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A vascular smooth muscle/cell membrane chromatography–offline-gas chromatography/mass spectrometry method for recognition, separation and identification of active components from traditional Chinese medicines

Xiaofang Hou, Mingzhe Zhou, Qiao Jiang, Sicen Wang, Langchong He *

School of Medicine, Xi'an Jiaotong University, 76 Yanta West Road, Xi'an 710061, Shaanxi, China

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ABSTRACT

We describe an analytical method of vascular smooth muscle cell membrane chromatography (VSM/CMC) combined with gas chromatography/mass spectrometry (GC/MS) for recognition, separation and identification of active components from traditional Chinese medicines (TCMs). VSM cells by means of primary culture with rat thoracic aortas were used for preparation of the stationary phase in the CMC model. Retention components by the VSM–CMC model were collected and then analyzed by GC/MS under the optimized conditions in offline conditions. After investigating the suitability and reliability of the VSM/CMC–offline-GC/MS method using nifedipine and nitrendipine as standard compounds, this method was applied in screening active components from the extracts of TCMs such as *Radix Angelicae Dahuricae* (RAD), *Rhizomza Seu Radix Notopterygii* (RSRN), *Radix Glehniae* (RG) and *Fructus Cnidii* (FC). Retention components from the extracts in the VSM–CMC model were imperatorin and osthole identified by the GC/MS method. *In vitro* pharmacological trials indicated that imperatorin and osthole could concentration dependently relax the rat thoracic artery pre-contracted by KCl ($P < 0.05$). The maximum relaxation effects (R_{max}) were $63 \pm 5\%$ and $40 \pm 6\%$ for imperatorin and osthole, respectively. The VSM/CMC–offline-GC/MS method is an effective screening system that can rapidly detect and enrich target components from a complex sample and then accurately identify them.

1. Introduction

Natural products can be the source of new drug discovery [1,2], but identifying the active components from the plants can be difficult. In general, traditional screening methods for testing activities and simultaneously isolating components require many procedures. High-throughput screening (HTS) methods with certain biological targets as models have been used for finding active compounds [3–5]. Many single components should be obtained by a set of isolation procedures if natural products are used as sources. A large library of compounds should be established before using HTS methods. HTS has entailed a huge workload and a low efficiency in drug discovery until now. Establishing a new method for promoting screening efficiency in the research and development of drugs is extremely important. Using HPLC for high-throughput screening have been quite a few [6,7]. Essentially, HPLC retention mechanism is dependent on the interaction between solute and stationary phase [8]. Wainer and co-workers reported the immobilization of a nicotinic acetylcholine receptor (nAChR) subtype in the immobilized artificial membrane (IAM) LC stationary phase for determination of ligand binding affinities at a nAChR [9,10]. Many membrane receptors or enzymes as biological targets have been widely used for screening *in vitro* models for the discovery of active components from medicinal herbs [11,12], and leading compounds from the library [13–16].

In our previous investigations, the model of cell membrane chromatography (CMC) in which cell membrane enriched certain receptors as the stationary phase [17,18] was applied to screen active components from a complex sample such as a medicinal plant [19–21], and to investigate interactions between drug and receptor [22–24]. In comparison with other chromatographic methods, gas chromatography/mass spectrometry (GC/MS) is a more efficient method for the separation and identification of unknown components in medicinal plants [25–29]. Little information is available for a combined method of the CMC model with GC/MS for screening active components from a complex sample. In receptor pharmacology studies, it has been recognized that L-calcium channel receptors (L-CCRs), which are mainly rich in cell membranes of myocardium [30] and vascular smooth muscle (VSM) [31–33], are the key targets of dihydropyridines [34–36] including nifedipine [37] and nitrendipine [38]. They can bind and block L-CCRs on the outside of membranes, stopping ecto-Ca^{2+}

* Corresponding author. Tel.: +86 29 82655451; fax: +86 29 82655451.
E-mail address: helc@mail.xjtu.edu.cn (L. He).

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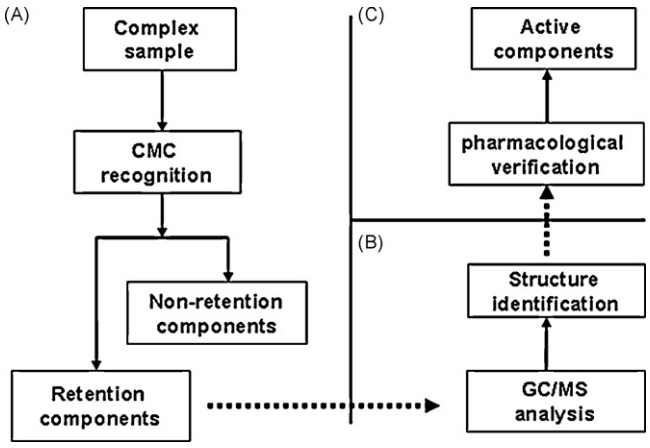

Fig. 1. Brief scheme of the analytical process using a VSM–CMC–offline-GC/MS method including three steps: (A) the retention components were obtained by the affinity recognition of VSM–CMC model from a complex sample; (B) the retention components were collected and analyzed by GC/MS to identify these chemical structures; (C) the pharmacological effects of active components identified were tested.

inflow and producing the vasodilations [39]. The binding interaction between the drugs and receptors has also been proved by the classic radioligand binding assays in float cell membrane of VSM [38,40] and in the tissues [41].

In this paper, the VSM cell membrane was firstly used in CMC model to prepare the stationary phase although other kinds of cell membrane had already been applied in different CMC models [19–24]. Drugs or components selectively acting on the receptors in the VSM cell membrane were retained. To improve identifiable function of CMC system for those retention components after the recognition from VSM–CMC model, the retention fractions were collected and analyzed by GC/MS. In this way, a VSM/CMC–offline-GC/MS method was established and applied for recognition, separation and identification of active components from traditional Chinese medicines (TCMs). Combining with a pharmacological verification trial, it will be an efficient scheme for fast screening target components from complex samples (Fig. 1).

2. Experimental

2.1. Materials

Silica gel (ZEX-II, 100–200 mesh) was obtained from Qingdao Meigao Chemical Company Limited (Qingdao, PR China). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco Incorporated (Grand Island, NY, USA). Dimethyl sulfoxide (DMSO), MTT, RPMI-1640 medium and trypsin were from Sigma (Saint Louis, MO, USA). HPLC grade methanol and acetic ether were purchased from Fisher Scientific (Pittsburgh, PA, USA). Metoprolol, atenolol, nifedipine, nitrendipine, imperatorin and osthole were supplied by the National Institute for the Pharmaceutical and Biological Products of China. *Radix Angelicae Dahuricae* (RAD), *Rhizomza Seu Radix Notopterygii* (RSRN), *Radix Glehniae* (RG), *Fructus Cnidii* (FC) were purchased from the TCM Store (Xi'an, PR China).

2.2. Preparation of standard solutions

Standard stock solutions of metoprolol, atenolol, nifedipine, nitrendipine, imperatorin and osthole (1 mg mL^{-1} each) were prepared separately in acetic ether. A mixed standard solution I of 1 mg mL^{-1} of metoprolol, atenolol, nifedipine, nitrendipine, and a mixed standard solution II composed of 1 mg mL^{-1} imperatorin, osthole, atenolol and nitrendipine were prepared.

2.3. Sample preparation

The extracts from RAD and FC were obtained by supercritical CO_2 extraction procedure under the pressure 30.5 MPa and temperature 45 °C for 3 h, respectively. The extracts from RSRN and RG were obtained by means of ultrasonic extraction. The procedure included the 50 g ground RSRN and RG powders were respectively put into 100 mL triangular flask. Each sample was extracted using 50 mL ether for two times (30 min per time) under the ultrasonic condition.

2.4. Preparation of VSM–CMSP

Male Sprague–Dawley rats (250–300 g) were from the Animal Center of Xi'an Jiaotong University (Xi'an, China). The tissue-sticking method was used to obtain primary cultures of vascular smooth muscle cells (VSMCs) from thoracic aortas [42]. The VSM cell membrane was prepared as previously described [43]. Cells (7×10^6) were washed thrice with normal saline solution by centrifuging at 650 × *g* for 5 min at 4 °C. Tris–HCl (50 mM, pH 7.4) was added to produce a cell suspension. The resulting homogenate was centrifuged at 200 × *g* for 5 min. The pellet was discarded and the supernatant centrifuged at 15,000 × *g* for 20 min at 4 °C. The supernatant was then discarded. Tris–HCl (50 mM, 10 mL, pH 7.4) was added to the pellet, and the mixture centrifuged under identical conditions. The membrane suspension was obtained after the pellet was suspended with distilled water. VSM cell membrane stationary phase (VSM–CMSP) was prepared according to literatures [20,18]. Briefly, 0.05 g silica was activated at 105 °C for 30 min and used as a carrier. It was then homogenized with the cell membrane suspension, i.e., the mixture was slowly added to it under a vacuum and with agitation at 4 °C. The mixture obtained was packed into the column by a wet method to yield a VSM–CMSP column (10 mm × 3.1 mm, 5 μm). The life-span of this VSM/CMC column was about 3 days under a continuous usage.

2.5. VSM/CMC assay

A HPLC system and a 32 Karat workstation (Beckman Coulter, Fullerton, CA, USA) were used. A VSM–CMSP column (10 mm × 3.1 mm, 5 μm) was used. The mobile phase was superpure water with a 0.2 mL/min flow rate at a column temperature of 37 °C. The detected wavelength is listed in Table 1. It took approximately 2 h to establish equilibrium of the chromatographic system before injection. Ten microliters of standard solutions or sample solutions were injected, respectively. All assays were carried out within the life-span of the VSM/CMC column. In the procedure of "recognition analysis", the retention fractions were collected in a 96-well plate using a Model SC-100 fraction collector (Beckman Coulter). Retention fractions as shown in the chromatograms were combined and evaporated with a SpeedVac concentrator (5301, Eppendorf, Germany). After extraction with 100 μL acetic ether and vortex mixing for 5 min, the extracts of retention fraction were analyzed by GC/MS.

2.6. GC/MS assay

A capillary gas chromatography–mass spectrometry (GCMS-QP2010 Shimadzu, Kyoto, Japan) with a Rtx-5MS capillary column (30 m × 0.25 mm. i.d., 0.25 μm film thickness, Restek, CA, USA) was used. Helium (purity 99.999%) was the carrier gas with a constant column flow of 2.0 mL/min. The initial temperature was 70 °C, held for 2 min; ramped at 10 °C/min up to 240 °C and held for 4 min. The inlet temperature was maintained at 240–280 °C. The mass spectrometer was operated in electron ionization mode at 70 eV. The mass range scanned from 40 *m*/*z* to 500 *m*/*z* for full-scan mode.

Table 1
Detection wavelengths of the analytical solutions.

Standard solutions	Metoprolol	Atenolol	Nifedipine	Nitrendipine	Imperatorin	Osthole
Wavelengths (nm)	244	276	240	236	248	322
Mixed standard solutions	I	II				
Wavelengths (nm)	210	240				
Sample solutions	RAD	RSRN	RG	FC		
Wavelengths (nm)	248	248	248	322		

Data were collected using GC/MS Analysis Station software. Data were analyzed using a NIST library (Shimadzu, Kyoto, Japan).

Under these conditions, the extracts of retention fractions recognized by VSM–CMC model, standard solutions, and samples were analyzed by GC/MS.

2.7. In vitro experiments

Isolated arteries from male Sprague–Dawley rats were cut into rings (2–3-mm long) and placed in 2-mL tissue chambers filled with K–H solution of the following composition (in mM): 115 NaCl, 4.6 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4{\cdot}7H_2O$, 2.5 $CaCl_2$, 25 $NaHCO_3$, 11.1 dextrose, according to the method described in reference [44]. Tissue baths were maintained at 37 °C, pH 7.4, and bubbled with a mixture of 95% O_2 and 5% CO_2. Rings were mounted on two stainless-steel hooks to fix them to the bottom of the chamber, and to a JH-2 force displacement transducer connected to a PowerLab 8SP unit (AD Instruments, UK) to record the isometric tension developed by aortic rings. Optimal tension was selected from preliminary experiments in which the rings were stretched so that the greatest response to phenylephrine (10^{-6} M) could be obtained.

KCl (60 mM) was added to the baths to induce pre-contraction of rat thoracic artery segments. After sustained tension was obtained, imperatorin and osthole (0.1 μM to 0.1 mM) was added cumulatively to the baths, and the concentration–response curves to imperatorin, osthole and nifedipine were constructed.

3. Results and discussion

3.1. Suitability and reliability of the VSM/CMC–offline-GC/MS method

The reproducibility of the different VSM/CMC columns was tested by the nifedipine standard solutions. The results showed that the RSD (%) of retention time (t_R) of nitrendipine peak was 14.60% when changing VSM/CMC columns ($n = 5$). The precision between the columns was to meet the assay requirements. Validation of the VSM/CMC–offline-GC/MS method was assessed with metoprolol, atenolol, nifedipine, nitrendipine standard solutions and their standard mixed solutions, respectively.

Fig. 2A shows the chromatograms of four standard solutions on the VSM–CMC model. Metoprolol (R_{01}) and atenolol (R_{02}) had almost no retention characteristics, but nifedipine (R_1) and nitrendipine (R_2) had obvious retention characteristics. The retention time of nifedipine and nitrendipine were 2.3 min and 8.4 min, respectively. Selected fractions (between the two dotted lines) were collected, extracted and injected into the GC/MS system for identification. Mass spectrometric data revealed that R_{01}, R_{02}, R_1 and R_2 peaks represented metoprolol (m), atenolol (a), nifedipine (nf) and nitrendipine (nt), respectively.

Fig. 3A is the chromatogram of the mixed standard solutions I on the VSM–CMC model. Four fractions (between the two dotted lines) were collected and labeled as R_0, R_1, R_2 and R_3. R_1 and R_2 had obvious retention characteristics and were the retention fractions. Mixed standard solutions I and each of the fractions were injected

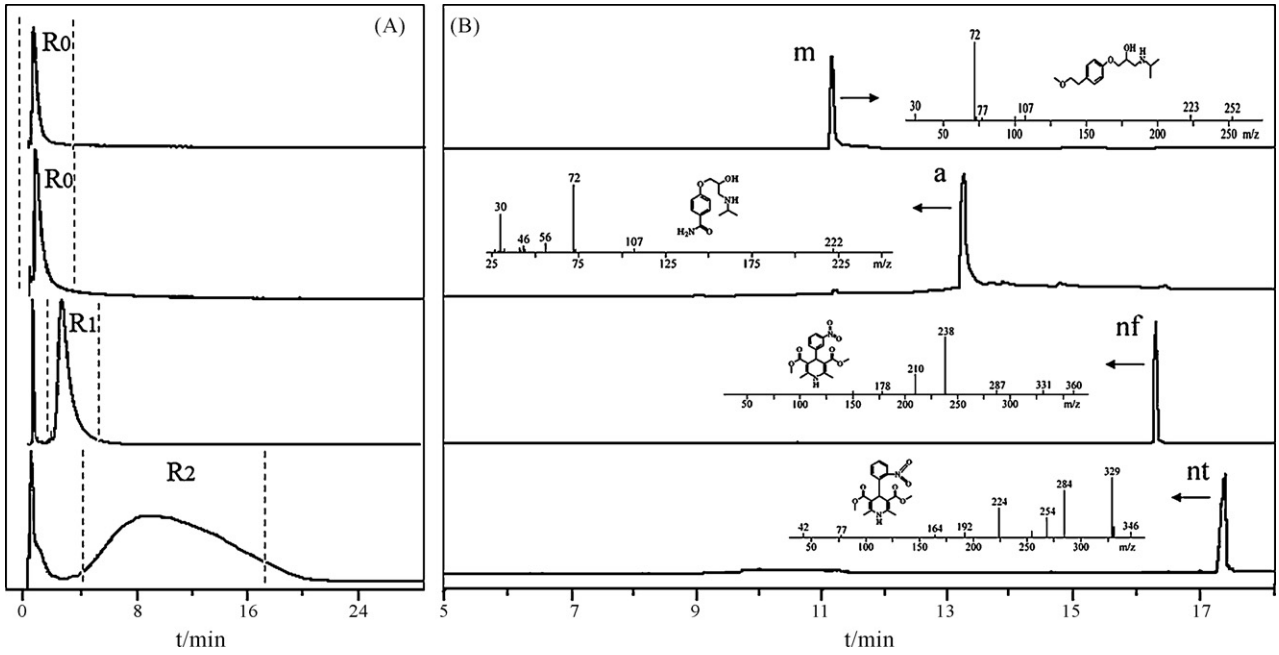

Fig. 2. Chromatograms of the standard solutions using a VSM–CMC–offline-GC/MS method. (A) VSM–CMC chromatograms of the standard solutions including metoprolol (R_{01}), atenolol (R_{02}), nifedipine (R_1) and nitrendipine (R_2), respectively; (B) GC/MS chromatograms of the corresponding fractions (between two dotted lines in VSM–CMC chromatograms) collected from the VSM–CMC model, and identified as metoprolol (m), atenolol (a), nifedipine (nf) and nitrendipine (nt) respectively.

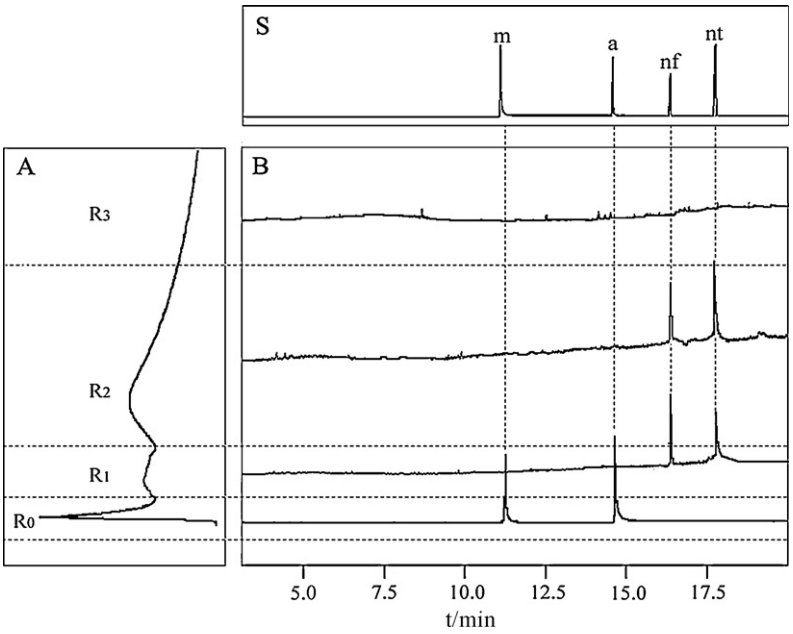

Fig. 3. Chromatograms of the mixed standard solutions I using a VSM–CMC–offline-GC/MS method: (A) VSM–CMC chromatogram of the mixed standard solutions I, and four fractions (between two dotted lines in VSM–CMC chromatograms) were collected and labeled as R_0, R_1, R_2 and R_3 respectively; (B) GC/MS chromatograms of the corresponding R_0, R_1, R_2 and R_3 fractions collected from the VSM–CMC model; (S) GC/MS chromatogram of the mixed standard solutions I containing metoprolol (m), atenolol (a), nifedipine (nf) and nitrendipine (nt) respectively. R_0 represents non-retention fraction, both R_1 and R_2 represent retention fractions of different retention time, and R_3 represent equilibrium fraction.

into the GC/MS system for analysis (Fig. 3S and B). By comparing the retention times of chromatograms in Fig. 3S and B, metoprolol (m) and atenolol (a) were present in the R_0 fraction, nifedipine (nf) and nitrendipine (nt) were in R_1 and R_2 fractions, and none were in the R_3 fraction.

These results suggested that nifedipine and nitrendipine showed better retention characteristics. They are calcium antagonists [45] and could interact with the L-type calcium channel receptor [46] abundant in the VSM cell membrane. Metoprolol (m) and atenolol (a) are β1-blockers had no retention characteristics because they selectively act on myocardial cells abundant with the β_1 receptor [47]. Retention components could therefore be recognized and separated by the VSM–CMC model. The retention time had positive correlation with interaction strength. The structure characteristics of corresponding fractions were identified by GC/MS.

3.2. Practical application

Some TCMs such as RAD, ASRN, RG and FC were commonly used as anti-cardiovascular diseases medicines. It was also found that there were some effective components on the vascular relaxation in these TCMs based on our previous works [48]. In this regard, they were selected as samples in the experiments. The VSM/CMC–offline-GC/MS method was first applied in screening active components from four types of TCMs (RAD, RSRN, RG, FC). Fig. 4 shows the analytical results of the supercritical CO_2 extracts from RAD using this method. R_1 peak, as shown in Fig. 4A_1, was considered to be a set of major retention components in the extracts on the VSM–CMC model. After that, R_1 fraction was collected, extracted and injected into the GC/MS system for identification by following two steps: (1) R_{1-1} peak was a main retention component of R_1 fraction in GC/MS chromatogram, as shown in Fig. 4B_1, and

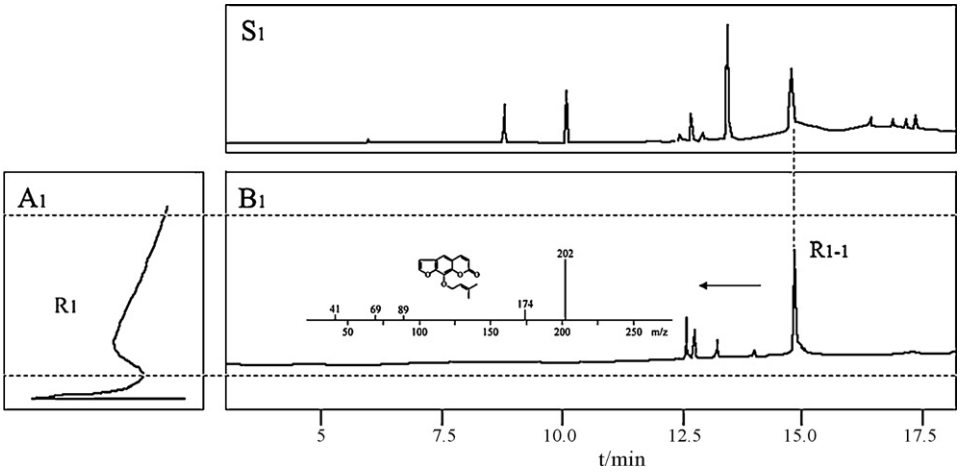

Fig. 4. Chromatograms of the extracts from RAD using a VSM–CMC–offline-GC/MS method: (A_1) VSM–CMC chromatogram of the supercritical CO_2 extract from RAD, and R_1 fraction was collected; (B_1) GC/MS chromatogram of R_1 fraction, and a main retention peak (R_{1-1}) was identified as imperatorin; (S_1) GC/MS chromatogram of the supercritical CO_2 extract from RAD as a comparison with B_1.

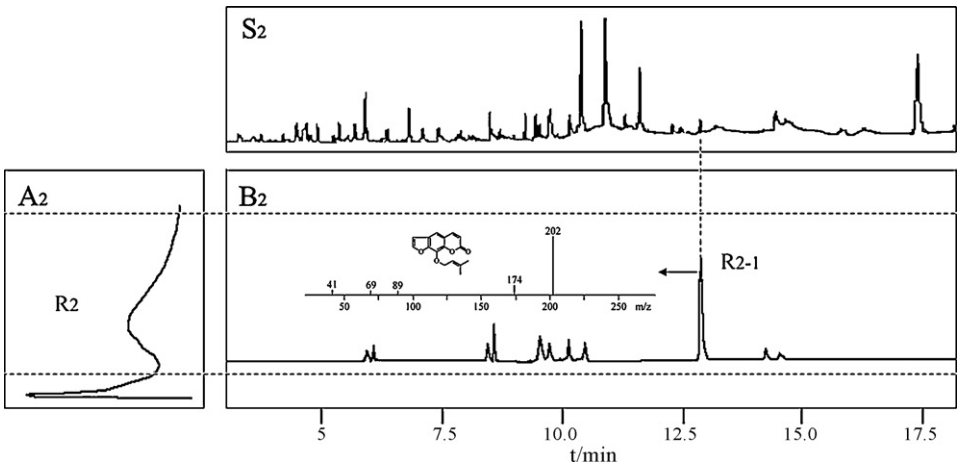

Fig. 5. Chromatograms of the extracts from RSRN using a VSM–CMC–offline-GC/MS method: (A_2) VSM–CMC chromatogram of the supercritical CO_2 extract from RSRN, and R_2 fraction was collected; (B_2) GC/MS chromatogram of R_2 fraction, and a main retention peak (R_{2-1}) was identified as imperatorin; (S_2) GC/MS chromatogram of the supercritical CO_2 extract from RSRN as a comparison with B_1.

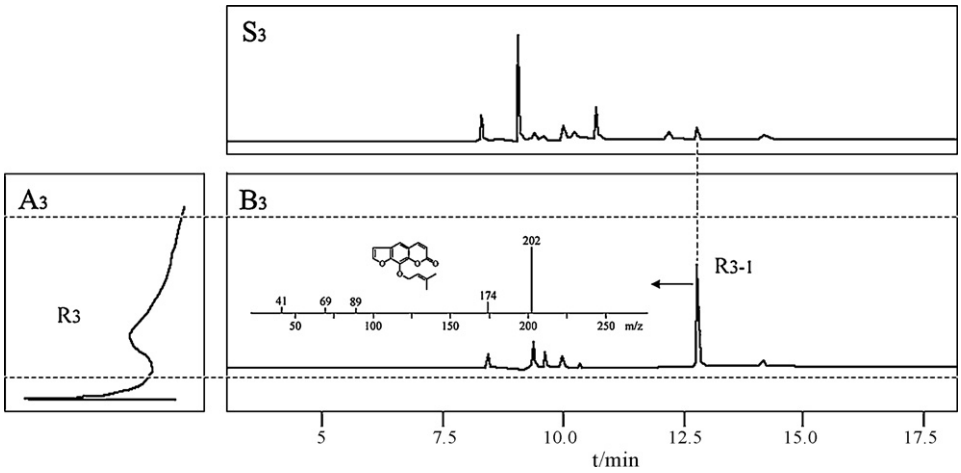

Fig. 6. Chromatograms of the extracts from RG using a VSM–CMC–offline-GC/MS method: (A_3) VSM–CMC chromatogram of the supercritical CO_2 extract from RG, and R_3 fraction was collected; (B_3) GC/MS chromatogram of R_3 fraction, and a main retention peak (R_{3-1}) was identified as imperatorin; (S_3) GC/MS chromatogram of the supercritical CO_2 extract from RG as a comparison with B_3.

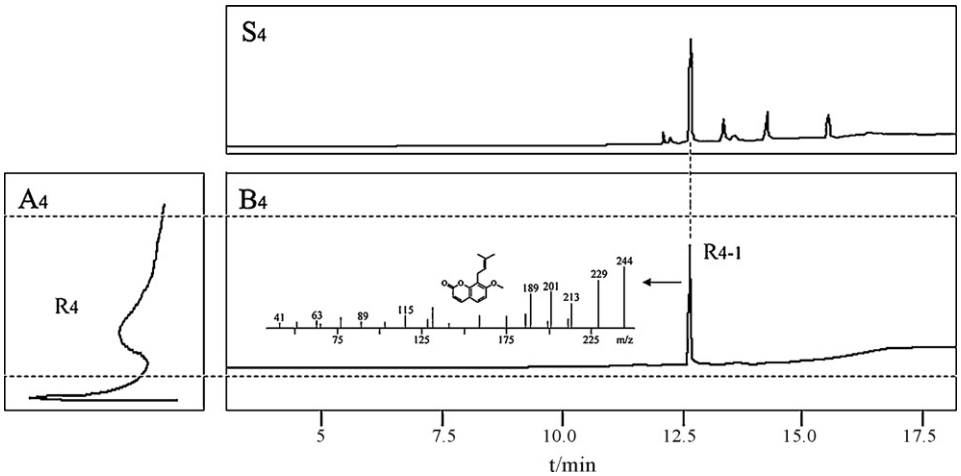

Fig. 7. Chromatograms of the extracts from FC using a VSM–CMC–offline-GC/MS method: (A_4) VSM–CMC chromatogram of the supercritical CO_2 extract from FC, and R_4 fraction was collected; (B_4) GC/MS chromatogram of R_4 fraction, and a main retention peak (R_{4-1}) was identified as imperatorin; (S_4) GC/MS chromatogram of the supercritical CO_2 extract from FC as a comparison with B_4.

Table 2
The enrichment ratios of both imperatorin and osthole after VSM–CMC model.

	Imperatorin (peak area ratio, %)			Osthole (peak area ratio, %)
	RAD	RSRN	RG	FC
In the extracts	19.8	1.4	3.1	45.4
In the fractions	56.8	12.5	23.2	99.1
Enrichment ratio	2.8	8.9	7.4	2.2

was identified as imperatorin by means of the mass spectrometer and its database in GC/MS system; (2) R_{1-1} retention component was to come from the supercritical CO_2 extracts of RAD by comparison with Fig. 4S_1. Furthermore, imperatorin as a major component was also enriched by using VSM–CMC model. The other examples are shown in Figs. 5–7. Imperatorin was an active component in the extracts from RSRN and RG; osthole was an active component in the FC extracts.

The application results reflected that the VSM/CMC–offline-GC/MS method can efficiently screen active components from a complex sample. The VSM–CMC model can recognize target components through affinity interactions between the components and L-calcium channel receptors on the VSM cell membrane. Recognition was verified using nifedipine and nitrendipine as controls on the VSM–CMC model because dihydropyridines block the movement of calcium into arteriolar smooth muscle by L-calcium channel receptors.

Until now, GC/MS is actually the most efficient method for separation and identification of unknown volatile components from a complex sample. There are 147,198 data for covering most of common compounds in natural plants including of the 4 TCMs used in this work. Moreover, the chemical structures have already been clear for most of the components in TCMs. The structure information of any separated component on GC/MS system can be obtained using this database with a different similarity. And then the component structure can be finally confirmed by comparing with the chromatographic behaviors between this component and its standard.

As listed in Table 2, according to the peak areas of imperatorin and osthole in the GC/MS chromatograms from Figs. 4–7, the relative contents of imperatorin and osthole in the extracts and in the fractions were calculated with area normalization method. It was from 1.4% to 19.8% in the extracts, increasing to 12.5–56.8% in the fractions for imperatorin, and was 45.4–99.1% for osthole. The enrichment ratio between the extracts and fractions increased from 2.2-times to 8.9-times for different samples. The VSM–CMC model could not only recognize imperatorin and osthole, but could also enrich them from different TCMs. It is particularly useful to the low contents of target components in screened samples.

3.3. Validation of screening results

To validate the screening results with the VSM/CMC–offline-GC/MS method, the mixed standard solution II containing atenolol, nitrendipine, imperatorin and osthole as standard compounds was used.

Fig. 8A is the chromatogram of the mixed standard solutions II on the VSM–CMC model. Four fractions were collected and labeled as R_0, R_1, R_2 and R_3. R_1 and R_2 were the retention fractions. Mixed standard solutions II and each of the fractions were injected into the GC/MS system (Fig. 8S and B). Comparing the retention times of chromatograms in Fig. 8S and B revealed that atenolol (a) was present in the R_0 fraction, osthole (o) and imperatorin (i) were in the R_1 fraction, osthole (o) and nitrendipine (nt) were in the R_2 fraction, and none were in the R_3 fraction.

Fig. 8 illustrates that nitrendipine (nt), imperatorin (i) and osthole (o) were in the retention fractions. The results verified that the VSM–CMC model could recognize and separate active components from mixed standard solutions.

3.4. Relaxation effects of imperatorin and osthole

As shown in Fig. 9, there were obvious relaxations in the rat thoracic artery pre-contracted by KCl for imperatorin and osthole in comparison with nifedipine as a control ($P < 0.05$). The maximum relaxation effects (R_{max}) were $63 \pm 5\%$ and $40 \pm 6\%$ for imperatorin and osthole, respectively. The results reflected that the VSM/CMC–offline-GC/MS method could efficiently screen active

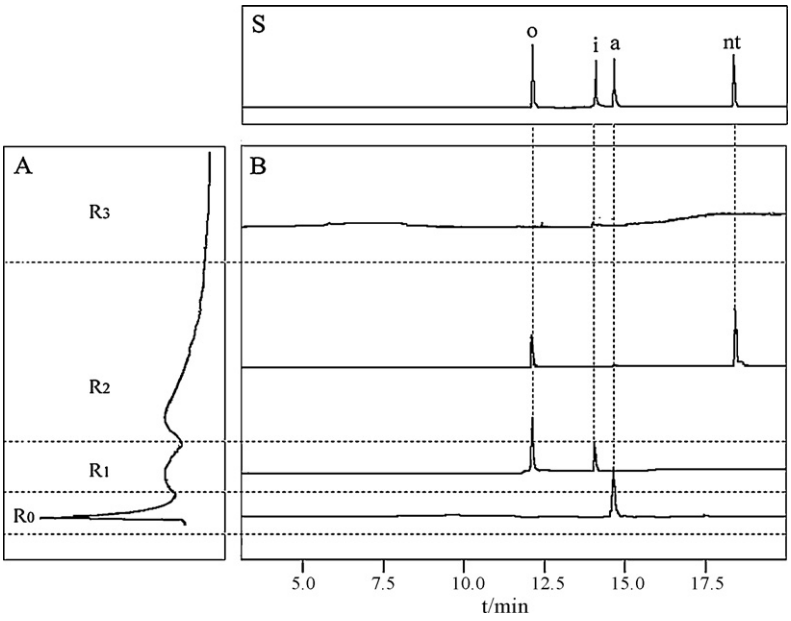

Fig. 8. Chromatograms of the mixed standard solutions II using a VSM–CMC–offline-GC/MS method: (A) VSM–CMC chromatogram of the mixed standard solutions II, and four fractions (between two dotted lines in VSM–CMC chromatograms) were collected and labeled as R_0, R_1, R_2 and R_3 respectively; (B) GC/MS chromatograms of the corresponding R_0, R_1, R_2 and R_3 fractions collected from the VSM–CMC model; (S) GC/MS chromatogram of the mixed standard solutions II containing imperatorin (i), osthole (o), atenolol (a) and nitrendipine (nt) respectively.

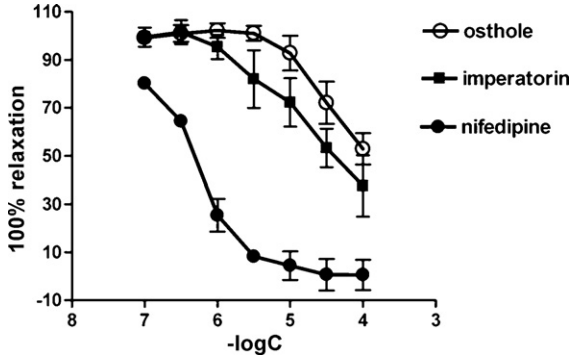

Fig. 9. Relaxation effects of osthole (○), imperatorin (■), and nifedipine (●); nifedipine used as a control. ($n = 8$).

components from a complex sample because of recognition of the VSM–CMC model.

4. Conclusion

In summary, screening active components using a clear biological target from a medicinal plant is still a laborious and time-consuming process for drug discovery. The VSM/CMC–offline-GC/MS method is only another way to rapidly recognize target components from a complex sample, and then accurately identify them. Its applications will enable screening procedures to be more efficient in some extent.

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