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Short communication

Binding study of drug with bovine serum album using a combined technique of microdialysis with flow-injection chemiluminescent detection

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Abstract

Microdialysis coupled with flow-injection chemiluminescence (FI–CL) has been developed to determine the binding parameters of a drug binding to protein by using antibiotic tetracycline hydrochloride binding to bovine serum albumin as a model system. The drug and protein were mixed in different molar ratios in 0.067 M 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. The concentration of unbound tetracycline hydrochloride in the microdialysate was determined by FIA–CL. In vitro recovery of tetracycline hydrochloride under experimental conditions was 30.0%. The data obtained by the present microdialysis–FI–CL system was analyzed using the Scatchard analysis and Klotz plot. The results show that the Scatchard plot and Klotz plot are linear with good correlation coefficient, indicating a good agreement of the experimental data and to the theoretical equation. The FIA chemiluminescence system combined with microdialysis developed in this work demonstrated its use for determination of interaction between drug and protein by using relatively simple instrument.

Keywords: Microdialysis; Drug–protein interaction; Flow-injection analysis; Chemiluminescence

1. Introduction

In pharmacology, effects of a chemical are related to the target site concentration of said chemical. In general, only the unbound drug is active and capable of diffusing across membrane, whereas bound drug hardly passes through the blood capillary walls to reach the action site. In this respect, plasma protein binding is an important factor in establishing pharmacokinitic and pharmacodynamic properties of a drug, as only the free fraction of the drug is pharmacologically active [1]. Some important pharmacokinitic properties such as hepatic metabolism rate, renal excretion rate, biomembrane permeation rate, and the steady state distribution volume, also depend on the unbound drug fraction.

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Various methods have been developed to study drug–protein phenomena. Because drug–protein binding is reversible and a kinetically rapid interaction, it should be analyzed without disturbing binding equilibrium. Until now, equilibrium dialysis [2–4] and ultrafiltration [4–6] have been commonly used to determine unbound drug concentrations in both in vivo and in vitro samples. However, these methods suffer from drawbacks. For example, equilibrium dialysis experiments require large quantities of the drug and are time-consuming due to the long periods required to reach equilibrium. The ultrafiltration method also requires large amounts of the drug and the drug–protein equilibrium may be altered by changes in sample concentration during filtration. It seems that the advent of high-performance frontal analysis (HPFA) [7–9] overcomes these problems. The HPFA has several advantages. For example it allows direct injection analysis that benefits automatic analysis, simultaneous determination of total and unbound drug concentrations. HPFA is easy to incorporate into an on-line HPLC system. HPFA permits determination of unbound concentrations of respective enantiomers when combined with chiral HPLC column. HPFA is suitable for the study of strong protein binding which is often difficult to achieve by the conventional methods. However, HPFA is not suitable for the analysis of weak protein binding because it requires a large injection volume. More recently solid phase microextraction [10] and affinity capillary electrochromatography were reported to study the drug–protein binding [11].

Microdialysis is a sampling technique, which was first introduced for brain study [12]. Now microdialysis has been applied to the general pharmacokinetic, toxicology, metabolism, biological process monitoring, etc [13–16]. Some investigators have used the technique to determine degree of binding of drugs to plasma protein in vitro and in vivo [17–29]. In most of these studies individual dialysate samples were collected off-time with a fraction collector, then analysed by HPLC with UV, electrochemical detection or fluoremetry, due to the low perfusate flow rates used (typically 1–5 μL/min or less), evaporation of samples during handing is inevitable. One of approaches overcoming problems associated with handing small volume samples is on-line coupling of the microdialysis system with the analytical system. 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.

It is now widely realized that chemiluminescence (CL) detection in analysis can be a means of achieving high sensitivity with simple instruments (no monochromator required) and rapidity in signal detection (normally 0.1–10 s) over a wide dynamic range with low detection limits. When coupled with flow-injection analysis (FIA), CL-based FIA methods provide cheap, rapid, simple, and reproducible means of detection, and therefore, has been extensively used in many fields [30–37]. In this work, a microdialysis–FIA–chemiluminescence system was developed to determine the binding parameters of a drug binding to protein by using antibiotic tetracycline hydrochloride binding to bovine serum albumin as a model system because tetracycline is regularly applied for the prevention or the treatment of mastitis and metritis in cows. In addition, tetracyclines are widely used antibiotics in aquaculture and in nutrition and feed additives in the field of agriculture. Hence, there is a growing need to monitor tetracyclines in the food industry. Study of tetracycline–BSA binding may provide the basis for estimating the tetracycline residue level.

2. Experimental

2.1. Chemicals and reagents

All the reagents were of analytical-reagent grade unless specified otherwise; potassium hexacyanoferrate(III) and potassium ferrocyanide were obtained from Chongqing Chemical Reagent Company. A 0.01 mol/L luminol solution was prepared by dissolving 1.772 g of luminol in 1000 mL of 0.01 mol/L NaOH. More diluted solutions were prepared in proper concentration of NaOH and used immediately. Tetracycline hydrochloride was available from the Sino-American Company. Stock solutions of drug was freshly prepared in phosphate buffer ($Na_2HPO_4{\cdot}2H_2O$, 0.067 M, KH_2PO_4, 0.067 M) adjusted to pH 7.4. 100 μM bovine serum album (BSA) (Sigma, American) was prepared in phosphate buffer.

2.2. Experimental procedure

2.2.1. Procedure for chemiluminescent determination of tetracycline

Flow lines were inserted into potassium ferrocyanide/NaOH/luminol solution, potassium hexacyanoferrate(III) solution, water carrier, respectively. The flow system (Fig. 1) consisted of two peristaltic pumps which delivered reagents streams and a water carrier stream at a flow rate of 4.0 mL/min (total flow rate) and one microdialysis syringe pump (controlled by a Type KH-1 microdialysis syringe pump controller from Institute of Chemistry, Chinese Academy of Sciences, Peking, PR China). The flow cell was made by coil a 40 cm length PTFE tubing (0.8 mm i.d.) into a spiral disk shape with a diameter of 2.5 cm and fixed the disk onto the surface of organic glass close to photomultiplier tube of the BPCL Ultra Weak Chemiluminescence Analyzer (Institute of Biophysics, Chinese Academy of Sciences, Beijing) with transparent glue. PTFE tubing (0.8 mm i.d.) was used to connect all components in the flow system. 15 μL of sample solution was injected into the carrier stream by an eight-way injection valve. The emitted CL was collected with photomultiplier tube (operated at −800 V) of the BPCL Ultra Weak Chemiluminescence Analyzer. The signal was recorded using an IBM-compatible computer, equipped with a data acquisition interface. Data acquisition and treatment were performed with BPCLWIN software running under Windows 98. For characterization of the on-line injection and chemiluminescent analysis system, the sample was directly introduced into the injection valve in a continuous manner. This was accomplished by directly connecting the syringe pump to the injection valve with the microdialysis probes, which were from Bioanalytical Systems (BAS) (West Lafayette, IN). In these experiments, aqueous standards were used. A series of working standard solution with different concentrations were prepared by diluting a concentrated fresh standard solution of tetracycline hydrochloride with phosphate buffer. The CL signal was measured by injection 15 μL of standard solution into water carrier stream by an eight-way injection valve. The CL emission intensities versus tetracycline hydrochloride concentration were used for the calibration.

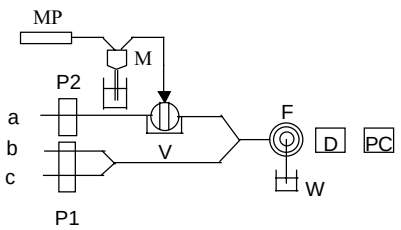

Fig. 1. Experimental set-up for studying the drug–protein binding using microdialysis–FIA–chemiluminescence detection system. P1, P2: peristaltic pump; V: injection valve; F: flow cell; W: waste liquid; D: PMT; PC: personal computer; a: water carrier stream; b: potassium hexacyanoferrate(III); c: potassium ferrocyanide and luminol in sodium hydroxide solution; M: microdialysis probe; MP: micropump and its controller system.

2.2.2. Procedure for microdialysis and protein binding studies

The microdialysis experiments were performed in two steps, the first step consisting of a primary probe calibration, studying the time needed to reach a stable dialysate concentration and the effect of dialysis medium concentration on the recovery value. The second step consisted of protein binding studies in vitro. They were performed in triplicate at 37 °C. The microdialysis manifold is shown schematically in Fig. 1. Recovery experiments were carried out by placing the dialysis probe in a 50 mL beaker containing buffer with known concentration of tetracycline hydrochloride varying between 10 and 60 μM. Probe was perfused with phosphate buffer at a 5 μL/min for 3 min. The relative recovery R was estimated according to the following equation

$$R = \frac{C_{\text{dialysate}}}{C_{\text{m}}} \quad (1)$$

where C_{m} was the surrounding concentration (total drug concentration), and $C_{\text{dialysate}}$ the drug concentration in microdialysate.

Then the same microdialysis probe was placed in 100 μM BSA solution containing 10–60 μM tetracycline hydrochloride. Probe was perfused with phosphate buffer without drug. Thirty minutes after the beginning of the perfusion at least three determinations were done and to obtain a mean $C_{\text{dialysate}}$ value corresponding to the studied medium. Free drug concentration (C_{u}) in the surrounding medium was determined

by dividing the concentration $C_{\text{dialysate}}$ by the recovery (R) according to the following equation

$$C_{\text{u}} = \frac{C_{\text{dialysate}}}{R} \quad (2)$$

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

$$(B\%) = \frac{(C_{\text{m}} - C_{\text{u}})}{C_{\text{m}}} \times 100\% \quad (3)$$

The binding parameters were estimated by the following equation for Scatchard analysis

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

or Eq. (5) for Klotz plot

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

where [A] being 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.

3. Results and discussion

3.1. Optimization of experimental conditions for the FI microdialysis sampling CL determination of tetracycline hydrochloride

A series of experiments was conducted to establish the optimum analytical conditions for the chemiluminescent oxidation of tetracycline hydrochloride by potassium hexacyanoferrate(III) and dissolved oxygen. The parameters optimized included reagent and sample flow rates, and oxidant and depression reagent and alkali concentrations. Luminol oxidation by potassium hexacyanoferrate(III) produces strong emission leading to high background. Primary experiments suggest that the addition of potassium ferrocyanide decreases the CL intensity of luminol reaction in the presence of potassium hexacyanoferrate(III). The potassium ferrocyanide concentration was varied in the range 25–150 mM in order to maximize the S/N ratio. The maximum S/N ratio was obtained with 50 mM potassium ferrocyanide. The effect of potassium hexacyanoferrate(III) was studied in the range from 10 to 100 μM. The greatest S/N ratio was obtained with 25 μM potassium hexacyanoferrate(III). Due to the nature of the luminol reaction, which is more favored under basic conditions, sodium hydroxide was introduced into the manifold in the reagent stream to improve the sensitivity of the system. The effect of sodium hydroxide concentration was examined in the range 0.1–0.5 M. The highest S/N ratio was obtained at the concentration of 0.4 M. Finally, luminol effect was also studied in the range 0.1–0.5 mM. Experiment results showed that the maximum S/N ratio was obtained with 0.2 mM luminol. Using the selected conditions given above and following the procedure described in the experimental section, the calibration graph of emission intensity vs. tetracycline hydrochloride concentration was linear in the range of 1–100 μg/mL expressed by the regression equation: $\Delta I = 15.5C + 41$ ($n = 6$), $r^2 = 0.9998$, ΔI being the chemiluminescence intensity and C the tetracycline hydrochloride concentration. The detection limit based on three times the baseline noise was 0.2 μg/mL tetracycline hydrochloride. The precision of the system was 1.4% R.S.D. ($n = 11$, 5 μg/mL tetracycline hydrochloride).

3.2. Probe calibration recovery

For use of microdialysis, the maximization of the extraction efficiency or relative recovery is the main concern. The perfusion flow-rate is an important factor that defines the performance of a microdialysis probe and has direct influence on the recovery of the probe. Hence, prior to use of any probe, the recovery as a function of the flow rate of the perfusate, or at least the recovery for the flow rate of interest, must be examined for the compound to be determined. The effect of perfusion rate on the recovery of the microdialysis probe used in this study at 37 °C with the probe length of 10 mm was studied in the range of 1–10 μL/min. The experimental results show that the recovery increases with decreasing the perfusion flow-rate. A low flow rate of the perfusion liquid through the probe gives a higher recovery, but fewer injections could be made during a given time period and at high flow rates low concentration of tetracycline hydrochloride reached the detection system. From the observed results, the 5 μL/min was selected as optimum dialysis flow rate and gave the relative recovery of 30%.

3.3. Drug protein binding

A major advantage of using in vitro microdialysis to study drug protein binding is that it allows precise control of important experimental variables, such as temperature. Another advantage of the technique is that it is a selective sampling technique in the sense that no plasma protein or other large molecules enter the perfusion fluid. Because of the concentration gradient of free drug from the outside to the inside of the microdialysis fiber, free drug will diffuse into the probe. The molecular weight cutoff of the dialysis membrane is such that protein and protein-bound drug cannot cross the membrane. Because little drug is actually removed from the sample, the overall drug concentration remains essentially 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. [21], the relative decrease in the tetracycline hydrochloride concentration in the tetracycline hydrochloride–BSA mixed solution can be calculated according to the following equation

$$A(\%) = \left[\frac{(v \times R \times t \times C_u)}{(V \times C_m)}\right] \times 100\% \quad (6)$$

where v is the perfusion flow rate, R the recovery, 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, $v = 5\,\mu L/min$, $R < 40\%$, $t < 45\,min$, $V = 50\,ml$, $C_u/C_m < 0.60$, A calculated by Eq. (6) is below 0.1%, indicating that the effect of microdialysis sampling on binding equilibrium can be neglected.

The protein binding was studied at four different concentrations (10, 20, 40 and 60 μM) (Table 1). The typical drug peaks observed in this FIA binding study are shown in Fig. 2. The results showed increased protein binding with decreased concentration. This was expected because the equilibrium shifted as the concentration varied. The result from literature is compared with the results from this study in Table 1. As can be seen, the results from this study showed favorable agreement with earlier published data [38].

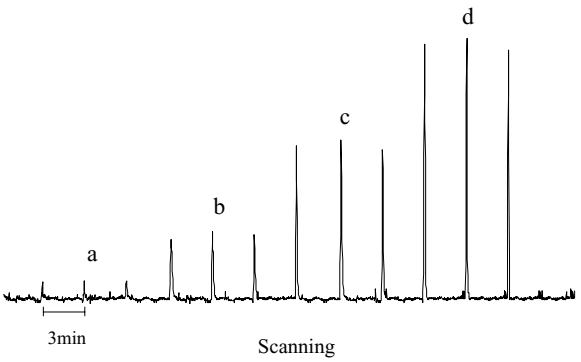

Fig. 2. Typical recording of the system's response to microdialysate from 100 μM BSA solution containing 10, 20, 40 and 60 μM tetracycline hydrochloride for a, b, c, d, respectively. Time interval between perfusing the probe with phosphate buffer and microdialysate collection is 3 min.

The Scatchard plot is presented in Fig. 3A. From the regression equation of the Scatchard plot, the slope was equal to 4.97×10^3, the y-intercept equal to 8.90×10^3. Therefore, the association constant between tetracycline hydrochloride and BSA was 4.97×10^3 L/mol, the number of binding sites per protein molecule was 1.8. However, the correlation coefficient (0.9732) given by Scatchard equation is not satisfied. So, the Klotz plot is tested and the result is shown in Fig. 3B. As can be seen, the slope was equal to 1.0×10^{-4}, the y-intercept equal to 4.92×10^{-1}. Hence, the association constant between tetracycline

Table 1
Data of the studied drug bound to bovine serum album as determined by microdialysis combined with FIA–CL detection

Concentration for binding study (μM)	Ratio of studied drug to BSA (μM:μM)	Unbound drug concentration (μM)	Binding fractions (%)	Literature value for binding fraction (%)
10	10:100	5.37	46.32	50 [38]
20	20:100	10.83	45.83	
40	40:100	22.08	44.81	
60	60:100	34.18	43.04	

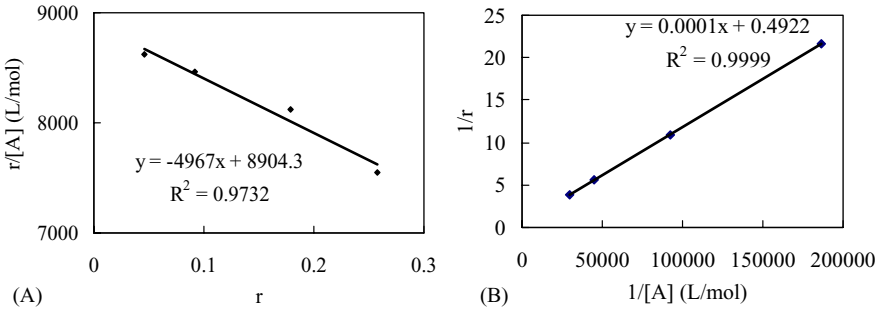

Fig. 3. Scatchard plot (A) and Klotz plot (B) for tetracycline hydrochloride binding to BSA.

hydrochloride and BSA was 4.92×10^3 L/mol, the number of binding sites per protein molecule was 2.0. The correlation coefficient (0.9999) given by Kloz equation is satisfied. Linearity of Scatchard plot and Kloz plot demonstrates the studied drug has only one type of binding site. From above results, it can be seen that the *nK* values calculated using Eqs. (4) and (5) for studied drug are quite similar, indicating a good agreement of the experimental data to the theoretical equation.

4. Conclusion

Microdialysis is a dynamic process between the concentration of drug in the dialysate and the concentration in the matrix surrounding the probe. It offers the advantage of not changing the volume of the sample and not greatly changing the concentration of the components in the sample. Therefore, the binding equilibrium is not disturbed by changes in the sample during the course of experiment. In the present system, the relative decrease in the tetracycline hydrochloride concentration in the tetracycline hydrochloride–BSA mixed solution is less than 0.1%. So the mount of analyte perfused by the perfusate normally is so small that it can be neglected without influencing the equilibrium system.

The FIA chemiluminescence system combined with microdialysis developed in this work demonstrated its use for determination of interaction between drug and protein by using relatively simple instrument. Since the membrane excludes proteins and other macromolecular, the dialysate can be analyzed without further clean-up, which is very suitable for the combination with FIA. Finally, because microdialysis probes are readily implanted intravenously, binding of drugs to proteins could be studied in vivo under physiological conditions following dosing, which is difficult for equilibrium dialysis and ultrafiltration.

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