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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF A NEW β-LACTAMASE INHIBITOR AND ITS METABOLITE IN COMBINATION THERAPY WITH PIPERACILLIN IN BIOLOGICAL MATERIALS

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SUMMARY

[2*S*-(2α,3β,5α)]-3-Methyl-7-oxo-3-(1*H*-1,2,3-triazol-1-yl-methyl)-4-thia-1-azabicyclo[3.2.0]-heptane-2-carboxylic acid 4,4-dioxide (YTR-830H) is a new β-lactamase inhibitor and the combination therapy of this compound with piperacillin is now under study. For the determination of the β-lactamase inhibitor and piperacillin in biological materials, plasma and visceral tissue homogenates were deproteinized, whereas diluted urine and filtered faeces homogenates were treated with a Sep-Pak C_{18} cartridge. In order to assay the inactive metabolite of β-lactamase inhibitor, each sample was treated with a Sep-Pak C_{18} cartridge. Aliquots of each preparation were chromatographed using ion-pair and reversed-phase chromatographic techniques on a high-performance liquid chromatograph equipped with a UV detector, set at 220 nm. The detection limits of β-lactamase inhibitor and piperacillin were 0.2 μg/ml in plasma, 2.5–5.0 μg/ml in urine and 0.2–0.5 μg/g in visceral tissue and faeces. Those of the metabolite were 1.0 μg/ml in plasma, 2.5–5.0 μg/ml in urine and 1.0 μg/g in visceral tissue and faeces. A precise and sensitive assay for the determination of the β-lactamase inhibitor, its metabolite and piperacillin is described, and their stabilities in several media are reported.

INTRODUCTION

[2*S*-(2α,3β,5α)]-3-Methyl-7-oxo-3-(1*H*-1,2,3-triazol-1-yl-methyl)-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid 4,4-dioxide (I, YTR-830H) is a new β-lactamase inhibitor, developed by Micetich et al. and Taiho Pharmaceutical (Tokushima, Japan) [1]. This antibiotic, which is now undergoing both fundamental and clinical studies as a combination therapy with piperacillin (PIPC), has been shown to extend the in vitro spectrum of β-lactam antibiotics to a number of resistant organisms. The enzymological and bacteriological activities of

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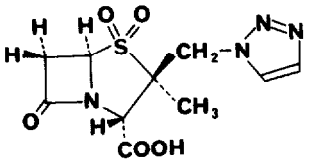
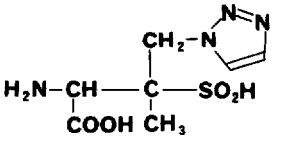
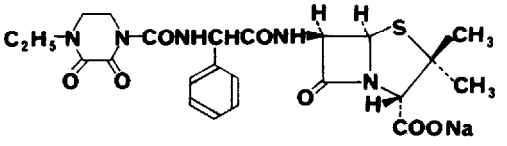


β-Lactamase inhibitor (I)



Metabolite (II)



Piperacillin (PIPC)

Fig. 1. Structures of β-lactamase inhibitor I, its metabolite II and piperacillin (PIPC).

this β-lactamase inhibitor are distinct from those of clavulanic acid and sulbactam. It has also been demonstrated to have relatively little intrinsic biological activity [2–17]. The β-lactamase inhibitor I is metabolized in the body to an inactive metabolite, 2-amino-3-sulphino-3-(1*H*-1,2,3-triazol-1-yl-methyl)butyric acid (II). The structures of I, II and PIPC are shown in Fig. 1.

Several high-performance liquid chromatographic (HPLC) methods are already available for measuring PIPC in biological materials [18–24]. For the pharmacokinetic study of I as a combination therapy with PIPC, it seems important to establish a method for the determination of the concentrations of I, II and PIPC in biological materials. We have examined preparations of I and PIPC separately from those of II because of their physicochemical properties. Subsequently, I and II were determined by ion-pair HPLC on a reversed-phase column and PIPC by reversed-phase HPLC with UV detection.

This paper describes the precise and rapid HPLC methods developed, which are not subject to interferences from biological constituents.

EXPERIMENTAL

Materials and reagents

The β-lactamase inhibitor I, its metabolite II and PIPC were synthesized and purified in our laboratory. Metabolite II was obtained by hydrolysis of I with

sodium hydroxide solution and subsequent purification using cation-exchange resin column (H-type) chromatography: m/z 249 $[M+1]^+$ ion in the fast atom bombardment (FAB) mass spectrum and δ 1.15 [$-CH_3$], 4.09 [$-CH$], 4.77 [$-CH_2$], 7.81 [$=CH$] and 8.08 [$=CH$] in the ^1H NMR spectrum measured in ^2H_2O containing NaO^2H. The internal standards, antipyrine, 3,4,5-trimethoxybenzoic acid, nicotinic acid and methyl *p*-hydroxybenzoate, and other chemicals used were all purchased from Wako (Osaka, Japan). The ion-pair chromatographic reagent PIC-A (low UV), containing a tetra-*n*-butylammonium salt, was obtained from Waters Assoc. (Milford, MA, U.S.A.). Methanol and acetonitrile were of liquid chromatographic reagent grade.

The PIC-A aqueous solution, used as a mobile phase, was prepared at a 5 m*M* concentration by adding the contents of one vial (ca. 20 ml) of commercial PIC reagent to 1.0 l of distilled water. The phosphate buffer (5 m*M*, pH 6.86), also used as a mobile phase, was prepared by dissolving 2.686 g of disodium hydrogenphosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$) and 1.020 g of potassium dihydrogenphosphate (KH_2PO_4) in 3.0 l of distilled water. The pH value was adjusted with KH_2PO_4 if necessary.

A Sep-Pak C_{18} cartridge, obtained from Waters Assoc., was activated with consecutive 5-ml volumes of methanol, distilled water and 50 m*M* KH_2PO_4 before use. A Milex-HV filter unit (0.45 μm) was purchased from Millipore (Bedford, MA, U.S.A.). Plasma and urine were collected from healthy men, beagle dogs and rats and visceral tissues were obtained from rats.

Instruments

An LC-6 high-performance liquid chromatograph equipped with an SPD-6AS variable-wavelength detector, a C-R3A Chromatopac data system and an SIL-6A automatic injector (Shimadzu, Kyoto, Japan) was used.

A Radial-Pak NOVA C_{18} chromatographic column (100 mm × 8 mm I.D., 4 μm particle size) (Waters Assoc.) was used. The mobile phases for the determination of I and II were acetonitrile–PIC-A aqueous solution, in the proportions 10:90 for I in human, rat and dog plasma, human urine and rat visceral tissues, except for the small intestine, 12:88 for I in rat urine and the small intestine and 15:85 for I in dog urine, and 2:98 for II in all samples. The mobile phase for the determination of PIPC in all samples was 5 m*M* phosphate buffer (pH 6.86)–acetonitrile (84:16). The flow-rate of each mobile phase was 2.0 ml/min. The column was maintained at room temperature and the compounds eluted were recorded by the detector at a constant wavelength of 220 nm; the attenuator was set at 0.02 a.u.f.s. for plasma, 0.04–0.32 a.u.f.s. for urine, 0.02–0.08 a.u.f.s. for visceral tissue and 0.02–0.16 a.u.f.s. for faeces.

The ultrafiltration technique using centrifugation with a Centriflow apparatus (Amicon, Lexington, MA, U.S.A.) was used for the determination of human plasma protein-binding levels of I and PIPC.

Preparation of samples

Blood samples were collected in heparinized containers and immediately cen-

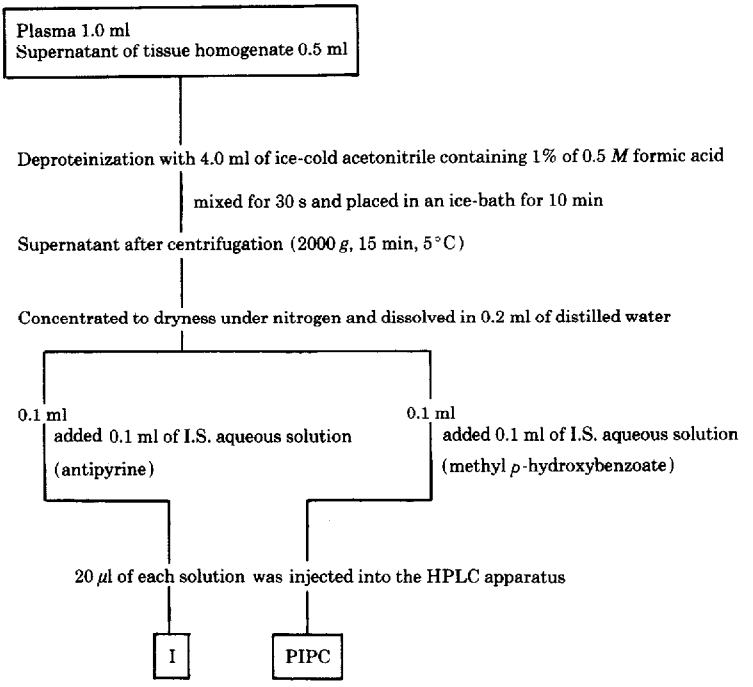


Fig. 2. Preparation procedure for β-lactamase inhibitor I and piperacillin (PIPC) from plasma and tissue homogenate.

trifuged for 15 min at 2000 *g* for human and dog and for 1–2 min at 2000 *g* for rat in a refrigerated centrifuge in order to separate the plasma. Visceral tissues were collected and rapidly exsanguinated or perfused with cold physiological saline to remove blood. The plasma, urine and visceral tissues samples were frozen at −80 °C until analysis, except for the rat plasma, which was prepared immediately for assay.

Before preparation, plasma samples were centrifuged and samples of 0.1–1.0 g of visceral tissue were homogenized in an ice-bath with 2.0 ml of physiological saline and then centrifuged at 2000 *g* and 5 °C for 15 min to obtain a clear supernatant. For the determination of I and PIPC in plasma and visceral tissue, each plasma sample (1.0 ml) or the supernatant of the tissue homogenate (0.5 ml) sample were deproteinized with ice-cold acetonitrile solution containing 1% of 0.5 *M* formic acid. To each final solution, the respective internal standards (I.S., 2 µg of antipyrine for the determination of I in plasma, 5 µg of antipyrine for I in visceral tissue or 5 µg of methyl *p*-hydroxybenzoate for PIPC in both plasma and tissue) were added. Subsequently, 20 µl of each solution were analysed by ion-pair HPLC for I and by reversed-phase HPLC for PIPC. For the assay of II, on the other hand, the supernatant of the organ homogenates (0.5 ml) was cleaned up by extraction followed by deproteinization, then the residue obtained after evaporation to dryness was dissolved in 0.1 *M* KH_2PO_4; the plasma sample (0.5 ml) was diluted with 0.1 *M* KH_2PO_4. Each preparation was treated with a Sep-Pak C_{18} cartridge and the internal standard (nicotinic acid, 50 µg for plasma and

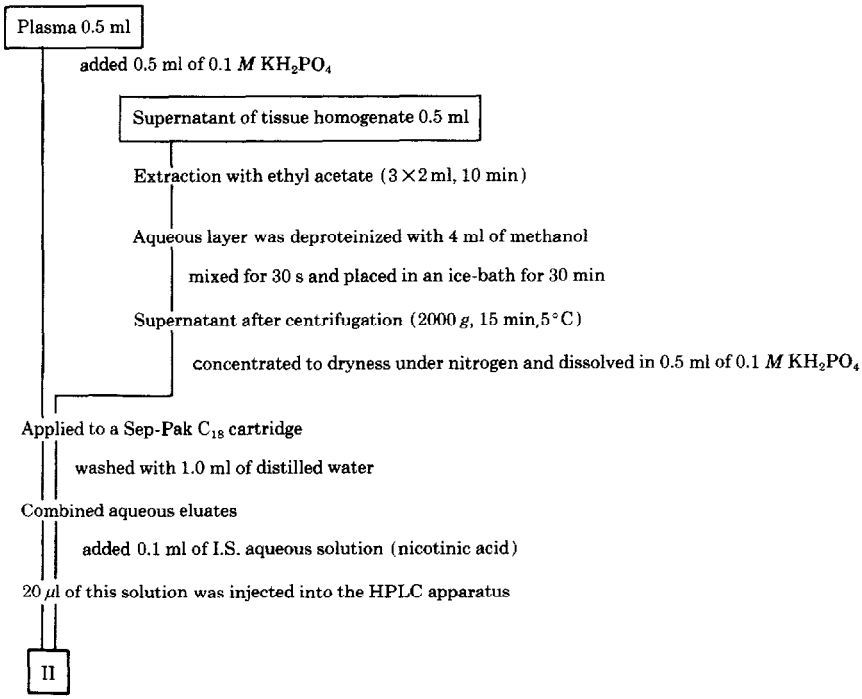


Fig. 3. Preparation procedure for metabolite II from plasma and tissue homogenate.

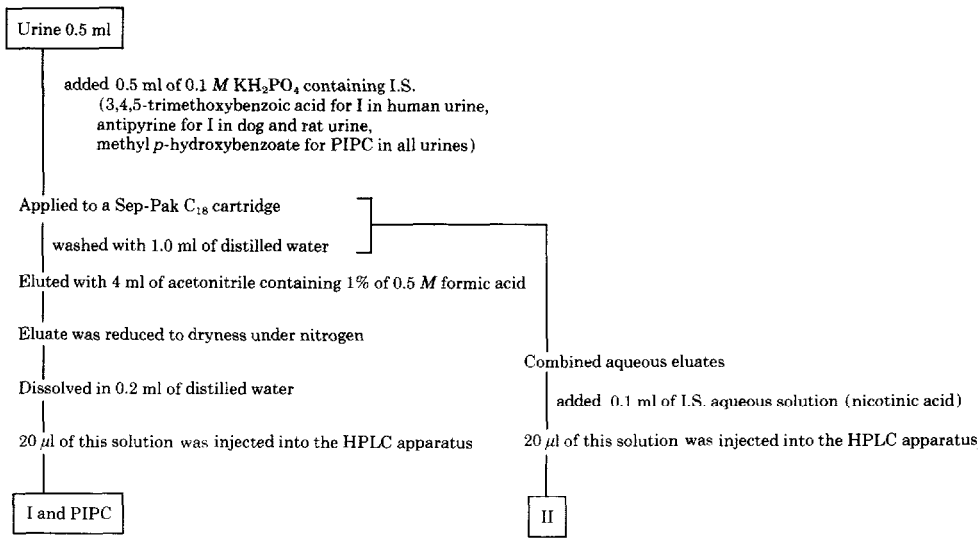


Fig. 4. Preparation procedure for β-lactamase inhibitor I, its metabolite II and piperacillin (PIPC) from urine.

visceral tissue) was added, then 20 μl of each preparation were subjected to analysis by ion-pair HPLC. The detailed procedures are given in Figs. 2 and 3.

Each centrifuged urine sample (1.0 ml) was diluted with 0.1 *M* KH_2PO_4 containing the internal standards (100 μg of 3,4,5-trimethoxybenzoic acid for I in human urine, 100 μg of antipyrine for I in dog and rat urine and 20 μg of methyl *p*-hydroxybenzoate for PIPC in all urine samples. The diluted urine sample was prepared with an activated Sep-Pak C_{18} cartridge, as shown in Fig. 4. Then 20 μl of each final solution were injected into the HPLC apparatus.

Each whole faeces sample was homogenized and 1.0 g of the homogenate was shaken vigorously with 4 ml of ice-cold 0.1 *M* phosphate buffer (pH 7.0) for 5 min. After refrigerated centrifugation at 2000 *g* for 10 min, the supernatant (1.5 ml) was filtered with a Milex-HV filter unit (0.45 μm). This filtrate (0.5 ml) was treated by the same procedure as for urine samples, then 20 μl of each final solution were injected into the HPLC apparatus.

Calibration graphs

A series of standard solutions of I, II and PIPC, containing 100, 50, 10, 5, 1.0, 0.5 and 0.2 μg/ml for plasma and visceral tissue homogenates samples and 1000, 500, 100, 50, 10, 5 and 2.5 μg/ml for urine samples in 0.1 *M* phosphate buffer (pH 7.0) containing the indicated proportions of the respective internal standards, were prepared. A 20-μl sample of each standard solution was analysed by the proposed HPLC method. Calibration graphs were obtained by plotting the ratio of the peak areas for each compound to that of the respective internal standard against concentration. Each calibration graph obtained was linear.

RESULTS AND DISCUSSION

Sample preparation

For the analysis of plasma and visceral tissue samples on a reversed-phase column it is necessary to remove proteins and desirable to remove lipophilic substances. Therefore, an initial attempt at extraction with ethyl acetate after washing with *n*-hexane was examined. However, the results obtained were not satisfactory as a poor recovery of I was obtained (ca. 63–77% from plasma and urine) following the addition of small amounts. II could not be extracted with ethyl acetate because its solubility in water is too high to allow its extraction from biological materials using an organic solvent.

In order to clean-up the plasma samples, a Sep-Pak C_{18} cartridge containing μBondapak C_{18}/Porasil (R/B) (Waters Assoc.) was then used. It was possible to support I and PIPC on this cartridge using 5 m*M* KH_2PO_4 solution and subsequent elution with acetonitrile containing 1% of 0.5 *M* formic acid. The recovery of I and PIPC using this application to the Sep-Pak C_{18} cartridge was 90–97%, with detection limits of 0.5 μg/ml in plasma and 2.5 μg/ml in urine. II was not supported on this cartridge, but it was found that II was recovered from the combined aqueous eluate (the initial 5 m*M* KH_2PO_4 eluate plus the second washing with water eluate), showing a recovery of ca. 80–100% with detection limits of 1.0 μg/ml in plasma and 2.5–5.0 μg/ml in urine.

Deproteinization by addition of methanol, ethanol or acetonitrile and their solutions containing 1% of 0.5 or 2.0 *M* formic accid or 1% of 1.0 or 5.0 *M* hydrochloric acid were next examined. It was found that the use of acetonitrile containing 1% of 0.5 *M* formic acid to prepare protein-free filtrates from the plasma and visceral tissue homogenates samples gave highly reproducible recoveries of I and PIPC. The detection limit of each compound was 0.2 μg/ml in plasma. The recovery of II was very low (ca. 30%) because of its apparent coprecipitation with protein. In addition, the apparent human plasma protein-binding levels of I and PIPC were ca. 80 and 20%, respectively, determined by the ultrafiltration technique.

Deproteinization with acetonitrile containing 1% of 0.5 *M* formic acid and subsequent preparation with a Sep-Pak C_{18} cartridge for plasma samples were also examined. This method gave recoveries of ca. 80% for I and PIPC and ca. 25% for II.

Filtration with a Milex filter unit was used for the preparation of urine and faeces homogenates. No adsorption on this filter and good HPLC separations of I, II and PIPC were observed. However, this method was not suitable for these samples because of the long analysis time and poor sensitivity.

On the basis of these findings, deproteinization with acetonitrile containing 1% of 0.5 *M* formic acid was employed for the preparation procedure for the analysis of I and PIPC in plasma and visceral tissue samples. This form of preparation is simple and fast. Application to the Sep-Pak C_{18} cartridge was used for the preparation procedure to determine II in all samples and I and PIPC in urine samples. Especially for the determination of II in visceral tissues, the centrifuged supernatant of homogenates was washed by extraction with ethyl acetate and the resulting aqueous layer was deproteinized with methanol before application to the Sep-Pak C_{18} cartridge.

This procedure for plasma and urine sample was also applied to other biological fluids.

Chromatography

The concentrations of I and II must be determined in the presence of PIPC. Therefore, a selective HPLC procedure is required for the determination of I, II and PIPC in all types of biological materials from subjects to whom the β-lactamase inhibitor I has been administered in combination with PIPC.

I, II and PIPC were well separated from biological constituents when most of the reversed-phase chromatographic columns were used. After various tests, a Radial-Pak NOVA C_{18} column was chosen for the separation, and isocratic systems consisting of acetonitrile–PIC-A aqueous solution containing 5 m*M* tetra-*n*-butylammonium salt was chosen as the mobile phase for the separation of I and II. A ratio of 10:90 was used for I in human, dog and rat plasma, human urine and rat tissue, except the small intestine. Ratios of 12:88 for I in rat urine and the small intestine, 15:85 for I in dog urine and 2:98 for II in all samples were also used. For the separation of PIPC in all samples, an isocratic system consisting of acetonitrile–5 m*M* phosphate buffer (pH 6.86) (16:84) was used as the mobile phase. Antipyrine (for I in all plasma, dog and rat urine and visceral tis-

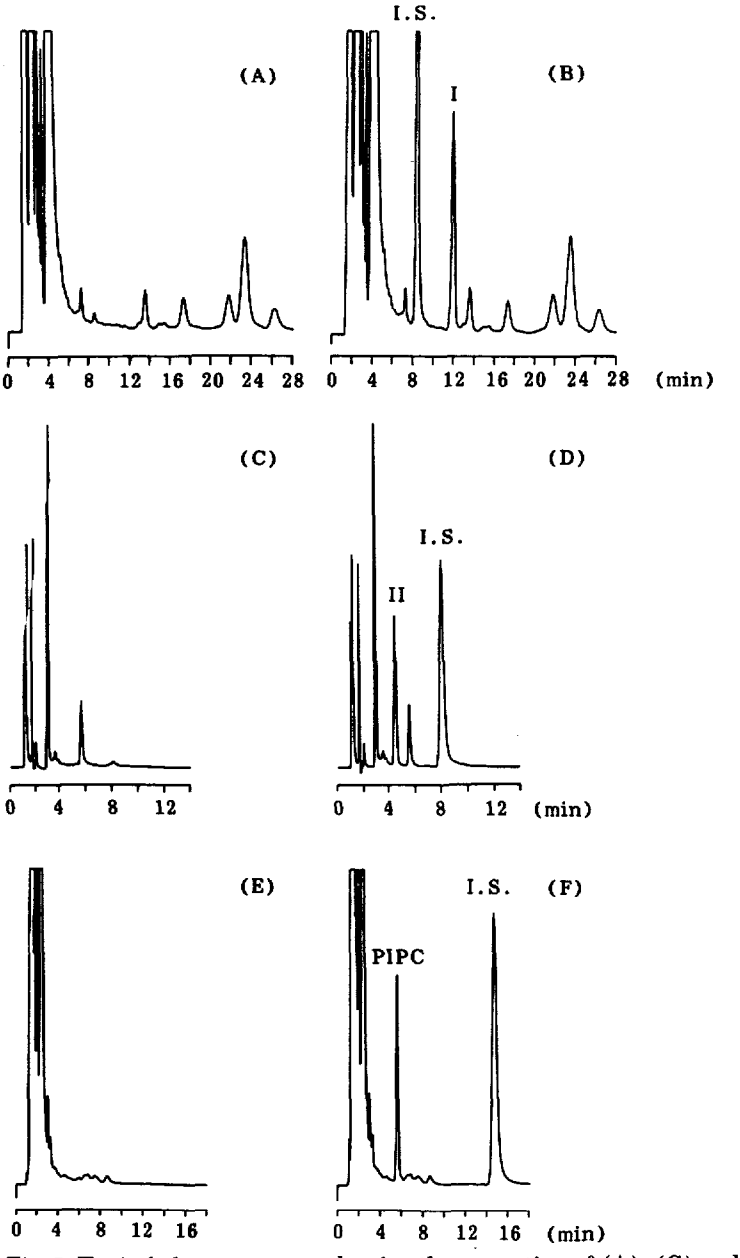


Fig. 5. Typical chromatograms showing the separation of (A), (C) and (E) blank human plasma, (B) I, (D) II and (F) PIPC, and the respective internal standards (I.S.; antipyrine for I, nicotinic acid for II and methyl *p*-hydroxybenzoate for PIPC) prepared from human plasma.

sue), 3,4,5-trimethoxybenzoic acid (for I in human urine), nicotinic acid (for II in all samples) and methyl *p*-hydroxybenzoate (for PIPC in all samples) were used as internal standards, as these compounds were well separated by HPLC and there were no interferences in the quantitation. Other conditions, e.g. a Shim-

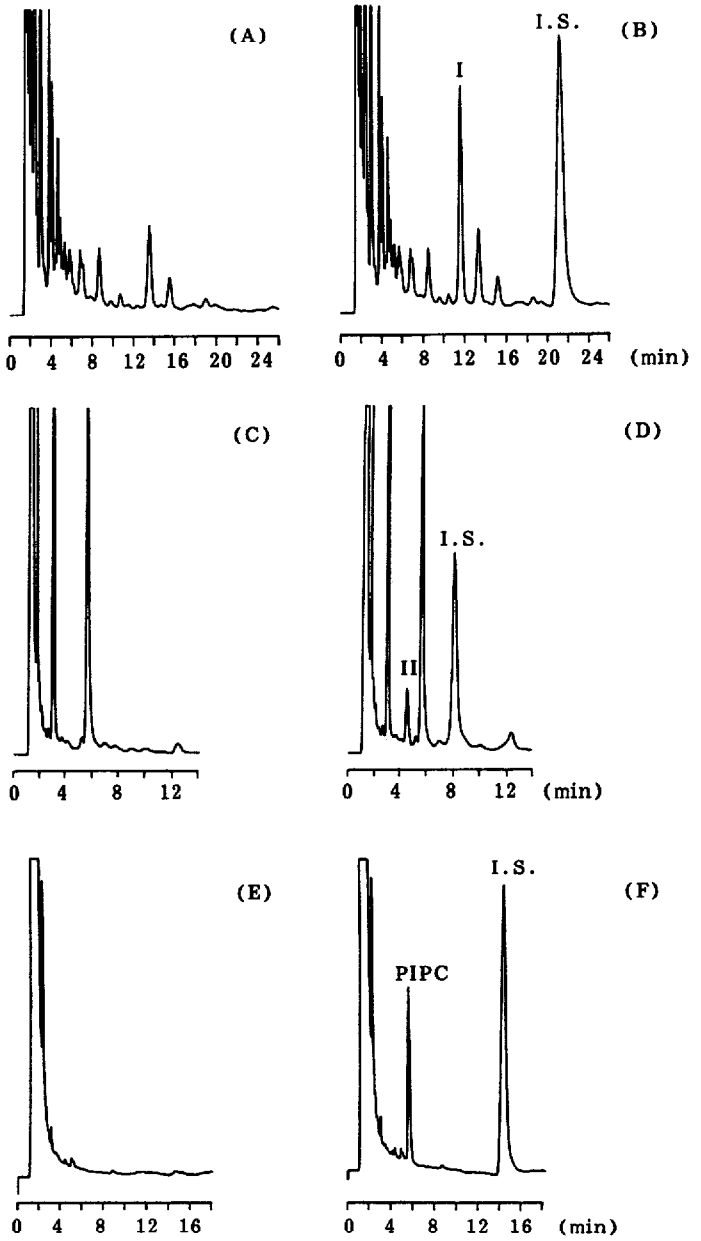


Fig. 6. Typical chromatograms showing the separation of (A), (C) and (E) blank human urine, (B) I, (D) II and (F) PIPC, and the respective internal standards (I.S.; 3,4,5-trimethoxybenzoic acid for I, nicotinic acid for II and methyl *p*-hydroxybenzoate for PIPC) prepared from human urine.

Pak CLC-ODS (Shimadzu) chromatographic column, could be employed with some samples, whereas a μBondapak C_{18} column gave poor separations with most samples.

Typical chromatograms showing the separation of blank and I, II and PIPC

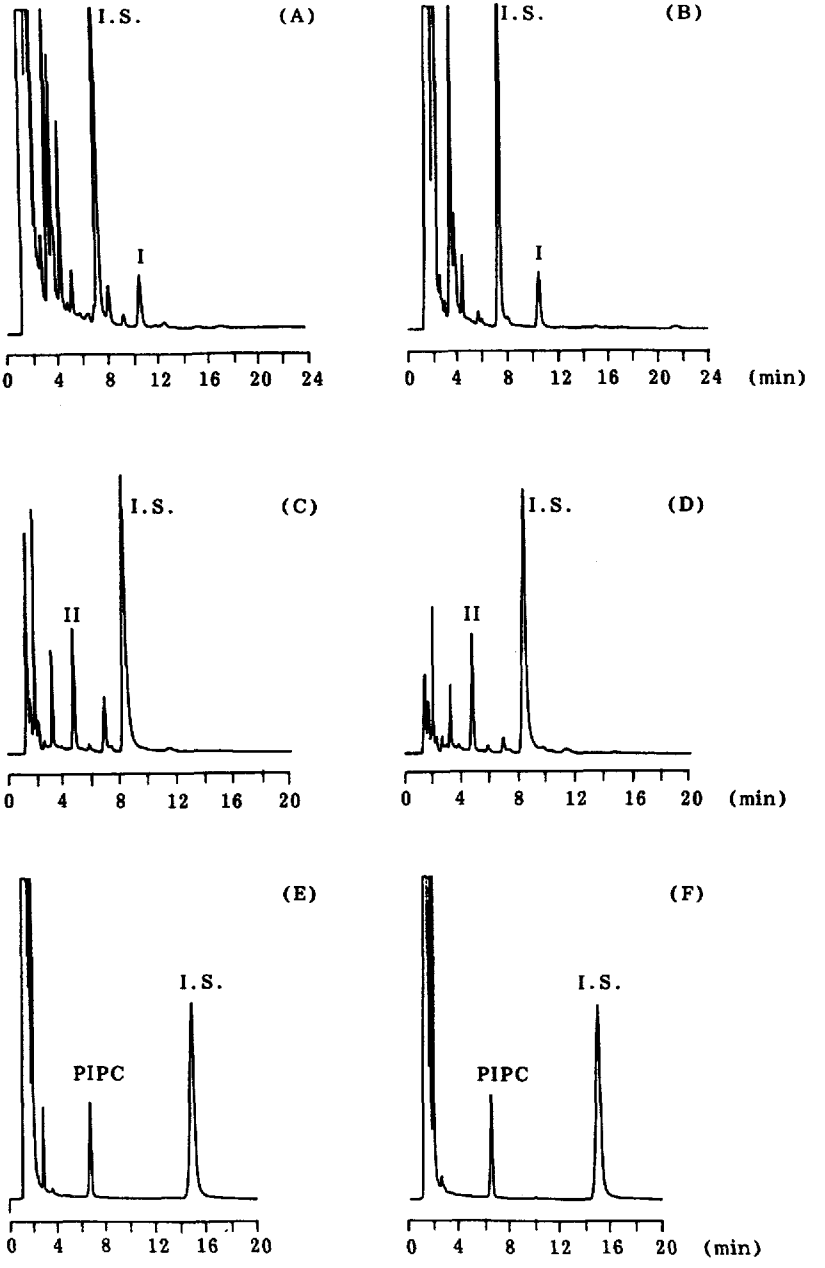


Fig. 7. Typical chromatograms showing the separation of (A) and (B) I, (C) and (D) II and (E) and (F) PIPC, and the respective internal standards (I.S.; antipyrine for I, nicotinic acid for II and methyl *p*-hydroxybenzoate for PIPC) prepared from rat (A), (C) and (E) liver and (B), (D) and (F) spleen.

prepared from human plasma and urine and from rat liver and spleen are shown in Figs. 5–7. The relative retention times of these compounds varied slightly from column to column. PIPC and its metabolites [19] had no appreciable influence

on the measurement of I and II. PIPC was not eluted by the present ion-pair chromatographic method. I and II, on the other hand, were eluted as the front peaks by revered-phase chromatography, which had no influence on the assay of PIPC. The results obtained for the chromatographic separations of human faeces, plasma and urine of dog and rat and rat visceral tissues correlated well with those obtained in Figs. 5–7. The chromatographic interferences from constituents endogenous to all biological materials were negligible. Hence the proposed method appears to be satisfactory for the determination of I, II and PIPC in human and animal biological materials when I is administered alone or in combination with PIPC.

Recovery, variability and sensitivity

Known amounts (0.2–1000 μg/ml) of I, II and PIPC were added to blank plasma and urine of human, dog and rat and to blank homogenates of various rat organs. As indicated in Table I, satisfactory recoveries and variabilities of I, II and PIPC were obtained with good coefficients of variation (C.V.). The results obtained for the recoveries of these compounds from dog and rat plasma and urine and rat organs (given in Table II) compared well with those obtained with human plasma and urine, showing 80–100% for each compound. In addition, the recoveries of I

TABLE I

VARIABILITY OF THE RECOVERY OF β-LACTAMASE INHIBITOR I, ITS METABOLITE II AND PIPERACILLIN (PIPC) FROM HUMAN PLASMA AND URINE

Each value is the mean of six determinations.

Biological fluid	Added (μg/ml)	Recovery (%)		
		I	II	PIPC
Plasma	100.0	93.8 ± 2.6	89.7 ± 1.7	92.9 ± 7.2
	50.0	92.5 ± 2.0	86.5 ± 1.9	100.4 ± 3.4
	10.0	93.5 ± 1.9	88.7 ± 2.6	93.2 ± 4.6
	5.0	88.4 ± 7.1	91.3 ± 6.4	99.1 ± 2.4
	1.0	83.2 ± 3.3	83.2 ± 4.2	89.3 ± 1.2
	0.5	81.5 ± 3.1	-	91.0 ± 2.5
	0.2	90.7 ± 7.7	-	96.5 ± 1.1
C.V. (%)		4.1–4.8	3.8–3.9	2.1–3.8
Detection limit (μg/ml)		0.2	1.0	0.2
Urine	1000.0	86.6 ± 6.1	84.2 ± 1.8	95.6 ± 1.3
	500.0	83.0 ± 5.0	84.6 ± 1.8	91.4 ± 1.6
	100.0	89.9 ± 5.7	90.3 ± 4.1	90.8 ± 2.0
	50.0	93.9 ± 3.9	92.9 ± 2.1	87.0 ± 0.7
	10.0	98.6 ± 5.7	87.9 ± 3.3	86.7 ± 1.5
	5.0	113.0 ± 6.5	82.5 ± 2.0	86.7 ± 1.5
	2.5	98.3 ± 11.3	89.5 ± 8.0	92.8 ± 4.2
C.V. (%)		6.2–7.4	3.4–3.7	1.4–1.5
Detection limit (μg/ml)		2.5–5.0	2.5–5.0	2.5

TABLE II

RECOVERY OF β-LACTAMASE INHIBITOR I, ITS METABOLITE II AND PIPERACILLIN (PIPC) FROM VISCERAL TISSUES OF RAT

Each value is the mean of three determinations. Amounts added: 10 μg/ml for I and PIPC and 20 μg/ml for II.

Tissue	Recovery (%)		
	I	II	PIPC
Kidney	95.5 ± 1.8	104.2 ± 5.2	97.8 ± 2.0
Liver	98.6 ± 3.1	98.9 ± 4.9	94.8 ± 2.7
Lung	98.6 ± 2.1	92.8 ± 7.1	101.7 ± 4.3
Stomach	98.4 ± 4.6	93.7 ± 7.1	104.5 ± 3.8
Spleen	99.4 ± 2.5	86.3 ± 6.2	100.2 ± 0.1
Thymus	96.6 ± 1.3	90.4 ± 6.6	95.0 ± 1.7
Small intestine	85.1 ± 1.1	87.2 ± 2.4	100.2 ± 3.2
Testis	96.1 ± 1.4	86.6 ± 1.9	95.9 ± 1.5
Muscle	98.2 ± 2.7	100.4 ± 5.7	91.0 ± 5.5
Skin	104.2 ± 2.3	91.7 ± 5.4	89.3 ± 5.3
Heart	98.3 ± 1.9	97.0 ± 3.0	91.5 ± 3.6
Brain	99.3 ± 0.4	90.9 ± 5.3	98.5 ± 2.4
Pancreas	98.3 ± 2.1	91.5 ± 0.6	91.3 ± 1.5
Detection limit (μg/g)	0.5	1.0	0.2

and PIPC from rat blood, employing the same deproteinization procedure as used for plasma samples, were ca. 81–94 and 75–80%, respectively.

The detection limits of I, II and PIPC in plasma, urine and visceral tissue using the present HPLC methods are given in Tables I and II. Those in faeces were 5.0 μg/g for I and II and 1.0 μg/g for PIPC. The time required for this assay was 15–25 min. The reproducibility of the method was ±3.1%.

Stability

I and PIPC were found to be stable in the final redissolving solution at room temperature for up to 24 h following the preparation procedure described here. Hence, the method can be used with an automatic injector for overnight runs.

The stability of I and PIPC in acetonitrile solution containing 1% of 0.5 *M* formic acid was also assessed at both room temperature and 0 °C. No loss of I at either temperature was observed, and the loss of PIPC at room temperature was ca. 5% after 4 h; at 5 °C it was ca. 3% after 4 h. These results demonstrate that in the procedure with acetonitrile containing 1% of 0.5 *M* formic acid, deproteinization must be carried out at a temperature of less than 5 °C.

Subsequently, the stability of I was evaluated in 0.1 *M* phosphate buffer (pH 7.0) and human and dog plasma and urine at −80 °C, −20 °C, 5 °C and room temperature. As summarized in Table III, no significant degradation in samples frozen at −80 °C was observed during ten weeks for human plasma and dog plasma and urine. At −20 °C, no degradation was observed after one week for buffer and human and dog plasma, five weeks for human urine and three days for dog urine.

TABLE III

STABILITY OF β-LACTAMASE INHIBITOR I IN 0.1 *M* PHOSPHATE BUFFER (pH 7.0) AND HUMAN AND DOG PLASMA AND URINE

Medium	Incubation temperature	I (%)								
		1 h	2 h	4 h	1 day	3 days	1 week	3 weeks	5 weeks	10 weeks
0.1 *M* Phosphate buffer (pH 7.0)	−20°C	–	–	–	97.0	97.1	97.1	93.1	87.0	95.0
	5°C	97.0	93.8	96.7	96.4	92.9	89.0	68.7	51.8	31.3
	RT	99.2	99.1	94.8	91.4	75.2	52.2	12.8	2.4	0.0
Human plasma	−80°C	–	–	–	96.7	101.1	104.0	96.7	107.3	103.2
	−20°C	–	–	–	92.6	96.4	95.0	81.2	80.6	53.7
	5°C	94.6	98.0	92.3	89.8	86.2	67.7	6.8	7.9	1.5
	RT	95.5	98.3	91.9	65.9	18.9	16.2	0.5	0.0	0.0
Human urine	−20°C	–	–	–	102.2	99.3	97.1	99.3	95.9	92.1
	5°C	100.4	100.3	98.5	102.3	104.6	96.5	93.5	87.2	79.4
	RT	99.7	99.1	98.4	99.2	95.8	90.1	70.2	50.4	28.8
Dog plasma	−80°C	–	–	–	102.2	102.8	98.8	94.8	106.8	101.1
	−20°C	–	–	–	98.3	102.3	94.6	83.6	78.3	74.5
	5°C	100.8	97.4	100.6	99.3	93.3	55.8	10.3	2.2	0.0
	RT	100.5	99.0	99.5	81.1	35.5	2.8	0.0	0.0	0.0
Dog urine	−80°C	–	–	–	98.4	95.3	95.3	96.1	100.1	94.8
	−20°C	–	–	–	95.2	100.8	89.0	77.1	45.5	41.8
	5°C	98.4	100.1	99.9	94.2	92.7	72.5	27.8	12.4	5.6
	RT	101.2	95.5	95.0	80.1	51.7	51.7	20.6	0.0	0.0

At 5°C, no degradation was observed after one day for buffer and dog plasma and urine, 2 h for human plasma and one week for human urine. After standing at room temperature, the periods were 4 h for buffer and dog plasma and urine, 2 h for human plasma and three days for human urine. In addition, the rates of degradation of I at 37°C for 24 h were ca. 70% in human plasma, 4.0% in human urine, 65% in dog plasma and 25% in 0.1 *M* phosphate buffer (pH 7.0).

The stability of II was investigated in 0.1 *M* phosphate buffer (pH 6.90), 0.1 *M* citrate buffer (pH 3.03) and 0.1 *M* borate buffer (pH 9.00) at room temperature and in human plasma and urine at 37°C. No significant degradation of II was observed under these incubation conditions.

In addition, the stability of I in rat blood and plasma at 37°C were examined. It was found that I is very unstable in both blood and plasma; the rates of degradation were ca. 30% for 0.5 h and ca. 60% for 2 h in blood and ca. 20% for 0.5 h and ca. 50% for 2 h in plasma. These results demonstrate that rat plasma must be collected and the sample prepared immediately.

Degradation of I during 24-h incubation of human faeces homogenates varied between 3 and 15% at 5°C or room temperature and between 5 and 45% at 37°C. These results were affected by the variable amounts of β-lactamases contained in faeces from day to day or from one specimen to another, but they also demonstrate that I is fairly stable in faeces.

The results obtained for the stabilities of I and II in visceral tissue homogenates, bile and other biological fluids may be identical with those obtained with plasma or urine. The degradation of I and II in frozen visceral tissues and biolog-

ical fluids at −80°C would be expected to be minimal for considerably longer periods. The stability of PIPC has already been reported [25].

Application

In some instances, the concentration of I and II must be determined in the presence of other antibiotics. Therefore, the HPLC separations of authentic samples of other penicillin and cephalosporin antibiotics, i.e. ampicillin (ABPC), amoxicillin (AMPC), mezlocillin (MZPC), cefoperazone (CPZ), cefotaxime (CTX), cefuroxime (CXM), cefmetazole (CMZ) and cefotetan (CTT), under the conditions reported here were examined. These antibiotics had no appreciable influence on the measurement of I and II.

The present method has high accuracy and sensitivity and should be useful for basic and clinical pharmacological investigations of the β-lactamase inhibitor I.

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