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REVIEW

ANTIBIOTIC MONITORING IN BODY FLUIDS

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1. INTRODUCTION

Many studies performed with animals or humans at the stage of development of a new antibiotic necessitate the quantitation of the drug in body fluids and include studies of toxicology, pharmacokinetics, bioavailability and metabolism. The concentration range associated with optimal therapeutic effect without toxic effects is investigated. The optimal antibiotic plasma level should exceed the minimal inhibitory concentration (MIC) by as large a margin as possible to ensure a sufficient level of antibiotics in infected tissues. The determination of plasma antibiotic levels in patients with varying degrees of impaired renal or hepatic function permits guidelines to be established for adjusting the dosage in such patients.

However, this conventional dose approach is not satisfactory for antibiotics with a narrow therapeutic margin such as aminoglycosides, chloramphenicol and vancomycin. In these cases, reliance on the recommended dosage regimen is not sufficient to ensure therapeutic concentrations and avoid the only slightly higher toxic concentrations caused by the wide inter- and intrapatient variability, the influence of disease state and the potential for drug interactions. This is especially important when treating patients with hepatic or renal impairment which lead to unpredictable variations in the metabolic or renal clearance. Also there exists some variability in pharmacokinetics in neonates and young infants, and the dosage should be individualized in such patients. The most rational approach to ensure appropriate dosage is then to measure plasma or serum levels of the hospitalized patient at frequent intervals. Obviously, measurements must be accurate, but also rapid to allow adjustment of the next dose.

Routine monitoring of antibiotic levels generally involves quantitation in the microgram concentration range as antibiotics are generally metabolized to only a small extent and the administered doses are high. Lower concentrations have to be measured mainly for pharmacokinetic studies. The antibiotic is present in a complex biological matrix [plasma, serum, urine, saliva, cerebrospinal fluid (CSF), tissue, bile] from which its concentration must be selectively determined. Among the analytical techniques suitable for routine monitoring of antibiotics in body fluids, the agar diffusion plate method has been traditionally used. Other microbiological techniques have been less frequently employed [1]. Some agar diffusion assay systems used routinely have been recently reviewed [1]. In recent years, high-performance liquid chromatography (HPLC) has become increasingly used for the determination of antibiotics.

Immunoassays have been essentially used for the determination of aminoglycosides. Other non-microbiological methods such as gas—liquid chromatography (GLC), high-performance thin-layer chromatography (HPTLC), radioenzymatic assay, spectrophotometry, fluorimetry or differential pulse polarography have been little employed.

HPLC procedures have been reviewed on several occasions [2—4]. Chromatographic procedures reported up to 1978 have also been reviewed [5]. The present review summarizes and comments on HPLC and non-isotopic immunoassay procedures recently reported. Methods older than six to seven years are not mentioned. The few GLC and HPTLC procedures recently reported are also reviewed. HPLC procedures are summarized in tables where the characteristics of the procedures are mentioned only for the quantitation in plasma or serum. It was rarely attempted to compare the sensitivity of the reported procedures because either the limit of detection (LD) was given with often no mention of the signal-to-noise ratio, or the limit of sensitivity (LS) or quantitation was given but not the precision of the procedure for the concentration limit.

1.1. High-performance liquid column chromatography

The choice of adequate conditions for an HPLC procedure is governed by the polarity and the ionizable groups of the antibiotic. Polar compounds are often directly determined in plasma and serum after protein precipitation. Acids or water-miscible organic solvents are used as precipitants. Organic solvents, less efficient precipitants, are mostly employed for highly protein-bound antibiotics to avoid co-precipitation. High organic solvent volumes must be added to ensure complete precipitation of proteins [6]. This simple procedure is also applied to lipophilic antibiotics such as chloramphenicol when coupled to sensitive and selective detection. But lipophilic substances are generally extracted from the biological matrix into organic solvents at pH where the ionization of their basic or acid function is suppressed. Either the organic extract is evaporated and the residue reconstituted or the drug is back-extracted in a buffer before analysis. Sample pre-treatment by solid-phase extraction using a small column and by ultrafiltration are little employed.

Alkyl bonded phase columns (C_8 or C_{18}) are essentially used with buffer—methanol or acetonitrile as mobile phase. The organic modifier content in the mobile phase depends on the polarity of the drug being investigated and on the type of the reversed-phase packing. The pH of the buffer is selected to provide sufficient retention in case of ionizable drugs. The column life-time is often extended when deproteinized samples are injected by adding a short guard column packed with large-sized particles of alkyl bonded phase.

Most antibiotics have relatively high molar absorptivity within the UV absorption range, so that UV detection permits determinations with sufficient sensitivity. There are some exceptions such as aminoglycosides and erythromycin. More specific systems such as fluorimetry and electrochemical detection are less widely used. Fluorimetry is essentially used after derivatization of the antibiotic. The derivatization is performed either in the sample before injection (pre-column mode) or on-line in the column effluent (post-column mode). The post-column procedure requires a more complicated chromatographic system.

The derivatizing reagent is added to the column effluent by a separate pump and the reaction is performed in a reaction coil.

1.2. Immunoassay techniques

Immunoassays have been recently described by Munro et al. [7]. Such assays depend upon the competition between a labelled antigen (antibiotic) and an unlabelled antigen (antibiotic to assay in serum) for a specific antibody. The more unlabelled antigen present in the reaction mixture, the less labelled antigen will be combined with antibody. Because antigen—antibody reactions are stoichiometric, the determination of either free or bound labelled antigen results in a direct calculation of the antibiotic level. Instead of radioactive markers used for the radioimmunoassay (RIA), non-isotopic immunoassays employ the following labels: fluorophores for the fluorescence polarization immunoassay (FPIA) and the quenching fluoroimmunoassay (FIA), enzymes for the enzyme immunoassay (EIA) and fluorogenic enzyme substrates for the substrate-labelled fluoroimmunoassay (SLFIA). Latex particles coated with an antibiotic protein conjugate are used for the latex agglutination inhibition assay (LAIA).

2. PENICILLINS

Penicillins, β-lactam antibiotics, possess broad antimicrobial activity against both gram-positive and gram-negative organisms. They consist of bulky side-chains attached to 6-aminopenicillanic acid (Fig. 1). All penicillins possess a carboxylic group (pK_a = 2—4). Penicillinase-resistant penicillins have no additional ionizable group within the pH range 2—8. Broad-spectrum penicillins such as ampicillin, amoxicillin and mecillinam possess a basic function (pK_a = 7—9.5). Other broad-spectrum penicillins such as ureidopenicillins, carbenicillin, ticarcillin and sulbenicillin are acidic compounds.

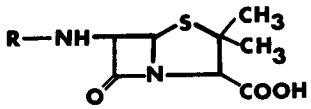

Fig. 1. Common nucleus of penicillins.

2.1. High-performance thin-layer chromatography

An HPTLC method has been developed for the determination of ampicillin in urine [8]. Chloroform extracts were analysed using silica gel 60 plates, dioxane—water—butanol—formic acid as solvent and densitometric determination at 480 nm. The calibration curve was linear over the range 0.05—1 μg/ml and coefficients of variation were not greater than 3%.

2.2. High-performance liquid chromatography

Numerous HPLC procedures have been described (Table 1). Penicillins do

TABLE 1

HPLC OF PENICILLINS

Drug and reference	Body fluid or tissue (amount used)	Extraction and/or deproteinization	Stationary phase	Mobile phase	Pre- or post-column derivatization	Detection wavelength (nm)	Limit of detection (LD) or of sensitivity (LS) (μg/ml)
Cloxacillin [9]	A. Serum (500 μl) Urine	Acetonitrile	C_8	Methanol—phosphate buffer, pH 4.5	—	UV 225	LD 0.3
	B. Serum (500 μl) Urine	Methylene chloride	C_8	Acetonitrile—phosphate buffer, pH 4.5	—	UV 210	LD 0.05
Cloxacillin Nafcillin [10]	Plasma Serum (100 μl)	Chloroform	C_{18}	Acetonitrile—phosphate buffer, pH 7	—	UV 195—218	LD 0.08 LS 0.5
Cloxacillin Dicloxacillin Flucloxacillin Oxacillin [11]*	A. Plasma Serum (100 μl)	Perchloric acid—methanol	C_8	Methanol—acetate buffer, pH 5.5	—	UV 220	LS 1
	B. Plasma Serum (100 μl)	Methylene chloride	C_8	Acetonitrile—acetate buffer, pH 6.6	—	UV 220	LS 0.4
Cloxacillin Dicloxacillin Methicillin Nafcillin Oxacillin [12]	Serum (400 μl)	Acetonitrile	C_{18}	Acetonitrile—ammonium acetate, pH 5.6	—	UV 254	LS 0.5
Amoxicillin [13]	Plasma (1000 μl) Urine	Perchloric acid—dichloroethane	C_8	Methanol—phosphate buffer, pH 8	Post-column: imidazole and mercuric chloride	UV 310	LD 0.025

(Continued on p. 366)

TABLE 1 (continued)

Drug and reference	Body fluid or tissue (amount used)	Extraction and/or deproteinization	Stationary phase	Mobile phase	Pre- or post-column derivatization	Detection wavelength (nm)	Limit of detection (LD) or of sensitivity (LS) (μg/ml)
[14]**	Urine	Dilution	C_{18}	Methanol—dilute acetic acid	Post-column: fluorescamine	Fluorescence 395/485	LS 25
[15]	Serum (500 μl) Urine	Ultrafiltration	C_{18}	Methanol—phosphate buffer, pH 3.2	—	UV 227	LD 0.5
[16]	Plasma (1000 μl)	Sep-Pak C_{18} cartridges	C_8	Methanol—phosphate buffer, pH 7	—	UV 225	LS 0.5
Mecillinam [17]	Urine	Dilution	C_{18}	Acetonitrile—phosphate buffer, pH 5	—	UV 220	Not stated
Amoxicillin Ampicillin [18]	Plasma (100 μl) Saliva Urine	Perchloric acid	C_8	Methanol—phosphate buffer, pH 4.6	—	UV 225	LS 0.5
[19]**	Plasma (500 μl)	Trichloroacetic acid	C_{18}	Methanol—water	Pre-column: mercury bichloride solution	Fluorescence 345—355/420—435	LS 0.0005 (ampicillin) LS 0.01 (amoxicillin)
Ampicillin Mecillinam [20]	Plasma (1000 μl) Lymph Urine	Trichloroacetic acid	C_8	Methanol—phosphate buffer, pH 8	Post-column: imidazole and mercuric chloride	UV 310	LS 0.1

Azlocillin [21]**	Urine	Dilution	C_{18}	Acetonitrile—ammonium carbonate—potassium phosphate (gradient)	—	UV 220	Not stated
[22]	Serum (20 μl)	Acetonitrile	C_{18}	Methanol—phosphate buffer, pH 3.5	—	UV 220	LD 0.4
Azlocillin Mezlocillin [23]	Plasma (1000 μl)	Chloroform and tetrabutyl-ammonium phosphate	C_{18}	Acetonitrile—phosphate buffer, pH 7	—	UV 210—220	LS 0.7—0.5
[24]	Plasma (1000 μl)	Sep-Pak C_{18} cartridges	C_{18}	Acetonitrile—phosphate buffer, pH 7	—	UV 220	LS 1.3
Piperacillin [25]	Plasma (1 ml)	Chloroform—1-pentanol	C_{18}	Methanol—acetate buffer, pH 4.8	—	UV 254	LS 0.05
Ticarcillin [26]	Serum (200 μl) Urine	Ethyl acetate	C_{18}	Acetonitrile—phosphate buffer, pH 2.05	—	UV 210	LD 1
Carbenicillin Sulbenicillin [27]	Urine	Filtration	C_{18}	Methanol—tetrabutyl-ammonium bromide solution	—	UV 254	Not stated

(Continued on p. 368)

TABLE 1 (continued)

Drug and reference	Body fluid or tissue (amount used)	Extraction and/or deproteinization	Stationary phase	Mobile phase	Pre- or post-column derivatization	Detection wavelength (nm)	Limit of detection (LD) or of sensitivity (LS) (μg/ml)
Carbenicillin Nafcillin Piperacillin Ticarcillin [28]	Serum (250 μl)	Chloroform—butanol	Phenyl	Methanol—ammonium acetate, pH 4.2 (gradient)	—	UV 250	Not stated

*Simultaneous determination of the penicillin, the penicilloic acid and the 5-hydroxymethyl metabolite.
**Simultaneous determination of the penicillin and the penicilloic acid.

not have specific chromophores, and eluents must be monitored at low wavelengths where many endogenous compounds interfere. Penicillins are unstable compounds, sensitive to nucleophilic and electrophilic attacks catalysed by bases and acids, respectively. They can undergo degradation during the analysis process. Clinical specimen are generally stored at −70°C before analysis.

Penicillinase-resistant penicillins such as isoxazolyl penicillins and nafcillin can be extracted from body fluids into organic solvents of medium polarity. Chloroform [10] and methylene chloride [9, 11] have been used to extract these penicillins from serum, plasma or urine. Overall recoveries from serum were 91—100% [10] and 79—83% [9]. Procedures involving protein precipitation have been also described [9, 11, 12]. Rudrik and Bawdon [12] have removed the acetonitrile used as protein precipitant with methylene chloride. Penicillins were then concentrated in the aqueous phase as serum lipids have been extracted into the organic phase. These penicillinase-resistant penicillins have been chromatographed as anionic compounds on C_8 or C_{18} columns with pH 4.5—7 buffers and methanol or acetonitrile. All methods [9—12] use an internal standard (generally a penicillin of the same group). The procedures described by Thijssen [11] (only that including protein precipitation) and Teare et al. [9] (that including extraction) appear more difficult to be carried out because of instability of penicillins in the reagents used for sample pretreatment. However, the method of Thijssen [11] is the only one that permits the concurrent determination of the isoxazolyl penicillin metabolites: the 5-hydroxymethyl metabolites and the penicilloic acids.

Penicillins such as amoxicillin, ampicillin and mecillinam are amphoteric compounds, which makes their extraction into organic solvents difficult. Thus serum or plasma proteins have been removed by precipitation [13, 18, 20], ultrafiltration [15] or elution on a Sep-Pak C_{18} cartridge [16]. Automatic injection is precluded in the case of protein precipitation because these penicillins are unstable in samples diluted with the acids used as precipitants. The Sep-Pak C_{18} cartridge does not appear to be efficient because a large peak was present on the chromatogram. About 30 min per sample was required for the chromatography. Urine has been diluted before injection [14, 15, 17, 18, 20]. Interfering urine components have been extracted into *n*-amyl alcohol—dichloroethane [13] to decrease the sensitivity limit (200 ng/ml). The chromatography of these penicillins has been performed on C_8 or C_{18} columns with mixtures of methanol and phosphate buffers. UV detection permitted penicillin concentrations to be determined down to 0.5 μg/ml of plasma or serum [15, 16, 18]. This limit of sensitivity has been decreased after pre- or post-column derivatization. Imidazole and mercuric chloride have been added to the effluent [13, 20]. The reaction was performed in a reaction coil at 40°C for 8 min. Air segmentation was used to reduce band-spreading. The reaction was not complete; this can be a source of imprecision as no internal standard was used. A rapid deterioration of the column characteristics was observed as up to 200 μl of the protein-free supernatant were injected. Pre-column derivatization was performed by adding a mercury bichloride solution to deproteinized plasma samples [19]. The mixture was heated at 40—50°C for 25 min. An ethyl acetate extract was then chromatographed and the fluorescence was measured. A post-column derivatization procedure [14] has

been also described for the determination of amoxicillin in urine. The reaction of derivatization was rapid but two separate pumps were required to add a buffer (pH 11.4) and the reagent, fluorescamine. The method cannot be applied to measure plasma levels due to its lack of sensitivity. None of these methods used internal standardization during sample pre-treatment.

Several HPLC methods have been reported for the determination of ureidopenicillins in body fluids [21—25, 28]. The main difference among these methods is whether proteins are removed by precipitation [22] or elution on a Sep-Pak cartridge [24], or the penicillin is extracted at acidic pH [25, 28]. An ion-pair extraction with tetrabutylammonium phosphate has been also described [23]: the absolute recovery was only 50% and the coefficients of variation obtained were in the range 4—27%. The two-step extraction procedure described by Brisson and Fourtillan [25] appears to be the most sensitive method. It permits the determination of piperacillin in plasma at concentrations as low as 50 ng/ml. Piperacillin was extracted into chloroform—pentanol and back-extracted into pH 7 phosphate buffer with a recovery of 84%. Gau and Horster [21] have described a procedure which allows the concurrent determination of azlocillin and its penicilloic acid metabolite in urine. A gradient elution was used to reduce the analysis time owing to the different polarities of both compounds. The method described by Annesley et al. [28] allows the simultaneous determination of different cephalosporins and penicillins in serum. Piperacillin, ticarcillin, carbenicillin and nafcillin have been determined by this method after extraction into chloroform—butanol. A gradient elution was used to allow rapid measurement of drugs. Another method has been described for the assay of ticarcillin using carbenicillin as internal standard [26]. The drugs have been extracted into ethyl acetate which was then evaporated. The absolute recoveries from serum were 76% for both compounds. Sharp and symmetrical peaks were obtained using a C_{18} column with an acidic eluent (pH 2.05) whereas ion-pair chromatography with tetrabutylammonium bromide resulted in two incompletely resolved peaks for carbenicillin and a broad peak for sulbenicillin, a chemically related compound [27]. This was attributed to the presence of diastereomers.

3. CEPHALOSPORINS

Cephalosporins are a family of β-lactam antibiotics commonly used for the treatment of infections caused by gram-positive and gram-negative bacilli. They are produced semisynthetically: various side-chains are substituted at C_3 and C_7 of 7-aminocephalosporanic acid (Fig. 2). They are highly polar and water-soluble compounds. In addition to the carboxylic group ($pK_a = 2—4$) of the 7-aminocephalosporanic acid, they can possess other ionizable groups (other acidic and/or amino groups).

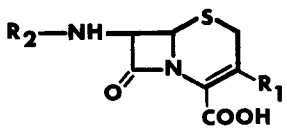

Fig. 2. Common nucleus of cephalosporins.

3.1. HPLC procedures applied to one or two cephalosporins

In recent years, a considerable number of HPLC procedures have been reported for the determination of cephalosporins in body fluids (Table 2). They are mostly based on plasma or serum protein precipitation and urine dilution before injection. Methanol or acetonitrile are often preferred to the strong precipitants trichloroacetic acid and perchloric acid. Proteins have been removed by ultrafiltration for cefmenoxime [39] and cefsulodin [60], but these procedures require large plasma volumes (1—2 ml). Samples have been purified on Sep-Pak cartridges or anion-exchange columns only for cefoperazone [44], moxalactam [76] and ceftizoxime [66]. Moxalactam and cefazolin have been extracted into isoamyl alcohol—chloroform [36] and moxalactam into ethyl acetate [79].

Cephalosporins have been chromatographed on reversed-phase packings, octadecylsilane bonded phase materials being mostly employed. Various mobile phases have been used consisting of methanol or acetonitrile as organic modifier and often weakly acidic phosphate or acetate buffers. Ion-pair chromatography has also been employed with pH 6—8 eluents by formation of ion pairs between the anionic form of the cephalosporin and generally tetrabutylammonium.

UV detection has been performed at 254 nm and for slightly higher sensitivity at 228—280 nm depending on the absorption maximum of the investigated cephalosporin. Sensitive determinations have been carried out with post-column derivatization with fluorescamine [34] or pre-column derivatization in the presence of hydrogen peroxide [19]. The eluent fluorescence was then monitored.

β-Lactam antibiotics are labile in weak basic and strong acid media. Some cephalosporins have been found to degrade during analysis process. Such problems have been circumvented by addition of pH 5—6.5 buffers to plasma or serum samples before freezing and/or to deproteinized samples before automatic injection for cefmenoxime [39], cefoxitin [58], cefsulodin [50, 60] and moxalactam [77]. Ethylenediaminetetraacetic acid was added in plasma and urine samples containing cefpimizole [43]. Moreover, plasma and urine samples have been stored at −70°C before analysis for cefotaxime [54], ceftazidime [63, 65] and moxalactam [78].

3.2. HPLC procedures applied to several cephalosporins

The procedures reported above are specific for the determination of one or two cephalosporins. A particular octadecylsilane packing is used with a particular eluent composition for each procedure. However, cephalosporins have similar physicochemical properties owing to their common 7-amino-cephalosporanic acid nucleus, and a few methods which permit to determine several cephalosporins with minor modifications from one cephalosporin to another have been recently described. Nygard and Wahba Khalil [80] have reported a simple and rapid procedure which includes an internal standard. The method involves plasma protein precipitation, elution on a C_{18} column and detection at 254 nm. The eluent was a mixture of various amounts of

TABLE 2

HPLC OF CEPHALOSPORINS

Drug and reference	Body fluid or tissue (amount used)	Extraction and/or deproteinization	Stationary phase	Mobile phase	Pre- or post-column derivatization	Detection wavelength (nm)	Limit of detection (LD) or of sensitivity (LS) (μg/ml)
Cefaclor [29]	Plasma (100 μl) Urine	Methanol	C_{18}	Methanol—water—acetic acid	—	UV 254	LD 0.5
Cefadroxil [30]	Plasma (1000 μl)	Acetonitrile	C_{18}	Methanol—acetate buffer, pH 4.8	—	UV 240	LD 0.15
Cefamandole [31]	Plasma (200 μl) Urine	Methanol—sodium acetate	C_{18}	Methanol—sodium acetate, pH 5.2	—	UV 270	LD 0.3
Cefatrizine [32, 33]	Serum (200 μl) Urine	Trichloroacetic acid	C_{18}	Methanol—acetonitrile [33]—phosphate buffer, pH 5	—	UV 254	LS 1
[34]	Serum (200 μl) Urine	Trichloroacetic acid	C_{18}	Acetonitrile—phosphate buffer, pH 7	Post-column: fluorescamine	Fluorescence 395/460	LS 0.1
Cefazolin [35]	Serum (500 μl)	Sodium tungstate—sulphuric acid	C_{18}	Methanol—acetic acid, pH 3	—	UV 272	Not stated
[36]*	Serum (500 μl)	2-Propanol + isoamyl alcohol in chloroform	Phenyl	Acetonitrile—tetra-butylammonium salt—phosphate buffer, pH 7.6	—	UV 270	LS 1

Cefmenoxime [37, 38]	Plasma Serum (200 μl) Urine	Acetonitrile	C_{18} [37] Phenyl [38]	Acetonitrile—dilute acetic acid [37] or phosphoric acid [38]	—	UV 254	LD 0.3—0.2
[39]	Plasma (1000 μl)	Ultrafiltration	C_{18}	Acetonitrile—acetate buffer, pH 5.3	—	UV 254	LD 0.05
[40]	Serum (500 μl) Urine	Methanol	C_{18}	Acetonitrile—acetate buffer, pH 4.4	—	UV 254	LD 0.05
[41]	Serum Plasma (500 μl) Urine	Perchloric acid	CN	Acetate buffer, pH 3.8	—	UV 254	LS 0.5
Cefmetazole [42]	Serum (50 μl)	Methanol—trichloroacetic acid	C_{18}	Acetonitrile—citrate buffer, pH 5.4	—	UV 254	LS 0.4
Cefpimizole [43]	Plasma (1000 μl) Urine	Acetonitrile and methylene chloride	C_{18}	Methanol—tetrabutylammonium—ethylenediamine-tetraacetic acid—acetic acid, pH 6	—	UV 254	LS 0.3 LD 0.05
Cefoperazone [44]	Serum (2000 μl)	Sep-Pak cartridges	C_{18}	Methanol—water	—	UV 228	LD 0.25
[45, 46]	Serum (100 μl [45]) (500 μl [46]) Tissue	Methanol [45] Acetonitrile [46]	C_{18} [46] Phenyl [45]	Acetonitrile—phosphate buffer—tetrabutylammonium [45]	—	UV 254	LD 1 [45] Not stated [46]
[47, 48]	Plasma or serum (100 μl) Urine CSF [47]	Methanol	C_{18}	Acetonitrile—triethylamine—acetic acid (gradient elution) [48]	—	UV 254	Not stated [47] LD 1 [48]

(Continued on p. 374)

TABLE 2 (continued)

Drug and reference	Body fluid or tissue (amount used)	Extraction and/or deproteinization	Stationary phase	Mobile phase	Pre- or post-column derivatization	Detection wavelength (nm)	Limit of detection (LD) or of sensitivity (LS) (μg/ml)
Cefotetan [49]**	Plasma (200 μl)	Acetonitrile	C_{18}	Acetonitrile—tetrabutylammonium bromide—phosphate buffer, pH 6.4	—	UV 280	LD 0.02
Cefotiam [50, 51]***	Plasma or serum (200 μl) Blood [51] Urine [50]	Methanol [50] Acetonitrile[51]	C_{18}	Methanol—tetrabutylammonium phosphate	—	UV 254 [50] 280 [51]	LS 0.5 [50] Not stated [51]
[50]	Plasma (200 μl) Urine	Trichloroacetic acid	C_{18}	Methanol—phosphate buffer, pH 8	—	UV 254	LS 0.2
[40]	Plasma (500 μl) Urine	Methanol	C_{18}	Acetonitrile—acetate buffer, pH 4.4	—	UV 254	LD 0.1
Ceforanide [52]	Plasma (500 μl)	Trichloroacetic acid + methylene chloride	C_{18}	Methanol—ammonium acetate buffer, pH 4	—	UV 254	LS 5
Cefotaxime [53—55] §	Serum (1000 μl) [53, 55], (100—300 μl) [54] Urine [55] Bile [54]	Trichloroacetic acid [53]—perchloric acid [54] Chloroform—acetone [55]	C_{18} [54, 55] C_8 [53]	Methanol or acetonitrile—phosphoric acid [53]—sodium dihydrogen phosphate [54]—acetic acid [55]	—	UV 310 [53] 254 [54] 262 [55]	LD 0.3 to 0.5
[50] §§	Plasma (200 μl) Urine	Methanol	C_{18}	Methanol—tetrabutylammonium salt—phosphate buffer	—	UV 254	LS 0.5

Cefoxitin [56—58]	Serum Plasma (100—200 μl) [56, 58] (500 μl) [57] Urine [58] CSF [56]	Ethanol [56] Trichloroacetic acid [57, 58]	C_{18}	Methanol or acetonitrile—ammonium carbonate [56]—phosphate buffer [57, 58]—acetic acid [57]	—	UV 238 [56] 235 [57] 254 [58]	LS 1 [56, 58] LD 1 [57]
[59]	Bile (1000 μl)	Acetate buffer, pH 4.6	C_8	Acetonitrile—water	—	UV 235	Not stated
Cefroxadin [50]	Plasma (200 μl) Urine	Trichloroacetic acid	C_8	Methanol—phosphoric acid	—	UV 254	LS 0.5
Cefsulodin [60]	Plasma (2000 μl)	Ultrafiltration	C_{18}	Acetonitrile—ammonium acetate buffer, pH 4.2	—	UV 254	LS 0.8
[61, 40]	Plasma (100 μl) [61] Serum (500 μl) [40] Urine	Methanol	C_8 [61] C_{18} [40]	Acetonitrile—ammonium acetate buffer, pH 5.2 [61]—acetate buffer, pH 4.4 [40]	—	UV 265 [61] 254 [40]	LD 1 [61] LD 0.2 [40]
[50, 51] §§§	Plasma or serum (200 μl) Blood [51] Urine [50]	Methanol [50] Acetonitrile [51]	C_8 [50] C_{18} [51]	Methanol—tetrabutylammonium phosphate	—	UV 254 [50] 280 [51]	LS 0.5 [50] Not stated [51]
Ceftazidime [62]	Plasma Serum (250 μl) Urine	Perchloric acid	C_{18}	Acetonitrile—ammonium dihydrogen phosphate—formic acid	—	UV 257	LS 0.6

(Continued on p. 376)

TABLE 2 (continued)

Drug and reference	Body fluid or tissue (amount used)	Extraction and/or deproteinization	Stationary phase	Mobile phase	Pre- or post-column derivatization	Detection wavelength (nm)	Limit of detection (LD) or of sensitivity (LS) (μg/ml)
[63—65]	Serum (100—150 μl) Urine CSF [65]	Methanol	C_{10} [63] C_{18} [64, 65]	Methanol or acetonitrile—ammonium dihydrogen phosphate, perchloric acid [63]—phosphate buffer [64]—acetic acid [65]	—	UV 254—257	LD 0.3 [63] LS 1 [64] LD 0.5 [65]
Ceftizoxime [66]	Serum (500 μl)	Anion-exchange column	C_{18}	Acetonitrile—acetic acid, pH 2.8	—	UV 270	Not stated
[67]	Serum (500 μl) Urine Bile	Acetonitrile	Phenyl	Acetonitrile—phosphate buffer, pH 2.6	—	UV 254	LS 2.5
Ceftriaxone [68]	Plasma (250 μl) Urine Saliva	Acetonitrile	NH_2	Acetonitrile—ammonium carbonate solution	—	UV 274	LD 0.4
[69]	Serum (200 μl) Urine CSF	Acetonitrile	C_8	Acetonitrile—hexadecyltrimethylammonium bromide—phosphate buffer, pH 7	—	UV 280	LS 0.5
Cefuroxime [70]	Serum (1000 μl)	Dimethylformamide	C_{18}	Methanol—aqueous acetic acid	—	UV 280	LS 1
[71]	Plasma (100 μl)	Perchloric acid	C_8	Methanol—potassium dihydrogen phosphate	—	UV 278	LS 0.5

[50]	Plasma (200 μl) Urine	Methanol	C_{18}	Methanol—tetrabutyl-ammonium salt—phosphate buffer	—	UV 254	LS 0.5
Cephalexin [72, 73, 50]	Plasma (100—500 μl) Urine Saliva [73]	Methanol [72, 73] Trichloroacetic acid [50]	C_{18} [72, 73] C_8 [50]	Methanol—acetic acid [72, 73]—phosphoric acid [50]	—	UV 254	LD 0.1 [72], 0.2 [73] LS 0.5 [50]
[19]	Plasma (500 μl)	Trichloroacetic acid	C_{18}	Methanol—water	Pre-column: hydrogen peroxide	Fluorescence 345/420	LS 0.002
Cephradine [19]	Plasma	Trichloroacetic acid	C_{18}	Methanol—water	Pre-column: hydrogen peroxide	Fluorescence 345/420	LS 0.01
[74]	Serum (1000 μl) Urine	Trichloroacetic acid	C_{18}	Methanol—acetate buffer	—	UV 264	Not stated
Moxalactam [75]**	Serum (not stated)	Ammonium sulphate	C_{18}	Methanol—dilute nitric acid	—	UV 254	Not stated
[76]**	Urine	Sep-Pak cartridges	C_{18}	Methanol—tetrabutyl-ammonium salt—phosphate buffer, pH 6	—	UV 254	LD 10
[36]*	Serum (500 μl)	2-propanol + iso-amyl alcohol in chloroform	Phenyl	Acetonitrile—tetra-butylammonium salt—phosphate buffer, pH 7.6	—	UV 270	LS 1
[77]** [78]	Plasma Serum (200—500 μl) Urine CSF	Methanol	C_{18}	Acetonitrile or methanol—ammonium acetate [77]—phosphate buffer, pH 6.5 [78]	—	UV 270 [77] 230 [78]	LD 1.5 [77] Not stated [78]

TABLE 2 (continued)

Drug and reference	Body fluid or tissue (amount used)	Extraction and/or deproteinization	Stationary phase	Mobile phase	Pre- or post-column derivatization	Detection wavelength (nm)	Limit of detection (LD) or of sensitivity (LS) (μg/ml)
[79]**	Plasma (500 μl) Urine	Hydrochloric acid + ethyl acetate	C_{18}	Acetonitrile–ammonium acetate buffer, pH 6.5	—	UV 275	LD 0.5

*Concurrent determination of cefazolin and moxalactam.
**Concurrent determination of the two epimers.
***Concurrent determination of cefotiam and either cefsulodin [50, 51], or cefotaxime [50], or cefuroxime [50].
§Concurrent determination of cefotaxime and desacetylcefotaxime.
§§Concurrent determination of cefotaxime and cefotiam.
§§§Concurrent determination of cefsulodin and cefotiam.

acetonitrile and pH 4.7 phosphate buffer. The reproducibility and accuracy of the method were shown over the range 5—500 μg/ml of plasma. A similar method has been described [81], which permits the accurate determination of different cephalosporins down to 0.2—0.5 μg/ml of plasma, such sensitivity being adequate for pharmacokinetic studies. The eluent was then a mixture of various amounts of methanol and dilute sulphuric acid. Such an acidic eluent (pH 2.2) was selected as it resulted in good retention of both acidic and amphoteric cephalosporins. The method was used in routine analysis with no degradation of the octadecyl silica as more than 500 injections could be performed with the same column.

A more time-consuming two-step extraction procedure has been described [82]. Non-ionized cephalosporins were extracted into chloroform—1-pentanol at acidic pH, then anionic cephalosporins were back-extracted into pH 7 phosphate buffer. The chromatography step (C_{18} column, methanol or acetonitrile—pH 4.8 acetate buffer) was facilitated owing to the negligible solvent front obtained. The quantitation (UV: 234—275 nm) was linear down to 0.075—0.15 μg/ml. A modification of this procedure has been proposed by Annesley et al. [28]. An internal standard was included, only 250 μl of plasma were used instead of 1 ml, the organic extract was evaporated and then reconstituted in ammonium acetate, and a mobile phase gradient was used. Amphoteric cephalosporins cannot be determined by these procedures since they are protonated at low pH and cannot be extracted.

As reported by White and Laufer [83], microbore systems should be applicable and advantageous for the determination of cephalosporins.

4. AZTREONAM (MONOBACTAM)

Aztreonam is a new, synthetic, β-lactam antibiotic in the recently defined family of monobactam antimicrobial compounds. Aztreonam possesses potent antibacterial activity against a broad spectrum of aerobic gram-negative bacteria.

Recently, an HPLC method [84] has been suggested for the determination of aztreonam in serum and urine. The method includes serum protein precipitation with acetonitrile (with almost quantitative recovery) or urine dilution, ion-pair chromatography on an octadecylsilane bonded phase column and detection at 293 nm. The limits of quantitation were 1 μg/ml in serum and 5 μg/ml in urine. A good agreement was found between the results obtained by this method and by a microbiological assay.

5. AMINOGLYCOSIDES

Aminoglycosides are highly effective agents against gram-negative infections. They are highly polar, water-soluble compounds. They possess several primary amino groups. They are weakly basic (pK_a between 7.5 and 8) and not metabolized to any extent.

5.1. Immunoassays

Radioimmunoassays have been widely utilized for the determination of aminoglycosides and various kits are commercially available. The techniques

proposed for the different aminoglycosides have been reviewed by Maitra et al. [85]. At present, there is a trend leading away from radioimmunoassays to non-isotopic immunoassays. Numerous non-isotopic immunoassays have been recently proposed for the determination of aminoglycosides in serum: EIA for gentamicin and tobramycin [86—90] and netilmicin [91—93], FIA for gentamicin [94—97], FPIA for gentamicin, tobramycin and amikacin [98, 99] and streptomycin [100], SLFIA for gentamicin [103], amikacin [101], dibekacin [102] and netilmicin [104], and LAIA for gentamicin [105, 106] and tobramycin [107]. These methods are rapid, they do not require a separation step and some of them have been automated [88, 92, 97, 99, 100]. Some kits are commercially available [86—93, 104]. Up to 100 assays per hour can be performed for the determination of gentamicin, tobramycin or netilmicin when the EIA assay is adapted to a microcentrifugal analyser [88, 92]. Small amounts of serum are required: 20—75 μl. Coefficients of variation of less than 10% are generally obtained except for the LAIA procedure which gives rise to coefficients of variation of less than 14% [105]. However, in most cases, non-linear standard curves are obtained. Aminoglycoside concentrations down to 0.5 μg/ml of serum can be determined. The results obtained correlate well with those obtained by radioimmunoassay procedures. A cross-reactivity with structurally related aminoglycosides occurs but drugs likely to be administered concurrently do not interfere. However, for the photometric end point procedures, various factors including bilirubin, haemoglobin and lipids or other light-scattering materials such as immune complexes, may potentially interfere when they are abnormally present. Endogenous chromophores may reduce FIA results by light-absorption effects and endogenous enzymes may influence EIA results. Witebsky and Selepak [87] have nevertheless shown that the EIA procedure is accurate for the determination of gentamicin in serum in the presence of bilirubin up to bilirubin concentrations of 45 mg/dl. Furthermore, the immunoassay results are influenced by lot-to-lot variations. So, the current gentamicin SLFIA has been modified by substituting monoclonal antibodies for conventional antiserum to gentamicin [103]. This reduces the possibility of lot-to-lot variation.

5.2. Gas—liquid chromatography

Mayhew and Gorbach [108, 109] have described a GLC procedure for the determination of aminoglycosides in serum. The method involves a time-consuming two-stage derivatization. Linear standard curves were obtained in the range 1.3-12.5 μg/ml with an electron-capture detector.

5.3. High-performance liquid chromatography

HPLC has been also utilized for the determination of aminoglycosides (Table 3). However, the detection is severely limited because the extinction coefficient of aminoglycosides is not optimum for sensitive UV detection. This has been circumvented by derivatizing the primary amino groups to yield fluorophores or chromophores.

Aminoglycosides are poorly soluble in non-polar organic solvents and their

TABLE 3

HPLC OF AMINOGLYCOSIDES

Drug and reference	Body fluid or tissue (amount used)	Extraction and/or deproteinization	Stationary phase	Mobile phase	Pre- or post-column derivatization	Detection wavelength (nm)	Limit of detection (LD) or of sensitivity (LS) (μg/ml)
Amikacin [110]	Serum (1000 μl)	Silica gel column	C_{18}	Methanol—acetonitrile—dilute tripotassium ethylenediaminetetra-acetate	Pre-column: OPA	Fluorescence 350/415	LS 1
[111]	Plasma Serum (25 μl)	Methanol	C_{18}	Acetonitrile—water	Pre-column: FDNB	UV 360	Not stated
[112]	Serum (50 μl)	Acetonitrile	C_8	Acetonitrile—phosphate buffer, pH 3.0	Pre-column: TNBS	UV 340	LD 0.5
Gentamicin [113]*	Plasma Serum (50—100 μl)	Acetonitrile	C_{18}	Acetonitrile—water	Pre-column: dansyl chloride	Fluorescence 220/470	LS 0.145
[114]*	Serum (50 μl)	Acetonitrile	C_{18}	Acetonitrile—water—tris(hydroxymethyl)-aminomethane, pH 7	Pre-column: FDNB	UV 365	LS 0.33
[115]	Serum (500 μl)	Acetonitrile	C_{18}	Acetonitrile—methylene chloride—water—methanol	Pre-column: BSC	UV 230	LS 0.2
[116]**	Serum (20 μl)	Methanol	C_{18}	Acetonitrile—disodium 1,2-ethanedisulphonate—sodium octane sulphonate—dilute acetic acid, pH 3.5	Post-column: OPA	Fluorescence 365/440	LS 0.5

(Continued on p. 382)

TABLE 3 (continued)

Drug and reference	Body fluid or tissue (amount used)	Extraction and/or deproteinization	Stationary phase	Mobile phase	Pre- or post-column derivatization	Detection wavelength (nm)	Limit of detection (LD) or of sensitivity (LS) (μg/ml)
[117]**	Plasma Serum (100 μl) Urine	Acetonitrile	C_8	Acetonitrile—tris-(hydroxymethyl)amino-methane—dilute hydro-chloric acid, pH 3	Pre-column: OPA	Fluorescence 340/418	LS 0.5
[118]	Plasma (100 μl) Urine	Acetonitrile	SCX	Acetonitrile—dilute phosphoric acid	Pre-column: fluorescamine	Fluorescence 275/418	LS 1
Tobramycin [119]	Serum (1000 μl)	Silica gel column	C_{18}	Methanol—dilute tri-potassium ethylene dinitrilotetraacetic acid	Pre-column: OPA	Fluorescence 365/420	LS 0.5
[120]	Serum (250 μl) Urine	Acetonitrile	CN	Methanol—acetonitrile—dilute tripotassium ethylenediaminetetra-acetic acid, pH 6.5	Pre-column: OPA	Fluorescence 340/418	LD 0.2
[121]	Serum (50 μl)	Acetonitrile	C_{18}	Acetonitrile—dilute acetic acid	Pre-column: FDNB	UV 365	LS 0.5
Amikacin Gentamicin Tobramycin [122]**	Serum (400 μl)	Cation-exchange column	C_{18}	Methanol—sodium pentane sulphonate—acetic acid—sodium sulphate	post-column: OPA	Fluorescence 340/418	Not stated
Amikacin Kanamycin [123]	Serum (200 μl)	Cation-exchange column	C_8	Heptane sulphonic acid (other components are not stated)	Post-column: OPA	Fluorescence Not stated	Not stated

Amikacin Dibekacin Gentamicin Netilmicin Sisomicin Tobramycin [124]***	Serum (20 μl)	Column switching	C_{18}	Methanol—dilute sodium acetate, pH 7.4	Pre-column: OPA	Fluorescence not stated/ 418	LS 1
Gentamicin Netilmicin Tobramycin [125]**	Serum (100—200 μl)	Acetonitrile	C_{18}	Methanol—triethyl-amine—Tris buffer, pH 7.9	Pre-column: OPA	Fluorescence not stated	LS 0.5
Gentamicin Sisomicin [126]*	Serum (50 μl)	Acetonitrile	C_{18}	Acetonitrile—dilute acetic acid	Pre-column: FDNB	UV 365	LS 0.5

*The four major components of gentamicin were eluted in two peaks.
**The four major components of gentamicin were eluted in three peaks.
***The four major components of gentamicin were eluted in four peaks.

extraction from body fluids is difficult. Direct derivatization is obstructed by albumin, therefore serum proteins have been removed by precipitation [111—118, 120, 121, 125, 126] or elution on small ion-exchange [122, 123] or silica gel [110, 119] columns. Proteins have been mostly precipitated with acetonitrile after dilution with an alkaline buffer to avoid co-precipitation of the aminoglycosides. The acetonitrile and interfering substances have been removed with methylene chloride. The drugs were then concentrated in the aqueous phase. Most methods require only 100 μl of sample or less.

When post-column derivatization has been applied, the protein-free supernatant has been directly injected onto a C_{18} column. Aminoglycosides are highly polar compounds and consequently little retained on alkyl bonded phase columns. Therefore, they have been eluted as ion pairs with alkylsulphonates to increase their retention. Alkylsulphonate reagent has been also added to the sample to be injected to eliminate split peaks [116, 122]. After pre-column derivatization, the separation step is easier to perform. The derivatives, much less polar than aminoglycosides, have been chromatographed in reversed-phase mode with eluents containing high percentages of organic modifier.

The derivatizing reagents that have been employed are as follows.

5.3.1. Fluorescence detection

5.3.1.1. o-Phthalaldehyde (OPA) [110, 116, 117, 119, 120, 122—125]. The reaction of derivatization is rapid, thereby allowing pre-column [110, 117, 119, 120, 124, 125] or post-column [116, 122, 123] derivatization. 2-Mercaptoethanol was added with OPA. Brij-35 solution was also added in the post-column mode to minimize the background noise. Pre-column derivatization has been performed in deproteinized samples and followed by extraction of the derivatives before injection [117, 120, 125]. It has also been performed on a silica gel column [110, 119], but amikacin and tobramycin were unstable in the eluate and gave rise to several derivatives. Heat treatment of the eluate (50—60°C for 5—10 min) permitted only one derivative for each drug to be obtained. Thereafter rapid cooling prevented a decrease in fluorescence.

Recently, a fully automated method has been reported for the determination of six aminoglycosides in serum [124]. The procedure involves sample clean-up on a short reversed-phase column, subsequent derivatization with OPA on this column, and on-line separation of derivatives on a reversed-phase column by column switching. Three pumps were required to deliver the rinsing solvent, the derivatizing reagent and the mobile phase.

5.3.1.2. Fluorescamine [118] and Dns chloride [113]. Pre-column derivatization procedures have been reported. Heat treatment is necessary only with Dns chloride (75°C for 15 min). Derivatized samples have been directly injected [118] or first extracted into ethyl acetate [113].

5.3.2. Ultraviolet detection

5.3.2.1. 1-Fluoro-2,4-dinitrobenzene [111, 114, 121, 126]. The derivatization step is time-consuming (80°C for 30—45 min) and consequently performed in the pre-column mode. The reaction was not complete after 45 min of incubation but the gentamicin derivative was found to be stable in derivatized samples within 4—5 h after derivatization [114]. The derivatives obtained are soluble

only in semi-polar organic solvents and cannot be extracted from the mixture. They have been injected directly onto the column [114, 121, 126] or purified on silica gel before injection [111].

5.3.2.2. Trinitrobenzene sulphonic acid [112] and benzene sulphonyl chloride [115]. These reagents have been utilized for pre-column derivatization. Heat treatments, 30 min at 70°C [112] and 10 min at 75°C [115], are required. The derivatives have been purified on a Bond-Elut C_{18} extraction column [112] or extracted into methylene chloride before injection [115].

6. CHLORAMPHENICOL AND THIAMPHENICOL

Chloramphenicol is the first broad-spectrum antibiotic discovered with activity against a host of aerobic and anaerobic, gram-positive and gram-negative bacteria. Thiamphenicol is a structurally related compound with a similar antimicrobial spectrum. Both are soluble in organic solvents. Chloramphenicol is only slightly soluble in aqueous buffers, thiamphenicol is a more soluble weak base.

6.1. Immunoassays and gas—liquid chromatography

A competitive enzyme-linked immunoassay has been described for the determination of chloramphenicol in serum [127]. The assay is specific and sensitive (limit of detection 1 ng/ml) but it requires at least 7 h to be performed. GLC methods using electron-capture [128, 129] or mass spectrometric [128] detectors have been also described. These procedures are accurate, highly specific and sensitive (limit of quantitation 5 ng/g of tissue with electron-capture detection). However, they require tedious sample pre-treatment: extraction and derivatization with Trisil.

6.2. High-performance liquid chromatography

Numerous rapid and simple HPLC methods have been described for the determination of chloramphenicol in biological fluids (Table 4). The essential difference among these methods is that either drug extraction or protein precipitation is applied before injection. Chloramphenicol, which is about 50% bound to plasma proteins [144], partly precipitates when using a very efficient precipitant: 63% of chloramphenicol was recovered after trichloroacetic acid precipitation [143], compared with about 100% after acetonitrile precipitation [140, 142]. Diethyl ether and ethyl acetate have been mainly used to extract chloramphenicol. After evaporation and reconstitution of the diethyl ether extract, the total recovery was only 51% when the sample was previously buffered at pH 3 [136], whereas the recovery was almost quantitative when the sample was buffered at pH 10.4 [137]. Almost quantitative recoveries were obtained after extraction of chloramphenicol into ethyl acetate at pH 4.6 [131, 134]. Chloramphenicol has been essentially chromatographed on C_{18} columns and then detected at 254 nm or 270—280 nm where it has an absorption maximum [141]. Only a few methods [130, 139] do not use internal standardization. Most methods require small plasma or serum volumes

TABLE 4

HPLC OF CHLORAMPHENICOL

Drug and reference	Body fluid or tissue (amount used)	Extraction and/or deproteinization	Stationary phase	Mobile phase	Detection wavelength (nm)	Limit of detection (LD) or of sensitivity (LS) (μg/ml)
Chloramphenicol [130, 131, 132, 133*, 134*]	Serum or plasma (500 μl [130], 50–100 μl [131–134]) CSF and urine [133]	Ethyl acetate	C_{18}	Acetonitrile or methanol–acetate buffer, pH 5.3–6.4 [131, 133, 134] or water [130, 132]	UV 270–280 [131–134] 254 [130]	LS 7.5 [131] LD 2 [132] LS 1 [134] Not stated [130, 133]
[135, 136* [137]	Serum or plasma (50–200 μl [135, 137]) (400 μl [136]) CSF and urine [135]	Diethyl ether	C_{18} [135, 137] C_8 [136]	Methanol–water [135, 137] or dilute phosphoric acid [136]	UV 278–280	LS 1 [135] Not stated [136] LS 5 [137]
[138]	Serum or plasma (100 μl) (CSF)	Chloroform–isopropanol	C_{18}	Acetonitrile–acetate buffer, pH 4.0	UV 280	LS 0.1–0.2
[139, 140, 141, 142*]	Serum or plasma (25–50 μl [140–142]) (200 μl [139]) CSF [141]	Acetonitrile	C_{18}	Methanol or ethanol phosphate buffer, pH 2.5 [139], pH 7 [140], pH 3 [141] or dilute acetic acid [142]	UV 278–280 [139, 140] 254 [141, 142]	LD 0.5 [139, 142] LS 1 [141] LS 0.5 [140]
[143]*	Plasma (250 μl) Urine	Trichloroacetic acid	C_{18}	Acetonitrile–acetate buffer, pH 5.7	UV 275	Not stated

*Simultaneous assay of chloramphenicol and chloramphenicol succinate.

(100 μl or less) and can be used to monitor chloramphenicol in paediatric patients.

Chloramphenicol is intravenously administered as its succinate ester which is partly hydrolysed in the body to free chloramphenicol. Several procedures [133, 134, 136, 142, 143] permit chloramphenicol and its ester to be assayed simultaneously. Three of these methods [133, 134, 143] allow the simultaneous determination of both chloramphenicol 1-monosuccinate and chloramphenicol 3-monosuccinate which exist in equilibrium in solution. The glucuronide metabolite of chloramphenicol is eluted near the solvent front [131, 145] and would not interfere with any assay. The method described by Aravind et al. [145] permits the analysis of chloramphenicol glucuronide in urine after incubation of urine samples for 3 h at 37°C with β-glucuronidase.

An HPLC procedure has been described for the determination of thiamphenicol in serum and CSF [146]. It involves protein precipitation with methanol, reversed-phase chromatography and UV detection at 254 nm.

7. MACROLIDE ANTIBIOTICS

Macrolide antibiotics are high-molecular-weight drugs soluble in organic solvents. A few HPLC procedures have been described for the quantitation of erythromycin and rosaramicin in body fluids (Table 5). An ether extract was chromatographed on a reversed-phase column with acetonitrile and/or methanol and a buffer pH 6.7—7 for erythromycin [147—149] and pH 4.0 for rosaramicin [150]. The method described by Tsuji [147] involves post-column derivatization with naphthotriazole disulphonate followed by on-line extraction of the excess of reagent into chloroform and fluorescence detection. This method requires complex instrumentation. The column is maintained at 70°C with subsequent risk of dissolution of the bonded phase material and a large serum volume (1—3 ml) is required. This method is nevertheless the only one that permits the simultaneous determination of erythromycin ethylsuccinate, a prodrug of erythromycin. Others [148, 149] have monitored the column effluent by using a dual-electrode electrochemical detector in the oxidative screen mode. Rosaramicin was quantitated by UV detection [150].

8. RIFAMPICIN (RIFAMYCIN)

Rifampicin is a semisynthetic antibiotic with a wide spectrum of antibacterial activity. It is established as a first-line antituberculosis agent.

A simple and sensitive HPLC method [151] has been described for the simultaneous determination of rifampicin, 25-desacetylrifampicin, 3-formylrifamycin and 3-formyl-25-desacetylrifamycin in plasma, saliva and urine. An organic extract was injected onto a silica gel column. The yields of extraction into isooctane—dichloromethane were 96% for rifampicin and 72% for the main metabolite 25-desacetylrifampicin. UV detection was performed at 254 nm. A good baseline separation was obtained for the four peaks and the limit of quantitation was 0.1 μg/ml. However, as silica columns are easily affected by the humidity of solvents, which results in variations in retention times, two other methods have been elaborated for the assay of rifampicin and 25-des-

TABLE 5

HPLC OF MACROLIDE ANTIBIOTICS

Drug and reference	Body fluid or tissue (amount used)	Extraction and/or deproteinization	Stationary phase	Mobile phase	Pre- or post-column derivatization	Detection wavelength (nm)	Limit of detection (LD) or of sensitivity (LS) (μg/ml)
Erythromycin [147]	Serum (1000–3000 μl)	Diethyl ether	C_{18}	Acetonitrile–ammonium acetate–water, pH 7.0	Post-column: naphthotriazole disulphonate	Fluorescence 360/440	LD 0.01
[148]	Plasma (200 μl) Urine	Diethyl ether	C_{18}	Acetonitrile–methanol–sodium acetate buffer pH 6.7	—	Electrochemical, dual electrode +0.7 V, +0.9 V	LD 0.03
[149]	Serum (250 μl)	Methyl *tert.*-butyl ether	Diphenyl	Acetonitrile–methanol–sodium perchlorate–ammonium acetate, pH 7.0	—	Electrochemical, dual electrode 0.7 V, 0.8 V	LS 0.05
Rosaramicin [150]	Serum Plasma (1000 μl)	Diethyl ether	C_{18}	Acetonitrile–acetate buffer, pH 4	—	UV 254	LS 0.01

acetylrifampicin [152, 153]. Both methods use reversed-phase columns but they involve time-consuming sample pre-treatment consisting of two extraction steps and evaporation of the organic extract.

Ascorbic acid was added before extraction [151, 152] to prevent oxidation of rifampicin and its metabolites.

9. TETRACYCLINES

Tetracyclines are a family of closely related antibiotics (Fig. 3); their spectrum is the broadest known. These compounds possess a great number of functional groups; they have three dissociation constants in the pH range 1—12. For tetracycline, the pK_a values corresponding to the acidic hydroxy group at position 3, the dimethylamino function and the hydroxy group at position 12 are 3.3, 7.5 and 9.4, respectively [154]. Fig. 4 illustrates the ionization stages of tetracycline as a function of pH.

Several HPLC procedures have been described for the determination of tetracyclines in body fluids (Table 6). Tetracyclines are polar compounds difficult to extract from body fluids. They exhibit poor chromatographic performannce because they strongly interact with alkyl bonded silica phases. However, they have favourable UV absorption characteristics as they have an absorption maximum between 345 and 370 nm [157] where few endogenous compounds absorb.

Tetracyclines have been extracted from plasma into acetyl acetate at pH 8—9 by simultaneous calcium chelation and ion-pair formation with phenylbutazone [159]. They have been also extracted into chloroform—1-heptanol at pH 9.4

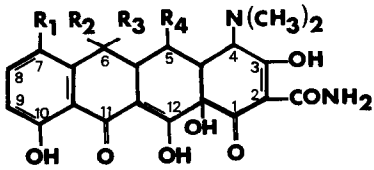

Fig. 3. Common nucleus of tetracyclines.

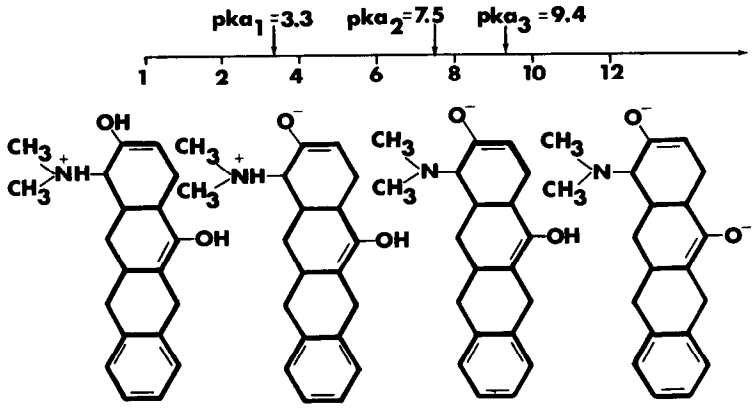

Fig. 4. Ionization stages of tetracycline as a function of pH [154]; only ionizable functions are shown.

TABLE 6

HPLC OF TETRACYCLINES

Drug and reference	Body fluid or tissue (amount used)	Extraction and/or deproteinization	Stationary phase	Mobile phase	Detection wavelength (nm)	Limit of detection (LD) or of sensitivity (LS) (μg/ml)
Doxycycline [155]	Serum (500 μl) Urine	Ethyl acetate	C_8	Acetonitrile—dilute citric acid	UV 350	LD 0.05
[156]	Serum (100 μl) Blood (50 μl) Tissue	Acetonitrile	C_8	Acetonitrile—phosphate buffer, pH 2.4	UV 357	Not stated
Tetracycline [157]	Plasma (500 μl) Urine	Chloroform—1-heptanol	C_2	Acetonitrile—dilute phosphoric acid	UV 357	LD 0.02
[158]	Urine	—	C_8	Isopropanol—diethanolamine buffer, pH 7.3—tetraammonium EDTA	UV 365	LD 0.4
Chlortetracycline Oxytetracycline Tetracycline [159]	Plasma (2000 μl) Urine Tissue	Ethyl acetate	C_{18}	Acetonitrile—dilute nitric acid—monosodium phosphate, pH 2.4	UV 355	LS 0.5
Doxycycline Tetracycline Pyrrolidinomethyltetracycline [160]	Serum Blood (50 μl) Tissue	Acetonitrile	C_8	Acetonitrile—dilute nitric acid—monosodium phosphate, pH 2.4	UV 357	LD 0.2
Lumecycline Tetracycline [161]	Plasma (500 μl) Urine	Trifluoroacetic acid	C_{18}	Acetonitrile—phosphate buffer, pH 2.2	UV 357	LS 0.05

as ion pairs with tetrabutylammonium cations [157]. Re-extractions have then been performed into dilute phosphoric acid [159] or perchloric acid [157] where the ionization of hydroxy groups is suppressed. Back-extraction has been performed with perchloric acid by Eksborg et al. [157] because the excess of tetrabutylammonium which perturbs the chromatography was removed from the aqueous phase by perchlorate ion-pair formation. However, ion-pair formation between perchlorate ions and dimethylammonium cations of tetracyclines might also occur. These ion pairs are highly soluble in organic solvents and an excess of perchloric acid was avoided to yield sufficient recoveries: more than 80% of tetracycline was found to be back-extracted [157]. A more simple extraction procedure [155] was described for doxycycline, the most lipophilic tetracycline. The method involves extraction from serum into ethyl acetate at pH 6.1, evaporation of the organic extract and redissolution of the residue into the mobile phase. The mean recovery was 87.8 ± 4.3% (S.D.).

Procedures based on only plasma or serum protein precipitation followed by direct injection of the supernatant onto the column have been also described [156, 160, 161]. Trifluoroacetic acid [161] and acetonitrile—pH 2.4 buffer [156, 160] were used as deproteinization agents with subsequent recoveries of about 70% for tetracycline [161] and 90—100% for doxycycline, tetracycline and pyrrolidinomethyltetracycline [156, 160]. Hermansson [161] used trifluoroacetic acid in spite of co-precipitation of tetracycline with plasma proteins because large volumes of supernatant (250 μl) were injected onto the column and the chromatography of acetonitrile-precipitated plasma samples resulted in elution of tetracycline in the solvent front. This might be due mainly to the high acetonitrile content in the sample compared with that in the mobile phase.

Tailing peaks are obtained for tetracyclines when using the conventional C_8 or C_{18} columns and acetonitrile—buffer eluents. Eksborg et al. [157] found it necessary to use silica gel bound to short alkyl chains (C_2) to obtain an optimal separation speed. However, broad and tailing peaks were obtained. C_8 and C_{18} columns have been used [156, 159], but pre-equilibration of the column with a solvent less polar than the mobile phase and containing a tetracycline was found necessary to avoid tailing peaks and obtain reproducible retention times. No such requirement was found by Hermansson [161] who obtained symmetrical peaks on a C_{18} column when the eluent had a high ionic strength. Likewise, sharp and symmetrical peaks were obtained on a C_8 column with acetonitrile—0.1 *M* citric acid as eluent [155].

Solvophobic and silanophilic interactions [162] between tetracyclines and the hydrocarbonaceous layer and the residual silanol groups might occur simultaneously and result in assymmetrical peaks as the retention of tetracyclines was found to pass through a minimum at 40—50% acetonitrile [163]. The addition of alkylammonium ions to the mobile phase has been shown to result in competition with tetracyclines for adsorption sites on reversed-phase columns and to provide more symmetrical peaks [163]. The addition of ethylenediaminetetraacetic acid to a pH 3 buffered eluent [154] and the addition of a tertiary amine to a pH 8 buffered eluent [164] have also been shown to improve peak symmetry by formation of zwitterion and ion pairs, respectively.

The stability of tetracyclines is also a critical point. They are unstable in acidic and alkaline medium. The extraction of tetracycline into chloroform—1-heptanol at pH 9.4 [157] must be rapidly performed to avoid degradation. Trifluoroacetic acid precipitated samples must be injected just after protein precipitation [161] and acetonitrile—pH 2.4 buffer precipitated samples must be entirely processed at 0°C [156]. Furthermore, over the pH range 2—6, all tetracyclines are subject to reversible epimerization. For the extraction of doxycycline into ethyl acetate at pH 6.1 [155], mild evaporation conditions and addition of ascorbic acid eliminated the epimerization reaction.

10. VANCOMYCIN

Vancomycin is a narrow-spectrum bactericidal antibiotic primarily used for the treatment of infections caused by penicillinase-producing staphylococci. It is an amphoteric glycopeptide antibiotic.

10.1. Immunoassays

Fluorescence polarization immunoassays have been developed for the quantitation of vancomycin [165—167]. The procedures are fast, fully automated and highly reproducible (coefficient of variation < 5%). They use less than 100 μl of serum and are free from interferences from many drugs tested. Interference from haemolysis, lipaemia and icteraemia is not significant since background readings are automatically taken on each serum sample. Good correlations have been obtained with the results obtained by HPLC and RIA techniques. The procedure described by Schwenzer et al. [165] permits vancomycin concentrations to be determined down to 0.6 μg/ml of serum.

10.2 High-performance liquid chromatography

An HPLC procedure [168] has also been described. Vancomycin is extracted from serum (400 μl) with an almost quantitative recovery using a CM-Sephadex column. It is then eluted on a C_{18} column and detected by UV absorption at 210 nm. Ristocetin is used as internal standard. Proteins were not precipitated as low recoveries were obtained. Other antibiotics that are frequently administered with vancomycin such as aminoglycosides do not interfere with this procedure.

11. COMPARISON OF ASSAY TECHNIQUES

The ideal assay of an antibiotic should be precise, accurate, sensitive, rapid, specific with regard to metabolites and co-administered antibiotics, unaffected by changes in endogenous constituents, capable of processing microsamples, simple to be performed and inexpensive. Each of the assay methods has its advantages and drawbacks which must be carefully considered in the selection of the most convenient solution for a particular laboratory and a particular antibiotic.

11.1. Microbiological assays

The microbiological assay provides versatility, simplicity, high capacity and inexpensiveness. It is easily applicable to new antimicrobial agents. The lowest detectable concentration is 0.1—1 μg/ml in most agar diffusion assays. This sensitivity, although usually sufficient for routine determinations, is inadequate for certain pharmacokinetic investigations. Furthermore, the microbiological assay is not as reproducible as HPLC and immunoassays. A major shortcoming is the long turn-around time: 4—18 h are required to give the desired information for conventional microbiological assays. The presence of another antimicrobial agent in the sample often interferes with the assay. As many hospitalized patients receive more than one antibiotic, this necessitates changes in procedure by using a test organism susceptible only to the drug being investigated or by the addition of a substance that inactivates the potentially interfering agents. Additional interferences may arise due to metabolites or degradation products. Another disadvantage, for pharmacokinetic studies, is that the technique cannot measure metabolite levels.

11.2. Immunoassays

Radioimmunoassays are sensitive, specific and accurate, and they require only a few microlitres of sample. However, they necessitate the use of radioactive isotopes and are therefore subject to specific regulations; they utilize expensive gamma counters, the reagents used have a relatively short shelf-life and a separation step is necessary. Therefore, these assays are increasingly being replaced by non-isotopic immunoassays which have most of the advantages of radioimmunoassays, are much more rapid, do not use radioactive materials and do not necessitate a separation step. The introduction of commercially available tests which require minimal expertise, permits small laboratories to carry out determinations. The relatively high costs of these kits are partially compensated by the saving in time. But a specific, high-titre antibody is required for each drug, and commercial tests are only available for a few antibiotics. Few laboratories have the expertise necessary to raise their own antisera and prepare their own labelled antibiotics. This severely limits the use of immunoassays. They are essentially applied to the quantitation of aminoglycosides and vancomycin. These drugs are not significantly metabolized, which avoids problems of interference, as immunoassays are not substance-specific so that metabolites or similar drugs can interfere. Unlike radioimmunoassays, non-isotopic immunoassays are prone to interference by non-specific factors present in body fluids, especially in samples from very ill patients. Different batches of antibodies can show considerable differences in activity, thus affecting reproducibility. It may be anticipated, however, that monoclonal antibodies may improve the reproducibility of immunoassays.

11.3. High-performance liquid chromatography

HPLC is the chomatographic procedure essentially used for antibiotic quantitation. GLC is cumbersome because it requires derivatization of the drug into

volatile compounds. Moreover most antibiotics cannot be volatilized. HPTLC has not yet found extensive application for therapeutic drug monitoring. It allows a much higher through-put than HPLC and GLC as many samples can be run simultaneously. However, it requires an expensive densitometer.

HPLC is accurate, sensitive, selective and rapid. It is uniquely suited to the separation of closely related compounds as compared to bioassays and immunoassays. It permits the simultaneous assay of the drug being investigated and its prodrug or its metabolites. It also permits precise pharmacokinetic investigations when the drug consists of a mixture of various components. For instance, both isomers of moxalactam [75, 77, 79] and the three major components of gentamicin [116, 117, 122, 124, 125] could be separated and quantitated in one analytical step. Furthermore, two antibiotics from the same family can be determined simultaneously. This allows direct comparison of pharmacokinetics or of penetration into tissues. Another advantage of HPLC is its unlimited potential. The technique can deal with various classes of drugs, even with high-molecular-weight compounds. It has succeeded in providing an assay method for most antibiotics in clinical use. Reversed-phase chromatography, which is essentially used, affords a great selectivity by the choice of mobile phase composition and packing type.

The time required to set up an HPLC method for a new antibiotic is becoming shorter. Many procedures have been described, and in some instances quite different procedures have been suggested for the same antibiotic because of the large number of possibilities that HPLC affords. However, each of these procedures can generally be applied to other drugs of the same family with some modifications. Examples are the recently reported procedures allowing the determination of various cephalosporins in body fluids with only minor modifications from one compound to another [28, 80—82]. The main modification concerns the content of organic modifier in the mobile phase. Another example is the determination of six aminoglycosides in serum by using the same column switching system [124]. It is evident that each of these general procedures can be extended to other drugs of the same family.

However, HPLC has limited capacity. Samples cannot be simultaneously assayed, unlike bioassays and immunoassays. HPLC requires sample clean-up which is time-consuming when the drug is extracted from the biological matrix. Some methods involve also time-consuming pre-column derivatization especially for determination of aminoglycoside levels. Another shortcoming of HPLC is its lack of versatility when different drugs have to be regularly analysed. Changing from one method to another is time-consuming. This can be overcome in a slightly complicated way using parallel columns with a single pump and appropriate valving. Different equilibrated columns are then available. Furthermore, HPLC requires expensive equipment. This, however, is partly compensated by its low running cost.

HPLC is a well advanced technique still continuing to develop. Systems for column switching are becoming increasingly used for routine determinations of drug levels in body fluids. Sample clean-up is then fully automated. Several aminoglycosides have been determined in serum with such a system [124] as mentioned above. Microbore columns with an internal diameter of 1 mm or less are currently under development. They permit smaller sample volumes to be

used because of less sample dilution on the column. Less packing material is used and the solvent consumption is reduced by at least 90% over that of a conventional column. Antibiotic levels have been determined in fermentation broths on microbore columns [83]. Finally, the use of new detectors such as mass spectrometric and diode-array UV detectors might give an abundance of information, particularly on the structure of the compounds in metabolism investigations.

12. CONCLUSIONS

Antibiotic levels have been mainly monitored in body fluids by microbiological assay. Because this technique requires a long turn-around time and lacks specificity, other assay techniques have been applied. HPLC and immunoassays appear as an alternative to current microbiological assays. HPLC has been applied to most antibiotics in clinical use and immunoassays have been essentially applied to aminoglycosides and vancomycin. For such assays, HPLC is more complex and time consuming, especially for aminoglycoside determinations, because of the inability of HPLC detectors to measure these drugs without derivatization. However, because of its unique selectivity, HPLC is the technique best suited for all classes of antibiotics in special situations such as pharmacokinetic studies or metabolism investigations.

13. LIST OF ABBREVIATIONS

BSC = benzene sulphonyl chloride
CSF = cerebrospinal fluid
Dns = 1,5-dimethylaminonaphthalene sulphonyl
EIA = enzyme immunoassay
FDNB = 1-fluoro-2,4-dinitrobenzene
FIA = fluoroimmunoassay
FPIA = fluorescence polarization immunoassay
GLC = gas—liquid chromatography
HPLC = high-performance liquid chromatography
HPTLC = high-performance thin-layer chromatography
LAIA = latex agglutination inhibition assay
LD = limit of detection
LS = limit of sensitivity
MIC = minimal inhibitory concentration
OPA = *o*-phthalaldehyde
RIA = radioimmunoassay
SLFIA = substrate-labelled fluoroimmunoassay
TNBS = trinitrobenzene sulphonic acid

14. SUMMARY

Analytical procedures recently described for the quantitative determination of antibiotics in body fluids are reviewed. High-performance liquid chromatography (HPLC) and immunoassays appear as an alternative to current micro-

biological assays. HPLC has been applied to most antibiotics in clinical use and a major part of the review deals with this technique. Attention is given to sample pretreatment, characteristics of chromatography and detection, and limit of sensitivity. Non-isotopic immunoassays have been essentially applied to aminoglycosides and vancomycin and are also reviewed. Advantages and drawbacks of HPLC and immunoassays are presented.

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