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Determination of ticarcillin epimers in plasma and urine with high-performance liquid chromatography

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Abstract

A high-performance liquid chromatogaphic method was developed for determining the concentrations of ticarcillin (TIPC) epimers in human plasma and urine. Samples were prepared for HPLC analysis with a solid-phase extraction method and the concentrations of TIPC epimers were determined using reversed-phase HPLC. The mobile phase was a mixture of 0.005 *M* phosphate buffer (pH 7.0) and methanol (12:1, v/v) with a flow-rate of 1.0 ml/min. TIPC epimers were detected at 254 nm. Baseline separation of the two epimers was observed for both plasma and urine samples with a detection limit of ca. 1 μg/ml with a *S/N* ratio of 3. No peaks interfering with either of the TIPC epimers were observed on the HPLC chromatograms for blank plasma and urine. The recovery was more than 80% for both plasma and urine samples. C.V. values for intra- and inter-day variabilities were 0.9–2.1 and 1.1–6.4%, respectively, at concentrations ranging between 5 and 200 μg/ml. The present method was used to determine the concentrations of TIPC epimers in plasma and urine following intravenous injection of TIPC to a human volunteer. It was found that both epimers were actively secreted into urine and that the secretion of TIPC was not stereoselective. Plasma protein binding was also measured, which revealed stereoselective binding of TIPC in human plasma.

Keywords: Enantiomer separation; Ticarcillin

1. Introduction

Stereoselectivity in pharmacokinetics is one of the most important aspects in the clinical use of chiral drugs. Since stereoisomers generally exhibit different pharmacokinetic properties as well as pharmacological activity, it is necessary to clarify the pharmacokinetics of the individual stereoisomers.

Ticarcillin (TIPC) is a semi-synthetic penicillin, which has been in clinical use for a number of years. As is the case for several other semi-synthetic penicillins, TIPC has been used as a mixture of two epimers (*R*- and *S*-TIPC). The chiral center is the (carboxy-3-thienylacetyl)amino group attached at the C-6 position of penicillanic acid (Fig. 1). Although the disposition of TIPC has been studied both in human and animals [1–12], the pharmacokinetics of each epimer are unknown, since previous studies

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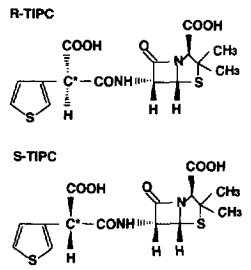

Fig. 1. Chemical structures of ticarcillin (TIPC) epimers. *: Chiral carbon in the side chain.

employed either microbiologic assay [1–4,6] or non-stereospecific HPLC methods [5,7–10,13–15]. In fact, stereospecific HPLC methods have been reported [16–18], but pharmacokinetic studies have not been conducted using stereospecific assay methods. Moreover, for carbenicillin (CBPC) and sulbenicillin (SBPC) which are epimeric β-lactams, it has been reported that renal secretion of CBPC is stereoselective in humans [19] and that stereoselective degradation of SBPC epimers takes place in human plasma [20]. Therefore, pharmacokinetic behavior may differ between TIPC epimers, but this has not been clarified, yet.

In the present study, a stereospecific HPLC method was developed for analysis of TIPC epimers in biological fluids. The method was applied to determine TIPC concentrations in plasma and urine following TIPC administration to a human volunteer. The method was also used to measure the plasma protein binding of TIPC epimers in vitro.

2. Experimental

2.1. Reagents

Ticarcillin disodium salt (Monapen®) and carbenicillin disodium salt (Geopen®) were purchased from Fujisawa Pharmaceutical Industries (Osaka, Japan) and Pfizer Pharmaceutical (Tokyo, Japan), respectively. The ratios of the *R*- to *S*-epimer (*R*/*S* ratio) were approximately 1.2 for both TIPC and CBPC. Methanol used was HPLC grade and obtained from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals were of reagent grade and used as received.

2.2. Isolation of TIPC epimers

TIPC epimers were resolved and isolated using a glass column (26 mm I.D.×1000 mm) packed with porous styrene–divinyl benzene copolymer beads. Two types of polymer beads, Diaion HP-20 (Mitsubishi Chemical Industries, Tokyo, Japan) and Amberlite XAD-2 (Organo Co., Tokyo, Japan) were used. Polymer beads were ground under wet conditions for ca. 15 min with a grinding machine (Ishikawa Kojo Mfg. Co. Ltd., Tokyo, Japan) prior to use. Diaion HP-20 and Amberlite XAD-2 were used for isolation of *R*-TIPC and *S*-TIPC, respectively.

Approximately 5 ml of *R*,*S*-TIPC aqueous solution (ca. 200 mg/ml, *R*/*S*=ca. 1.2) was loaded and eluted with distilled water at a flow-rate of ca. 3 ml/min. Eluent was collected (8 ml each) with a fraction collector and the fractions containing each epimer were freeze-dried. The absolute configuration of each fraction was assigned with ^{1}H NMR according to the method reported by Bird and Steele [21]. Each epimer thus obtained was injected into the HPLC system in order to determine the specific retention time.

2.3. Plasma and urine collection

Blood and urine were obtained after overnight fast from two healthy male volunteers (22 years of age) without TIPC administration. Blood was immediately centrifuged and plasma was pooled and stored at −80°C until use. Urine was also stored at −80°C prior to use. Plasma and urine thus obtained were used as blank samples.

2.4. Sample preparation for HPLC analysis

A solid-phase extraction column (Isolute™-SAX 200 mg/3 ml of reservoir volume; International Sorbent Technology, Hengoed, Mid-Glamorgan, UK) was used for plasma and ultrafiltrate samples, and the same type of solid-phase extraction column with a different bed volume (Isolute™-SAX 100 mg/1 ml of reservoir volume) was used for urine samples.

A Isolute™-SAX column was pre-conditioned with 2 ml of methanol followed by 2 ml of distilled

water. Two hundred microliters of CBPC aqueous solution (120 μg/ml) were added as an internal standard to 0.5 ml of plasma or diluted urine sample obtained from the in vivo administration study, which was then mixed with 5 ml of 0.05 *M* CH_3COONH_4. One hundred microliters of CBPC aqueous solution (120 μg/ml) were added to 0.1 ml of plasma or ultrafiltrate obtained from the plasma protein binding study, which was then mixed with 3 ml of 0.05 *M* CH_3COONH_4. The mixture was loaded onto a pre-conditioned SAX column and was drawn through the column under vacuum. The column was flushed with 3 ml of 0.5 *M* CH_3COOH–CH_3CN (1:1, v/v) and then with 2 ml of 0.1 *M* CH_3COONH_4–CH_3OH (1:1, v/v), which were discarded. This procedure was necessary to eliminate interfering substances on the HPLC chromatogram. The sample was then eluted with 0.5 ml (for urine) or 1.0 ml (for plasma and ultrafiltrate) of 10% LiCl–CH_3OH (3:2, v/v), and an aliquot of the final eluent was injected onto the HPLC system. All the solutions were kept cold on ice to prevent possible degradation of TIPC and CBPC.

2.5. HPLC conditions

A high-performance liquid chromatograph was used to determine the concentrations of TIPC epimers. The HPLC system consisted of a dual piston pump (Model LC10AD), a UV detector (Model SPD-10A) and an integrator (Model C-R4A), all from Shimadzu Co., Kyoto, Japan. A Cosmosil® column ($5C_{18}$-AR, 250×4.6 mm I.D., Nacalai Tesque Co., Kyoto, Japan) was used as an analytical column. Mobile phase composition was 0.005 *M* phosphate buffer (pH 7.0)–CH_3OH (12:1, v/v) and the flow-rate was 1.0 ml/min. The temperature of the column was kept at 19°C, and TIPC epimers were detected at 254 nm.

2.6. Calibration curves

A standard TIPC solution of 5–10 mg/ml in distilled water was prepared on each day of assay. Appropriate volumes of the standard solution were added to plasma or diluted urine in order to obtain TIPC concentrations ($R+S$) of 0, 10, 100 and 200 μg/ml in plasma and 0, 10, 50, 150 and 250 μg/ml in urine. Plasma and urine samples thus obtained were prepared for HPLC analysis according to the same procedures as described above. The peak-area ratio of each TIPC epimer to CBPC (*R* plus *S*) was plotted against the concentration of TIPC to construct a calibration curve.

2.7. Recovery study

Plasma and diluted urine were spiked to contain TIPC concentrations ($R+S$) of 10–200 μg/ml and were analyzed with HPLC as described above. Recovery was estimated by comparison of the peak areas for samples to those for standard aqueous solutions.

2.8. Intra- and inter-day variability study

On the same day, plasma and diluted urine were spiked to contain TIPC concentrations ($R+S$) of 10–200 μg/ml and were analyzed by HPLC. Coefficients of variation following repeated analyses were used as estimates for intra-day variability. On the other hand, plasma and diluted urine were spiked to contain TIPC concentrations ($R+S$) of 5–200 μg/ml and were analyzed by HPLC on separate days. Coefficients of variation were used as estimates for the day-to-day variability. CBPC was used as an internal standard as described for the construction of calibration curves.

2.9. Plasma protein binding study

For both the control and probenecid studies, plasma was obtained from the volunteer at 15 min before TIPC injection and was used for in vitro binding studies. Binding of TIPC was measured in vitro with an ultrafiltration method. Amicon Centrifree® was used as an ultrafiltration device with a type YMT membrane (Amicon Division, W.R. Grace and Co., MA, USA). A 1-ml volume of the plasma sample (pH adjusted to 7.4±0.1 with 1 *M* HCl) was mixed with 50 μl of various concentrations of TIPC ($R/S=1.20$) aqueous solution, and an aliquot (0.1 ml) was prepared for HPLC as previously described to determine the total (bound plus unbound) concentration of each epimer. The remainder of the sample (ca. 0.95 ml) was trans-

ferred into the ultrafiltration device and centrifuged at 1000 *g* for 4 min at 37°C. A 100-μl aliquot of the ultrafiltrate was prepared for HPLC analysis in a similar manner as described above for the plasma samples in order to determine the unbound concentration of each epimer. Unbound fraction (f_u) was calculated as C_f/C_t, where C_f is the concentration of each epimer in the ultrafiltrate and C_t is the concentration of each epimer in plasma. A customized Himac 15D centrifuge (Hitachi, Tokyo, Japan) was used to control the temperature during ultrafiltration.

2.10. Volunteer study

This study was approved by the Ethical Review Board of School of Pharmaceutical Sciences, Kitasato University. A male volunteer participated in the present study, and the volunteer gave written informed consent. The volunteer was 23 years of age with the body weight of 73 kg. He had no evidence of disease as determined by physical examination, urinalysis and blood chemical tests. Control and probenecid studies were conducted with one week washout period.

For the control study, 2 g of TIPC (Monapen®; *R*-TIPC/*S*-TIPC=1.20) was dissolved in 40 ml of saline and injected into a forearm vein over a 4-min injection time. Blood was collected in heparinized disposable syringes from the vein of the contralateral forearm before TIPC injection and at 0.25, 0.5, 0.75, 1, 1.5, 2.5, 3.5 and 5 h following injection of TIPC. Plasma was immediately obtained by centrifugation and stored at −80°C until it was analyzed. Urine was collected before TIPC injection as well as at the following time intervals after the injection: 0 to 0.5, 0.5 to 1, 1 to 2, 2 to 3, 3 to 4, 4 to 6, 6 to 12, 12 to 24 and 24 to 27 h. Immediately after each collection, urine volume was recorded and stored at −80°C until it was analyzed. On the day of analysis urine was thawed and diluted 50-fold with deionized water prior to sample preparation.

For the probenecid study, 1 g of probenecid (Probenemid® tablets, Banyu Pharm. Co., Tokyo, Japan) was orally administered at 12 and 1 h before TIPC injection. TIPC was administered in the same manner as in the control study. Blood was collected before TIPC injection and at 0.25, 0.5, 0.75, 1, 1.5, 2.5, 3.5, 5 and 9 h after the injection. Urine was collected at the same time intervals as for the control study. Urine and plasma samples were stored in the same manner as for the control study.

2.11. Determination of GFR

Creatinine concentrations in plasma and urine were determined with a Creatinine-Test Wako kit (Wako Pure Chemicals, Osaka, Japan). The GFR was determined as the urinary excretion rate of creatinine divided by the creatinine concentration in plasma.

2.12. Pharmacokinetic analysis

TIPC concentration in plasma versus time curves were analyzed by a non-linear least squares method (MULTI) [22] with a weight of $1/C^2$ for each data point, where C is the concentration in plasma. In the control study, drug concentration profiles in plasma were analyzed with a one-compartment open model which showed a better fit compared with that obtained with a two-compartment open model. In the probenecid study, however, drug concentration profiles in plasma were analyzed with a two-compartment open model. When probenecid was coadministered, a small distribution phase was observed and the two-compartment open model gave smaller values for AIC (Akaike's information criterion) [22]. Total body clearance (Cl) was calculated as the dose divided by the area under the curve.

The amount of each epimer excreted in urine during each collection interval was determined as the concentration of each epimer in the urine multiplied by the urine volume. Renal clearance (Cl_r) was calculated as the renal excretion rate divided by the concentration in plasma at the midpoint of each urine collection interval. Renal clearance for unbound drug ($Cl_{r,u}$) was calculated as Cl_r/f_u. The f_u value at a given concentration was calculated using binding parameter values obtained in the plasma protein binding study [19].

3. Results and discussion

HPLC chromatograms for blank human plasma and the human plasma obtained following TIPC administration to a human volunteer are shown in

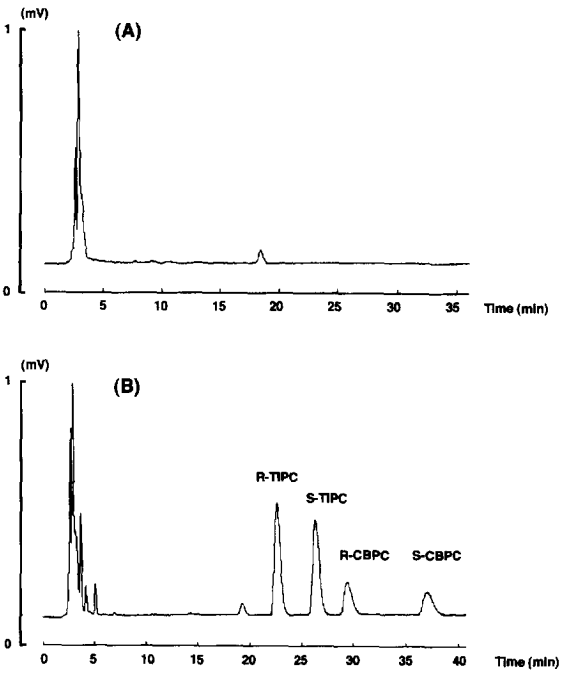

Fig. 2. Chromatograms for (A) blank human plasma and (B) the plasma obtained from a healthy male volunteer at 2.5 h after intravenous injection of 2 g of TIPC. The concentrations in plasma were 21.9 and 20.5 mg/ml for *R*- and *S*-TIPC, respectively.

Fig. 2A Fig. 2B, respectively. As shown in Fig. 2B, the two peaks corresponding to *R*- and *S*-TIPC were baseline separated. TIPC and CBPC were also baseline separated. Also, there were no peaks interfering with either of the TIPC epimers on the chromatogram for blank plasma, as shown in Fig. 2A. It was confirmed that the *R*-epimers of CBPC and TIPC eluted prior to the *S*-epimers under the present HPLC conditions by injecting isolated epimers. HPLC chromatograms for urine samples were very similar to those for plasma samples with baseline separation of the two epimers and without any interfering peaks for blank urine (data not shown).

Calibration curves were linear for plasma over the concentration (*R*-TIPC+*S*-TIPC) range between 0–200 μg/ml with the correlation coefficients being greater than 0.999 for both *R*- and *S*-TIPC. Calibration curves for urine samples were also linear for the concentrations ranging between 0 and 250 μg/ml with the correlation coefficients being similar to those observed for plasma. Moreover, the concentrations of TIPC epimers were calculated using these calibration curves. The results showed that the deviations between the calculated and actual concentrations were 2.5–8.9% and 0.4–0.6% at approximately 5 and 100 μg/ml, respectively, for plasma and urine samples.

The recoveries for both epimers in human plasma and urine are summarized in Table 1. Although the recovery of the *R*-epimer appeared to be slightly less than that of the *S*-epimer, more than 80% of each epimer was recovered, and the recovery was quite consistent as reflected in the small S.E. values.

The intra- and inter-day variabilities were also measured and the results are summarized in Table 2 Table 3. The variability was acceptable, as reflected by the small C.V. values.

In order to clarify the possibility of epimerization during the present sample preparation procedure, TIPC epimers were partially resolved and were analyzed according to the present analytical method. When the epimeric mixture of 83.1% of *R*-TIPC ($R/(R+S)=0.83$) was dissolved in human plasma at a concentration of 100 μg/ml and prepared for HPLC analysis, the ratio of *R*-TIPC decreased to 81.5±0.5% (mean±S.D., $n=3$). The result suggested that 1.9% of *R*-TIPC epimerized to *S*-TIPC during the present sample preparation procedure. Epimerization of *S*-TIPC was also checked using an epimeric

Table 1
Accuracy and precision for the determination of TIPC epimers in human plasma and urine

Epimer	Concentration (μg/ml)		Recovery	*n*
	Theory	Measured	(mean±S.E.) (%)	
Plasma				
R	5.5	4.6	83.6±2.0	6
S	4.6	3.9	84.8±2.6	6
R	56.3	47.0	83.4±1.8	4
S	47.7	40.6	85.1±1.9	4
R	109.9	90.4	82.3±0.8	6
S	93.1	78.0	83.8±0.7	6
Urine				
R	5.3	4.7	87.6±2.8	3
S	4.5	4.1	90.2±2.5	3
R	55.2	49.1	89.0±2.8	3
S	46.8	41.8	89.4±2.7	3
R	112.5	102.8	91.4±1.0	3
S	95.2	88.6	93.1±1.5	3

Table 2
Intra-day variability for the determination of TIPC epimers in human plasma and urine

Concentration (*R*+*S*) (μg/ml)	Epimer	C.V. (%)[a]
Plasma		
10	*R*	1.4
	S	1.1
200	*R*	1.0
	S	0.9
Urine		
50	*R*	2.0
	S	1.8
150	*R*	1.8
	S	2.1

[a] Coefficient of variation. Each sample was analyzed 3 times.

mixture of 72.1% of *S*-TIPC in plasma at a concentration of 100 μg/ml. The ratio of *S*-TIPC decreased to 69.6±0.3% (mean±S.D., $n=3$) after being prepared for HPLC, suggesting that 1.8% of *S*-TIPC epimerized to *R*-TIPC. These results suggested that epimerization of TIPC during the present sample preparation procedure was at most 1.9%, which does not appear to affect the results obtained in the present study.

Protein binding was measured by ultrafiltration, and the results are shown in Fig. 3. Unbound fraction

Table 3
Inter-day variability for the determination of TIPC epimers in human plasma and urine

Concentration (*R*+*S*) (μg/ml)	Epimer	C.V. (%)[a]	*n*[b]
Plasma			
10	*R*	4.4	5
	S	4.4	5
100	*R*	1.4	5
	S	1.1	5
200	*R*	2.2	5
	S	1.8	5
Urine			
5	*R*	3.7	6
	S	6.4	6
50	*R*	1.8	6
	S	1.8	6
150	*R*	2.0	6
	S	1.3	6

[a] Coefficient of variation.
[b] Concentrations were measured on *n* separate days.

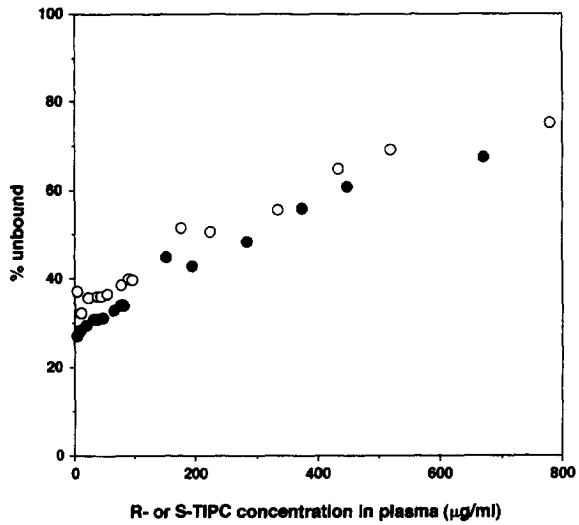

Fig. 3. Unbound fraction of *R*-TIPC (○) and *S*-TIPC (●) in human plasma.

was greater for *R*-TIPC, indicating that the binding is stereoselective with the binding of *S*-TIPC being favorable. Ratio of the f_u for *R*-TIPC to that for *S*-TIPC [$f_u(R)/f_u(S)$] was approximately 1.2. Scatchard plot showed a straight line for each epimer, indicating a single class of binding sites for both epimers. Apparent binding parameters values are as follows; $n=0.78$ and $K=3.90\times10^3\ M^{-1}$ for *R*-TIPC and $n=0.92$ and $K=3.86\times10^3\ M^{-1}$ for *S*-TIPC, where n is the number of binding sites and K is the binding constant. Binding of TIPC epimers in the probenecid treated plasma was very similar to that observed in the plasma without probenecid.

Stereoselective plasma protein binding has been reported for various enantiomers and diastereomers [23,24], including CBPC (a β-lactam antibiotic) [19] and Moxalactam (an oxacephem antibiotic) [25]. CBPC and Moxalactam are clinically used as a mixture of two epimers and the binding in human plasma has been reported to be favorable for the *S*-epimer for both antibiotics. The present sudy has revealed that the plasma protein binding of TIPC, which is a semi-synthetic epimeric β-lactam antibiotic, is also stereoselective with the binding of the *S*-epimer being favorable.

R-TIPC and *S*-TIPC concentration profiles in plasma following TIPC injection with and without probenecid coadministration are shown in Figs. 4A

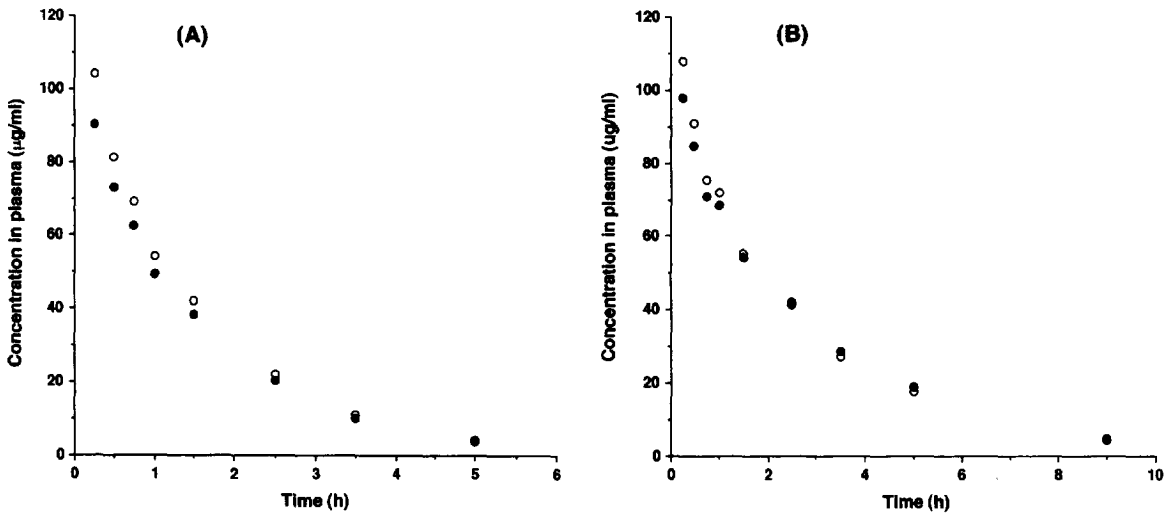

Fig. 4. Concentration of *R*-TIPC (○) and *S*-TIPC (●) in plasma following intravenous injection of 2 g of TIPC (*R*:*S*=1.20) to a healthy male volunteer with (B) and without (A) probenecid coadministration. Each point represents the mean of duplicate determinations.

and B. In both studies, *R*-TIPC concentrations were slightly greater than *S*-TIPC concentrations, which was because *R*-TIPC content was greater in the preparation that was administered. *R*-TIPC and *S*-TIPC concentrations became similar as time progressed. This was reflected in the total body clearance (*Cl*), with the *Cl* of *R*-TIPC being greater than that of *S*-TIPC. The *Cl* values were 119 and 109 ml/min per body for *R*-TIPC and *S*-TIPC, respectively, in the control study. Probably, the greater *Cl* for *R*-TIPC was, at least partly, due to the greater f_u of *R*-TIPC in plasma as described above.

In the probenecid study, *Cl* values were 60.7 and 50.6 ml/min per body for *R*-TIPC and *S*-TIPC, respectively. The *Cl* values of both epimers were significantly smaller than those in the control study, suggesting that elimination of both epimers from the body is inhibited by probenecid. This is consistent with the previous studies [1,2,8], where disappearance of TIPC from the body was delayed when probenecid was coadministered.

Urinary excretion rates of TIPC for each time interval are summarized in Table 4. More than 80% of both epimers were recovered in urine within 24 h in both control and probenecid studies, suggesting that urinary excretion is the major elimination pathway for TIPC. On the other hand, urinary excretion rates of both epimers were significantly reduced when probenecid was coadministered, indicating that renal secretion of TIPC epimers are inhibited in the presence of probenecid.

Cl_r values obtained were 99.0±9.8 and 93.1±9.5 ml/min per body (mean±S.E. of four time intervals shown in Table 4) for *R*-TIPC and *S*-TIPC, respectively. $Cl_{r,u}$ values were 279±34 and 295±36 ml/min per body (mean±S.E. of four time intervals shown in Table 4), respectively, which were significantly greater than the GFR (110±4 ml/min per body, mean±S.E., n=4). The differences between $Cl_{r,u}$ and GFR were 169±30 and 185±32 ml/min

Table 4
Urinary excretion rates of *R*-TIPC and *S*-TIPC following intravenous injection of 2 g of TIPC to a healthy volunteer with and without probenecid coadministration

Study group	Time period (h)	Urinary excretion rate (mg/min)	
		R-TIPC	*S*-TIPC
Without probenecid	0.5–1	5.99	5.14
	1–2	3.36	2.84
	2–3	2.35	2.02
	3–4	1.33	1.17
With probenecid	0.5–1	3.96	3.34
	1–2	2.44	2.20
	2–3	1.55	1.42
	3–4	1.38	1.29

per body (mean±S.E. of four time intervals shown in Table 4) for *R*-TIPC and *S*-TIPC, respectively, which indicated that the difference in renal secretion between the epimers were not statistically significant. The results suggested that both TIPC epimers are secreted in the urine and that renal secretion of TIPC may not be stereoselective.

When probenecid was coadministered, Cl_r values were significantly reduced compared with that in the control study; Cl_r for *R*-TIPC and *S*-TIPC were 46.1±3.4 and 41.5±3.0 ml/min per body (mean±S.E. of four time intervals shown in Table 4), respectively, in the probenecid study. The results indicate that both epimers are normally secreted via an organic anion transporter in the renal tubles. Although Cl_r for *R*-TIPC was greater than that for *S*-TIPC, $Cl_{r,u}$ values of *R*-TIPC and *S*-TIPC were almost identical and were approximately equal to the GFR; $Cl_{r,u}$ values were 124±9 and 128±9 ml/min per body (mean±S.E. of four time intervals shown in Table 4) for *R*-TIPC and *S*-TIPC, respectively, and the GFR was 120±9 ml/min per body (mean±S.E., $n=4$) in the probenecid study. The results suggested that renal tubular secretion is almost completely inhibited by probenecid and that both epimers are excreted only by glomerular filtration. Therefore, the differences in Cl_r in the probenecid study were simply due to the differences in plasma protein binding.

In the present study, a stereoselective HPLC method was established to determine the concentrations of TIPC epimers in human plasma and urine. Using the present HPLC method, it was revealed that binding of TIPC epimers in human plasma was stereoselective with the binding of the *S*-epimer being favorable. Stereoselective degradation in plasma, which was significant for sulbenicillin, was not observed for TIPC (data not shown). On the other hand, renal tubular secretion of TIPC appeared to be less stereoselective than that of CBPC. The present study should lead to a better understanding of pharmacokinetics of epimeric β-lactam antibiotics.

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