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DETERMINATION OF CEFODIZIME IN BIOLOGICAL MATERIALS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Cefodizime (THR-221) is a new semi-synthetic cephalosporin. A high-performance liquid chromatographic method has been developed for the determination of cefodizime in biological materials. A plasma or serum sample was deproteinized with methanol and the resulting methanol eluate was concentrated to a volume of 0.5 ml. Urine and bile samples were diluted with buffer and each diluted sample was filtered. Faeces samples were homogenized and the supernate obtained after centrifugation was filtered. Visceral tissue samples were homogenized, the centrifuged supernate was deproteinized with methanol, and the methanol eluate was concentrated to a volume of 0.5 ml. Aliquots of each preparation were chromatographed on a reversed-phase column with an ion-pair chromatographic technique on a high-performance liquid chromatograph equipped with an UV detector set at 264 nm. The detection limits for cefodizime were 0.1 μg/ml in plasma or serum, 0.3 μg/ml in bile, and 0.5 μg/ml in urine, 0.5 μg/g in faeces and visceral tissue. This precise and sensitive assay for the determination of cefodizime is described, and its stability in several media is reported.

INTRODUCTION

Cefodizime [THR-221; (6*R*,7*R*)-7-[(*Z*)-2-(2-amino-4-thiazolyl)-2-methoxyiminoacetamido]-3-[[[5-(carboxymethyl)-4-methyl-2-thiazolyl]thio]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid, disodium salt] is a semi-synthetic cephalosporin derivative containing a *syn*-methoxyimino-aminothiazolyl group at the 7-position and a mercaptothiazolyl group at the 3′-position (Fig. 1), developed by Hoechst (Frankfurt, F.R.G.) and Roussel Uclaf (Paris, France).

This antibiotic, which is now under clinical study in Japan, has been demonstrated to have a broad spectrum of activity against Gram-positive and Gram-negative organisms, to be stable against β-lactamases, and to have a longer half-life in blood than other cephem antibiotics [1–12].

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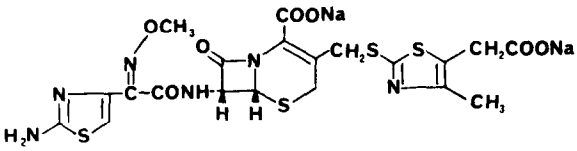
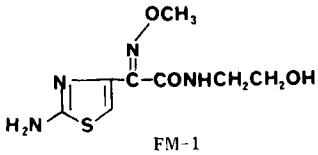
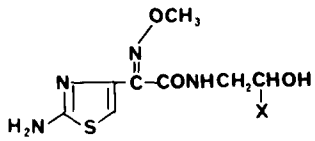


Cefodizime (THR-221)



FM-1



FM-2 (X, unidentified)

Fig. 1. Chemical structures of cefodizime and its in vitro metabolites.

Microbiological methods are widely used for the analysis of other cephalosporins and have been shown to be convenient in this instance. Published studies have also employed microbiological assay methods for determining the concentration of cefodizime in biological fluids [7,9,10].

We have examined the determination of cefodizime in biological materials by ion-pair high-performance liquid chromatography (HPLC) on a reversed-phase column, with UV detection. It was found that no metabolites of cefodizime were present in plasma, urine and other biological materials in in vivo experiments. However, it was also found that two metabolites in human faeces were present after incubation at 37°C for 24 h (Fig. 1). The chemical structure of one of these metabolites has not yet been definitely identified.

The present paper describes this precise and rapid HPLC method, which is not subject to interference from biological constituents, metabolites of cefodizime or other co-administered antibiotics.

EXPERIMENTAL

Materials and reagents

Cefodizime (THR-221) was obtained from Hoechst. The internal standard, 3,5-dinitrobenzoic acid, and other chemicals used, were all purchased from Wako (Osaka, Japan). The ion-pair chromatographic reagent, PIC B-6®, was obtained from Waters Assoc. (Milford, MA, U.S.A.). Methanol and acetonitrile were of

liquid-chromatographic reagent grade. Antibiotics used were synthesized in our laboratory.

The phosphate buffer (0.1 *M*, pH 7.0) used as a diluting solution was prepared by dissolving 35.81 g of disodium hydrogenphosphate 12-hydrate and 13.61 g of potassium dihydrogenphosphate in 1 l of distilled water, and the 1% phosphate buffer (pH 6.0) used for homogenization of faeces was prepared by dissolving 6.0 g of disodium hydrogenphosphate 12-hydrate and 7.0 g of potassium dihydrogenphosphate in 1 l of distilled water. The respective pH values were adjusted with sodium hydroxide or phosphoric acid if necessary.

Instruments

An LC-4 high-performance liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with an SPD-2AS variable-wavelength detector (Shimadzu), a C-R2A Chromatopac data system (Shimadzu) and a WISP 710B automatic injector (Waters Assoc.) was used.

A Radial-Pak NOVA C_{18} chromatographic column (100×8 mm I.D., 4 μm particle size) (Waters Assoc.) was used. The mobile phase was prepared with 20 ml of PIC B-6 reagent (Waters Assoc.) and 1000 ml of acetonitrile–distilled water (20:80), or with 20 ml of acetic acid and 1000 ml of acetonitrile–distilled water (20:80) containing 5 m*M* sodium 1-hexanesulphonate; the flow-rate was 2.0 ml/min. The column was maintained at room temperature, and compounds thus eluted were recorded by the detector at a constant wavelength of 264 nm; the attenuator was set at 0.02 or 0.04 a.u.f.s.

Preparation of samples

Blood samples were collected in heparinized containers and immediately centrifuged for 15 min at 2000 *g* 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 or serum, urine, bile, other biological fluids and visceral tissue samples were frozen at −20°C until analysis.

Each plasma or serum sample (0.5 ml), after recentrifugation if necessary, was transferred to a 10-ml centrifuge tube and diluted with 2 ml of ice-cold methanol solution containing 5 μg of 3,5-dinitrobenzoic acid as an internal standard. The sample was vortexed for 30 s, placed in an ice-bath for 30 min, and then centrifuged for 15 min at 2000 *g* in a refrigerated centrifuge to remove proteins. The clear supernatant was concentrated under nitrogen at water temperature to a volume of 0.5 ml, then 20 μl of this solution were injected into the HPLC apparatus.

Each centrifuged urine sample (1.0 ml) was diluted with 4 ml of 0.1 *M* phosphate buffer (pH 7.0) containing 100 μg of the internal standard, mixed well and filtered with a Milex-HA filter unit (0.45 μm, Millipore, Bedford, MA, U.S.A.); then 20 μl of this filtrate were subjected to HPLC analysis.

To 1 ml of 0.1 *M* phosphate buffer (pH 7.0) containing 15 μg of the internal standard were added 0.5 ml of centrifuged bile or other biological fluid samples. The mixture was stirred, and filtered with a Milex-HA filter unit (0.45 μm); then 20 μl of this preparation were injected into the HPLC apparatus.

Each whole faeces sample was homogenized, and 1.0 g of this homogenate was vigorously shaken with 4 ml of ice-cold ethanol–1% phosphate buffer (pH 6.0) (2:1) for 5 min. After refrigerated centrifugation at 2000 *g* for 10 min, the supernate (1.5 ml) was filtered with a Milex-HV filter unit (0.45 μm). This filtrate (0.5 ml) was mixed with 0.5 ml of 0.1 *M* phosphate buffer (pH 7.0) containing 10 μg/ml of the internal standard, and 20 μl of this final solution were injected into the HPLC apparatus.

A 0.1–1.0-g sample of visceral tissue was homogenized in an ice-bath with 4 ml of 0.1 *M* phosphate buffer (pH 7.0) and then centrifuged at 2000 *g* and 5 °C for 15 min. To 0.5 ml of the resulting supernate, 2 ml of ice-cold methanol solution containing 10 μg of internal standard were added. The solution was vigorously vortexed and then left to stand at 5 °C for 30 min. Subsequently, the supernatant obtained after centrifugation (2000 *g*, 5 °C, 15 min) was concentrated under nitrogen at water temperature to a volume of 0.5 ml, and 20 μl of this concentrate were analysed by the HPLC method.

Calibration curves

A series of cefodizime standard solutions containing 200, 100, 50, 10, 5, 1.0 and 0.5 μg/ml for samples of plasma, bile, other biological fluids and faeces and visceral tissue homogenates, and 400, 200, 100, 50, 10 and 5 μg/ml for urine samples in 1 ml of 0.1 *M* phosphate buffer (pH 7.0) containing indicated proportions of the internal standard, 3,5-dinitrobenzoic acid (10 μg for the former samples and 20 μg for the latter samples) was prepared. A 20-μl sample of each standard solution was analysed by the present HPLC method, and calibration curves were obtained by plotting the ratio of the peak area of cefodizime to that of the internal standard against the concentration of cefodizime. Each calibration curve was linear (former curve: $y=0.075\,783\,3x-0.023\,702\,6$, $r=0.999\,987$; latter curve: $y=0.007\,599\,1x-0.015\,470\,7$, $r=0.999\,937$).

RESULTS AND DISCUSSION

Sample preparation

Several methods of sample preparation were investigated on the basis that the aqueous solubility of cefodizime is too high to allow its extraction from biological materials using organic solvent.

Samples of urine and bile or other biological fluids can be injected onto an HPLC column, but for 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 chloroform–acetone was examined. However, the results were not satisfactory, since cefodizime did not remain quantitatively in the aqueous layer. This procedure gave poor recovery, ca. 70–75%, of cefodizime from plasma. 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 cefodizime on this cartridge using 5 m*M* potassium dihydrogenphosphate and subsequent elution with methanol, whereas this preparation was not adaptable for plasma samples because of

the very strong protein-binding level of cefodizime The recovery of cefodizime from plasma using this application onto the Sep-Pak C_{18} cartridge was ca. 60%. In addition, the apparent human plasma protein-binding level of cefodizime was ca. 85%, as determined by the ultrafiltration technique employing centrifugation with a Centriflow apparatus (Amicon, Lexington, MA, U.S.A.).

Deproteinization by addition of methanol, acetonitrile, trichloroacetic acid (TCA), or perchloric acid (PCA) was further examined. It was found that cefodizime was significantly degraded by PCA and TCA, and that the respective recoveries of cefodizime from plasma were ca. 55% and 70%. On the other hand, deproteinization with acetonitrile gave a good recovery for plasma samples, but no constant recovery in some organ samples, the level varying between 60 and 100%. The use of methanol to prepare protein-free filtrates from the plasma and visceral tissue homogenate samples gave highly reproducible recoveries. Thus, deproteinization with methanol was employed for the preparation procedure of plasma and visceral tissues. This form of preparation is simple and fast.

Filtration with a Milex filter unit, on the other hand, was used for the preparation of urine, faeces homogenates, bile and other biological fluid samples. No adsorption of cefodizime on this filter was observed.

Chromatography

In some instances, the concentration of cefodizime must be determined in the presence of other antibiotics. Microbiological determination appears convenient, but its lack of specificity is a potential drawback under these circumstances. Therefore, a selective HPLC procedure is required for the determination of cefodizime in all types of biological material from subjects to whom this antibiotic has been administered, either alone or in combination with other antibiotics.

Cefodizime was well separated from biological constituents when most reversed-phase chromatographic columns were used. After various tests, a Radial Pak NOVA C_{18} column was chosen for the separation, and a solvent system consisting of 20 ml of PIC B-6 reagent and 1000 ml of acetonitrile–distilled water (20:80) or 20 ml of acetic acid and 1000 ml of acetonitrile–distilled water (20:80) containing 5 m*M* sodium 1-hexanesulphonate was chosen as the mobile phase, with an internal standard of 3,5-dinitrobenzoic acid. This ion-pair procedure was found to be suitable for the separation of cefodizime from biological materials when cefodizime was administered alone or in combination with other antibiotics. Other conditions, e.g. the use of μBondapak C_{18} (Waters Assoc.) as a chromatographic column and acetonitrile–5–20 m*M* potassium dihydrogenphosphate as the mobile phase, resulted in poor separation in some samples.

The HPLC separations of authentic samples of cefodizime and other antibiotics, i.e. piperacilline (PIPC), cefmetazole (CMZ), cefotetan (CTT), cefoperazone (CPZ) and cefotaxime (CTX) under the present HPLC conditions are shown in Fig. 2. The retention times of cefodizime, PIPC, CMZ, CTT, CPZ and CTX were 7.7, 22.2, 4.0, 2.6, 4.4, and 3.4 min, respectively, and that of the internal standard was 10.2 min. The relative retention times of these compounds varied slightly from column to column. As indicated by these chromatographic patterns,

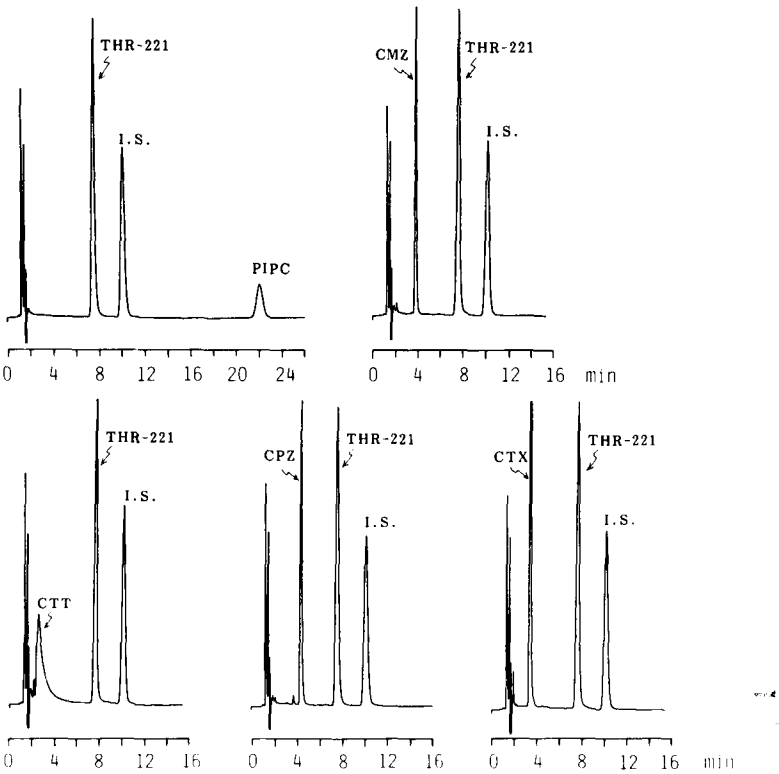


Fig. 2. Chromatograms showing the separation of authentic samples of cefodizime (THR-221), piperacilline (PIPC), cefmetazole (CMZ), cefotetan (CTT), cefoperazone (CPZ), cefotaxime (CTX) and internal standard (I.S., 3,5-dinitrobenzoic acid). Details of the HPLC conditions are described in the text.

other antibiotics had no appreciable influence on the measurement of cefodizime with the present method.

Typical chromatograms showing the separation of control and cefodizime prepared from human plasma and urine, and from several rat visceral tissues following administration of cefodizime (intravenous injection of 1 g to humans and 15 mg/kg to rats) are shown in Figs. 3 and 4. The degree of chromatographic interference from constituents endogenous to all biological materials was negligible. Thus, the present method appears to be satisfactory for the determination of cefodizime in human and animal biological materials.

Recovery, sensitivity and accuracy

Known amounts (0.5–200 μg/ml) of cefodizime were added to control plasma, urine and other biological fluids from humans, and control homogenates of various organs of rats, respectively, and the recovery of cefodizime was determined. As listed in Tables I and II, the recoveries of cefodizime prepared from human plasma and rat organs using the methanol deproteinization technique were essentially quantitative, with coefficients of variation (C.V.) averaging ca. 1–2%. The

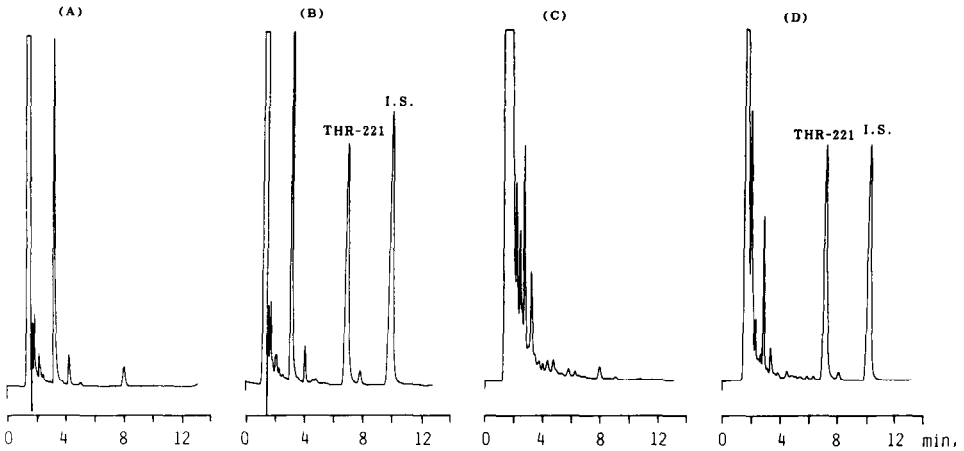


Fig. 3. Chromatograms showing the separation of control human (A) plasma and (C) urine, and cefodizime (THR-221) and internal standard (I.S., 3,5-dinitrobenzoic acid) prepared from human (B) plasma and (D) urine. Details of the HPLC conditions are described in the text.

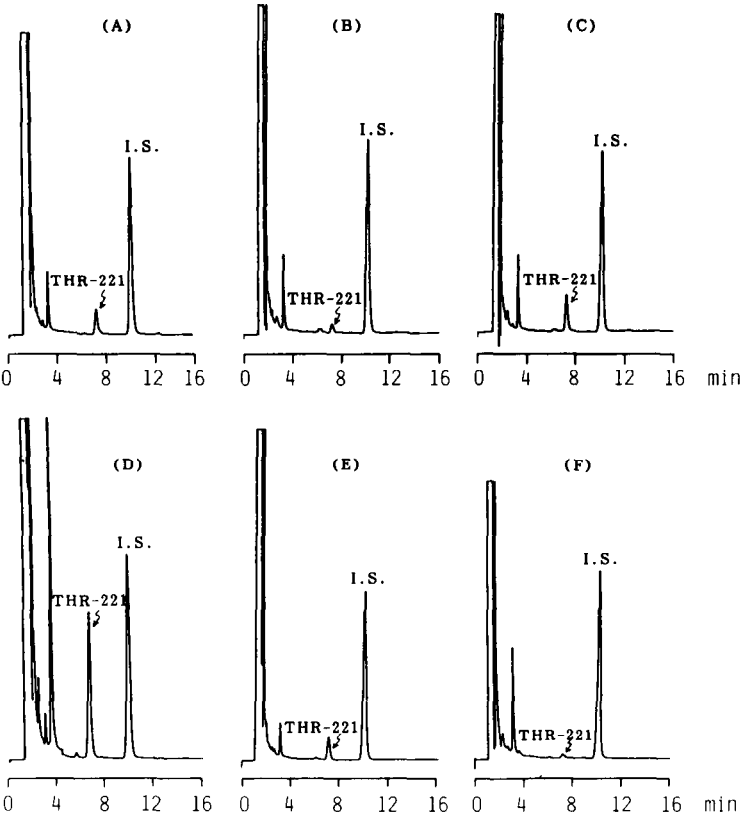


Fig. 4. Chromatograms showing the separation of cefodizime (THR-221) and internal standard (I.S., 3,5-dinitrobenzoic acid) prepared from visceral tissues of rats: (A) liver, (B) spleen, (C) lung, (D) kidney, (E) heart and (F) brain. Details of the HPLC conditions are described in the text.

TABLE I

RECOVERY OF CEFODIZIME FROM HUMAN PLASMA

Cefodizime added (μg/ml)	Recovery (%)			
	1	2	3	Mean ± S.D.
0.5	94.2	92.8	92.0	93.0 ± 1.1
1.0	93.1	92.0	95.1	93.4 ± 1.6
5.0	93.2	94.4	94.0	93.9 ± 0.5
10.0	93.3	95.4	93.5	94.1 ± 0.9
50.0	98.5	97.5	98.4	98.1 ± 0.5
100.0	99.7	97.9	100.7	99.4 ± 1.2
200.0	101.6	96.4	99.5	99.2 ± 2.1

recoveries from urine and other biological fluids by use of the filtration technique were also quantitative. It is not ethically possible to obtain several kinds of human visceral tissue; however, results with visceral tissues from humans gave similar results to those obtained with animals, indicating the applicability of the present method to human tissues.

The detection limits of cefodizime using this HPLC method were 0.1 μg/ml of plasma, 0.3 μg/ml of bile and other biological fluids, 0.5 μg/ml of urine, and 0.5 μg/g of faeces or visceral tissue, respectively. The time required for this assay was 15 min and the reproducibility of the method was ±3.1%.

Stability

Cefodizime was found to be stable in solution at room temperature for up to 15 h following the preparation procedure described in this report; hence, the present method can be used with an automatic injector for overnight runs.

The stability of cefodizime in methanol solution was also assessed at both room temperature and 5 °C. The loss of cefodizime at room temperature was ca 50% after 12 h, and at 5 °C it was ca. 6% after 24 h. These results demonstrate that the procedure of methanol deproteinization must be carried out at a temperature of less than 5 °C.

TABLE II

RECOVERY OF CEFODIZIME FROM VISCERAL TISSUES OF RATS

Tissue	Cefodizime added (μg/ml)	Recovery (%)		
		1	2	Mean ± S.D.
Kidney	10.0	103.6	101.5	102.6 ± 1.5
Small intestine	10.0	99.1	99.7	99.4 ± 0.4
Liver	10.0	96.0	100.8	98.4 ± 3.4
Stomach	10.0	104.2	101.7	103.0 ± 1.8
Spleen	10.0	99.7	100.5	100.1 ± 0.6
Thymus	10.0	101.7	99.6	100.7 ± 1.5

TABLE III

STABILITIES OF CEFODIZIME IN 0.1 *M* PHOSPHATE BUFFER (pH 7.0), HUMAN PLASMA AND URINE

Medium	Incubation condition	Cefodizime contained (%)									
		1 h	2 h	4 h	1 day	3 days	5 days	1 week	3 weeks	5 weeks	10 weeks
0.1 *M* Phosphate buffer (pH 7.0)	−20°C	−*	−	−	101.6	102.0	101.2	102.8	103.3	98.7	96.4
	5°C	98.7	100.4	102.7	99.3	100.4	98.9	98.8	93.8	89.3	78.6
	Room temp.	101.6	103.1	100.9	99.7	87.3	81.3	72.3	27.8	15.7	4.6
Plasma	−20°C	−	−	−	97.9	113.5	103.0	102.4	101.4	100.1	50.4
	5°C	101.7	98.4	100.9	99.6	106.4	101.0	104.0	102.4	98.2	48.6
	Room temp.	99.7	101.4	101.3	98.4	102.8	94.7	90.9	79.1	64.5	16.0
Urine	−20°C	−	−	−	95.8	94.7	96.2	94.9	96.6	85.0	46.7
	5°C	98.2	98.1	96.2	96.7	93.5	97.9	97.3	98.2	86.3	46.0
	Room temp	97.2	96.5	97.2	95.2	92.2	91.7	88.8	46.9	1.4	0.0

*Not measured.

Subsequently, the stability of cefodizime was evaluated in 0.1 *M* phosphate buffer (pH 7.0), human plasma and urine at −20°C, 5°C, and room temperature. As summarized in Table III, no significant drug degradation in frozen samples was observed during 3 weeks for buffer, 10 weeks for plasma and 5 weeks for urine; at 5°C, no degradation was observed in 3 weeks for buffer, 1 week for plasma and 5 weeks for urine, and after standing at room temperature, the periods were 1 day for buffer and plasma and 3 days for urine samples, respectively. In addition, the rates of degradation of cefodizime at 37°C for 24 h were ca. 15% in human plasma and 2.5% in human urine, whereas no significant degradation of cefodizime in 0.1 *M* phosphate buffer (pH 7.0) was observed under these incubation conditions.

Degradation of cefodizime during 24-h incubation in human faeces homogenates varied between 0 and 20% at 5°C or at room temperature, and between 0 and 100% 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. In this incubation study of cefodizime in faeces homogenates at 37°C, it was found that cefodizime changes into small amounts of two metabolites, FM-1 and FM-2. However, as these metabolites were found to have too fast an elution time, they had absolutely no influence on the measurement of cefodizime using the present method. A chromatogram showing the separation of these in vitro metabolites under the following HPLC conditions is shown in Fig. 5: column, μBondapak C_{18}; mobile phase, 0.5% triethylamine aqueous solution (pH 2.25, adjusted with phosphoric acid); flow-rate, 1.5 ml/min; detector, UV 254 nm. Cefodizime was not eluted under these HPLC conditions. Each fraction eluting at retention times of 5.0 (FM-2) and 6.6 min (FM-1) from the HPLC column was collected separately and identified. Their chemical structures identified on the basis of spectral data of ^{1}H nuclear magnetic resonance (^{1}H NMR) spectroscopy and fast atom bombardment (FAB) mass spectrometry are presented in Fig. 1. FM-1: *m/z* 245 $[M+1]^+$ ion in the FAB mass spectrum and δ ca. 3.2 [$CH_2\times2$], δ 3.8 ($C=N-OCH_3$], δ 6.8 [=CH in an aminothiazol moiety], δ 7.2 [NH_2 in an aminothiazol moiety] and δ 8.42 [CONH] in ^{1}H NMR spectrum ([$^2H_6$]dimethylsulphoxide); and FM-2: δ 3.3 [CH_2], δ 3.9]$C=N-OCH_3$], δ 5.2

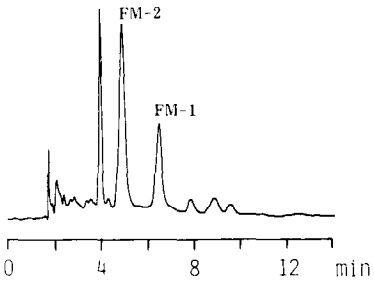


Fig. 5. Chromatograms showing the separation of metabolites of cefodizime (THR-221) observed after incubation of human faeces homogenates at 37°C for 24 h following addition of cefodizime. Details of the HPLC conditions are described in the text.

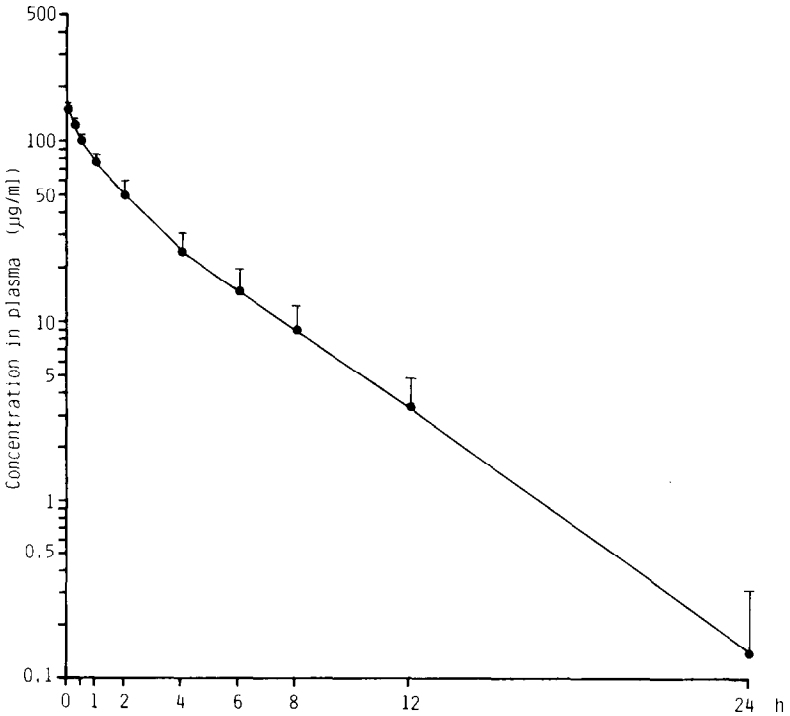


Fig. 6. Plasma level of cefodizime (THR-221) determined using the present HPLC method after intravenous injection of 1 g of cefodizime to four healthy volunteers. Each point is presented as the mean ± S.D.

[-OCH (X)-] and δ 6.9 [=CH in an aminothiazol moiety] in the ^{1}H NMR spectrum (^2H_2O). However, in this study, the concentrations of these metabolites could not be determined, since their authentic compounds have not yet been synthesized.

The results obtained for the stabilities of cefodizime in visceral tissue homogenates, or bile and other biological fluids were identical with those obtained with plasma or urine, respectively. The degradation of cefodizime in frozen visceral tissues and biological fluids would be expected to be minimal for considerably longer periods.

Application

The time course of changes in the concentrations of cefodizime in plasma after intravenous injection of 1 g of cefodizime was measured by the present method. Fig. 6 shows the mean ± S.D. plasma concentrations of cefodizime obtained from four healthy volunteers. The terminal half-life was ca. 2.4 h [13].

Subsequently, the concentrations of cefodizime in plasma and serum after intravenous injection of 15 mg/kg cefodizime to male Wistar rats (180 g body weight), determined by the described method, were compared. No significant difference between the concentrations in plasma and serum was observed.

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