Comprehensive two-dimensional high-performance liquid chromatography system with immobilized liposome chromatography column and reversed-phase column for separation of complex traditional Chinese medicine Longdan Xiegan Decoction

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Abstract

A comprehensive two-dimensional HPLC system with an immobilized liposome chromatography (ILC) column in conjunction with an RP column (in tandem) was developed for the screening and analysis of the membrane-permeable compounds in a traditional Chinese medicine prescription Longdan Xiegan Decoction (LXD). More than 50 components in LXD were resolved using the developed separation system. Eight flavonoids and two iridoids were identified interacting with the ILC column; a system that mimics biomembranes. The results show that the developed comprehensive two-dimensional chromatography system can be used for identifying membrane permeable natural products in complex matrixes such as extracts of traditional Chinese medicine prescriptions.

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1. Introduction

With an increasing demand for efficient separation and analysis of complex matrixes in environmental, biological and natural products samples, development of comprehensive two-dimensional-liquid chromatography (2D-LC) is needed to improve separation capability of HPLC technology\cite{1}. Recently 2D-LC systems have been used to separate molecules in biological systems\cite{2–5}, polymers\cite{6}, traditional Chinese medicines (TCMs)\cite{7–9} and other complex mixtures\cite{10}.

TCMs are very complex mixtures containing hundreds or even thousands of constituents of different structural types and concentrations. Only a few of them have been elucidated as being responsible for their pharmacological activity and/or toxicity\cite{11}. In view of this consideration, a rapid screening of bioactive compounds in TCMs is essential to drug discovery and the understanding of the pharmacological profiles of the TCMs. Recently we have developed screening methods using LC to probe the interaction of components in TCMs extracts with immobilized HSA, α\textsubscript{1}-acid glycoprotein (α\textsubscript{1}-AGP) and liposome\cite{12–14}. In addition microdialysis-HPLC has also been used for probing the interaction of extracts of TCMs with DNA, α\textsubscript{1}-AGP and HSA in the homogenous phase\cite{15–17}. 2D-LC, especially using a biochromatography column, offers a convenient method for the study of interactions between the multi-components in TCMs and tethered biological targets.

Immobilized liposome chromatography (ILC) is regarded as a powerful tool to study drug–membrane interactions in LC\cite{14,18}. Liposome\cite{19–21} formed by phosphatidylcholine, the main components found in cell membrane, was noncovalently or covalently immobilized on soft gel particles or silica particles as chromatographic stationary phase. The immobilized liposomes are used as models to probe the penetration ability of compounds through biological membranes, and the permeability of these compounds has been considered as one of the most important parameters to evaluate their bioactivity\cite{18–20}. ILC may therefore be utilized for the study of the interaction of the components in TCMs with a biological membrane\cite{21–23}. 

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Longdan Xiegan Decoction (LXD) is one of the most popular formulated TCM prescriptions widely used in clinical medication for anti-inflammation, anti-infection, anti-bacteria, anti-allergy, liver protection, cholangue and immunostimulant effects [24]. LXD consists of 10 medicinal materials including Gentianae Radix, Scutellariae Radix, Gardeniae Fructus, Rehmanniae Radix, Alismatis Rhizoma, Plantaninis Semen, Angelicae Sinensis Radix, Clematitis Armandii Caulis, Glycyrrhizae Radix et Rhizoma and Bupleuri Radix. The chemical components of some ingredient herbs in LXD were triterpenoids, flavonoids, iridoidal glycosides, pigments and volatile oils, organic acids, amino acids, and inorganic compounds [7,25–27]. So far there is not much focus on research into the chemical and pharmacological interpretation of its bioactive components as a whole formulation.

In the present work, we extended a comprehensive 2D chromatography system coupled with the diode array detector (DAD) and atmospheric pressure chemical ionization (APCI) mass spectrometry for fingerprinting analysis of LXD. An ILC column was used in the first dimension to probe the interaction of components in the TCM extract with the coated liposome in the column, then an ODS column was chosen in the second dimension for its relatively powerful separation and resolution capacity for analysis of the fractions eluted from the ILC column.

2. Experimental

2.1. Reagents and materials

Silica gel (5 μm, 300 Å) from Chrom Expert (Sacramento, CA, USA), phosphatidylcholine (PC) from Shanghai Chemical Reagents (Shanghai, China) was used. Acetonitrile was of chromatographic grade, distilled water used in all experiments was purified by a Milli-Q system (Milford, Bedford, MA, USA). Other chemicals were of analytical grade. Longdan Xiegan Decoction was prepared in our laboratory and the ingredient crude drugs were purchased from Huayu Chinese Medicinal Material Co. Ltd. (Shanghai, China), which were authenticated by Dr. Lihong Wu, Shanghai R&D Centre for Standardization of Chinese Medicines (Shanghai, China). The reference compounds of geniposide, gentiopicroside, liquiritin and baicalin were prepared in our laboratory and characterized by UV, MS and NMR spectral data. Buffer A (pH 7.4) was 10 mM sodium phosphate solution containing 50 mM NaCl. All solvents and sample solutions were filtered through a 0.45 μm nylon membrane before use.

2.2. Preparation of ILC column

PC (0.1–1 g) was dissolved in chloroform and 0.4–4 g of silica gel was added to the solution. The solution was shaken for 30 min, and the solvent was removed in a round-bottomed flask by rotary evaporation. The dried silica was kept under high vacuum overnight to remove the remaining solvent. The PC film coated porous silica gel was swollen in buffer A for 2–4 h to form the liposome, and then was washed three times with buffer A to remove free and loosely coated liposome. Liposome immobilized stationary phase was packed into a stainless steel column (150 mm x 4.6 mm i.d.) using the slurry packing method.

2.3. Preparation of samples

The powdered samples of the mixture of Gentianae Radix (2.4 g), Scutellariae Radix (1.2 g), Gardeniae Fructus (1.2 g), Rehmanniae Radix (2.4 g), Alismatis Rhizoma (2.4 g), Plantaninis Semen (1.2 g), Angelicae Sinensis Radix (1.2 g), Clematitis Armandii Caulis (1.2 g), Glycyrrhizae Radix et Rhizoma (1.2 g) and Bupleuri Radix (2.4 g) were immersed in 168 mL of water for 1 h and then decocated to boil for 1 h. The extract was filtered, and then 10 mL of the filtrate was extracted with an equal volume of ethyl acetate three times to obtain ethyl acetate and the aqueous fractions. Solvent from the ethyl acetate fraction was removed by rotary evaporation at 40 °C under vacuum, and the residue was dissolved in 10 mL of methanol. The ethyl acetate extract and the remaining aqueous solution were filtered through a 0.45 μm membrane and stored at 4 °C for subsequent experiments.

2.4. Chromatographic conditions

Chromatographic conditions for 2D-LC; the separation on the ILC column was accomplished within 400 min by isocratic elution with 10 mM ammonium acetate solution (pH 6.8) at a flow rate of 0.05 mL/min. The ODS column was eluted by a linear gradient in the second dimension, which was started from 10% A (90% B) to 70% A (30% B) in 7 min, and then returned to the initial mobile phase of 10% A (90% B) and held for 3 min for re-equilibration at a flow rate of 2.0 mL/min. Mobile phase A was acetonitrile and B was 0.1% (v/v) acetic acid.

In APCI-MS, the analytes obtained from the second dimensional column was 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, the nebulizing gas flow was 2.5 L/min, the APCI, CDL and block temperature was set at 400 °C, 250 °C and 200 °C, respectively. The mass range [m/z] was from 50 to 1000 and the scan speed was set at 0.5 s.

2.5. Apparatus and instruments

The main scheme of the 2D-LC system was described in our previous report [28]. Mobile phase was delivered through the injector into the ILC column. The outlet of the ILC column was attached to an eight-port valve on which two loops are equipped. One loop was connected to a fraction collector, and the effluent in the other loop was pumped out by a secondary pump to pass through the second column. The effluent of column 2 was monitored at 210 nm by an SPD-M10Avp diode array detector and the outlet of the diode array detector was directly connected to APCI-MS.

The two-dimension separation was performed on a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) consisting of two LC-10ADvp pumps, a SPD-M10Avp diode array detector (DAD), a SCL-10Avp system controller and LC–MS solution workstation. The 2D-LC was controlled by an in-house custom program. The chromatographic data were collected using a WDL-95 chromatography workstation controlled by an in-house custom program. The obtained chromatographic data with normalization of peak time was also treated by a in-house custom software. 2D Chromatograms were graphically represented with the aid of a software from Fortner Transform (version 3.4, Fortner, Savoy, IL, USA). 3D landscape images were displayed using a program written in-house with MATLAB 5.3 software (The Math Works, Sherborn, MA, USA).

3. Results and discussion

In comprehensive 2D-LC, the analytes were successively passed through two different types of columns. Each fraction from the first dimensional column was collected and injected into the second column. The analysis time of the second dimension was short enough to meet the rate of fractionation from the first-dimensional separation. In the present study, the separation process on the ILC column was accomplished in 70 min and equal volumes were collected at 10 min intervals. Accordingly the
separation on the ODS column was completed in 10 min for one run to match the cycling rhythm of the 2D-chromatography process. Fig. 1 shows the separation of the LXD extract on an ILC column and an ODS column, respectively. In this figure, different patterns of chromatograms could be observed, which provide the different selectivity for separation of the components in LXD. It can be seen that the ODS column showed better resolution capacity and efficient separations compared with the ILC column. The total analysis time was largely determined by the speed of the second dimension separation system. In order to obtain high 2D resolution in a short time, the ILC column was used for the first dimensional separation and an ODS column was chosen for the second dimensional separation.

3.1. Analysis of LXD fractions

In order to obtain improved separation performance, the total extract of LXD was divided into two parts by partitioning with ethyl acetate and water. The chromatograms from the fractions of ethyl acetate and water of LXD on ILC column are shown in Fig. 2. There are more intense peaks in the ethyl acetate fraction (Fig. 2a) than those of the aqueous fraction (Fig. 2b). This indicates that the main components in the ethyl acetate fraction of the LXD extract have significant interactions with immobilized liposome. The detected components can be regarded as permeable components; however, further study is required to prove whether or not they have practical pharmacological activities. As the remaining components in aqueous solution showed weak retention on the ILC column, they were excluded for further 2D separation in this study.

3.2. Optimization of the comprehensive 2D chromatography

In the comprehensive 2D-LC system, loop volume is determined by the multiplication of flow rate on the first column and run time on the second column. Thus flow rate of the first dimension separation can be obtained by dividing loop volume by the run time on the second column. In this work, the loop volume was set at 0.5 mL and the duration for each cycle was set at 10 min, so the flow rate of the first dimension separation was 0.05 mL/min.

The column efficiency was fairly low in general due to the intrinsic property of the immobilized biomacromolecules used as the stationary phase which leads to poor peak separation. As a result, a single peak that eluted from the ILC column may contain several co-eluting compounds. Comprehensive 2D-LC facilitates the further separation of the fractions from the first dimension by a high performance column in the second dimension, and hence it can be used to investigate the complex mixtures in TCMs. Therefore, it is a key step to choose a suitable column for the second dimensional separation. It is well known that reversed-phase material is readily available and can offer good performance at high flow rates and possesses a relatively large peak capacity which has been widely used in the analysis of TCMs and other botanical metabolites. A Kromasil ODS column and a monolithic ODS column were used for the comparison of their peaks separation capacity. Kromasil ODS column gave a better separation than the monolithic ODS column under the same chromatographic conditions (Fig. 3 and Fig. 1b) despite needing a relatively longer separation time for each run. The optimized experimental parameters were such that the flow rate in the secondary dimension was 2 mL/min, using acetonitrile and 0.1% (v/v) acetic acid as the mobile phase and the detection wavelength at 210 nm.
3.3. Identification of compounds in the comprehensive two-dimensional chromatography

Fig. 4 shows the typical contour plot for the ethyl acetate fraction of the LXD extract obtained on the comprehensive 2D-LC system. The x- and y-axes of this contour plot represent the retention times on the ILC column and the ODS column, respectively. The height or area of peaks is expressed as the size and darkness of spots in the 2D plot which are determined by the relative UV absorbance. It was shown that the spots were thick dotted in the first five cycles, which meant that those components have a weaker interaction with the ILC stationary phase, while the late-eluting peaks possessed stronger interaction properties [22]. Due to the complexity of the components in a formulated TCM preparation, some peaks were not separated at the baseline and the spots were dispersed (Fig. 4). The identification of the components in these peaks was aided by using MS detection. In our established two-dimensional chromatographic system, more than 50 components in the ethyl acetate fraction of the LXD extract were separated as shown in a 3D plot (Fig. 5). Ten of them (1–10) were identified as geniposide (1), gentiopicroside (2), oroxylin A-7-O-glucuronide (3), wogonoside (4), 7-O-β-D-glucuronopyranosylchrysin (5), baicalin (6), ononin (7), liquiritin apioside (8), 3′,4′-dihydroxy-5,6-dimethoxy-7-O-glucosidoflavone (9), and liquiritin (10). Among these compounds, 1, 2, 6, and 10 were unambiguously identified by direct comparison with the authentic compounds and the others were tentatively identified by referencing to literature data [29,30]. Their structures along with their UV and MS spectra are shown in Fig. 6.

Peaks 1 and 2 (Fig. 3) exhibited [M – H]− ions at m/z 386.8 and 356.0 and were characterized as geniposide (1) and gentiopicroside (2) respectively by comparison with reference standards. Similarly, peaks 6 and 10 were identified as baicalin (6) and liquiritin (10) respectively by direct comparison with the authentic compounds. Peaks 3 and 4 afforded the same [M – H]− ion at m/z 459.0 and the fragment ion at m/z 282.9 by losing a glucuronic acid unit [M – H – 176]− in the mass spectra, 3 and 4 were identified as oroxylin A-7-O-glucuronide (3) and wogonoside (4), respectively by comparing their UV absorption patterns. Compound 3 gave a stronger shoulder peak at 312 nm which is absent in the UV spectrum of compound 4 [31]. Peak 7 gave a molecular ion [M + H]− at m/z 439.9 and a fragment ion at m/z 286.9, produced from losing a side chain of a glucosyl moiety [M – H – 162]−. By referencing to the literature mass and UV spectral data, it was putatively identified as ononin (7) [32]. Peak 8 gave its UV maxima at 278, 325 nm indicating a characteristic absorption of flavanone. Its mass spectrum displayed a molecular ion at m/z 549.0 [M – H]− and two fragment ions at m/z 417.0 [M − H – 132]− and 255.0 [M − H – 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, 8). The mass spectra of peak 5, showed a molecular ion [M – H]− at 428.9 and a fragment ion at 253.0 by losing a glucuronol moiety (176 Da). So, 5 was putatively identified as 7′-O-β-D-glucuronopyranosylchrysin (5). Peak 9 gave a molecular ion [M − H]− at 491.0 and a fragment ion at 328.9 by losing a glucose (162 Da), and was identified as 3′,4′-dihydroxy-5,6-dimethoxy-7-O-glucosidoflavone (9).
1. Geniposide

2. Gentioficroside

3. Oroxylin A-7-O-glucuronide

4. Wogonoside

5. 7-O-β-D-Glucuronopyranosylchrysin

Fig. 6. UV and mass spectra with the structures identified as the main components, interacted with ILC, detected in Longdan Xiegan Decoction.
6. Baicalin

7. Ononin

8. Liquiritin apioside (Liquiritigenin-4'-O-[ -D- Apiofuranosyl ]-(1→2)- -D-glucoside])

9. 3',4'-Dihydroxy-5,6-dimethoxy-7-O-glucoside flavone

10. Liquiritin

Fig. 6. (Continued).
4. Conclusions

In this study, eight flavonoids and two iridoids showing interactive activity with the ILC column were identified by a 2D HPLC system with an IL column and an RP column in tandem aided by UV and APCI/MS. The flavonoids are regarded as the principal pharmacologically active components in Scutellariae Radix and Glycyrrhizae Radix et Rhizoma, and the iridoids are believed to be responsible for the activities of Gentianae Radix and Gardeniae Fructus. The method developed in this study was proven quick and effective for screening and analysis of membrane-permeable compounds in complex herbal mixtures using immobilized liposome as a membrane model. By comparing with the conventional biochromatography, the 2D system has demonstrated obvious advantages for biological fingerprinting analysis of a complex TCM prescription. It pre-selects the compounds with strong binding property to an ILC column and separates the target peaks on an ODS column with high sensitivity and powerful resolution capacity.

The partitioning capacity provides information about the interaction between the lipids and the substances studied. Nevertheless the retention behavior of a compound on ILC column merely reflects a non-specific–non-polar interactions for there are no specific receptors in liposome. The compounds identified are bioactive in terms of membrane permeability but their pharmacological effects need to be evaluated by more extensive bioassays using in vitro or in vivo models. In addition, the aqueous-soluble ingredients which are non-retainable on the ILC column may be ignored in such a condition. Consequently some alternative screening methods should be combined to get more reliable results.

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