STUDIES ON PROTEIN BINDING OF ANTIBIOTICS

IV. EFFECT OF THE BINDING OF DRUG TO 100,000×g SUPERNATANT FLUID OF RABBIT LIVER HOMOGENATES ON URINARY EXCRETION

YASUO WATANABE, RIEKO KITAYAMA, TOSHIO HAYASHI, YOSHIFUMI NAKASHIMA, MASASHI NOGUCHI, TAKASHI YASUDA, ISAMU SAIKAWA and KIHACHIRO SHIMIZU*

Research Laboratory, Toyama Chemical Co., Ltd., Toyama, Japan
*Department of Internal Medicine, Tokyo Women's Medical College, Tokyo, Japan

(Received for publication May 15, 1982)

The extent of the binding of β-lactam antibiotics to 100,000×g supernatant fluid of rabbit liver homogenates was investigated.

Urinary excretion tended to decrease as the extent of the binding to 100,000×g supernatant fluid increased. Predicting urinary excretion was achieved using the binding to 100,000×g supernatant fluid and other physicochemical factors. A good coincidence resulted from multiple regression analysis. [^{14}C]Benzylpenicillin binding proteins in rabbit liver were investigated and revealed that the affinity of cefoperazone was much stronger than that of cefazolin.

The extent to which a β-lactam antibiotic is excreted in the bile and urine may be influenced by a number of physicochemical factors, including molecular weight, polarity, chemical structure and lipid solubility. In fact, several investigators have pointed out that molecular weight may be of importance for biliary excretion.[1~4] Compounds of relatively low molecular weight are poorly excreted in the bile whereas those of greater molecular weight are more extensively eliminated by the hepatic route. However, the fate of a drug cannot be explained by molecular weight alone. Other physicochemical factors also cannot explain the elimination of the drug itself.

The effect of serum protein binding on the elimination of a drug has been frequently discussed.[5~8] But the serum protein binding did not appear to be a substantial determinant in drug fate.

It is also thought that the extent of the binding of a β-lactam antibiotic to tissue is one of the most important determinants of drug excretion in the body. However, the relationship between the binding to the tissue and urinary elimination remains obscure. The purpose of the present study, therefore, is to characterize the effect of the binding of antibiotics to 100,000×g supernatant fluid on urinary excretion in rabbits.

Materials and Methods

Antibiotics

The following β-lactam antibiotics were used.

Penicillins: Apalcillin (APPC, Sumitomo Chemical Co., Ltd.), piperacillin and ampicillin (PIPC and ABPC, Toyama Chemical Co., Ltd.), carbenicillin (CBPC, Taito Pfizer Co., Ltd.), sulbenicillin (SBPC, Takeda Pharmaceutical Co., Ltd.), mezlocillin (MZPC, Bayer Co., Ltd.), benzylpenicillin (PCG, Banyu Seiyaku Co., Ltd.), ticarcillin (TIPC, Fujisawa Pharmaceutical Co., Ltd.).

Cephems: Cefoperazone (CPZ, Toyama Chemical Co., Ltd.), cefotiam and cefmenoxime (CTM and CMX, Takeda Pharmaceutical Co., Ltd.), cefmetazole (CMZ, Sankyo Co., Ltd.), cefazolin (CEZ, Fujisawa Pharmaceutical Co., Ltd.), cefoxitin (CFX, Daiichi Pharmaceutical Co., Ltd.), ceftezole (CTZ,

Chugai Pharmaceutical Co., Ltd.), cephaloridine (CER, Torii Pharmaceutical Co., Ltd.) and cefotetan (CTT, Yamanouchi Pharmaceutical Co., Ltd.).

Preparation of 100,000×*g* Supernatant Fluid of Rabbit Liver

Japanese white male rabbits, weighing 2.0~2.5 kg, were anesthetized with ether. To remove the blood present in the liver, the livers were perfused *in situ via* the portal vein with cold 0.85% NaCl, blotted and weighed. A 50% homogenate was prepared in 1/15 M phosphate buffer, pH 7.0, using a Teflon-glass motor driven homogenizer. The homogenate was centrifuged at 24,000×*g* for 30 minutes and the supernatant was then centrifuged at 100,000×*g* for 2 hours at 4°C. The supernatant solution was pipetted off without the lipid layer and stored at −70°C. Protein was quantified by the LOWRY method.[9]

Determination of Binding Rate

The extent of the binding to 100,000×*g* supernatant fluid of liver homogenates and serum protein was measured by a centrifugal ultrafiltration method. One volume of drug dissolved in 1/15 M phosphate buffer (pH 7.0) was mixed with 9 volumes of samples and poured into a Visking cellulose tube bag (Visking Company: size 8/32). The bag was hung in a 10-ml glass tube and centrifuged at 1,000×*g* for 30 minutes. The concentration of drug in the ultrafiltrate was then measured by a high pressure liquid chromatograph (HPLC, Shimadzu, LC-2 model) or bioassay. The reference experiment was performed by using a buffer in place of the samples. The binding rate was calculated according to the following equation:

$$\text{Binding rate } (\%) = (1 - X/Y) \times 100$$

where X is the drug concentration in the filtrate of the sample and Y is that of the reference.

Drug Administration and Sampling of Urine

Drugs, except 20 mg/kg intramuscular doses of PIPC and CBPC, were administered intravenously at one dose of 20 mg/kg. Rabbit urine samples were taken by cannulating both the ureters. Samples were collected into 1/15 M phosphate buffer (pH 6.0) to avoid decomposition and stored at −20°C until assayed.

Measurement of Antibiotic Concentration

Measurement of antibiotic concentration was performed with HPLC, except for MZPC, TIPC and SBPC. Samples were run on a column (250×4 mmϕ) of LiChrosorb RP-18 at an ambient temperature and a flow rate of 1.0 ml/minute. The eluate was monitored at 254 nm. Measurement for MZPC, TIPC and SBPC was performed by the paper disk method, using *Bacillus subtilis* ATCC 6633 as a test organism. Samples were diluted in 1/15 M phosphate buffer (pH 6.0). Standards were prepared with 1/15 M phosphate buffer at pH 6.0 for the assay of urine concentrations.

Reversed Phase Thin-layer Chromatography

The silica gel plates (Merck: Silica Gel 60 F-254) were heated at 110°C for 1 hour and cooled. A stationary non-polar phase then obtained by impregnating the silica gel layer with silicone oil (Toshiba Silicone Co., Tokyo). The impregnation was carried out by developing the plates in a 5% silicone solution in ether. Spots (10 μl) of the antibiotic solutions (1 mg/ml in water) were then applied and the plates were placed in the developing buffer. The buffer used was a sodium acetate-veronal buffer (pH 7.0) containing 3% acetone and saturated with silicone oil. Development was continued until the solvent front reached the 10 cm mark. The wet plates were dried, and then antibiotics were detected by iodine or UV absorption.

Multiple Regression Analysis[10]

The following equation was used for multiple regression analysis by the method of least squares.

$$Y_i = \beta_0 + \sum_{j=1}^{k} \beta_j X_{ij} \quad i = 1, 2, \cdots n$$

where Y_i is the predicted urinary excretion of drug$_i$, X_{ij} is the factor of drug, β_j (j=0, 1, 2, . . . k) the parameters to be predicted, and n is the sample number.

Binding of [^{14}C]PCG to 100,000×*g* Supernatant Fluid and Competition with CPZ and CEZ

A sample (30 μl), partially purified by CM Sephadex C-50, was mixed with 6 μl of 1 mM [^{14}C]PCG

potassium salt (0.3 μCi) and 3 μl of solution of non-radioactive CPZ or CEZ at concentrations of 0.2, 1, 5, 25 and 125-fold (molar ratio to [^{14}C]PCG). The mixture was incubated for 30 minutes at 37°C and was mixed with 30 μl of 0.2 mM tris-HCl buffer, pH 6.8, containing 3% (w/v) sodium dodecyl sulfate (SDS), 30% (w/v) glycerol and 0.02% (w/v) bromophenol blue, and heated in boiling water for 2 minutes. The whole solution was then subjected to SDS slab gel electrophoresis. Gels were run in a buffer composed of 0.6% (w/v) tris (Sigma Chemical Co., Ltd.), 2.88% (w/v) glycine (Wako Pure Chemical Co., Ltd., Osaka) and 0.1% (w/v) sodium dodecyl sulfate, at a constant current of 20 mA for 1~1.5 hours and then at 25 mA for 5~6 hours at 4°C. After electrophoresis the gel was fixed in 500 ml of 50% (w/v) methanol - 7% (w/v) acetic acid for 1 hour at room temperature, and washed three times for 0.5 hour each with 500 ml of a solution of 5% (v/v) methanol - 7% (v/v) acetic acid. The gel solution was replaced with dimethyl sulfoxide by three successive treatments of the gel with 300 ml of dimethyl sulfoxide for 30 minutes. Finally, 2,5-diphenyloxazole (Dotite, Wako Pure Chemical Co., Ltd., Osaka) was incorporated into the gel by treating it with 160 ml of 22.2% (w/v) 2,5-diphenyloxazole in dimethyl sulfoxide for 3 hours. The dimethyl sulfoxide was removed by washing the gel in running water for 1 hour and the gel was dried *in vacuo* on Toyo paper No. 2. A fluorogram was prepared by exposing the gel to X-ray film (Fuji RX-S, Fuji Photo Film Co., Ltd., Tokyo) at −80°C for illumination to sensitize it for fluorography.

Results

The Binding to 100,000×*g* Supernatant Fluid and Serum Protein of Drugs and Their Physicochemical Properties

The extent of the binding of β-lactam antibiotics to 100,000×*g* supernatant fluid of rabbit liver homogenates and serum protein, molecular weight, Rf value and urinary excretion (0~6 hours) are listed in Tables 1 and 2. Of the 8 penicillins, the extent of the binding to 100,000×*g* supernatant fluid varied from 4.6% for SBPC to 71.6% for APPC. Other penicillins, except SBPC and TIPC, were more than 20% bound. On the other hand, the extent of the binding of cephems was less than that for penicillins. CTM, CER and CMZ were <10% bound, and CEZ, CFX, CTZ and CMX were between 10 and 20% bound, and CPZ and CTT had greater binding of 27.3% and 27.1% respectively. Serum protein binding for penicillins varied from 19.7% for MZPC to 83.5% for APPC, and from 39.2% for CFX to

Table 1. Extent of binding of antibiotics to rabbit liver supernatant fluid (100,000×*g*) and serum protein, Rf value, molecular weight, and urinary excretion.

Penicillins

Antibiotic	Binding (%)		Rf	MW	Urinary excretion (%) (0~6 hours)
	Liver supernatant fluid* (100,000×*g*)	Serum protein			
APPC	71.6	83.5	0	522	36.1
PIPC	24.0	21.4	0.14	518	52.8
ABPC	33.9	22.5	0.38	349	81.1
PCG	52.9	54.5	0.32	334	74.7
CBPC	29.6	57.8	0.73	378	65.5
SBPC	4.6	37.5	0.70	414	84.8
MZPC	26.4	19.7	0.40	558	55.9
TIPC	10.4	48.1	0.78	384	78.8

* Protein concentration is 4.1% to 4.7%. Mean 6-hour urine excretion after 20 mg/kg i.v. doses, except 20 mg/kg i.m. doses of PIPC and CBPC, are expressed. Values given are means of 3 rabbits. Other definitions are described in the text.

Table 2. Extent of binding of antibiotics to rabbit liver supernatant fluid (100,000×*g*) and serum protein, Rf value, molecular weight, and urinaty excretion.

Cephems

Antibiotic	Binding (%)		Rf	MW	Urinary excretion (%) (0~6 hours)
	Liver supernatant fluid* (100,000×*g*)	Serum protein			
CPZ	27.3	95.7	0.24	646	47.4
CTM	7.6	52.1	0.19	526	79.6
CER	5.7	41.9	0.13	416	79.3
CMZ	5.6	62.9	0.58	472	91.5
CEZ	12.1	97.3	0.51	455	81.3
CFX	11.6	39.2	0.68	427	74.6
CTZ	12.9	97.8	0.66	440	91.2
CMX	19.4	98.6	0.61	512	75.7
CTT	27.1	60.4	0.81	576	71.7

* Protein concentration is 4.1% to 4.7%.
Mean 6-hour urine excretion after 20 mg/kg i.v. doses are expressed. Values given are means of 3 rabbits.
Other definitions are described in the text.

98.6% for CMX for cephems. The hydrophobic character of β-lactams was expressed as the Rf value, measured by means of reversed phase thin-layer chromatography. Lower values indicated higher hydrophobicity. The Rf values were widely distributed from 0 for APPC to 0.78 for TIPC for penicillins, and for cephems, from 0.13 for CER to 0.81 for CTT. Molecular weight was expressed as free acid. The drugs with a molecular weight of between 334 and 558 for penicillins, and between 416 and 646 for cephems were studied. Molecular weight of <400 were ABPC, PCG, CBPC and TIPC, 400~500 were SBPC, CER, CMZ, CFX, CEZ and CTZ, >500 were APPC, PIPC, MZPC, CPZ, CTM, CMX and CTT. Urinary excretion of APPC and CPZ was 36.1% and 47.4%, respectively, showing that both drugs are mainly or largely excreted in the bile. Other drugs were above 50% in the urine.

Relationship between the Binding to 100,000×*g* Supernatant Fluid of Rabbit Liver Homogenates and Urinary Excretion

There was a significant relationship in penicillins ($P<0.1$), where the fraction of the dose found in the urine tended to decrease as the extent of binding to 100,000×*g* supernatant fluid increased. However, this relationship was inferior to that between molecular weight and urinary excretion, and between Rf value and urinary excretion. In cephems, a good relationship ($P<0.05$) existed between the binding to 100,000×*g* supernatant fluid and urinary excretion (Figs. 1 and 2). This relationship was also inferior to that between molecular weight and urinary excretion.

Prediction of Urinary Excretion

Using the binding to 100,000×*g* supernatant fluid and serum protein, molecular weight and Rf value, the prediction of urinary excretion was achieved by use of multiple regression analysis. These results are shown in Tables 3 and 4. Each equation (1), which involved all factors used, was given for penicillins and cephems. Each equation could be further simplified by the forward selection procedure.[10] From the results of partial F-test, equation (2), the most recently entered variable to the regression, was statistically significant. The coefficient of determination for penicillin was 90.3%, and

Fig. 1. Relationship between binding of penicillins to 100,000×g supernatant fluid of rabbit liver and urinary excretion.

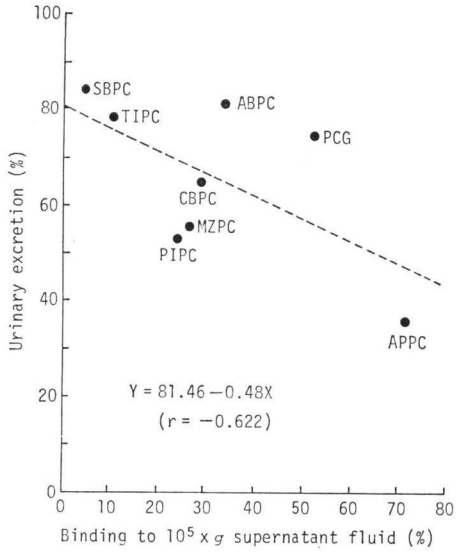


Fig. 2. Relationship between binding of cephems to 100,000×g supernatant fluid of rabbit liver and urinary excretion.

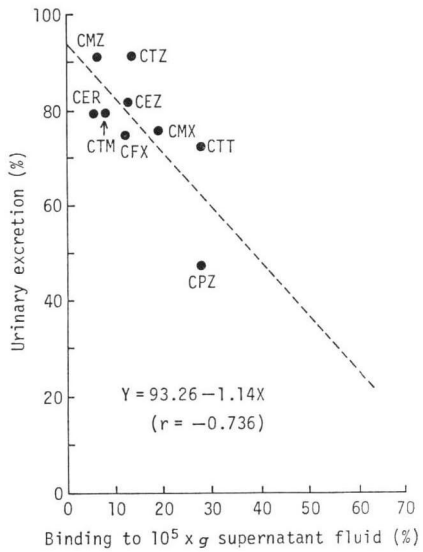


Table 3. Multiple regression analysis in rabbit. Penicillins

$$Y=143.6-0.294X_1-0.153X_2+2.01X_3-0.144X_4 \quad (1)$$

(Coefficient of determination: 92.3%)

$$Y=139.7-0.413X_1-0.140X_4 \quad (2)$$

(Coefficient of determination: 90.3%)

Y : urinary excretion
X_1 : binding to supernatant fluid (100,000× g) of rabbit liver
X_2 : serum protein binding
X_3 : Rf value
X_4 : molecular weight

Antibiotic	Urinary excretion (%)	
	Observed value	Predicted value
APPC	36.1	37.2±13.4*
PIPC	52.8	57.4± 8.7
ABPC	81.1	76.9± 8.2
PCG	74.7	71.1±11.1
CBPC	65.5	74.6± 6.8
SBPC	84.8	79.9± 9.4
MZPC	55.9	50.8±10.7
TIPC	78.8	81.7± 8.6

* 95% confidence limit.

Table 4. Multiple regression analysis in rabbit. Cephems

$$Y=70.56-1.66X_1+0.11X_2+31.54X_3+0.0139X_4 \quad (1)$$

(Coefficient of determination: 86.9%)

$$Y=78.38-1.477X_1+31.32X_3+0.0089X_4 \quad (2)$$

(Coefficient of determination: 83.2%)

Y : urinary excretion
X_1 : binding to supernatant fluid (100,000× g) of rabbit liver
X_2 : serum protein binding
X_3 : Rf value
X_4 : molecular weight

Antibiotic	Urinary excretion (%)	
	Observed value	Predicted value
CPZ	47.4	51.3±14.8*
CTM	79.6	77.8±12.9
CER	79.3	77.7±14.4
CMZ	91.5	92.5±13.9
CEZ	81.3	80.5± 7.0
CFX	74.6	86.3± 9.2
CTZ	91.2	83.9± 8.6
CMX	75.7	73.4± 7.4
CTT	71.7	68.8±12.4

* 95% confidence limit.

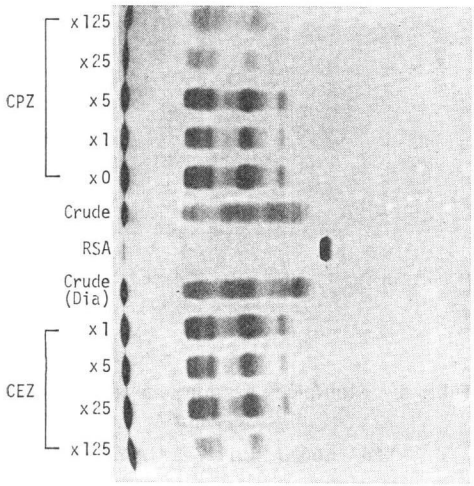
Fig. 3. Competition between CPZ and [^{14}C]PCG and between CEZ and [^{14}C]PCG on 100,000×g supernatant fluid.

The separation of 100,000×g supernatant fluid from rabbit liver, partially purified by CM Sephadex C-50, is performed by SDS-polyacrylamide gel electrophoresis. Concentrations of antibiotics are expressed as molar ratios to labeled benzylpenicillin. The exact conditions are described in the text.

RSA: rabbit serum albumin.

Crude: 100,000×g supernatant fluid.

Crude (Dia): 100,000×g supernatant fluid dialyzed against water.



83.2% for cephems. The urinary excretion of penicillin was predicted by the binding to 100,000×g supernatant fluid and molecular weight. For cephems the prediction was by the binding to 100,000×g supernatant fluid, Rf value and molecular weight. The binding to 100,000×g supernatant fluid was essential for prediction in the case of both penicillins and cephems. The predicted values obtained from each equation (2) coincided well with the observed values.

Binding of [^{14}C]PCG to 100,000×g Supernatant Fluid and Competition with CPZ and CEZ

Fig. 3 shows the [^{14}C]PCG binding proteins in rabbit liver and competition between [^{14}C]PCG and CPZ, and between [^{14}C]PCG and CEZ. [^{14}C]PCG binding protein were not definitely fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. However, the results of competition study elucidated a much stronger affinity for CPZ than for CEZ.

Discussion

A number of studies have shown that the physicochemical properties of drugs have an important and sometimes dominant influence on their excretion. HIROM *et al.* have studied the excretion in the bile and urine after intravenous injection of 16 organic anions with molecular weights 355~752, using rats, guinea pigs and rabbits. They concluded that there was a threshold molecular weight for appreciable biliary excretion of anions, varing with species: about 325±50 for rats, 400±50 for guinea pigs and 475±50 for rabbits.[1] WRIGHT *et al.* examined the biliary excretion of 18 cephalosporin derivatives in cannulated rats. The molecular weight of the compound had a dominant influence on the degree of biliary excretion, in spite of the widely differing physical chemical properties imparted by the different structures. Below a molecular weight of about 450, less than 15% of the dose was found in the bile of cannulated rats. However, about 450, the biliary excretion increased with increasing molecular weight[3]. Our results also indicated that molecular weight was superior to other physicochemical determinant (such as lipophilicity and serum protein binding) in predicting drug fate in rabbits. But the drug fate in rabbits cannot be explained clearly by molecular weight alone.

From the prediction of urinary excretion by multiple regression analysis, and the relationship found between the binding to 100,000×g supernatant fluid and urinary excretion, it became apparent that the binding to tissue was a important determinant in the pharmacologic behavior of β-lactam antibiotics. KORNGUTH *et al.* suggested that the tissue binding would tend to pull drug into tissue and counteract the effect of binding to serum proteins. Several antibiotics, including CEX, CET and PCG, have been found to bind *in vitro* to specific components in the 100,000×g supernatant fluid of rat liver homogenates.[11] Their components present in the cytisol of animals and humans have been shown to bind many endogeneous and exogeneous compounds.[12] The major binding protein has been named "ligandin". In this

experiment, we did not purify the ligandin and did not determine the extent of the binding of drug to ligandin, because large amounts of liver were necessary to determine the binding rate.

The equation for the prediction of urinary excretion by multiple regression analysis involved the binding to 100,000×g supernatant fluid for both penicillins and cephems. Therefore, the binding to 100,000×g supernatant fluid should be scrutinized closely when considering drug fate in rabbits. This equation might be able to predict the urinary excretion of other β-lactam antibiotics. In fact, our unpublished data showed that it was able to predict the urinary excretion of latamoxef,[13] cefbuperazone[14] and oxacillin. However, it could not predict that of cefpiramide.[15] This indicates that factors other than those mentioned above should be considered.

The results of the competition with [^{14}C]PCG to tissue cleared that CPZ has a higher affinity than CEZ. It is necessary to conduct further studies to resolve how this phenomenon interacts with drug fate.

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