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# Reversible and Oriented Immobilization of Histidine-Tagged Protein on Silica Surface and in the Characterization by Frontal Analysis



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# Reversible and Oriented Immobilization of Histidine-Tagged Protein on Silica Surface and in the Characterization by Frontal Analysis

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This approach utilizes N, N'-bis (carboxymethyl)-L-Lysine (ANTA) coordinate bivalent metal cation $Ni^{2+}$ leaving free coordination sites for reversible combining gene recombinant histidine-tagged $\beta_2$-adrenoceptor onto the macropore silica. The amount of transient metal nickel ion on the support was determined by atomic absorption spectrophotometry. Established novel protein oriented immobilization $\beta_2$-AR column was evaluated by five $\beta_2$-adrenoceptor agonists applying frontal analysis. The association equilibrium constant for ligands on the column was $1.98\times10^4$ $M^{-1}$ for salbutamol, $3.43\times10^4$ $M^{-1}$ for clenbuterol, $2.09\times10^4$ $M^{-1}$ for tulobuterol, $1.84\times10^4$ $M^{-1}$ for terbutaline, $1.71\times10^4$ $M^{-1}$ for methoxyphenamine and corresponding concentration of binding site were $7.46\times10^{-6}$ M, $1.82\times10^{-5}$ M, $2.16\times10^{-5}$ M, $8.29\times10^{-6}$ M and $3.88\times10^{-5}$ M, respectively. The results obtained from breakthrough and nonlinear fitting indicated that all drugs have a single binding site on the $\beta_2$-adrenoceptor column. The present combining histidine-tagged protein method was reliable and exact to reveal interactions between receptor and drugs.

## Introduction

Immobilization of biological macromolecules with the entire retention of their activity is an interesting subject owing to its application in the field of pharmaceutical research and bioreactors.[1-3] Protein immobilization technology has been widely applied and most techniques resulted in various orientations which causes considerable activity loss.[4-6]Controlling the molecular structure and orientation of protein on a solid surface without destroying their biologically activity have many advantages, such as retain active binding sites and increase stability. One strategy was to produce recombinant proteins containing histidine residues in either amino or carboxyl end of the protein and the proteins were immobilized by metal chelating; and histidine tags have only six amino acids and structurally and functionally benign, which achieve reversible orient immobilization of histidine-tagged protein; the interaction between histidine and chelated ions such as $Cu^{2+}$, $Co^{2+}$ and $Ni^{2+}$ etc. have high binding stability and low dissociation constant; immobilized proteins can be washout by competitively eluted with imidazole, so the manner is a reversible interaction.[7-9] Several similar approaches using chelated metal ions have been reported, such as lipid interfaces, gold surfaces, magnetic nanoparticles and some high polymer.[10-12] Nitrilotriacetic acid (NTA)/metal/histidine-tag (HT) were mainly used in the biosciences for isolation and purification of proteins.[13,14] In many researches, NTA was most covalently bound to gold surface previously modified with an activated self-assembled monolayer for electrochemical study or some magnetic nanoparticles still for purification or acted as fluorescence labeling of his-tag proteins.[15,16] In the present study, ANTA group was coupled to active macropore silica as a metal-chelating linker and his-tag protein was orient immobilized on the support, furthermore chromatography study was developed by correlative analytic techniques. The specific immobilization properties of the material prepared were tested using recombined $\beta_2$-adrenoceptor ($\beta_2$-AR), which is important in the treatment of many diseases and the crystal structure has been determined.[17-19] This has enabled the preparation of wild recombinant His-$\beta_2$-AR within a six-histidine tag at the N-terminus or C-terminus by genetic engineering, the position of his tag would apart from the active sites and accurate interactions between receptor and ligands were obtained.

## Materials and Methods

### Chemicals and instruments

Salbutamol was from Changzhou yabang pharmaceutical Co. Ltd, clenbuterol was from Wuhan huamei science and technology Ltd., tulobuterol and methoxyphenamine were from Wuhan beierka biomedical Co. Ltd, terbutaline was from Shanghai xiarui pharmaceutical technology Co. Ltd. N, N'-bis (carboxymethyl)-L-Lysine (ANTA) was from Jiaxing aisen chemical Co. Ltd. Globulin free bovine serum albumin (BSA, A0281) was obtained as lyophilized powder from Sigma-Aldrich (St. Louis, MO, USA). $Ni^{2+}$ Sepharose 6 Fast Flow (10×58 mm) and Sephadex G25 pre-packed column (10×100 mm) were purchased from GE Healthcare Life Sciences (Uppsala, Sweden). Macroporous silica gel (SPS 300-7, pore size 300 Å, particle size 7.0 mm) was from Fuji Silysia Chemical Company Limited (Tokyo, Japan).

The HPLC system employed in this work consisted of a binary pump, a column oven from Agilent Technologies (Santa Clara, USA) and a diode array detector (Waldbronn, Germany). The data were collected and processed by a Chemstation 5.2 software installation.

### Expression and purification of $\beta_2$-AR

The full gene of Sus scrofa $\beta_2$-AR (Refseq: MN_001128436) was cloned in our previous work and the protein was expressed in *E.coli* BL21 (DE3) with an N-terminal His-tag.[20] The transformed *E. coli* strain was grown overnight at 37 ℃ in 50 mL Luria-Bertani (LB) medium containing 100 μg/L ampicillin sodium salt. When the value of $OD_{600}$ reached 0.4–0.6, isopropyl-$\beta$-D-thiogalactopyranoside (IPTG) was added to and the final concentration was 2.0 mM, then the cultured solution was incubated at 30 ℃ for 6.0 h. The cells containing His-tagged $\beta_2$-AR were separated from the culture media by centrifugation at 11000 rpm for 10 min at 4.0 ℃, and the supernatant was discarded. The *E. coli* pellet was collected and obtained 30.0 g *E. coli* extract for the next experiment. The *E. coli* was suspended at a concentration of 0.1 g/mL with 20 mM phosphate buffer containing 500 mM NaCl (buffer A, pH 7.4) prior to cell disruption in an ice-water bath using an ultrasonic processor at 200 W. The processed suspension was centrifuged at 10,000 rpm for 30 min at 4.0 ℃, the precipitation was discarded and supernatant was filtrated by 0.22 μm membrane for further purification.

Nickel-chelated Sepharose high-performance affinity media, quaternary sepharose fast flow anion exchangers and size-exclusion chromatography were sequentially used to purify the recombinant $\beta_2$-AR expressed as His-tagged protein in *E. coli*. A total of 30 g *E. coli* extract was loaded onto the column (10×58 mm) containing Ni-chelated Sepharose high performance affinity media. To purify the $\beta_2$-AR, flushed the column sequentially using buffer A, 10% buffer B (buffer A in the presence of 500 mM imidazole, pH 7.4) and 50% buffer B at a flow rate of 1.5 mL/ min. 50% buffer B extract concluded $\beta_2$-AR was further purified on the quaternary sepharose fast flow column (16×25 mm) and 18 % buffer C (20 mM phosphate buffer containing 800 mM NaCl, pH 7.4) was used to equilibrate the column until the baseline was stable. A gradient elution consisting of buffer C increased from 18 to 40% over a period of 20 min at a flow rate of 5.0 mL/min was used to collect the $\beta_2$-AR fraction. The collection was desalinated using the Sephadex G25 column (10×100 mm) to acquire a purified $\beta_2$-AR solution. Sodiumdodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) was used to determine the purity and molecular weight of $\beta_2$-AR.

### Specific immobilization of $\beta_2$-AR

The $\beta_2$-AR was oriented immobilized on macroporous silica gel using an ANTA-$Ni^{2+}$ chelating method (Figure 1). In detail, Aminopropyl silica gel was prepared according to the method in our previous report.[21] 2 g macro-pore silica gel was pretreated with 50 ml 10% hydrochloric acid and dried at 110 ℃, then the silica gel was reacted with γ-aminopropyl triethoxysilane in dried toluene, and activated by N, N'-carbonyldiimidazole. The amino-nitrilotriacetic-Ni (II) complex (ANTA-$Ni^{2+}$) was formed by reaction of N, N'-bis (carboxymethyl)-L-Lysine (ANTA) with an excess of Nickel (II) sulfate stirred in 20 mM HEPES in aqueous solution overnight.[22] Then activated aminopropyl silica gel was acted with ANTA-$Ni^{2+}$ solution for one night, then collected $\beta_2$-AR elution and phosphate buffer (pH 7.4) were added to combine with the silica gel. The resulting mixture was stirred for 2 h at 4.0 ℃ ice bath conditions, after filtration and washing with buffer solution several times to produce the immobilized $\beta_2$-AR stationary phase.

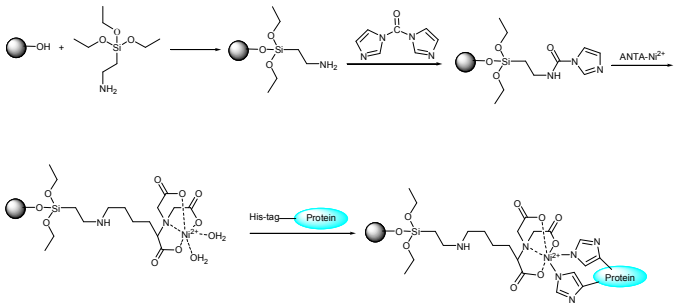


**Fig.1.** Diagram for oriented immobilizing His-tagged $\beta_2$-AR on the surface of ANTA-Ni (Ⅱ) chelated silica gel.

### Chromatography

The column containing the oriented immobilized $\beta_2$-AR was prepared by packing the stationary phase into a stainless steel column (4.6 mm ×5.0 cm) using the slurry method at a pressure of $4\times10^7$ Pa. Phosphate buffer (20 mM, pH 7.4) was used as the slurry solution. The flow rate was 0.3 ml/min. The ligands were detected using UV detector at respectively max absorbance wavelength. The column was placed into a column oven at 37 ℃.

### Frontal analysis

All ligands were dissolved in 20 mM phosphate-buffered solution as 10 mM stock solutions and further diluted with phosphate-buffered solution (20 mM, pH 7.4) to obtain a chromatographic mobile phase that contained serial concentration of ligands (0.5, 1, 10, 30, 60 and 100 μM for methoxyphenamine; 0.5, 1, 3, 10, 20, 40 and 60 μM for terbutaline; 2, 5, 10, 30, 50, 70 and 90 μM for tulobuterol; 1, 3, 5, 8, 10, 30 and 50 μM for clenbuterol; 0.5, 1, 3, 5, 10, 20 and 40 μM for salbutamol). All the chromatographic mobile phases were totally

degassed by ultrasonic wave before use. Frontal analysis was performed at a flow rate of 0.3 ml/min. A correction for the system void time was made using sodium nitrite as a non-retained solute. The retained compounds were eluted and the column regenerated between studies by passing phosphate buffer solution (20 mM, pH 7.4) through the column.

**Model of equilibrium isotherm**

The adsorbed isotherm of five ligands was determined by frontal analysis at 37 ℃, respectively. The Langmuir (Eq. (1)) and bi-Langmuir (Eq. (2)) model was used to describe adsorption of five drugs on the $\beta_2$-AR column.

$$q = \frac{q_s b C}{1 + bC} \quad (1)$$

$$q = \frac{q_{s1} b_1 C}{1 + b_1 C} + \frac{q_{s2} b_2 C}{1 + b_2 C} \quad (2)$$

In equation $q$ is the monolayer capacity, $q_s$ is the monolayer saturation capacity and $b$ is the adsorption constant of the studied compound. While $q_{s1}$ and $q_{s2}$ are the saturation capacities for the first and second type of sites, respectively, $b_1$ and $b_2$ are the adsorption constants on the first and second type of sites, respectively, and C is the concentration of solute. Non-linear regression of experimental data to adsorption isotherm models was done using OriginLab software.

**Determination of association constants and binding sites**

Frontal analysis is a classical method to characterize the binding of small molecules to a protein. This assay can be applied in measuring the association constant ($K_a$) and the number of active binding sites ($m_L$) of a ligand to an immobilized receptor by the affinity chromatography.[23] If an applied analyte (A) binds to only a single type of ligand site (L) and this process has fast association/dissociation kinetics, Eq. (1) can be used to relate the true number of binding sites on the column ($m_L$) to the apparent moles of solute ($m_{Lapp}$) required to reach the mean point of the breakthrough curve. [23]

$$\frac{1}{m_{Lapp}} = \frac{1}{m_L K_a [A]} + \frac{1}{m_L} \quad (3)$$

In this relationship, $K_a$ is the association equilibrium constant for the binding of A to L, and [A] is the concentration of solute applied to the column. This equation predicts that a plot of $1/m_{Lapp}$ versus 1/[A] will give a straight line with a slope equal to $1/(K_a m_L)$ and an intercept of $1/m_L$. This can be obtained $K_a$ from the ratio of the intercept to the slope and $m_L$ from the inverse of intercept.

If multiple binding sites for A are detected, expanded versions of Eq. (3) can be used. For instance, if a column contains two classes of binding sites, $L_1$ and $L_2$, the relationship between $1/m_{Lapp}$ and [A] takes the following form[23]

$$\frac{1}{m_{Lapp}} = \frac{1 + K_{a1}[A] + \beta_2 K_{a1}[A] + \beta_2 K_{a1}^2 [A]^2}{m_{Ltot}\{(\alpha_1 + \beta_2 - \alpha_1\beta_2) K_{a1}[A] + \beta_2 K_{a1}^2 [A]^2\}} \quad (4)$$

In Eq. (4), $K_{a1}$ is the association equilibrium constant for the highest affinity site ($L_1$), and $\alpha_1$ is the fraction of all binding sites that belong to this group (i.e., $\alpha_1 = m_{L1,tot}/m_{L,tot}$, where $m_{L1,tot}$ is the total moles of site $L_1$ in the column). The term $\beta_2$ is the ratio of association equilibrium constants for the low versus high affinity sites, where $\beta_2 = K_{a2}/K_{a1}$ and $0 < K_{a2} < K_{a1}$.

Unlike Eq. (3), the expression in Eq. (4) does not predict a linear relationship between $1/m_{Lapp}$ and [A]. However, this equation does approximate a straight line at low analyte concentrations, as shown in Eq. (4).

$$\lim_{[A]\to 0} \frac{1}{m_{Lapp}} = \frac{1}{m_{Ltot}(\alpha_1 + \beta_2 - \alpha_1\beta_2) K_{a1}[A]} + \frac{\alpha_1 + \beta_2^2 - \alpha_1\beta_2^2}{m_{Ltot}(\alpha_1 + \beta_2 - \alpha_1\beta_2)^2} \quad (5)$$

By Eqs. (4) and (5) it is thus possible to examine frontal analysis data for a two-site system and obtain quantitative information on the relative amount of each site (as represented by $\alpha_1$) and their affinities (as represented by $K_{a1}$ and $\beta_2$).

## Results and Discussion

**Purification of his-tagged $\beta_2$-AR**

The total protein in the desalted $\beta_2$-AR solution was determined by Bradford method. Using BSA as a standard substance, at the concentration of 15.625, 31.25, 62.5, 125, 250, 500μg/mL and the calibration curve was plotted. The linear regression equation was y = 0.0005x + 0.3111, with a correlation coefficient of 0.9993. The concentration of the total protein in the solution was 95.68μg/mL. Determination of molecular weight and purity in the solution was obtained by Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and BandScan software as our previous procedure.[20] This confirmed $\beta_2$-AR in solution was to be 66.5 kDa and the concentration of $\beta_2$-AR was approximately 91μg /mL.

**Quantification of Nickel ion in the silica gel**

Nickel ion was digested from silica gel by striping buffer solution (100 mmol/L EDTA, 0.5 mol/L NaCl, 20 mmol/L Tris-HCl, pH 7.9) and the contents of nickel ion were determined by atomic absorption spectrophotometry.[24] The calibration curves of the content of $Ni^{2+}$ were presented and the linear regression equation was y = 0.0065x + 0.0159, linear coefficient was $R^2$=0.9962. The results were calculated to be 309.8μg/g which was much higher than that of reference.[24] Metal nickel ion and his tag protein can be combined according to 1:1 ratio on molar basis, so the support can immobilize his tag protein theoretical maximum was 5.28μM/g.

**Immobilization of His-tagged $\beta_2$-AR**

Proposed immobilization approach is a reversible, oriented and density controlled binding manner. Comparing with random immobilization method in our previous experiments, this better

retained $\beta_2$-AR bioactive characteristics and increased the binding sites on silica gel surface. The concentration of immobilized His-tag $\beta_2$-AR on silica gel was calculated by detecting the free concentration of $\beta_2$-AR in the solution before and after the metal ion chelation reaction through the Bradford method. The amount of $\beta_2$-AR attached on the gel was 389.56pmol/g. This was bigger than that of random immobilization and other orient immobilization reported earlier (200.17pmol/g).[20] The results indicated that this method can immobilize number of recombination protein, the quantity of target protein on the support will increase if we make much more protein reacted with the support.[21,25] Existing reports of metal chelate oriented immobilization protein methods mainly applied for purification of protein and some electrochemistry studies.[14,26] This work applied the technology to control protein orientation on support. Moreover, other recombination protein including his-tag can also be deposited by this method on macropore silica and for further research.

**Specificity and stability of $\beta_2$-AR column**

The specificity of the $\beta_2$-AR column was tested by determining the retention times of specific drugs on the receptor. The retention times of specific ligands salbutamol, clenbuterol, tobuterol, terbutaline and methoxyphenamine were listed in table 1, whereas the specific ligands of the $\alpha_1$- adrenoceptor naftopidil and terazosin hydrochloride cannot retain on the column. The varied retention times illustrated that $\beta_2$-AR had the specificity for recognizing its ligands after the immobilization and packing procedures.

**Table 1** Retention time and capacity factors of five ligands in different columns. The void time was determined by sodium nitrite (2.03 min) on the two columns.

| Ligands | $\beta_2$-AR column | | Blank column | |
|---|---|---|---|---|
| | Retention time (min) | capacity factors ($k$') | Retention time (min) | capacity factors ($k$') |
| Salbutamol | 5.22 | 1.57 | 7.70 | 2.79 |
| Clenbuterol | 12.61 | 5.21 | 25.73 | 11.67 |
| Tobuterol | 10.25 | 4.05 | 20.69 | 9.19 |
| Terbutaline | 4.56 | 1.25 | 5.72 | 1.82 |
| Methoxyphenamine | 11.93 | 4.88 | 20.90 | 9.30 |

It was noted that all ligands have a retention on the blank column compared with the void time, and the retention time of them was longer on the blank support than that on the $\beta_2$-AR column, the results were different from previous experiments, this may be arrived from different combine mechanism.[27] In this report protein was orient immobilized on the support surface and the conjugation was occurred between nickel ions and nitrogen atoms of his tag, similarly all ligands have nitrogen atoms, so which may be the reason they have a retention on the blank column. Comparing the retention time of every ligand on the two columns, the values were different and retention time of every ligand on the blank column were longer than that on the $\beta_2$-AR column. The results indicate that the anchoring strength between ligands and the blank support was higher than that between ligands and the $\beta_2$-AR column, conformed that weak interaction occurred between ligands and protein and more strong force between ligands and metal ions. It was also confirmed that $\beta_2$-AR was immobilized on the silica gel by metal ions.

The stability of $\beta_2$-AR was investigated by detecting the retention time of salbutamol, clenbuterol and tobuterol for 25 consecutive days. The relative standard deviations (RSD) of the retention times on those days were 1.23%, 3.56% and 2.16%. Meanwhile, the peak profiles of the three drugs have no obviously diversities, indicating that the immobilized $\beta_2$-AR retained bioactivity and stable over the testing period, it can be concluded that the $\beta_2$-AR column had a good stability for at least 25 days.

**Determination of $K_a$ and $m_L$ by front analysis**

Interactions between ligands and $\beta_2$-AR column were performed by front analysis in which serial concentrations of known ligands were added to the mobile phase and passed through the column. Representative chromatographic curves produced by this method using clenbuterol as mobile phase additive are presented in Figure 2. As indicated by this example, the mean position of these curves shifted to the left as the concentration of applied clenbuterol increased. Isotherm data of five ligands can also be acquired from breakthrough curves. Fig. 3 showed this best fits curve model of typical drug methoxyphenamine and the isotherm of methoxyphenamine was best fitted to the Langmuir model through nonlinear fitting by OriginLab 8.0, which manifested this drug has only one type of binding site with $\beta_2$-AR by front analysis. Other drugs have analogous curves and isotherm were also best fitted to the Langmuir model (not shown here), which indicated them have only one type of binding site on the same column.

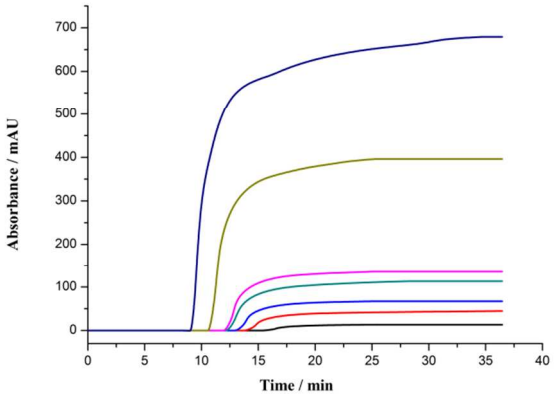


**Fig.2.** Frontal analysis curves for clenbuterol to the $\beta_2$-AR column at pH 7.4 and 37℃, where the concentration applied clenbuterol (from right to left) are 1.0, 3.0, 5.0, 8.0, 10, 30 and 50 μM.

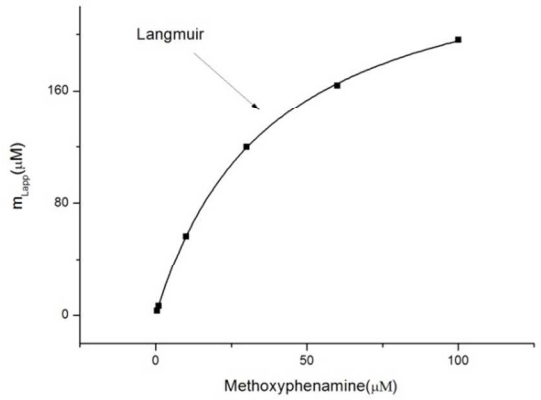


**Fig.3.** Adsorption data of Methoxyphenamine measured by frontal analysis and best isotherm model processed by non-linear regression.

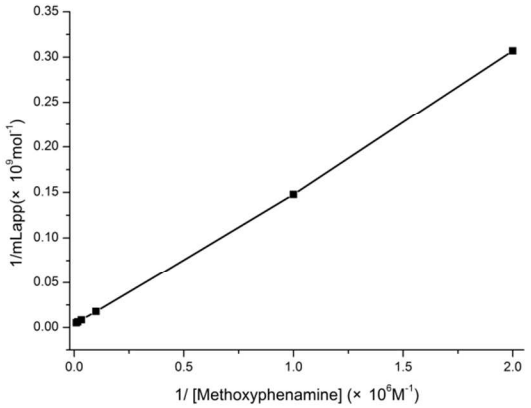


**Fig.4.** Double-reciprocal frontal analysis plots for the application of methoxyphenamine to an immobilized $\beta_2$-AR column and the concentration of methoxyphenamine were 0.5, 1, 10, 30, 60 and 100 μM, respectively. The regression equation was y = 0.1508x + 0.0026 and correlation coefficients 0.9995.

The plots obtained by $1/m_{Lapp}$ versus 1/ [methoxyphenamine] gave linear relationship at this studies, which present in Fig. 4 with correlation coefficients 0.9995 and others were gave linear relationships as much ($R^2 \geq 0.99$). The results suggested that only a single type of binding site was present for all ligands on the immobilized $\beta_2$-AR. Meanwhile association constants and binding site of ligands were analyzed using Equation (3) and the data were listed in table 2. The affinity rank order of the five ligands measured from this table was clenbuterol > tulobuterol> salbutamol > terbutaline > Methoxyphenamine and the results were equally to the research from the research of *Baker*.[28] Comparing the $K_a$ values calculated by the affinity chromatographic method were generally lower than the corresponding values from radio ligand binding studies. Although the $K_a$ values differed, linear regression analysis shows that the data from chromatography present a positive response to logarithms of the $K_a$ values from radio ligand binding assay with a correlation coefficient of $R^2 = 0.9709$ and a regression equation of y = 0.1842x + 0.8716 which was shown in Figure 5 indicating that the observed differences was quantitative not qualitative. It can be concluded this method could survey interaction between ligands and $\beta_2$-AR and accuracy evaluate intensity of the effects.

**Table 2** Linear best-fit parameters for frontal analysis studies performed with salbutamol, terbutaline, clenbuterol, tulobuterol and methoxyphenamine on $\beta_2$-AR column.

| Ligands | Association constant ($K_a$) ($\times 10^4$L/mol) | Binding site ($m_L$) ($\times 10^{-5}$ mol/L) |
|---|---|---|
| Salbutamol | 1.98 | 0.75 |
| Terbutaline | 1.84 | 0.83 |
| Clenbuterol | 2.37 | 1.82 |
| Tulobuterol | 2.09 | 2.16 |
| Methoxyphenamine | 1.71 | 3.88 |

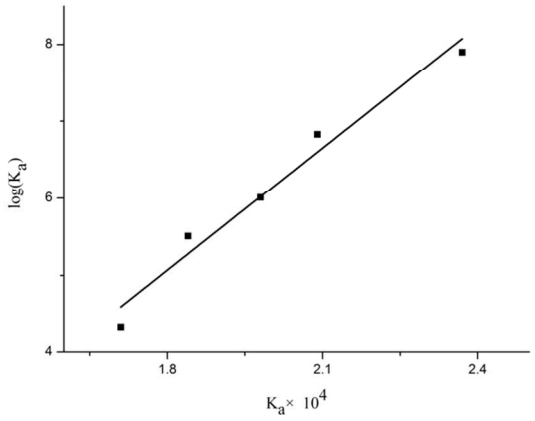


**Fig.5.** Correlation between the calculated $K_a$ by $\beta_2$-AR column and logarithm of the published value from the report of Baker. The regression equation was y = 0.1842x + 0.8716 with the correlation coefficients 0.9709.

## Conclusions

ANTA-$Ni^{2+}$ was introduced into macropore silica gel and applying chelate metal ion can special combine his tag of recombination $\beta_2$-AR and the protein was orient immobilized on the support. Corresponding ligands was applied to research the interaction with $\beta_2$-AR by front analysis. Association constant ($K_a$) and binding sites ($m_L$) of five ligands were obtained from the breakthrough curves. The $K_a$ values are substantially different from varying ligands, indicating that the present affinity method can be rapidly used to determined receptor–ligand interaction. Due to the chelating manner

between metal nickel ion and protein was reversed, the support can be repeatedly used rather than that support which combine protein by covalent bonding. Meanwhile the recombination protein can also be adopted as long as the protein still retain bioactive when protein was eluted from the support. So the technique for chelating is an economic and protein orient controlled method. Similar other protein including histidine-tagged can also be deposited by this method which offered a new route for protein orient immobilization and further studies.

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## Notes and references

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