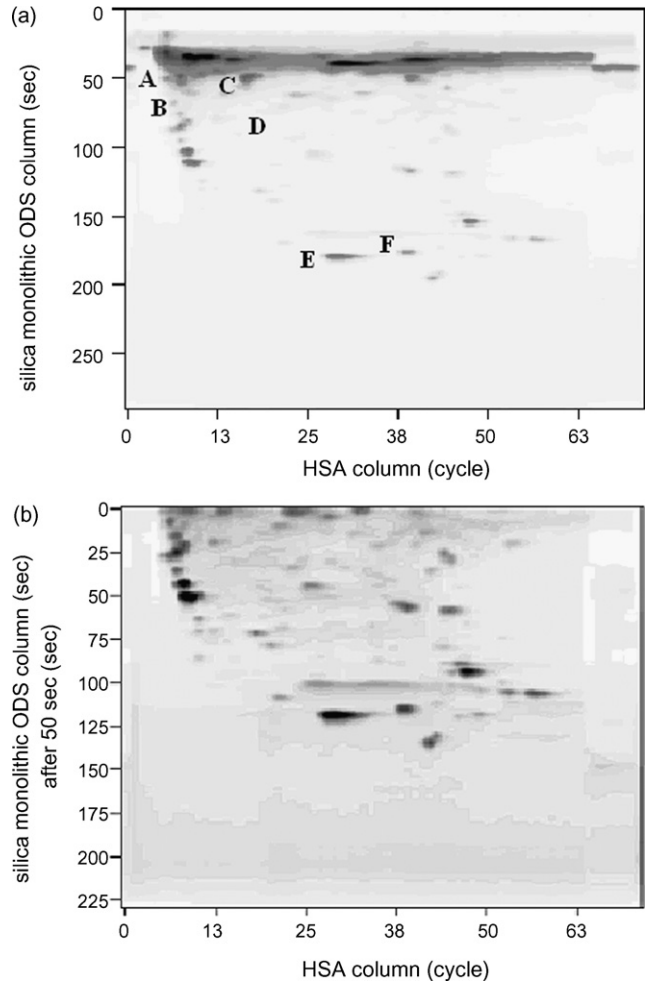

Fig. 3. 2D chromatogram of LXD. (a) Chromatogram of whole cycle and (b) chromatogram after 50 s in each cycle. Chromatographic conditions for the HSA column: gradient elution from 1% ACN to 15% ACN in 10 mM ammonium acetate buffer (pH 7.0); flow rate, 0.1 mL/min. Chromatographic conditions for the silica monolithic ODS column: linear gradient elution from 1% ACN to 60% ACN in 3.5 min, and then returning to the initial mobile phase and holding for 1.5 min for re-equilibration; flow rate, 3.0 mL/min; injection volume, 5 μL; detection wavelength, 254 nm. Cycle time for the second dimension is 5 min. Compounds A–F: liquiritin apioside, licuroside, ononin, wogonoside, liquiritin, baicalin.

The use of on line 2D biochromatography simplifies the sample preparation and handling, and further increases the precision of the method. Six peaks marked as A–F could be identified as shown Fig. 3. However the disadvantage of such method is that peaks of the high-abundance components are overlapping, and are hard to resolve within the limited separation time on the second dimension as shown in Fig. 2b.

In order to investigate these overlapped compounds displayed on the HSA-immobilized column, the off-line 2D separation method was also applied in this study, and the fractions collected from the first dimension were separated in the second dimension under an optimized condition. Under the off-line mode, the fractions from the first dimension HSA-immobilised column were collected with an automatic collector. After concentration, the fractions were re-dissolved and injected separately into the conventional ODS column for further separation. In this work, the peak capacity of the HSA-immobilised column was about 15 in 50 min. The fractions from the HSA column was collected every 8 min from the beginning to 56 min, and totally 7 fractions were collected for separation on the ODS column. Most components were separated in 40 min in ODS column as showed in Fig. 4. It can be seen that over 100 peaks can be detected in total.

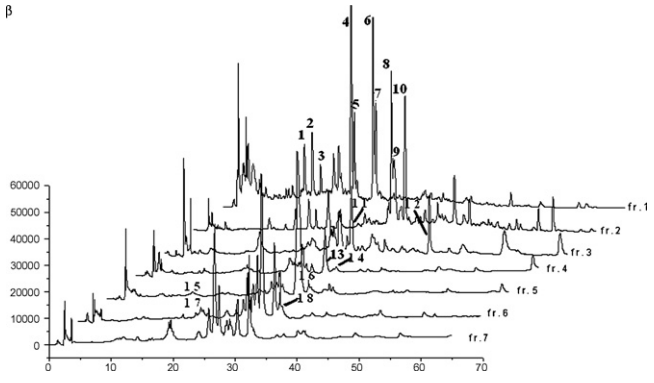

Fig. 4. Chromatograms on ODS column for separation of the collected fractions from HSA column. Chromatographic conditions are the same as in Fig. 1c.

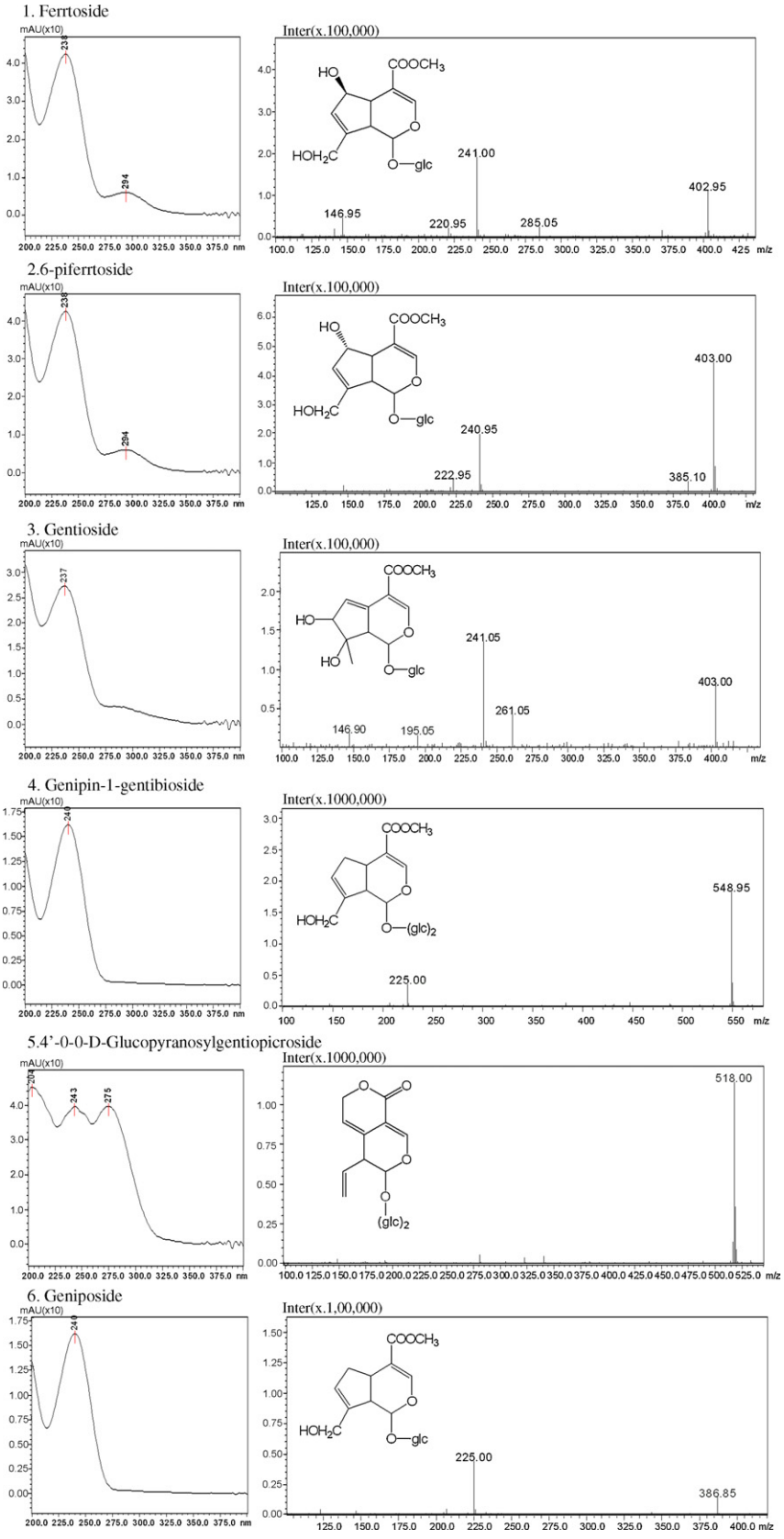

Fig. 5. UV, MS spectra and identified structures of the main components detected in LXD.

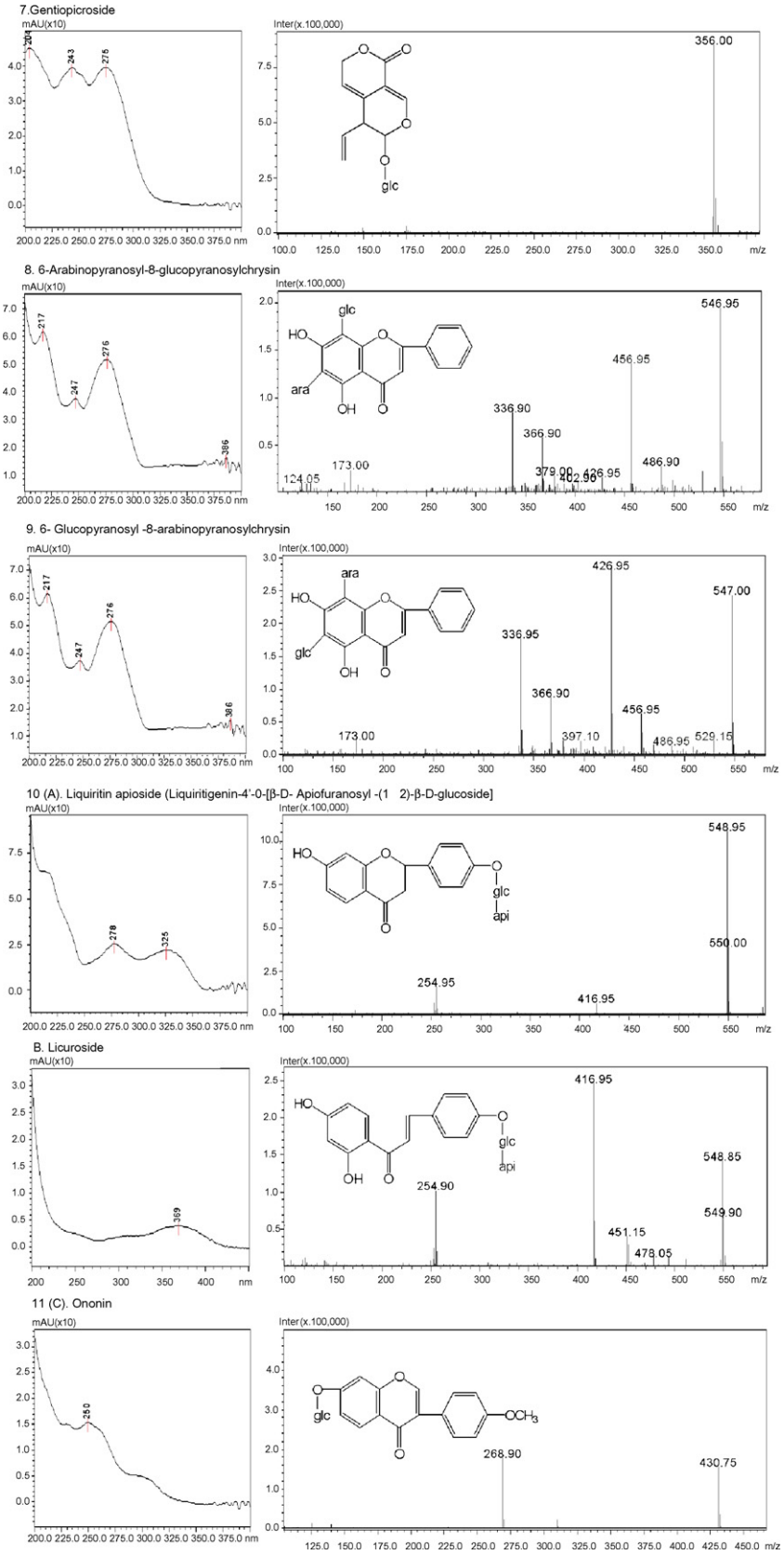

Fig. 5. (*Continued*)

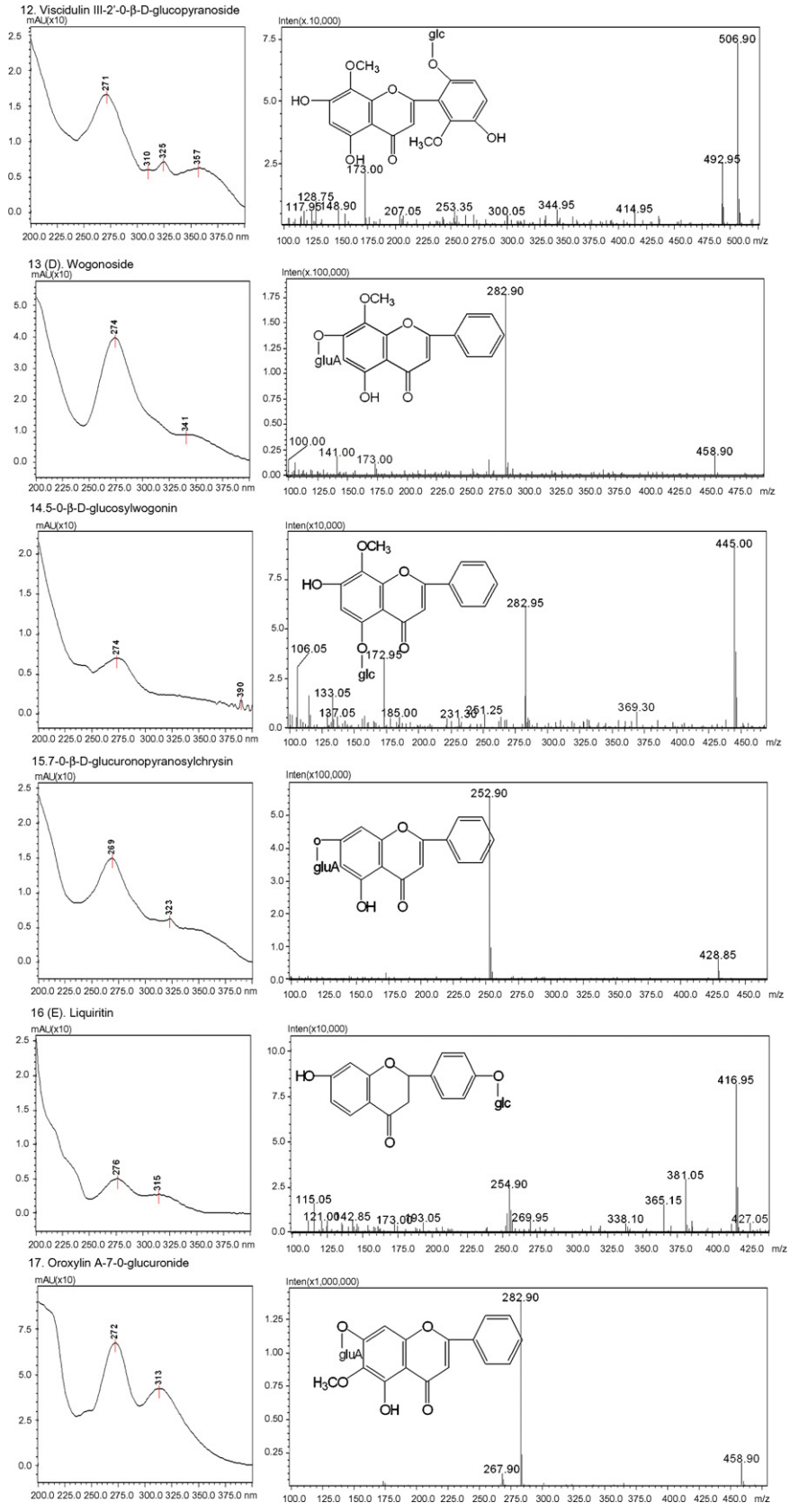

Fig. 5. (*Continued*)

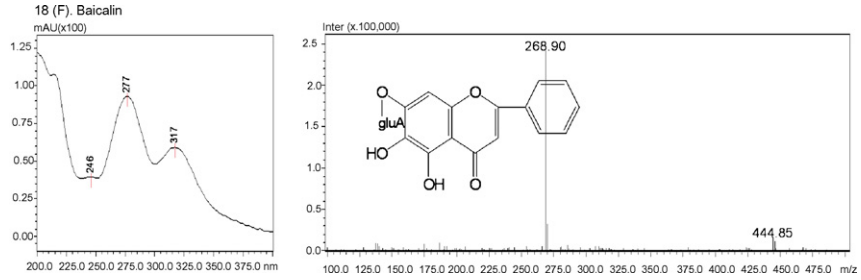

Fig. 5. (*Continued*)

The main constituents in LXD are iridoidal glycosides, triterpenoids and flavonoids. By comparing the UV and MS spectra of the detected peaks in the chromatograms of LXD (Fig. 4) with those of reference substances or reported data in literatures [23–25], 18 peaks were identified as shown in Fig. 4. Among those compounds, **1**, **2**, **4**, **6**, **7**, **8**, **16** and **18** were unambiguously identified by direct comparison with the authentic compounds and the others were putatively authenticated by referencing to the reported data. Fig. 5 shows their structures, UV and MS spectra of the identified components (**1–18**) of LXD.

Peaks 1, 2 and 3 showed similar UV and MS spectra. All the three compounds showed a $[M-H]^-$ ion at *m/z* 403 and gave a fragment ion at *m/z* 241 by losing a glucosyl moiety. By comparing with the retention behaviors in HPLC, the UV absorptions and MS spectra with reference standards and the reported data, peaks 1 and 2 were identified as ferrtoside (**1**) and 6-epiferrtoside (**2**), respectively, and peak 3 was putatively identified as gentioside (**3**). Peak 5 generated a molecular ion at *m/z* 518 $[M-H]^-$, 162 Da more than that of peak 7. Both 5 and 7 gave very similar UV pattern displaying the maximum absorption at 204, 243 and 274 nm, which is the characteristic absorption of secoiridoid. Thus, they were identified as 4′-*O*-β-D-glucopyranosylgentiopicroside (**5**) and gentiopicroside (**7**).

Similarly, peaks 4 and 6 were identified as genipin-1-gentiobioside (**4**) and geniposide (**6**), respectively by comparing their UV maxima and MS spectra, the former showed its molecular ion $[M-H]^-$ at *m/z* 549, 162 Da more than that of the latter which gave its $[M-H]^-$ ion at *m/z* 387.

Peaks 8 and 9 were identified as a pair of flavone isomers with different substitution patterns of glucose and arabinose. In the MS spectra, both of them gave molecular ions at *m/z* 547 $[M-H]^-$, and the fragment ions at *m/z* 487 $[M-H\text{-}60]^-$, 457 $[M-H\text{-}90]^-$, 427 $[M-H\text{-}120]^-$, 367 $[M–H\text{-}180]^-$ and 337 $[M–H\text{-}210]^-$ characteristic fragmentation of C-flavonoid [26]. By comparing with the retention behaviors and the MS fragmentations with those of the available reference compounds, peak 8 was exclusively identified as 6-arabinopyranosyl-8-glucopyranosylchrysin (**8**), and accordingly, peak 9 was elucidated as its isomer, 6-glucopyranosyl-8-arabinopyranosylchrysin (**9**).

Peak 10 gave its UV maxima absorbance at 278, 325 nm indicating a characteristic absorption of flavanone. Compound **10** displayed a molecular ion at *m/z* 549 $[M-H]^-$ and fragment ions at *m/z* 417 $[M-H\text{-}132]^-$, and 255 $[M-H\text{-}132\text{-}162]^-$, derived from the successive losses of a pentosyl (132 Da) and a hexosyl (162 Da) moieties, so, this compound was presumed to be liquiritigenin-4′-*O*-[β-D-apiosyl-(1 → 2)-β-D-glucoside] (liquiritin apioside, **10**).

Peak B also demonstrated a molecular ion $[M-H]^-$ at *m/z* 549, and fragment ions at *m/z* 417 $[M-H\text{-apiosyl}]^-$ and 255 $[M-H\text{-apiosyl-glucosyl}]^-$, the same as those of compound **10**. Nevertheless, peak B displayed a UV maximum at 370 nm which is a typical chalcone absorption. On the basis of the above results, peak B was identified as licuroside.

Both compounds **11** and **14** showed two prominent peaks in their mass spectra, the molecular ion peaks $[M-H]^-$ at *m/z* 430.75 and *m/z* 445.00, and the fragment ion peak at *m/z* 268.90 and *m/z* 282.95, respectively, produced from losing a side chain of a glucosyl $[M-H\text{-}162]^-$. According to the mass and UV spectral data, they were putatively identified as ononin (**11**) and 5-*O*-β-D-glucopyranosylwogonin (**14**), respectively.

The mass spectra of peaks 12 and 16 showed prominent molecular ions $[M-H]^-$ at *m/z* 506.90 and *m/z* 416.95, the weak fragment ions at $[M-H\text{-}162]^-$ at *m/z* 344.95 and *m/z* 254.90, respectively. According to the mass and UV spectral data, they were presumed to be viscidulin III-2′-*O*-β-D-glucopyranoside (**12**) and liquiritin (**16**).

Peaks 13, 15, 17, and 18 were identified as glucuronopyranosyl flavones based on their UV maximum absorptions and MS spectra. All these four compounds showed molecular ions $[M-H]^-$ and fragment ions at $[M-H\text{-}176]^-$ by losing a glucuronic acid moiety. So, compounds **13**, **15**, **17** and **18** were elucidated as wogonoside, 7-*O*-β-D-glucuronopyranosylchrysin, oroxylin A-7-*O*-glucuronide and baicalin, respectively. The above identified compounds in LXD are showed in Fig. 5.

4. Conclusions

In this study, 19 compounds (7 iridoids and 12 flavonoids) with interaction activity to the immobilized HSA column were identified by on-line/off-line 2D biochromatography. These compounds are among those principal ingredients found in Gentianae Radix, Scutellarae Radix, Gardeniae Fructus, and Glycyrrizae Radix et Rhizoma. Flavonoids bind more strongly with HSA than iridoids because they have a higher retention values than the iridoids. The flavonoids are regarded as the principal active components for the pharmacological activity in Scutellarae Radix and Glycyrrizae Radix et Rhizoma [27–28]. The method developed in this study was proven effective for screen-

ing and analysis of bioactive compounds in TCMs, especially in its off-line mode.

By comparing with the routine biochromatography, the 2D biochromatography system has also been demonstrated to have advantages of finding strong binding bioactive components providing an enhanced peak capacity, good sensitivity and powerful resolution for biological fingerprinting analysis of complex prescription in TCMs.

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