Journal of Pharmaceutical and Biomedical Analysis 51 (2010) 1032–1036

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

A combined cell membrane chromatography and online HPLC/MS method for screening compounds from *Radix Caulophylli* acting on the human α_{1A}-adrenoceptor

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ARTICLE INFO

Article history:
Received 2 September 2009
Received in revised form 5 November 2009
Accepted 7 November 2009
Available online 13 November 2009

Keywords:
Cell membrane chromatography
High performance liquid chromatography/mass spectrometry
Online analysis
α_{1A}-Adrenoceptor
Radix Caulophylli

ABSTRACT

We have developed an online analytical method that combines α_{1A}-adrenoceptor (α_{1A}AR) cell membrane chromatography (α_{1A}AR-CMC) with high performance liquid chromatography and mass spectrometry (HPLC/MS) for the identification of active components from *Radix Caulophylli* acting on the human α_{1A}AR. Fractions retained by the α_{1A}AR-CMC column were captured into a loop and the components were directly analyzed by combining an 8 port column switcher with an HPLC/MS system for separation and preliminary identification. Using methoxamine as a positive control drug, magnoflorine and caulophine from *Radix Caulophylli* were identified as the active molecules acting on the α_{1A}AR. This new α_{1A}AR-CMC-online-HPLC/MS method can be applied for screening active components acting on α_{1A}AR from traditional Chinese medicines exemplified by *Radix Caulophylli*. This method will be of great utility in drug discovery using natural medicinal herbs as a source of novel compounds.

1. Introduction

Cell membrane receptors, including G protein-coupled receptors (GPCRs), are currently the predominant targets for new drug development [1]. The α_1 adrenoceptors comprise three of nine well-characterized GPCRs that are activated by epinephrine and norepinephrine. Agonists acting on the α_1 adrenoceptors produce numerous physiological effects and are used therapeutically in several indications. Many known α_1 adrenoceptor agonists including phetolamine, 5-methylurapidil, methoxamine, and oxymetazoline are selective for α_{1A} [2–4]. The α_{1A}-adrenoceptor (α_{1A}AR) is widely distributed in many tissues and organs including cerebrum, heart, blood vessel, liver, kidney, prostatic gland, and spleen [5]. Ligand binding to α_{1A}AR is thought to activate several signaling pathways [6]. Cell lines expressing human α_{1A}AR have recently been used for binding studies of α_{1A}AR ligands. Hieble et al. studied the affinity of α_{1A}AR antagonists using Chinese hamster ovary (CHO-K_1) cells expressing human α_{1A}AR [7]. Han Qide and Zhang Youyi's research team at Peking University has established an alternative cell line, HEK293/α_{1A}, that also expresses high levels of human α_{1A}AR [8].

HPLC retention mechanism is based on the interaction between solute and stationary phase [9]. 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 [10,11]. Cell membrane chromatography (CMC), a novel chromatographic technique developed by He et al. [12–14], affords an effective research tool for studying drug–receptor interactions [8,15,16]. This system has been successfully applied to the screening of active components in natural products [17–19].

Radix Caulophylli is a traditional medicine and alkaloids from *Radix Caulophylli* are known to have specific pharmacological activities [20]. However, the active compounds have not been systematically studied. The present work therefore aimed to establish a HEK293/α_{1A}-adrenoceptor cell membrane chromatography (α_{1A}CMC) system for screening of alkaloids from *Radix Caulophylli*. This was achieved by combining the CMC system with a two-dimensional liquid chromatography technique. The α_{1A}AR-CMC system was first validated using a known selective α_{1A}-AR antagonist. A column switching technique was then used to combine the α_{1A}AR-CMC system with online HPLC/MS analysis for the direct separation and identification of active components. This method was successfully applied to the screening of active compounds present in a total *Radix Caulophylli* alkaloid preparation.

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doi:10.1016/j.jpba.2009.11.007

2. Experimental

2.1. Chemicals and materials

Silica gel (ZEX-II, 100–200 mesh) was obtained from Qingdao Meigao Chemical Co., Ltd. (Qingdao, China). Methoxamine, atenolol and aphrodine were from Sigma (St. Louis, MO, USA); tetracycline and berberine were from the National Institute for the Control of Pharmaceutical and Biological Products of China (NICPBP, Beijing, China). The HEK293 α_{1A}AR high expression cell line was a gift from Prof. Youyi Zhang at Peking University Third Hospital (Beijing, China). Dulbecco's modified Eagle's medium (Gibco-BRL, Rockville, MD, USA) containing 4.5 g/l glucose and supplemented with 10% fetal calf serum was from Sigma (St. Louis, MO, USA). Penicillin (50 U/mL), dimethyl sulfoxide (DMSO), MTT, RPMI-1640 medium and trypsin were also from Sigma. HPLC grade methanol and acetic ether were from Fisher Scientific (Pittsburgh, PA, USA). *Radix Caulophylli* was collected in the Qinba Mountains area (Shaanxi province, China) in October 2008 and dried at room temperature. Herbs were authenticated by the Department of Pharmacognosy at Xi'an Jiaotong University (Xi'an, China). A reference sample has been deposited at the Specimen Laboratory, Research and Engineering Center for Natural Medicine, Xi'an Jiaotong University, Xi'an, China.

2.2. Instruments

The high performance liquid chromatography tandem mass spectrometry (HPLC/MS) system was from Shimadzu Corporation (Kyoto, Japan). This included three LC-20AD pumps, a DGU-20A_3 degasser, a SIL-20A autosampler, a CTO-20A column oven, a SPD-20A UV/vis detector, a SPD-M20A diode array detector, a LCMS2010EV mass spectrometer, and an LCMS solution workstation. A VICI$_{AG}$ 8G-0911V 8 port 2-position switch valve (Valco Instrument Co. Inc., Houston, USA) was used as the column switcher and two 2.4 mL loops were used as the capture loop. A α_{1A}AR CMC column (10 mm × 2.0 mm I.D.) was used as the first dimension column and a Shimadzu Shim-pack VP-ODS column (150 mm × 2.0 mm I.D., 5 μm; Kyoto, Japan) was used as the second dimension column, respectively.

2.3. Preparation of standard solutions

Standard stock solutions of methoxamine, atenolol, aphrodine, tetracycline and berberine (1 mg/mL each) were separately prepared in methanol. A mixed standards solution (0.01 mg/mL) of methoxamine, atenolol, aphrodine, tetracycline and berberine was prepared in methanol.

2.4. Sample preparation

Total alkaloid of *Radix Caulophylli* was prepared as references [18] as follows: air-dried root materials (3 kg) were ground and subsequently extracted with 95% aq. EtOH for 2 h and the process was repeated twice. The combined alcoholic extract was concentrated under the reduced pressure to obtain a dark brown viscous mass. The concentrated extract was extracted with 2% HCl overnight while being stirred and the extraction was repeated once. The combined acidic extract was passed through positive ion exchange resin (LSD001) column and was eluated with 4% NH_4OH–MeOH, following 2% HCl–MeOH until the elute was tested negative for alkaloids in the Dragendorff test. The elute was evaporated to dryness to yield total alkaloid of *Radix Caulophylli*. Sample solutions of *Radix Caulophylli* total alkaloids (1 mg/mL) were prepared in methanol and stored at 4 °C in the dark. Working solutions (0.1 mg/mL or 0.01 mg/mL) were diluted with mobile phase on the day of the experiment.

2.5. α_{1A} CMC system

Cultured HEK293 α_{1A}AR high expression cells (7×10^6) were washed 3× with phosphate buffer saline (50 mM, pH 7.4) centrifuging each time at 110 × *g* for 10 min at 4 °C. Tris–HCl (50 mmol/L, pH 7.4) was added to produce a cell suspension; this was ruptured by sonication (30 min). The resulting homogenate was clarified by centrifugation (1000 × *g*, 10 min), the pellet was discarded, and cell membranes were recovered by centrifugation (12,000 × *g*, 20 min, 4 °C). The pellet was resuspended in 10 mL Tris–HCl (50 mmol/L, pH 7.4) and membranes were again collected by centrifugation (12,000 × *g*, 20 min, 4 °C). The pellet was suspended into 5 mL distilled water. Cell membranes were then immobilized by a previously reported technique [14]. Briefly, the α_{1A}AR cell membrane stationary phase (CMSP) was prepared by adsorption of the cell membrane suspension (5 mL) onto activated silica (0.05 g) under vacuum at 4 °C with gentle agitation. The α_{1A}AR CMSP was packed into a column (10 mm × 2.0 mm I.D.) using a wet packing procedure. Other chromatographic conditions were 5 mmol/L aqueous ammonium acetate as the mobile phase, 0.2 mL/min flow rate, a UV detector, and a column temperature of 37 ± 0.5 °C.

2.6. HPLC/MS system

HPLC conditions were a VP-ODS column (150 mm × 4.6 mm I.D., 5 μm), a mobile phase of methanol:0.1% aqueous formic acid (35:65, v/v) with a flow rate of 1.0 mL/min and a column temperature of 37 °C. MS conditions were as follows: nebulizer gas, N_2 (purity 99.999%); flow rate, 1.5 L/min; drying gas, N_2 (purity 99.999%); pressure, 0.1 MPa; interface temperature, 250 °C; heat block temperature, 200 °C; detector voltage, 1.5 kV; positive ionization mode.

2.7. Application of the α_{1A}AR-CMC-online-HPLC/MS system

The α_{1A}AR-CMC system was combined with an online HPLC/MS system by means of an 8 port column switcher. Fractions retained by the α_{1A}AR-CMC system were separately captured and then eluted onto the HPLC/MS system. This combined α_{1A}AR-CMC-online-HPLC/MS method was applied to the screening of components acting on α_{1A}AR; methoxamine was used as a positive control. Standard solutions and total alkaloids of *Radix Caulophylli* were analyzed separately.

2.8. Comptetive displacement assay

The log *k* values of caulophine were respectively measured on the α_{1A}AR-CMC system with different methoxamine concentrations (*C*) in the range 0–1.5×10^{-5} mol/L in the mobile phase. *k*-Values of caulophine and methoxamine were measured once the CMC system reached equilibrium with the mobile phase at each methoxamine concentration. The log *k* versus *C* curve of caulophine and methoxamine was plotted.

The affinity constant can be obtained by taking the reciprocal of *k* a linear plot of 1/*k* versus the concentration [M] [21,22]:

$$\frac{1}{k} = \frac{K_m}{K_c[S_{tot}]}[M] + \frac{1}{K_c[S_{tot}]} \quad (1)$$

The affinity constant for methoxamine (K_m) is obtained dividing the slope by the intercept. The concentration of common binding sites could be determined using the same substance both as a marker and as the analyte. Since $K_m = K_c$, Eq. (1) reduces to and $[S_{tot}]$ is the reciprocal of the slope:

$$\frac{1}{k} = \frac{1}{[S_{tot}]}[M] + \frac{1}{K_c[S_{tot}]} \quad (2)$$

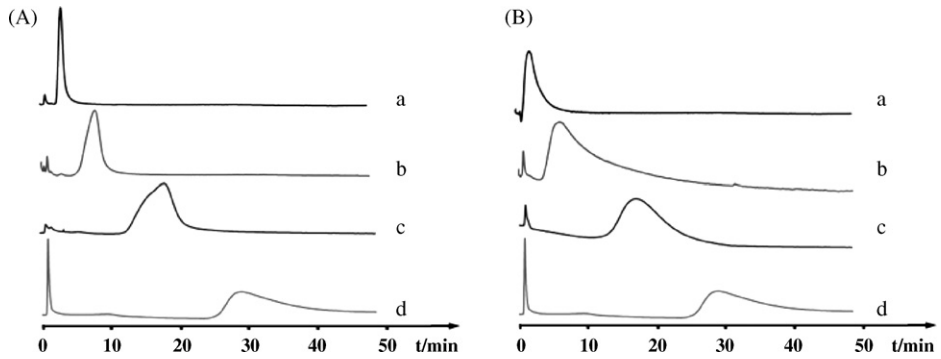

Fig. 1. Chromatogram of phentolamine on the α_{1A}AR-CMC system using either phosphate buffer (A) or ammonium acetate buffer (B) as the mobile phase. (a) 50 mmol L^{-1}; (b) 15 mmol L^{-1}; (c) 5 mmol L^{-1}; (d) 0 mmol L^{-1}.

The affinity constant for caulophine (K_c) is further calculated using Eq. (1).

3. Results and discussion

3.1. α_{1A}AR-CMC model

The α_{1A}AR-CMC method was first developed by Wang et al. to study the interactions of ligands with the α_{1A}AR [16]. Phosphate buffer was the mobile phase in this study. In the present investigation we aimed to develop a combined α_{1A}AR-CMC-online-HPLC/MS method, precluding the use of non-volatile salts in the mobile phase. We therefore compared the chromatographic characteristics of the alpha-adrenergic antagonist phentolamine in the α_{1A}AR-CMC system at different concentrations of either ammonium acetate or phosphate buffer in the mobile phase. There was no significant difference in performance between the two buffers (Fig. 1); 5 mmol/L ammonium acetate was therefore selected as the mobile phase for the α_{1A}AR-CMC system.

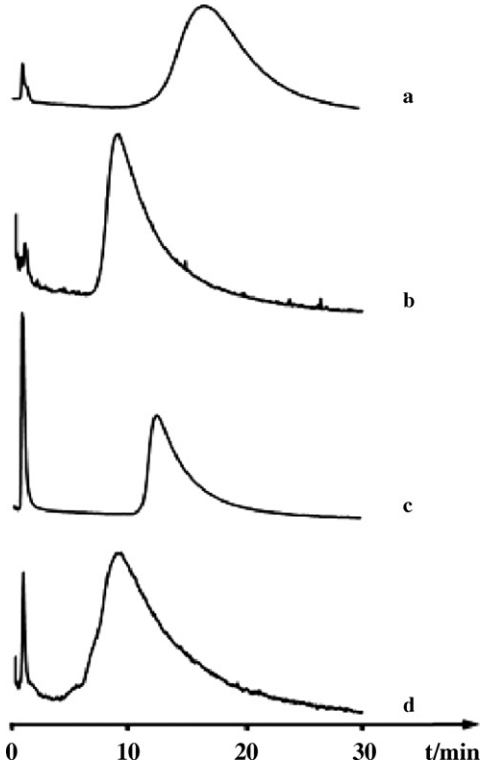

Fig. 2. Chromatogram of four α_{1A}AR ligands on the α_{1A}AR-CMC system. Ligands were: (a) phenolamine; (b) 5-MU; (c) methoxamine; (d) oxymetazoline.

Four α_{1A}AR ligands were studied on the α_{1A}AR-CMC system. As shown in Fig. 2, all four ligands were significantly retained, indicating that the α_{1A}AR-CMC system is able to specifically interact with α_{1A}AR ligands.

3.2. Combined α_{1A}AR-CMC-online-HPLC/MS

The α_{1A}AR-CMC system was combined with online HPLC/MS analysis by the use of a column switching device. As shown in Fig. 3A, the first fraction retained by the α_{1A}AR-CMC column (C_1) was captured into a loop, and was then pumped onto a ODS analytical column (C_2) for qualitative analysis (Fig. 3B). At the same

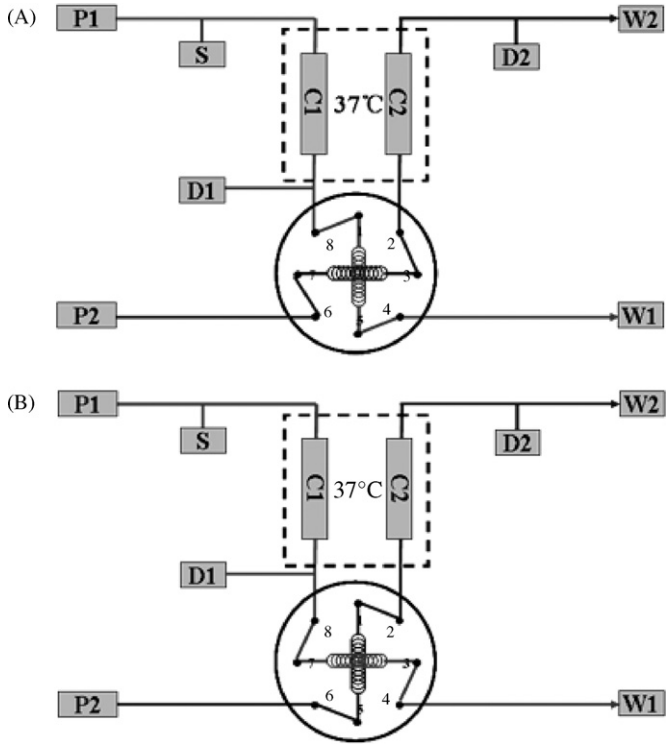

Fig. 3. Outline of the combined α_{1A}AR-CMC-online-HPLC/MS method. (A) Affinity procedure using the α_{1A}AR-CMC system. The first retention fraction was captured into the first loop after the α_{1A}AR-CMC column (C_1) from a complex sample (S); establishing the equilibrium procedure of HPLC/MS system from the second loop to an analytical column (C_2) with mobile phase of second D. (B) Analytical identification procedure using HPLC/MS. The first captured fraction was analyzed using C_{18}-HPLC/MS to separate and analyze their structural characteristics; the second retention fraction (if any) was captured into the second loop after the α_{1A}AR-/CMC column (C_1) from the same sample (S). D_1, UV/vis detector; D_{MS}, DAD and MS detector; P_1 and P_2, pumps.

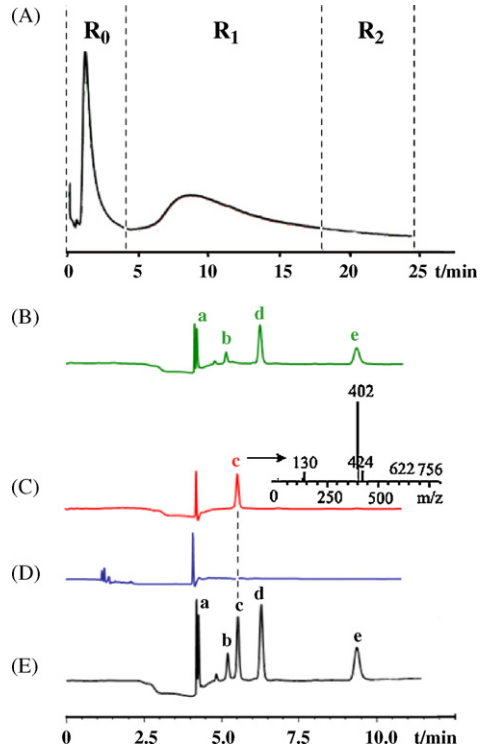

Fig. 4. Chromatograms of mixed standards using the combined α_{1A}AR-CMC-online-HPLC/MS method. (A) α_{1A}AR-CMC chromatogram of the mixed standards. (B) HPLC/MS chromatograms of the fractions R_0 captured into the loops. (C) HPLC/MS chromatograms of the fractions R_1. (D) HPLC/MS chromatograms of the fractions R_2. (E) HPLC/MS chromatogram of the mixed standards solution. (a) Atenolol; (b) tetracycline; (c) methoxamine; (d) aphrodine; (e) berberine.

time, the second retention fraction was collected into another loop and pumped into column C_2 for analysis. A mixed standards solution containing methoxamine, atenolol, tetracycline, aphrodine, and berberine was used to verify the suitability and reliability of the α_{1A}AR-CMC-online-HPLC/MS system (Fig. 4). Of the five standards, only methoxamine is selectively acts on α_1AR. The chromatogram of the mixed standards solution on α_{1A}AR-CMC is presented in Fig. 4A. Three fractions (indicated by dotted lines) were sequentially captured into loops and then switched onto column C_2 for chromatographic separation (Fig. 4B–D) and MS identification (Fig. 4C). This experiment demonstrated that methoxamine was specifically retained by the α_{1A}AR-CMC system from the solution of mixed standards and could be simultaneously analyzed by HPLC/MS.

3.3. Practical application

Alkaloids are the principal type of active components found in natural herbs. The α_{1A}AR-CMC-online-HPLC/MS method was first applied to the screening of total alkaloids from *Radix Caulophylli* for compounds binding to α_{1A}AR. Chromatograms of total alkaloids obtained using the α_{1A}AR-CMC-online-HPLC/MS method are presented in Fig. 5. This revealed that there was only one significant retention fraction (Fig. 5A); this fraction was captured and switched onto the HPLC/MS system online for further separation and identification. As shown in Fig. 5B–D, peaks *b* and *c* emerged as the two principal components of the retention fraction. From UV and MS data (Fig. 5C), and the literature [23,24], peaks *b* and *c* were respectively identified as magnoflorine and caulophine. Comparison with Fig. 5C indicated that magnoflorine and caulophine were indeed present in the total *Radix Caulophylli* alkaloid preparation. The chemical structures of magnoflorine and caulophine are shown in Fig. 6.

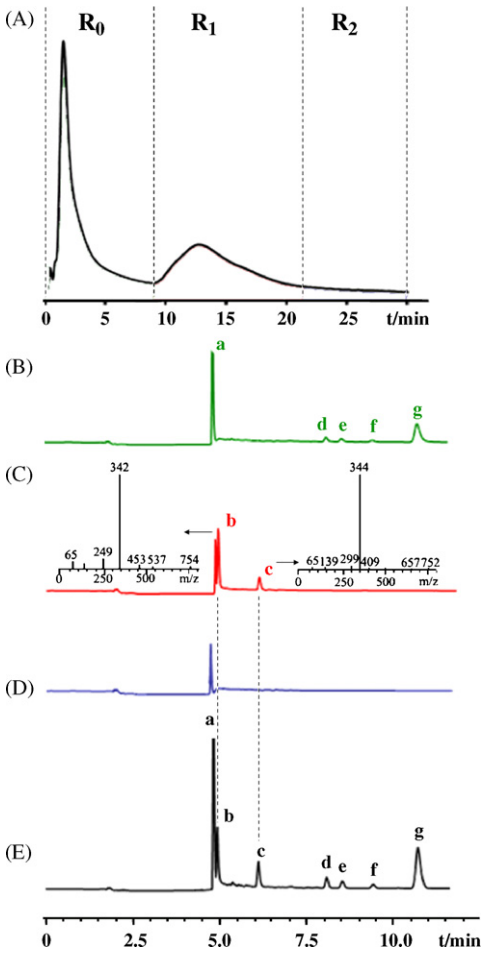

Fig. 5. Chromatograms of total alkaloids of *Radix Caulophylli* using the α_{1A}AR-CMC-online-HPLC/MS method. (A) α_{1A}AR-CMC chromatogram of total alkaloids. (B) HPLC/MS chromatograms of the fractions R_0 captured into the loops. (C) HPLC/MS chromatograms of the fractions R_1. (D) HPLC/MS chromatograms of the fractions R_2. (E) HPLC/MS chromatogram of the mixed standards solution.

3.4. Interactions between caulophine and cell membrane receptors

The interaction between caulophine and cell membrane receptors was quantified by competition experiments: this allowed a $\log k$ versus C displacement curve to be plotted (Fig. 7). The $\log k$ values of caulophine decreased when the concentration of methoxamine increased, indicating that binding of caulophine to α_{1A}AR on the α_{1A}AR-CMC system could be displaced by methoxamine (Fig. 7B). This result suggests that α_{1A}AR is likely to be a common target from both molecules. When the concentration of methoxamine exceeded 1.0×10^{-5} mol/L, $\log k$ values of caulophine became

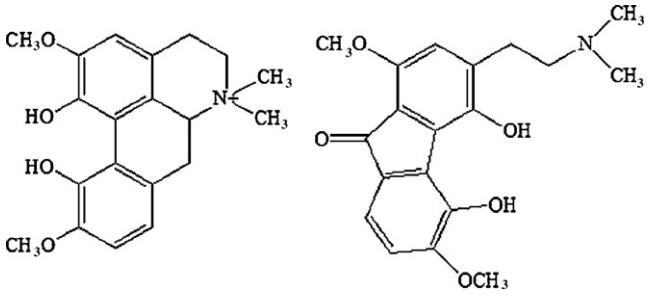

Fig. 6. Chemical structures of magnoflorine (left) and caulophine (right).

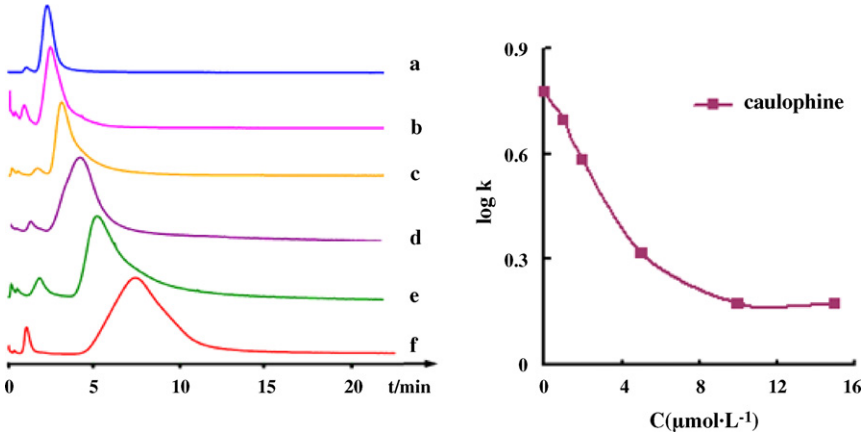

Fig. 7. Competitive displacement chromatograms (left) and log k versus C curves (right) of caulophine on the α_{1A}AR-CMC system in the presence of different mobile phase methoxamine concentrations. Concentrations were: (a) 1.5×10^{-5} mol/L; (b) 1.0×10^{-5} mol/L; (c) 0.5×10^{-5} mol/L; (d) 0.2×10^{-5} mol/L; (e) 0.1×10^{-5} mol/L; (f) 0×10^{-5} mol/L.

invariable, indicating that dynamic equilibrium had been achieved. This also suggests that caulophine interacts with α_{1A}AR in a manner similar to methoxamine.

The affinity constants of methoxamine can be calculated by determining the ratio of the slope to the intercept a linear plot for $1/k$ versus C of caulophine plot. The affinity constant of methoxamine binding to α_{1A}AR was obtained as 2.67×10^5 M^{-1}, and that of caulophine was 3.10×10^3 M^{-1}. It was indicated that caulophine was low-affinity to α_{1A}AR.

4. Conclusions

We have developed a combined α_{1A}AR-CMC-online-HPLC/MS method; this was successfully used to screen total alkaloids of the traditional Chinese medicine *Radix Caulophylli* for active components able to bind to α_{1A}AR. By combining specific retention in the α_{1A}AR-CMC system with accurate identification by online HPLC/MS analysis, this method affords a means for drug discovery by screening natural medicinal herbs for new pharmacologically active molecules targeting specific receptors such as α_{1A}AR.

Acknowledgements

This work was supported by National Natural Science Foundation of China (Grant Numbers 30801450 and 30730110) and Program for New Century Excellent Talents in University (NCET-08-0437).

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