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Binding kinetics of five drugs to beta2-adrenoceptor using peak profiling method and nonlinear chromatography

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ABSTRACT

Investigations of drug-protein interactions have advanced our knowledge of ways to design more rational drugs. In addition to extensive thermodynamic studies, ongoing works are needed to enhance the exploration of drug-protein binding kinetics. In this work, the beta2-adrenoceptor (β_2-AR) was immobilized on *N*, *N*'-carbonyldiimidazole activated amino polystyrene microspheres to prepare an affinity column (4.6 mm × 5.0 cm, 8 μm). The β_2-AR column was utilized to determine the binding kinetics of five drugs to the receptor. Introducing peak profiling method into this receptor chromatographic analysis, we determined the dissociation rate constants (k_d) of salbutamol, terbutaline, methoxyphenamine, isoprenaline hydrochloride and ephedrine hydrochloride to β_2-AR to be 15 (±1), 22 (±1), 3.3 (±0.2), 2.3 (±0.2) and 2.1 (±0.1) s^{-1}, respectively. The employment of nonlinear chromatography (NLC) in this case exhibited the same rank order of k_d values for the five drugs bound to β_2-AR. We confirmed that both the peak profiling method and NLC were capable of routine measurement of receptor-drug binding kinetics. Compared with the peak profiling method, NLC was advantageous in the simultaneous assessment of the kinetic and apparent thermodynamic parameters. It will become a powerful method for high throughput drug-receptor interaction analysis.

1. Introduction

The association or dissociation equilibrium constants of drug-protein binding (K_A or K_D) are routinely measured to describe the affinities between drugs and proteins as a key part of drug discovery [1,2]. In many cases, the kinetic rate constants (k_a and k_d) of drugs binding to proteins can vary by several orders of magnitude even when they demonstrate similar K_A values [3]. Methods of affinity determination are limited in their abilities to provide an adequate basis for the selection of lead compounds. This necessitates more comprehensive evaluations of drug-protein binding interactions, particularly through a kinetics study [4].

The kinetic data of drug-protein binding are traditionally acquired by radioactive filter binding or scintillation proximity assays [5,6]. Such assays suffer from the necessary usage of radioactive ligands. Numerous other methods have also been employed for determining kinetic data of drug-protein interactions, such as surface plasmon resonance (SPR), stopped flow techniques, filtration assays and microdialysis [7–9]. In addition to these methodologies, high performance affinity chromatography (HPAC) is becoming one of the commonly used methods to determine the kinetics of drug-protein interactions with weak to moderate affinities (association equilibrium constant, $K_A < 10^6\ M^{-1}$) and k_d values in the range of 10^{-2}–$10^2\ s^{-1}$ [10–12]. In this context, band-broadening measurements, including the number of theoretical plates (N) for an affinity column and the retention time of the analyte (t_R), are determined for both drugs and a non-retained solute on an immobilized protein column [13]. Two kinds of theoretical methods, peak profiling and nonlinear chromatography (NLC) are modeled to utilize these data for calculating the dissociation rate constants. The success of these methodologies relies on their applications in real samples [14]. However, such applications are very limited and far behind the requirements to achieve high-throughput drug-protein interaction analysis.

Beta2 adrenergic receptor (β_2-AR) acts as the main therapeutic target for drugs against diseases of the heart and respiratory system

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[15–17]. Our earlier reports have illustrated that the receptor-conjugated macroporous silica gel is a new stationary phase [18,19] for drug-receptor interaction analyses. This stationary phase has some intrinsic problems. For example, biocompatibility is low due to the utilization of inorganic support. The analysis efficiency of drug-receptor interactions of this stationary phase needs improvement. This situation motivates us to search for friendlier solid supports and a more efficient theoretical model to create a high-throughput methodology for constructing immobilized β_2-AR. Here, we introduced amino polystyrene microspheres to construct the β_2-AR stationary phase. The throughput of the resulting stationary phase was evaluated by investigating the thermodynamic and kinetic interactions between five drugs and β_2-AR using peak profiling method and nonlinear chromatography.

2. Material and methods

2.1. Reagents and instruments

Standards of salbutamol (batch No. 100328–200703), terbutaline (batch No. 100273–201202), methoxyphenamine (batch No. 100403–201101), isoprenaline hydrochloride (batch No. 100166–201004) and ephedrine hydrochloride (batch No. 171241–201508) were purchased from the National Institutes for Food and Drug Control (Beijing, China). Yeast extract, peptone, ampicillin, and isopropylthio-β-D-galactoside (IPTG) were from Amresco (Houston, Texas, USA). *N*, *N*'-carbonyldiimidazole was obtained from Sigma-Aldrich (St. Louis, MO, USA). Amino polystyrene microspheres (pore size 300 Å, particle size 8 μm) were acquired from Wuxi Knowledge & Benefit Sphere Tech. Co., Ltd. (Wuxi, China). Ni-chelated sepharose high-performance affinity media and quaternary sepharose fast flow anion exchangers were from GE Healthcare Life Sciences (Uppsala, Sweden). Other chemical reagents were analytically pure unless stated.

An AKTA10 low-pressure chromatographic apparatus from GE Healthcare Life Sciences (Uppsala, Sweden) was utilized to purify β_2-AR. The ZZXT-A type packing machine was supplied by Dalian Elite Analytical Instruments Co., Ltd. (Dalian, China). The chromatographic system for drug-receptor interaction analysis was the Agilent 1100 series apparatus (Agilent Technologies, Santa Clara, USA), which contains a binary pump, a column oven and a diode array detector (Waldbronn, Germany).

2.2. Preparation of the immobilized β_2-AR column

The histidine-tagged β_2-AR was expressed and purified from the genetically engineered bacteria *E. coli* BL21 (DE3)-pET32a-β_2-AR, which was previously preserved in our laboratory [20]. The separation and purification of the receptor were achieved using a sequential two-step column chromatographic assay as reported previously, i.e., Ni-chelated sepharose high performance affinity chromatography and quaternary sepharose fast flow anion exchange chromatography. The purity of the β_2-AR product was estimated to be 96% by sodium dodecyl sulfate polyacrylamide gel electrophoresis [20].

The purified β_2-AR was immobilized on the *N*, *N*'-carbonyldiimidazole (CDI) activated amino polystyrene microspheres through the covalent bond between the amino acid of the receptor and the microsphere [21]. Initially, 1.0 g of amino polystyrene microspheres (amino content: 456 μmol/g microsphere) was immersed in dry acetonitrile (30 ml) containing 6.0 mmol CDI. The reaction was kept at room temperature for 2.0 h under vigorous agitation. The resulting microspheres were filtrated and washed sequentially with dry acetonitrile (100 ml) and phosphate buffer (0.02 M, pH 7.4, 100 ml). Thereafter, 30 ml of β_2-AR was attached on the activated microspheres by reacting for 2.0 h at room temperature. Using the slurry method, the column containing β_2-AR was prepared by packing the affinity microspheres into a stainless steel column (4.6 mm × 5.0 cm) under a pressure of 400 Pa. The control column was prepared under identical conditions, but no β_2-AR was added during the immobilization step.

2.3. Chromatographic experiments

All the experiments were performed on an Agilent 1100 series high-performance liquid chromatographic apparatus. Phosphate buffer (0.02 M, pH 7.4) was filtered through a 0.45 μm cellulose acetate filter, and the filtrate was utilized as the mobile phase. Solutions of the five drugs were prepared by water. Salbutamol and terbutaline were detected at 276 nm. The detection wavelengths for methoxyphenamine, isoprenaline hydrochloride, ephedrine hydrochloride and sodium nitrate were set at 213, 280, 257 and 254 nm, respectively. All chromatographic studies were carried out in triplicate at 25 °C.

2.4. Peak profiling experiments

Peak profiling experiments were performed on the immobilized β_2-AR column with a sampling volume of 5.0 μl of each drug. The applied concentrations of salbutamol, terbutaline, methoxyphenamine, isoprenaline hydrochloride, ephedrine hydrochloride and sodium nitrate were 1.0, 0.5, 1.5, 2.5, 4.0 and 10.0 mM. Multiple flow rates of 0.4, 0.8, 1.2, 2.0 and 2.4 ml/min were implemented for all the five drugs. The first and second statistical moments (i.e., the retention time and variance) of the chromatographic peaks were determined using PeakFit 4.12 (Systat Software, San Jose, CA) with an exponentially modified Gaussian (EMG) fit and a linear progressive baseline. The residual option in PeakFit software was employed to determine the best fit for the chromatographic peaks.

2.5. Nonlinear chromatography

In this experiment, 5.0 μl of a series of concentrations of salbutamol, terbutaline, methoxyphenamine, isoprenaline hydrochloride and ephedrine hydrochloride was directly injected into the immobilized β_2-AR columns to record their elution profiles. The concentrations were 0.1, 0.2, 0.4, 0.8, 1.0 and 2.0 mM for salbutamol and terbutaline; 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0 and 5.0 mM for methoxyphenamine; 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 mM for isoprenaline hydrochloride; and 2.5, 5.0, 10.0, 15.0, 20.0, 25.0, and 50.0 mM for ephedrine hydrochloride. The data were processed using NLC function in Peak Fit 4.12.

3. Results and discussions

3.1. Characterization of the affinity column

The activity and specificity of the β_2-AR column were investigated by analyzing the retention behaviors of sodium nitrite (non-retained analyte), terazosin (specific ligand of α_1-AR), salbutamol and methoxyphenamine on the column. The retention times of the ligands were 1.3 ± 0.1, 1.2 ± 0.2, 5.6 ± 0.1 and 20.1 ± 0.1 min with peak widths of 0.5 ± 0.1, 0.5 ± 0.1, 1.1 ± 0.1 and 4.2 ± 0.1 min for the four ligands. The discrepancy in retention times and peak widths confirmed the bioactivity of the receptor. The specificity can also be confirmed by the fact that all the ligands gave the same retention ((1.2 ± 0.2) min) on a control column without β_2-AR.