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Short communication

Screening of permeable compounds in Flos Lonicerae Japonicae with liposome using ultrafiltration and HPLC

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ABSTRACT

A new method for the screening of membrane-permeable compounds in traditional Chinese medicines (TCMs), using ultrafiltration and HPLC analysis, has been proposed. We hypothesized that exposure of a TCM extract to a liposome membrane, the concentration of membrane-permeable compounds in the solution should decrease. Using this approach, the permeability of compounds in Flos Lonicerae Japonicae (Japanese honeysuckle) was investigated. By comparing chromatograms of samples prepared before and after interaction with a liposome membrane, eight permeable compounds of Flos Lonicerae Japonicae were identified, and all of them were proven to be biologically active. Based on the significance of these results, this method could be a novel approach for identifying potentially bioactive components in other TCMs.

1. Introduction

Traditional Chinese medicines (TCMs) have attracted ever-increasing attention due to their long history in clinical practice and reliable therapeutic efficacy. On the other hand, only limited information is known about the biologically active components in TCMs. Because of this lack of analysis, screening of the bioactive compounds in TCMs has become essential, not only for drug discovery and an improvement of our understanding of the therapeutic principles of TCMs, but also to ensure the appropriate level of quality control of TCMs. The traditional method for screening bioactive components involves extraction and isolation of TCMs followed by pharmacological screening of the purified compounds. This technique consumes time and samples, it is laborious, and it is inefficient for the direct screening of bioactive compounds from TCMs.

Immobilized liposome chromatography (ILC) [1] is regarded as a powerful tool for studying drug–membrane interactions in vitro and has been utilized in the screening and analysis of the membrane-permeable compounds in TCMs [2–4]. Liposomes are structurally similar to biological membranes because of their lipid bilayer structure. This similarity allows the liposomes to mimic the biological membrane and predict the penetrability of the compounds into cells. How much each compound penetrates the membrane is a key factor in evaluating the bioactivity of the compound. Unfortunately, ILC separation is lower than conventional HPLC; and, the identification of compounds is problematic.

In this paper, a new method for the screening of permeable compounds in Flos Lonicerae Japonicae (Jinyinhua in Chinese), one of the most commonly used TCMs for the treatment of fever, inflammation, and infectious diseases for thousands of years [5], was developed by employing liposome membranes, centrifugal ultrafiltration and HPLC. In ultrafiltration, the components of TCMs pass through the filter when not treated with liposomes; however, they are retained when treated. Centrifugal ultrafiltration is also a more rapid technique than equilibrium dialysis or microdialysis, which also employ semi-permeable membranes. Preliminary results indicate that multiple permeable compounds were simultaneously predicted through comparing the chromatograms of the Flos Lonicerae Japonicae extracts before and after interaction with the liposome membrane.

2. Experimental

2.1. Reagents and materials

Reference substances of chlorogenic acid (**1**), caffeic acid (**2**), rutin (**3**), hyperoside (**4**), luteolin-7-*O*-glucoside (**5**), 3,5-dicaffeoyl quinic acid (**6**), quercetin (**7**) and luteolin (**8**) were all purchased from Tauto Biotech (Shanghai, China). The purity of each compound was determined to be above 98% by HPLC analysis. The chemical structures of the reference compounds are shown in Fig. 1. Flos Lonicerae Japonicae was purchased from local drug stores in Shanghai, China. Hydrogenated soybean phosphatidylcholine (HSPC, Epikuron 200SH) was purchased from Degussa (Freising,

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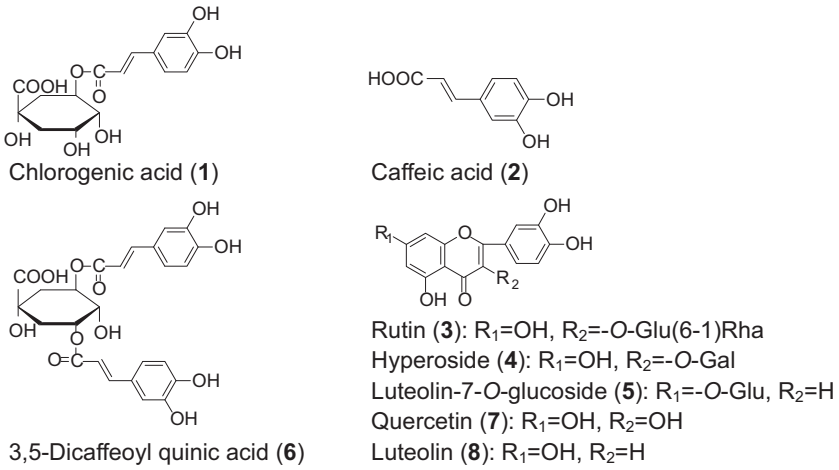

Fig. 1. Chemical structures of the identified compounds in Flos Lonicerae Japonicae.

Germany). 1,2-distearoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt) (DSPG) was purchased from Avanti Polar Lipids, Inc. (Alabaster, USA). Acetonitrile and methanol were of HPLC grade from Merck (Darmstadt, Germany), distilled water was purified by a Milli-Q system (Millipore, MA, USA), and all other chemicals were of analytical grade.

2.2. HPLC apparatus and conditions

The HPLC analysis was performed on a Hitachi L-2000 series HPLC apparatus, equipped with an L-2130 pump, L-2200 autosampler, L-2300 column oven and L-2455 diode array detector. The system was operated by a Hitachi D-2000 Elite HPLC system manager. HPLC separation was performed using a YMC-Pack ODS-A column (250 mm × 4.6 mm I.D., 5 μm) connected with a security guard cartridge (C18, 4 mm × 3.0 mm, Phenomenex, USA) at the temperature of 30 °C. The mobile phase consisted of 0.1% (v/v) aqueous phosphonic acid (A), acetonitrile (B) and methanol (C). DAD was set at 330 nm for phenolic acids (**1**, **2** and **6**) and 360 nm for flavonoids (**3**–**5**, **7** and **8**). An aliquot of 10 μL of each sample solution was injected and eluted by the following program at the flow rate of 1.0 mL/min: 0–20 min, 90% A–10% B → 84% A–16% B; 20–40 min, 84% A–16% B; 40–45 min, 84% A–16% B → 52% A–48% C; 45–65 min, 52% A–48% C; and this was followed by a 10 min equilibrium period prior to the injection of next sample.

2.3. Preparation of standard solutions

Mixed standard, stock solutions containing chlorogenic acid, caffeic acid, rutin, hyperoside, luteolin-7-O-glucoside, 3,5-dicaffeoyl quinic acid, quercetin and luteolin were prepared by dissolving accurately weighed reference compounds in methanol. Working standard solutions were prepared by diluting the mixed standard solution with methanol. The standard stock and working solutions were all prepared in dark brown, calibrated flasks and stored at 4 °C.

2.4. Preparation of the extract of Flos Lonicerae Japonicae

The Flos Lonicerae Japonicae sample was powdered to homogeneous size in a mill and sieved through a 60-mesh filter. The collected powder (5.0 g) was suspended in 200 mL 70% ethanol (v/v), ultrasonically extracted for 45 min and the extract there of was centrifuged at 15,000 rpm for 15 min. The solvent of the supernatant was removed with rotary evaporation under vacuum at 45 °C, and the residue was dissolved in 20 mL PBS and stored at 4 °C.

2.5. Method validation

2.5.1. Calibration curves, limits of detection and quantification

The stock solution of eight reference compounds was diluted to the appropriate concentration for construction of calibration curves. The mixed standard solution was injected as six separate concentrations. Calibration curves were constructed by plotting the mean peak areas versus the concentration of standards. The limit of detection (LOD) and quantification (LOQ), under these chromatographic conditions, were separately determined at an S/N of 3 and 10, respectively.

2.5.2. Precision, accuracy and stability

The measurement of intraday and interday variability was utilized to determine the precision of this newly developed method. The intraday precision was examined by analyzing, in triplicate, the mixed standard solutions at three concentration levels within 1 day; interday precision was determined for 3 consecutive days. The relative standard deviation (RSD) was taken as a measure of precision. A recovery test was used to evaluate the accuracy of the method. The Flos Lonicerae Japonicae powder was spiked with accurate standards at 80%, 100% and 120% of the amount found in the extract. Those samples were then extracted and analyzed for comparison. The stability of the sample solutions under different pH conditions (pH = 5.4, 7.0, 7.4) was tested at 4 °C for 48 h, room temperature for 12 h, and 37 °C for 2 h, respectively. The analytes showed the most stability in PBS (RSD ≤ 2.71%) over the tested period.

2.6. Preparation of liposomes

Liposomes were prepared by a thin lipid film hydration method [6]. HSPC and DSPG, at a molar ratio of 10:1, were dissolved in a chloroform/methanol mixture (2:1, v/v) and dispersed by sonication. Chloroform and methanol were completely removed from the lipid film under vacuum, and the film was hydrated with 10 mM phosphate-buffered saline (PBS) in a water bath, at 60 °C for 20 min. The suspension was then extruded using an extrusion device (Avanti) at 60 °C; this was equipped with a double polycarbonate membrane (0.45 μm), followed by a 0.2 μm pore size membrane. The ultimate concentration of HSPC was 30 mM. The particle sizes of the liposomes were measured in samples diluted with PBS using Nicomp 380ZLS (NICOMP Particle Sizing Systems, Santa Barbara, California, USA). The mean particle size of liposomes was found between 170 and 220 nm.

Table 1
Calibration curves, LODs and LOQs of 8 analytes.

Analyte	Calibration curve[a]	r	Test range (μg/mL)	LOD[b] (μg/mL)	LOQ[c] (μg/mL)
1	$y = 14609x + 276$	0.9993	2.06–1030	0.041	0.129
2	$y = 24223x + 93$	0.9996	0.201–100.4	0.033	0.100
3	$y = 6873x - 48$	0.9999	0.200–99.8	0.033	0.099
4	$y = 9580x - 93$	0.9995	0.201–100.6	0.033	0.101
5	$y = 14809x - 220$	0.9995	0.206–103	0.034	0.103
6	$y = 21453x - 173$	0.9997	1.016–508	0.064	0.169
7	$y = 21182x - 218$	0.9997	0.102–50.8	0.017	0.051
8	$y = 19342x - 205$	0.9997	0.099–49.5	0.016	0.050

[a] y: peak area; x: concentration of the analyte (μg/mL).
[b] LOD, limit of detection.
[c] LOQ, limit of quantification.

2.7. Permeation experiment

The liposome (0.1 mL) solution and the extract of the Flos Lonicerae Japonicae (0.9 mL) were mixed and reacted at 37 °C for 20 min. The mixture was centrifuged at 4000 rpm for 10 min using Amicon Ultra-4 filter devices (Millipore), with molecular weight cutoffs of 30 kDa, and the filtrate was analyzed by HPLC. PBS (0.1 mL) was added to the extract to provide the unreacted control sample.

3. Results and discussion

3.1. Optimization of chromatographic conditions

To obtain the optimum HPLC conditions, various mobile phase compositions were tested. The mixture of 0.1% aqueous phosphonic acid, acetonitrile and methanol was chosen as the gradient eluting solvent system due to the desired separation and acceptable tailing factor given within the run time of 65 min. As the main components in Flos Lonicerae Japonicae show different UV absorption properties, different wavelengths were simultaneously detected to monitor the compounds in a single run (330 nm for phenolic acids **1**, **2**, and **6** and 360 nm for flavonoids **3**–**5**, **7**, and **8**). The column temperature also significantly affected the chromatographic behavior. When optimizing the temperature between 20 and 35 °C, all components achieved baseline separation at the column temperature of 30 °C. Representative chromatograms of the mixed standards and extract are shown in Fig. 2(A and B).

3.2. Validation of the HPLC method

All calibration curves showed good linear regression ($r \geq 0.9993$) within the tested ranges (Table 1), and the intraday and interday variations were less than 2.18% for all analytes. As shown in Table 2, the HPLC method provided good accuracy with recoveries ranging from 98.13% to 102.49% for the analytes. In other words, the quantification of the compounds in Flos Lonicerae Japonicae, using this HPLC-DAD method, is precise, accurate and sensitive.

3.3. Interaction of Flos Lonicerae Japonicae with liposome membrane

All or part of the liposome membrane-permeable compounds in the extract that interacted with the liposomes were retained during the ultrafiltration process. This result gave a decrease in the peak area of the interactive compounds. Alternatively, those that did not bind to liposomes maintained their peak areas because they freely passed through the filter membrane. Due to these differences, the permeability of compounds in the extract could be obtained by comparing the chromatograms before and after

Table 2
Precisions, repeatabilities, and recoveries of 8 analytes.

Analyte	Concentration (μg/mL)	Precision		Recovery ($n = 3$)	
		Intraday ($n = 3$) RSD (%)	Interday ($n = 3$) RSD (%)	Recovery (%)	RSD (%)
1	2.06	0.53	0.98	99.61 ± 2.25	2.26
	51.50	0.27	1.01		
	515	0.35	0.45		
2	0.20	2.18	1.34	99.44 ± 1.22	1.23
	5.02	1.00	0.94		
	50.20	0.50	0.61		
3	0.20	0.81	1.71	102.49 ± 2.16	2.11
	4.99	0.85	0.75		
	49.90	0.55	1.22		
4	0.20	1.87	1.50	101.76 ± 1.51	1.49
	5.03	0.13	1.95		
	50.30	0.49	0.20		
5	0.21	1.07	1.94	101.17 ± 3.53	3.49
	5.15	1.21	0.53		
	51.50	0.63	0.39		
6	1.02	2.00	1.09	98.13 ± 2.39	2.44
	25.40	1.04	0.81		
	254	1.63	1.24		
7	0.10	1.48	1.51	100.96 ± 1.98	1.96
	2.54	1.15	0.81		
	25.40	0.42	0.74		
8	0.10	0.55	1.30	101.08 ± 3.04	3.01
	2.48	2.20	1.36		
	24.75	0.78	0.55		

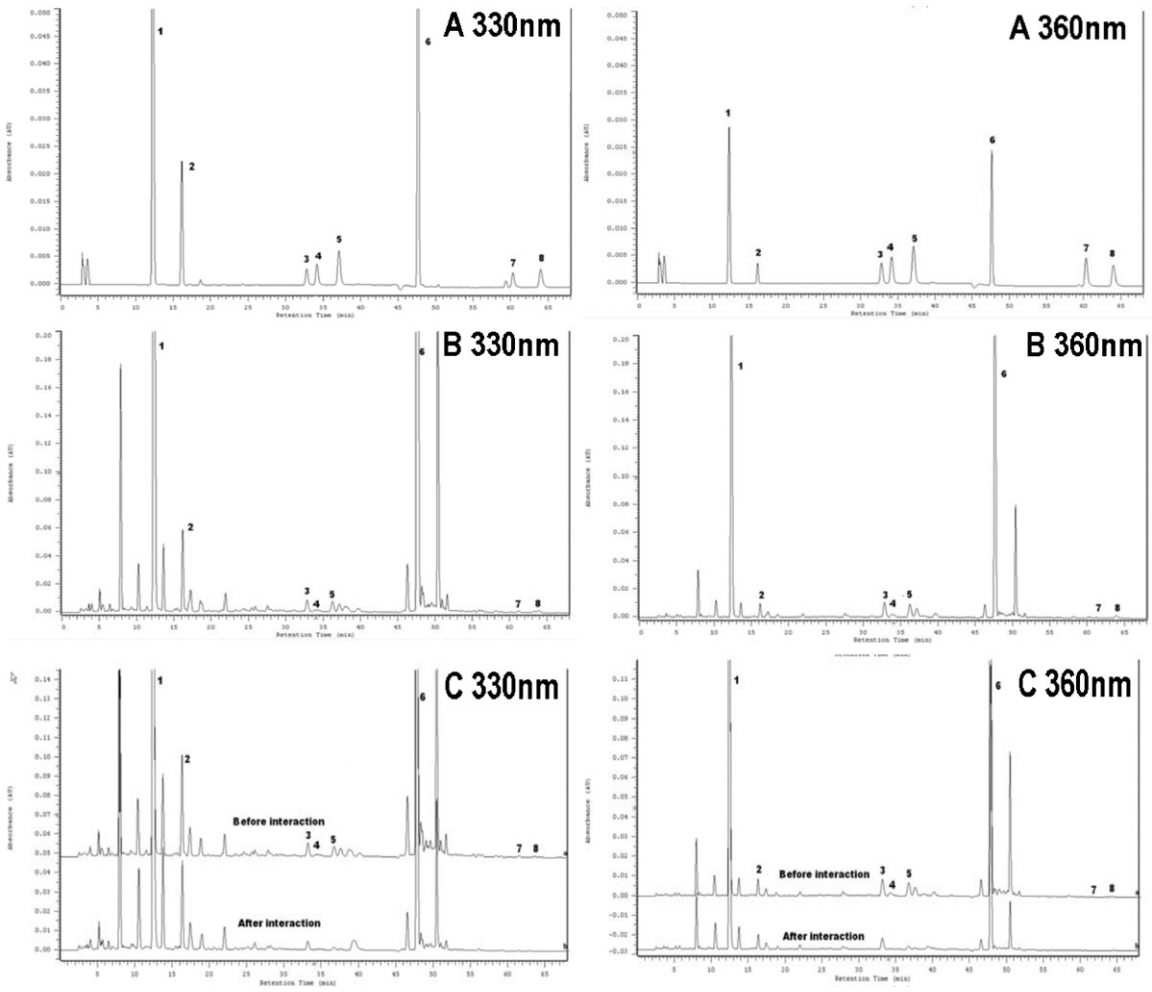

Fig. 2. (A) Representative chromatograms of mixed standards detected at 330 nm and 360 nm; (B) representative chromatograms of the Flos Lonicerae Japonicae extract detected at 330 nm and 360 nm; (C) representative chromatograms of the Flos Lonicerae Japonicae extract before and after interaction with liposome membrane at pH 7.0 detected at 330 nm and 360 nm.

interaction. The peaks labeled with numbers **1**–**8** in the chromatograms, shown in Fig. 2(C), were identified as chlorogenic acid, caffeic acid, rutin, hyperoside, luteolin-7-O-glucoside, 3,5-dicaffeoyl quinic acid, quercetin and luteolin. Identification of the compounds was performed by comparing retention times and online UV spectra with those of available standards. Identified peaks were then further confirmed by spiking samples with standard mixtures. Of the eight permeable compounds identified by the proposed method, all were proven to be bioactive compounds [5].

According to theory, the binding degree increases when the interaction with liposome membrane is stronger. The binding degrees of our compounds in the extract with the liposome membrane were determined using the following equation: Binding degree = $(C_o - C)/C_o$. C_o and C are the concentrations of each compound before and after the interaction with liposome membrane. The binding degrees of the above eight compounds are listed in Table 3.

Table 3
Binding degrees of 8 compounds in the extract of Flos Lonicerae Japonicae.

Peak no.	Binding degree (%) (n = 3)		
	pH = 5.4	pH = 7.0	pH = 7.4
1	12.60 ± 1.76	3.25 ± 0.14	2.19 ± 0.70
2	66.39 ± 0.51	8.97 ± 2.53	1.73 ± 1.50
3	30.38 ± 0.09	29.13 ± 0.42	8.64 ± 3.06
4	68.25 ± 0.47	56.07 ± 1.47	12.11 ± 3.25
5	83.37 ± 0.07	76.90 ± 0.50	29.96 ± 2.56
6	81.04 ± 0.03	54.86 ± 0.97	24.60 ± 1.82
7	84.81 ± 0.18	75.05 ± 0.61	–
8	87.51 ± 0.21	76.33 ± 0.05	–

The results indicated the binding degrees were influenced by the polarities of compounds. More specifically, the weaker polarity compounds had the higher binding degrees. For example, at pH 7.0, the binding degree of peak 1 was only 3.25%, while peak 7 was 75.05%. Another difference that we noticed involved the relationship between the binding degree and molecular configuration. The binding degree of 3,5-dicaffeoyl quinic acid (**6**) was found to be higher than chlorogenic acid (**1**). Comparison of these structures indicated that chlorogenic acid has only one caffeoyl group while 3,5-dicaffeoyl quinic acid has two. The caffeoyl group has been reported to be the active group in caffeoyl quinic acid derivatives and is thought to increase the activity of these types of compounds [7].

In comparing the structures of rutin (**3**), hyperoside (**4**), quercetin (**7**), luteolin-7-*O*-glucoside (**5**) and luteolin (**8**), it was found that the binding degrees of flavonoid glycosides were lower than their flavonoid aglycones, which was in agreement with previous reports [8,9]. Moreover, the effect of pH was also investigated for its interaction with Flos Lonicerae Japonicae and the liposome membrane. At pH 5.4, all of the compounds had high binding degrees. This is possibly because the compounds were either phe-

nolic acids or flavonoids and were in their molecule state at pH 5.4.

4. Conclusions

In the present study, a rapid, simple method employing a liposome membrane for the screening of membrane-permeable compounds in TCMs followed centrifugal ultrafiltration and HPLC-DAD analysis was proposed and successfully applied for the investigation of potential bioactive compounds in Flos Lonicerae Japonicae. In addition, the characteristics of the multiple components of TCMs could be resolved and compared through this procedure. Based on these findings, this new method could be used as a first step in the screening of bioactive compounds in TCMs. Moreover, the liposome membranes can be replaced with proteins [10], biomembranes [11], or even live cells [12,13] to interact with TCMs, which could lead to other avenues of detection.

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