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APPLICATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF SOME ANTIBIOTICS IN CLINICAL MICROBIOLOGY

J. KNÖLLER, W. KÖNIG*, W. SCHÖNFELD, K.D. BREMM and M. KÖLLER

Lehrstuhl für Medizinische Mikrobiologie und Immunologie, AG Infektabwehrmechanismen, Ruhr Universität, Universitätsstrasse 150, 4630 Bochum (F.R.G.)

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SUMMARY

During recent years high-performance liquid chromatography has become an excellent tool for the determination of antibiotics in biological fluids. Compared with biological assays, the major benefits of this method are specificity and rapidity. In particular, the determination of biologically inactive metabolites emphasizes that this technique plays an outstanding role for the analysis of antibiotics. This paper describes how the method can be used in the analysis of several antibiotics and demonstrates the efficacy of this method for clinical microbiology. Methods for the determination in biological fluids of acylaminopenicillins (azlocillin, mezlocillin, piperacillin and aspoxicillin), quinolones (ciprofloxacine, norfloxacine and ofloxacine), a penem (imipenem) and a cephalosporin (cefixime) are summarized. Furthermore, their application to in vitro studies and their trial in clinical studies are described.

INTRODUCTION

In the past ten years new techniques based on analytical methodology have been established in microbiology [1]. High-performance liquid chromatography (HPLC) in particular has become an important tool for routine determination of antimicrobial agents in body fluids. The major benefits of HPLC are specificity, rapidity and sensitivity. A further advantage from the pharmacokinetic point of view is its potential for the detection and quantitation of metabolic degradation products, such as penicilloic and penilloic acid.

Owing to the short time required for HPLC analysis and its great potential for the separation and detection of antibiotic drugs, many reports have focused on the techniques for the determination of various antimicrobial drugs in biological fluids and tissues [1–5].

This paper summarizes the results of our reversed-phase HPLC studies of an-

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timicrobial agents and describes the development of standard methods as well as their application and validation by clinical studies. Degradation studies are dealt with only briefly because they have been reported elsewhere [5–13].

EXPERIMENTAL

Reagents

Azlocillin and mezlocillin, together with the appropriate penicilloates and penilloates, and ciprofloxacine were kindly provided by Bayer (Leverkusen, F.R.G.). Norfloxacine, imipenem and cilastatin were kindly provided by Merck Sharp & Dohme (Munich, F.R.G.) and aspoxicillin and cefixime by E. Merck (Darmstadt, F.R.G.). Ofloxacine was purchased from Hoechst (Frankfurt, F.R.G.) and piperacillin from Cyanamide-Lederle (Munich, F.R.G.).

Methanol, dipotassium hydrogenphosphate and phosphoric acid were purchased from Riedel de Haen (Seelze, F.R.G.) and acetonitrile was purchased from Baker Chemicals (Gross-Gerau, F.R.G.).

Buffer solutions

Soerensen buffer consisted of 66.6 m*M* dipotassium hydrogenphosphate adjusted to pH 7.40 with 66.6 m*M* potassium dihydrogenphosphate.

The phosphate-buffered saline (PBS) comprised 120 m*M* sodium chloride–10 m*M* disodium hydrogenphosphate dihydrate–3 m*M* potassium dihydrogenphosphate adjusted to pH 7.40.

Phosphate buffer 1, for β-lactam antibiotics, was 57.4 m*M* dipotassium hydrogenphosphate adjusted to the appropriate pH with phosphoric acid.

Phosphate buffer 2, for oxoquinolinecarboxylic acids, was 15 m*M* phosphoric acid adjusted to pH 3.0 with tetrabutylammonium hydroxide. This buffer can be replaced by 4.4 m*M* tetrabutylammonium hydrogensulphate with the same proportions of methanol and acetonitrile as indicated for the analysis of the oxoquinolinecarboxylic acid derivatives.

Phosphate buffer 3, for imipenem, was 15 m*M* phosphoric acid adjusted to pH 7.0 with tetrabutylammonium hydroxide.

Study design

Degradation studies in buffer solutions were performed in Soerensen buffer. Studies of the pH dependency were performed in borate buffer after Theorell and Stenhagen [22].

Sample preparation

Buffer, serum and plasma samples (1:2 up to 1:10) as well as urine samples (1:10 up to 1:100) were diluted with Soerensen buffer (pH 7.40) or with 0.03 *M* phosphoric acid (in the case of the oxoquinolinecarboxylic acid derivatives). After centrifugation, 20 μl of the supernatant were subjected to HPLC.

The human lung and gut samples were cut with a scalpel, placed in a glass vial with 1–3 ml of Soerensen buffer and homogenized in an Ultra-Turrax (Janke &

Kunkel, Staufen, F.R.G.) for 10–20 s. After centrifugation (three times at 9600 *g* for 5 min), 20 µl of the resultant supernatant were subjected to HPLC.

The human chondral tissue samples were also cut with a scalpel into very small pieces, placed in a glass vial containing 3–6 ml of Soerensen buffer and homogenized in an Ultra-Turrax (Janke & Kunkel) in an ice-bath for 2–3 min. After centrifugation (four or five times at 9600 *g* for 5 min), the supernatant (100 µl) was injected onto the HPLC column.

Human pleural samples were diluted with Soerensen buffer and homogenized with a Sonifier W185 (Branson Ultrasonics, Plainview, NJ, U.S.A.). After centrifugation, 20 µl of the supernatant were injected.

Apparatus

HPLC was performed with a Constametric III-G pump, a variable-wavelength UV detector (Spectromonitor D), an LDC 301 computing integrator (LDC-Milton Roy, Hasselroth, F.R.G.) and an SP 8780 XR autosampler (Spectra-Physics, Darmstadt, F.R.G.). The column (200 mm×4 mm I.D.) was packed with the reversed-phase material Nucleosil C_{18} (5 µm) (Macherey and Nagel, Düren, F.R.G.).

Detection modes

Table I summarizes the detection procedures for the antibiotic drugs analysed in our laboratory.

HPLC procedure

The solvent system consisted of a mixture (Table II) of methanol or acetonitrile and phosphate buffer (adjusted to the appropriate pH with phosphoric acid) as listed under *Buffer solutions*. After mixing, the solvent was again adjusted with phosphoric acid to the final pH value. The mobile phases were degassed and delivered at a flow-rate of 1 ml/min at room temperature. After sample preparation, 20 µl of the centrifuged supernatants were applied to the column. Column performance was frequently checked by analysing standards after every twentieth serum or urine sample. About 250–300 samples could be analysed with one column.

Standards (5, 25, 50, 100 and 250 µg/ml) of the penicillins (and their metabolites), imipenem and cefixime were prepared by serial dilution of a stock solution (prepared daily) containing 1 mg/ml drug with Soerensen buffer (pH 7.40). Quinolone standards (250, 100, 50, 25, 10, 5 and 2.5 ng/ml) were prepared by serial dilution of a stock solution (1 mg/ml) with 0.03 *M* phosphoric acid. Calibration curves were obtained by plotting the peak area against the concentrations of the standard solutions. The linear relationship between the peak area and the antibiotic concentration ranged between 1 and 250 µg/ml (10 and 250 ng/ml for the quinolones) with a correlation coefficient of 0.998. The detection limits were as follows: 0.1 µg/ml for the acylaminopenicillins together with their penicilloic acid, 0.8 µg/ml for the appropriate penilloic acids, 0.5 µg/ml for aspoxicillin, 0.1 µg/ml for cefixime, 0.3 µg/ml for imipenem, 2.5 ng/ml for ciprofloxacine and norfloxacine and 10 ng/ml for ofloxacine. All samples were stored at −70 °C until analysis and kept cold at 4 °C prior to injection.

TABLE I

DETECTION MODES USED

Antibiotic	Detection	Wavelength (nm)
Azlocillin	UV	220
Mezlocillin	UV	220
Piperacillin	UV	220
Aspoxicillin	UV	220
Cefixime	UV	230
Imipenem	UV	313
Ciprofloxacine	Fluorometry	ex., 278, em., 446
Norfloxacine	Fluorometry	ex., 278, em., 446
Ofloxacine	Fluorometry	ex., 278, em., 446

TABLE II

MOBILE PHASES USED

After mixing, the solvent was adjusted with phosphoric acid to the appropriate pH value. For β-lactam antibiotics, phosphate buffer 1 was used, and for oxoquinolinecarboxylic acids, phosphate buffer 2 was used.

Antibiotic	Eluent	pH
Azlocillin	16% Acetonitrile	5.0
Mezlocillin	24% Acetonitrile	4.0
Piperacillin	23% Acetonitrile	5.2
Aspoxicillin	8% Methanol	5.8
Cefixime	15% Methanol	5.2
Imipenem	Phosphate buffer 3	7.0
Ofloxacine	13% Methanol and 7% acetonitrile	3.0
Ciprofloxacine	13% Methanol and 7% acetonitrile	3.0
Norfloxacine	13% Methanol and 7% acetonitrile	3.0

Peaks were identified by their retention times. The concentrations were determined by external standardization. Data obtained for mezlocillin should give information about precision and accuracy of the developed HPLC methods and should serve as an example for the other penicillins. The linear relationship between the peak height (as well as peak area) and the mezlocillin concentration ranged between 2.5 and 250 μg/ml (correlation coefficient 0.999) with the following coefficients of variation (C.V.): 2.30% at a cefixime concentration of 2.5 μg/ml; 1.40% at 100 μg/ml; 0.8% at 250 μg/ml.

RESULTS AND DISCUSSION

Acylaminopenicillins

The following degradation studies of the acylureidopenicillins azlocillin, mezlocillin and piperacillin were performed.

Mezlocillin (which was analysed as representative of the other penicillins) was

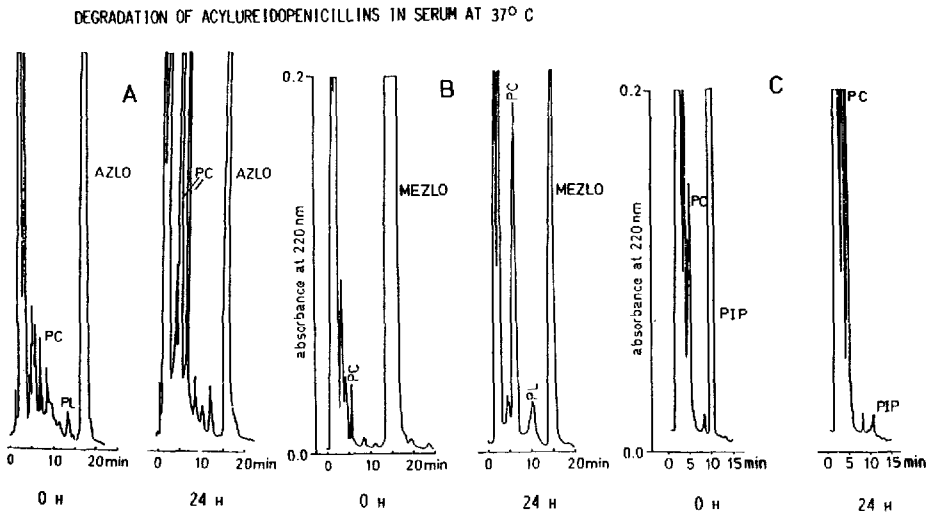

Fig. 1. Representative chromatograms for the acylureidopenicillins. (A) Serum samples spiked with azlocillin (50 μg/ml), (B) mezlocillin (100 μg/ml) and (C) piperacillin (50 μg/ml), before and after a 24-h incubation period in serum. Peaks: PC = penicilloate; PL = penilloate.

degraded as follows [7]: 50% of the mezlocillin was degraded in serum at −20°C after four weeks and up to 72% after six months; at 4°C ca. 55% was decomposed after seven days and up to 98% after four weeks. In buffer solution (pH 7.40) a higher stability was observed: 45% of the mezlocillin was degraded at −20°C after three months and up to 63% after six months; 50% was degraded at 4°C after 2 weeks and up to 91% after six months.

Comparative studies (Fig. 1) were then carried out at 37°C in serum and buffer solutions. Azlocillin [6,11] was the most stable penicillin in serum (52% degradation after 24 h) followed by mezlocillin (70% after 24 h), whereas piperacillin was completely decomposed under the same conditions [10]. Surprisingly, no degradation of the acylureidopenicillins in urine (pH values between 4.5 and 5.5) was observed during an incubation period of 24 h at 37°C, so we investigated their degradation with respect to their pH dependency. These drugs remained stable between pH 4.0 and 6.0, but rapid degradation took place above pH 8.0 (especially at pH 9.0 and 10.0). At high pH values penicilloic acid was the main metabolite, whereas under acidic conditions (pH 3.0) penilloic acid was formed.

The HPLC methods described above were applied not only to in vitro studies but also under clinical conditions with different biological samples, such as plasma, serum, urine, saliva, aqueous humour, bronchial lavage fluid and tissue samples (e.g. lung tissue and cartilaginous tissue). No interference with other serum components was observed in spiked samples, in native serum and urine samples of patients as well as of healthy volunteers. Furthermore, false penicillin quantitation due to coeluting peaks of the biological matrix could not be detected. The determination of mezlocillin in pleurocentesis as well as of azlocillin in lung tissue, gut tissue and chondral tissue [14] are examples of the use of HPLC assays in clinical studies.

Azlocillin and the metabolites penicilloate and penilloate were determined in serum and lung tissue [11]. At 30 min before lung resection eighteen patients (fifteen males, three females; aged between 45 and 65 years) were treated with 5 g of azlocillin intravenously); all patients suffered from lung cancer. After 30 min a mean azlocillin serum level of 322 μg/ml ($n=18$) was determined; the half-life of azlocillin was 77 min. Penicilloate was detected up to 9% (of the detected azlocillin), and the penilloate concentration remained between 1 and 5 μg/ml. The amount of azlocillin in the lung was 92 μg/g of tissue, which is a sufficient concentration for therapy. The penicilloate amounted to 14% of the detected azlocillin in frozen tissue samples and 4% in samples that were analysed on the day of resection.

In colon surgery antibiotic prophylaxis is in general use. All patients ($n=11$) were treated first with 0.5 g of metronidazol (20-min infusion) followed by a 20-min infusion with 4 g of azlocillin. Azlocillin serum levels between 219 and 343 μg/ml were determined after 30 min. In the gut samples, the azlocillin concentrations were between 18.3 and 42.4 μg/g of tissue, indicating that sufficient penetration of the antibiotic into the tissue had occurred.

Prophylactic treatment with azlocillin was carried out under surgery because bacterial infection of chondral tissue [14] after surgical intervention is often followed by the loss of joint function. At 45, 60 and 120 min after intravenous infusion of 75 mg of azlocillin per kg body weight to ten patients (4–10 years old) undergoing thoracoplastic surgery, the cartilage (0.5–3 g) and serum samples were withdrawn and analysed by HPLC. In serum, the concentrations of azlocillin were ca. 478 ± 126 μg/ml after 45 min, decreasing to 120 ± 46 μg/ml after 120 min. Tissue levels of 24 ± 16 μg/g (45 min) and 29 ± 21 μg/g (120 min) were measured.

Besides investigating whether a sufficiently high drug concentration is produced within the infectious focus, the analysis of antibiotics is undertaken to assess whether the chemotherapy is reasonable. Therefore, information about the concentration of mezlocillin in the pleural effusion provides an efficient treatment of bronchopulmonary infections. All patients ($n=9$) were treated with an infusion of mezlocillin (10 g) for 30 min. Serum levels were determined to be 475 ± 91 μg/ml ($n=7$) after 30 min, falling to 310 ± 87 μg/ml after 4 h. In the pleural effusion high and long-lasting levels of mezlocillin could be detected: 68.8 ± 34 μg/ml ($n=7$) after 30 min and up to 104 ± 40 μg/ml ($n=7$) after 4 h.

Aspoxicillin

Aspoxicillin (TA-058) is a new semisynthetic β-lactam antibiotic with a chemical similarity to other penicillins, such as mezlocillin and piperacillin. Its pharmacokinetic behaviour, first studied in Japan, was investigated and compared with that of piperacillin after intravenous and intramuscular injection. Plasma and urine levels of eight healthy male volunteers (22–33 years old) were determined by HPLC (Fig. 2).

After intravenous injection of 1 g of aspoxicillin, intravenous concentrations of 68 ± 13 μg/ml were measured after 10 min, steadily decreasing with a half-life of ca. 60 min. In comparison, piperacillin concentrations amounted to 74 ± 21

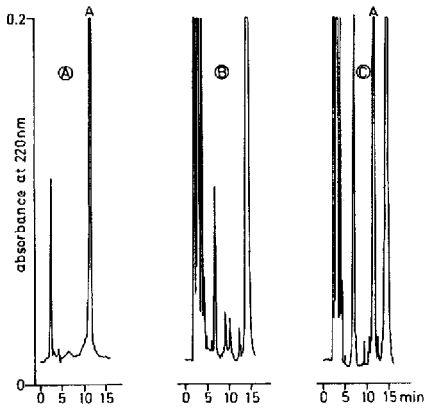

Fig. 2. Representative chromatograms of (A) buffer sample spiked with aspoxicillin (50 μg/ml), (B) blank serum (diluted 1:5 with Soerensen buffer) and (C) serum sample spiked with aspoxicillin (50 μg/ml). Peak A = aspoxicillin.

μg/ml after 10 min. The recovery in urine within 24 h was ca. 97% of the dose for aspoxicillin and 72% for piperacillin. Intramuscular administration led to plasma aspoxicillin levels of 30 μg/ml after 30–45 min. The half-life was comparable with that obtained after intravenous injection, and the recovery in urine was ca. 83% within 24 h.

We also studied the stability of aspoxicillin [10] in serum and buffer at different temperatures over three months. Furthermore, the degradation of aspoxicillin versus piperacillin was determined in serum and buffer at 37 °C. Aspoxicillin remained stable only at −70 °C: degradation was observed at −20 °C and 4 °C. At 37 °C, 20% of aspoxicillin was degraded in serum after 24 h, whereas piperacillin was completely degraded under the same conditions, as shown above.

Imipenem

Imipenem (N-formimidoyl thienamycin) was found to have the widest antimicrobial potency of β-lactam antibiotics, including the third-generation cephalosporins and the new acylaminopenicillins [15].

Carbapenem antibiotics were extensively metabolized by a dipeptidase (dehydropeptidase-I) located in the brush border membrane of mammalian kidneys. The activity of this enzyme resulted in low recoveries in urine. Coadministration of the selective enzyme inhibitor cilastatin markedly increased the urinary recovery of imipenem. We investigated imipenem degradation and its metabolism, including extrarenal metabolism, by HPLC [12].

After incubation with the microsomal kidney fraction (rabbit) at 37 °C for 2 h, 40% of the drug was degraded to two metabolites (Fig. 3), which showed different UV maxima (275 and 308 nm). We suggested that these products are substances with an open lactam ring structure. The generation of these metabolites by the renal enzyme was completely blocked by cilastatin, a specific dipeptidase inhibitor [16]. In serum, 76.6 ± 3.6% of the imipenem was recovered after 1 h and 52.6 ± 3.7% after 2 h. This recovery could be improved by 13% by preincubation

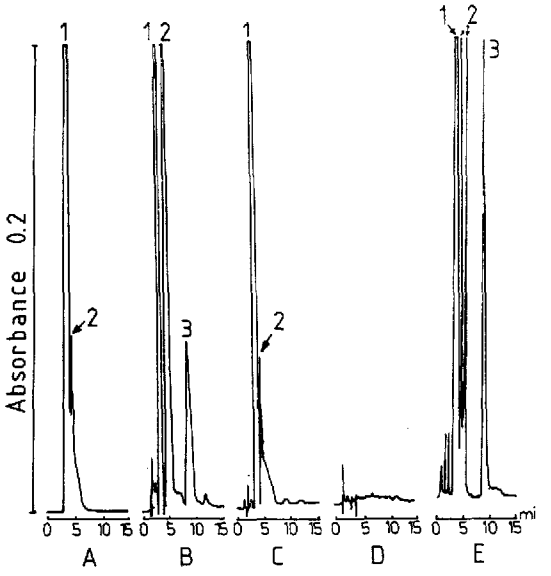

Fig. 3. Representative chromatograms of imipenem (0.5 mg/ml) after incubation for 3 h at 37°C: (A) in water; (B) in the microsomal kidney fraction; (C) as B, but with a preincubation of the microsomal kidney fraction with cilastatin (2 μg/ml); (D) renal microsomal fraction without imipenem; (E) after incubation for 1 h at room temperature in PBS buffer. Peaks: 1 = imipenem; 2 and 3 = metabolites.

of serum with EDTA. These results indicate a possible involvement of serum dipeptidases in the systemic breakdown of the imipenem molecule.

Cefixime

Cefixime (FK-027) is a β-lactamase-resistant third-generation cephalosporin antibiotic for oral use. It exhibits a good in vitro activity against most gram-negative pathogens and a wide range of gram-positive organisms. The cephems comprise a wide group of compounds containing over thirty antibiotics of medical importance. HPLC also was used to assay these substances for drug monitoring extensively [17]. We developed a rapid and sensitive HPLC method (Fig. 4) for the determination of cefixime in serum, buffer and urine. The in vitro stability of cefixime [13] was studied in serum and buffer during storage at various temperatures (4°C, −20°C and −70°C) for three months. Furthermore, its stability in serum, buffer and urine at 37°C was studied over the time period of 24 h.

Degradation was only observed at 4°C in serum. Cefixime was stable for three months at −70°C and even at −20°C. The data obtained at 37°C over 24 h, showed cefixime to be stable in serum (compared with the data obtained with the acylureidopenicillins) as well as in buffer and urine, where no degradation could be observed.

Oxoquinolinecarboxylic acid derivatives

In recent years an increasing number of condensed carboxylic acids with antimicrobial activity have been developed, almost all based on a 1,4-dihydroxy-4-

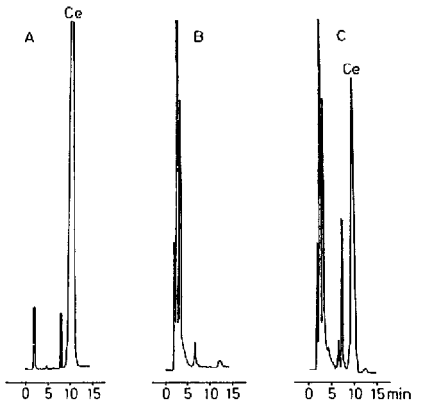

Fig. 4. Representative chromatograms of (A) buffer control to which cefixime (125 μg/ml) had been added, (B) a blank serum (diluted 1:10 with Soerensen buffer) and (C) after one month of storage at 4 °C (serum sample to which 250 μg/ml cefixime had been added). Peak Ce = cefixime.

oxo-3-quinolinecarboxylic acid skeleton. For convenience, the abbreviated name quinolones or oxoquinolinecarboxylic acids has been used for the members of this antibiotic group. The biological activity of the quinolones is directed against DNA replication: they inhibit the DNA-gyrase of bacteria.

Because the metabolites are also supposed to be active in biological assays, it was necessary to develop a method suitable for distinguishing between the parent substances and their metabolites. This was confirmed by Joos et al. [18]: they showed a good correlation between a biological assay and HPLC for ciprofloxacine concentrations in serum, but they obtained significant differences between these two methods with drug concentrations in urine. The results obtained with the bioassay were markedly higher than the HPLC values, which might be due to microbiologically active metabolites excreted by the kidney.

Several studies dealing with the determination of norfloxacine and ciprofloxacine in serum [19,20], urine, faeces, bronchial, pleural fluids and saliva showed no interference from other serum components or compounds. Thus chromatographic identification with fluorescence detection is a very specific method for the analysis of these substances.

Because of the broad antimicrobial spectrum of ciprofloxacine [21], this drug is qualified for the prophylaxis of acute chronic bronchitis. In the course of a clinical study its clinical efficacy and its compatibility were examined. Moreover, we were interested in analysing the drug concentrations within the focus of infection. All patients ($n=17$) were treated with a 500-μg ciprofloxacine tablet after breakfast as well as after supper. In serum, drug concentrations of 945 ± 758 ng/ml ($n=6$) after 30 min were determined and amounted to 3358 ± 826 ng/ml after 60 min. At 4–6 h after the first tablet, 2143 ± 636 ng/ml ciprofloxacine were measured in the sputum, decreasing to 1663 ± 944 ng/ml after 8–12 h after the first tablet had been taken.

Ciprofloxacine was also studied for chemoprophylaxis under immunosuppressive therapy in patients suffering from leukaemia. Patients ($n=43$) were treated

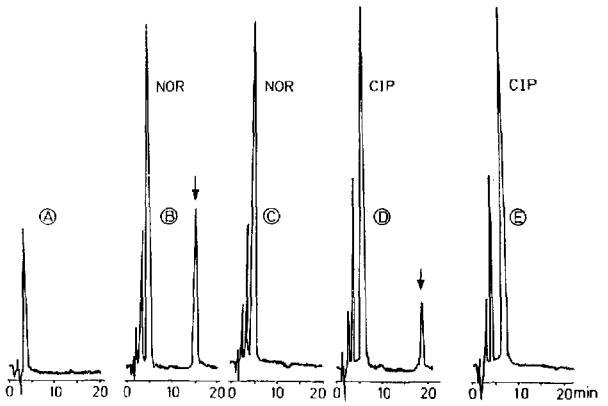

Fig. 5. Representative chromatograms of (A) blank serum, (B) serum after 24 h incubation with 1 μg/ml norfloxacine at 37°C, (C) serum of a patient treated with 500-mg norfloxacine tablet, (D) serum after 24 h incubation with 1 μg/ml ciprofloxacine at 37°C and (E) serum of a patient treated with 500-mg ciprofloxacine tablet. Chromatograms B and D each show an additional peak, with a retention time of 15 and 18 min, respectively, which are probably degradation compounds. Peaks: NOR = norfloxacine; CIP = ciprofloxacine.

with ciprofloxacine tablets containing either 750 mg (group A) or 500 mg (group B). The ciprofloxacine concentrations (Fig. 5) analysed in the serum ranged between 843 and 1433 ng/ml (group A) and between 552 and 843 ng/ml (group B) after 90 min. They reached values between 436 and 1318 ng/ml (A) and between 431 and 561 ng/ml (B) after 180 min.

In saliva, sufficiently high concentrations of ciprofloxacine were determined: the results obtained ranged between 244 and 420 ng/ml (B) and between 395 and 637 ng/ml (A) after 90 min decreasing to values between 178 and 216 ng/ml (B) as well as 287 and 470 ng/ml (A) after 180 min.

CONCLUSION

HPLC has become a valuable adjunct to biological assays for the determination of antibiotics in body fluids and tissues. With regard to drug monitoring and pharmacokinetic studies HPLC has outstanding advantages, especially for the analysis of inactive (e.g. penicilloic acids) and active metabolites (such as metabolites of ciprofloxacine). The HPLC technique is suitable for all groups of antibiotic drugs [1] such as penicillins, cephalosporins, penems, aminoglycosides and oxoquinolinecarboxylic acids, as well as β-lactamase inhibitors, etc. As pointed out, the HPLC assay usually requires aqueous buffer systems (sometimes containing ion-pair reagents) of different pH values with various proportions of organic solvents, such as methanol and acetonitrile. In contrast to other methods [3], the only sample preparation required is dilution with Soerensen buffer. The usual detection methods for antibiotic drugs are based on UV absorption [3]. A variable-wavelength detector is useful in the region 210–235 nm; the absorption is more intense within this region, though antibiotics such as acylaminopenicillins, cephalosporins and chloramphenicol have absorption maxima at ca 250–270,

270–280 and 345–370 nm, respectively. Fluorescence detection also plays an important role [21], especially for the oxoquinolinecarboxylic acid derivatives, owing to the natural fluorescence of these compounds. Thus, HPLC has become a valuable completion to biological assays in clinical microbiology and may provide further approaches for rapid diagnosis in microbiology.

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