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ORIGINAL PAPER

Cell membrane chromatography competitive binding analysis for characterization of α_{1A} adrenoreceptor binding interactions

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Abstract A new high α_{1A} adrenoreceptor (α_{1A}AR) expression cell membrane chromatography (CMC) method was developed for characterization of α_{1A}AR binding interactions. HEK293 α_{1A} cell line, which expresses stably high levels of α_{1A}AR, was used to prepare the stationary phase in the CMC model. The HEK293 α_{1A}/CMC-offline-HPLC system was applied to specifically recognize the ligands which interact with the α_{1A}AR, and the dissociation equilibrium constants (K_D) obtained from the model were $(1.87\pm0.13)\times10^{-6}$ M for tamsulosin, $(2.86\pm0.20)\times10^{-6}$ M for 5-methylurapidil, $(3.01\pm0.19)\times10^{-6}$ M for doxazosin, $(3.44\pm0.19)\times10^{-6}$ M for terazosin, $(3.50\pm0.21)\times10^{-6}$ M for alfuzosin, and $(7.57\pm0.31)\times10^{-6}$ M for phentolamine, respectively. The competitive binding study between tamsulosin and terazosin indicated that the two drugs interacted at the common binding site of α_{1A}AR. However, that was not the case between tamsulosin and oxymetazoline. The results had a positive correlation with those from radioligand binding assay and indicated that the CMC method combined modified competitive binding could be a quick and efficient way for characterizing the drug–receptor interactions.

Keywords HEK293 α_{1A} adrenoreceptor · Cell membrane chromatography · Modified competitive binding · Dissociation equilibrium constant · Binding site

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Introduction

Alpha1 adrenoreceptor (α_1AR) comprises a family of the G-protein-coupled receptors that was physiologically activated by the catecholamines, epinephrine, and norepinephrine [1]. Three cDNAs encoding α_1AR subtypes (α_{1A}, α_{1B}, and α_{1D}) have been cloned from rat, human, and other sources, expressed in cell lines, and indentified pharmacologically in native tissues [2, 3]. Benign prostate hyperplasia (BPH) is a common disease and impacts health-related quality of life profoundly. It has been reported that the α_{1A}AR is predominant in the human prostate [4, 5]. Tamsulosin, terazosin, alfuzosin, doxazosin, and 5-methylurapidil, namely α_{1A}AR antagonists, have a greater selectivity in prostate smooth muscle [6], and it has been used in the treatment of many clinical BPH symptoms, such as micturition, nocturia increase, dysuresia, etc. [7, 8]. In order to fully characterize the mechanism of the drugs and screen for long-acting a_{1A}AR antagonists, investigation of drug–receptor interactions becomes increasingly important. The interactions are usually examined by means of radioligand binding assay (RBA) [9]. Although it has many advantages such as high sensitivity and accuracy, it can result in potential radioactive pollution, and it failed to exactly simulate the fundamental processes of drug action dynamically.

Recently, receptor affinity chromatography, carried out through frontal analysis or nonlinear studies, has been a new technique for characterizing binding sites, drug discovery, as well as drug development [10–12]. Based on this thought, the cell membrane chromatography (CMC) method has been proposed and applied to investigate the affinity interaction between drug and membrane receptor [13–15], and fast screen the α_{1A}AR antagonists from

complex samples [16, 17]. We have established a CMC method using frontal analysis to investigate the K_D values for calcium antagonist–L-type calcium channel interactions [18]. However, the influence of detection wavelengths and the estimation of CMC column dead volume might make the method inaccurate. Moreover, the CMC method should be developed to differentiate specific binding from nonspecific binding because only specific binding can activate the signal transduction pathway.

In this study, HEK293 α_{1A} cell line, which expresses stably high levels of α_{1A}AR, was used to prepare the cell membrane stationary phase (CMSP) in the CMC model. An HEK293 α_{1A}/CMC system was established for determining the affinity of the α_{1A}AR antagonists binding to the α_{1A}AR. In an effort to obtain further insight into the binding sites of ligands to the α_{1A}AR on the CMSP, a competitive binding study was modified and developed. To ensure that the location of the binding region which tamsulosin occupied varied from that of oxymetazoline, a series of two breakthrough curves were constructed. The specificity, selectivity, sensitivity, reproducibility, and robustness of the HEK293 α_{1A}/CMC method were also investigated.

Experimental

Materials and reagents

Tamsulosin, terazosin, alfuzosin, doxazosin, 5-methylurapidil, phentolamine, oxymetazoline, nitrendipine, and nilotinib as standard drugs were provided by the National Institute for the Control of Pharmaceuticals and Biological Products (Beijing, China). The HEK293 a_{1A} high expression cell line was a gift from Prof. Youyi Zhang at Peking University Third Hospital (Beijing, China). Dulbecco minimal essential medium (DMEM) and G418 were purchased from Invitrogen Corporation (Grand Island, NE, USA). Methanol was of LC grade (Honeywell, NJ, USA). Silica gel (ZEX-II, 200–300 mesh) was obtained from Qingdao Meigao Chemical (Qingdao, China), and C_{18} solid-phase extraction columns were purchased from Supelco (500 mg, 3 mL^{-1}, Sigma-Aldrich, Bellefonte, PA, USA). All other reagents and solvents were of analytical reagent grade and used without further purification unless otherwise noted. The phosphate-buffered saline (PBS, 50 mM) was prepared by dissolving disodium hydrogen phosphate (8.954 g) in the newly double-distilled water (500 mL) and diluted into various concentrations before using (adjust pH 7.4 by phosphonic acid).

Apparatus and conditions

CMC analysis was performed on a Shimadzu LC-20A apparatus that consisted of two LC-20AD pumps, a DGU-20A3 degasser, an SIL-20A auto sampler, a CTO-20A column oven, and an SPD-M20A diode array detector (Shimadzu, Kyoto, Japan). The data were acquired by the LCsolution software (Shimadzu, Kyoto, Japan) and processed by GraphPad Prism version 5.0 (San Diego, CA, USA).

The CMC mobile phase consisted of double-distilled water or PBS was delivered at a flow rate of 0.2 mL min^{-1}. The HPLC conditions were a Dikma C_{18} column (150×4.6 mm, 5 μm), a mobile phase of methanol–water–triethylamine (70:29.8:0.2, *v*/*v*/*v*, adjust pH to 6.0 by acetic acid), with 1.0 mL min^{-1} flow rate. The detection wavelengths for the ligands were 280 nm for tamsulosin, 246 nm for terazosin, 246 nm for alfuzosin, 248 nm for doxazosin, 283 nm for 5-methylurapidil, 277 nm for phentolamine, and 280 nm for phentolamine.

Preparation of sample solutions

The stock solutions (5×10^{-3} mol L^{-1}) of tamsulosin, terazosin, alfuzosin, doxazosin, 5-methylurapidil, phentolamine, oxymetazoline, nitrendipine, and nilotinib were prepared by separately dissolving the standard drugs in methanol. Standard solutions at various concentrations were prepared by diluting the stock solutions with the mobile phase for each one.

Cell culture and preparation of CMSP

The pREP8/bovine a_{1A}AR plasmid was transfected into HEK293 cell lines by the calcium phosphate precipitation method [19, 20]. HEK293 cells stably expressing α_{1A}AR were cultured in DMEM (contained 10% fetal bovine serum, 100 U mL^{-1} penicillin, 100 U mL^{-1} streptomycin, and 0.1 mg mL^{-1} G418). Cells were grown at 37 °C in a humidified atmosphere with 5% CO_2, and cells from exponentially growing cultures (10^7) were harvested using trypsin and incubated for 10 min at 4 °C. Cultured HEK293 α_{1A}AR cells were washed three times with 5 mM PBS by centrifuging at 3,000×*g* for 10 min, and the pellet was re-

Table 1 Biological characteristics of HEK293 and HEK293 α_{1A}AR CMSP

CMSP	Total protein (mg g^{-1} silica)	α_{1A}AR (pg g^{-1} silica)	EGFR (pg g^{-1} silica)	VEGFR-2 (pg g^{-1} silica)
HEK293	47.3±2.5	73.3±5.0	38.9±3.0	32.2±2.8
HEK293 α_{1A}AR	61.6±2.9	381.1±15.1	43.3±3.3	30.0±2.4

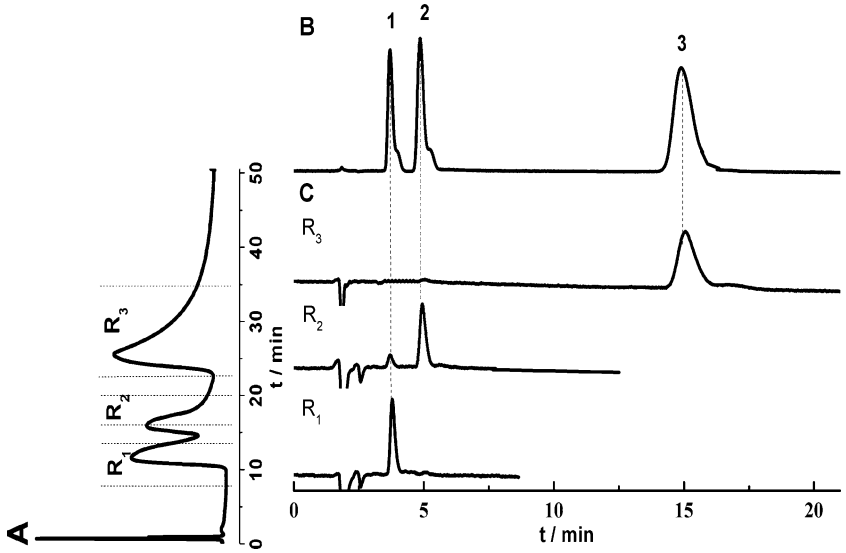


Fig. 1 Chromatograms of mixed standard solution using an HEK293 α_{1A}/CMC-offline-HPLC method. (**A**) HEK293 α_{1A}/CMC chromatogram of mixed standard solution including R_1, R_2, and R_3 fractions; (**B**) HPLC chromatograms of R_1, R_2, and R_3 fractions; (**S**) HPLC chromatogram of mixed standard solution of phentolamine (*1*), tamsulosin (*2*), and doxazosin (*3*). HEK293 α_{1A}/CMC column, 10×2.0 mm; flow rate, 0.2 mL min^{-1}; column temperature, 37 °C; sample concentration, 5×10^{-4} mol L^{-1} for each ligand; sample volume, 5 μL; detection wavelength, 254 nm; mobile phase, 2 mM PBS, pH 7.4. RP-HPLC column, Dikma C_{18} (150×4.6 mm, 5 μm); flow rate, 1.0 mL min^{-1}; column temperature, 37 °C; detection wavelength, 254 nm; mobile phase, methanol–water–triethylamine (70:29.8:0.2, *v*/*v*/*v*, adjust pH to 6.0 with acetic acid)

suspended with 50 mM Tris–HCl (pH 7.4), followed by ultrasonic destruction for 30 min. The resulting homogenate was clarified by centrifugation at 1,000×*g* for 10 min, and the supernatant was centrifuged at 12,000×*g* for 10 min. The precipitate was then suspended with 5 mM PBS [16, 17]. Briefly, the HEK293 α_{1A}AR CMSP was prepared by adsorption of the cell membrane suspension (5 mL) on the activated silica (0.05 g) under vacuum and with a gentle agitation. The CMSP was placed overnight and then washed with 5 mM PBS five times. Finally, the mixture obtained was packed into a column (10×2.0 mm I.D.) using a wet packing method (10 MPa, 5 min). All the procedure was performed at 4 °C. The HEK293 CMSP was also obtained by the same procedure.

The total protein was determined using BCA protein assay reagent kit (Jiankangyuan Biotech, Zhuhai, China), and the content of the α_{1A}AR in the HEK293 and HEK293 α_{1A}AR CMSP was determined using human α_{1A}AR kit (Cusabio Biotech, Wuhan, China) by ELISA [21]. Experiments were performed in triplicate. The absorbance was quantitatively measured at 450 nm. Likewise, the human epidermal growth factor receptor (EGFR) and

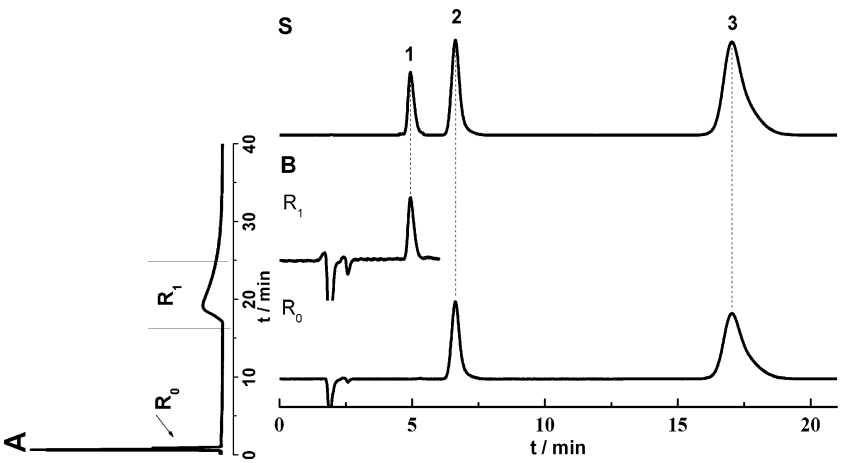


Fig. 2 Chromatograms of mixed standard solution using an HEK293 α_{1A}/CMC-offline-HPLC method. **A** HEK293 α_{1A}/CMC chromatogram of mixed standard solution including R_0 and R_1 fractions; (**B**) HPLC chromatograms of R_0 and R_1 fractions; (**S**) HPLC chromatogram of mixed standard solution of tamsulosin (*1*), nitrendipine (*2*), and nilotinib (*3*). The chromatographic conditions were same to those of Fig. 1

vascular endothelial growth factor receptor-2 (VEGFR-2) were also evaluated by the commercially available EGFR and VEGFR-2-ELISA kit [22, 23].

HEK293 α_{1A}/CMC-offline-HPLC applications

A mixed standard solution containing phentolamine, tamsulosin, and doxazosin at a concentration of 5×10^{-4} mol L^{-1} for each one was used to validate the specificity of the HEK293 α_{1A}/CMC-offline-HPLC system. Fractions retained by the α_{1A}AR CMSP were separately concentrated and then injected into the HPLC system. This method was also applied to screen the mixed standard solution of tamsulosin, nitrendipine, and nilotinib acting on the α_{1A}AR. To examine whether tamsulosin is interacting with the receptors on the HEK293 CMSP, a series of two retention profiles was constructed.

Determination of K_D values

One assumption made is that the drug interacts reversibly with a single type of the binding site on the α_{1A}AR. K_D values were measured in an HEK293 α_{1A}/CMC system, performed by continuously pumping tamsulosin ranging from 1.12×10^{-7} to 3.60×10^{-6} mol L^{-1} through an α_{1A}/CMC column. The breakthrough curves of tamsulosin with different concentrations were recorded. Next, the standard solutions of tamsulosin, terazosin, alfuzosin, doxazosin, 5-methylurapidil, phentolamine, and oxymetazoline (10^{-4} mol L^{-1} for each one) were injected into the column, respectively. The capacity factor (k') of CMC chromatographic peak in elution curve was calculated using the following equation:

$$k' = (t_R - t_0)/t_0 \quad (1)$$

where t_R is the retention time of ligand, and t_0 is the dead time of non-retained solvent.

According to Eq. 2 [18, 24],

$$\frac{1}{k' - X} = \frac{K_D V_M}{K_M [R]_s}[L]_m + \frac{K_D V_M}{[R]_s} \quad (2)$$

where K_D and K_M are the equilibrium dissociation constants for the analyte and marker in the mobile phase, respectively. $[L]_m$, $[R]_s$, and V_m are the molar concentration of ligands in the effluent, immobilized receptors at the surface of the stationary phase, and the dead volume of the column, respectively. The term X was introduced to the part of k' value to eliminate the errors and obtained by iterative testing [24, 25]. When using tamsulosin both as a marker and as an analyte, Eq. 2 reduces to

$$\frac{1}{k'} = \frac{V_M}{[R]_s}[L]_m + \frac{K_D V_M}{[R]_s} \quad (3)$$

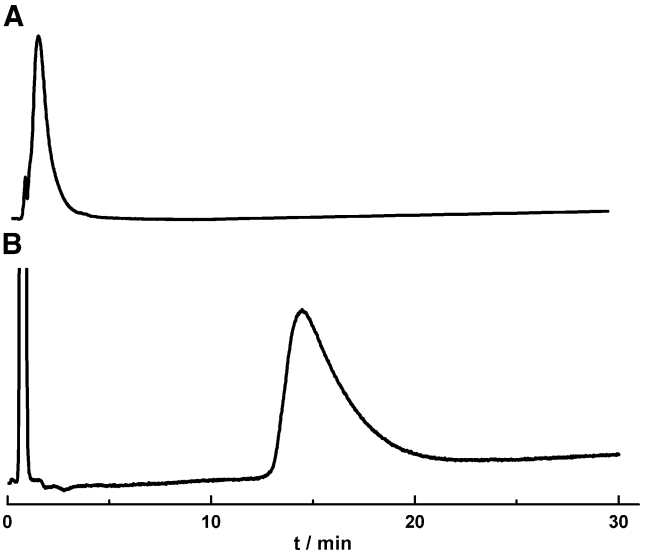


Fig. 3 The CMC chromatograms of tamsulosin on the HEK293 (**A**) and HEK293 α_{1A} (**B**) column. The chromatographic conditions were same to those of Fig. 1

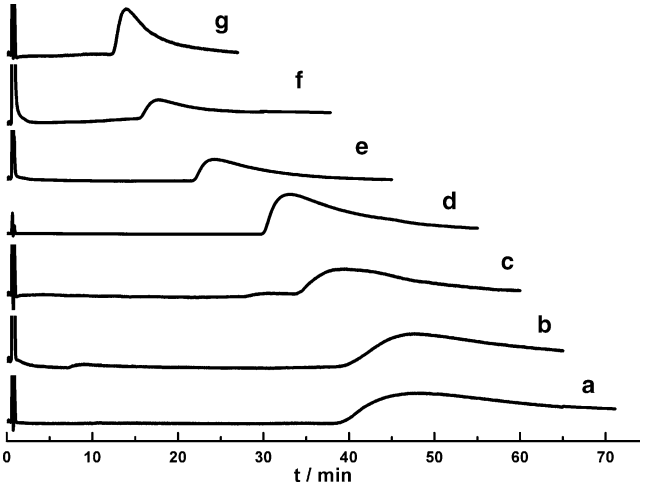
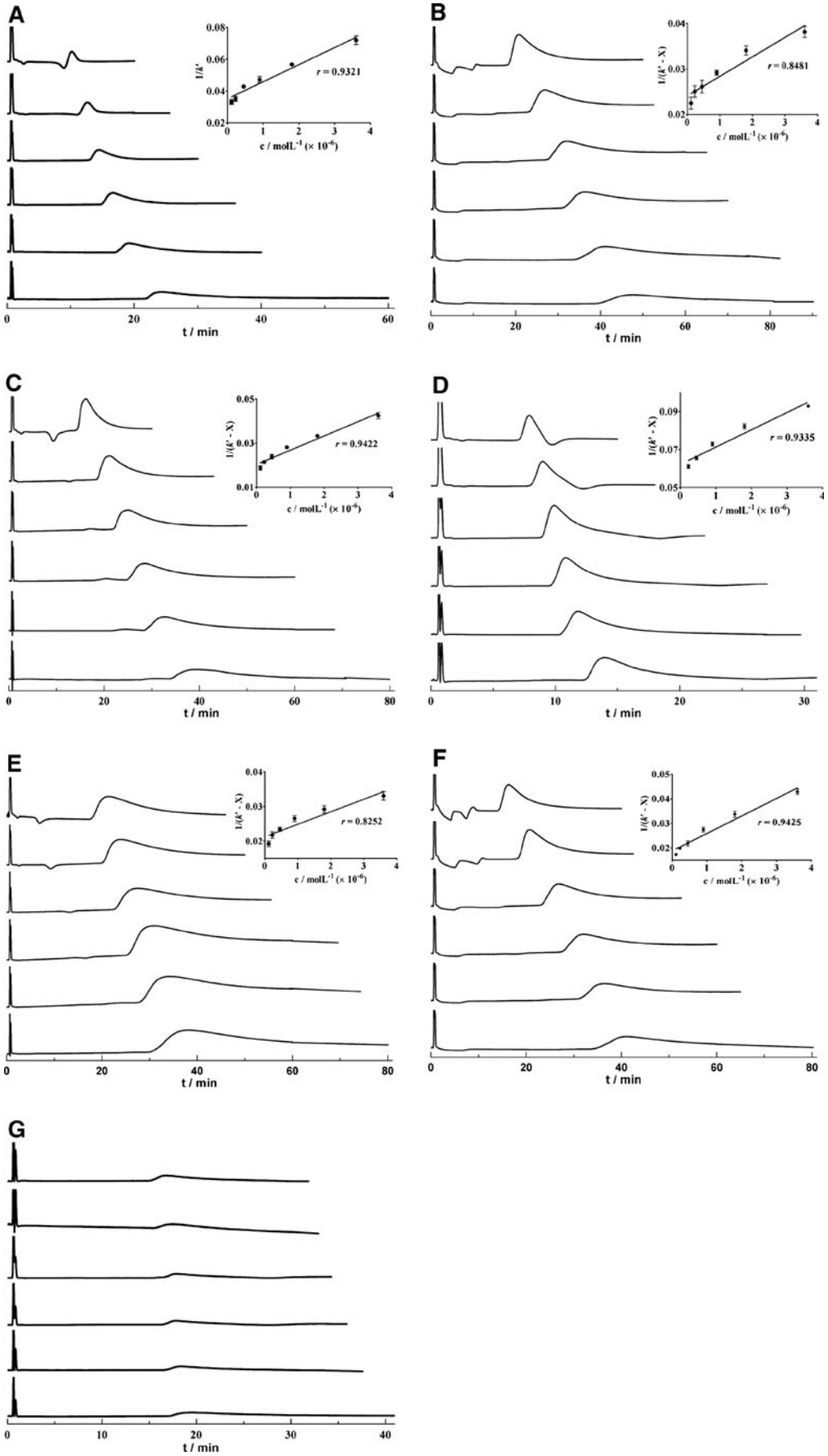


Fig. 4 The CMC chromatograms of alfuzosin (**a**), doxazosin (**b**), 5-methylurapidil (**c**), terazosin (**d**), tamsulosin (**e**), oxymetazoline (**f**), and phentolamine (**g**) on the HEK293 α_{1A}/CMC column. The chromatographic conditions were same to those of Fig. 1, and the detection wavelengths for seven ligands can be found in the text

Fig. 5 Elution profiles of tamsulosin (**A**), terazosin (**B**), 5-methylurapidil (**C**), phentolamine (**D**), alfuzosin (**E**), doxazosin (**F**), and oxymetazoline (**G**) on the HEK293 α_{1A}/CMC column with different concentrations of tamsulosin in the mobile phase and regression curves achieved by plotting $1/k'$ or $1/(k' - X)$ versus $[L]_m$. The six concentrations were 1.12×10^{-7}, 2.25×10^{-7}, 4.50×10^{-7}, 8.99×10^{-7}, 1.80×10^{-6}, and 3.60×10^{-6} mol L^{-1}, respectively. Each *point with a bar* represents the mean ±SEM (n=3). The chromatographic conditions were same to those of Fig. 1, and the detection wavelengths for seven ligands can be found in the text ▶


A
B
C
D
E
F
G
t / min
c / molL⁻¹ (× 10⁻⁶)
r = 0.9321
r = 0.8481
r = 0.9422
r = 0.9335
r = 0.8252
r = 0.9425

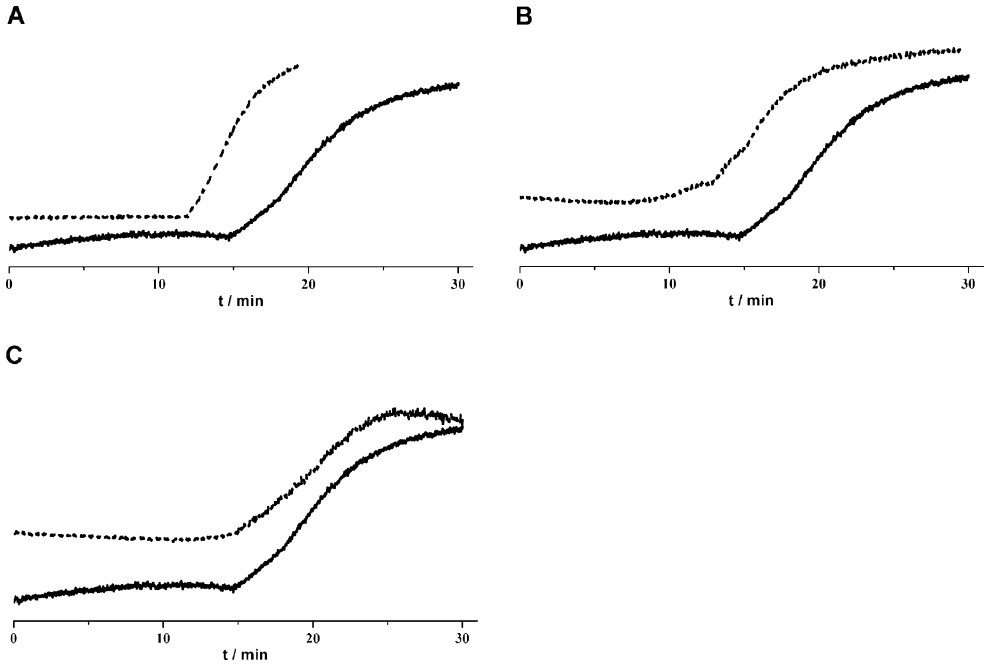


Fig. 6 Competitive binding breakthrough curves of tamsulosin (10^{-6} mol L^{-1}). The *straight* and *dashed lines* represent the breakthrough curves using the normal α_{1A}/CMC column and the column after its saturation with tamsulosin (**A**), terazosin (**B**), and oxymetazoline (**C**; 5×10^{-7} mol L^{-1}). HEK293 α_{1A}/CMC column, 10×2.0 mm; flow rate, 0.2 mL min^{-1}; column temperature, 37 °C; mobile phase, 5 mM PBS, pH 7.4; detection wavelength, 280 nm

$[R]_s/V_M$ is obtained from the slope and substituted to Eq. 2. So, the K_D values of the ligands were obtained from the linear regression by plotting $1/(k' - X)$ versus $[L]_m$. All data were expressed as means±standard errors of the means (SEM, $n\geq3$), and values of $p<0.01$ were regarded as indicative of statistical significance.

Competitive binding study

For further competitive studies, the mobile phase that contained tamsulosin, terazosin, and oxymetazoline (5×10^{-7} mol L^{-1} for each one) was separately pumped through the α_{1A}/CMC column to saturate the binding sites of α_{1A}AR. Afterwards, tamsulosin was propelled towards the column. The breakthrough curves were recorded and compared to those obtained from the normal column without saturation.

Results and discussion

Biological characteristics of CMSP

In order to characterize the CMSP, the total protein and α_{1A}AR in the cell membrane suspension before and after adsorption on the activated silica were determined, respectively. The difference values, i.e., the content of the total protein and α_{1A}AR in the HEK293 and HEK293 α_{1A}AR CMSP, are listed in Table 1. Other endogenous receptor protein including EGFR and VEGFR-2 was lowly detected in two types of CMSP, respectively. As a result, the HEK293 α_{1A}AR CMSP containing high-level α_{1A}AR was successfully prepared.

Application of an HEK293 α_{1A}/CMC-offline-HPLC system

A mixed standard solution containing phentolamine, tamsulosin, and doxazosin was used to validate the specificity of the HEK293 α_{1A}/CMC-offline-HPLC system. As shown in Fig. 1A, there were three significant retention fractions named R_1, R_2, and R_3 (R_1, 11.7 min; R_2, 15.9 min; and R_3, 25.5 min). Then, the three fractions were collected and concentrated by using C_{18} solid-phase extraction columns, respectively, followed by injecting into HPLC system for further separation and analysis. The retention times were R_1 3.7 min, R_2 4.9 min, and R_3 14.9 min, respectively (Fig. 1B). Compared with the HPLC chromatogram of the standard solution (Fig. 1S), the retained components were finally identified as phentolamine, tamsulosin, and doxazosin, respectively.

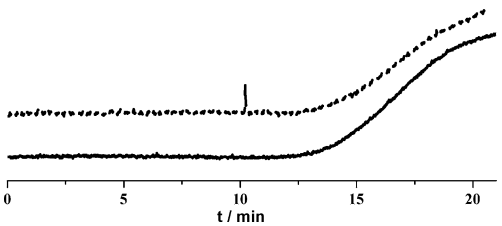


Fig. 7 Competitive binding breakthrough curves of terazosin (10^{-6} mol L^{-1}). The *straight* and *dashed lines* represent the breakthrough curves using the normal α_{1A}/CMC column and the column after its saturation with oxymetazoline (5×10^{-7} mol L^{-1}). The CMC conditions were same to those of Fig. 6

Table 2 The correlation analysis of K_D values obtained from the HEK293 α_{1A}/CMC and pKi values from RBA technique

Ligands	K_D/M (CMC, $\times 10^{-6}$)	RBA (pKi)		
		Thiyagarajan [8]	Martin et al. [26]	Richardson et al. [27]
Tamsulosin	1.87±0.13	9.82	10.40	9.2
5-Methylurapidil	2.86±0.20	8.51	8.89	8.2
Doxazosin	3.01±0.19	8.60	8.89	8.2
Terazosin	3.44±0.19	7.62	8.53	6.9
Alfuzosin	3.50±0.21	7.29	8.42	7.0
Correlation coefficients		0.9343	0.9582	0.8782
P values		0.0047	0.0024	0.0091

A mixed standard solution containing tamsulosin, nitrendipine, and nilotinib was applied to verify the selectivity of the α_{1A}/CMC system. Of the three drugs, nitrendipine is an antagonist acting on L-type calcium channel, and nilotinib is a tyrosine protein kinase inhibitor interacting with BCR-ABL. As shown in Fig. 2, only tamsulosin, an α_{1A}AR antagonist, can be recognized by the system. Also, there was no obvious retention when tamsulosin was injected into an HEK293/CMC system (Fig. 3). It can be concluded that the α_{1A}AR on the CMSP plays a key role, and the system can specifically recognize the components which interact with the α_{1A}AR.

Affinity on the α_{1A}AR

As shown in Fig. 4, the k' values of ligands on the HEK293 α_{1A}/CMC column varied from each other, as a result of the different affinities that the ligands interacted with α_{1A}AR, and the retention times of six ligands decreased with continuously increasing the concentration of tamsulosin ranging from 1.12×10^{-7} to 3.60×10^{-6} mol L^{-1} in the mobile phase (Fig. 5A–F). The degree of the shift is relevant to the affinity the ligands interact with α_{1A}AR and the efficient concentration of the binding sites that both the markers and the ligands occupy. Particularly, the injected ligands have other binding sites which do not interact with the marker in the mobile phase. So, the term X is equal to a critical k' of the ligand at a marker concentration, above which the retention time of the ligand no longer decreased with the increase of the marker's concentration. The X values were 12.35, 9.98, 6.11, 9.83, and 10.45 for terazosin, 5-methylurapidil, phentolamine, alfuzosin, and doxazosin, respectively. In accordance with Eq. 2, the corresponding graph of the reciprocal values of $(k' - X)$ versus $[L]_m$ was obtained with three replicates on three different α_{1A}/CMC columns. The calculated K_D values were $(1.87\pm0.13)\times10^{-6}$ M for tamsulosin, $(2.86\pm0.20)\times10^{-6}$ M for 5-methylurapidil, $(3.01\pm0.19)\times10^{-6}$ M for doxazosin, $(3.44\pm0.19)\times10^{-6}$ M for terazosin, $(3.50\pm0.21)\times10^{-6}$ M for alfuzosin, and $(7.57\pm0.31)\times10^{-6}$ M for phentolamine, respectively, and each plot for the ligands gave a linear relationship, with the correlation coefficients varied from 0.8252 to 0.9425 over the six different concentrations tested (Fig. 5A–F). So far as we know, tamsulosin, 5-methylurapidil, terazosin, alfuzosin, and doxazosin selectively interact with α_1AR. The first two have a modest selectivity for α_{1A}AR over other subtypes, while the last three demonstrate similar selectivity for all three a_1AR subtypes [26, 27]. However, little decrease in the retention time of oxymetazoline was observed (Fig. 5G). The behavior suggested that the ligand interacted

Table 3 Robustness evaluation of α_{1A}/CMC method

Chromatographic changes	Level	k'
Mobile phase composition (mM, PBS)		
1.8	−0.2	22.4
1.9	−0.1	20.3
2.0	0	20.6
2.1	0.1	19.5
2.2	0.2	18.9
Mean±SEM		20.3±1.3
pH value		
7.2	−0.2	19.8
7.3	−0.1	19.2
7.4	0	20.6
7.5	0.1	20.9
7.6	0.2	21.8
Mean±SEM		20.5±1.0
Sample volume (10^{-4} mol L^{-1}, μL)		
1	−4	21.8
2	−3	21.5
5	0	20.6
10	5	18.5
20	15	16.9
Mean±SEM		19.9±2.1

with α_{1A}AR in a manner different from tamsulosin, and in fact, it is a nonselective α_1AR agonist.

We have established a CMC method based on frontal analysis for determining the K_D values of calcium antagonist–L-type calcium channel interactions [18]. However, the results were influenced by the molar absorption coefficients of the ligands. Furthermore, the estimation of the dead volume was a complex work. Here, we described a new model combined modified competitive binding, and we only have to obtain the k' values of the analytes and the concentrations of the marker in the mobile phase, which greatly simplified the calculation process.

Displacement study on the α_{1A}AR

As shown in Fig. 6, tamsulosin was pumped through the column resulting in the displacement of tamsulosin, terazosin, and oxymetazoline on the α_{1A}AR binding sites. The mean position of tamsulosin's breakthrough curve on the α_{1A}/CMC column, which has been saturated with tamsulosin and terazosin, is shifted to the left compared to that of tamsulosin on the normal column. However, that was not the case for oxymetazoline. This means that the terazosin occupies certain binding sites of α_{1A}AR on the column thus hindering the association of tamsulosin, while oxymetazoline interacted with α_{1A}AR in a manner different from tamsulosin. Next, terazosin was pumped through the column saturated with oxymetazoline, which led to the few change in breakthrough times (Fig. 7). So, the two drugs occupy the different class of binding sites on α_{1A}AR as well.

Correlation with the RBA method

As shown in Table 2, the pKi values of tamsulosin ranked first, followed by 5-methylurapidil and doxazosin, whereas terazosin and alfuzosin ranked last [8, 26, 27]. The binding affinities of the five ligands were compared with that reported previously. Due to the use of radioactive nuclides including [^{3}H], [^{125}I], etc., the sensitivity of the method is greatly higher than that obtained from CMC method by two to three orders of magnitude. Even so, the K_D values calculated using the CMC method correlated positively with those published pKi values obtained from the RBA technique with r^2 values of 0.9343, 0.9582, and 0.8782 ($p<0.05$), respectively. Because of its easiness, simplicity, and accuracy, the CMC method is possessed of popularization and application value.

Methodology of the HEK293 α_{1A}/CMC

The standard solution of tamsulosin was kept at 4 °C and was analyzed at selected time intervals after storage over the study period. After 24 h, 48 h, 72 h, and 1 week, a decrease by 3.50%, 6.29%, 11.13%, and 31.32% in retention time was observed, respectively. Thus, the activity of CMC column can keep relatively stable in 2 days.

The limits of detection for the ligands were determined at a signal-to-noise ratio of 3, and they were 5.77×10^{-7}, 2.93×10^{-7}, 4.12×10^{-7}, 3.58×10^{-7}, 5.12×10^{-7}, 5.45×10^{-7}, and 8.02×10^{-7} mol L^{-1} for tamsulosin, terazosin, alfuzosin, doxazosin, 5-methylurapidil, phentolamine, and oxymetazoline, respectively.

The reproducibility of the α_{1A}/CMC method was performed by continually injecting standard solution (10^{-4} mol L^{-1}) of phentolamine, tamsulosin, and doxazosin into five different α_{1A}/CMC columns (n=50), and the results showed that the retention time decreased by merely 18.95%, 11.68%, and 17.08%, respectively, which were used to eliminate the errors caused by decay of membrane protein activity. In addition, the relative standard deviations of the retention times were 6.16%, 8.86%, and 9.95%, respectively.

The robustness of the method was examined by replicate injections (n=6) of standard solution of tamsulosin at a concentration of 10^{-4} mol L^{-1} with slight changes made to the mobile phase composition, pH value, and sample volume (Table 3). Few changes in k' values were observed.

Conclusion

The CMC method combined competitive binding provides a powerful technique for the characterization of the α_{1A}AR binding interactions without disturbing the receptor's quaternary structure. There was a positive correlation between K_D and the published pKi values, and the binding sites on the α_{1A}AR have been validated successfully.

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