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Review

Determination of third-generation cephalosporins by high-performance liquid chromatography in connection with pharmacokinetic studies

F. Péhourcq[a,*], C. Jarry[b]

[a]*Laboratoire de Pharmacologie Clinique, Université Victor Segalen, 146 Rue Léo-Saignat, 33076 Bordeaux Cedex, France*
[b]*Laboratoire de Chimie Physique, Université Victor Segalen, 146 Rue Léo-Saignat, 33076 Bordeaux Cedex, France*

Abstract

The third-generation cephalosporins are semisynthetic β-lactam antibiotics, including several oral and parenteral agents with extended activity against Gram-negative pathogens. They are generally determined either by microbiological techniques or by high-performance liquid chromatography (HPLC). The major drawback of bioassays is the lack of specificity, especially when a biotransformation of the cephalosporin molecule leads to active metabolites, or when the antibacterial therapy is based on association with drugs. Thus, for many years, numerous reversed-phase HPLC procedures have been proposed to overcome these difficulties. This review presents different HPLC methods proposed for the quantification in biological fluids of fourteen third-generation cephalosporins, ranged between parenteral and oral compounds. The sensitivity and specificity of these chromatographic procedures are discussed with regard to the pharmacokinetic properties of the antibiotics studied.

Keywords: Reviews; Antibiotics; Cephalosporins; Lactams

Contents

*Corresponding author.

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1. Introduction

Cephalosporins are among the safest and the most effective broad spectrum bactericidal antimicrobial agents available to the clinician, and have therefore become the most widely prescribed of all antibiotics.

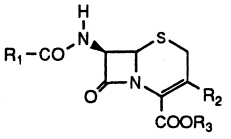
All of these semi-synthetic antibiotics derive from the 7-aminocephalosporanic acid composed of a β-lactam ring fused with a dihydrothiazine ring (Table 1), but differ in the nature of the substituents attached at the 3- and/or 7-positions of the cephem ring. These substitutions affect either the pharmacokinetic properties (3-position) or the antibacterial spectrum (7-position) of the cephalosporins [1]. Traditionally, the cephalosporins are divided into first-, second- and third-generation compounds and, recently, new fourth-generation agents were synthesized [2,3]. Compared with the previous first- and second-generation agents, the third-generation cephalosporins are characterized by a broader antibacterial spectrum, which is related to their enhanced stability to β-lactamases. The susceptibility pattern of the various pathogenic bacteria is quite similar for all compounds; however, growing numbers of resistance reports have been established, due, in particular, to the increasing use of cephalosporins [4].

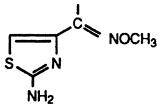
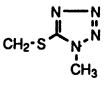
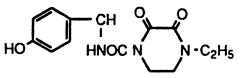
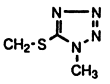
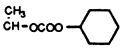
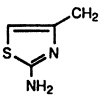
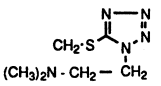
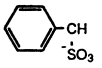
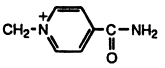
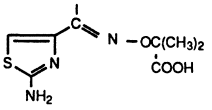
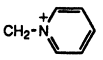
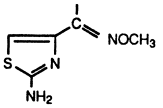
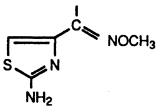
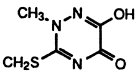
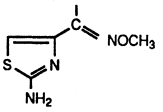
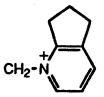
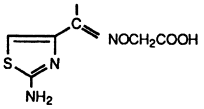
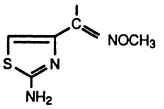
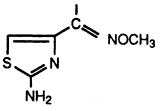
The aim of this review is to provide information on the different HPLC methods proposed to quantify third-generation cephalosporins, ranged between parenteral and oral compounds, in biological fluids. Most of the information is presented in tabular form; however, chromatographic procedures are highlighted and discussed in the text.

Several third-generation cephalosporins have been introduced into clinical use in the past fifteen years, including parenteral and oral agents. Among the currently administered parenteral cephalosporins, eleven compounds were retained for this review: cefmenoxime, cefoperazone, cefotaxime, cefotetan, cefotiam, cefsulodine, ceftazidime, ceftizoxime, ceftriaxone and latamoxef (moxalactam) (Table 1); cefpirome, considered by some authors to be a fourth-generation agent, was also added [5].

In the last few years, efforts have been made to develop new orally active cephalosporins with advantageous pharmacokinetic properties such as a high bioavailability and a long biological half-life. A high bioavailability results in higher and less variable systemic drug concentrations. But, as the cephalosporins have a low liposolubility, they scarcely cross lipidic membranes, such as the intestinal mucosa, and their systemic availability after oral administration is limited [6]. Two synthetic chemical approaches have been developed in order to successfully circumvent this problem [7]. The first one was achieved by the introduction of a structural element that is closely related to an amino acid residue, permitting the molecule to be transported by a dipeptide transport system in the brush-border membrane and, consequently, allowing active absorption [8]. This class of molecules forms the non-ester type compounds, such as cefixime (Table 1). The second approach involved the synthesis of prodrug esters: the hydrophilic carboxyl group at the 4-position of the antibiotic is masked by an ester group that can be hydrolyzed easily. This led to microbiologically inactive prodrug esters that are absorbed from the small intestine (pH 5–7) and hydrolyzed by esterases

Table 1
Chemical structures of third-generation cephalosporins (the structures of cefotaxime, cefotetan and latamoxef are depicted in Figs. 1–3)



Compound (R_3)	R_1	R_2
CEFMENOXIME H		
CEFOPERAZONE H		
CEFOTIAM 		
CEFSULODINE H		
CEFTAZIDIME H		
CEFTIZOXIME H		H
CEFTRIAXONE H		
CEFPIROME		
CEFIXIME H		$CH = CH_2$
CEFPODOXIME 		$CH_2{-}OCH_3$
CEFETAMET $CH_2{-}O - COC(CH_3)_3$		CH_3

to form an active parent compound. Cefetamet pivoxil and cefpodoxime proxetil belong to this ester-type group (Table 1).

Numerous studies have examined the pharmacokinetic properties of cephalosporins in patients, providing a guide to optimum dosage of these antimicrobial agents. This was particularly useful for patients with renal or hepatic insufficiencies, as well as for neonates or elderly subjects. The third-generation cephalosporins present different pharmacokinetic properties [1,9], e.g. protein binding percentages and half-lives, and they differ in their major route of elimination. Furthermore, the third-generation cephalosporins seem to penetrate adequately into the cerebrospinal fluid (CSF) and, consequently, they appear to be appropriate agents for the treatment of meningitidis [1]. Thus, it is important to quantify these drugs in biological fluids, and the HPLC methods seem to be of particular interest for determining concentrations of the active compound in plasma/serum, urine, bile and CSF.

2. RP-HPLC determination of third-generation cephalosporins

Before 1982, microbiological assays were widely used for the measurement of antibiotics in biological fluids. The major drawback of bioassays is their lack of specificity when assaying samples from patients treated with several antibiotics or with an antimicrobial agent that produces active metabolites. In addition to this lack of specificity, these assays usually require an overnight incubation.

High-performance liquid chromatography (HPLC) was developed during the 1970s and 1980s essentially in terms of technological improvements. Its sensitivity, precision and specificity made it suitable for pharmacokinetic studies on a large variety of therapeutic agents. At the other end, in routine hospital use, clinical research and frequent therapeutic drug monitoring clearly indicate the need for rapid and accurate methods.

During the last few years, many RP-HPLC methods, using various stationary phases, mobile phases and sample preparation procedures, have been described for the determination of cephalosporins in biological fluids [10–15]. Recently. two methods using a combination of capillary HPLC with mass spectrometry have been developed [16,17]. The separation and identification of nine cephalosporins were performed by capillary liquid chromatography–electrospray ionization mass spectrometry [16]. Additionally, Kobayashi et al. [17] have used capillary HPLC–fast atom bombardment (FAB) mass spectrometry for the determination of mass spectra of ten third-generation cephalosporins. Hence, they were able to successfully identify ceftriaxone in human serum using an octadecyl reversed-phase cartridge extraction method. No conventional gas chromatography–mass spectrometry data of cephem antibiotics in their underivatised forms are available, since cephalosporins are polar, non-volatile and thermolabile compounds.

The development of a liquid chromatographic method for the measurement of cephalosporins in biological fluids may be divided into different steps.

2.1. Choice of HPLC conditions: Analytical column, mobile phase and detection

The physicochemical properties of the analyte greatly influence the choice of the stationary phase. The third-generation cephalosporins possess strongly polar substituents and, consequently, can be separated by ion-exchange chromatography. Reversed-phase partition chromatography is preferentially used for the separation of less polar analytes possessing either alkyl and/or halogen substituents. Nevertheless, in the last fifteen years, more than 90% of the published HPLC methodologies applied to third-generation cephalosporins involved reversed-phase conditions. This may be explained by the easy use of non-polar coated silicas (C_8, C_{18}), due to their great stability. Even the most polar third generation cephalosporin, ceftriaxone, may be analyzed on this type of stationary phase by ion-pairing chromatography.

As depicted in Table 1, all cephalosporins possess a carboxylic group and most present another acidic (cefsulodine) or basic function (cefotiam). As a result, at pH 8, all cephalosporins are present mainly in an ionic form, while at pH values around three, the dissociation of the carboxylic group is partly suppressed [12]. Hence, in the pH range of three to eight, their chromatographic retention will greatly depend on the dissociation capacity of the sub-

stituents attached at the 3- and/or 7-positions on the cephem ring.

The ionic strength of the mobile phase is also an important factor. In agreement with the findings of Van de Venne et al. [18], at pH 8, the increase in buffer cations results in an increase in the adsorption of ionized acids on the silica and, consequently, in the retention of all cephalosporins. This phenomenon is due to compensation of the negative charges of the cephalosporin molecule by these cations. At pH 3, if the ionic strength increases, the retention decreases, for cephalosporins having an amino function [12]. This may be explained by a competitive interaction of the cations from the buffer with the protonated amino substituent of the cephalosporin towards the residual silanols from the stationary phase. Such a phenomenon, called the dual-retention model, was previously reported by Bij et al. [19].

So, the retention of ionized cephalosporins is a complex mechanism involving, at the same time, the pH and the ionic strength of the eluent, and the acidity of the solute (pK_a). Generally, in the described HPLC methods, most cephalosporins are chromatographed on a reversed-phase analytical column with an acidic eluent of low ionic strength.

UV absorbance is commonly used for the quantitation of third-generation cephalosporins. The detector, an ultraviolet photometer, possesses either a fixed or a variable wavelength (spectrophotometer). Most of the solvents used in HPLC have wide windows in the UV–visible region, making them compatible with UV detectors, even at very short wavelengths. Acetonitrile is a solvent that is frequently used, in particular, at wavelength down to 195 nm.

2.2. Preparation of samples

Biological fluids are complex mixtures, composed of e.g. proteins and lipids. Beyond the deleterious effects that these compounds have on the pump, the injector and the column packing material, their presence will frequently interfere with the separation of the cephalosporins. Consequently, sample preparation is always required before injection onto the analytical column. For the quantitation of cephalosporins, different types of sample pretreatment, including dilution of sample in an appropriate solvent or buffer, protein precipitation and solid-phase extraction on cartridges, have been used. The choice of pretreatment strategy and the amount of sample clean-up are dictated by the efficiency and the selectivity of the chromatographic technique. As the therapeutic concentrations of third-generation cephalosporins are usually in the μg/ml range, concentration techniques are usually not required.

2.2.1. Dilution of samples

This method is mainly applicable when the biological samples contain low amounts of protein, such as urine, cerebrospinal fluid or bile. The dilution solvent may be water or an appropriate buffer. The pH of the buffer must be chosen according to the stability range of the cephalosporin being studied. Since the ionic strength may affect the retention time of the analyte, it may be useful to adjust it. In some cases, specimens from CSF were injected directly onto the column, without prior sample processing [20].

2.2.2. Protein precipitation

Protein precipitation is a very popular method of sample pretreatment and is easy to perform. The majority of high-molecular-mass proteins and fibrin may be removed from plasma samples to prevent damage to the column's filter. The problems associated with precipitation procedures are sample dilution, incomplete protein precipitation, drug coprecipitation and acid-catalyzed degradation of labile drugs [21,22].

Trichloroacetic acid, perchloric acid, methanol, ethanol and acetonitrile have been frequently used for deproteinization. Acidic precipitation procedures might threaten the structural integrity of the cephalosporins [23]. The injection of a highly alcoholic solution might transiently disturb the resolution of the column, causing broadening of the cephalosporin peaks. Under these conditions, peak areas and retention times remained unchanged. The broadening was more marked with ethanol than with methanol [22].

Another disadvantage of the deproteinization methods stems from the dilution, leading to a decrease in the sensitivity. When using an organic solvent, one way to overcome this problem is to back-extract the excess solvent. In many techniques, after the deproteinization of plasma or serum using

an equal volume of acetonitrile, the remaining acetonitrile was removed from the supernatant by methylene chloride. Then, the cephalosporin was concentrated in the supernatant [13,24].

2.2.3. Solid–liquid extraction

Some cephalosporins were isolated by first adsorbing them into either an ion-exchange resin, if they were charged [25], or a bonded reversed-phase packing material, C_8 [26] or C_{18} [27], followed by sequential elution. In these methodologies, if the interaction of cephalosporin with packing material in the pre-column was stronger than the interaction between cephalosporin and protein, the recovery was good, nearly 100% [10]. Cephalosporins are weak acids and bind to albumin to different extents [1]. Solid-phase extraction of drugs can be automated [28–30]. In the column-switching technique, a fraction of the effluent from a primary column was selectively transferred to an analytical column. The primary column produced an on-line sample.

2.3. Stability of the third-generation cephalosporins

In solution, cephalosporins are rather unstable compounds, compared with other soluble drugs. The stability of the cephalosporin solutions depends on several factors, such as the temperature and the pH of the solution. Generally, the degradation of cephalosporins was studied under different conditions, e.g. in calibration solutions, during the course of the analytical process and in frozen samples. In a few methods, time-consuming processes, such as extraction and concentration, were excluded, to minimize drug decomposition. Degradation may also occur during the deproteinization step.

2.4. Use of an internal standard

The use of an internal standard (I.S.) is a controversial point, since one can never be sure that, for a given sample, the extraction recoveries of the I.S. and the drug will be identical. Hence, when no extraction of the drug from the biological fluid is achieved, and when the injected volume is found to be reproducible from one injection to another, an I.S. is not absolutely necessary. Nevertheless, if an I.S. is used, it should have physicochemical properties and chromatographic behaviour that are close to those of the third-generation cephalosporin being studied. No I.S. was used with the HPLC methods described here. In some cases [10], the analyzed drug might be used simultaneously as an external standard. Chromatographic or detection conditions might undergo fluctuations that are not discernible without reference to an I.S.; validation samples prepared from solutions that are different from those used for calibration (external standard) were assayed at the beginning and at the end of each series of determinations.

3. Measurement of cephalosporins: Application to pharmacokinetic studies

3.1. Parenteral third-generation cephalosporins

3.1.1. Cefmenoxime

Cefmenoxime is an aminothiazolyl cephalosporin. It has a similar chemical structure to cefotaxime, ceftriaxone and ceftizoxime, differing only in the substituent at the 3-position of the nucleus (Table 1).

The described HPLC methods for cefmenoxime were simple and rapid, since no extraction step was required (Table 2). The clear supernatant was injected directly onto a reversed-phase column (C_{18} or CN type). These methods proved to be useful for drug concentration analysis in human serum and urine specimens. The sensitivity seemed to be good enough (from 0.05 μg/ml [22] to 0.6 μg/ml [33]) for clinical applications, considering the usual dosages of this cephalosporin.

The stability of cefmenoxime in solution was studied [22]. From a methanol deproteinized sample, degradation was only 5% after 4.7 h, and the authors concluded that the degradation that occurred during the deproteinization process (using methanol) was negligible.

3.1.2. Cefoperazone

Cefoperazone is not absorbed when given orally, and the drug must be administered by intramuscular or intravenous injection. It is often co-administered with aminoglycosides or imidazoles to treat *Pseudomonas* and mixed (aerobic/anaerobic) infections [34].

Table 2
HPLC assays for cefmenoxime

Reference	Specimen	Column	Mobile phase	Detection	Sample pretreatment	I.S.
[21]	Serum, urine	Nucleosil C_{18}, 5 μm (15×0.4 cm)	ACN–water–acetic acid (50:10:1, v/v/v)	UV, 254 nm	D: MeOH	Cefuroxime
[31]	Serum urine	C_{18}	ACN–25 m*M* acetic acid (32:69, v/v)	UV, 254 nm	D: ACN	None
[32]	Serum	μBondapak CN (30 cm×3.9 mm)	Acetate buffer, pH 3.8	UV, 254 nm	D: perchloric acid	*p*-Anisic acid
[33]	Serum	μBondapak C_{18} (30 cm×3.9 mm)	ACN–0.05 *M* ammonium acetate (20:80, v/v) plus Pic-A	UV, 254 nm	D	Cefoxitin

ACN=acetonitrile, D=deproteinization, Pic-A=tetrabutylammonium sulfate and MeOH=methanol.

The cited HPLC methods (Table 3) involved a simple and rapid deproteinization of serum or plasma samples by an organic solvent containing an I.S. According to the technique of Signs et al. [37], the use of a methanol–sodium acetate mixture (70:30, v/v) as a precipitant increased the drug's solubility. At pH 5.2, near the pK_a of cefoperazone, this weak acid was partially ionized, leading to an increase of its solubility in a heterogenous extraction solvent.

Cefoperazone is found to be relatively stable in plasma and urine samples. At −20°C, no degradation was noticed in plasma after ten weeks and in urine after 28 weeks [38].

The sensitivity of the RP-HPLC methods was low enough (around 1 μg/ml) to permit the measurement of cefoperazone in its therapeutic window (5–250 μg/ml) [39].

3.1.3. Cefotaxime

In the liver, cefotaxime forms a metabolite, desacetylcefotaxime (Fig. 1 Table 4), which is microbiologically active and can be detected in plasma in significant amounts [40]. Therefore, it is of particular interest to determine both cefotaxime and desacetylcefotaxime in biological fluids. Because of its good specificity, HPLC presents an advantage in comparison with microbiological assays for the measurement of the parent compound in the presence of its active metabolite. With the exceptions of refs. [43,47], most of the HPLC techniques described allowed the simultaneous determination of cefotaxime and desacetylcefotaxime in serum and urine.

The two compounds were always detected in the UV mode at a wavelength, often near 240 nm, that corresponds to the maximal absorption. According to

Table 3
HPLC assays for cefoperazone

Reference	Specimen	Column	Mobile phase	Detection	Sample pretreatment	I.S.
[35]	Serum, tissue	μBondapak phenyl (30 cm×3.9 mm)	ACN–0.005 *M* TBAB buffer (20:80, v/v)	UV, 254 nm	D: MeOH	Cefoxitin
[36]	Serum urine	μBondapak C_{18} (30 cm×3.9 mm)	Gradient 1.2 m*M* triethylamine–42 m*M* acetic acid–ACN	UV, 254 nm	D: MeOH	None
[37]	Plasma	μBondapak C_{18} (30 cm×3.9 mm)	ACN–MeOH–0.01 *M* sodium acetate (15.2:0.8:84, v/v/v)	UV, 254 nm	D: MeOH–sodium acetate	Cephaloridine
[38]	Serum	C_{18}, 5 μm (25 cm×4.6 mm)	ACN–TMAC–orthophosphoric acid–water (30:0.1:0.03:69.87, v/v)	UV, 254 nm	D: ACN	Ticarcillin

ACN=acetonitrile, TBAB=tetrabutylammonium bromide, TMAC=tetramethylammonium chloride, D=deproteinization and MeOH=methanol.

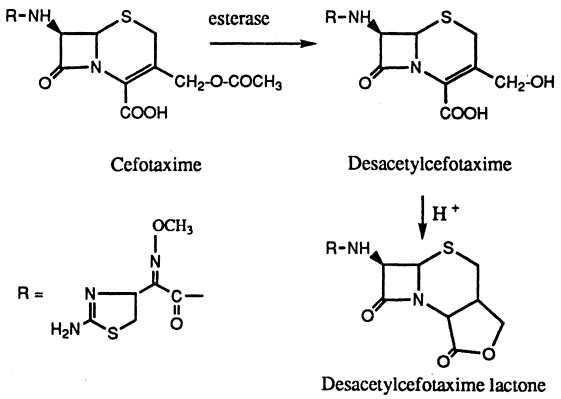


Fig. 1. Chemical structures of cefotaxime and derived compounds.

ref. [42], cefotaxime and its metabolite were detected at 310 nm. Although there was a loss of sensitivity, the authors concluded that this was preferable for routine analysis, because it avoided possible interferences from endogenous serum compounds, which absorbed at 240 nm. Under these chromatographic conditions, the sensitivity (0.3 μg/ml) was still good in relation to the concentrations reached under therapeutic conditions. However, the volume of sample was too large (1 ml) for this method to be used in paediatrics or in patients with renal failure, where sample size is a major concern.

Under strongly acidic conditions, cefotaxime is rapidly hydrolyzed and a heterocyclization (lactoni-

Table 4
HPLC assays for cefotaxime

Reference	Specimen	Column	Mobile phase	Detection	Sample pretreatment	I.S.
[41]	Serum	μ-Bondapak C_{18} (30 cm×3.9 mm)	ACN–0.01 *M* acetate buffer, pH 4 (5:95, v/v)	UV, 254 nm	D: ACN	Hydroxyethyl-theophylline
[42]	Serum	LiChrosorb RP-8, 5 μm (25 cm×4.6 mm)	MeOH–2 m*M* phosphoric acid (28:72, v/v)	UV, 310 nm	D: trichloroacetic acid	None
[43]	Serum, urine	μ-Bondapak C_{18} (30 cm×3.9 mm)	MeOH–0.01 *M* acetate buffer, pH 4.8 (15:85, v/v)	UV, 234 nm	D: HCl M: chloroform–1-pentanol Bex: phosphate buffer, pH 7	None
[44]	Serum urine, bile, saliva	Spherisorb ODS (10 cm×3 mm)	MeOH–water–acetic acid (12:87:1, v/v)	UV, 262 nm	D: chloroform–acetone	None
[45]	Serum, bile	LiChrosorb RP-18 7 μm (25 cm×4 mm)	ACN–MeOH–0.02 *M* phosphate buffer (10:7:83, v/v)	UV, 254 nm	D: perchloric acid Sd+sodium acetate	None
[46]	Serum	LiChrosorb RP-18, 7 μm (25 cm×4 mm)	MeOH–Pic-A	UV, 254 nm	D: phosphoric acid–methanol	Cephalexin
[47]	Serum	Radial-Pak C_{18} (10 cm×8 mm)	MeOH–acetic acid, 0.75% (30:70, v/v), pH 5.5 plus TEA	UV, 254 nm	D: ACN	4′-Nitroacetanilide
[24]	Plasma, urine	Radial-Pak C_{18} (10 cm×8 mm)	ACN–water (17:83, v/v), plus Pic-A	UV, 270 nm	D: 2-propanol Sd: chloroform–4% isoamyl alcohol Sf	Cephaloridine
[48]	Plasma, urine	μ-Bondapak C_{18} (30 cm×3.9 mm)	ACN–0.007 *M* phosphoric acid (15:85, v/v)	UV, 254 nm	D: ACN Sd: ACN–1-butanol Sf	None
[49]	Serum	RP-8, 10 μm (25 cm×4.6 mm)	MeOH–0.02 *M* phosphate buffer, pH 4.5 (23:77, v/v)	UV, 245 nm	D: MeOH	Cephradine

ACN=acetonitrile, TEA=triethylamine, MeOH=methanol, M=mixed, Bex=back extraction, Pic-A=tetrabutylammonium sulfate, D=deproteinization, Sd=supernatant after deproteinization and Sf=final supernatant injection.

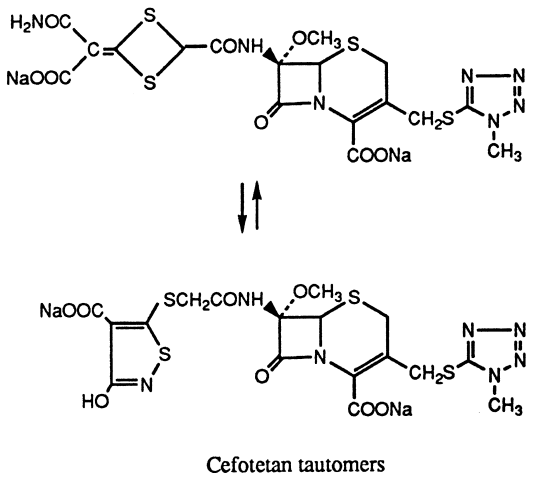


Fig. 2. Chemical structures of cefotetan tautomers.

sation) of desacetylcefotaxime occurs (Fig. 1). The rapid formation of lactones from 3-hydroxymethylcephalosporins by acids has been known for a long time [50]. Degradation studies have shown that the optimum pH range of stability is 4.3–6.5 [51–53]. Thus, acid deproteinization of plasma samples with trichloroacetic acid [42], perchloric acid [45] or phosphoric acid–methanol [46] converted significant amounts of cefotaxime to desacetylcefotaxime and, finally, to the lactone. As the measurement of unchanged compound is favourable, it is recommended that this type of sample pretreatment is avoided.

Moreover, measurable degradation of cefotaxime to desacetylcefotaxime was observed when the serum samples were stored at room temperature. At −20°C, no degradation was noticed over a three-week period [24], however, when storing samples for several months, a temperature of −70°C is necessary [45]. The plasma samples must not be haemolyzed, because the blood esterases rapidly hydrolyze cefotaxime to desacetylcefotaxime; this may explain the unexpectedly high levels of metabolite in ref. [54].

3.1.4. Cefotetan

Cefotetan is commercially available as two epimers, while in weakly alkaline solutions, a tautomeric form is also present (Fig. 2 Table 5). One HPLC method described the separation and quantitation of both epimers in human plasma and urine [56], establishing different pharmacokinetic behaviours for the epimers after intravenous administration ($t_{1/2}$=3 versus 4 h). However, as both epimers have similar antibacterial activity, these pharmacokinetic differences have little clinical importance. With regard to the tautomeric equilibrium, the tautomeric form was barely detectable in human plasma samples (mean of 4.5% of the parent drug), and accounted for approximately 3.5% of the dose excreted in urine [58–60]. The cefotetan/cefotetan tautomer ratio was not influenced by the severity of renal impairment [58,61].

Cefotetan was chromatographed in a reversed-phase mode with a mobile phase composed of a binary mixture of organic solvent and phosphate buffer. In two published cases, sample pretreatment involved the use of dichloromethane to remove the remaining proteins after deproteinization [56,57]. The limits of detection were low enough (0.3 to 2 μg/ml) to permit the use of these methods in pharmacokinetic studies. Serum concentrations of

Table 5
HPLC assays for cefotetan

Reference	Specimen	Column	Mobile phase	Detection	Sample pretreatment	I.S.
[55]	Serum, urine	Nucleosil C_{18}	ACN–0.1 *M* phosphate buffer, pH 3 (8:92, v/v)	UV, 280 nm	D: trichloroacetic acid	Cephazolin
[56]	Serum, urine	Lichrosorb RP-18	ACN–phosphate buffer, pH 6.4 (7.5:92.5, v/v)	UV, 280 nm	D: ACN Sd+dichloromethane	None
[57]	Serum	μBondapak C_{18} (30 cm×3.9 mm)	MeOH–0.1 *M* phosphate buffer, pH 3	UV, 229 nm	D:ACN Sd+dichloromethane	None

ACN=acetonitrile, MeOH=methanol and D=deproteinization.

10.5 μg/ml were recorded 12 h after single intravenous doses (1 g) [62].

3.1.5. Cefotiam

Cefotiam shows a broad-spectrum antibacterial activity against both Gram-positive and Gram-negative bacteria, and is combined with cefsulodine to treat systemic infections in which a broader anti-infective spectrum is needed [63]. Consequently, three fast HPLC methods [22,46,64] described the simultaneous determination of cefotiam with cefsulodine (Table 6); they involved direct injection of the deproteinized plasma/serum onto the analytical column. With acetonitrile as a precipitant agent, the recovery of cefotiam from injected solutions was excellent (around 100%) [64]. Kinetic degradation on storage was studied by Lecaillon et al. [46]. It appeared that plasma samples containing cefotiam were stable for fourteen days at 5°C and for more than nine months at −20°C, which represented a noticeable result in comparison with others cephalosporins.

3.1.6. Cefsulodine

Cefsulodine is a potent cephalosporin derivative with an antibacterial activity against *Pseudomonas aeruginosa* that is comparable to that of gentamicin [67]. In plasma, its stability seems to be greatly improved by the addition of phosphate buffer, to bring the final pH to around six [21,66] (see Table 7). Alkaline conditions were found to facilitate the hydrolysis. At room temperature in unbuffered plasma, the degradation half-life is roughly 8 h. In frozen plasma samples, cefsulodine was found to be stable for only about 1.5 months at −20°C. It was possible to increase the stability on storage (up to three months) by adjusting the pH, e.g. by the addition of a pH 5 buffer to plasma samples before freezing [46]. In the absence of phosphate buffer, plasma solutions were stable for three months at −70°C [64]. Moreover, decomposition of cesulodine might also occur during the deproteinization process. Pseudo-first-order degradation kinetics was established during a process based on deproteinization with methanol [22]. The time required for a 5% degradation was about 0.8 h, compared with cefotiam (3.8 h) and cefmenoxime (4.7 h).

In the first HPLC method described [21], the authors avoided the use of precipitant reagents such as trichloroacetic acid (TCA) or organic solvents. They assumed that all procedures involving deproteinization with such agents were unsatisfactory with respect to coprecipitation of cefsulodin, incomplete protein precipitation (TCA) and chromatographic difficulties. They used an ultrafiltration method on the diluted plasma sample that was suitable for the removal of proteins and that allowed the free and total drug concentrations in plasma to be determined, if desired. Ikura et al. [22] used methanol for deproteinization since this organic solvent did not affect the pH of the sample solution. In their study, Ackers et al. [66] also precipitated proteins using cold methanol and they showed that the methanol-treated specimens were stable for at least 5

Table 6
HPLC assays for cefotiam

Reference	Specimen	Column	Mobile phase	Detection	Sample pretreatment	I.S.
[22]	Serum, urine	Nucleosil C_{18}, 5μm (15 cm×4 mm)	ACN–0.1 *M* acetate buffer, pH 4.4 (5:95, v/v)	UV, 254 nm	D: MeOH	Ceftezole
[46]	Plasma, urine	LiChrosorb RP-18, 5 μm (25 cm×4 mm) at 40°C	MeOH–0.02 *M* phosphate buffer (24:76, v/v)	UV, 254 nm	D: TCA	Cephalexin
[64]	Serum	μ-Bondapak C_{18} (30 cm×3.9 mm)	MeOH–water (35:65, v/v) plus Pic-A	UV, 280 nm	D: ACN	Cefazoline
[65]	Plasma, saliva	Ultrasphere ODS at 35°C	Dioxane–MeOH–0.05 *M* phosphate buffer, pH 6.5 (2.4:24.4:73.2, v/v)	UV, 254 nm	D: MeOH	None

ACN=acetonitrile, MeOH=methanol, Pic-A=tetrabutylammonium sulfate, D=deproteinization and TCA=trichloroacetic acid.

Table 7
HPLC assays for cefsulodine

Reference	Specimen	Column	Mobile phase	Detection	Sample pretreatment	I.S.
[21]	Plasma	μ-Bondapak C_{18} (30 cm×3.9 mm)	ACN–0.02 *M* ammonium acetate, pH 4.2 (4.5:95.5, v/v)	UV, 254 nm	Ultrafiltration	*p*-Fluoro-α-methylbenzylamine hydrochloride
[22]	Serum, urine	Nucleosil C_{18}, 5 μm (15 cm×4 mm)	ACN–0.1 *M* acetate buffer, pH 4.4 (5:95, v/v)	UV, 254 nm	D: MeOH	Ceftezole
[46]	Plasma, urine	LiChrosorb RP-18, 5 μm (25 cm×4 mm) at 40°C	MeOH–0.02 *M* phosphate buffer (24:76, v/v)	UV, 254 nm	D: TCA	Cephalexin
[64]	Serum	μBondapak C_{18} (30 cm×3.9 mm)	MeOH–water (35:65, v/v) plus Pic-A	UV, 280 nm	D: ACN	Cefazoline
[66]	Plasma, urine	Zorbax C_8 (30 cm×4.6 mm) at 30°C	MeOH–0.035 *M* ammonium acetate, pH 5.2 (4.5:95.5, v/v)	UV, 265 nm	D: MeOH	None

ACN=acetonitrile, MeOH=methanol, D=deproteinization, TCA=trichloroacetic acid and Pic-A=tetrabutylammonium sulfate.

h at 4°C, or overnight at −70°C. The two latter procedures were simpler and faster than those described by Granneman and Senello [21].

3.1.7. Ceftazidime

Ceftazidime was measured using different RP-HPLC methods (Table 8). Six of them involved acid [68] or methanol [69–73] precipitation. In their study, Fasching et al. [72] compared two different methods of deproteinization: a mixture of acetic acid–methanol or only methanol. As a result of the influence of pH on the dissociation of the drug–protein complex, drug recoveries of greater than 90% were obtained with the first method. Bompadre et al. [29] proposed the on-line solid-phase extraction of ceftazidime prior to the RP-HPLC determination. This method involved the direct injection of a 50-μl serum sample into the HPLC system and, conse-

Table 8
HPLC assays for ceftazidime

Reference	Specimen	Column	Mobile phase	Detection	Sample pretreatment	I.S.
[68]	Plasma, urine	Hypersil ODS, 5 μm	ACN–0.05 *M* ammonium phosphate–formic acid (93:7:0.01, v/v)	UV, 257 nm	D: perchloric acid	Cephalexin
[69]	Serum, urine	μBondapak C_{18} (30 cm×3.9 mm)	MeOH–0.15 *M* phosphate buffer, pH 6.5 (82:18, v/v)	UV, 255 nm	D: MeOH	8-Chlorotheophylline
[70]	Serum, urine	MicroPak MCH 10 (30 cm×4 mm) at 50°C	MeOH–50 m*M* phosphate buffer (20:80, v/v), 117 μ*M* perchloric acid	UV, 257 nm	D: MeOH	None
[71]	Serum, urine, CSF, PDF	μBondapak C_{18} (30 cm×3.9 mm)	ACN–acetic acid–water (6:1:93, v/v), pH 4	UV, 254 nm	D: MeOH	Hydrochloro-thiazide
[72]	Serum	μBondapak C_{18} (30 cm×3.9 mm)	ACN–acetic acid (10:90, v/v)	UV, 275 nm	D: (MeOH–acetic acid)	None
[73]	Serum, urine, CSF	LiChrosorb 10 μm C_{18} (25 cm×4.6 mm)	MeOH–0.1 *M* sodium phosphate buffer (6:94, v/v)	UV, 254 nm	D: MeOH	Tinidazole
[29]	Serum	HP ODS, 5 μm (15 cm×4 mm)	ACN–10 m*M* phosphate buffer, pH 5 (4:96, v/v)	UV, 258 nm	Column-switching (C_8 column)	None

ACN=acetonitrile, CSF=cerebrospinal fluid, MeOH=methanol and D=deproteinization.

quently, overcame the deproteinization step. A 96% recovery of ceftazidime was observed while the sensitivity (1 μg/ml) was sufficient for human pharmacokinetic studies and for clinical purposes.

Ceftazidime is unstable in serum at ambient temperature. Hwang et al. [71] have studied the stability of ceftazidime under different conditions. At ambient temperature after 24 and 48 h of storage, the recoveries of ceftazidime were 29 and 4%, respectively. At −15°C after 30 days, the recovery was 86%, and after storage at −70°C, it was 91%. The marked deterioration of ceftazidime in serum samples at ambient temperature precluded the storage of samples for any appreciable length of time at −70°C, unless they were analyzed immediately. In all of the HPLC methods described, the sensitivity was sufficient (from 0.3 μg/ml [70] to 1.5 μg/ml [72]) to perform pharmacokinetic studies. After a 1-g intravenous (i.v.) infusion and a l-g intramuscular (I.M.) dose, the maximal concentrations were 59–83 and 29–39 μg/ml, respectively [74].

3.1.8. Ceftizoxime

Ceftizoxime is an aminothiazolyl cephalosporin (Table 9). It is not metabolized and is cleared from the body by the kidneys, by both filtration and secretion [77]. Therefore, measurement of serum levels may be desirable in the management of patients with renal insufficiency.

Few HPLC assays have been developed for the determination of this compound in serum samples. All methods used UV detection and reversed-phase conditions. The major differences were in sample preparation procedures. Protein precipitation with acid [76] or organic solvent [75], anion-exchange [25] or solid-phase extraction [27] have been used in sample clean-up. Mc Cormick et al. [75] have chosen the deproteinization of serum with acetonitrile to avoid acidic conditions that might affect the stability of this cephalosporin. Then, the acetonitrile was removed using dichloromethane and ceftizoxime was concentrated in the upper aqueous phase.

3.1.9. Ceftriaxone

In contrast to other cephalosporins, ceftriaxone possesses a greatly extended elimination half-life (6–8 h), which has resulted in a recommended once daily administration schedule [78]. Consequently, 24 h after injection, the mean plasma concentrations are higher than 10 μg/ml [79]. This permitted the development of HPLC micromethods (volume of sample between 50 and 250 μl) with sufficient sensitivity to determine ceftriaxone concentrations, especially in neonates (Table 10)

Ceftriaxone is a highly polar cephalosporin that is soluble in water. No retention was achieved on a C_8 or C_{18} reversed-phase column, even when water was used as the mobile phase. As an acid, ceftriaxone is capable of forming lipophilic ion pairs with quaternary ammonium salts. Consequently, the technique of ion-pair reversed-phase chromatography was generally chosen for developing new HPLC methods, and quaternary ammonium ions are used as counterions [tetrapentylammonium bromide (TPAB) [80], hexadecylmethylammonium bromide (HDTMAB) [20,83,84] tetrabutylammonium hydrogensulfate (THBS) [82]], having good lipophilic properties. The quaternary ammonium salts were sometimes buffered

Table 9
HPLC assays for ceftizoxime

Reference	Specimen	Column	Mobile phase	Detection	Sample pretreatment	I.S.
[25]	Serum	μBondapak C_{18} (30 cm×3.9 mm)	ACN–acetic acid (13:87, v/v)	UV, 270 nm	Solid–liquid column, column: DEAE–Sephadex A-25	None
[75]	Serum	μBondapak C_{18} (30 cm×3.9 mm)	ACN–water–acetic acid (13:84.2:2.8, v/v)	UV, 310 nm	D:ACN Sd+dichloromethane, Sf	Cefotaxime
[27]	Serum	8 ODS C_{18}, 5 μm	MeOH–water–acetic acid (40:60:0.5, v/v)	UV, 262 nm	Solid–liquid extraction, column:ODS C_{18}, 5 μm	Cephalosporin
[76]	Serum	Ultrasphere CN	MeOH–acetic acid (15:85, v/v)	UV, 270 nm	D: perchloric acid	None

ACN=acetonitrile, MeOH=methanol, D=deproteinization, Sd=supernatant after deproteinization and Sf=final supernatant injection.

Table 10
HPLC assays for ceftriaxone

Reference	Specimen	Column	Mobile phase	Detection	Sample pretreatment	I.S.
[80]	Plasma, urine, bile	LiChrosorb RP-18 (15 cm×3.2 mm)	ACN–20 m*M* phosphate buffer, pH 7,–TPAB (200:800:3.89, v/v/w)	UV, 274 nm	D: ethanol	4-*N*-Nitrobenzoic acid
[81]	Plasma urine, saliva	LiChrosorb NH_2 (25 cm×4 mm)	ACN–water–ammonium carbonate (10%, w/v) (70:26:4, v/v)	UV, 274 nm	D: ACN	None
[20]	Plasma	LiChrosorb RP-8, 5 μm (25 cm×4 mm) at 40°C	ACN–12.5 m*M* phosphate buffer, pH 7–HDTMAB (40:60:2.73 g, v/v/w)	UV, 280 nm	D: ACN Sd+dichloromethane Sf	None
[82]	Plasma	ODS, 10 μm (25 cm×4.6 mm)	MeOH–phosphate buffer–THBS (20:80:1.75 g, v/v/w)	ED, 1.15 V	D: MeOH	Cefotaxime
[83]	Serum, urine, CSF	μBondapak C_{18} (30 cm×3.9 mm)	ACN–10 m*M* potassium phosphate, pH 9 (46:54, v/v), ion pairing reagent: 10 m*M* HDTMAB	UV, 274 nm	D: ACN	Moxalactam
[86]	Serum, urine	ODS C_{18}, 5 μm (15 cm×4.6 mm)	ACN–10 m*M* potassium phosphate, pH 8.8 (50:50, v/v), ion pairing reagent: 5 m*M*HDTMAB	UV, 280 nm	D: ACN	None

ACN=acetonitrile, CSF=cerebrospinal fluid, TPAB=tetrapentylammonium bromide, HDTMAB=hexadecyltrimethylammonium bromide, THBS= tetrabutylammonium hydrogensulfate, D=deproteinization, Sd=supernatant after deproteinization, Sf=final supernatant injection and MeOH=methanol.

at pH 7 to prevent damage to the packing material and to enhance the stability of ceftriaxone (maximum stability in the range of pH 7–7.8 [80]). Another approach was selected by Granich and Krogstad [83], who chose to adjust the pH of the mobile phase to nine, in order to eliminate the peak trailing exhibited in ion pair reversed-phase chromatography. As the use of an alkaline pH caused damage to the silica column, a silica saturation precolumn was introduced between the pump and the injector. The peak shape could also be improved by increasing the concentration of ion-pairing reagent, but this approach may lead to dissolution of the packing material from the analytical column. Moreover, extreme care was necessary to maintain the integrity of the HPLC system when ammonium salts were used and, consequently, some authors flushed the entire system with an acetonitrile–water mixture, to dissolve salt deposits [80]. Another procedure involved the separation of ceftriaxone on a polar alkylamino bonded-phase column (NH_2) [81], with the column being rapidly conditioned; no blocking of frits, phase shrinkage or back-pressure problems were reported.

Since ceftriaxone is a highly polar compound, it cannot be extracted from biological fluids by classical techniques such as liquid–liquid extraction. Sample preparation always involved deproteinization with ethanol [80], acetonitrile [80,20,83,84] or methanol [82]. As it has been reported that cefriaxone is quite unstable in ethanolic solution, the sample solution had to be analyzed within 2 h of preparation [85]. Solvents such as acetonitrile and methanol were preferred as they improved the stability.

Although the UV spectrum of ceftriaxone has its maximum at 240 nm, plasma samples were always measured at 274 or 280 nm, in order to avoid interfering peaks. Detection by an electrochemical detector using a glassy carbon electrode at a potential of 1.15 V was used by Ti et al. [82]. Under these conditions, ceftriaxone and the I.S. were well resolved from endogenous compounds.

3.1.10. Latamoxef (moxalactam)

Latamoxef has another asymmetric carbon atom on the lateral chain, a structure allowing stereoisomerism (Fig. 3 Table 11). The drug is a mixture of the *R*- and *S*-epimers in about a 1:1 ratio. Since the *R*-epimer is twice as active as the *S*-form and presents different pharmacokinetic properties [91], a chromatographic system permitting the determination of each individual diastereomer is necessary. In more than half of the reported HPLC methods, great care

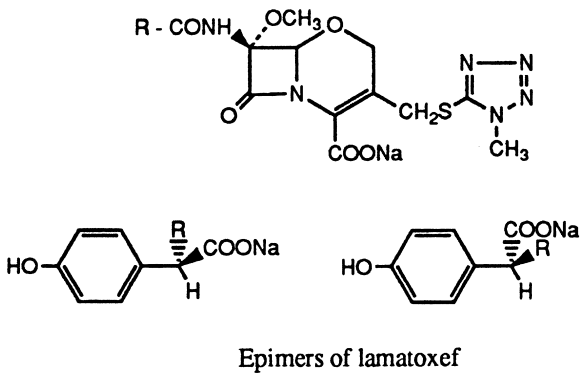


Fig. 3. Chemical structures of latamoxef epimers.

was taken with respect to their possible interconversion when separating the *R*- and *S*-epimers [86,87]. The two isomers readily interconverted in aqueous solution and this interconversion was faster at elevated temperatures, and at low pH. The accurate measurement of *R*/*S* ratios required rapid processing of fresh samples at a pH value between five and six. These pH conditions are also ideal for enhancing the stability of moxalactam, which is unstable at pH values above eight and in acidic conditions [86,87]. Moreover, as moxalactam was found to be unstable at room temperature, the samples were either immediately analyzed or were stored at −70°C.

Moxalactam is always chromatographed under reversed-phase conditions, the pH of the mobile phase being between five and seven (Table 11). With the exception to two methods [90,95], the described procedures for sample preparation were rapid and simple. After deproteinization, a miscible solvent was used to remove the remaining proteins and the initial denaturant provided a good sample for HPLC analysis [86]. In addition, some methods [86,87,90]

Table 11
HPLC assays for latamoxef (moxalactam)

Reference	Specimen	Column	Mobile phase	Detection	Sample pretreatment	I.S.
[86]	Plasma, tissue	μBondapak CN (30 cm×3.9 mm)	ACN–water (20:80, v/v), plus Pic-A	UV, 270 nm	D: isopropanol Sd+dichloromethane Sf+ammonium acetate	None
[87]	Plasma, urine	Nucleosil C_{18} 10 μm (30 cm×4 mm)	MeOH–0.05 *M* phosphate buffer (5:95, v/v), pH 6.5	UV	Sep-Pak cartridges	None
[88]	Plasma Urine	Chromegabond C_{18} (30 cm×4.6 mm) Zorbax TSM 6 μm	ACN–0.1 *M* ammonium acetate, pH 6.5 (95:5, v/v) MeOH–5 m*M* *n*-heptylamine, pH 6 (11:89, v/v)	UV, 270 nm	D: MeOH Sd+0.1 *M* citrate buffer dilution with mobile phase	None
[89]	Plasma, urine, CSF	ODS-HC-SIL-X (25 cm×4.6 mm)	MeOH–0.01 *M* phosphate buffer, pH 6.5 (4:96, v/v)	UV, 230 nm	D: MeOH	8-Chloro-theophylline
[90]	Plasma, urine	μBondapak C_{18} (30 cm×3.9 mm)	ACN–phosphate buffer, pH 7–Pic-A (17:82.2:0.8, v/v)	UV, 280 nm	O: ethyl acetate evaporation	None
[91]	Serum	Hypersil C_{18}, 5 μm	MeOH–0.1% nitric acid (14:86, v/v)	UV, 254 nm	D: ammonium sulfate	None
[92]	Plasma, bile	LiChrosorb C_8	MeOH–0.01 *M* phosphate buffer, pH 7 (10:90, v/v)	UV, 268 nm	D: perchloric acid (0.5%)–MeOH	None
[93]	Plasma, CSF	RP-MicroPak MCA	ACN–0.05 *M* ammonium citrate, pH 6.5 (95.2:4.8, v/v)	UV	D: MeOH	None
[94]	Plasma, CSF	μBondapak/phenyl (30 cm×3.9 mm)	idem Ref. [87]	UV, 280 nm	idem Ref. [86]	None
[95]	Plasma	μBondapak C_{18} (30 cm×3.9 mm)	ACN–0.05 *M* ammonium acetate, pH 5.5 (3.8:96.2, v/v)	UV, 280 nm	idem Ref. [90]	Allopurinol

ACN=acetonitrile, CSF=cerebrospinal fluid, Pic-A=tetrabutylammonium sulfate, D=deproteinization, O=organic solvent, Sd=supernatant after deproteinization, Sf=final supernatant injection and MeOH=methanol.

required a large volume of plasma (≥1 ml) and, consequently, were not adapted for monitoring moxalactam in paediatric patients.

3.1.11. Cefpirome

Cefpirome has an expanded activity spectrum against Gram-positive and Gram-negative bacteria that are resistant to cefotaxime and ceftazidime [96]. Due to its extremely broad activity spectrum, this injectable cephalosporin is classed by some authors in the fourth-generation [5]. The expanded activity and safety profile of this drug make it a potentially valuable antimicrobial agent for use in infants and children. The described HPLC techniques (Table 12) were generally micromethods, requiring only a few microliters of sample and with sufficient sensitivity (<1 μg/ml) [99–101].

The method described by Kearns et al. [101] is an adaptation for milk and urine of their previously developed microanalytical HPLC technique in serum [98]. No changes in selectivity and sensitivity for cefpirome were observed between the two methods, which differed in the ion-pairing agents used [triethylamine instead of tetrabutylammonium bromide (TBAB)] and in the composition of the mobile phase. The authors used isopropanol as the protein-precipitating agent and found low recovery data from serum samples (88.7 and 86.7% for 50 and 100 μg/ml, respectively). They suggested that isopropanol protein precipitation did not completely free the drug from protein-binding sites. The enhancement of drug recovery by precipitation was obtained using a mixture of acid and methanol. For the deproteinization of serum, Uihlein et al. [99] used perchloric acid (7%)–methanol (1:1, v/v). Moreover, they subjected the tube to ultrasonic waves while adding the serum in order to minimize inclusions during albumin precipitation.

3.2. Oral third-generation cephalosporins

3.2.1. Cefixime

Cefixime, an aminothiazolyl cephalosporin is distinguished by its 3 h elimination half-life, which permits twice daily administration, or, in many instances, once daily administration. It has a bioavailability of about 50% after oral administration [102]. Following a single oral dose of 200 mg, peak plasma concentrations were attained in 3 or 4 h and were about 2.0 to 2.6 μg/ml [103]. In the described techniques (Table 13), the detection limit was sufficiently low; 0.05 μg/ml ([30,108]) or 0.1 μg/ml [105,107].

Sample preparation involved different procedures that were applicable to the determination of a highly polar compound such as cefixime. A simple deproteinization step, using acetonitrile [106] or TCA [105,108], was used, but the stability of cefixime

Table 12
HPLC assays for cefpirome

Reference	Specimen	Column	Mobile phase	Detection	Sample pretreatment	I.S.
[97]	Serum	Nova-Pak C_{18}	MeOH–acetic acid 1% (25:75, v/v)	UV, 270 nm	D: ACN	None
[98]	Serum	μBondapak C_{18} (30 cm×3.9 mm)	MeOH–0.05 *M* acetate buffer–TBAB 4‰ (v/v), pH 5.1 (30:70, v/v)	UV, 240 nm	D: isopropanol Sd: evaporation	β-Hydroxypropyltheophylline
[99]	Serum, urine	Spherisorb ODS II (12.5 cm×4.6 mm)	ACN–0.1 *M* phosphoric acid, pH 2.8 (6:94, v/v)	UV, 270 nm	D: perchloric acid 7%–MeOH	None
[100]	Plasma	μBondapak C_{18} (30 cm×3.9 mm)	MeOH–0.05 *M* acetate buffer–TBAB 4‰ (v/v), pH 5.1 (18:82, v/v)	UV, 240 nm	D: ACN Sd: evaporation	Cefaclor
[101]	Milk	ODS, 5 μm (10 cm×2.1 mm) at 50°C	MeOH–TEA (0.3%) (12:88, v/v)	UV, 240 nm	idem Ref. [98]	idem Ref. [98]
	Urine		MeOH–water (10:90, v/v)			

ACN=acetonitrile, TBAB=tetrabutylammonium bromide, D=deproteinization, TEA=triethylamine, Sd=supernatant after deproteinization and MeOH=methanol.

Table 13
HPLC assays for cefixime

Reference	Specimen	Column	Mobile phase	Detection	Sample pretreatment	I.S.
[104]	Serum, urine	TSK–LS410 ODS, 5 μm (15 cm×4.6 mm)	MeOH–0.03 *M* phosphate buffer, pH 2.5 (27:73, v/v)	UV, 295 nm	D: ethanol	None
[105]	Serum	Nova-Pak C_{18} (10 cm×8 mm)	ACN–12.5 m*M* phosphate buffer, pH 2.7 (17:83, v/v)	UV, 280 nm	D: TCA 6%	7-Hydroxycoumarin
	Urine			313 nm		
[106]	Serum	Ultrasphere C_8 (15 cm×4.6 mm)	idem Ref. [105]	UV, 240 nm	D: ACN	Cephalexine
[107]	Serum, tissue	Nucleosil C_{18}, 5 μm (20 cm×4 mm)	MeOH–57.4 m*M* phosphate buffer, pH 5.2 (15:85, v/v)	UV, 230 nm	Dilution with buffer, pH 7.4	None
[108]	Serum, CSF	Ultrasphere C_8 (15 cm×4.6 mm)	ACN–0.01 *M* phosphate buffer, pH 2.7 (15:85, v/v)	UV, 280 nm	D: TCA 6%	7-Hydroxycoumarin
[30]	Plasma, urine	Hitachi Gel 3056 (ODS)	ACN–0.01 *M* phosphoric acid–0.1 *M* monopotassium phosphate–water (13:20:1:66, v/v)	UV, 286 nm	Solid–liquid extraction (column-switching)	None
				314 nm		

ACN=acetonitrile, MeOH=methanol, TCA=trichloroacetic acid, D=deproteinization and CSF=cerebrospinal fluid.

under acidic conditions was not studied. Falkowski et al. [105] determined cefixime recovery from human serum to be about 60%, using the TCA sample preparation procedure. Consequently, to have sufficient sensitivity, they needed a large volume of serum (250 μl), which was not suitable for paediatric applications. Two methods [30,104] involved a column-switching technique for the analysis of cefixime. The absolute recoveries in plasma and urine were better than 99.1 and 98.6%, respectively, and the sensitivity of these methods (0.05 μg/ml) was markedly increased.

3.2.2. Cefetamet pivoxil

Cefetamet pivoxil is the pivaloyloxymethylester of the semisynthetic third-generation aminothiazolyl cephalosporin, cefetamet (Table 1). This ester, which is inactive in vitro, is rapidly hydrolyzed by esterase to release the active cephalosporin [109].

One method (Table 14) was described for the

Table 14
HPLC assays for cefetamet

Reference	Specimen	Column	Mobile phase	Detection	Sample pretreatment	I.S.
[111]	Plasma — cefetamet	Spherisorb ODS 1, 5μm (12 cm×4 mm)	ACN–4 m*M* perchloric acid (17:83, v/v)	UV, 265 nm	D: perchloric acid	None
	— cefetamet pivoxyl	Nucleosil 5 C_{18}	ACN–0.1 *M* phosphate buffer, pH 6.5 (40:60, v/v)			
	Urine	Spherisorb ODS 1, 5μm (12.5 cm×4 mm)	ACN–4 m*M* perchloric acid (15:85, v/v)			

ACN=acetonitrile and D=deproteinization.

simultaneous determination of both cefetamet and cefetamet pivoxyl. Cefetamet pivoxyl is a lipophilic compound that is insoluble at neutral pH values; these properties influence its bioavailability [110]. Wyss and Bucheli [111] studied the dependence of the stability on plasma pH and on addition of an esterase inhibitor. As cefetamet pivoxyl was stable at pH 6.5 in plasma with 2.5 mg/l sodium fluoride, they developed two different HPLC procedures to measure cefetamet and cefetamet pivoxyl in plasma, using a common sample work-up. The mobile phase was a mixture of phosphate buffer, pH 6.5, and acetonitrile, to enhance the stability of the ester. Plasma samples were analyzed after deproteinization with perchloric acid, and the mean recoveries of cefetamet and cefetamet pivoxyl were 87.4 and 37.9%, respectively. Under these conditions, the limit of quantitation of cefetamet pivoxyl in plasma samples was 0.5 μg/ml. This HPLC method was applied to pharmacokinetic studies [112], but cefetamet pivoxyl could not be detected in any biological samples, which was consistent with the rapid hydrolysis of this ester.

3.2.3. Cefpodoxime proxetil

Cefpodoxime proxetil is a prodrug that is rapidly hydrolyzed by intestinal wall esterases into its active form, cefpodoxime (Table 15). Then, it is well absorbed from the gastrointestinal tract. After the administration of single- and multiple doses of cefpodoxime proxetil in the dose range of 100 to 400 mg of cefpodoxime equivalents, the average peak plasma concentrations ranged from 1.0 to 4.5 μg/ml [113]. Because of these low concentrations, pharmacokinetic studies on this cephalosporin required highly sensitive and accurate methodology. In the described HPLC methods (Table 15), the limits of quantitation in plasma samples ranged from 0.01 [26] to 0.2 μg/ml [115].

Cefpodoxime is eliminated in part by a renal mechanism. After administration of doses ranging from 100 to 800 mg, approximately 24 to 41% of the initial dose was recovered in urine [113]. Three HPLC techniques have been published that describe the procedure used to quantify cefpodoxime levels in human urine. Generally, urine pretreatment was simple and rapid; urine was diluted with water [115] or buffer [114] before injection onto the HPLC system. Regarding the stability of cefpodoxime at ambient temperature, dilution with phosphate buffer at pH 5.5 was suitable [114]. In contrast to some other cephalosporins, cefpodoxime was found to be stable for one year at −20°C in both plasma and urine [26,28]

4. Conclusion

As third-generation cephalosporins are among the most widely prescribed of all antibiotics, measure-

Table 15
HPLC assays for cefpodoxime

Reference	Specimen	Column	Mobile phase	Detection	Sample pretreatment	I.S.
[114]	Serum	Nucleosil C_{18}, 5 μm (25 cm×4 mm)	ACN–10 m*M* acetate buffer, pH 4 (9:90, v/v)	UV, 260 nm	D: ACN Sd+dichloromethane Sf+10 m*M* acetate buffer, pH 4	None
	Urine		(10:90, v/v)			
[28]	Urine	Phenomenex IB-SIL C_{18}	ACN–0.05 *M* sodium acetate buffer (7:93, v/v)	UV, 254 nm	Solid–liquid extraction (column-switching)	None
[115]	Serum, urine	Ultrasphere XL-ODS	ACN–21.5 m*M* ammonium acetate, pH 5 (7:93, v/v)	UV, 254 nm	D: ACN Sd+dichloromethane Sf	None
[26]	Plasma	Phenomenex IB-SIL C_{18}	ACN–MeOH–0.05 *M* sodium acetate, pH 6 (4:4:92, v/v)	UV, 254 nm	Solid-phase extraction (C_8)	Cefaclor

ACN=acetonitrile, MeOH=methanol, D=deproteinization, Sd=supernatant after deproteinization and Sf=final supernatant injection.

ment of them in biological samples was essential, with regard to their pharmacokinetic profiles. Due to HPLC specificity and sensitivity, HPLC procedures were found to be more suitable than microbiological ones. The purpose of this review was to provide information on the different HPLC methods described during the last fifteen years. These methods were discussed in terms of HPLC conditions (analytical column, mobile phase and detection) and the preparation of biological samples.

From a chemical point of view, the third-generation cephalosporins form a homogeneous class of compounds. Their physicochemical properties have led to relatively weak stability in solution. Therefore, in most of the described chromatographic assays, the stability of the cephalosporin was studied under different analytical conditions e.g. storage temperature and pH media. In this work, particular emphasis was placed on problems related to sample volume and preparation, with regard to the range of concentrations found in therapeutic and pharmacokinetic studies.

5. List of abbreviations

ACN	acetonitrile
B	bile
Bex	back-extraction
C	centrifugation
CSF	cerebrospinal fluid
D	deproteinization
Dil	dilution
E	evaporation of the organic layer
ED	electrochemical detection
HDTMAB	hexadecyltrimethylammonium bromide
I.S.	internal standard
M	mixed
MeOH	methanol
0	organic solvent
PDF	Peritoneal dialysis fluid
Pic-A	tetrabutylammonium sulfate
RP	reversed-phase
Sd	supernatant after deproteinization
Sf	final supernatant injection
TBAB	tetrabutylammonium bromide
TEA	triethylamine
THBS	tetrabutylammonium hydrogensulfate
TMAC	tetramethylammonium chloride
TPAB	tetrapentylammonium bromide

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