Department of Pharmaceutics[1], Shenyang Pharmaceutical University, Shenyang, New Drug Research and Development Center[2], North China Pharmaceutical Group Corporation, Shijiazhuang, P.R. China

The determination of *in vitro* pingyangmycin hydrochloride plasma protein binding by microdialysis

Z. B. Gao[1], P. T. Ding[1], H. Xu[1], L. Zhang[2], J. Wei[1], D. W. Chen[1]

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Dr. Ping-tian Ding, Department of Pharmaceutics, Shenyang Pharmaceutical University, Wenhua Road 103, Shenyang 110016, P.R. China
zl51@163.com

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Microdialysis sampling was used to study the binding of pingyangmycin hydrochloride (PYM) to plasma proteins in canis familiaris blood. *In vitro* plasma protein binding fractions were evaluated in a series of PYM concentration. The results showed decreased protein binding with increased concentration. The data was analyzed using the Scatchard analysis and Klotz plot. The results showed that the Scatchard plot and Klotz plot were linear with good correlation coefficient, indicating a good agreement of the experimental data to the theoretical equation.

1. Introduction

Most drugs are bound to plasma proteins, mainly to a serum albumin. Binding of drugs to plasma protein is an important factor in determining their pharmacokinetic and/or pharmacodynamic behavior. Plasma protein binding studies of drugs have typically been performed by equilibrium dialysis (Dinora et al. 2005), ultrafiltration (Heinze and Holzgrabe 2006) and ultracentrifugation (Barre et al. 1985). The present study selected pingyangmycin hydrochloride as a model drug and a method is descriped for determining the *in vitro* plasma protein binding of pingyangmycin hydrochloride, using canis familiaris plasma.

2. Investigations, results and discussion

In the present study, the no-net-flux method was used to determine the recovery of the microdialysis probe. After the determination of the perfusate concentration (C_p) and dialysate concentration (C_d) of PYM using the HPLC system, linearity between C_p and the net increase ($Cd - Cp$) of PYM concentration in the dialysate was established, and the slope of the line corresponded to the recovery (R). Fig. 1 shows the calculation of the recovery of the microdialysis probe in canis familiaris plasma with a concentration of 30.30 $\mu g \cdot mL^{-1}$. According to the definition of recovery (Gao et al., in press), the following equation was obtained: $C_d - C_p = -0.3622\ C_p + 4.9353$ ($r^2 = 0.9972$). The calculated recovery was 36.22% (n = 3, SD = 0.62%). The recoveries of the microdialysis probe in different PYM concentration in canis familiaris plasma were shown in the Table. There was no significant difference among these recoveries ($P > 0.05$). The concentration at the intersection of this line with X-axis corresponded to the free drug concentration in plasma. The bound fraction of the drug (B %) was calculated as follows:

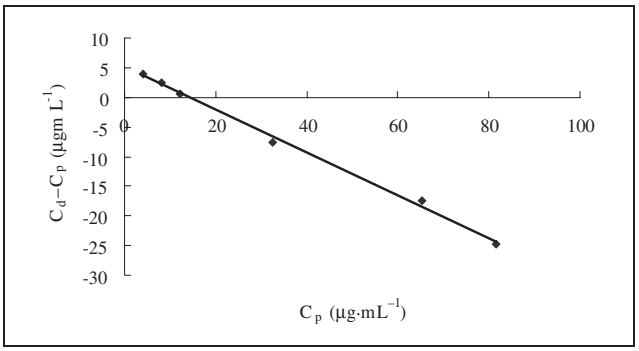

Fig. 1: In vitro recovery of the microdialysis probe in rabbit plasma with a PYM concentration of 30.30 μg ml^{-1} (n = 3)

$$B\ \% = \left(C_{plasma} - C_u\right)/C_{plasma} \times 100 \qquad (1)$$

Where C_{plasma} was the total drug concentration in plasma and C_u the unbound drug concentration.

According to Eq. (1), the *in vitro* protein binding was measured at five different concentrations. The result (Table) showed decreased protein binding with increased concentration. This was expected because the equilibrium shifted as the concentration varied.

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

$$r/[A] = nK - rK \qquad (2)$$

or the following equation for Klotz plot:

$$1/r = 1/n + (1/nK) \times (1/[A]) \qquad (3)$$

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. Once r/[A] (or 1/r) and r (or 1/[A]) were determined, r/[A] (or 1/r) was regressed on r (or 1/[A]) using the linear equation $Y = mX + b$

Table: Data of in vitro PYM bound to canis familiaris plasma protein determined by microdialysis

Concentration of PYM ($\mu g \cdot mL^{-1}$)	In vitro recovery of microdialysis probe (%)	Unbound drug concentration ($\mu g \cdot mL^{-1}$)	Bound fraction (%)
10.10	36.77	4.41	56.34
20.20	36.41	8.94	55.76
30.30	36.22	13.63	55.03
40.40	36.37	18.49	54.26
60.60	36.68	28.42	53.11

(where m = slope and b = Y-intercept), from which n and K could be estimated (Huang et al. 2001).

The Scatchard plot was shown in Fig. 2A. From the regression equation of the Scatchard plot, the modulus of the slope was 8.758×10^3, the *y*-intercept 3.146×10^3. The correlation coefficient (r^2) was 0.9964. Therefore, the association constant between pingyangmycin hydrochloride and canis familiaris plasma protein was 8.758×10^3 L/mol, the number of binding sites per protein molecule was 3.592×10^{-1}. The result of the Klotz plot is shown in Fig. 2B. As can be seen, the modulus of the slope was 3.0×10^{-4}, the y-intercept 2.784. Hence, the association constant between pingyangmycin hydrochloride and canis familiaris plasma protein was 9.280×10^3 L/mol, the number of binding sites per protein molecule was 3.592×10^{-1}. The correlation coefficient ($r^2 = 1$) given by Kloz equation was satisfied. Linearity of Scatchard plot and Klotz plot demonstrated the studied drug had only one type of binding site. The nK values calculated using Eqs. (2) and (3) for the studied drug were quite similar, indicating a good agreement of the experimental data with the theoretical equation. Compared with high affinity drugs, for example warfarin and fenoprofen (nK is about 10^6 M^{-1}), the nK value of the studied drug was very small (3.15×10^3 M^{-1} for Scatchard plot and 3.33×10^3 M^{-1} for Klotz plot), showing that the studied drug was a lightly binding drug. The plasma concentration of albumin was 421.32 ± 17.41 μmol L^{-1}. Then, the plasma concentration of active albumin that had PYM binding ability was 151.34 ± 17.57 μmol L^{-1}.

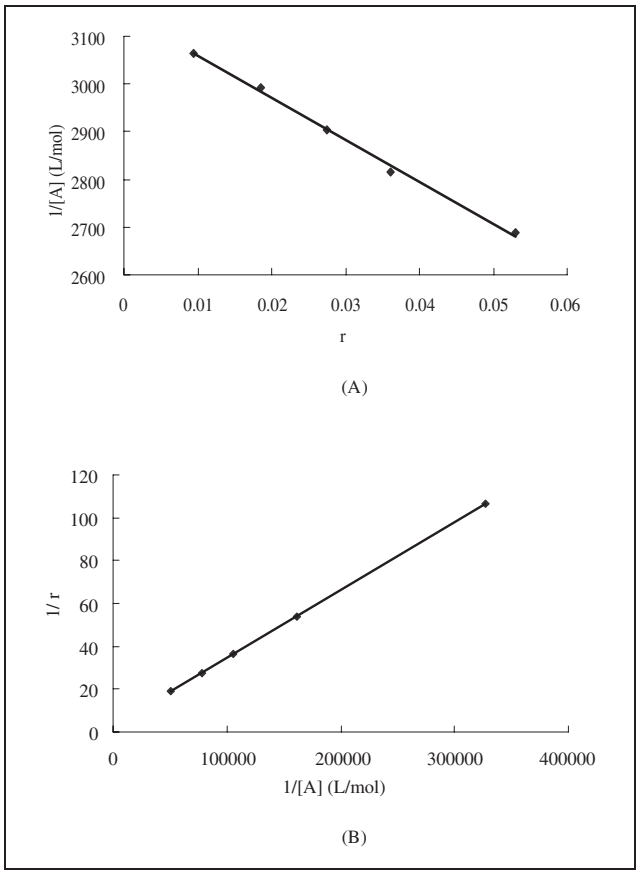

Fig. 2: Scatccard plot (A) and Klotz plot (B) for PYM bound to canis familiaris plasma protein

3. Experimental

3.1. Materials

PYM was obtained from Taihe Pharmaceutical Co. (Tianjin, China). Sodium 1-pentanesulfonate was from Tedia Company Inc. (Fairfield, OH, USA). All other chemicals were of analytical grade. Water for normal saline preparation was triple distilled.

3.2. Microdialysis

Microdialysis sampling system was composed of a KH-1 microdialysis syringe pump (Chemistry Institute of Academy Sinica, Beijing, China) with a 1.0 mL glass syringe and concentric vascular microdialysis probes (MD-2310, 10 mm dialysis membrane) obtained from Bioanalytical System Inc. (West Lafayette, IN, USA).

3.3. Recovery of the microdialysis probe and in vitro plasma protein binding percentage of pingyangmycin hydrochloride

Blank blood was collected in heparinized tubes by vein sampling and immediately centrifuged to separate the plasma. Plasma thus obtained from several canis familiaris was pooled and subsequently spiked with pingyangmycin hydrochloride and vortexed for 5 min to obtain the following concentrations: 10.10, 20.20, 30.30, 40.40 and 60.60 $\mu g \cdot mL^{-1}$.

In canis familiaris plasma, the concentration of albumin was measured by using a 7600 Clinical Analyzer (Hitachi High-Technologies Co., Japan). Probe characterization was described previously (Ding et al. 2000; Gao et al. in press). Briefly, the probe was perfused with a series of PYM solutions ranging from 1.02 to 97.92 $\mu g \cdot mL^{-1}$, while its dialysis membrane was immersed into 15 mL plasma containing PYM of different concentration. Each sample solution was brought to 37 °C in a water bath set at a 700 rpm stirring condition. Perfusion solutions with a serial PYM concentration were perfused through the probe at a constant flow-rate (3 μL min^{-1}) using the perfusion pump. After equilibration for 30 min, the perfusate was collected and the perfusate (C_p) and dialysate (C_d) concentrations of PYM were determined using the HPLC system.

3.4. Chromatographic instruments and conditions

Levels of PYM in dialysate were measured by HPLC (Shimadzu LC-10AD, Japan) with a Discovery® RP-amide C16 column (4.6 mm × 25 cm, Supelco, USA) and a UV absorbance detector (Shimadzu SPD-10A, Japan). The column temperature was 40 °C. The mobile phase consisted of a mixture of methanol acetonitrile solution A (8 : 4: 88, v/v/v). Solution A was prepared by dissolving 960 mg of sodium 1–pentanesulfonate and 1.86 g of edetate disodium in 1000 mL of deaerated 0.08 N acetic acid, adjusted with ammonium hydroxide to a pH of 4.3. The mobile phase was filtered through a Millipore 0.22 μm filter and degassed prior to use. The flow rate of the mobile phase was 1.0 mL · min^{-1} and a UV detection wavelength of 291 nm was used for all experiments. The volume injected into the chromatography system was 20 μL.

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