ELSEVIER

Journal of Chromatography B, 652 (1994) 43–49

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

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

Mariko Ishida[a], Yasuyuki Tsuda[a], Youichi Onuki[a,☆] Tomoo Itoh[a], Hideyo Shimada[b], Hideo Yamada[a,*]

[a]*Department of Pharmacokinetics and Biopharmaceutics, School of Pharmaceutical Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108, Japan*

[b]*Department of Clinical Pharmacology, School of Pharmaceutical Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108, Japan*

(First received June 15th, 1993; revised manuscript received October 26th, 1993)

Abstract

A high-performance liquid chromatographic method was developed for determining the concentrations of carbenicillin (CBPC) epimers in plasma and urine. Samples were prepared for HPLC analysis by solid-phase extraction and the concentrations of CBPC epimers were determined using reversed-phase HPLC with a mixture of 0.05 *M* ammonium acetate and methanol as a mobile phase. Baseline separation of the two epimers was observed for both plasma and urine samples with a detection limit of *ca.* 10 μg/ml. No peaks interfering with either of the CBPC epimers were observed on the HPLC chromatograms for blank plasma and urine. The presented method was used to determine the protein binding of CBPC epimers *in vitro* in human and rabbit plasma. The stereoselectivity of the binding of CBPC appeared to be reversed in human and rabbit plasma.

1. Introduction

Carbenicillin (CBPC) 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, CBPC has been used as a mixture of two epimers (*R*- and *S*-CBPC). The chiral center is the (carboxyphenylacetyl)amino group attached at the C-6 position of penicillanic acid (Fig. 1). Although the dispo-

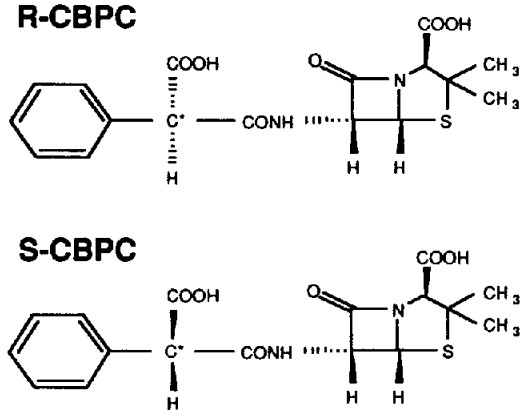

Fig. 1. Chemical structures of carbenicillin (CBPC) epimers. *: Chiral carbon in the side chain.

* Corresponding author.

☆ Present address: Central Research Labs., SS Pharmaceuticals Co., Nanpeidai 1143, Narita-shi, Chiba-ken 286, Japan.

0378-4347/94/$07.00
SSDI 0378-4347(93)E0378-4

sition of CBPC has been extensively studied both in human and animals [1–4], the pharmacokinetics of each epimer is unknown, since almost all previous studies employed a microbiologic assay due to lack of a reliable and convenient stereospecific analytical method. The HPLC method previously reported was unable to determine the concentrations of each epimer because of unsatisfactory resolution [5]. Since antimicrobial activity has been reported to be different between epimers for other chiral β-lactam antibiotics [6,7], it is important to understand the pharmacokinetic behavior of each epimer.

In the present study, a stereospecific HPLC method was developed for analysis of CBPC epimers in biological fluids. The method was applied to determine the plasma protein binding of each epimer *in vitro*.

2. Experimental

2.1. Reagents

Carbenicillin disodium salt was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and used as received. The ratio of the *R*- to *S*-epimer (*R*/*S* ratio) was approximately 1.1. 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 CBPC epimers

CBPC epimers were resolved and isolated using a glass column (100 × 3 cm I.D.) packed with porous polystyrene (Diaion HP-20, 250–800 μm in particle size, Mitsubishi Chemical Industries, Tokyo, Japan)[8]. Approximately 3.5 ml of *R*,*S*-CBPC aqueous solution (*ca.* 200 mg/ml, $R/S = 1.1$) was loaded and eluted with distilled water at a flow-rate of *ca.* 2.5 ml/min. Eluent fractions were 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 [9]. Each epimer thus obtained was injected onto the HPLC in order to determine the specific retention time.

2.3. Plasma and urine collection

Blood and urine were obtained after overnight fast from four healthy male volunteers (20–23 years of age). Blood was immediately centrifuged at 1000 *g* for 10 min and plasma was pooled and stored at −70°C until use. Urine was also stored at −70°C prior to use.

Rats and rabbits used in the present study were male Sprague–Dawley (270–300 g) and male Japan White (2.5–3.0 kg) obtained from Japan SLC (Shizuoka, Japan) and Gokita Breeding (Tokyo, Japan), respectively. Rat plasma was collected through a cannula inserted into a jugular artery and rabbit plasma was collected from an ear vein with heparinized syringes. Urine was obtained from the urinary bladder under anesthesia.

2.4. Sample preparation for HPLC

Solid-phase extraction columns (SAX-Bond Elut, Analytichem International, CA, USA) were pre-conditioned with 2 ml of methanol followed by 2 ml of distilled water. Human plasma samples were filtered through a Cosmonice filter (0.45 μm, type W, Nihon Millipore Kogyo, Japan) and aliquots (0.5 ml) were mixed with 5 ml of 0.05 *M* ammonium acetate ($AcONH_4$). The mixture was loaded on preconditioned SAX columns and drawn through under vacuum. The columns were then flushed with 3 ml of 0.5 *M* acetic acid–acetonitrile (1:1, v/v) followed by 2 ml of 0.1 *M* $AcONH_4$–methanol (1:1, v/v); both fractions were discarded. This procedure was necessary to eliminate interfering peaks. Samples were then eluted with 0.5 ml of 10% LiCl–methanol (3:2, v/v) and 20-μl portions of the final eluent were injected onto the HPLC system.

For the analysis of CBPC epimers in rat and

rabbit plasma, the volume was reduced to 0.1 and 0.3 ml, respectively. The volume of 0.05 *M* $AcONH_4$ was also reduced to 1.0 ml for rat plasma and to 3.0 ml for rabbit plasma. The remaining procedures were the same as for human plasma.

Urine was diluted 40-fold with distilled water, and the diluted sample was filtered through a Cosmonice filter. Urine was diluted before analysis because CBPC concentrations were found to be much greater than those in plasma following CBPC injection to healthy volunteers in a preliminary study. A portion of the filtered sample (0.5 ml) was mixed with 5 ml of 0.05 *M* $AcONH_4$ and the mixture was prepared for HPLC analysis with the same procedures as described for plasma, except that 1.0 ml of 10% LiCl–CH_3OH (3:2, v/v) was used for rat and rabbit urine samples to elute CBPC from the SAX columns.

2.5. HPLC conditions

The HPLC system consisted of a dual piston pump (Model LC-10AD), a UV detector (Model SPD-10A) and an integrator (Model C-R4A), all from Shimadzu Co. (Kyoto, Japan). The analytical column used was Cosmosil $5C_{18}$-AR (250 × 4.6 mm I.D., 5 μm particle size, Nacalai Tesque Co., Kyoto, Japan). Mobile phase compositions were 0.05 *M* $AcONH_4$–methanol 9:1, 6:1 and 7:1 (v/v) for human, rabbit and rat samples, respectively. The flow-rate was 1.2 ml/min, and each epimer was detected at 254 nm.

2.6. Calibration curves

A standard CBPC 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 CBPC concentrations ($R + S$) of 10, 25, 50, 100, 200 and 500 μg/ml in plasma and 25, 50, 100, 200, 400, 600, 800 and 1000 μg/ml in urine. Plasma and urine samples thus obtained were prepared for HPLC analysis according to the same procedures as described above.

With a mixture of two epimers, the ratio of the two epimers (*R*/*S* ratio) determined with ^{1}H-NMR was compared with the peak-area ratio of *R*- and *S*-CBPC on a HPLC chromatogram [8,9]. Since the *R*/*S* ratio obtained with ^{1}H-NMR was almost identical to that observed with HPLC, it was concluded that the molar absorptivities of the two CBPC epimers were essentially identical on the present HPLC system.

2.7. Recovery study

Plasma and diluted urine were spiked to contain 25–100 μg/ml of CBPC and were analyzed with HPLC as described above. Recovery was estimated by comparison of the peak areas for samples with those for standard aqueous solutions.

2.8. Inter-day variability study

On separate days, plasma and diluted urine were spiked to contain 25–400 μg/ml of CBPC and were analyzed by HPLC. Coefficients of variation for peak areas were used as estimates for the day-to-day variability.

2.9. Plasma protein binding study

Binding of CBPC in plasma was measured *in vitro* with an ultrafiltration method using Amicon Centrifree with a YMT membrane (Amicon Div., W.R. Grace and Co., Beverly, MA, USA). Plasma (2.5 ml) was mixed with 125 μl of CBPC solutions of various concentrations and an aliquot (0.3 ml) was analyzed with HPLC as previously described in order to determine the total (bound + unbound) concentration of each epimer in plasma. The remainder of the sample (*ca.* 2.2 ml) was centrifuged at 1000 *g* for 7 min at 37°C and a 300-μl aliquot of the filtrate was analyzed with HPLC in the same manner as for plasma samples to determine the unbound concentration of each epimer. A customized Himac 15D centrifuge (Hitachi, Tokyo, Japan) was used to control the temperature during ultrafiltration.

The concentrations of each epimer in plasma ranged from 20 to 200 μg/ml.

3. Results and discussion

HPLC chromatograms for blank human plasma and the human plasma spiked with CBPC are shown in Figs. 2A and B, respectively. As shown in Fig. 2B, the two peaks corresponding to *R*- and *S*-CBPC were baseline separated. When each isolated epimer was injected onto the HPLC system, the retention times for the *R*- and *S*-epimer were approximately 18 and 24 min, respectively. Also, there were no peaks interfering with either of the CBPC epimers on the chromatogram for blank plasma, as shown in Fig. 2A. In Fig. 3 the chromatogram is shown for plasma obtained from a healthy male volunteer following CBPC injection [10]. The chromatogram is very similar to that in Fig. 2B.

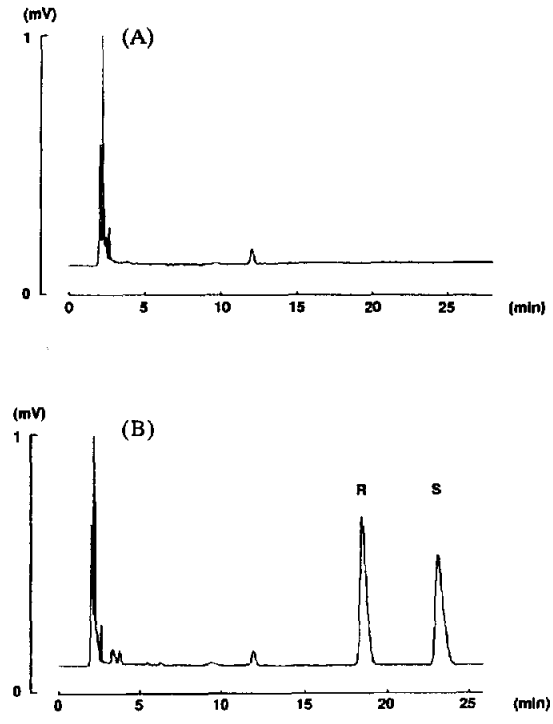

Fig. 2. Chromatograms for (A) blank human plasma and (B) human plasma spiked with CBPC. Five hundred microliters of plasma was spiked with 20 μl of CBPC aqueous solution (5.2 mg/ml). R: *R*-epimer and S: *S*-epimer.

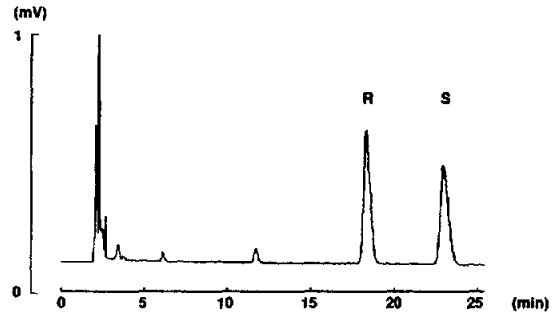

Fig. 3. Chromatogram for the plasma sample obtained from a healthy male volunteer at 0.5 h after intravenous injection of 2 g of CBPC. The concntrations in plasma were 88 and 83 μg/ml for *R*- and *S*-CBPC, respectively.

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 (not shown). Moreover, when each epimer in plasma or urine was prepared for HPLC analysis with the present method, no peaks corresponding to the other epimer were observed on a HPLC chromatogram. Therefore, it was concluded that no epimerization occurred during the present sample preparation procedures.

For rabbit plasma spiked with CBPC, each epimer was again baseline separated and no interfering peaks were observed for rabbit blank plasma. Similar results were obtained for rabbit urine, rat plasma and rat urine samples (not shown).

The calibration curves were linear for human plasma as well as for rat and rabbit plasma over the concentration range 10–250 μg/ml, the correlation coefficients being greater than 0.999 for both *R*- and *S*-CBPC. Calibration curves for urine samples were also linear for concentrations ranging between 25 and 500 μg/ml with correlation coefficients similar to those observed for plasma analysis.

The recoveries for both epimers in human plasma and urine are summarized in Table 1. Although the recovery of the *S*-epimer appeared to be slightly less than that of the *R*-epimer, almost 90% of each epimer was recovered, and

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

Epimer	Concentration (μg/ml)		Recovery (Mean ± S.E.)(%)	n
	Theory	Measured		
Plasma				
R	13.0	11.8	90.5 ± 5.8	3
S	11.9	10.0	84.4 ± 0.5	3
R	26.2	24.9	95.2 ± 3.5	3
S	23.8	21.5	90.4 ± 1.5	3
R	52.4	47.0	89.7 ± 1.0	20
S	47.6	42.4	89.0 ± 0.9	20
Urine				
R	52.4	48.4	92.3 ± 0.7	20
S	47.6	42.4	91.1 ± 0.7	20

CBPC concentrations ($R + S$) were 25, 50 and 100 μg/ml for plasma and 100 μg/ml for urine.

the recovery was quite consistent as reflected in the small S.E. values. It was confirmed that the recovery could be increased by using 0.5 ml of 20% LiCl–methanol (3:2, v/v) as an eluent to elute CBPC from the solid-phase extraction column, although this eluent composition seemed to deteriorate the HPLC column performance more rapidly. The day-to-day variability was also measured and the results are summarized in Table 2. The variability was acceptable, as reflected by the small C.V. values.

Protein binding was measured by ultrafiltration, and the unbound fraction in plasma 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. The results are shown in Figs. 4 and 5 for human and rabbit plasma, respectively. In human plasma the unbound fraction was greater for the *R*-epimer, whereas in rabbit plasma it was greater for the *S*-epimer. These results indicate that in human plasma binding of the *S*-epimer is favored while in rabbit plasma the stereoselectivity in binding is opposite to that in human plasma.

Table 2
Inter-day variability for the determination of CBPC epimers in human plasma and urine

Concentration (μg/ml)	Epimer	Plasma		Urine	
		C.V. (%)[a]	n[b]	C.V. (%)[a]	n[b]
25	*R*	5.9	4	10.1	10
	S	7.3	4	8.3	10
50	*R*	5.9	6	6.3	4
	S	4.7	6	6.0	4
100	*R*	N.D.[c]	N.D.	6.7	9
	S	N.D.	N.D.	8.4	9
200	*R*	2.9	6	6.0	9
	S	1.8	6	6.3	9
400	*R*	3.5	5	7.8	10
	S	2.4	5	8.0	10

[a] Coefficient of variation.
[b] Peak areas were measured on *n* separate days.
[c] N.D. = not determined.

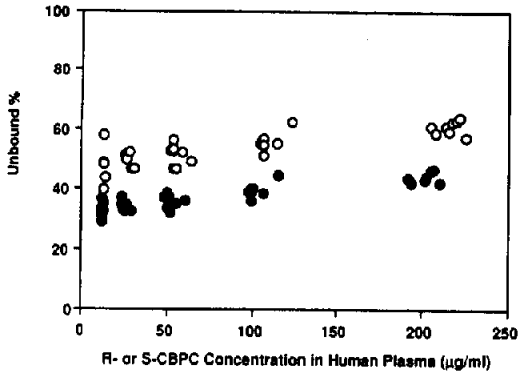

Fig. 4. Unbound fraction of *R*-CBPC (○) and *S*-CBPC (●) in human plasma.

Stereoselective plasma protein binding has been reported for various enantiomers and diastereomers [11,12], including moxalactam [13]. Moxalactam is an oxacephem antibiotic which is clinically used as a mixture of two epimers. Its binding in human plasma *in vitro* has been reported to be favorable for the *S*-epimer. The present study has revealed that the plasma protein binding of CBPC, which is a semi-synthetic epimeric β-lactam antibiotic, is also stereoselective.

Since the *R*-epimer eluted prior to the *S*-epimer on reversed-phase HPLC (Fig. 2B), the *S*-epimer is likely to be more lipophilic. Since CBPC has been reported to bind mainly to albumin in plasma [14], the extent of interaction with albumin may be different for the two CBPC epimers due to differences in their lipophilicity. However, the difference in lipophilicity probably does not solely determine plasma protein binding, as suggested by the opposite stereoselectivity between human and rabbit plasma. The difference in stereoselectivity between various animal species is one of the important issues in the pharmacokinetics and pharmacodynamics of chiral drugs which will have to be clarified in future studies.

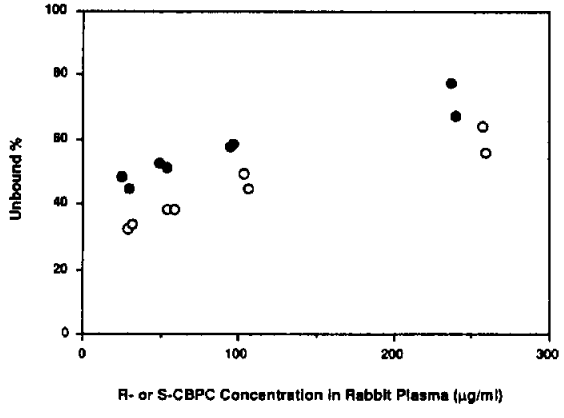

Fig. 5. Unbound fraction of *R*-CBPC (○) and *S*-CBPC (●) in rabbit plasma.

In the present study, a stereoselective HPLC method was established to determine the concentrations of CBPC epimers in human plasma and urine. The method was also applicable for the determination of CBPC epimers in plasma and urine samples obtained from rabbits and rats. Using the present HPLC method, binding of CBPC epimers in human and rabbit plasma was measured. There appeared to be a difference in stereoselectivity in plasma protein binding between the two species. The present HPLC method was successfully employed to clarify the differences in pharmacokinetic behavior between the CBPC epimers [10], especially in the renal excretion process, which is the major elimination pathway for CBPC. Furthermore, the present HPLC method may be used to study the stereoselective pharmacokinetics of other semi-synthetic epimeric β-lactam antibiotics, such as sulbenicillin and ticarcillin, which may in turn lead to more effective development and use of epimeric antibiotics.

4. References

[1] G. Benoni, M.E. Bertazonni and F.G. Cantelli, *Drug Exp. Clin. Res.*, VIII(1) (1991) 71–77.
[2] B.R. Meyers, S.Z. Hirschman, L. Strougo and E. Srulevitch, *Antimicro. Agents and Chemother.*, 17 (1980) 608–611.
[3] S.J. Pancoast and H.C. Neu, *Clin. Pharmacol. Ther.*, 24 (1978) 108–116.
[4] J.F.M. Nouws, G. Zir, C.A.M. Van Ginneken and T.B. Vree, *J. Vet. Pharmacol. Ther.*, 7 (1984) 35–43.
[5] K. Yamaoka, S. Narita, T. Nakagawa and T. Uno, *J. Chromatogr.*, 168 (1979) 187–193.
[6] H. Nomura, K. Kawamura, M. Shinohara, Y. Masuda, Y. Okada and S. Fujii, *J. Takeda Res. Lab.*, 31 (1972) 442–452.

[7] K. Butler, A.R. English, V.A. Ray and A.E. Timreck, *J. Inf. Dis.*, 122 (1970) Suppl. S1.
[8] N. Hashimoto and H. Tanaka, *J. Pharm. Sci.*, 74 (1985) 68–71.
[9] A.E. Bird and B.R. Steele, *J. Chem. Soc.*, Perkin I (1982) 563–569.
[10] T. Itoh, M. Ishida, Y. Onuki, Y. Tsuda, H. Shimada and H. Yamada, *Antimicrob. Agents Chemother.*, 37 (1993) 2327–2332.
[11] F. Jamali, R. Mehvar and F.M. Pasutto, *J. Pharm. Sci.*, 78 (1989) 695–715.
[12] D.E. Drayer, *Clin. Pharmacol. Ther.*, 40 (1986) 125–133.
[13] H. Yamada, T. Ichihashi, K. Hirano and H. Kinoshita, *J. Pharm. Sci.*, 70 (1981) 112–113.
[14] H. Rosenkranz and D. Forster, *Infection*, 7 (1979) 102–108.