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Journal of Pharmaceutical and Biomedical Analysis
33 (2003) 765–773
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Flow-injection chemiluminescence detection for studying protein binding of terbutaline sulfate with on-line microdialysis sampling

Zhouping Wang, Zhujun Zhang *, Zhifeng Fu, Dinglong Chen, Xiao Zhang

Department of Chemistry, Institute of Analytical Science, Southwest China Normal University, Beibei, Chongqing, 400715, P.R. China

Received 24 April 2003; received in revised form 9 June 2003; accepted 11 June 2003

Abstract

The binding of terbutaline sulfate to bovine serum albumin was studied in vitro using the technique of microdialysis sampling combined with flow-injection chemiluminescence analysis (FIA-CL). In the presence of formaldehyde, terbutaline sulfate can be oxidized by $KMnO_4$ to produce high chemiluminescence emission in sulfate acid media. The concentration of terbutaline sulfate is proportional with the CL intensity in the range of 1×10^{-7}–2×10^{-5} mol l^{-1} with a detection limit of 3×10^{-8} mol l^{-1}. The drug and protein were mixed in different molar ratios in 0.067 mol l^{-1} phosphate buffer, pH 7.4, and incubated at 37 °C in a water bath. The microdialysis probe was utilized to sample the mixed solution at a perfusion rate of 5 μl min^{-1} and the dialytic efficiency of terbutaline sulfate under the experimental conditions was 26.3%. The data obtained by proposed microdialysis flow-injection chemiluminescence method was analyzed with Scrathard analysis and Klotz plot. The estimated association constant (K) and the number of the binding site (n) on one molecule of BSA by Scrathard analysis were 4.11×10^4 l mol^{-1} and 1.06, respectively. The proposed system proved that FIA-CL coupled with on-line microdialysis sampling is a simple and reliable technique for the study of drug–protein interaction.

Keywords: Microdislysis sampling; Chemiluminescence analysis; Terbutaline sulfate; Bovine serum albumin

1. Introduction

The study of protein interaction with drug and the determination of related parameters are of great practical and theoretical importance in pharmacokinetics and pharmacodynamic properties study [1]. When a drug is administered, in general, most of it enters the blood stream and is transported in two forms. One dissolved in blood in the form of free drug (unbound), and the other combined with various elements in blood (bound), such as plasma proteins and blood cells [2]. The main factor determining the ratio of bound to unbound drug in plasma is the reversible interaction between drug and protein molecules. Many plasma proteins, particularly albumin and α-acid glycoprotein, can bind with drugs. Since only the

* Corresponding author. Tel./fax: +86-23-6825-3863.
E-mail address: zzj18@hotmail.com (Z. Zhang).

0731-7085/03/$ - see front matter
doi:10.1016/S0731-7085(03)00416-3

unbound drug can reach target tissue region through passive diffusion to generate pharmacological action, so, the concentration of unbound drug is closely related to the concentration of drug in action site, namely, pharmaceutical effect. The unbound fraction, ratio of the concentration of unbound to total drug, is often more useful than the bound fraction. The distribution of drug as well as the relation between pharmacological activity and total drug concentration in plasma are all influenced by the binding action of plasma proteins. In this respect, plasma protein binding is an important factor in establishing pharmacokinetic and pharmacodynamic properties of a drug. The ratio of bound to unbound drug is very important to research specific properties of drug, such as the metabolic rates in the liver, the excretory rate of the kidney and the steady state distribution volume [1,3,4]. In addition, recent research results show that protein binding of a racemic drug is potentially different between the enantiomers, which may result in the enantioselectivity in disposition property [5–7].

Because drug-protein binding is reversible and a kinetically rapid interaction, it should be analyzed without disturbing binding equilibrium. Based on the procedures of separating mixture of the bound and free drug, various methods, mainly including equilibrium dialysis [8,9], ultrafiltration [3,10], and high-performance frontal analysis (HPFA) [11,12], followed by HPLC or CE have been developed for indirect study of drug–protein interaction. Hage and Tweed reviewed the chromatographic and electrophoretic methods for the study of drug–protein interaction with 161 references [13]. Spectrophotometry and fluorometry have extensively used for direct study of drug–protein interaction based on monitoring the change of a physicochemical property of the drug–protein system [14,15]. Ion-selective electrode and quartz crystal resonant sensor techniques have also been developed for the direct study of drug–protein interaction [16,17]. Each of them often presents different advantages and disadvantages [3,4,13].

Recently, microdialysis has proven itself to be a flexible and powerful tool for monitoring in vivo and in vitro chemistry [18,19]. Such technique has been successfully applied to biomedical, pharmaceutical, and neuroscience studies [20–22]. However, to the best of our knowledge, only few reports have dealt with the technique for the determining the degree of binding of drugs to plasma protein in vitro [23–27]. Moreover, most of them often suffer from the disadvantage of sample loss during handling due to an off-line operation. As an alternative to it, Shi et al. [26] and Huang et al. [4] proposed an on-line sampling method coupling microdialysis with detector system by FIA. This method minimizes the delay between sample collection and analysis and eliminates the problems of evaporation that can occur during any physical manipulation of small volume samples.

In recent years extremely sensitive analytical technique based on chemiluminescence (CL) systems have received considerable attention. Simplicity of detection, low detection limit, wide calibration ranges and short analysis times are some of the characteristics that make the method attractive. When coupled with flow-injection analysis (FIA), the CL-based FIA method provides cheap, rapid, simple and reproducible means of detection and, therefore, has been successfully applied to many drugs detection [28–31]. When coupled with microdialysis sampling and used for the study of protein binding of drug, it offers the merits of rapidity, sensitivity and simple instrument [4]. Hitherto, nevertheless, few literatures involved in the application of microdialysis sampling combined with flow-injection CL detection for the study of protein binding of drug [4].

Terbutaline sulfate (±-α-[(tert-butylamino)-methyl]-3,5-dihydroxy-benzyl alcohol sulfate) is a synthetic β_2-adrenergic agonist that is widely used as a bronchodilator for the treatment of bronchial asthma, chronic bronchitis and emphysema. Until now, many analytical methods have been developed for the determination of terbutaline sulfate in various substrates, which mainly included colorimetric [32], liquid chromatography (LC) [33,34], liquid chromatography-mass spectrometry (LC-MS) [35], capillary electrophoresis-mass spectrometry (CE-MS) [36,37], ^{1}H-NMR and RP-LC [38], and plastic membrane electrode [39] techniques. However, there is a lack of information concerning

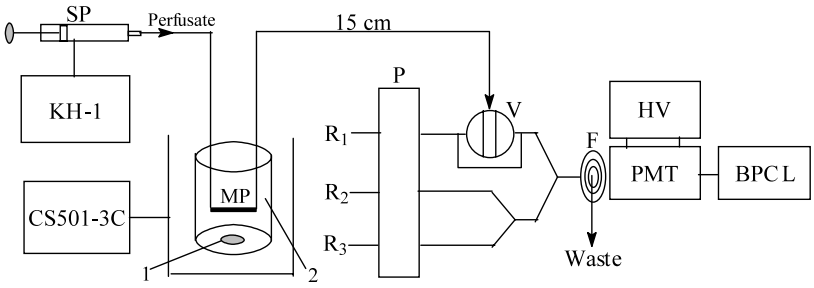

Fig. 1. Schematic diagram of studying the drug–protein binding using flow-injection CL detection with on-line microdialysis sampling: 1, stirrer; 2, Terbutaline sulfate + BSA mixture solution; SP, microdialysis syringe pump; MP, microdialysis probe; R_1, water carrier; R_2, $KMnO_4$ solution (in H_2SO_4); R_3, HCHO solution; P, peristaltic pump; V, injection valve; F, flow cell; KH-1, Model KH-1 syringe micropump controller system; CS501-3C, CS501-3C super thermostat water bath controller; PMT, photomultiplier tube; HV, negative high-voltage supply; BPCL, luminescence analyzer controlled by personal computer.

chemiluminscence (CL) analysis of terbutaline sulfate.

In present work, a novel FIA-CL method for the determination of terbutaline sulfate based on the CL reaction of acid $KMnO_4$ with terbutaline sulfate in the presence of formaldehyde is proposed. It then was applied for in vitro study of protein binding of terbutaline sulfate coupled with on-line microdialysis sampling. The drug and protein were mixed in different molar ratios in 0.067 mol l^{-1} phosphate buffer, pH 7.4, and incubated at 37 °C in a water-bath. The microdialysis probe was utilized to sample the mixed solution at a perfusion rate of 5 μl min^{-1} and the dialytic efficiency of terbutaline sulfate under the experimental conditions was 26.3%. The relative parameters obtained agreed well with literature values and demonstrated good accuracy and precision. Compared with other methods established for the study of drug–protein interaction, the proposed system offers several advantages of simplicity, rapidity and sensitivity.

2. Experimental

2.1. Chemicals and reagents

All the reagents were of analytical reagent grade unless specified otherwise. The ultrapurified water prepared by a Milli-Q system (Millipore) was used throughout.

Bovine serum albumin (BSA) was purchased from Bio Life Science & Technology, Shanghai, China and its solutions were prepared in phosphate buffer. A stock solution of terbutaline sulfate (Drug and biological products examination bureau of China, Beijing, China) (1×10^{-4} mol l^{-1}) was daily prepared in amber-colored measuring flask and stored in the refrigerator (4 °C). Working standard solutions were prepared from the stock solution by appropriate dilution in phosphate buffer (Na_2HPO_4–KH_2PO_4, 0.067 mol l^{-1}, pH 7.4) immediately before use. Stock solution of potassium permanganate (0.01 mol l^{-1}) was prepared by dissolving 1.58 g of potassium permanganate in 1 l of 0.1 mol l^{-1} H_2SO_4. Formaldehyde solution (3%, v/v) was diluted daily from its original solution.

2.2. Apparatus

Microdialysis sampling was performed using a MF-7051 Microdialysis Probe from Bioanalytical Systems (0.12 mm i.d., 0.32 mm o.d., 5 cm membrane length, exclusion limit 3035 Dak, BSA, West Lafayette, IN). A Model KH-1 syringe micropump (1000 μl) and its controller system were used for delivery of perfusates. The flow rate was fed on 5 μl min^{-1}. The microdialysis manifold is shown schematically in Fig. 1. The PTFE tubing

of 0.25 mm i.d. and 0.8 mm o.d. was used to connect the microdialysis probe directly to the eight-way injection valve. CL signal measurements were carried out with a computerized BPCL-type ultra weak CL analyzer (Institute of Biophysics, Chinese Academic of Sciences, Beijing, China). The emitted CL was collected with CR-105 photomultiplier tube (operated at -800 V, Hammamatsu, Tokyo, Japan) of CL analyzer. Data acquisition and treatment were performed with BPCL software running under Windows 98. The flow system (Fig. 1) consisted of a peristaltic pump which delivers each reagent solution at equal slow rate (2.5 ml min^{-1}) through flow tubes and an eight-way injection valve with a 50 μl loop, through which sample solution was injected into the carrier stream. The flow cell was made by coiling 30 cm of colorless glass tube (1 mm i.d. and 2 mm o.d.) into a spiral disk shape with a diameter of 2 cm and placed closed to the window of the photomutiplier tube. PTFE tubing of 0.25 mm i.d. and 0.8 mm o.d. was used for all connections.

2.3. Procedures

2.3.1. Optimization of CL system

Flow lines were inserted into $KMnO_4$ (in 2.5 mol l^{-1} H_2SO_4) solution, formaldehyde solution and water carrier, respectively. With the injection valve in the load position, the pump was started at a constant speed of 2.5 ml min^{-1} on each flow line to wash the whole flow system until a stable baseline was recorded. The 25 μl terbutaline sulfate solution was injected into the carrier stream (water) through injection valve. This stream was merged with the mixture of $KMnO_4$ and formaldehyde solution in flow cell, producing CL emission. To establish the optimal conditions for the CL system, the relative CL intensity was measured as a function of the concentrations of $KMnO_4$, H_2SO_4 and formaldehyde. In these experiments, the terbutaline sulfate standard solution was directly introduced into the injection valve in continuously. The CL emission intensities versus terbutaline sulfate concentration were used for the calibration.

2.3.2. Drug–protein interaction studies in vitro

In order to use the microdialysis probe for the drug–protein interaction experiment, its dialytic efficiency for terbutaline sulfate in different flow rates of perfusate and under various temperatures must be calibrated firstly. As Fig. 1 shown, calibration experiments of dialytic efficiency were carried out by placing the microdialysis probe into a stirred 50 ml 5×10^{-6} mol l^{-1} terbutaline sulfate solution (contained in a 50 ml beaker) that was maintained at 37 °C in a water bath of Model CS501 super thermostat water bath (Chongqing, China). The probe was perfused with phosphate buffer at a 5 μl min^{-1} for 5 min. The relative dialytic efficiency *RDE* was estimated according to the following equation:

$$RDE = C_{out}/C_m \quad (1)$$

where C_{out} was the terbutaline sulfate concentration in microdialysate, and C_m the drug concentration surrounding the probe.

After the probe calibration was completed, the protein binding studies in vitro was conducted immediately. The same microdialysis probe was placed in the mixture of 10 μmol l^{-1} BSA and different concentrations of terbutaline sulfate (3–11 μmol l^{-1}) and connected to the flow injection CL system, and then perfused with phosphate buffer at a 5 μl min^{-1} for 5 min to obtain 25 μl samples in sample loop. For each terbutaline sulfate concentration, the dialysate collection began after an equilibration time of 30 min. For every change of Terbutaline sulfate concentration, the first dialysate was discarded to avoid the residual influence of the previous concentration. At least three determinations were done for each terbutaline sulfate concentration to obtain a mean C_{out} value corresponding to the studied medium. Unbound drug concentration (C_u) in the surrounding medium was determined by dividing the concentration C_{out} by the dialytic efficiency (*RDE*) according to the following equation:

$$C_u = C_{out}/RDE \quad (2)$$

The bound fraction of drug *B* (%) was calculated as follows:

$$B(\%) = (C_m - C_u)/C_m \times 100\% \quad (3)$$

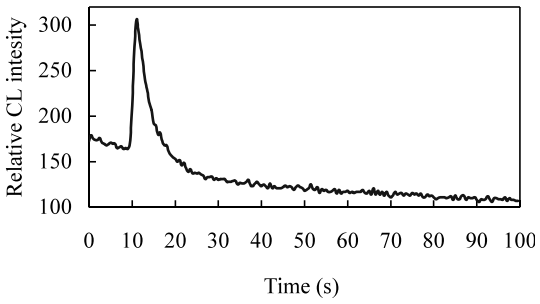

Fig. 2. Kinetic curve of the CL reaction. Terbutaline sulfate was added to the mixture of $KMnO_4$ and HCHO solutions.

The binding parameters were estimated by Eq. (4) for Scatchard analysis [13,26] or Eq. (5) for Klotz plot

$$\frac{r}{[C_u]} = nK - rK \quad (4)$$

$$\frac{1}{r} = \frac{1}{n} + \left(\frac{1}{nK}\right)\left(\frac{1}{[C_u]}\right) \quad (5)$$

where $[C_u]$ is the concentration of the free drug, r the ratio of bound drug to the protein in molar concentration, n the number of binding sites on one protein molecule and K the association constant. Once $r/[C_u]$ (or $1/r$) and r (or $1/[C_u]$) were determined, $r/[C_u]$ (or $1/r$) was regressed on r (or $1/[C_u]$) using the linear equation $Y = mX + b$ (where m = slope and $b = Y$-intercept), from which n and K could be estimated.

3. Results and discussions

3.1. Kinetic figure of the CL reaction

The kinetic figure of the CL emission was examined in a static method. The signal profile is shown in Fig. 2, indicting that the luminescence reaction is rapid. Terbutaline sulfate solution was injected into the mixture of $KMnO_4$ and HCHO solutions at the 9.5th second. The CL signal was recorded by BPCL ultra-weak luminescence analyzer and the intensity reached a maximum $\sim$1.5 s (9.5–11 s) later. Then the CL intensity became weaker and below the original value after $\sim$7 s (11–18 s).

3.2. Conditions of the CL detection system

3.2.1. Effect of H_2SO_4 concentration on the CL intensity

The CL emission of $KMnO_4$ often involves the acid medium. So, various acid including HNO_3, H_2SO_4, HCl, H_3PO_4, $HClO_4$ and polyphosphoric acid were introduced into the CL system as reaction medium with a concentration of 1 mol l^{-1}. The results show that the maximum CL signal was achieved in polyphosphoric acid and then H_2SO_4. Due to better signal/noise ratio and reproducibility was obtained when H_2SO_4 was used, therefore, H_2SO_4 was chosen as the CL media for further experiment. The effect of H_2SO_4 concentration on the CL intensity was studied over the range of 0.2–6.0 mol l^{-1}. The results show that the CL intensity increased with increasing concentration in the range of 0.2–2.5 mol l^{-1}. Above the concentration of 2.5 mol l^{-1}, the CL intensity declined. Thus, the 2.5 mol l^{-1} H_2SO_4 was used throughout for subsequent work.

3.2.2. Effect of $KMnO_4$ concentration on the CL intensity

In this CL system, $KMnO_4$ was used as the oxidant. The dependence of the CL intensity on the concentration was examined for 2×10^{-7} mol l^{-1} terbutaline sulfate. It was found that the highest CL intensity was obtained when the concentration of $KMnO_4$ was 7×10^{-5} mol l^{-1}. Above the concentration of 7×10^{-5} mol l^{-1}, the CL intensity declined probably due to the inner filter effect. So, the $KMnO_4$ concentration of 7×10^{-5} mol l^{-1} was selected as optimum.

3.2.3. Effect of HCHO concentration on the CL intensity

The effect of HCHO concentration on CL intensity was investigated in the range of 1–5% (v/v). The result showed that the highest CL intensity was at 3% HCHO concentration. Therefore, the HCHO concentration of 3% was chosen for further studies.

3.2.4. Effect of flow rate on the CL intensity

The flow rate is an important factor in flow injection CL system. According to the kinetic

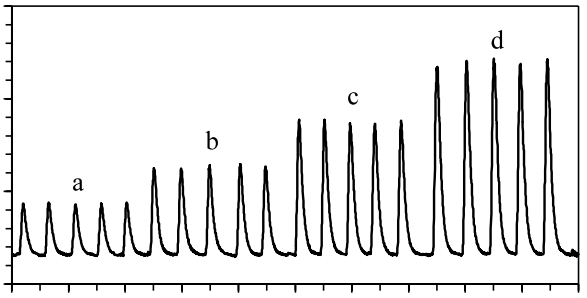

Fig. 3. A typical recoding output of the proposed CL system for the measurement of terbutaline sulfate (a, 3×10^{-6} mol l^{-1}; b, 5×10^{-6} mol l^{-1}; c, 7×10^{-6} mol l^{-1}; d, 10×10^{-5} mol l^{-1}). $KMnO_4$, 7×10^{-5} mol l^{-1}; H_2SO_4, 2.5 mol l^{-1}; HCHO, 3% (v/v); flow rate, 2.5 ml min^{-1}; high voltage, -650 V.

curve (Fig. 2), the CL emission of $KMnO_4$–terbutaline sulfate–HCHO system is rapid. Therefore, an optimum flow rate is necessary for the maximum collection of the emitted light in the flow cell to deliver the product. The effect of flow rate on the CL emission was tested in the range of 0.1–4.0 ml min^{-1}. The result showed that the highest CL intensity was achieved when the flow rate of each line was fed at 2.5 ml min^{-1}.

3.2.5. Analytical characteristics of the CL system for terbutaline sulfate determination

Under the optimum conditions described previously, the calibration graph of concentrations of terbutaline sulfate versus CL peak height was linear in the range of 1×10^{-7}–2×10^{-5} mol l^{-1} ($I = 61.5 + 14.8$[terbutaline sulfate] $\times 10^6$ (mol l^{-1}), $r = 0.9996$, $n = 10$), and the calibration of the measurement was performed with ATr sulfate concentration of 0.1, 0.3, 0.5, 0.7, 1, 3, 5, 7, 10, 20 mol l^{-1}. The relative standard deviation (R.S.D.) for 1×10^{-6} mol l^{-1} terbutaline sulfate measurement was 2.7% ($n = 11$). The detection limit, defined as three times of the S.D. for the reagent blank signal, was 3×10^{-8} mol l^{-1} (3σ) terbutaline sulfate. A typical recording output of the proposed CL system for the measurement of different concentrations of terbutaline sulfate was shown in Fig. 3.

3.3. Dialytic efficiency calibration of microdialysis probe

Before the use of microdialysis probe, the dialytic efficiency of the probe must be investigated for the analyte to be determined. It is often influenced by various factors, such as temperature, perfusion flow rate, probe membrane length and type of analyte. When the probe and analyte are fixed, temperature and perfusion flow rate became two important factors determining the dialytic efficiency of microdialysis probe. In this work, the dialytic efficiency of the microdialysis probe is necessary to assess free concentration of the studied drug in the surrounding medium.

3.3.1. Effect of perfusate flow rate on the dialytic efficiency of microdialysis probe

The effect of perfusate flow rate on the dialytic efficiency of microdialysis probe was examined in the range of 1–9 μl min^{-1} at 37 °C. It was seen that the dialytic efficiency of the probe decreased with the increment of perfusate flow rate. A low flow rate of the perfusate through the probe generates a higher dialytic efficiency, but fewer samples could be made during a same time period and at high flow rates low concentration of terbutaline sulfate reached the detection system. According to the observed results, the 5 μl $min^{-1}$was chosen as optimum dialysis flow rate and gave the dialytic efficiency of $26.3 \pm 2.7\%$ ($n = 3$).

3.3.2. Effect of temperature on the dialytic efficiency of microdialysis probe

The effect of temperature on the dialytic efficiency of microdialysis probe was investigated at 25, 37 and 40 °C with a perfusate flow rate of 5 μl min^{-1}, respectively. The results show that higher temperature produced higher dialytic efficiency. Since the dialytic efficiency of the probe varied in different temperature, the dialysis system must be controlled strictly at 37 °C (±0.2 °C) in order to get and maintain an accurate dialytic efficiency in physiologically normal temperature (37 °C).

Table 1
The binding fractions in BSA solution

Ratio of studied drug to BSA (μmol l^{-1}: μmol l^{-1})	Unbound drug concentration (μmol l^{-1})[a]	Bound drug concentration (μmol l^{-1})[a]	Binding fraction (%)[a]	Literature value (%)
3:10	2.14±0.028	0.86±0.013	28.67±0.12	
5:10	3.64±0.117	1.36±0.053	27.20±0.34	
7:10	5.15±0.023	1.85±0.029	26.43±0.39	25 [37]
9:10	6.69±0.085	2.31±0.057	25.67±0.47	
11:10	8.29±0.029	2.71±0.058	24.64±0.32	

[a] Mean±S.D. ($n = 3$).

Table 2
Binding parameters for terbutaline sulfate–BSA interaction

Data analysis	T (°C)	pH	n[a]	K ($\times 10^4$)[a]	nK ($\times 10^4$ l mol^{-1})[a,b]	r^2*
Eq. (4)	37±0.2	7.4	1.06±0.037	4.11±0.227	4.36±0.135	0.9996
Eq. (5)	37±0.2	7.4	1.12±0.042	3.85±0.045	4.31±0.062	0.9843

[a] Mean±S.D. ($n = 3$).
[b] $nK = r/C_u$, where r and C_u represent the number of bound drug molecule per one protein molecule and unbound drug concentration, respectively.
* r^2 is the regression coefficient.

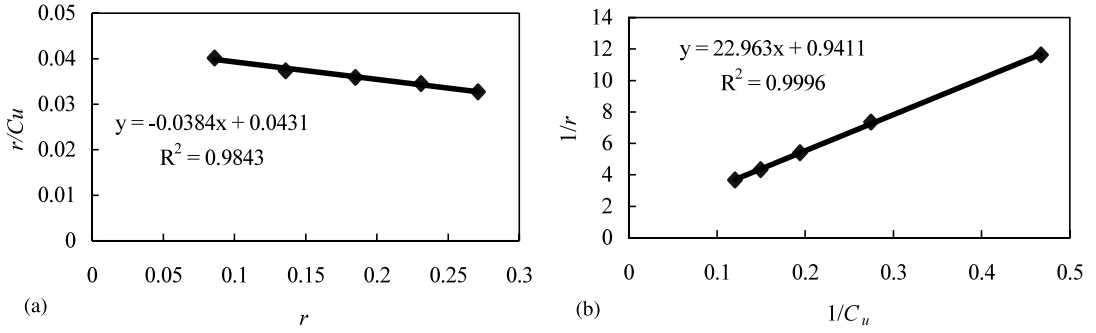

Fig. 4. Scatchard plot (a) and Klotz plot (b) of BSA binding of terbutaline sulfate.

3.4. Interaction between terbutaline sulfate and BSA

Microdialysis is a selective sampling technique in the sense that no plasma protein or other large molecules enter the perfusate. Moreover, it allows precise control of important experimental variables, such as temperature. The dialysis membrane has certain molecular weight cutoff. It allows free drug crossing the membrane and diffusing into the probe due to the concentration gradient of free drug from the outside to the inside of the microdialysis probe membrane, but hardly for protein and protein-bound drug. Because little drug is actually removed from the sample, the overall drug concentration remains apparently constant during the experiment. Also, because microdialysis does not change the fluid volume, the protein concentration remains constant. Therefore, the drug binding equilibrium is not disturbed by this technique. According to method proposed by Wang et al. [25] the relative decrease in the terbutaline sulfate concentration in the terbutaline sulfate–BSA mixed solution can be calculated

according to the following equation:

$$A(\%) = ((v \times RDE \times t \times C_u)/(V \times C_m)) \times 100\% \quad (6)$$

where v is the perfusion flow rate, RDE the relative dialytical efficiency, t the sampling period, V the volume of the mixed solution, C_u the unbound drug concentration, and C_m the drug concentration surrounding the probe, respectively. In present experiment, $v = 5$ μl min^{-1}, $RDE < 30\%$, $t < 60$ min, $V = 50$ ml, $C_u/C_m < 0.75$, A calculated by Eq. (6) is below 0.2%, indicating that the effect of microdialysis sampling on binding equilibrium can be neglected.

Table 1 listed the binding data of studied drug in drug-BSA mixed solution with different molar ratios of studied drug to BSA sampled by microdialysis and analyzed by FIA-CL. The binding parameters for terbutaline sulfate–BSA interaction calculated using Eqs. (4) and (5) was shown in Table 2. The bound parameters of terbutaline sulfate reported here were consistent with those published in literature [40]. This agreement indicates the reliability of the present method. Moreover, the nK values using Eqs. (4) and (5) for studied drug are quite similar, and the Scatchard plot and Klotz plot are linear (Fig. 4), indicating that studied drug has only one type of binding site. At the same time, compared with high affinity drugs, for example, warfarin and fenoprofen (nK is about 10^6 l mol^{-1}), the nK value for terbutaline sulfate is very small, showing that terbutaline sulfate is a very lightly binding drug.

4. Conclusion

In this work, the flow-injection CL system with on-line microdialysis sampling was successfully applied to studying protein binding for terbutaline sulfate. The estimated association constant (K) and the number of the binding site (n) on one molecule of BSA by Scrathard analysis were 4.11×10^4 l mol^{-1} and 1.06, respectively. The bound parameters of terbutaline sulfate reported here were consistent with those published in literatures. This agreement indicates the reliability of the present method. From the observed results, it can be seen that the method provided a fast and simple technique for the study of drug–protein interaction compared with the classic technique (such as equilibrium dialysis) and is a valid method for the determination of the binding characteristics of drug to protein, offering the advantage of no disturbing binding equilibrium. The method demonstrates further potentials for applications to determining the degree of binding, the affinity constant and the binding number for small molecules, environmental toxicant and macromolecules. For in vivo study, the proposed method should be coupled with HPLC or CE in order to avoid the interference of other species existing in body fluids.

Acknowledgements

This study was support by the National Natural Science Foundation of China (No. 20175039).

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