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Short communication

Coupling microdialysis with flow-injection chemiluminescence detection for a protein–drug interaction study

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Abstract

The interaction of metronidazole (MTZ) and human serum albumin (HSA) was studied using the coupling system of on-line microdialysis sampling with flow-injection chemiluminescence detection (FI-MD-CL). The interested drug and HSA 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. Then the microdialysis probe was put into the MTZ–HSA mixed solution and sampled at a perfusion rate of 5 μL min^{-1}. The microdialysates was determined using flow-injection chemiluminescence. In vitro recovery (R) of MTZ under experimental conditions was approximately 25.2% with a R.S.D. of about 3.2%. The values estimated for the binding constant (K) and the number of the binding sites (n) were found to be 1.50×10^3 L mol^{-1} and 1.89, respectively. The values of nK obtained using Scatchard analysis and Klotz plot were found to be quite similar. The method provided a reliable and simple technique for the study of drug–protein interaction.

Keywords: Interaction; Metronidazole; Human serum albumin; Microdialysis; Flow-injection; Chemiluminescence

1. Introduction

Most drugs undergo a greater or lesser extent of reversible binding to plasma proteins and blood cells such as albumin and α_1-acid glycoprotein [1], plasma protein binding is an important factor in establishing pharmacokinetic and pharmacology properties of drugs. So, studies on drug–protein interaction are important in pharmacology and pharmacokinetics [2]. To develop a simple and reliable method to study the drug–protein interaction is of great practical and theoretical importance.

Various methods have been used to evaluate drug–protein interaction, including equilibrium dialysis [3,4], ultrafiltration [5,6], spectrometry [7,8], ion-selective electrode [9,10], etc. However, these conventional methods suffer from a number of problems [5,11]. Equilibrium dialysis experiments require large quantities of drug and long periods since it is necessary to achieve the dialysis equilibrium. Ultrafiltration method also requires large amounts of drug, and the drug–protein equilibrium may be broken during filtration. High performance frontal analysis (HPFA), which was established by Nakagawa and coworkers [12–15] using a restricted-access type LC column and reversed-phase chromatography combined with size-exclusion technique, has a number of advantages in studying drug–protein interaction. Unluckily, HPFA requires a skilled operator and a column with suitable hydrophobic strength to allow the elution of drug from the column by mobile phase. Moreover, due to a large injection volume required, HPFA is not suitable for the analysis of weak protein binding. HPLC and CE for study of drug–protein interaction have been well reviewed by Hage and Tweed [16].

Microdialysis has been exploited extensively for in vivo or in vitro analysis [17–19], and the technique has been used not only as standard technique in the neurosciences, but also as functional studies in many other fields including pharmacokinetic, toxicology, bioprocess monitoring, etc. The pharmacokinetic applications of microdialysis have been well covered in two recent reviews [20,21]. Microdialysis sampling offers some merits. It is time-saving and even simpler than equilibrium dialysis.

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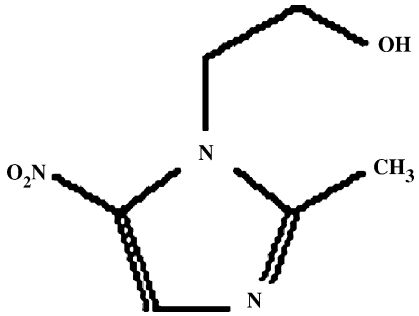

Fig. 1. Molecular structure of metronidazole.

Another merit is that the technique enables to on-line hyphenate with many analytical techniques such as LC, CE, MS, etc. and implements automatic analysis easily. Because the bound drug cannot pass through the semipermeable membrane, only the unbound drug can be determined by microdialysis sampling so that no any other sample pretreatments required.

In most of the studies adopted microdialysis sampling technique, individual dialysate samples were collected with a separate collector, and then analyzed with various methods. Namely, they were off-line analysis, which suffers some drawbacks. Whereas, on-line coupling of microdialysis sampling with the analytical system can not only minimize the sample loss, but also decrease the delay between sample collection and analysis, improve sensitivity and reproducibility. However, it is very unluckily that only a few papers related to the drug–protein interaction study using on-line combining microdialysis sampling to the analytical system were reported until now [11,22–24].

Chemiluminescence (CL) is an attractive detection method for its very low detection limit and wide linear range. The combination with flow-injection analysis (FIA) has made chemiluminescence detection more attractive. Compared to the common use of electrochemical detection of microdialysate, however, few studies concerning microdialysate analysis based on chemiluminescence detection [11,24–28] were reported. Only four references employing the FI-CL detection for microdialysate analysis have been published to date [11,23–25].

Metronidazole (MTZ, its structure shown in Fig. 1) is a widespread clinical used drug due to its activity against protozoal and anaerobic bacteria. Numerous methods for the determination of MTZ have been published [29–32]. However, no studies concerning MTZ analysis using chemiluminescence analysis were reported so far. Based on MTZ increases the chemiluminescence intensity during luminol oxidation by $K_3[Fe(CN)_6]$ in the presence of $K_4[Fe(CN)_6]$ in NaOH medium, the MTZ in microdialysates was determined using chemiluminescence detection in the present work.

From the above discussion, the necessity of FI-MD-CL on-line detection system for microdialysate analysis can hardly be overemphasized at present for that the potentials of the system are not fully investigated. In this work, the system was used to determine the interaction of MTZ and HSA with several merits. The major advantage is that the binding equilibrium is not disturbed during the experiment for that the concentration of analytes in the mixed solution did not change remarkably. Secondly the important experimental variables can be precisely controlled so that no pretreatment steps are needed. The third is that it allows precise control of the sample volume. The binding constant (K) and the number of the binding sites (n) of the MTZ to HSA were calculated by two equations (Scatchard and Klotz plots). The binding fraction estimated agrees well with the literature value.

2. Experimental

2.1. Reagents and standard solutions

All the reagents were of analytical-reagent grade and doubly distilled water was used throughout. $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$ were purchased from Chongqing Chemical Reagent Company. A 0.01 mol L^{-1} luminol stock solution was prepared by dissolving 1.772 g of luminol in 1000 mL of 0.01 mol L^{-1} NaOH. More diluted solutions were prepared in proper concentration of NaOH and used immediately. Stock solution of MTZ (obtained from National Institute of the Control of Pharmacological and Biological Products, NICPBP) containing drug 1 mmol L^{-1} was freshly prepared in phosphate buffer ($Na_2HPO_4 \cdot 12H_2O$, 0.067 mol L^{-1}; KH_2PO_4, 0.067 mol L^{-1}). A total of 100 μmol L^{-1} HSA (purchased from Institute of Pharmacological Products of Shanghai) was prepared in phosphate buffer.

2.2. Apparatus and instruments

The FI-CL system (shown in Fig. 2) consisted of two peristaltic pumps that delivered reagents streams at a flow rate of 1.0 mL min^{-1} and a water carrier stream at a flow rate of 2.0 mL min^{-1} (total flow rate of 4.0 mL min^{-1}). PTFE tubing (0.25 mm i.d. and 0.8 mm o.d.) was used to connect all components in the flow system. The emitted chemiluminescence was collected by photomultiplier tube (PMT, operate at −800 V) of a BPCL Ultra Weak Luminescence Analyzer (Institute of Biophysics, Chinese Academy of Sciences, Peking, PR China). The flow cell was a flat spiral-coiled colorless PTFE tubing (0.8 mm i.d., 2.5 cm diameter, without gaps between loops) and was fixed onto the surface of organic glass close to PMT of the Analyzer with transparent glue. Two three-channel peristaltic pumps and

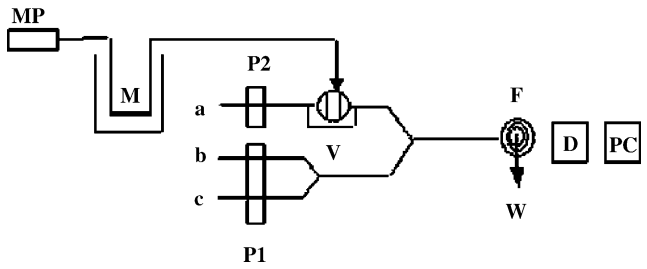

Fig. 2. Schematic diagram of the FI-MD-CL on-line detection system for in vitro determining of drug–protein interaction. P1 and P2, peristaltic pump; V, injection valve; F, flow cell; W, waste; D, detector; PC, personal computer; MP, syringe pump and its controller system; M, microdialysis probe. (a) Water carrier stream; (b) 50 μmol L^{-1} $K_3[Fe(CN)_6]$ and 10 mmol L^{-1} $K_4[Fe(CN)_6]$; (c) 20 μmol L^{-1} luminol in 0.1 mol L^{-1} NaOH solution.

an eight-channel injector valve were used to construct the FI-CL system.

The microdialysis system comprises a KH-1 microdialysis syringe pump controller (Institute of Chemistry, Chinese Academy of Sciences, Peking, PR China), a MF-7051 microdialysis probe from Bioanalytical Systems (0.12 mm i.d., 0.32 mm o.d., 5 cm membrane length, 3035 Dak, MWCO, BAS, West Lafayette, IN, USA) and a microdialysis syringe pump (1000 μL) which was used for delivery of perfusates. The microdialysis manifold is shown schematically in Fig. 2.

2.3. Procedures

2.3.1. Optimization of the FI-CL system

As shown in Fig. 2, flow lines were inserted into luminol/NaOH solution, $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ solution, water carrier, respectively. The pumps were started to wash the whole flow system until a stable baseline was recorded. Then 15 μL sample was injected into the carrier stream and mixed with the reagent streams in the flow cell, producing a CL signal. For characterization of the FI-CL system, the sample was directly introduced into the injection valve in a continuous manner, which was accomplished by connecting the syringe pump directly to the injection valve. To establish the optimal conditions for the FI-CL analysis of MTZ, the ratio of the peak height of CL signal to noise (S/N) was measured as a function of the concentration of $K_4[Fe(CN)_6]$, $K_3[Fe(CN)_6]$, NaOH, and luminol. In these experiments, drug standards were used instead of microdialysates from drug–protein mixed solution. A series of working standard solutions with different concentrations were prepared by diluting a concentrated fresh standard solution of MTZ with phosphate buffer. The concentration of MTZ was quantified by CL emission intensities.

2.3.2. Microdialysis sampling

The microdialysis sampling was performed by perfusing the probe with phosphate buffer at a flow rate of 5 μL min^{-1}. Before the probe was put into the MTZ–HSA mixed solution, the mixed solution should be incubated at 37 °C in a water-bath (Model CS501-3C super thermostat, Chongqing, PR China) for more than 10 min. Simultaneously, the probe must be washed by the phosphate buffer for several minutes to eliminate air in the probe, which was used for protection of the dialysis membrane. Sampling from the solutions was started after an equilibration time of 30 min.

2.3.3. Recovery of microdialysis

The recovery (R), also called the dialysate extraction fraction, was determined by placing the microdialysis probe into a stirred 50 μmol L^{-1} MTZ (contained in a 50 mL beaker) that was maintained at 37 °C in the water-bath. The operation conditions were the same as for the microdialysis sampling described above. Probe was pumped with phosphate buffer at a 5 μL min^{-1} for 3 min. R was estimated according to the following equation:

$$R = \frac{C_d}{C_m} \quad (1)$$

where C_d is the drug concentration in microdialysate and C_m is the drug concentration surrounding the probe, respectively.

2.3.4. Drug–protein binding experiment

Once the recovery of microdialysis was determined, the same microdialysis probe was immersed into 100 μmol L^{-1} HSA solution containing 40–120 μmol L^{-1} MTZ and perfused with phosphate buffer at a flow rate of 5 μL min^{-1}. The collection interval was 3 min (170 s loading time and 10 s injection time) to yield about 15 μL sample. Then the procedure of FI-CL measurements was followed as described above.

For every change of MTZ concentration, the first dialysate was discarded to avoid the residual effect of the previous concentration. For each MTZ concentration at least three determinations were done to obtain a mean C_d value. Free drug concentration (C_u) in the surrounding medium was determined by dividing the concentration C_d by the recovery (R) according to the following equation:

$$C_u = \frac{C_d}{R} \quad (2)$$

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

$$B\% = \frac{C_m - C_u}{C_m} \times 100\% \quad (3)$$

The binding parameters were estimated by the following equations:

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

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

where r is the ratio of bound drug to the protein in molar concentration, n the number of binding sites on one protein molecule, and K is 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 $Y = mX + b$, from which n and K could be estimated. Eq. (4) is used for Scatchard analysis and Eq. (5) for Klotz plot.

3. Results and discussions

3.1. Characterization of the FI-CL system

The FI-CL system was characterized before applying in the on-line determining the interaction of MTZ and HSA. The principle of the determination of MTZ is that it increases the radiation emitted during the oxidation of luminol by $K_3[Fe(CN)_6]$ in the presence of $K_4[Fe(CN)_6]$ in NaOH medium. The effects of NaOH, $K_3[Fe(CN)_6]$, $K_4[Fe(CN)_6]$, and luminol concentrations on signal to noise (S/N) ratio and performance of the system for MTZ measurements were investigated.

The effect of $K_3[Fe(CN)_6]$ concentration on the S/N ratio was studied in the range from 10 to 200 μmol L^{-1}. The maximum S/N ratio was obtained with 50 μmol L^{-1} of $K_3[Fe(CN)_6]$. Since luminol oxidation by $K_3[Fe(CN)_6]$ produces strong emission, high background is inevitable. According to Shevlin and Neufeld

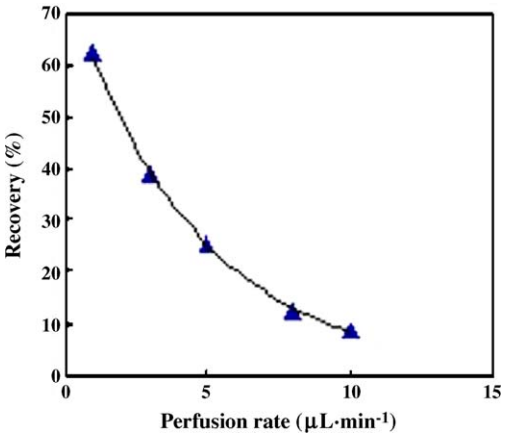

Fig. 3. The effect of perfusion rate on microdialysis probe recovery.

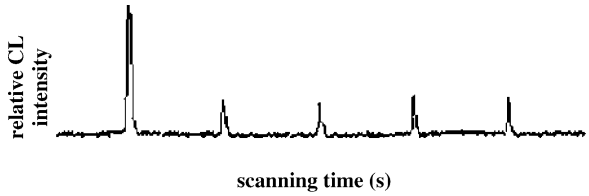

Fig. 4. Typical recording of the system's response to dialysate of mixed solution containing 80 μmol L^{-1} of MTZ standard and 100 μmol L^{-1} of HSA ($n = 4$). For conditions of the system see the text. The first peak is the recording of previous concentration and is discarded.

[33], the addition of $K_4[Fe(CN)_6]$ can decrease the CL intensity of luminol reaction with $K_3[Fe(CN)_6]$. So, $K_4[Fe(CN)_6]$ was examined in the range from 1 to 50 mmol L^{-1} for use with the present CL system. The greatest S/N ratio was obtained with 10 mmol L^{-1} $K_4[Fe(CN)_6]$. The effect of NaOH concentration on the S/N ratio was studied at different concentrations from 0.01 to 0.5 mol L^{-1}. The maximum S/N ratio was obtained when using 0.1 mol L^{-1} NaOH. The effect of luminol concentration on the S/N ratio was investigated for the range of 10–200 μmol L^{-1}. 20 μmol L^{-1} of luminol was found to be optimum for the S/N ratio.

Under the optimum conditions, the calibration graph of CL intensity versus MTZ concentration was linear in the range of 0.68–170 μg mL^{-1} with the regression equation of $\Delta I = 27.1C + 88.93$ ($n = 6$, $r^2 = 0.9970$) over the concentration range of 0.68–17 μg mL^{-1} and $\Delta I = 5.83C + 443.09$ ($n = 5$, $r^2 = 0.9931$) over the concentration range of 17–170 μg mL^{-1}, ΔI being the CL intensity and C the MTZ concentration (μg mL^{-1}). The detection limit based on three times the baseline noise was 0.23 μg mL^{-1} MTZ. The precision of the system was 3.7% R.S.D. ($n = 8$, 100 μg mL^{-1} MTZ).

3.2. Probe calibration recovery

The recovery (R) of probe, a key parameter in the microdialysis method for the determination of drug–protein interaction, is affected by many factors such as the perfusion rate, membrane length, temperature, type of analyte, etc. The effects of the perfusion rate and temperature on R were studied in this work. Fig. 3 demonstrates the experimental results of the effect of perfusion rate on R of the probe used in this study at 37 °C. It can be seen that recovery decreased if perfusion rate increased, and vice versa. A low flow rate of the perfusion gives a higher recovery, but few injections could be made during a given time period and vice versa. Considering sensitivity and experimental time, flow rate of 5.0 μL min^{-1} was selected as the perfusion flow rate.

Temperature is also a key factor that affects the recovery of probe since microdialysis is a dynamic sampling method based on analyte diffusion across a semipermeable membrane and diffusion processes are temperature dependent. In the experiment, the recoveries at 5.0 μL min^{-1} in different temperature were determined too. The recoveries were 8.2 ± 4.3% ($n = 3$) and 25.2 ± 3.2% ($n = 3$), for 21.0 and 37.0 °C, respectively. As seen, higher temperature produces higher recovery, which is in accord with our previous work [34]. In this experiment, the temperature of 37 °C was adopted. Under the selective conditions, a recovery of 25.2 ± 3.2% ($n = 3$) was available.

3.3. Interaction between MTZ and HSA

Using the system in Fig. 2, the interaction of MTZ and HSA was studied using the FI-MD-CL system as above-mentioned processes. The binding fractions of MTZ in HSA solution are listed in Table 1, which is in agreement with the literatures where a 10–20% binding degree in plasma has been reported [35]. Fig. 4 shows the typical recording of the system's response to dialysate of mixed solution containing 80 μmol L^{-1} of MTZ standard and 100 μmol L^{-1} of HSA.

The interaction parameters of MTZ to HSA calculated using Eqs. (4) and (5) are presented in Table 2, the correlation coefficients obtained are also listed. The values of nK are quite similar. Both the Scatchard plot (refer to Fig. 5) and Klotz plot (refer to Fig. 6) are linear, showing that MTZ has only one type of binding sites on HSA. The Scatchard equation is widely applied in the study of interaction of drug and protein more than others, so the results obtained by this equation are accepted. On the other

Table 1
Unbound MTZ concentrations and binding fraction in HSA solutions

Ratio of MTZ to HSA (μmol L^{-1}:μmol L^{-1})	C_d (μmol L^{-1})	C_u (μmol L^{-1})	C_b (μmol L^{-1})	Binding fractions (%)
40:100	7.93	31.46	8.54	21.35
60:100	11.97	47.49	12.51	20.85
80:100	16.01	63.52	16.48	20.60
100:100	20.10	79.77	20.23	20.23
120:100	24.24	96.18	23.82	19.85

Table 2
Binding parameters for MTZ–HSA interaction

Data analysis	T (°C)	Solution	n	K (L mol^{-1})	nK (L mol^{-1})	r^{2}[a]
Eq. (4)	37.0 ± 0.5	pH 7.4	1.89	1.50×10^{3}	2.84×10^{3}	0.9893
Eq. (5)	37.0 ± 0.5	pH 7.4	1.85	1.54×10^{3}	2.85×10^{3}	0.9998

[a] r^{2} is the regression coefficient.

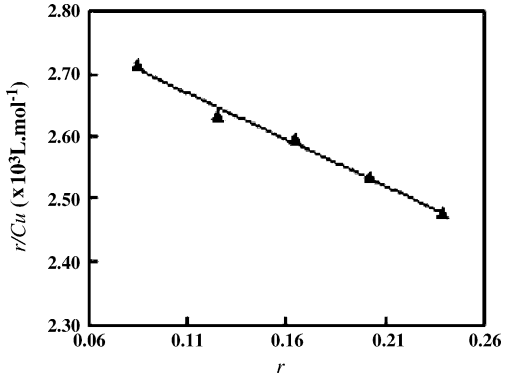

Fig. 5. Scatchard plot of MTZ–HSA interaction.

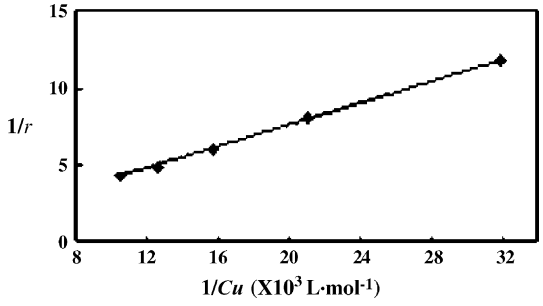

Fig. 6. Klotz plot of MTZ–HSA interaction.

hand, compared with high affinity drugs such as warfarin and ibuprofen (nK is about 10^{6} L mol^{-1}), the nK value for MTZ is very small, indicating that MTZ is a lightly binding drug.

4. Conclusion

Measuring interaction of drug and protein using microdialysis sampling combined with on-line flow-injection chemiluminescence detection is simple and reliable, and also to be automated. The interaction of MTZ and HSA was successfully determined using the FI-MD-CL system with relatively simple instrument and cheap reagents. The estimated association constant (K) and the number of the binding sites (n) values are 1.50×10^{3} L mol^{-1} and 1.89, respectively. The binding fraction is about 20%, which agrees well with the literature value. The in vitro recovery is about 25.2% with a R.S.D. of about 3.2%.

From the observed results, it is can be seen that FI-MD-CL system is a valid method of determining the in vitro drug–protein interaction. In addition, the proposed system can not only determine the in vitro drug–protein interaction, but also evaluate the in vivo drug–protein interaction by implanting or inserting the microdialysis probe into intravenous blood vessel or organism, which is difficult for equilibrium dialysis and ultrafiltration. Moreover, the system can be readily adapted to other analytes by varying the chemiluminescent reagents and can also be used to determine the in vivo pharmacokinetics, in vitro drug dissolution testing and in vivo contents of biochemical events.

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