STUDIES ON PROTEIN BINDING OF ANTIBIOTICS

I. EFFECT OF CEFAZOLIN ON PROTEIN BINDING AND PHARMACOKINETICS OF CEFOPERAZONE

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The effect of cefazolin (CEZ) on the protein binding and the pharmacokinetics of cefoperazone (T-1551) was investigated. For the simultaneous determination of T-1551 and CEZ, high pressure liquid chromatography (HPLC) was used. The extent of protein binding, the number of binding sites and the association constant to human serum albumin were 90.4%, 0.87 and 2.16×10^4 in T-1551, and 89.2%, 0.78 and 2.46×10^4 in CEZ, respectively. T-1551 and CEZ appeared to bind to the same site on the protein, since each drug competitively inhibited the binding of the other to serum protein. The mode of the binding of T-1551 to serum protein was similar to that of CEZ. When T-1551 and CEZ were co-administered, the serum level of T-1551 was lower than that with the single administration, while, urinary excretion was higher. These results suggested that the concomitantly administered drugs influenced one another's binding to serum protein *in vivo* and subsequently an increase in the concentration of the unbound drug in the serum made the drug available for glomerular filtration. It seemed that the high protein binding of T-1551 to serum was an important factor affecting its pharmacokinetics.

Cefoperazone (T-1551) is the sodium 7-[D(−)-α-(4-ethyl-2,3-dioxo-1-piperazinecarboxamido)-α-(4-hydroxyphenyl) acetamido]-3-[(1-methyl-1H-tetrazol-5-yl) thiomethyl]-3-cephem-4-carboxylate, which has a broad spectrum of antibacterial activity.[1] T-1551 binds well to rabbit, monkey and human serum protein but binds poorly to dog serum protein, like CEZ. When T-1551 is parenterally administered to animals, the serum level is lower in mice, rats and dogs than that of CEZ, whereas, higher in rabbits and monkeys. T-1551 is mainly excreted in an unchanged form through the liver in mice and rats, but, excretion in rabbits, dogs and monkeys is mainly through the kidneys. It has been disclosed that T-1551 has a wide range of variation among species on the animal studies.[2]

In humans, T-1551 has a high serum level, a long biological half-life and a high protein binding rate, much like CEZ, although both drugs have different excretion patterns. It has been reported that about 15~30% of T-1551 is excreted unchanged in the urine and as for CEZ, more than 90% is excreted.[3]

Materials and Methods

Antibiotics and other reagents

Cefoperazone (T-1551) was synthesized by Toyama Chemical Co., Ltd. and cefazolin (CEZ, Fujisawa, Japan) was purchased commercially. Human serum albumin was obtained from Sigma Chemical Co., Ltd.

Animals and humans

The rabbits used in this study were Japanese original white adult males weighing 2.0~2.5 kg. Three

normal adults weighing 58.0~72.0 kg were used in the cross over.

Determination of binding rate

The centrifugal ultrafiltration technique was used to determine the binding rates of T-1551 and CEZ. One volume of drug dissolved in 1/15 M phosphate buffer (pH 7.0) was mixed with 9 volumes of serum and was 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. Then, the concentration of drug in the ultrafiltrate was measured by HPLC.

The reference experiment was performed by using a buffer in place of the serum. The binding rate was calculated according to the following equation:

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

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

Measurement of antibiotic concentration

Measurement was performed with a high pressure liquid chromatograph (Shimadzu. LC-2 model). Serum (0.5 ml) was added to 0.5 ml of CH_3OH to deproteinize. The mixture was shaken for 2~3 minutes in a 10-ml glass tube and centrifuged for 10 minutes at 1,200×*g* at 5°C. The supernatant obtained was filtered through 0.5 μm Millipore filter and 6 μl of the filtrate was injected into HPLC. Samples were run on a column (250 mm×4 mmφ) of LiChrosorb RP-18 at an ambient temperature and a flow rate of 1.0 ml/min. The mobile phase consisted of 12.5% CH_3CN, 1.4% 1 M CH_3COOH, 2.7% 1 M $CH_3COOH\cdot N(C_2H_5)_3$ in water. The eluate was monitored at 254 nm. Urine and bile samples were measured using the same procedure except for the addition of CH_3OH (Chart 1).

Determination of maximum binding number and association constant

The binding rate of T-1551 to human serum albumin was measured over a range of the final concentrations of drug extending from 3.6×10^{-5} M to 1.2×10^{-3} M. Similarly, the binding of CEZ was also measured at the final concentrations of the drug ranging from 3.6×10^{-5} M to 1.2×10^{-3} M. The final concentration of albumin was 5.8×10^{-4} M in all studies. KLOTZ *et al.*'s formula[4] was applied, as shown below, to calculate the maximum binding number (n) and the association constant (K). A straight line was obtained in plotting $1/r$ *vs.* $1/D$, so n and K were calculated from a slope and a section.

$$1/r=\frac{1}{n\cdot K\cdot D}+\frac{1}{n}$$

D: free drug concentration
r: amount of bound drug per one molecule of albumin
n: maximum binding number
K: association constant (M^{-1})
A molecular weight of albumin was assumed as 69,000.

Nature of displacement

The binding rate of T-1551 at various concentrations was measured in the presence of the fixed concentration of CEZ (final concentration: 5.8×10^{-4} M), *i.e.*, T-1551 and serum were incubated at 37°C for 1 hour and a fixed concentration of CEZ was added to this mixture. The binding rate of T-1551 was determined by centrifugal ultrafiltration technique after incubating at 37°C for 1 hour. The binding

Chart 1. Assay procedure of T-1551 and CEZ.

Serum 0.5 ml	Urine and bile
↓ +CH_3OH 0.5 ml	↓
Shaking (2~3 min.)	Centrifugation (5°C,
↓	↓ 1,200×*g*, 10 min.)
Centrifugation	Supernatant
↓ (5°C, 1,200×*g*, 10 min.)	↓
Supernatant	Filtration (0.5 μm)
↓	↓
Filtration (0.5 μm)	HPLC (6 μl)
↓	
HPLC (6 μl)	

Table 1. Recovery of T-1551 and CEZ.

Concentration (μg/ml)	T-1551	CEZ
200	102.0%	99.6%
100	99.5	99.4
50	95.7	100.0
25	99.6	98.7

Rabbit serum was used.

rate of CEZ was also measured by mixing the fixed concentration of T-1551 (final concentration: 5.8×10^{-4} M). A similar investigation was performed with rabbit serum.

Drug administration

T-1551 and CEZ was dissolved in saline and intravenously administered. Single administration: a single dose of 20 mg/kg of each drug was administered to rabbits and 1 g of each drug to humans. Simultaneous administration: 20 mg/kg of both drugs were simultaneously administered to rabbits and 1 g of both drugs to humans.

Sampling of serum, urine and bile

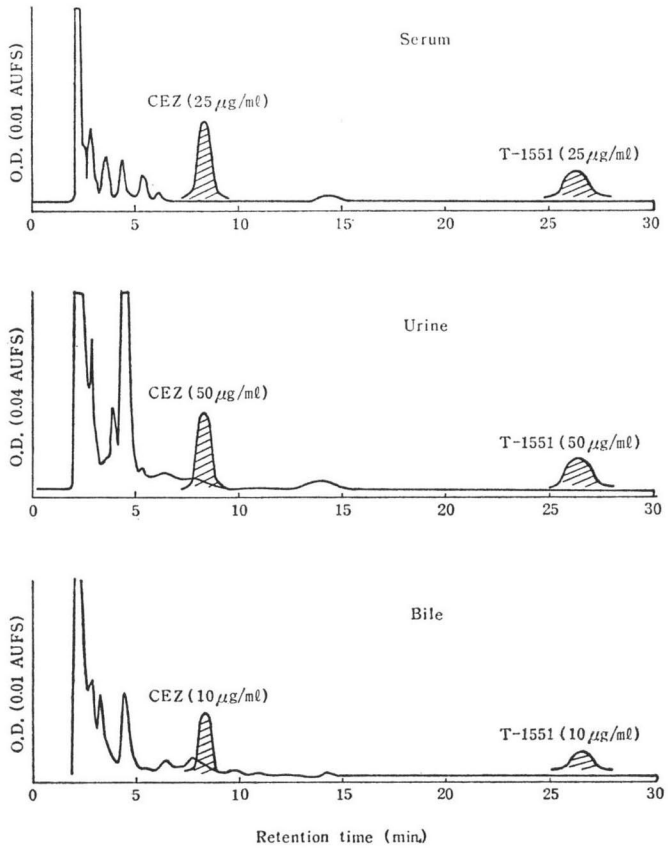
In rabbits, urine and bile samples were taken by cannulating both the ureters and bile duct under ether anesthesia. Both drugs were then administered 2 hours post-operatively. Samples were collected into 1/15 M phosphate buffer (pH 6.0) to avoid decomposition. After administration blood samples were taken from the auricular artery. In humans, blood and urine samples were taken at intervals up to 4 hours after administration.

In both cases, the blood was centrifuged and the serum obtained was stored at below −20°C until used.

Pharmacokinetic analysis

The serum concentration obtained in humans was calculated according to a 2-compartment open model[5].

Fig. 1. Typical chromatograms of T-1551 and CEZ.



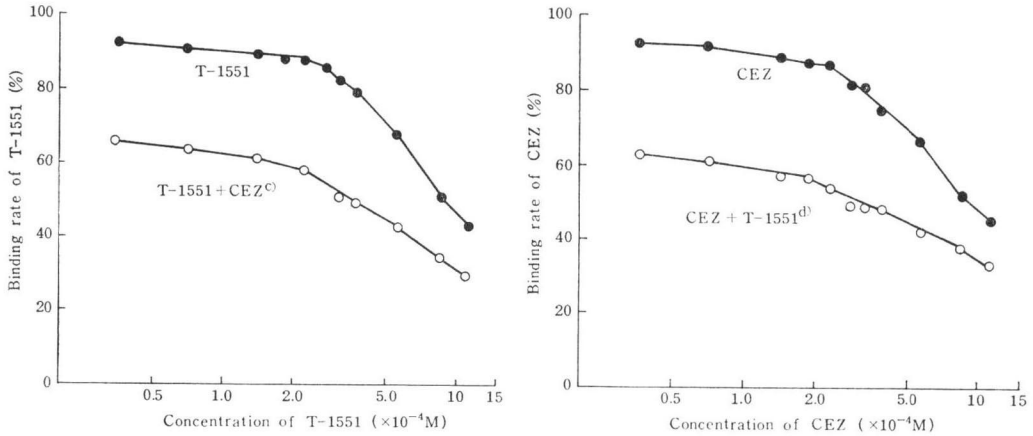
Serum, urine and bile obtained from rabbit were used.
Instrument: Shimadzu LC-2, Column: LiChrosorb RP-18
Mobile phase: 12.5% CH_3CN, 1.4% 1 M CH_3COOH, 2.7% 1 M $CH_3COOH \cdot N(C_2H_5)_3$ in water
Column temperature: Ambient, Flow rate: 1.0 ml/min.

Results

1. Separation of T-1551 and CEZ by HPLC

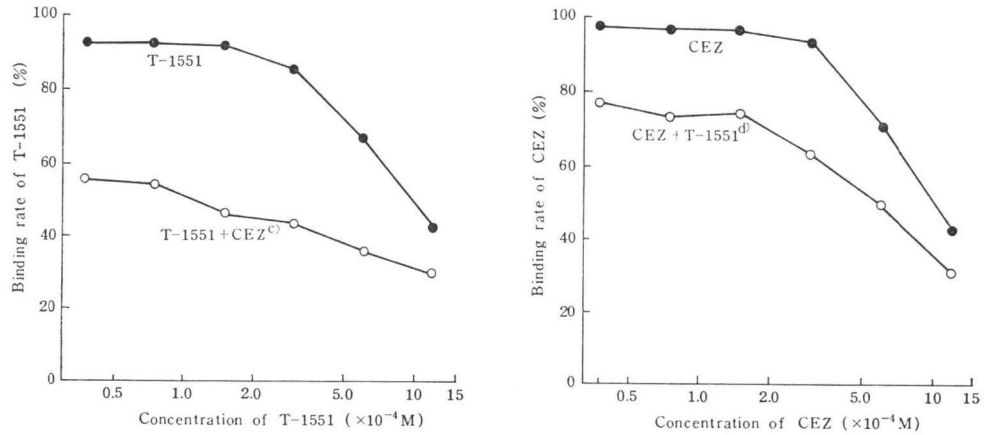
The typical chromatograms of both drugs are shown in Fig. 1. The retention times of T-1551 and CEZ in the serum, urine and bile were about 27 and 8 minutes, respectively. Both samples were well

Fig. 2. Competitive binding between T-1551 and CEZ.
(1) Effect of CEZ on binding of T-1551[a]. (2) Effect of T-1551 on binding of CEZ[b].



Protein: Rabbit serum. Method: Centrifugal ultrafiltration
a) Binding rate of T-1551 in the absence and presence of CEZ
b) Binding rate of CEZ in the absence and presence of T-1551
c) CEZ: 5.9×10^{-4} M (280 μg/ml)
d) T-1551: 6.0×10^{-4} M (400 μg/ml)

Fig. 3. Competitive binding between T-1551 and CEZ.
(1) Effect of CEZ on binding of T-1551[a]. (2) Effect of T-1551 on binding of CEZ[b].



Protein: Human serum albumin (5.8×10^{-4} M: 4%). Method: Centrifugal ultrafiltration
a) Binding rate of T-1551 in the absence and presence of CEZ
b) Binding rate of CEZ in the absence and presence of T-1551
c) CEZ: 5.8×10^{-4} M (276.7 μg/ml)
d) T-1551: 5.8×10^{-4} M (387.4 μg/ml)

separated from each other. The concentrations of T-1551 and CEZ as low as 1.0 μg/ml were able to be measured.

2. Recovery of T-1551 and CEZ

Recoveries of both drugs, which were simultaneously added to rabbit serum so as to give each concentration of 25, 50, 100 and 200 μg/ml, averaged about 100%, ranging from 95.7% to 102.0% in T-1551 and 98.8%~100.0% in CEZ (Table 1).

3. *In Vitro* Competitive Binding between T-1551 and CEZ

(1) Rabbit serum

The extent of T-1551 is 93~92% bound to rabbit serum below the concentration of 1.5×10^{-4} M (100 μg/ml) and CEZ is 97~93% below 2.9×10^{-4} M (140 μg/ml). The percentage of bound T-1551 and CEZ in the serum decreased greatly with an increase in drug concentration, suggesting that the drug-binding capacity of serum protein became saturated at high drug concentration and that the free drug concentration rose rapidly.

When a fixed concentration of CEZ was added to the solution of T-1551, the binding rate of T-1551 decreased remarkably, even at the concentrations below 1.5×10^{-4} M. Similar results were obtained with CEZ. These results suggested that T-1551 and CEZ competed with the same binding sites on the protein (Fig. 2).

(2) Human albumin

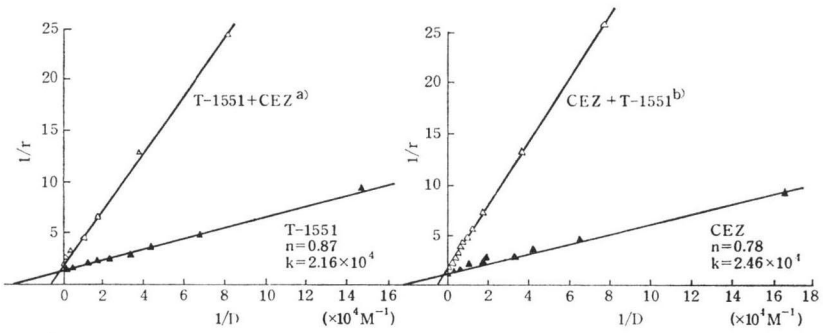
The results obtained in human serum albumin are shown in Fig. 3. The extent of T-1551 was 92~87% below the concentration of about 2.3×10^{-4} M (155 μg/ml) and decreased with an increase in drug concentration. Similarly, CEZ was 93~86% below about 2.3×10^{-4} M (110 μg/ml) and there was a marked reduction in binding above that concentration (Fig. 3).

By using KLOTZ *et al.*'s formula, a linear relationship was demonstrated between the reciprocal of the concentration of unbound drug and the reciprocal of the moles of drug bound per mole of albumin (Fig. 4).

The number of binding sites (n) and the association constant (K) were 0.87 and 2.16×10^4 in T-1551 and 0.78 and 2.46×10^4 in CEZ, respectively. Thus, the degree of the binding of T-1551 to serum protein

Fig. 4. KLOTZ plots for binding of T-1551 and CEZ to human serum albumin.

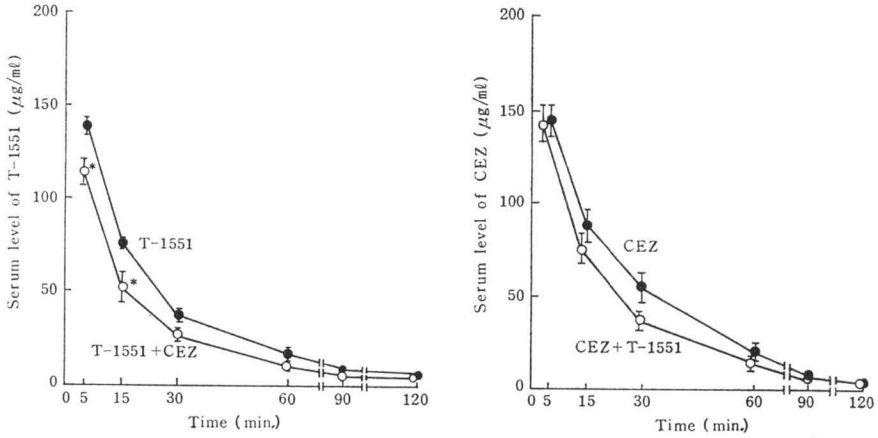
(1) Effect of CEZ on binding of T-1551. (2) Effect of T-1551 on binding of CEZ.



r: Amount of bound drug per one molecular
D: Free drug concentration
n: Maximum binding number
K: Association constant

Human serum albumin: 5.8×10^{-4} M
a) CEZ: 5.8×10^{-4} M
b) T-1551: 5.8×10^{-4} M

Fig. 5. Serum level of T-1551 and CEZ in rabbits.
(1) Effect of CEZ on serum level of T-1551. (2) Effect of T-1551 on serum level of CEZ.



Serum level of each drug in the simultaneous administration was compared with that in the single administration.
Dose: 20 mg/kg of each drug
1 group: 5 rabbits
*: significant difference at $P<0.05$

was similar to that of CEZ. Both drugs appeared to bind to the same sites on the protein, since each drug competitively inhibited the binding of the other. In our preliminary experiments, when T-1551 (50 μg/ml) and CEZ (50~100 μg/ml) were mixed, the binding rates of both drugs did not change.

4. Serum Level and Urinary and Biliary Excretion

(1) Rabbit

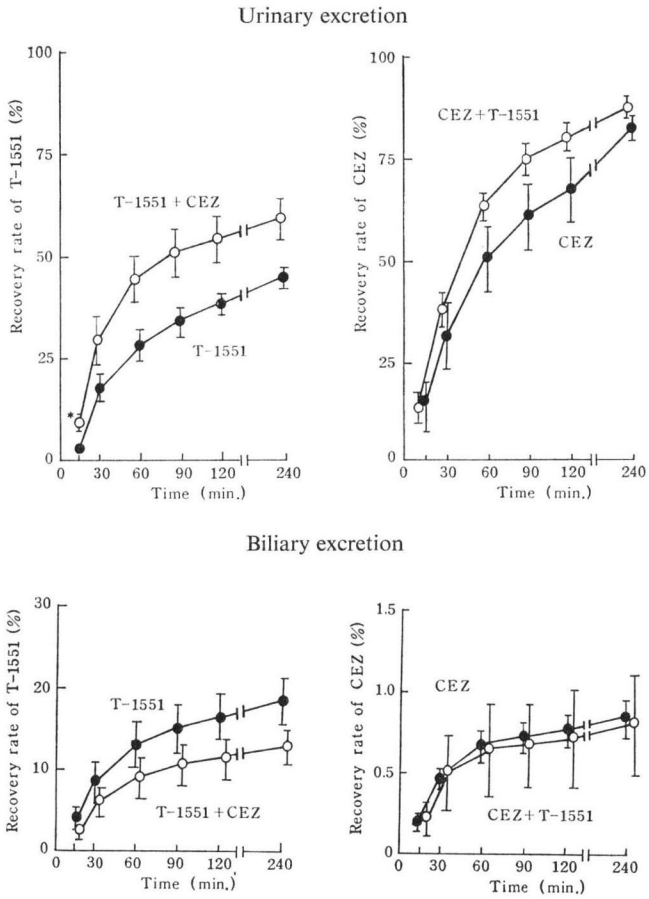
The serum level of T-1551 with the simultaneous administration was lower than that with the single administration. In particular, the levels at 5 and 15 minutes after the simultaneous administration were significantly lower ($P<0.05$). The serum level of CEZ with the simultaneous administration was also lower than that with the single administration. There were no significant differences at any time between these two drugs (Fig. 5).

Within 15 minutes after administration, 3.1±1.0% of T-1551 was excreted in the single administration and 9.2±2.1% in the simultaneous administration, which were significantly different ($P<0.05$). Cumulative urinary excretion of T-1551 up to 4 hours amounted to 44.4±2.4% of the dose in the single administration and 59.2±5.2% in the simultaneous administration ($P<0.05$). Biliary excretion of T-1551 was 19.7±2.7% in the single administration and 12.8±2.5% in the simultaneous administration up to 4 hours. These values were not significantly different. In contrast, cumulative urinary excretion of CEZ up to 4 hours with the simultaneous administration was higher than that with the single administration, though it was not significantly different. Biliary excretion was 0.83±0.13% in the single adminisitration and 0.80±0.32% in the simultaneous administration, which was much lower than those of T-1551 in each case (Fig. 6).

(2) Humans

The serum level of T-1551 with the simultaneous administration was lower than that with the single administration, as seen in rabbits. In particular, there was significant difference between these two groups at 5, 15 and 30 minutes, which showed 207.4±11.9 μg/ml, 157.1±12.3 μg/ml and 116.0±8.6 μg/

Fig. 6. Urinary and biliary excretion of T-1551 and CEZ in rabbits.



Urinary and biliary excretion of each drug for a single administration were compared with those for the simultaneous administration.
Dose: 20 mg/kg of each drug, 1 group: 5 rabbits
*: significant difference at $P<0.05$

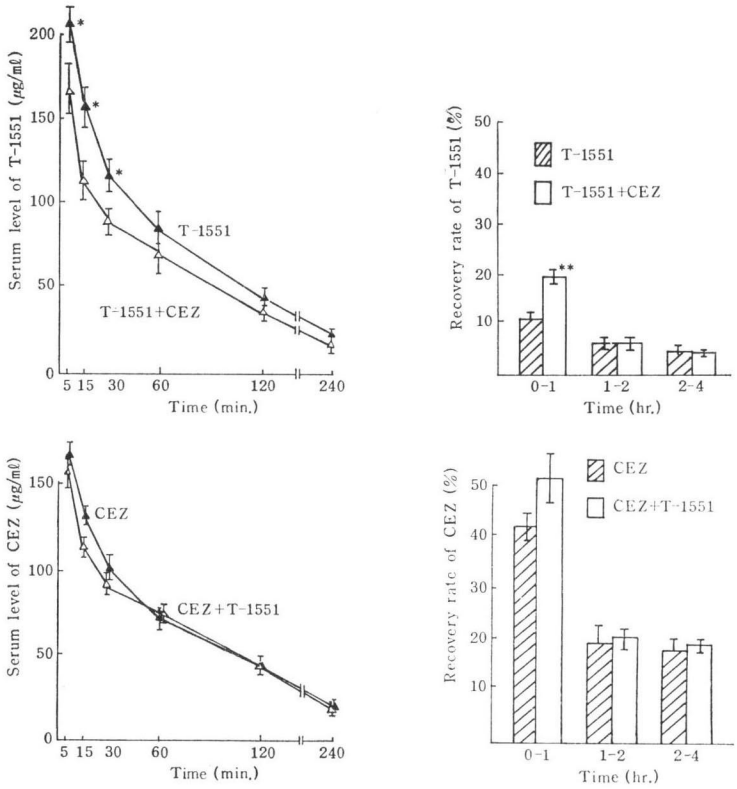
ml in the single administration and 167.4±15.5 μg/ml, 113.5±10.2 μg/ml and 86.3±7.9 μg/ml in the simultaneous administration, respectively.

On the other hand, the level of CEZ with the simultaneous administration was somewhat lower than that with the single administration up to 30 minutes, but both serum levels became similar after 1 hour.

Urinary excretion of T-1551 within 1 hour was 11.5±1.3% in the single administration and 20.4±1.4% in the simultaneous administration. These values were statistically different at $P<0.01$, however, there were no differences after 1 to 2 hours and 2 to 4 hours. Cumulative urinary excretion within 4 hours after administration were 22.0±1.8% in a single administration ($P<0.05$). Accordingly, this statistically difference on the total excretion was due to the excretion rate obtained within 1 hour.

In CEZ, urinary excretion within 1 hour was 42.3±3.1% in the single administration and 51.7±4.8% in the simultaneous administration. There was no difference at any duration between these two groups (Fig. 7).

Fig. 7. Serum level and urinary excretion of T-1551 and CEZ in humans.



Serum level and urinary excretion of each drug in the simultaneous administration were compared with those in the single administration.
Dose: 1 g/body of each drug
Single administration group consisted of 6 persons
Simultaneous administration group consisted of 3 persons
* Significant difference at $P<0.05$
** Significant difference at $P<0.01$

Table 2. Pharmacokinetic parameter of T-1551 and CEZ in humans.

		T-1551		CEZ	
		alone	with CEZ	alone	with T-1551
α	hr^{-1}	3.49±0.27	5.74±0.82*	4.00±0.46	5.05±0.44
β	hr^{-1}	0.43±0.03	0.48±0.04	0.43±0.02	0.44±0.02
K_{12}	hr^{-1}	1.24±0.12	2.31±0.52	1.43±0.27	1.68±0.16
K_{21}	hr^{-1}	1.76±0.19	3.00±0.37	2.25±0.19	3.09±0.40
K_{13}	hr^{-1}	0.81±0.06	0.91±0.01	0.75±0.04	0.72±0.02
V_I	l	4.20±0.27	4.90±0.62	5.00±0.31	5.35±0.32
V_{II}	l	2.79±0.20	3.66±0.45	2.99±0.33	2.93±0.26
V_d	l	6.99±0.44	8.58±0.84	7.99±0.36	8.29±0.20
$T_{1/2}$	min	98.8 ±6.7	87.8 ±6.9	98.0 ±4.2	95.4 ±4.42

Each parameter was calculated by a 2-compartment open model.
* Significant difference at $P<0.01$

5. Pharmacokinetic Analysis

α, K_{12}, K_{21}, K_{13} and Vd of T-1551 with the simultaneous administration showed higher values in comparison with those for a single administration. The biological half lives of both drugs, however, did not change greatly. These results suggested a rapid distribution of the drug to the tissues and a rapid elimination from the body due to an increase of free drug concentration. Similar results were obtained with CEZ (Table 2).

Discussion

In general, the following factors can be considered when the urinary excretion of drug is low. (1) Due to the drug's high protein-binding activity, the bound portion is not filtered during its passage through the glomerulus. (2) The drug possesses higher affinity for the tissue protein in the liver. (3) Molecular weight and the presence of polar groups in the drug, *etc.*

We have reported on the renal excretion mechanism of T-1551 in humans. When T-1551 and probenecid were concomitantly administered, the serum level and renal excretion of T-1551 were not greatly affected with probenecid. From these results, T-1551 appears to be mainly filtered through the glomerulus[6].

In recent years, several investigations have been performed on the mechanism of the biliary excretion of drugs. HIROM *et al.*[7] measured the biliary excretion rate of 16 organic anions and concluded that there was a threshold molecular weight for appreciable biliary excretion, which varies with species; about 325 ± 50 for the rat, 400 ± 50 for the guinea pig and 475 ± 50 for the rabbit. In addition, RYRFELDT *et al.*[8] investigated the relation between polarity and biliary excretion as for 10 penicillins in rats and reported that a relation existed between increasing polarity in the side-chain of the penicillin molecule and its biliary excretion.

However, its mechanims of biliary excretion was not examined throughly. Moreover, although there are some antibiotics such as apalcillin (PC-904)[9], piperacillin (T-1220)[10] and cefotiam (SCE-963)[11], which are excreted into the bile at a higher rate, their biliary excretion mechanism have not yet been clarified.

Our final purpose is to clarify the excretion mechanism of the drug. Therefore, in the present study, protein binding was examined as the fate of drugs in the body is greatly influenced by their binding to serum protein.

The relation between the protein binding and the pharmacokinetics of a drug has been frequently reported[12~14] and it is generally related that the distribution of a drug having a higher protein binding is restricted and the excretion from tissues is delayed.

The simultaneous administration of drugs influences their absorption, excretion and distribution, and has an important significance in their efficacy and toxicity. These have been recognized in the simultaneous administration of warfarin and phenylbutazone[15], bilirubin and sulfonamide[16], methotrexate and sulfonamide[17], *etc.*, which causes severe adverse clinical reactions. In the *in vivo* study of the competitive effect, ANTON *et al.*[18] showed that the serum levels of sulfaethylthiadiazole (SET) in rats were reduced by the simultaneous administration of sulfinpyrazone (SPZ), and that tissue levels of SET increased.

However, there have been scant reports on the *in vivo* interactions between antibiotics. One reason is due to the difficulty in determining more than two kinds of antibiotics, using bioassay methods. ISHIKAWA *et al.*[19] measured the concentration of dicloxacillin (MDIPC) by using *Staphylococcus* with high resistance against ampicillin (ABPC) and the concentration of ABPC by using *B. subtilis* ATCC 6633 after extraction of MDIPC with ethylether. NISHIDA *et al.*[20] also measured the concentration of ABPC and cloxacillin (MCIPC) by means of bioautography applying thin-layer chromatography. However, it is considered to be difficult to determine simultaneously by bioassay two kinds of antibiotics having a similar broad antibacterial spectrum. Therefore, we studied the conditions for applying high pressure liquid chromatography (HPLC), which has proven recently most effective in separating com-

pounds, and found that the method was available for the purpose of this study. HPLC has advantages over bioassay with regard to its accuracy and rapidity. By using HPLC, we calculated the n and K values of T-1551 and CEZ, and concluded that both drugs have the same degree of binding to human serum albumin. In addition, it seemed that both drugs were bound to the same sites of the serum protein.

Furthermore, we investigated how the results obtained in *in vitro* study reflected on rabbits and human, and observed that the serum level with the simultaneous administration was below that in a single administration, while renal excretion was higher. From these findings we concluded that both drugs competed with each other at high concentrations after administration, the level of unbound drug rapidly increased and subsequently the free drug was transferred rapidly into the tissues and urinary excretion of the drug became high. These were substantiated through the calculation by the computer on the basis of the total drug concentration in the serum. In other words, these results suggested that co-administered drugs influenced one another's binding to serum protein *in vivo* and subsequently an increase in the concentration of unbound drug in the serum made the drug available for glomerular filtration.

In rabbits, urinary excretion of T-1551 with the simultaneous administration was higher than that with the single administration. While, its biliary excretion was a little lower. These results suggested that the free T-1551 did not have a great influence on its biliary excretion, although the free drug in the serum made more drug available for glomerular filtration.

In conclusion, the high protein binding of T-1551 to serum is an important factor affecting its renal excretion, however, further study is necessary to clarify the difference between these two drugs.

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