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Frontal analysis of cell-membrane chromatography for determination of drug-α_{1D} adrenergic receptor affinity

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ABSTRACT

The aim of the present study was to determine drug-α_{1D} adrenergic receptor (AR) affinity by frontal analysis of cell-membrane chromatography (CMC). The cell-membrane stationary phase (CMSP) was prepared by immobilizing rat aorta cell membranes on porous silica, and the resulting CMSP was used to determine drug binding affinity to α_{1D}-AR by frontal analysis. The CMSP of rat aorta was stable and reproducible. Relative binding affinities (dissociation constant, K_d) were determined by frontal chromatography for prazosin (166.13 ± 18.36 nmol), BMY7378 (537.40 ± 30.84 nmol), phentolamine (646.92 ± 23.17 nmol), 5-methylurapidil (725.66 ± 25.48 nmol), oxymetazoline (910.56 ± 40.62 nmol) and methoxamine (1299.27 ± 51.73 nmol). These results were consistent with the affinity rank order and showed a good correlation with the affinity of the same compounds for the cloned α_{1D}-AR subtype obtained from radioligand-binding assay. The study demonstrates that frontal analysis of CMC may be used for direct determination of drug–receptor binding interactions, and that CMC is an alternative reliable method to quantitatively study ligand–receptor interactions.

1. Introduction

Cell-membrane chromatography (CMC), a novel bioaffinity chromatographic technique originated by He et al. in 1996 [1–3], can be applied to the study of drug–receptor interactions. In previous studies [4–6], human embryonic kidney (HEK) 293 cells that expressed cDNA of α_{1A}, α_{1B} or α_{1D} adrenergic receptor (AR) subtypes were cultured, and the cell-membrane stationary phase (CMSP) was prepared by immobilizing cell membranes on the surface of silica, which acted as carrier. Then, the interactions between nine ligands of the α_1-AR and α_{1A}, α_{1B} or α_{1D}-AR in the CMSP were investigated using CMC. The ligand–receptor affinity was shown by chromatographic parameters (capacity factor, k'). The results of the above studies showed that the prepared CMSP and CMC method were useful in evaluating drug–receptor affinity. In addition, the ligand-binding affinity to muscarinic acetylcholine receptor has been evaluated by CMC by immobilizing rat cerebral cell membrane and guinea pig jejunum membrane on the surface of a silica carrier [7,8]. These results have also shown that CMC can be used to evaluate drug–receptor affinity of drug candidates.

Functional assay and radioligand-binding assay undoubtedly contribute to a better understanding of the interaction between ligands and receptors, but they have some disadvantages. CMC is expected to become a reliable method to study ligand–receptor interactions. However, CMC have been not used to assay drug dissociation constant (K_d) and total receptor (B_t) exactly and directly, compared with radioligand-binding assay (RLBA) and functional assay. To improve CMC, we combined it with frontal affinity chromatography to determine K_d and B_t.

The α_1-AR, which is activated by adrenaline and noradrenaline, is a membrane protein and a member of the G-protein coupled receptor superfamily. According to recommendations from NC-IUPHAR, based on pharmacological and molecular evidence, the α_1-AR is divided into three subtypes: α_{1A}, α_{1B} and α_{1D} [9]. The α_1-AR family is of particular therapeutic interest because its constituents mediate a variety of physiological effects in many tissues, such as neurotransmission, vasoconstriction, cardiac inotropy and chronotropy, and glycogenolysis. In the past two decades, with the cloning of three α_1-AR subtypes, there was a great thrust to understand the expression of α_1-AR subtypes. The α_{1D} subtype is expressed in a variety of tissues, including vascular smooth muscle, cerebral cortex [10,11], and probably rat lung [12]. In rat aorta and iliac artery, it appears to be the predominant subtype that mediates vasoconstriction [13,14].

The aim of this research was to prepare the rat aorta cell-membrane stationary phase, determine the affinity (K_d) of drug-α_{1D} adrenergic receptor interaction and total receptor (B_t) by frontal analysis, and create an alternative reliable method to study quantitatively ligand–receptor interactions.

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2. Experimental

2.1. Chemicals

Prazosin, 8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspirol[4,5]decane-7,9-dionedihydrochloride (BMY7378), 5-methylurapidil, oxymetazoline, phentolamine, and methoxamine were purchased from Sigma–Aldrich, Inc. (St. Louis, MO, USA); *N*-(2-(2-cyclopropylmethoxy)ethyl)-5-chloro-α-dimethyl-1*H*-indole-3-thylamine (RS17053) was provided by Tocris Bioscience (Ellisville, MO, USA). Macroporous spherical silica (7 μm, 100 Å) was purchased from the Institute of Chemistry of the Chinese Academy of Sciences (Beijing, China). The water used in the study was prepared using a Milli-Q Water Purification System (Milli-Pore, Bedford, MA, USA).

2.2. Animals

Sprague–Dawley rats (5–7 weeks old) were supplied by the Experimental Animal Centre of Xi'an Jiaotong University (Xi'an, China). All experimental protocols involving animals were reviewed and approved by the Institutional Animal Experimentation Committee of Xi'an Jiaotong University.

2.3. Membrane preparation

Rats were sedated with CO_2 and decapitated. The rat aorta was removed immediately, washed thoroughly, cut into small pieces, and added to ice-cold phosphate-buffered saline (PBS; 8.0 g L^{-1} NaCl, 1.15 g L^{-1} Na_2HPO_4, 0.2 g L^{-1} KCl, 0.2 g L^{-1} KH_2PO_4, pH 7.0). The tissue suspension was then homogenized at 4 °C (twice, each time for 1 min) with a Polytron homogenizer equipped with a PT35ST probe. The crude homogenate was centrifuged at 1000 × *g* to remove cellular debris. The supernatant was centrifuged for 20 min at 12 000 × *g* (Hermel ZK401 high-speed refrigerated centrifuge; Berthold Hermel AG, Gosheim, Germany). The buff-colored layer around the pellet was gently re-suspended in the above buffer, and centrifuged as before. All procedures were performed at 4 °C.

2.4. Preparation of CMSP

The CMSP was prepared by immobilizing the rat aorta cell membranes on the surface of silica (7 μm, 100 Å), which acted as a carrier, and was used for rapid on-line chromatography. The preparation procedure was similar to that described previously [1,2]. In brief, 600 mg macroporous spherical silica activated at 120 °C was placed in a 10-mL reaction tube, followed by a suspension of rat aorta cell membranes (generally, the concentration of membrane protein was about 2.12 mg mL^{-1}). The adsorption of the rat aorta cell membranes to the activated silica surface took place at 4 °C, until equilibrium was reached. The whole adsorption process was carried out under vacuum and ultrasonication. The reaction mixture was diluted with an equal volume of deionized water. The cell-membrane phospholipids were able to fuse spontaneously with each other (self-fusion) on the silica surface in the aqueous solution, until a resealed cell-membrane layer was obtained. The residual free cell membranes were washed with Tris–HCl buffer (pH 7.4). The prepared CMSP particles in the buffer were packed into a chromatography column (10 mm × 1.2 mm I.D.) under low pressure, by the slurry method.

2.5. Chromatographic system

The HPLC system consisted of an SCL-10Avp system controller, an LC-10AT*vp* pump, and an SPD-10A*vp* UV–vis detector (Shimadzu, Kyoto, Japan). The data were acquired by LCsolution software (version 1.0, Shimadzu, Kyoto, Japan). The mobile phase consisted of PBS (5 mM, pH 7.4) and ligand, and the flow rate was 0.2 mL min^{-1}. The ligands were detected using UV absorption at 278 nm (BMY7378, phentolamine and RS17053), 250 nm (prazosin), 282 nm (oxymetazoline), 284 nm (5-methylurapidil), or 292 nm (methoxamine). The column was placed into a column oven at a temperature of 37 °C.

2.6. Frontal chromatography studies

All ligands were dissolved in ethanol at a concentration of 10 mM as stock solutions, and further diluted with PBS to obtain a chromatographic mobile phase that contained serial concentration of ligands (oxymetazoline, 200, 400, 600, 800 and 1000 nM; 5-methylurapidil, 200, 400, 600, 800 and 1000 nM; prazosin, 80, 100, 200, 300 and 400 nM; phentolamine, 400, 600, 700, 800 and 1000 nM; BMY7378, 100, 200, 400, 600 and 800 nM; methoxamine, 200, 400, 600, 800 and 1000 nM). The chromatographic mobile phase was degassed before use. Frontal analysis was performed using the chromatographic mobile phase at a flow rate of 0.2 mL min^{-1}. A correction for the system void time was made by performing similar experiments using acetone as a non-retained solute. The retained compounds were eluted and the column regenerated between studies by passing PBS through the column. All experiments were performed in triplicate under each set of tested conditions.

2.7. Calculation of dissociation constants and binding sites

Frontal affinity chromatography can be used to characterize the binding of small molecules to an immobilized membrane-bound target and to determine binding affinities (K_d) and total receptor (B_t). K_d was calculated using a previously described approach [15–17]. In brief, K_d, as well as B_t of the immobilized cell membrane of the rat aorta can be calculated using Eq. (1):

$$[D] \times (V - V_{min}) = B_t \times [D] \times (K_d + [D])^{-1} \quad (1)$$

where [*D*] is the concentration of ligand, *V* is the retention volume of ligand measured at the midpoint of the breakthrough curve, and V_{min} is the retention volume of ligand at the highest applied concentration of the displacer ligand. *V* and V_{min} were calculated using a previously described approach [18]. From the plot of $[D] \times (V - V_{min})$ versus [*D*], ligand K_d can be obtained. The data were analyzed by non-linear regression with the sigmoidal response curve using GraphPad Prism 4 software (GraphPad Software, La Jolla, CA, USA).

2.8. Statistical analysis

The relation between the calculated K_d and the affinities of agents (published pK_i values obtained from RLBA at clonal a_{1D}-AR) for the receptor subtype were analyzed by correlation analysis. Correlation constants (r^2) were calculated by linear regression analysis using GraphPad Prism 4. All data were expressed as means ± SD and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Activity and specificity of the CMSP column

In the CMC experiment, because the silica surface was completely coated by cell membranes in CMSP, the ligand–CMSP interaction can reflect the ligand–receptor affinity of the cell membrane. In rat aorta, the a_{1D}-AR appears to be the predominant subtype that mediates vasoconstriction. Therefore, the selective α_{1D}-AR agents should have high affinity for the CMSP of the rat

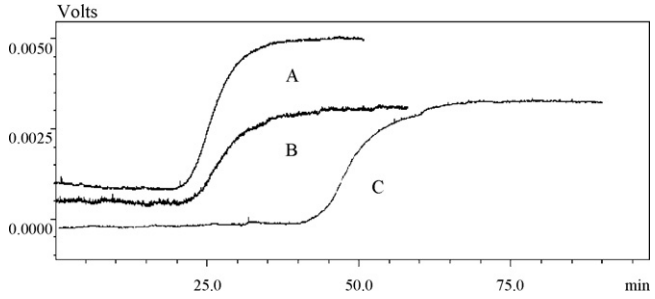

Fig. 1. Frontal chromatographic profiles of acetone 50 nM (A), RS17053 800 nM (B) and BMY 7378 800 nM (C) on the CMSP column (10 mm × 2.0 mm I.D.).

aorta, and the selective α_{1A}-AR or α_{1B}-AR agents should have low affinity. The activity and specificity of CMSP of the rat aorta were investigated by frontal analysis experiments with RS17053 and BMY7378. BMY7378 is a highly selective, high-affinity α_{1D}-AR antagonist; RS17053 is a selective α_{1A}-AR antagonist, which has low affinity for α_{1D}-AR by functional assay and RLBA. In this experiment, an 800 nM solution of BMY7378 or an 800 nM solution of RS17053 in chromatographic buffer was applied to the CMSP column of the rat aorta (chromatographic bed 10 mm × 2.0 mm I.D.) at 0.2 mL min^{-1}, and the characteristic frontal chromatographic curves were monitored by UV–vis detection at 278 nm. Fig. 1 clearly shows that the selective α_{1D}-AR antagonist BMY7378 had strong retention in the CMSP column of the rat aorta, but the selective α_{1A}-AR antagonist RS17053 had almost no retention. This indicated that the immobilized CMSP of the rat aorta was active, and the specific interaction was present in the immobilized CMSP, which was the ligand–α_{1D}-AR interaction.

3.2. Chromatographic conditions

Phosphate is one of the main components of cellular fluid. In mammals, cellular fluid has a pH in the range 6.9–7.4, and phosphate buffer is effective in maintaining this pH range. Being the mobile phase of the CMC system, PBS can simulate the cellular micro-environment. The interaction between ligand and immobilized receptors in the CMC system can simulate the fundamental processes of drug action more exactly and precisely. Therefore, PBS (pH 7.4) was selected as the mobile phase for the CMC system. To reach equilibrium between ligand and immobilized receptors in the CMC system, a flow rate of 0.2 mL min^{-1} was selected. The ligands were detected at the wavelength of absorption maximum.

Under these chromatographic conditions, an 800 nM solution of BMY7378 in chromatographic buffer was applied to the CMSP column of the rat aorta (chromatographic bed 10 mm × 2.0 mm I.D.) at 0, 12, 24, 36 and 48 h, and the characteristic frontal chromatographic curves were monitored by UV–vis detection at 278 nm. The obtained retention volume is shown in Table 1. This indicated that, under these chromatographic conditions, the CMSP was stable over a 48 h period.

Table 1
The obtained retain volume in the CMSP column (10 mm × 2.0 mm I.D.) at different time (flow rate: 0.2 ml min^{-1}; concentration of BMY7378: 0.8 μM).

Time (h)	Retain volume (mL)
0	18.48
12	18.53
24	18.40
36	18.56
48	18.45

In this part of the study, the washing protocol was also optimized. For high-affinity compounds such as prazosin and BMY7378, the column must be washed with the chromatographic buffer between each run for a minimum of 6 h to completely remove the bound ligand.

3.3. CMSP column

The FAC analysis time depends on flow rate, column length, column inner diameter and the immobilized protein density. In CMC, the immobilized protein density should be constant, because protein of cell membrane is constant. In our previous experiment, a 50 mm × 2.0 mm I.D. chromatography column was selected to study ligand–receptor affinity. In order to reduce the amount of protein in the column and shorten the FAC analysis time, a column length of 10 mm was chosen, and the inner diameter was change from 2.0 to 0.8 mm. The frontal chromatographic curves obtained for the columns of different inner diameter showed that the FAC analysis time was shortened by reducing the inner diameter, and the small-inner-diameter column can increased sensitivity. The small-inner-diameter column will be of benefit to CMC–MS in the future.

3.4. Determination of K_d and B_t

The CMSP column of the rat aorta was characterized using frontal chromatography in which serial concentrations of known ligands were added to the mobile phase and passed through the column. Representative chromatographic curves produced by frontal chromatography studies using BMY7378 are presented in Fig. 2. The relationship between the concentration of the ligand and the retention volume was analyzed using Eq. (1) to calculate the K_d of the ligand for the immobilized α_{1D}-AR. This technique has been applied previously to the study of numerous ligand–protein interactions. Using this approach, the calculated K_d values were 166.13 nM (prazosin), 537.40 nM (BMY7378), 646.92 nM (phentolamine), 725.66 nM (5-methyl-urapidil), 910.56 nM (oxymetazoline), and 1299.27 nM (methoxamine) as shown in Table 2. The average B_t value was 10.26 ± 5.00 pmol bed^{-1}, and the calculated r^2 values were 0.9628 (prazosin), 0.9697 (BMY7378), 0.9712 (phentolamine), 0.9859 (5-methylurapidil), 0.9968 (oxymetazoline), and 0.9951(methoxamine). The affinity rank order measured from the CMSP column of the rat aorta for six α_1-AR ligands was prazosin > BMY7378 > phentolamine > 5-methylurapidil > oxymetazoline > methoxamine.

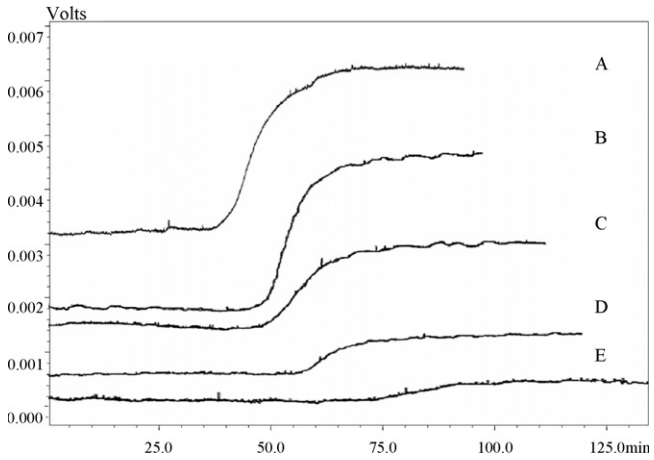

Fig. 2. Frontal chromatographic profiles of BMY 7378 on the CMSP column (10 mm × 1.2 mm I.D.) of the rat aorta ((A) 100 nM; (B) 200 nM; (C) 400 nM; (D) 600 nM; (E) 800 nM).

Table 2
Dissociation constant, K_d, of ligands at α_{1D}-AR in the CMSP column of the rat aorta and pK_i of ligands at clonal α_{1D}-AR.

Ligand	K_d (nM)	pK_i		
		pK_i[a]	pK_i[b]	pK_i[c]
Prazosin	166.13 ± 18.36	10.0	9.2	10.18
BMY7378	537.40 ± 30.84		8.5	
Phentolamine	646.92 ± 23.17	8.28		8.17
5-Methylurapidil	725.66 ± 25.48	7.86	7.1	8.00
Oxymetazoline	910.56 ± 40.62	6.47	6.6	6.44
Methoxamine	1299.27 ± 51.73	5.24	4.5	4.91

Literature values shown from [a][19], [b][20] and [c][21].

3.5. Correlation analysis between K_d at the CMSP of the rat aorta and published α_{1D} pK_i values

Full ranking scale data were analyzed using rank order correlation methods. The results are shown in Fig. 3. The calculated K_d values were positively correlated with published pK_i[a] [19], pK_i[b] [20] and pK_i[c] [21] values measured with RLBA using stably overexpressed cloned α_{1D}-AR ($r^2 = 0.9787$, $p = 0.0013$; $r^2 = 0.9621$, $p = 0.0032$; $r^2 = 0.9828$, $p = 0.0010$). The correlation coefficient indicated that there was a strong positive correlation between determined K_d using CMC and published pK_i values, and the K_d showed the same rank order as those of published pK_i values. The selective α_1-AR antagonist prazosin, which had the strongest affinity, ranked first. BMY7378, a highly selective α_{1D}-AR antagonist, ranked second. The non-selective α adrenoceptor antagonist phentolamine and highly selective α_{1A} antagonist 5-methylurapidil ranked third and fourth, respectively.

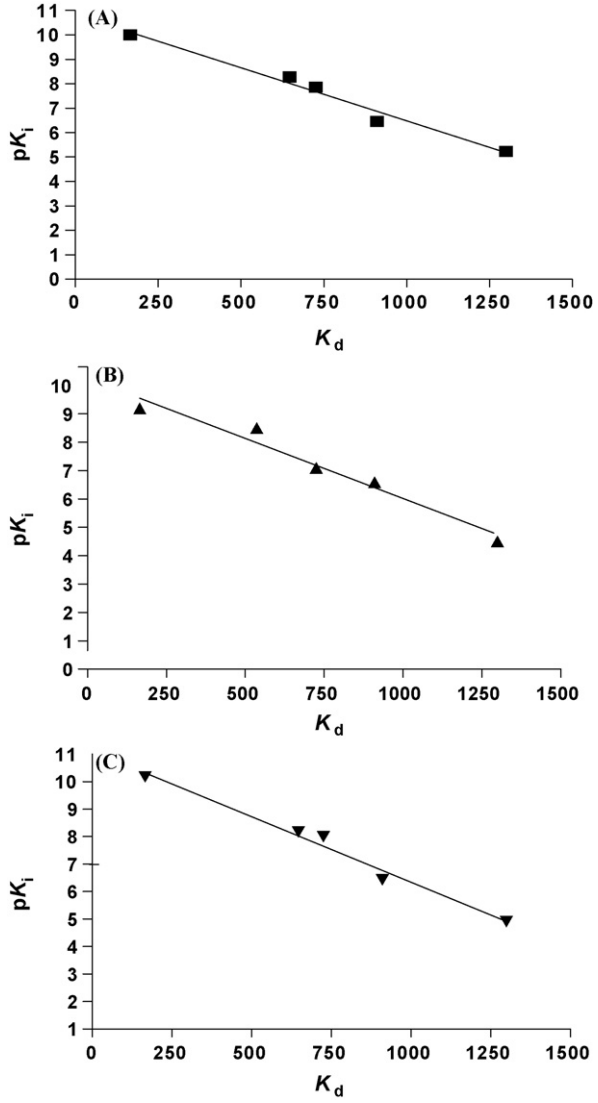

Fig. 3. Correlation between the calculated K_d and published cloned a_{1D} adrenoceptor pK_i values with an alpha level of 0.05 (two-tailed). Correlation constants (r^2) were calculated by linear regression analysis using GraphPad Prism 4.0: (A) K_d vs pK_i[a] ($r^2 = 0.9787$, p = 0.0013); (B) K_d vs pK_i[b] ($r^2 = 0.9621$, $p = 0.0032$); (C) K_d vs pK_i[c] ($r^2 = 0.9828$, $p = 0.0010$). Literature values shown from [a][19], [b][20] and [c][21].

4. Discussion

The results of this study demonstrated that the CMSP of the rat aorta was prepared successfully and determined correctly the binding affinity of six test ligands. The K_d values determined by the CMC system were not consistent with the binding affinity constants reported previously, especially the high binding affinity ligands prazosin and BMY7378. However, the affinity rank order of the six ligands was consistent with that reported previously, and the calculated K_d values were positively correlated with the binding affinity constants reported in the literature. Despite these drawbacks, these results demonstrate that the CMC system is convenient and feasible for determining the ligand–receptor K_d values.

The K_d values determined by the CMC system show that selective α_1 adrenoceptor antagonist prazosin, which had the strongest affinity, ranked first, the highly selective α_{1D} antagonist BMY7378 ranked second, and the highly selective α_{1A} antagonist 5-methylurapidil ranked fourth. In other words, prazosin and BMY7378 had strong receptor affinity in the rat aorta, but 5-methylurapidil had low affinity. This supports the hypothesis that a_{1D}-AR is the predominant subtype in the rat aorta. It also demonstrates that CMC can be used to identify receptor subtypes in tissue, like functional assay and RLBA.

In previous studies, CMC has been applied to the study of drug–receptor interactions and affinity [4–8], which can be shown by chromatographic parameters, such as capacity factors. However, CMC cannot be used to assay K_d. In the present study, our results suggest that frontal analysis of CMC may be used for determination of K_d, and that CMC is an alternative reliable method to quantitatively study ligand–receptor interactions.

Currently, receptor functional assay and RLBA are the methods that are most often used in studying ligand–receptor interactions. Functional assay has high measurement errors, low accuracy, and low sensitivity in quantitative analysis. RLBA might result in radioactive pollution and a need to separate free from bound ligands. Moreover, RLBA requires that ligand dissociation takes much longer than the time required to perform the separation step. In contrast to receptor functional assay and RLBA, CMC has several advantages. The receptors may be stably bound and may therefore be reused over a long period of time. It may be possible to perform multiple experiments with hard-to-obtain receptors, using an immobilized format.

Furthermore, one of the most important advantages of CMC is its utility for screening for active ingredients in plant extracts and natural product-like libraries. CMSP has the dual effect of biological membrane activity and chromatographic separation, which has been demonstrated by scanning electron microscopy, surface energy spectrometry, enzyme assay, and LC [1,2]. Therefore, chromatographic separation and receptor-based assays for screening for biologically active substances can be carried out simultaneously. As a reliable tool, CMC has been used successfully to isolate and analyze active leading compounds or effective components in Chinese traditional medicine extracts, such as *Angelica sinensis* [22], *Herba epimedii* [23], *Leontice robustum* [24], and *Cladonia alpestris* [25].

5. Conclusion

A method for measurement of drug-α_{1D}-AR affinity by frontal analysis of CMC was developed. CMSP columns that were prepared by immobilizing rat aorta cell membranes on porous silica possessed stability, reproducibility and affinity for α_{1D}-AR agents. The columns can be used for frontal affinity chromatography. The different α_{1D}-AR agents showed different affinity on the columns, and the relative binding affinity (K_d) of α_{1D}-AR agents showed a good correlation with the affinity of the same compounds for the cloned α_{1D}-AR subtype obtained from RLBA. In other words, the combination of CMC and frontal affinity chromatography can be used to determine K_d. In the future, we will pursue to prepare the different CMSP columns and to determining different drug–receptor affinities, and screen active ingredients in plant extracts and natural product-like libraries.

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