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Screening the bioactive compounds in aqueous extract of *Coptidis rhizoma* which specifically bind to rabbit lung tissues β_2-adrenoceptor using an affinity chromatographic selection method

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ABSTRACT

A receptor affinity chromatographic selection method was developed for screening the bioactive compounds binding to β_2-adrenoceptor (β_2-AR) in *Coptidis rhizome*. The bioactive compounds were analyzed by molecular recognition with a β_2-AR affinity column. The retention compounds eluted from the β_2-AR column were separated online with reverse-phase high-performance liquid chromatography by column switching technology, and identified by a coupled ion-trap mass spectrometer. Four compounds were screened as the bioactive compounds of *Coptidis rhizome* and identified as 2,9,10-trimethoxy-3-hydroxyl-protoberberine (jateorhizine), 2,3-methylenedioxy-9-methoxy-protoberberine, 2,3,9,10-tetramethoxy-protoberberine (palmatine) and 2,3-methylenedioxy-9,10-dimethoxy-protoberberine (berberine). The association constants of jatrorrhizine, palmatine and berberine to the β_2-AR were determined by the zonal elution method with standards. Berberine and palmatine had only one type of binding site on the immobilized β_2-AR. Their association constants were $(2.28 \pm 0.11) \times 10^4$/M and $(3.00 \pm 0.10) \times 10^4$/M, respectively. Jatrorrhizine had at least two type of binding sites on the immobilized β_2-AR, and the corresponding association constants were $(2.20 \pm 0.09) \times 10^{-4}$/M and $(6.78 \pm 0.001) \times 10^5$/M.

1. Introduction

Bioactive compounds from medicinal plants have been shown to be an important source for innovation in drug discovery [1,2]. More than 60% of approved and potential drug application candidates are bioactive compounds or chemically related to the bioactive compounds of medicinal plants. These candidates have been successful in reducing pain and suffering, and reducing death rates, even influencing the pharmaceutical industry [3].

Historically, the technologies for screening bioactive compounds from medicinal plants included classic pharmacological tools [4,5], combinatorial chemistry [6], ultra-high-throughput screening approaches [7] and the use of structural information in virtual ligand screening and structure-based drug design [8,9]. Many novel medicines have been developed on the basis of these approaches (at least in part). However, the investment in these technologies has not yet reversed the downward trend in the number of new chemical entities reaching the market [10]. Accordingly, there continues to be a need for new approaches to rapidly screen the bioactive compounds from complex matrices such as medicinal plants and traditional Chinese medicines (TCMs).

High-performance liquid chromatography (HPLC) is an efficient method for screening and analyzing complex matrices due to its high resolution and selectivity. The retention behavior based on interactions of the solutes with the stationary phase can be obtained, but information on their bioactivities is unknown due to the lack of correlation between the bioactivities and the retentions in conventional HPLC. To overcome this problem, techniques combining HPLC with other bioassays have been developed [11]. For instance, affinity chromatography with immobilized biomacromolecules as stationary phases was introduced to screen and analyze bioactive compounds from TCMs [12].

G-protein-coupled receptors (GPCR) are the targets for a broad range of drugs encompassing diverse therapeutic applications. Nearly 50% of marketed medicines function via GPCRs, including about 20% of the 50 best-selling medications [13]. One type of GPCR, β_2-adrenoceptor (β_2-AR), is mainly distributed in blood ves-

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sels, the heart and the lungs [14,15]. The β_2-AR has important roles in the treatment of heart disease, obesity, and diseases of the respiratory system [16–18]. Wainer et al. [19–21] absorbed the receptor on the surface of an immobilized artificial membrane with phosphatidylcholine silica beads to obtain an immobilized receptor-based stationary phase. They proved that the stationary phase could be applied in screening ligands of the β_2-AR.

In our previous work [22], a β_2-AR affinity stationary phase was obtained by immobilizing β_2-AR onto the surface of macro-pore silica gel through covalent bonds. This stationary phase was also used to determine the association constant of terbutaline and salbutamol binding to the β_2-AR. The results showed that β_2-AR immobilized through covalent bonds could maintain its biological activity and selectivity, and could be used to investigate the interaction between the receptor and ligands.

Several studies showed that the synthetic and natural isoquinoline-like compounds exhibited β_2-adrenergic agonistic and antagonistic activity [23]. Nikulin et al. had synthesized a series of trimetoquinol analogues. Furthermore, their studies also concluded that 1-benzyl-1,2,3,4-tetrahydroisoquinoline-6,7-diols could used as a novel affinity and photoaffinity probes for β-adrenoceptor subtypes [24]. Pyo et al. had synthesized (*R*)-(+)- and (*S*)-(−)-higenamine and their analogues. Their further investigation showed that all the synthetic compounds showed dose-dependent inhibitory activities to ADP, collagen and epinephrine induced platelet aggregation [25].

This contribution was designed to establish an online method for screening the bioactive compounds from *Coptidis rhizome*, one of the well-known TCM, by a β_2-AR stationary phase coupled with column switching high-performance liquid chromatography–mass spectrometry (HPLC–MS).

2. Experimental

2.1. Instruments and materials

A ZZXT-A packing machine was supplied by Dalian Yilite Analytic Instruments Company Ltd (Dalian, China). A BT01-100 peristaltic pump was obtained from Baoding Lange Constant Flowing Pump Company Ltd (Baoding, China). Digitoxin was from Shanghai Chemical Reagents Supplier Company Ltd (Shanghai, China); 6B agarose was from Bio-sep-technique Company Ltd, Xi'an Jiaotong University, (Xi'an, China). Dithiothreitol (DTT), benzyl sulfonylfluride and 1,4-butylene-glycol-1,2-dicyclohydrogen glyceride were from Wuxi Huili Synthetic Materials Company Ltd (Wuxi, China). *N*,*N*′-carbonyldiimidazole, leupetin and soybean trypsin inhibitor were all from Sigma–Aldrich (St. Louis, MO, USA). Macro-pore silica gel (SPS 300-7, pore size 300 Å, particle size 7.0 μm) was from Fuji Silysia Chemical Company Ltd (Tokyo, Japan). All other reagents were analytically pure.

2.2. Preparation and specific properties of the β_2-AR column

With reference to our previous reports [22], the β_2-AR was purified from rabbit lung tissues, immobilized on the surface of silica gel, and a β_2-AR column prepared (50 mm × 4.6 mm; particle size, 7.0 μm). The specific properties of the β_2-AR column were investigated by determining the association constants of terbutaline by frontal analysis. The results showed that the association constant of terbutaline binding to the β_2-AR on the stationary phase was 9.54 ± 0.23/M, demonstrated that the column has the properties of recognizing β_2-AR ligands. The mobile phase for this experiment consisted of 10.0 mmol/L Tris–HCl, 1.0 mmol/L ethylenediamine tetra-acetic acid (EDTA) and 2.0 mmol/L $MgCl_2$ (prepared by dissolving specified quantities of Tris, EDTA and $MgCl_2$ in 1 L of water and adjust the pH to 7.2 with HCl). The flow rate was set at 0.3 mL/min. The results showed that the β_2-AR column could specifically recognize its ligands.

2.3. Extraction of Coptidis rhizoma

The rhizome of *Coptidis rhizoma* (*Coptis chinensis* Franch) was purchased from the Xi'an Wan Shou herbal pieces Co. Ltd. The source of the herb was Sichuan province and harvested on August 2008. The authentication of this herb was performed by comparison with appropriate voucher specimens at the herbaria and by performing both physical and chemical properties identifications according to The Pharmacopoeia of People's Republic of China (2005 edition). An aliquot of 50 g dried *Coptidis rhizoma* was immersed in an eightfold volume of water for 30 min before being refluxed twice (90 min each). The suspension was filtered, and the resulting solution concentrated to 100 mL for use.

2.4. Screening for the bioactive compounds in Coptidis rhizoma

The bioactive compounds in *Coptidis rhizoma* were screened by β_2-AR column coupled with column switching HPLC–MS. The chromatographic system consisted of an Agilent 1100 Series HPLC apparatus (vacuum degasser, quaternary gradient pump, autosampler, column thermostat, diode array detector) and an additional isocratic Agilent 1100 pump (Waldbroel, Frankfurt, Germany). The water extraction of *Coptidis rhizome* was screened on the β_2-AR column (50 mm × 4.6 mm; particle size, 7.0 μm). The retention peak was switched into an Agilent SB-C_{18} reverse-phase HPLC column (4.6 mm × 150 mm; particle size, 5 μm) coupled with a trap mass spectrometer through a time-controlled six-way valve (Waldbroel) for online separation and identification.

For the screening experiment, the mobile phase consisted of 1.0 mmol/L EDTA, 2.0 mmol/L $MgCl_2$ and 10.0 mmol/L Tris–HCl (pH 7.2). The flow rate was 0.3 mL/min, the wavelength of the detection was 345 nm for all analytes, and the column temperature was 37 °C. The separating experiment was carried on an Agilent reverse-phase column by isocratic elution. The mobile phase was a solution of acetonitrile and water (50:50, v/v) with the addition of 5.0 mmol/L ammonium formate (pH 3.5), and the flow rate was set at 0.6 mL/min.

MS detection was on an Agilent SL trap system. The electrospray ion source-dependent conditions were identical for all analytes with a capillary voltage of 4500 V in the positive ion mode. Nitrogen as a nebulizer was set at 40 psi. The flow rate of dry gas was 7.0 L/min, and the temperature of the dry gas was 350 °C. The collision gas (He) for the MS/MS mode in the trap was set at a flow of 4 (instrument units). The high voltage of capillary was 4000 V, and its end plate offset was −500 V. The scan range was from m/z 50 to 1000.

3. Results and discussion

3.1. Selection of Coptidis rhizoma

Coptidis rhizome is the root of *Coptis chinensis* Franch. *Coptidis rhizome* has been widely used as a clearing heat and detoxifying agent Extensive studies exhibited that the herb had many pharmacological effects with strong clinical implications, including antiviral, antibacterial, antineoplastic, antiinflammatory, antioxidative, antihypertensive, antihyperglycemic and cholesterol-lowering effects [26–31]. Generally, the chemical compounds in *Coptidis rhizoma* consisted of berberine, jatrorrhizine, coptisine, and palmatine. Most of the compounds are now used as antibiotics, anti-arrhythmia agents, and to treat expanding coronary vessels [32].

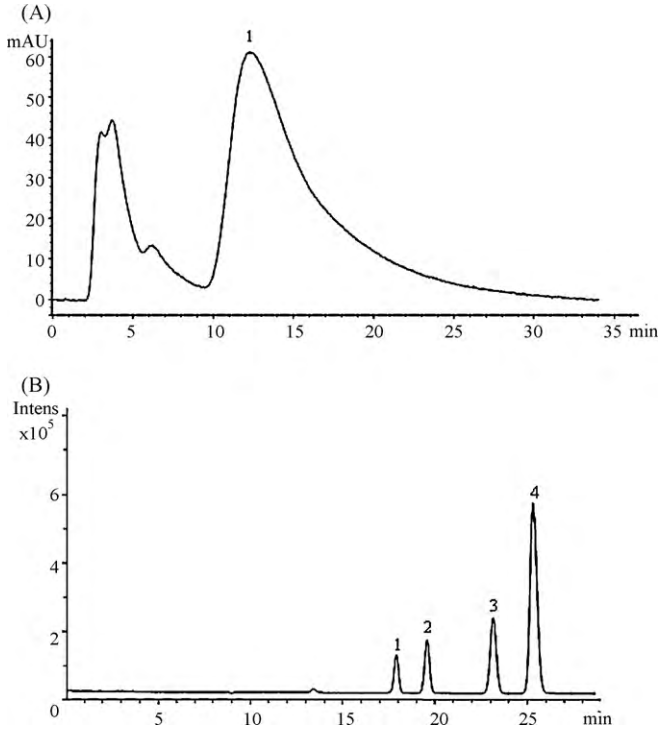

Fig. 1. Chromatograms of Coptidis rhizome extraction and total ion current of the retention compounds. (A) Chromatograms of *Coptidis rhizoma* extraction on the β_2-AR column. Peak 1 was the analyte of interest, and was transferred to the C_{18} column for separation and identification. (B) Total ion current of the retention compounds. (1) jatrorrhizine; (2) 2,3-methylenedioxy-9-methoxy-protoberberine; (3) palmatine; (4) berberine.

The β_2-AR is a member of a family of seven transmembrane proteins coupled to G-proteins. The β_2-AR is closely related to physiological processes in the cardiovascular and respiratory systems, so it has become the main target of drugs to treat diseases affecting these systems. From this viewpoint, *Coptidis rhizoma* was proposed to contain bioactive compounds that interact with the β_2-AR.

The β_2-AR has been reported to have three types of binding sites. One involves an interaction (presumably a salt bridge) between the amine group of the ligand and the carboxylate side chain of Asp^{113} in the third hydrophobic domain. The other two types of binding interaction are through hydrogen bonds, but are different. One is between the hydroxyl side chain of Ser^{204} and the *meta*-hydroxyl group of the ligand, and the second is between the hydroxyl side chain of Ser^{207} and the *para*-hydroxyl group of the ligand [33]. A model for the agonist binding site of β_2-AR has emerged in which the ligand is bound within the hydrophobic core of the protein in the transmembrane helices and anchored by specific molecular interactions. Asp binds to nitrogen while the two Ser residues interact with hydroxyl groups on the phenyl ring of the β_2-agonist molecule [10,34]. Thus, the specific molecular requirements for ligand binding to the β_2-AR are an amine group, aromatic ring and catechol hydroxyl groups. Reports showed that the predominant chemical compounds in *Coptidis rhizoma* are alkaloids of isoquinoline (which readily form the quaternary ammonium salt) [35].

3.2. Screening the bioactive compounds in Coptidis rhizoma

The chromatogram of the water extract of *Coptidis rhizoma* on the β_2-AR column before switching is shown in Fig. 1A. The extraction had some retention compounds on the β_2-AR column (annotated as peak 1). The profile of peak 1 was much wider than normal affinity peaks. This indicated that several co-elutes were included in peak 1 which also had a specific interaction with the β_2-AR column. Further separation should therefore be processed before identification of the compounds included in the peaks. For this purpose, column switching was designed to switch peak 1 to a C_{18} column for online separation and removal of the inorganic salts from the mobile phase in the β_2-AR column. Finally, the eluate from the reverse column was detected and identified by ion trap mass spectrometer. The corresponding total ion current is shown in Fig. 1B. The retention peak on the β_2-AR column was separated to four peaks, and the m/z values of their strongest intensity peaks were 337.9 $[M+H]^+$, 306.1 $[M+H]^+$, 351.8 $[M+H]^+$ and 335.9 $[M+H]^+$ (Fig. 1B). From this result, it could be seen that the four compounds comprised nitrogen, and that their molecular weights were 336.9, 305.1, 351.8 and 334.9. The MS^n technique of trap mass was used for obtaining the daughter ions of the four ions. MS^2 and MS^3 of the four ions (m/z) were: 322.7 and 294.4; 243.9 and 145.7; 336.7 and 308.2; and 320.7 and 292.6. For ions of 337.9 $[M+H]^+$, 351.8 $[M+H]^+$ and 335.9 $[M+H]^+$, the information of father ions and daughter ions were identical to the information of jatrorrhizine, palmatine and berberine standards (structures shown in Fig. 2A, C, and D). For the ion of m/z 306.1, the retention time, total ion current, extraction ion chromatogram, and fragment ions indicated that it was also an alkaloid compound. According to the reports of Chen et al. [36] and Bian et al. [37], it may be identified as 2,3-methylenedioxy-9-methoxy-protoberberine (structure shown in Fig. 2B).

3.3. Comparison with other screening methods

Wang et al. [12] initially introduced the novel strategy for screening and analyzing the biologically active components in *Angelica sinensis* using immobilized human serum albumin on silica as the stationary phase. Mao et al. [38] introduced the technique for screening and analyzing permeable compounds in TCMs. More than ten peaks were resolved from the methanol extract from *Radix Angelica sinensis* based on their interactions with a coated liposome stationary phase. He et al. [39] detailed a technique with an immobilized cell membrane as the stationary phase for screening the bioactive components of TCMs. In general, serum albumin and liposome are only the transporters (not the effectors) for a drug to exert its therapeutic effect. Screening technologies based on serum albumin and liposome therefore does not reflect the pharmacological activity of a compound. A screening technology based upon the cell membrane could reflect the bioactivity of a compound, but has the disadvantage of poor specificity due to the many types of receptor on the surface of cell membranes.

Recently, a method based on a microdialysis sampling coupled with HPLC was established for screening and analyzing the bioactive compounds in TCMs [40]. The results indicated that berberine, palmatine and jatrorrhizine were the bioactive compounds in *Coptidis rhizoma* that bind to calf thymus DNA. In the report of Zou et al. [41], the DNA-immobilized affinity HPLC column was prepared and applied to the biological fingerprinting analyses of *Coptidis rhizoma*. The results demonstrated that seven compounds in *Coptidis rhizoma* (including berberine, palmatine and jatrorrhizine) were active in binding to immobilized DNA, and that the method could be an effective alternative for screening and analyzing the multiple DNA-binding active compounds in complex samples such as natural products.

In the present study, the β_2-AR stationary phase coupled with column switching–HPLC–MS was used to screen the bioactive compounds in *Coptidis rhizoma*. Four compounds, including berberine, palmatine and jatrorrhizine, were the bioactive compounds that bind to the β_2-AR. The proposed method at least had three advantages compared with the above methods. First, the β_2-AR was immobilized on the macro-pore silica gel by covalent bonds. This not only avoids the loss of the β_2-AR on the surface of the stationary phase, but also improves the stability of the β_2-AR [42]. Second,

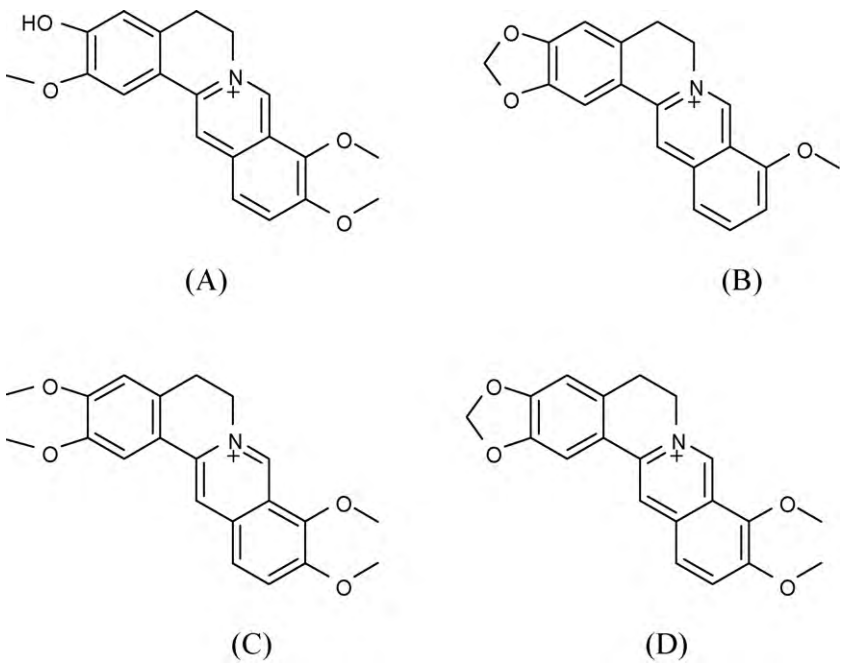

Fig. 2. Structures of (A) jatrorrhizine, (B) 2,3-methylenedioxy-9-methoxy-protoberberine, (C) palmatine, (D) berberine.

molecular recognition of the receptor to ligands had high selectivity. Finally, by online coupling with MS, this method not only had exquisite sensitivity, but also provided structural information of the bioactive compounds.

3.4. Determination of the association constants of berberine, palmatine and jatrorrhizine with the β_2-AR

In the method of zonal elution, a known concentration of a competing agent (I) can combine with the ligand (L). If (I) and the injection solute (A) competitively bind to a single site of (L), then Eqs. (1) and (2) showed the mass balance of the chromatographic procedure, and Eq. (3) can be used to represent the retention of (A) on the column [43]:

$$L + A \rightleftarrows LA \quad [LA] = K_A[L][A] \tag{1}$$

$$L + I \rightleftarrows LI \quad [LI] = K_A[L][I] \tag{2}$$

$$\frac{1}{k' - X} = \frac{V_m K_I[I]}{K_A m_L} + \frac{V_m}{K_A m_L} \tag{3}$$

where V_m is the void volume of the chromatographic system (i.e., the elution volume of a non-retained solute), m_L is the moles of binding sites in the column involved in the competition of (A) with (L), and [I] is the concentration of the competing agent in the mobile phase. K_A is the association equilibrium constant for the binding of (A) to (L) and K_I is the association equilibrium constant for the interaction of (I) at the same site. The term k' is the capacity for the injected solute, or $k' = t_R/t_m - 1$, where t_R is the measured retention time of the solute and t_m is the void time of the chromatographic system. The term X is a constant that represents the portion of k' due to the binding of A to the sites at which (I) does not compete, or due to the sites for which the contribution to k' is already known through independent measurements.

If (A) and (I) are identical, $X = 0$, K_A equates K_I, and Eq. (3) can be simplified to Eq. (4):

$$\frac{1}{k^1} = \frac{V_m[I]}{m_L} + \frac{V_m}{K_A m_L} \tag{4}$$

According to the theory described above, the interaction between berberine, palmatine, and jatrorrhizine with the β_2-AR was investigated by the immobilized β_2-AR column through self-competitive studies. Their capacity factors in the β_2-AR column are shown in Table 1. The capacity factors of berberine, palmatine, and jatrorrhizine decreased with increase of their concentration in the mobile phase, and did not reach saturation. According to Eq. (4), curves could be plotted by the corresponding $1/k'$ of berberine and palmatine versus their concentration in the mobile phase (Fig. 3A and B). In Fig. 3A and B, the k' of berberine and palmatine presented a good linear relationship with their corresponding concentration of berberine and palmatine in the mobile phase, and the correlation coefficients were 0.9951 and 0.9967. This suggested only one type of binding site between berberine and palmatine and the β_2-AR on the column. According to Eq. (4), the association constants of berberine and palmatine on the β_2-AR column were $(2.28 \pm 0.11) \times 10^4$/M and $(3.00 \pm 0.10) \times 10^4$/M, and the concentrations of binding sites were $(1.47 \pm 0.06) \times 10^{-4}$ M and $(1.10 \pm 0.04) \times 10^{-4}$ M.

For the self-competitive study of jatrorrhizine, the plot of $1/k'$ versus its concentration gave a weak linear relationship over the

Table 1
Influence of the mobile phase concentrations of berberine, palmatine and jatrorrhizine on their corresponding capacity factors of injected berberine (k'_b), palmatine (k'_p) and jatrorrhizine(k'_j).

[I]×10^{-6} M	$k'_{berberine}$	$k'_{palmatine}$	$k'_{jatrorrhizine}$
0	3.42	3.34	3.14
1	3.35	3.30	2.83
2	3.26	3.13	2.69
4	3.04	2.9	2.51
6	2.89	2.82	2.26
8	2.78	2.62	2.08
10	2.69	2.5	2.01
15	2.5	2.24	1.83
20	2.34	2.10	1.70
25	2.14	1.90	1.58

The values shown for k'_b, k'_p and k'_j represent the average results of triplicate injections. [I] were the concentrations of berberine, palmatine and jatrorrhizine in the mobile phase when determine their corresponding capacity factors, respectively.

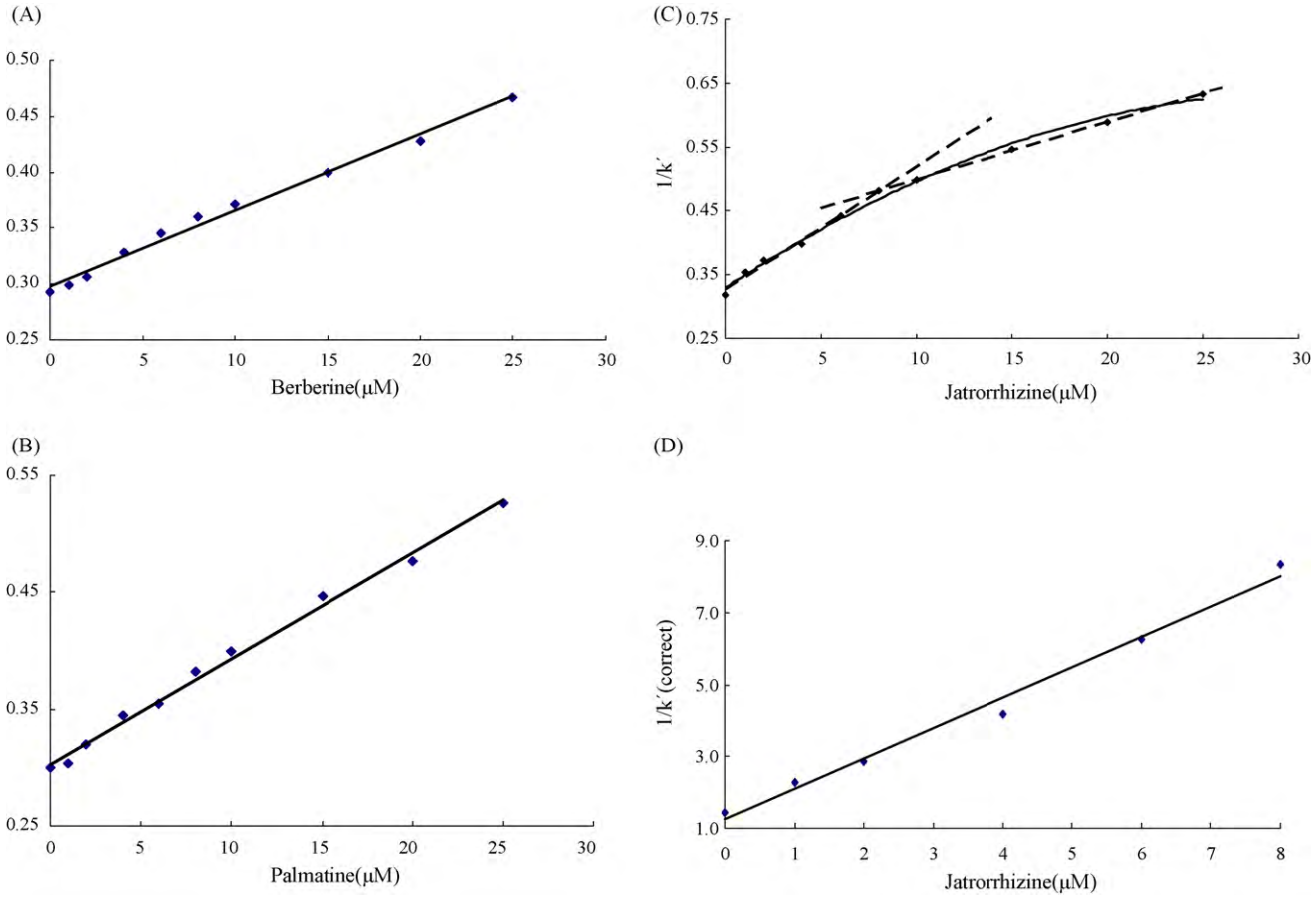

Fig. 3. Plots of zonal eluting experiments. (A) Change in $1/k'$ with the mobile phase concentration of berbeine when berberine was the injection solute. All data were from an immobilized β_2-AR column. The equation of the best-fit line was $y=(6800\pm317\,M^{-1})x+(0.298\pm0.014)$. The correlation coefficient was 0.9951; (B) Change in $1/k'$ with the mobile phase concentration of palmatine when palmatine was the injection solute. All data are from an immobilized β_2-AR column. The equation of the best-fit line was $y=(9100\pm284\,M^{-1})x+(0.303\pm0.012)$. The correlation coefficient was 0.9967; (C) Change in $1/k'$ with the mobile phase concentration of jatrorrhizine when jatrorrhizine was the injection solute. All data were from an immobilized β_2-AR column. The best-fit line shown for the first six and the last five points of the jatrorrhizine data were $y=(19{,}200\pm463\,M^{-1})x+(0.327\pm0.016)$ and $y=(9000\pm352\,M^{-1})x+(0.409\pm0.013)$, respectively. The corresponding correlation coefficients were 0.9945 and 0.9997; (D) Change in corrected $1/k'$ with the mobile phase concentration of jatrorrhizine when jatrorrhizine was the injection solute. The equation of the best-fit line was $y=(847{,}000\pm437\,M^{-1})x+(1.25\pm0.104)$ and the correlation coefficient was 0.9943.

entire concentration range (Fig. 3C). This indicated that jatrorrhizine had at least two separate types of binding site on the β_2-AR column. A linear relationship between $1/k'$ and its concentration in the range 8–25 μM was found to fit well with the linear relationship given by Eq. (4). Assuming that the two types of binding site are high-affinity sites and low-affinity sites, the binding sites of low-affinity sites were calculated to be $(1.11\pm0.02)\times10^{-4}$ M by Eq. (4) at 8–25 μM, and the corresponding association constant was $(2.20\pm0.09)\times10^{-4}$/M. The properties of the high-affinity sites could be investigated by the reported method in the range of 0–8 μM [44]. First, the theoretical value of the capacity factors in the range of 0–8 μM was calculated by the regression equation of 8–25 μM according to Eq. (4). The resulting values were subtracted from the experimental capacity factors of 0–8 μM in Table 1. The obtained capacity factors were used to plot the curve of $1/k'$ versus the concentration of jatrorrhizine in the mobile phase (Fig. 3D). According to Eq. (4), the high-affinity binding sites were calculated to be $(1.18\pm0.002)\times10^{-6}$ M, and the corresponding association constant was $(6.78\pm0.001)\times10^{5}$/M.

4. Conclusions

A rapid and valid method based on immobilized β_2-AR coupled with column switching–HPLC–MS was developed and applied to screen the bioactive compounds of *Coptidis rhizoma*. Four compounds, including berberine, palmatine and jatrorrhizine, were the bioactive compounds binding to the β_2-AR. Berberine and palmatine had only one type of binding site to the β_2-AR, but jatrorrhizine had at least two separate binding sites to the β_2-AR. The results detailed above demonstrated that this receptor HPLC affinity selection-mass spectrometric method could probe the interaction between the β_2-AR and multiple bioactive compounds. It could be used as an effective alternative for screening multiple receptor binding bioactive compounds in complex samples such as TCMs.

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