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High-performance Liquid Chromatography Analysis of Mezlocillin, Piperacillin, their Degradation Products, and of Ioxitalamic Acid in Plasma and Urine of Healthy Volunteers

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Summary: *In plasma and urine of 10 healthy volunteers after intravenous administration of 4 g mezlocillin and piperacillin, respectively, the parent compounds as well as degradation products were assayed by high-performance liquid chromatography. Ioxitalamic acid, a renal contrast medium, was administered simultaneously, in order to measure the glomerular filtration rate, and to control the collection of 24-h urine. As metabolite of mezlocillin the corresponding* *penicilloic acid only was found, whereas in the case of piperacillin a further degradation product was observed. Half of the doses given was recovered in the urine as unchanged drugs, and in addition 5–10% as metabolites. No differences were found in the pharmacokinetic behaviour of both antibiotics.*

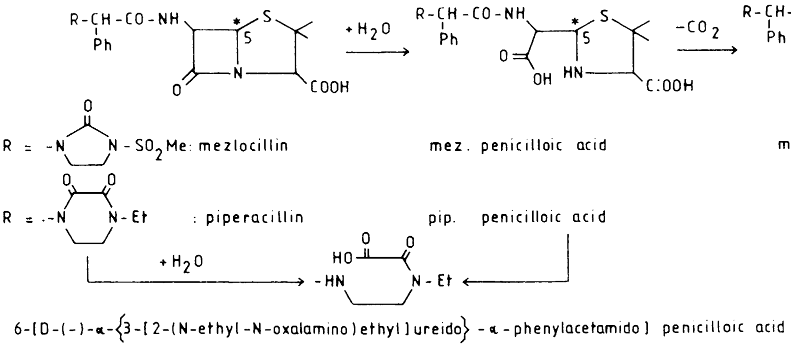
Zusammenfassung: *Hochdruckflüssigkeitschromatographische Bestimmung von Mezlocillin, Piperacillin, ihrer Abbauprodukte und von Ioxitalaminsäure im Plasma und Urin gesunder Probanden*

Nach intravenöser Applikation von je 4 g Mezlocillin bzw. Piperacillin wurden mit Hilfe der Hochdruckflüssigkeitschromatographie die Muttersubstanzen sowie Abbauprodukte dieser Ureidopenicilline im Plasma und Urin von 10 freiwilligen Probanden bestimmt. Um die Nierenfunktion zu überprüfen und die vollständige Urinsammlung über 24 h zu kontrollieren, erhielten die Versuchspersonen simultan Ioxitalaminsäure, ein Kontrastmittel, infundiert. Als einziger Metabolit vom Mezlocillin wurden geringe Mengen der entsprechenden Penicilloinsäure gefunden. Im Falle von Piperacillin wurde ein weiteres Abbauprodukt beobachtet. Etwa die Hälfte der gegebenen Antibiotika-Dosen wurde unverändert im Urin wiedergefunden, zusätzlich noch 5–10% in Form von Abbauprodukten. Bei den pharmakokinetischen Parametern beider Penicilline wurden keine signifikanten Unterschiede festgestellt.

Key words: *Acylureidopenicillins · Diagnostics · Ioxitalamic acid, clinical pharmacokinetics · Mezlocillin, clinical pharmacokinetics · Piperacillin, clinical pharmacokinetics*

1. Introduction

Mezlocillin (6-[D-2-(2-oxo-3-mesylimidazolidine-1-carboxamido)-2-phenylacetamido]penicillanic acid) and piperacillin (6-[D(–)-α-(4-ethyl-2,3-dioxo-l-piperazinyl-carbonyl-amino)-2-phenylacetamido] penicillanic acid) are acylureido penicillins with broad antibacterial activity and with only slight differences between their structures (Scheme 1). Penicillins are in general partially metabolized, mainly to inactive penicilloic acids [1]. This also was shown for mezlocillin, but in vitro degradation of mezlocillin in plasma specimens may have influenced some results, leading to false high concentrations of mezlocillin penicilloic acids [3]. So far, no metabolites of piperacillin have been detected in plasma, though some inactive degradation products have been observed in urine and bile [2].



Scheme 1: Chemical structures of mezlocillin, piperacillin, and degradation products.

The aim of the present study was to establish the pharmacokinetics of mezlocillin and piperacillin after intravenous infusion of 4 g over 30 min, and to quantify their probable main metabolites, the corresponding penicilloic acids. For analysis a liquid chromatographic method was chosen instead of bioassay, because these ring-open penicillin derivatives are not antibacterially active. Care was taken with the sampling and storage of plasma samples because of in vitro instability of mezlocillin and other penicillins in body fluids [3].

2. Material and methods

2.1. Subjects

The study included 10 healthy subjects (5 male, 5 female, aged between 22 and 34 years, median 29 years). Their weights ranged between 50 and 86 kg (median 66 kg), and their heights between 154 and 188 cm (median 174 cm). The health of the volunteers was established from their medical history, physical examination and laboratory screening (differential blood count, platelet count, serum creatinine, SGOT, SGPT, GGT and urine analysis). No subject was hypersensitive to penicillins or contrast media. Pregnancy was excluded by the regular use of contraceptives.

2.2. Administration of antibiotics

After giving informed written consent the subjects received 4 g of mezlocillin and after 6 weeks 4 g of piperacillin. Concurrently, the subjects received 5 ml Telebrix 300®, equivalent to 2.4 g ioxitalamic acid, as an internal standard for comparison of the two antibiotics. The injection of this contrast medium allows to measure the renal function as glomerular filtration rate, and to control the collection of the 24-h urine. The antibiotics and ioxitalamic acid were dissolved together in 50 ml water for injections and infused intravenously within 30 min at a constant rate.

2.3. Blood and urine collections for assay

Venous blood samples (6 to 8 ml) were withdrawn into heparinized syringes from a contralateral arm vein through an indwelling needle before and at specified intervals after infusion. These samples were taken at 15 min after start of the infusion, at the end of infusion and at 10, 20, 30, 45, 60, 90 min, 2, 3, 4, 6 and 8 hours thereafter. Blood samples were centrifuged at 4 °C within 30 min and the plasma was stored at −70 °C until analysis. Urine samples were collected before the start of the experiments and during the periods 0 to 4.5, 4.5 to 8.5, 8.5 to 12.5, and 12.5 to 24.5 h after the start of infusion. The pH (ranging from 5.0 to 6.5) and volumes of all urine samples were measured, and the specimens were stored at −70 °C. The excretion of creatinine in the fractionated urine collections over 24 h was assayed by the autoanalyser modification of the method of Jaffe (4).

2.4. Chromatographic assay

2.4.1. Reagents and chemicals

Mezlocillin with the respective penicilloic acid and penilloic acid, piperacillin with its penicilloic acid and ioxitalamic acid (5-acetamido-N-(2-hydroxy-ethyl)-2,4,6-triiodo isophtalamic acid) were supplied by the respective manufacturers. As diagnostic agent meglumine ioxitalamate (Telebrix 300®; manufacturer: Byk Gulden, Konstanz, FR Germany) was used. Acetonitrile (HPLC grade S) was purchased from Zinsser, Frankfurt/Main (FR Germany), tetrabutylammonium hydrogensulfate from Fluka, Neu Ulm (FR Germany). All other chemicals (analytical grade) were obtained from E. Merck, Darmstadt (FR Germany). Water was purified with a Milli-Q water purification system (Millipore, Eschborn, FR Germany).

Stock solutions of the antibiotics, their metabolites and of ioxitalamic acid were prepared in water to yield final concentrations of 1 mg/ml, and stored in aliquots at −20 °C. The stock solutions were than diluted with drug-free plasma to provide assay standards of 50 µg/ml for mezlocillin and piperacillin and 20 µg/ml for the penicilloic acids of both penicillins. For checking the linearity of the assay dilutions of 100–0.78 µg/ml for mezlocillin and piperacillin in plasma and 20–0.63 µg/ml for mezlocillin penicilloic acid were prepared. Lack of sufficient material did not allow to prepare a standard series of piperacillin penicilloic acid. For urine samples the standard solutions were prepared in 50 mmol/l sodium phosphate buffer (pH 6.5).

2.4.2. Sample treatment

Plasma samples were treated according to a published procedure [5] with minor modifications. In brief, 200 µl plasma were buffered with 200 µl 50 mmol/l sodium phosphate (pH 6.0) and deproteinized with 400 µl acetonitrile. The latter was than extracted into 2 ml dichloromethane, and 10–20 µl of the aqueous phase, containing the penicillins and their metabolites, were injected into the chromatograph. Urine was centrifuged and diluted tenfold with 50 mmol/l sodium phosphate buffer (pH 6.5). All biological samples were stored at −70 °C (up to 7 weeks) and thawed in iced water just prior to analysis.

2.4.3. Chromatography

The chromatographic system consisted of a pump M 6000A, an automatic injector WISP 710B (fitted with a cooling kit, in order to maintain 8 °C for the samples), a RCSS compression module equipped with a cartridge (100 × 5 mm I.D.) packed with Novapak® C-18 4–5 µm silica, a fixed-wavelength detector M 441, a data module M 730 and a system controller M 720 (all from Waters Assoc., Eschborn, FR Germany).

The flow rate was maintained at 1.0 ml/min, the resulting back-pressure was 6000 kPa. The eluent was monitored at 214 nm (Zn-lamp) for the determination of mezlocillin and at 229 nm (Cd-lamp) for the determination of piperacillin. For mezlocillin analysis the mobile phase was prepared by combining 760 ml of 12.5 mmol/l sodium phosphate buffer (pH 6.8), 240 ml acetonitrile and 150 mg tetrabutylammonium hydrogen sulfate. The apparent pH was adjusted to 7.3 with 10 N sodium hydroxide. Part of the mezlocillin samples was analysed using a HIBAR® column (125 × 4 mm I.D.) filled with LiChrospher® RP-18 5 µm silica (E. Merck; for mobile phase composition see Fig. 1). For piperacillin assay 1 g tetrabutylammonium hydrogen sulfate was used and the pH adjusted to 6.5.

For determination of ioxitalamic acid a HIBAR column (125 × 4 mm I.D.), prepacked with LiChrosorb® RP-18 5 µm silica (E. Merck), was used for separation. The flow rate was maintained at 1.0 ml/min, the back-pressure was 11000 kPa. The mobile phase was a mixture of 910 ml water, 90 ml acetonitrile, 600 µl acetic acid, 350 mg tetrabutylammonium hydrogen sulfate. The pH was adjusted to 4.8 with 10 N sodium hydroxide. The eluent was monitored at 254 nm (Hg-lamp), the retention time of ioxitalamic acid was about 4.5 min.

2.4.4. Pharmacokinetic analysis

Plasma level data were analysed by the open, two-compartment model. The decline in drug plasma levels in the postinfusion phase was fitted by a computer program for each subject using an iterative relative least-squares regression analysis. A Fortran program was used in the computation. The basic equation of the mathematical model was

$$C_p^t = A \cdot e^{-\alpha t} + B \cdot e^{-\beta t},$$

where C_p^t represents the plasma concentrations at time t after the dose. α and β (min^{-1}) are hybrid constants of the fast and the slow disposition process, respectively, and A and B (mg/l) are the zero-time intercepts of the two components of the biexponential curves. The calculated pharmacokinetic constants were corrected for infusion time [6]. A number of parameters have been calculated, especially following: area under the plasma concentration-time curve ($AUC_{0-\infty}$): apparent steady-state volume of distribution (V_{SS}), total body clearance (Cl_{tot}), and terminal plasma half-life ($t_{1/2\beta}$).

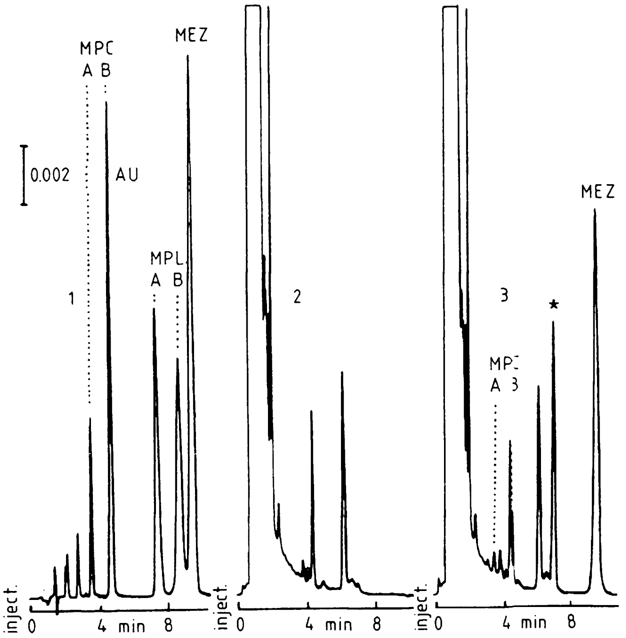


Fig. 1: Chromatograms of (1) an aqueous standard solution of mezlocillin (MEZ, 100 ng) mezlocillin penicilloic acid (MPC; 84 ng mixture of epimers A and B), and mezlocillin penilloic acid (MPL; 150 ng mixture of epimers A and B), (2) a plasma blank and (3) plasma of a volunteer 4 h after intravenous injection (3 min) of 5 g mezlocillin. The asterisk (*) marks an unidentified substance that appears in treated plasma or aqueous samples, and disappears within 2 days at room temperature (see chromatogram 2). Concentrations: MEZ = 15 µg/ml, MPC = 2.5 µg/ml. AU = absorption units.

Chromatographic conditions: Column: HIBAR LiChrospher RP-18 5 µm; (125 × 4 mm I.D.); mobile phase: 2.8 g sodium dihydrogen phosphate monohydrate, 255 mg tetrabutylammonium hydrogen sulfate, 750 ml water, 250 ml acetonitrile, apparent pH 6.2; flow rate: 1 ml/min; pressure 11000 kPa.

3. Results

3.1. Chromatography

In Fig. 1 chromatograms of a standard mixture of mezlocillin and its degradation products, mezlocillin penicilloic and penilloic acid, and of extracted human plasma are depicted. Each mezlocillin metabolite elutes as two distinct peaks because of epimerization at the carbon atom in position 5 (see Scheme 1) [7]. The earlier eluting isomers A are the minor components in freshly prepared aqueous solutions, but become dominant when standing at room temperature for many hours as it was also described for amoxycillin penicilloic acid [8]. Isocratic separation of all compounds within 10 minutes, as seen in Fig. 1, was enabled by application of reversed phase ion pair chromatography. For LiChrospher RP 18 silica a good compromise between separation of mezlocillin and its degradation products from interfering plasma components, and run time was found with 255 mg/l tetrabutylammonium hydrogensulfate in the mobile phase and pH 6.2. For Novapak C-18 silica a lower content of tetrabutylammonium hydrogen sulfate (150 mg/l) and a higher pH (7.3) proved to give better resolution (see Materials and methods for more details). As metabolite of mezlocillin in plasma and urine, we found mezlocillin penicilloic acid only. Since it has two carboxylic groups, the retention time is more sensitive to changes in concentration of tetrabutylammonium salt and pH than that of mezlocillin itself.

Fig. 2 shows chromatograms of a standard mixture of piperacillin and piperacillin penicilloic acid, prepared by incubation of piperacillin with β-lactamase, and of plasma samples of a volunteer after injection of 4 g piperacillin. Like penicilloic acid derived from mezlocillin, piperacillin penicilloic acid elutes as two separated epimers, and is hardly to determine in plasma. On the other hand, two higher unidentified peaks appear at shorter retention times.

3.2. Evaluation of the assay

The chromatographic peaks were quantitated by the area method. The areas of the two unidentified metabolites of pi-

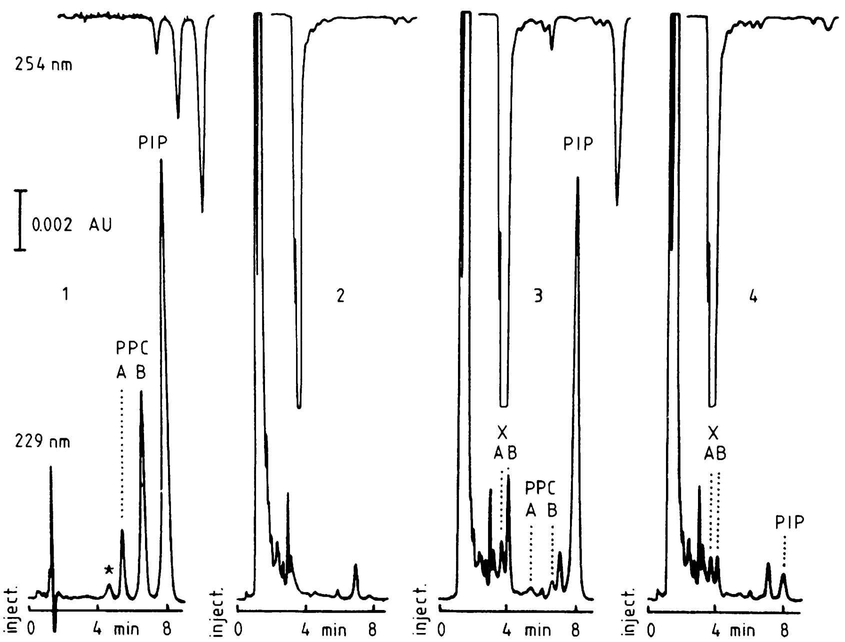


Fig. 2: Chromatograms of (**1**) an aqueous standard solution of piperacillin (PIP, 250 ng) and piperacillin penicilloic acid (PPC, ca. 200 ng mixture of epimers A and B; the asterisk marks an impurity, presumably a hydrolysis product of piperacillin), (**2**) a plasma blank, (**3**) plasma of a volunteer 1 h and (**4**) 4 h after the end of an intravenous infusion (30 min) of 4 g piperacillin. X marks two unidentified peaks (A and B), probably a mixture of epimers. concentrations: (**3**) PIP = 47 μg/ml, PPC = 2,3 μg/ml, X = 9,8 μg/ml when quantified as PIP. (**4**) PIP = 2,6 μg/ml; X = 4,8 μg/ml. Chromatographic conditions: see Material and methods. AU = absorption units.

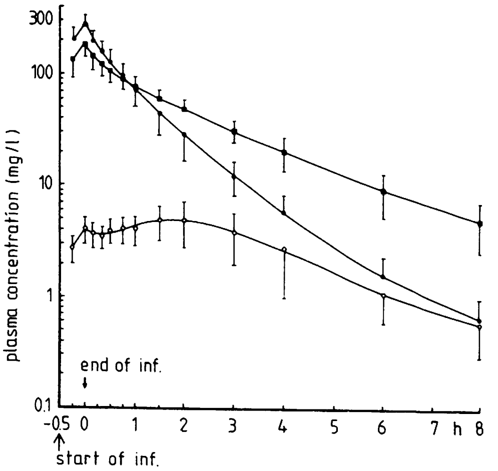


Fig. 3: Concentration-time course of mezlocillin (●) mezlocillin penicilloic acid (○) and of ioxitalamic acid (■) (mean, SD) after intravenous infusion (30 min) of 4 g mezlocillin and 2.4 g ioxitalamic acid to 10 healthy volunteers.

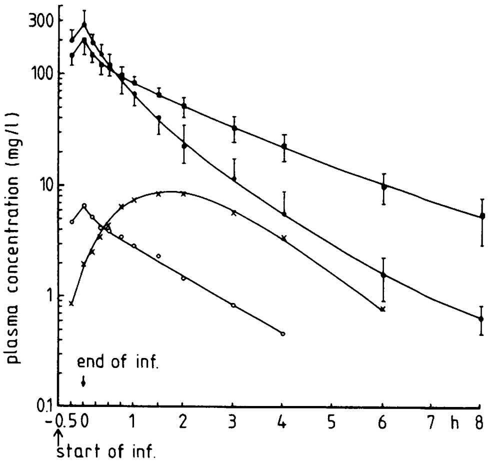


Fig. 4: Concentration-time course of piperacillin (●), piperacillin penicilloic acid (○), compound X (×), and of ioxitalamic acid (■) (mean, SD) after intravenous infusion (30 min) of 4 g piperacillin and 2.4 g ioxitalamic acid in 10 healthy volunteers.

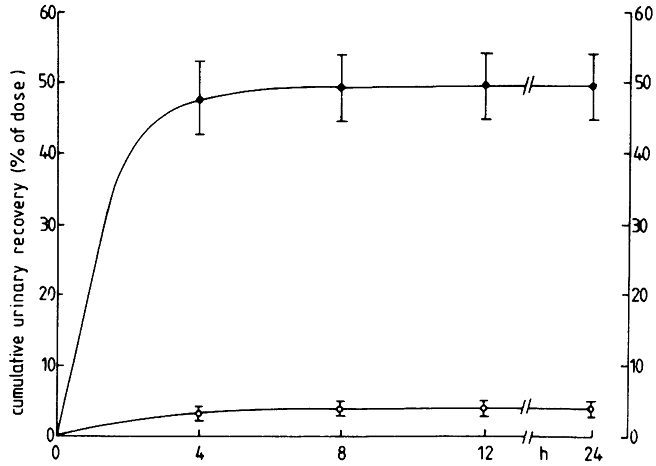


Fig. 5: Cumulative urinary recovery (mean, SD) of mezlocillin (●) and mezlocillin penicilloic acid (○) in 10 healthy volunteers after intravenous infusion (30 min) of 4 g mezlocillin.

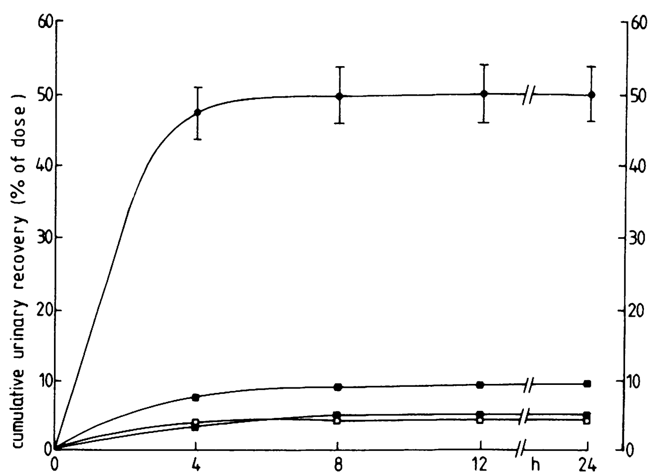


Fig. 6: Cumulative urinary recovery (mean, SD) of piperacillin (●) piperacillin penicilloic acid (□), and of compound X (▪) in 10 healthy volunteers after intravenous infusion (30 min) of 4 g piperacillin. ■ = Sum of ▪ and □.

peracillin were summarized and quantified as piperacillin. The recovery from plasma was 101.1 ± 1.3% for mezlocillin (concentration 100 μg/ml; n = 9), for mezlocillin penicilloic acid 100.8 ± 2.8% (concentration 42 μg/ml; n = 9), for piperacillin 104.3 ± 4.0 (concentration 50 μg/ml; n = 15) and for piperacillin penicilloic acid 91 to 93% (concentration 20 μg/ml; n = 3). The recovery from urine was not checked and set 100%, as the only sample treatment step was dilution of urine with buffer.

Some plasma specimens of the mezlocillin study were determined at two different days and the following reproducibility was found: mezlocillin 102.7 ± 4.8% (concentration range 3.95 to 328 μg/ml), mezlocillin penicilloic acid 107 ± 20% (concentration range 0.80 to 3.6 μg/ml). The precision of the piperacillin assay was checked with spiked plasma. The results are shown in Table 1.

3.3. Pharmacokinetics

Fig. 3 and 5 show the mean (± SD) plasma concentrations and the cumulative urinary excretion of mezlocillin and its ring-open metabolite, Fig. 4 and 6 the respective data of piperacillin. In addition, the plasma concentrations of ioxitalamic acid are depicted. In both cases, the mean plasma concentrations of the metabolites were always by far lower than the values of the respective parent compounds. Apparently neither mezlocillin nor piperacillin is metabolized in vivo to a greater extent. These findings are also illustrated in Table 2. In both studies we found nearly identical values for ioxitalamic acid in plasma, and in the beginning also for mezlocillin and piperacillin. With the time elapsed, the concentrations of mezlocillin remained slightly higher than those of piperacillin.

Table 1: Precision of the determination of piperacillin in plasma. (Piperacillin added: 25, 5, 0.5 μg/ml.)

Day	Piperacillin (μg/ml)		
1	25.2	5.69	0.60
2	26.0	5.85	0.69
3	25.3	5.29	0.62
4	26.1	5.96	0.69
5	26.2	5.74	0.70
Mean	25.8	5.71	0.66
SD (%)	0.5 (1.8%)	0.25 (4.5%)	0.05 (7.0%)

The pharmacokinetic parameters of mezlocillin, piperacillin, and of ioxitalamic acid are summarized in Table 3. All substances exhibit distribution volumes nearly 20% of body weight which agrees good with the extracellular space. Like iotalamic acid [9], ioxitalamic acid shows the same plasma clearance as inulin (ca. 120 ml/min), whereas those of mezlocillin and piperacillin are about 250 ml/min. On the other hand, the half-life of ioxitalamic acid in plasma is about 2 h, whereas the half-lives of both antibiotics are 1 h. The urinary recovery of ioxitalamic acid within 24 h was 90% in each study so that complete 24-h urine collection can be assumed. Also the excretion of creatinine was normal in all subjects. About 50% of the antibiotic doses given were recovered in the urine as active parent compounds, and 9% as metabolites in the case of piperacillin and 4% in the case of mezlocillin.

4. Discussion

4.1. Chromatographic assay

Besides the traditional bioassay, mezlocillin and piperacillin have been also determined in biological fluids by reversed phase HPLC [10–15]. But in all these cases the parent compounds only were assayed, as the ring-open metabolites are much more polar and elute with the front. Using gradient elution technique mezlocillin [16], azlocillin [17], apalcillin [18], and the respective penicilloic acids could be determined simultaneously in serum and urine. Unfortunately, all assays are considerably time consuming. In the present assay incorporation of ion-pair chromatography enabled iso-

Table 2: Mean (± SD) concentrations of mezlocillin and piperacillin after infusion (30 min) of 4 g, and of simultaneously administered ioxitalamic acid (2.4 g) in plasma of ten healthy volunteers. ITXpip = ioxitalamic acid in the piperacillin study; ITXmez = ioxitalamic acid in the mezlocillin study; MEZ = mezlocillin; PIP = piperacillin.

Time	ITXpip (μg/ml)	ITXmez (μg/ml)	$\frac{\text{ITXmez}}{\text{ITXpip}}$ 100 (%)	MEZ (μg/ml)	PIP (μg/ml)	$\frac{\text{MEZ}}{\text{PIP}}$ 100 (%)
-15 min 1)	141 ± 27	134 ± 40	95.0	200 ± 58	198 ± 42	110.0
0 min 2)	194 ± 51	187 ± 45	96.4	271 ± 61	272 ± 75	99.6
10 min	142 ± 18	144 ± 35	101.4	198 ± 46	187 ± 33	105.9
20 min	126 ± 16	121 ± 27	104.3	158 ± 39	148 ± 26	106.8
30 min	110 ± 15	108 ± 24	98.2	129 ± 34	118 ± 26	109.3
45 min	94.9 ± 15.9	91.3 ± 17.9	96.2	97.7 ± 25.8	87.7 ± 22.5	111.4
60 min	79.5 ± 11.5	78.5 ± 16.0	98.7	73.0 ± 21.8	65.2 ± 14.5	112.0
90 min	62.5 ± 9.9	61.0 ± 12.2	97.6	45.1 ± 16.5	39.6 ± 11.2	113.9
2 h	49.3 ± 9.9	49.2 ± 10.8	99.8	29.5 ± 13.4	24.1 ± 8.4	122.4
3 h	32.0 ± 8.2	32.0 ± 7.5	100.0	12.5 ± 4.7	11.5 ± 5.8	108.7
4 h	22.1 ± 6.0	21.1 ± 6.9	95.5	6.0 ± 2.4	5.5 ± 3.1	109.1
6 h	10.4 ± 3.0	9.6 ± 3.9	92.3	1.6 ± 0.8	1.5 ± 0.6	106.7
8 h	5.1 ± 2.3	5.0 ± 2.4	98.0	0.6 ± 0.4	0.6 ± 0.2	100.0
Mean ± SD			98.0 ± 3.1			108.2 ± 6.2

1) 15 min after the start of infusion (the antibiotics were infused at a constant rate over 30 min).
2) At the end of infusion.

Table 3: Comparative pharmacokinetic parameters (mean ± SD) of mezlocillin (4 g), piperacillin (4 g), and simultaneously administered ioxitalamic acid (2.4 g) given as short intravenous infusion (30 min) to 10 healthy volunteers. Abbreviations: ITXmez = ioxitalamic acid in the mezlocillin test; ITXpip = ioxitalamic acid in the piperacillin test; MEZ = mezlocillin; PIP = piperacillin; V_{SS} = steady-state volume of distribution; AUC = area under the plasma concentration-time curve; Cl_t = total body clearance; t_β = terminal plasma half-life; V_{SS}% b.w. = V_{SS} in percentage of body weight; Cl_t 70 kg = Cl_t normalized to 70 kg body weight; $U_{0-24\,h}$ = 24-h urinary recovery.

	V_{SS} (l)	V_{SS}% b.w. (%)	AUC (mg/l h)	Cl_t (ml/min)	Cl_t70 kg (ml/min/70 kg)	t_β (min)	$U_{0-24\,h}$ 1) (% of dose)
ITXmez	15.5 ± 3.2	23.5 ± 3.6	349 ± 71	118 ± 22	127 ± 27	106 ± 15	93.1 ± 7.1
ITXpip	15.2 ± 2.6	23.3 ± 4.5	359 ± 59	114 ± 18	123 ± 27	107 ± 14	87.2 ± 11.4
MEZ	14.3 ± 3.8	21.8 ± 5.1	303 ± 76	231 ± 50	247 ± 52	63.9 ± 11.0	49.6 ± 4.7 2)
PIP	14.5 ± 2.7	22.2 ± 4.7	282 ± 61	246 ± 50	262 ± 54	66.4 ± 13.0	49.6 ± 3.8 3)

1) 24-h creatinine excretion: mezlocillin study (1801 ± 530 mg (male): 1215 ± 609 (female)). Piperacillin study (2102 ± 130 mg (male), 1309 ± 412 (female)).
2) In addition: 4.1 ± 1.0% as mezlocillin penicilloic acid.
3) In addition: 4.2 ± 0.7% as piperacillin penicilloic acid, and 5.0 ± 1.5% as compound X.