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HOSPITAL ROUTINE ANALYSIS OF PENICILLINS, THIRD-GENERATION CEPHALOSPORINS AND AZTREONAM BY CONVENTIONAL AND HIGH-SPEED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic procedure for the measurement of fifteen β-lactam antibiotics in body fluids is described, with special reference to high-speed techniques. The procedure involves a unique sample preparation before analysis for all the following fifteen compounds: benzylpenicillin, ampicillin, cloxacillin, ticarcillin, mezlocillin, azlocillin, piperacillin, cefotaxime and its desacetyl metabolite, cefsulodin, cefoperazone, cefmenoxime, ceftazidime, ceftriaxone and the monobactam aztreonam; thus all biological samples arriving at the laboratory can be treated in batch. Of these fifteen antibiotics, eleven can be chromatographed with the same type of mobile phase, which consists of a mixture of ammonium acetate and acetonitrile in various ratios. Three others need ion-pairing chromatography because of their polarity, and ticarcillin requires citric acid. High-speed high-performance liquid chromatography seems to be particularly suitable for the routine analysis of β-lactam antibiotics because columns equilibrate more rapidly, retention times are much shorter, detection limits are lower and the longer lifetime of columns reduces analysis costs.

INTRODUCTION

β-Lactam antibiotics are the most widely used class of antimicrobial agents. Numerous new molecules belonging to this group of antibiotics are developed every year, and pharmacokinetic studies are necessary for a better understanding of these new agents. Such clinical research, and the increasing tendency of therapeutic drug monitoring in routine hospital use, clearly indicate the need for a rapid and reliable method. Microbiological assays are still widely used for the measurement of antibiotics in biological fluids, but they cause problems when the biotransformation of a drug leads to active metabolites, or when the antibacterial therapy is based on the association of drugs. The advantages of high-performance

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liquid chromatography (HPLC), namely its selectivity, sensitivity and rapidity, have been widely reported, and many methods using various stationary phases, mobile phases and sample preparation procedures have been described [1–4].

This paper describes an HPLC procedure for the analysis of a number of β-lactam antibiotics, including aztreonam, the recently introduced monobactam. The proposed method includes a simple and unique extraction procedure for all of the studied antibiotics and the same type of mobile phase for almost all of them. A similar report [5] has recently been published dealing essentially with first- and second-generation cephalosporins. Our study includes the following antibiotics: benzylpenicillin, ampicillin, cloxacillin, ticarcillin, azlocillin, mezlocillin, piperacillin, cefotaxime and its main metabolite desacetylcefotaxime, cefoperazone, cefsulodin, ceftazidime, cefmenoxime, ceftriaxone and aztreonam.

In addition, in our study, high-speed HPLC procedures are described and compared with conventional HPLC.

EXPERIMENTAL

Reagents and chemicals

Antibiotics of known potency were purchased from the following suppliers: benzylpenicillin (penicillin G), Specia, Paris, France; ampicillin, Bristol, Paris, France; cloxacillin and ticarcillin, Beecham, Paris, France; mezlocillin and azlocillin, Bayer, Puteaux, France; piperacillin, Lederle, Oullins, France; cefotaxime and desacetylcefotaxime, Roussel, Paris, France; cefoperazone, Pfizer, Orsay, France; ceftazidime, Glaxo, Greenford, U.K.; ceftriaxone, Roche, Neuilly, France; and aztreonam, Squibb, Princeton, NJ, U.S.A.

Acetonitrile, ammonium acetate, methylene chloride, hexadecyltrimethylammonium bromide, tetrabutylammonium bromide, acetic acid and citric acid were all of analytical grade (E. Merck, Darmstadt, F.R.G.). Water was obtained daily from a Milli-Ro-Milli-Q System (Millipore, Velizy, France).

Chromatographic equipment

The isocratic liquid chromatograph consisted of a 112 solvent delivery module (Beckman, Fullerton, CA, U.S.A.), a Model 210 sample injection valve with a variable loop (Beckman) and a Model 160 variable-wavelength detector (Beckman). Chromatograms were processed by a Model ICR1-B recording data processor (Intersmat, Courtry, France).

Penicillins were quantitated at 214 nm and cephalosporins and aztreonam at 254 nm.

Conventional chromatography. Conventional chromatography was performed on columns (250 mm or 150 mm × 4.6 mm I.D.) filled with 5-μm diameter octadecylsilane-coated silica particles (Ultrasphere ODS, Beckman). The 150-mm columns were loaded with 20 μl of sample and the 250-mm columns with 50 μl. Separations of ceftriaxone and cefotaxime and its metabolite were achieved on the 250-mm columns. Flow-rates were set at 1 ml/min for cefotaxime and 2 ml/min for ceftriaxone. All other separations (i.e. any β-lactam tested except

TABLE I

MOBILE PHASE COMPOSITIONS

Abbreviations: HDTMAB, hexadecyltrimethylammonium bromide; TBAB, tetrabutylammonium bromide; PBS, phosphate-buffered saline (pH 7).

Antibiotic	Acetonitrile (%)	Ammonium acetate (m*M*)	HDTMAB or TBAB (m*M*)	Citrate buffer (pH 2) or PBS (m*M*)	pH★
Penicillin G	24	20			5
Ampicillin	8	20			5
Cloxacillin	28	20			5
Ticarcillin	36	-		10 (citrate)	2 (HCl)
Mezlocillin	24	20			5
Azlocillin	20	20			5
Piperacillin	20	20			5
Cefotaxime and desacetylcefotaxime	8	20			5
Cefoperazone	20	20			5
Cefsulodin	10	-	5 (TBAB)		3.7
Cefmenoxime	12	20			5
Ceftriaxone	50	-	11 (HDTMAB)	10 (PBS)	8
Ceftazidime	9	20			5
Aztreonam	33	10	5 (TBAB)		7

★pH adjusted with glacial acetic acid, except for ticarcillin (hydrochloric acid).

ticarcillin and azlocillin) were performed on the 150-mm column with a flow-rate set at 1 ml/min.

High-speed chromatography. High-speed chromatography was performed on a high-speed analytical column (75 mm × 4.6 mm I.D.) filled with 3-μm diameter octadecylsilane-coated silica particles. A 5-μl loop was used to load them. All of the studied antibiotics except two (cefsulodin and aztreonam) were analysed by high-speed HPLC with the same mobile phases as for conventional chromatography. The flow-rate was in these cases 1 ml/min, except for cloxacillin (2 ml/min).

Composition of the mobile phases

Among the fifteen antimicrobial agents, eleven were analysed with the same type of mobile phase, a mixture of 20 m*M* ammonium acetate and acetonitrile adjusted to pH 5 with filtered glacial acetic acid. The organic/aqueous ratios used for particular antibiotics are given in Table I.

Of the four remaining molecules that were not analysed with this type of mobile phase, three (cefsulodin, ceftriaxone and aztreonam) clearly needed ion-pairing chromatography (this was not the case for ticarcillin). Suitable counter-ions were either hexadecyltrimethylammonium bromide or tetrabutylammonium bromide (Table I). For two of the fifteen antibiotics, ammonium acetate, was replaced by another salt: ticarcillin was analysed with a 10 m*M* citrate buffer at pH 2 (Table I) and ceftriaxone with a 10 m*M* phosphate-buffered saline at pH 7.

Extraction procedure

We developed a sample preparation procedure that was suitable for all fifteen compounds. It was slightly different from a method that we applied for the measurement of vancomycin [6,7].

Serum. An aliquot of serum (0.5 ml) was mixed thoroughly with an equal volume of acetonitrile in a 7-ml screw-capped glass tube on a vortex mixer (The Vortex Manufacturer, Cleveland, OH, U.S.A.). The tube was then gently shaken by rotation for 10 min (20 rpm). The resulting mixture was centrifuged for 10 min at 1000 *g*. The supernatant was transferred with a Pasteur pipette to another screw-capped glass tube, and seven aliquots of methylene chloride were added. The mixture was allowed to equilibrate for 10 min and then gently shaken by rotation for 10 min (20 rpm). After centrifugation (10 min at 1000 *g*), a 50-μl, 20-μl or 5-μl aliquot of the upper aqueous layer was injected into the column.

Urine and bile. All urine and bile samples were centrifuged, diluted (1:20 and 1:10, respectively) and injected into the chromatograph.

Establishment of the standard curves and detection limit

Quantitation was based on peak heights, as measured by the integrator. The limit of detection was defined as the lowest antibiotic concentration resulting in a signal-to-noise ratio of 4. A standard curve was prepared for any antibiotics by spiking normal sera with increasing amounts of the antibiotics. Urinary and biliary measurements were standardized by supplementing urine and bile samples free of antibiotics with the appropriate antibiotic.

The linearity of the method was assessed from the detection limit of each antibiotic (i.e. 0.05, 0.1, 0.2 or 0.5 μg/ml) to 350 μg/ml. Ten standards were used to prepare each calibration curve.

The extraction recovery was defined by the ratio of the peak height resulting from a supplemented serum to the peak height resulting from an aqueous solution at the same antibiotic concentration. The extraction recovery for any antibiotic was established at 20 μg/ml. Twenty replicates were run for each compound.

Reproducibilities

Both within- and between-day reproducibilities were tested for each of the studied β-lactams. Two concentrations of each antibiotic were included in this study, the first high (150 μg/ml) and the second low (5 μg/ml). Ten aliquots of each sample were tested in the same day and the resulting coefficient of variation (C.V.) indicated the within-day reproducibilities. Aliquots of the same sample were tested once a day during ten days and the resulting C.V. indicated the between-day reproducibilities.

Selectivity

Interferences studies were carried out with many substances that could be coadministered with the β-lactam (analgesics, salicylate, phenobarbital, carbamazepine, phenytoin, primidone, valproic acid, theophyllin, digitoxin, furosemide, quinidine, digoxin, procainamide, lidocaine and all of the actually available aminoglycosides).

TABLE II

CHARACTERISTICS OF METHODS DEVELOPED ON 150-mm OR 250-mm COLUMNS

Antibiotic	Retention time (min)	Within-day reproducibility (%)		Between-day reproducibility (%)		Detection limit (μg/ml)
		5 μg/ml	150 μg/ml	5 μg/ml	150 μg/ml	
Penicillin G	4.8	3.1	3.0	6.1	6.0	0.5
Ampicillin	6.3	4.2	4.0	5.8	6.0	0.5
Cloxacillin	6.2	4.4	4.4	5.7	5.5	0.5
Ticarcillin	-	-	-	-	-	-
Mezlocillin	5.2	3.1	3.4	4.5	4.3	0.5
Azlocillin	-	-	-	-	-	-
Piperacillin	5.6	3.9	3.2	4.7	4.3	0.5
Cefotaxime	8.4	3.5	3.5	3.9	3.7	0.2
Desacetylcefotaxime	2.9	3.8	3.6	4.2	3.9	0.2
Cefsulodin	4.5	3.5	3.5	3.9	3.9	-
Cefmenoxime	7.6	3.7	3.1	4.1	4.2	0.2
Ceftriaxone	17.5	4.2	3.9	5.2	4.8	0.2
Ceftazidime	6.3	4.2	3.9	5.3	4.8	0.2
Aztreonam	4.2	3.8	3.8	4.8	4.4	0.5

As two β-lactams were analysed with the same mobile phase, the selectivity factor α between the two peaks was defined as $\alpha = k_2/k_1$, where k_2 and k_1 are the capacity factors of the later- and the earlier-eluting peak, respectively. The capacity factor of one peak was defined by relating the retention time (t_R) of the peak to the retention time of an unretained peak (t_0): thus, $k = (t_R - t_0)/t_0$.

RESULTS

Sample preparation procedure

The procedure described for serum samples may be applied simultaneously to all the fifteen compounds, including desacetylcefotaxime. After precipitation of the proteins by acetonitrile, the latter is removed by methylene chloride while the antibiotic is concentrated in the upper aqueous layer. Thus, the extraction recoveries for all β-lactams and the monobactam vary between 98 and 117%. This clean-up procedure permits the processing of about twenty samples in 1 h.

Reproducibilities and detection limits

Within- and between-day reproducibilities are shown in Table II and Table III. The highest between-day value is 6.1%, when measuring benzylpenicillin by conventional chromatography. By high-speed chromatography, the highest between-day value is 3.3% (cloxacillin and ceftriaxone). Considering overall within- and between-day reproducibilities, the mean values obtained by high-speed HPLC are better than those obtained by conventional HPLC: $1.36 \pm 0.38\%$ versus $3.8 \pm 0.4\%$ for within-day reproducibilities and $2.58 \pm 0.64\%$ versus $4.75 \pm 0.77\%$ for between-day reproducibilities. Detection limits are indicated in Table II for conventional

TABLE III

CHARACTERISTICS OF METHODS DEVELOPED ON HIGH-SPEED COLUMNS

Antibiotic	Retention time (min)	Within-day reproducibility (%)		Between-day reproducibility (%)		Detection limit (μg/ml)
		5 μg/ml	150 μg/ml	5 μg/ml	150 μg/ml	
Penicillin G	1.5	1.0	0.9	3.3	3.1	0.1
Ampicillin	3.1	1.2	1.1	3.3	3.0	0.1
Cloxacillin	3.5	1.0	1.0	3.3	3.3	0.1
Ticarcillin	1.6	1.3	1.2	3.0	2.8	0.1
Mezlocillin	3.0	1.2	1.2	2.7	2.1	0.1
Azlocillin	2.2	1.0	1.0	2.5	2.2	0.1
Piperacillin	2.4	1.6	1.4	2.6	2.5	0.1
Cefotaxime	2.0	1.6	1.4	1.3	1.3	0.05
Cefoperazone	1.7	1.3	1.3	2.0	1.9	0.05
Cefsulodin	-	-	-	-	-	-
Cefmenoxime	2.2	1.5	1.4	1.9	1.7	0.05
Ceftriaxone	2.0	2.4	2.1	3.3	3.3	0.05
Ceftazidime	2.7	2.0	1.7	3.0	2.7	0.05
Aztreonam	-	-	-	-	-	-

chromatography and in Table III for high-speed chromatography. At these limits, high-speed HPLC gave slightly higher C.V. values than at 5 μg/ml, but never exceeding 3.5%.

Selectivity

No interferences could be observed with any of the studied substances that could be coadministered with the β-lactams. All chromatograms were always carefully checked for skewed peaks, shouldering peaks or tailing peaks. We have been measuring β-lactam antibiotics for four years by HPLC in our laboratory and we have rarely observed atypical chromatograms. When this did occur, it was mainly due to samples taken from patients suffering from renal insufficiency. The resolution was then enhanced by a slight modification of either the organic content of the mobile phase or the pH.

Some of the antibiotics studied were analysed using the same mobile phase: benzylpenicillin and mezlocillin, and azlocillin, piperacillin and cefoperazone (Table I). The selectivity factor between benzylpenicillin and mezlocillin measured by high-speed HPLC was calculated as 2, ensuring a good resolution between the two antibiotics. The same occurred with azlocillin and cefoperazone ($\alpha = 1.67$), and with piperacillin and cefoperazone ($\alpha = 2$). The worse selectivity factor (1.17) between piperacillin and azlocillin should not lead to interference because these two ureidopenicillins should not be coadministered to a patient.

Chromatograms and retention times

Typical chromatograms from the conventional column (150 or 250 mm) are shown in Fig. 1. The antibiotic retention times are indicated in Table II. All the

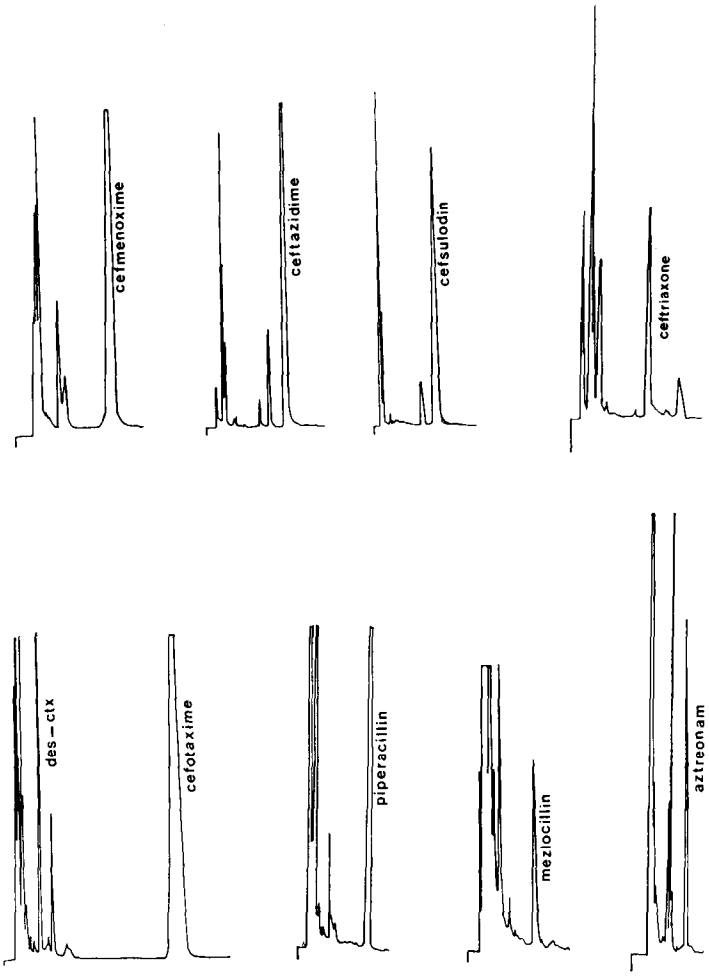

Fig. 1. Representative chromatograms for each of the β-lactam antibiotics analysed with a 250-mm column (cefotaxime, desacetylcefotaxime (des-ctx) and ceftriaxone) and a 150-mm column (all other antibiotics). Absorbance range, 0.05 a.u.f.s.; concentration range, from 20 μg/ml (aztreonam) to 50 μg/ml (cefotaxime). Retention times are summarized in Table II.

chromatograms looked clean and antibiotic peaks were well resolved from endogenous peaks. Blank serum samples (chromatograms not shown) were always carefully checked, and no interfering endogenous peaks could be detected even at the low range setting of the photometer. This appears to be very important for the measurement of a low concentration of an antibiotic. Ticarcillin and azlocillin were not analysed on these types of column. Ceftriaxone eluted in 17.5 min because of the strong counter-ion added to the mobile phase to make the molecule less polar. The shortest retention time was 4.2 min (aztreonam).

The chromatograms depicted in Fig. 2 are representative of high-speed chromatography. Cefsulodin and aztreonam were not analysed on this type of column. The corresponding retention times, indicated in Table III, were much shorter

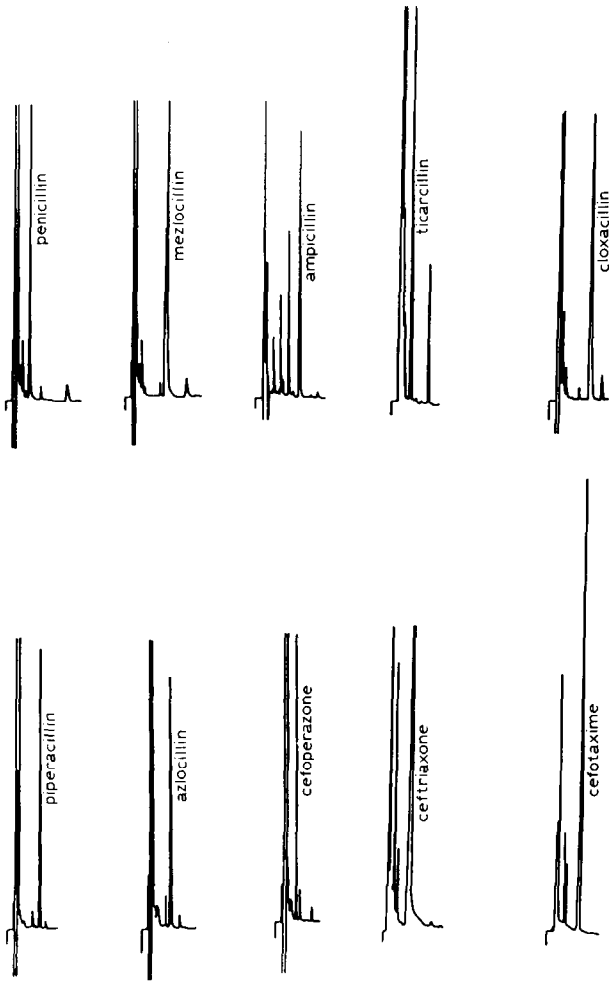

Fig. 2. Representative chromatograms for each of the β-lactam antibiotics analysed with a high-speed column. Absorbance range, 0.1 a.u.f.s.; concentration of each antibiotic, 50 μg/ml. Retention times are summarized in Table III.

than those obtained by conventional HPLC. Thus, the longest retention time obtained by high-speed HPLC, 3.5 min for cloxacillin, is shorter even than the shortest retention in conventional chromatography, 4.2 min for aztreonam. The mean values were: by high-speed chromatography, 2.32 ± 0.63 min; by conventional chromatography, 6.6 ± 3.7 min.

DISCUSSION

Drug monitoring by HPLC generally implies two steps: the former consists of biological sample handling and the latter relates to the choice of the stationary and mobile phases and the chromatographic conditions. Numerous procedures for the measurement of antimicrobial agents have been described [1–5,8] and

various approaches to sample handling have been proposed. Some authors only precipitate the proteins with an organic solvent, such as methanol or acetonitrile, or with an aqueous acid [1,9–13]. In some cases this deproteinization is followed by an organic extraction [14,15]. Direct extraction by an organic solvent followed by an aqueous back-extraction has also been described [2,16–18]. Ion-exchange chromatography on small prepacked columns also leads to excellent sample clean-up [19,20].

Our aim, during the investigational steps of this work, was to standardize to a maximum the overall procedure without losing the reliability required for routine use in a microbiology laboratory. We propose here a serum treatment for the measurement of fifteen β-lactam antibiotics that can be applied simultaneously to all the samples sent to the laboratory, whatever the antibiotic to measure. Thirty samples can thus be handled in 1.5 h. The good reproducibilities of the assays for all the compounds studied indicate that an internal standard to overcome sample-to-sample variation is not necessary.

The second important step during HPLC assay development relates to the stationary and mobile phases. Reversed-phase HPLC is particularly suitable for β-lactam antibiotics because of their chemical structure. Thus, only one type of stationary phase is necessary. Among the fifteen examples studied here, eleven could be chromatographed with only one type of mobile phase, a mixture of acetonitrile and ammonium acetate in various ratios. Thus, the column could be rapidly equilibrated when different β-lactams had to be analysed successively. It would take much longer to equilibrate the column if another salt or another organic solvent were needed when switching antibiotics. This also limits the number of chemicals required.

Normal (as opposed to ion-pairing) reversed-phase chromatography to monitor aztreonam, cefsulodin and ceftriaxone never gave fully satisfactory results. It thus appeared necessary to use a counter-ion because of the great polarity of those compounds. Ion-pairing chromatography for ceftriaxone has been described [15]. With the aim of facilitating the routine monitoring of these three compounds, we decided to reserve one column exclusively for ion-pairing chromatography. The impregnation of an analytical column with a counter-ion takes a long time, and also, once the column is well impregnated, the rinsing time is long, so considerable loss of time would result if the same column were used for normal and ion-pairing reversed-phase chromatography.

High-speed HPLC

The degree to which a column keeps zones narrow is termed the "efficiency", and one way to enhance the narrowness of peaks is to increase the rate of transfer of the molecules from mobile phase to stationary phase. Modern HPLC achieves this by using very small packing particles [21]. Thus, the use of 3-μm diameter particles of silica leads to an increased column efficiency and improved resolution. This results in a lowering of the limit of detection (Tables II and III) compared with chromatography on 150- or 250-mm columns. The detection limit for benzylpenicillin, ampicillin, cloxacillin and mezlocillin has been lowered by a fac-

tor of a fifth and for cefotaxime, cefmenoxime, ceftriaxone and ceftazidime by a factor of a quarter.

The retention time of an antibiotic on a high-speed column has been greatly shortened while the value of k has remained the same as on conventional columns, from one half (ampicillin) to one eight (ceftriaxone). This implies a considerable time-saving and an improved routine work-flow. The rapid equilibrating and rinsing of these short columns make them particularly well suited to the routine of a laboratory where a lot of small series of different antibiotics must be handled.

As some biological fluids, such as cerebrospinal fluid (CSF), do not need to be chemically handled, they can be directly injected into the column. CSF levels of antibiotics can be measured in less than 1 h with the high-speed columns. As only 5 μl of sample are injected, this makes high-speed HPLC particularly useful for paediatric use. This also contributes to a doubling of the column lifetime, thus lowering the cost of the analysis.

CONCLUSION

This paper describes the liquid chromatographic analysis of fifteen β-lactam antibiotics, with special reference to high-speed HPLC. Procedures are simple, rapid and may represent an interesting alternative to other procedures [7] for routine analysis and for pharmacokinetic studies.

Nevertheless, HPLC should not totally supersede the microbiological assays that furnish interesting information when samples are measured by the two methods, as some active metabolites of antibiotics have not yet been chromatographically resolved [18,22].

As the HPLC method measures a chemical entity, it is selective for one compound, and this allows reliable antibiotic monitoring even in the case of associated antimicrobial chemotherapy. This selectivity is also an advantage when measuring molecules that metabolize [22,23], such as cefotaxime, whose main metabolite is desacetylcefotaxime (Fig. 1).

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