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Review

High-performance liquid chromatography of antibiotics

F. JEHL*, C. GALLION and H. MONTEIL

Institute of Bacteriology, University Hospital Center, 3 Rue Koeberlé, 67000 Strasbourg (France)

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CONTENTS

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LIST OF ABBREVIATIONS

C	Centrifugation
CH_3CN	Acetronitrile
C.V.	Coefficient of variation
D	Deproteinization
DMF	Dimethylformamide
DNFB	Dinitrofluorobenzene
ED	Electrochemical detection
ELISA	Enzyme-linked immunosorbent assay
EMIT	Enzyme-multiplied immunoassay technique
ESD	Disodium ethanesulphonate
FPIA	Fluorescence polarization immunoassay
HDTMAB	Hexadecyltrimethylammonium bromide
HPLC	High-performance liquid column chromatography
M	Mix
NPPM	Non-published personal method
O	Organic solvent
OSS	Sodium octanesulphonate
PA	Perchloric acid
PSS	Sodium pentanesulphonate
RPC	Reversed-phase chromatography
S	Serum
Sl	Supernatant after deproteinization
SDS	Sodium dodecyl sulphate
Si	Final supernatant injection

TBAB	Tetrabutylammonium bromide
TCA	Trichloroacetic acid
TEA	Triethylamine
THF	Tetrahydrofuran
THBS	Tetrabutylammonium hydrogensulphate
UV	Ultraviolet

1 INTRODUCTION

1.1. Use of high-performance liquid chromatography in clinical chemistry

High-performance liquid chromatography (HPLC) is a technique that was developed during the 1960s and improved during the 1970s and 1980s, essentially in terms of technological improvements. Its sensitivity and precision and its applicability to an enormous variety of compounds have meant that column liquid chromatography is widely used in clinical laboratories for the monitoring of a variety of therapeutic agents in hospital routine analysis. HPLC is also a powerful tool for the development of new drugs, particularly in the fields of pharmacokinetic investigations and metabolism studies. HPLC is being increasingly used for the routine analysis of a number of endogenous constituents, such as steroids (glucocorticoids, aldosterone, estrogens, vitamin D), amino acids, bilirubin and conjugates, tyrosine, tryptophan and metabolites, organic acids (pyruvate, lactate) and nucleic acid components. No doubt other endogenous compounds will be monitored by HPLC in the future.

1.2. Classes of drugs monitored by HPLC and application to antibacterial agents

Of particular interest are the anticonvulsants (primidone, phenobarbital, phenytoin, etc.) and some of their metabolites, theophylline and antiarrhythmics (lidocaine, procainamide, propranolol, quinidine, etc.), tricyclic antidepressants (amitriptyline, desipramine, imipramine, etc.) and their metabolites, antineoplastic drugs (*e.g.* methotrexate, 6-mercaptopurine, 5-fluorouracil, doxorubicin, vinca alkaloids), hypnotics and sedatives. It is evident that almost all therapeutic drugs may be analysed by HPLC [for more information, see *J. Chromatogr.*, 340 (1985)].

Among these drugs, the antibiotics represent one of the largest classes of therapeutic agents. They are usually divided in several families showing very different chemical structures, and some of them are extensively metabolized. Patients often receive multiple antimicrobials that may be chemically similar or different, or which may have overlapping spectra of activity. Sometimes, microbiologically active metabolites of antimicrobials may be present in biological fluids. In such situations typical bioassay procedures are inadequate because of poor specificity,

and this has made HPLC the most suitable methodology for monitoring antibiotics, as evidenced by the increasing number of reports of HPLC analysis of antibiotics.

Sections 2–4 of this review deal only with our own work in this area. In later sections we focus on a large number of specific methodologies applied to the different existing antibiotics.

2 MONITORING OF ANTIBIOTICS CONVENTIONAL METHODS AND THEIR DRAWBACKS

2.1. Immunological assays

2.1.1. Radioimmunoassays

The most serious disadvantage of radioimmunoassays (RIAs) is the necessity to manipulate radiolabelled antigens, and this is not possible within every clinical laboratory. Even though such assays are more specific than microbiological assays, interferences can occur, *e.g.* with high concentrations of β-lactam antibiotics when aminoglycosides are measured.

2.1.2. Enzyme immunoassays

Enzyme immunoassays (EIAs) are more practicable than RIAs Whatever the technique considered [enzyme-multiplied immunoassay technique (EMIT), enzyme-linked immunosorbent assay (ELISA) and fluorescence polarization immunoassay (FPIA)] they have been shown to be accurate in clinical situations, but with some limitations. Interferences may occur when aminoglycosides are measured in the presence of high concentrations of β-lactam antibiotics, and there may be problems with severe hemolytic, lipemic or icteric samples. In general, one has to remember that immunological assays are indirect assays, and when problems occur with what could be called difficult samples (*e.g.* uremic, lipemic or icteric samples) they cannot be overcome easily. Direct methods such as HPLC offer the advantage of a visual translation (chromatograms) of the physicochemical events that occur in the detector cell and analytical column.

2.2. Microbiological assays

Microbiological assays of antibiotics are relative rather than absolute They are based on the determination of the level of an antibiotic by a definable microbiological response to a series of standard antibiotic concentrations by a strain of test organisms. There is a wide variety of assays, all of which are influenced by one or more of the following factors: general assay design, choice of assay medium, preparation of antibiotic standards, physical factors, method of calculation of results, choice of test organism. The major drawback of microbiological assays is their lack of specificity when assaying samples from patients treated with several antibiotics or with antimicrobial agent that produces active metabolites. They

also suffer from poor reproducibility and accuracy and are somewhat time-consuming and often result in delayed response (6–24 h).

3 HOW TO DEVELOP HPLC METHODOLOGIES FOR ANTIBIOTICS

The development of a chromatographic method for monitoring any drug in biological fluids must be divided into several steps. Each step should be resolved before going on to the next one.

3.1. Choice of the stationary phase

Retention mechanisms in HPLC are superimposed in the classical mechanism of conventional atmospheric chromatography. Thus one may find adsorption chromatography, partition chromatography, ion-exchange chromatography, gel permeation (exclusion) and affinity chromatography. Some physiochemical properties of the analyte may be taken into consideration by the investigator in choosing the stationary phase, such as the following.

The molecular mass: if it is greater than 2000, exclusion chromatography could be preferred, but this is not an absolute rule.

The structure of the analyte: strongly polar substituents (alkali, acids or ionizable groups) may require ion-exchange chromatography; weakly polar substituents (alkyl groups, halogens) may be preferentially chromatographed by reversed-phase partition chromatography; substituents of intermediary polarity can be analysed by either partition or adsorption chromatography.

The solubility of the drug.

It is interesting to note that *ca.* 80% of the HPLC methodologies for antibiotics published in the past five years involved reversed-phase partition. This may be explained by the great stability and ease of use of non-polar coated silica (C_2, C_8, C_{18}). Moreover, even relatively polar antibiotics may be analysed on these stationary phases either by ion-pairing chromatography or by ionization suppression.

HPLC methodologies now exist for almost all antibiotics used in therapy, and the easiest way to select a stationary phase is to refer to existing publications.

3.2. Types of detection

The most popular HPLC detectors are concentration-dependent. There are two general types: selective and universal. Selective detectors, *e.g.* absorbance and fluorescence detectors, are sensitive and especially useful for trace analysis.

In HPLC of antibiotics, by far the most frequently used detector is the ultraviolet (UV) photometer, with either fixed or variable wavelength (spectrophotometer). UV absorbance is commonly used for the quantitation of most of antibiotics, including penicillins, cephalosporins, quinolones, glycopeptides, cyclines,

macrolides, lincosamides, chloramphenicol, etc. The major exception is the class of aminoglycosides, most of which are monitored by fluorescence, except for neomycin and novobiocine. Macrolides may be measured by electrochemical detection (ED) (erythromycin). 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 wavelength. Water and acetonitrile are important solvents that can be used at wavelength down to 195 nm. Thus, one could say that a photometer or spectrophotometer should be the most suitable detector in a laboratory commencing the HPLC assay of antibiotics. Photometers are less expensive and, provided that they are equipped with interchangable sources (mercury lamp and zinc lamp) and filters (214, 254 and 280 nm), they represent the most interesting alternative.

3.3 Types of preparation of biological samples

Biological fluids are typically complex mixtures, composed of a wide variety of proteins, carbohydrates, lipids, etc. Beyond the deleterious effects these materials have on the injector, the column packing material, and the pumps, their presence will frequently interfere with the separation of the antibiotic under study. Consequently, some form of sample preparation is almost invariably required.

In general, there are four groups of methods for sample pretreatment prior to the assay of antibiotics. These include simple dilution of sample in an appropriate solvent, protein precipitation, solvent extraction and use of cartridges of various types that allow what could be called 'pre-chromatography' (enrichment) of the sample. Generally, the type of extraction and the amount of sample clean-up is dictated by the efficiency and the selectivity of the chromatographic technique used for analysis. The more specific and efficient the chromatographic procedure, the less sample extraction and clean-up are necessary to obtain the desired results. One has to note that sometimes the extraction steps are also necessary to improve the detection limit of the assay by concentrating the antibiotic.

3.3.1. Dilution of biological samples

This method is mainly applicable to biological fluids in which high concentration of antibiotic may be found with no or low amounts of protein, such as urine and bile. It should not be applied to serum or plasma samples. In urine or bile, dilution by an appropriate solvent is often suitable to eliminate baseline background and thus interferences and to lower the initially very high antibiotic concentration in the range of linearity of the method.

The dilution solvent may be water, but because the ionic strength may affect the retention time of an antibiotic, it may be useful to adjust the ionic strengths of diluting solvents to those of the biological samples under investigation.

3.3.2 Protein precipitation

This is a very popular method of sample pretreatment for antibiotic determination, and is really easy to perform. Either acids or organic solvents may be used. Acids commonly include perchloric acid, trichloroacetic acid and trifluoroacetic acid. Organic solvents used are acetonitrile, methanol, 2-propanol and dimethylformamide. These techniques require mixing of only a small volume of serum (50–100 μl) with the deproteinizating agent in the same amount or in excess. It is thus particularly suited to pediatric samples. However, acid precipitation leaves many interfering substances in the sample, and one has to verify that no interferences occur by injection of the deproteinization supernatant of a blank serum. In any event, either with acids or with organic solvents, this technique suffers from sample dilution when low concentrations of antibiotics are measured. One way to overcome this problem when using an organic solvent is to back-extract the excess of the solvent In our laboratory, we commonly deproteinize samples with an equal volume of acetonitrile; the remaining acetonitrile is then removed from the supernatant by methylene chloride, leading to an increased concentration of the antibiotic in the supernatant [1–5].

Another problem resulting from acid deproteinization is that the pH change may be deleterious to some antibiotics, such as β-lactams or quinolones. Of particular interest may be deproteinization with the same organic solvent as is used in the mobile phase.

As an alternative to precipitation, one may remove proteins by ultrafiltration. Nevertheless, it is important to consider that small protein molecules may not be removed by the ultrafilter, and also that other interfering materials may pass through the filter.

3.3.3. Solvent extraction

Extraction methods for isolating and concentrating antibiotics from a sample matrix may vary from a simple one-step solvent extraction to complicated back-extractions. Organic solvents selectively extract the antibiotic from the specimen by solvent partitioning. By appropriate solvent selection (hexane, chloroform, diethyl ether, methylene chloride, etc.) and manipulation of the specimen pH, a satisfactory separation of the antibiotic from most of the other material in the specimen can frequently be achieved.

In a one-step method, the organic extractant is evaporated to dryness and an aliquot of the reconstituted extraction residue is injected into the chromatograph. Many solvents may be used for reconstitution, but the best way is to use an aliquot of the mobile phase itself, if possible. A two-step method would imply a back-extraction of the antibiotic into an aqueous solution with a pH at which the drug is ionized and water-soluble. This latter step further purifies the specimen and may be used to concentrate the analyte if a smaller amount of the aqueous phase is used.

Examples of solvent extraction are given in Section 5.2.1.

3.3.4 Cartridges

Many compounds may be isolated by first adsorbing them into either an ion-exchange resin if they are charged, or a bonded reversed-phase packing material, followed by sequential elution. For example, aminoglycosides are very polar and difficult to extract with an organic solvent. However, they are easily adsorbed onto Amberlite resin or silicic acid, then eluted and analysed [6]. Antibiotics consisting of a mixture of several molecules, such as teicoplanin, can easily be extracted from biological matrix by reversed-phase cartridges [7].

3.4. Establishment of the mobile phase

This step must be conducted with simple solutions of the antibiotic under study: pure aqueous solutions when possible, or pure methanolic solution for example, to ensure that the peak that appears corresponds to the antibiotic. The approach will vary depending on the context.

If there are published techniques available concerning antibiotic (or any antibiotic belonging to the same chemical family) under investigation one should first test the mobile phase used in this previously described methodology. It will usually be necessary to adapt this to the particular laboratory and technical environment involved. Indeed, minor changes to the general equipment may have major consequences for what is the final purpose of the chromatographer, that is a well resolved symmetrical peak of the antibiotic. Minor changes may concern, for example, the quality of the water or organic solvents in the mobile phase, the quality of the stationary phase (*e.g.* end-capping of reversed phase), the length of the analytical column and the quality of the chemicals used to buffer the mobile phase.

Nevertheless, one has to note that sometimes two antibiotics belonging to the same family have very different chromatographic behaviour and necessitate somewhat different mobile phases. For example, ceftriaxone and cefotaxime are both cephalosporins. Cefotaxime is easily measurable by reversed-phase chromatography, but ceftriaxone needs ion-pairing reversed-phase chromatography. This latter technique consists of adding a non-polar counter ion to the mobile phase to form a non-polar ion pair with ceftriaxone, which is then more retained on the analytical column and better resolved

The second eventuality, which is less frequent, is that no paper has been published on the antibiotic. The investigator must then start with a mobile phase having a weak elution strength, *e.g.* on a reversed-phase analytical column an amount of 5–10% of non-polar solvent (acetonitrile, methanol) in ultra-pure water. The mobile phase eluting strength must then be adjusted so that the retention time for the peak of interest has k values between 1 and 10 [k is the capacity factor equal to $(t_R - t_0)/t_0$, where t_R is the retention time of antibiotic and t_0 the retention time of an unretained peak, such as the solvent]. It may happen that even with a weakly elutive mobile phase, the peak of an antibiotic will appear as

an unretained, tailing peak; then, ion-pairing chromatography or ionization control must be considered. Indeed, modifiers of mobile phase pH or ionic strength (buffer, salt) as well as counter ions greatly influence the mobile phase selectivity. Thus, different pH values or concentrations of different salts must be tested until a suitable k value and symmetrical peaks are obtained.

Once a sample preparation procedure has been developed, the investigator will have to adapt the pre-established mobile phase to the chromatography of the antibiotic in the presence of the co-extracted endogenous compounds. Indeed, there are no absolutely selective sample preparations and it may happen that endogenous substances may co-elute with or elute quite near the antobiotic peak, resulting in interferences. Thus it may become necessary to manipulate the mobile phase to 'force' the antibiotic to elute in a zone where no endogenous peak appears in an extracted blank serum (or any biological fluid under investigation). The ways to improve the definitive mobile phase are numerous and include slight variations of pH, an increase or decrease of the ionic strength and changes in the percentage of the non-polar organic modifier in the mobile phase. Sometimes two of these parameters have to be changed. If the interference takes the form of a 'shoulder' or tailing of the antibiotic peak, it may sometimes be sufficient to increase the column efficacy, *e.g.* by using a longer analytical column of the same type to increase the number of theoretical plates.

One has to remember that the last two steps of the development of an HPLC methodology may be conducted together. Indeed, an interfering peak may sometimes be avoided by changing one extraction solvent or by changing the precipitating agent of the sample preparation step

3.5. Is there always a need for an internal standard?

This is a controversial point. Internal standards are needed when the extraction recovery of the developed sample preparation is highly variable. Most often, this is the case when multiple-step solvent extraction or cartridge extraction is used. The internal standard should have physicochemical properties and chromatographic behaviour close to those of the antibiotic to be measured. The introduction of an internal standard into HPLC methodology implies that the entire developed procedure must be suitable for both molecules, which leads to additional difficulties. Moreover, one can never be sure that, for a given sample, the extraction recoveries of the internal standard and the antibiotic will be identical. From sample to sample, the internal standard and the antibiotic recoveries may vary in opposite directions, and this may lead to false results. This seldom occurs, but care should be taken. In fact, if the within- and between-day reproducibilities and linearities (see next section) of the developed methodology are proven, there is not an absolute need for an internal standard.

4 QUALITY CONTROL FOR A NEWLY DEVELOPED HPLC METHODOLOGY FOR ANTIBIOTICS

4.1. Precision

4.1.1. Repeatability

This parameter may be checked by injecting the final supernatant from a deproteinization or a solvent extraction of one sample at least ten times, and calculating the coefficient of variation (C.V.) of peaks height or peak area. The C.V. should be kept under 5%. This parameter is useful to investigate the reliability of all aspects of the HPLC system: stability of flow-rate, detector lamp, mobile phases, detector response etc.). It does not reflect the reproducibility of the sample preparation procedure.

4.1.2. Within-day reproducibility

A large number (at least 30) of aliquots of the *same* sample should be analysed the same day by the same operator under exactly the same conditions (lamp, mobile phase, column, etc.). The resulting C.V indicates the within-day reproducibility, which should be kept below 10%. This parameter reflects the reproducibility of the extraction procedures.

4.1 3. Between-day reproducibility

Thirty aliquots of the same sample used for within-day study should be analysed one by one during 30 days, using each time the calibration curve of the day. The resulting C.V. indicates the between-day reproducibility and should be kept below 10%.

4.2. Detection limit

The limit of detection may be defined as the lowest concentration of antibiotic resulting in a signal-to-noise ratio of 4.

4.3. Linearity

The linearity of the method should be investigated from the concentration corresponding to the detection limit to the highest concentration likely to be found in biological fluids (including toxic levels). If the relation between peak height or area is not linear up to these highest values, the limit of linearity must be determined. Thus, any sample found to have a concentration above this limit should be diluted to be kept in the linearity range. Every concentration used in the linearity study should be the mean of at least six samples.

4.4. Selectivity

During the development of a new HPLC methodology, a blank serum free of antibiotic is used to check for the absence of interferences from endogenous compounds. It is generally obtained from an healthy volunteer with normal renal and hepatic functions. In practice, when antibiotics are measured in a hospital routine context, a lot of samples are coming from patients with renal or hepatic dysfunction. Such samples often have atypical chromatographic patterns with larger endogenous peaks and additional endogenous compounds that create unexpected interferences. Thus, it is of great interest to include such atypical blank serum sample in the development stages, from different sources.

In clinical therapeutics, numerous drugs may be coadministered with a given antibiotic. Therefore it is necessary to evaluate the chromatographic behaviour of these drugs under the conditions newly developed for this antibiotic. They must have different retention times and they cannot interfere with the antibiotic. The choice of the drugs to be included in these interference studies will be a function of those available in the hospital and of the treatment habits of the physicians. Practically, it will be easier to inject simple solutions (in water, buffer or methanol) of drugs to locate accurately the corresponding peak. If it happens that a drug elutes close to the antibiotic, the investigator must then verify whether the extraction procedure is suitable for this drug. Indeed, it is possible that the sample clean-up treatment would eliminate the interfering drug.

4.5. Accuracy

Theoretically, the newly developed HPLC method should be compared with the reference method or any method considered as reference. Up to now, microbiological assays are considered as reference methods for antibiotics.

This comparison should include many supplemented serum samples with a known content of antibiotic, the concentrations ranging from the detection limit of the method to the highest value of the linearity. A correlation study should be undertaken between the two methods This correlation may also include biological samples from treated patients, but bias may arise if the antibiotic under study is biotransformed into active metabolites. In this case, one should refer to immunoenzymic or radioimmunological assays.

5. HOSPITAL ROUTINE ANALYSIS OF ANTIBIOTICS BY HPLC

5.1. Why measure levels of antibiotics?

In the early 1970s it became apparent that it would be desirable to be able to assay antibiotics accurately and rapidly, particularly in blood from patients receiving antimicrobial substances with narrow toxic/therapeutic ratios. Thus, the

major reason for rapid serum assays in hospitals was to regulate therapy with aminoglycosides and vancomycin. Nevertheless, even for drugs with a high therapeutic ratio (β-lactams), analyses can be justified when there is evidence that the serum level may be markedly different from usual or cannot be predicted even roughly owing to the complexity of the clinical situation, as may be the case for critically ill patients in whom absorption from an intramuscular injection or oral administration may vary, or patients with impaired or changing renal function.

When pharmacological studies require accurate, but not necessarily rapid or specific assays, clinical assays may sometimes be less accurate but must be specific and sufficiently rapid to allow dosage adjustments within the time limit of the dosing interval.

5.2. Standardization of the methodologies for each class of antibiotics

5.2.1. Standardization of clean-up of biological samples

Since we have been routinely measuring antibiotics by HPLC, it has become evident that the daily batch of analyses consists of numerous small series of different antibiotics rather than one or two large series of the same antibiotic. Moreover, if one considers that the samples coming from the different clinical departments of a hospital are generally not brought into the laboratory at the same time, one may easily imagine the resulting difficulties of achieving all the analyses within the same day if the HPLC analytical procedures are not standardized.

The first step is to standardize the clean-up procedure. As examples, we describe below standardized extractions for β-lactam antibiotics and quinolones For β-lactam antibiotics we developed an easy-to-perform and reliable procedure suitable for sixteen antibiotics (Table 1). Note that this procedure is also suitable for vancomycin (procedure 1). With respect to the quinolone family, eight of them (Table 2) can be extracted by procedures (procedures 2–5) that all begin with the same steps and differ only in the nature of the solvent used for the last step of back-extraction.

Thus, 24 antimicrobial agents are easily extracted by only two procedures. Evidently a universal clean-up procedure suitable for all available antibacterial agents does not exist, and we have had to develop specific methods for some antibiotics (procedures 6–9).

Nevertheless, by means of this maximum standardization, we have greatly enhanced the preparation steps in our laboratory. We prepare the samples in their order of arrival at the laboratory, together with the frozen ($-80°C$), ready-to-use corresponding calibration standards that are always available in our freezing chamber.

We shall now describe examples of standardized extraction procedures that we have developed in our laboratory for β-lactam antibiotics and quinolones [1–5,8,9], and of non-standard procedures for various other antibiotics [7]

TABLE 1

STANDARDIZATION OF HPLC METHODS FOR MOST β-LACTAM ANTIBIOTICS (EXTRACTION PROCEDURE 1)

Antibiotic	Mobile phase		
	Acetonitrile (%)	Ammonium acetate (m*M*)	pH adjusted with acetic acid
Cloxacillin	28	20	5
Penicillin G	24	20	5
Azlocillin	24	20	5
Mezlocillin	24	20	5
Piperacillin	24	20	5
Ampicillin	8	20	5
Cefoperazone	20	20	5
Cefotaxime (alone)	12	20	5
Cefotaxime + desacetylcefotaxime	8	20	5
Ceftazidime	7	20	4
Cefpiramide	15	20	5
Cefadroxyl	5	20	5
Cefmenoxime	12	20	5
Cefpodoxime	7	20	5
Cefpirome	10	20	5
Vancomycin	8	20	5.4

TABLE 2

STANDARDIZATION OF HPLC METHODS FOR SOME QUINOLONES

In all cases, the pH was adjusted to 2 with phosphoric acid

Quinolone	Extraction procedure	Mobile phase		
		Acetonitrile (%)	Sodium dihydrogen-phosphate (m*M*)	Tetrabutyl ammonium bromide (m*M*)
Norfloxacin	2	10	20	2.5
Pefloxacin	2	10	20	2.5
Ofloxacin	2	10	–	5
Ciprofloxacin	3	9	–	5
Difloxacin	5	13	10	5
Fleroxacin	2	10	10	5
Temafloxacin	2	19	10	5
Tosufloxacin	4	18	10	5

β-Lactams antibiotics: procedure 1 Serum (500 μl) is deproteinized with 500 μl of acetonitrile. After centrifugation, 3.5 ml of methylene chloride are added to the supernatant. The mixture is gently shaken by rotation for 10 min (20 rpm) and then centrifuged for 10 min at 1000 *g*. An aliquot of the upper aqueous layer is injected into the chromatograph (Figs. 1–3).

This procedure may be applied to all the antibiotics listed in Table 1.

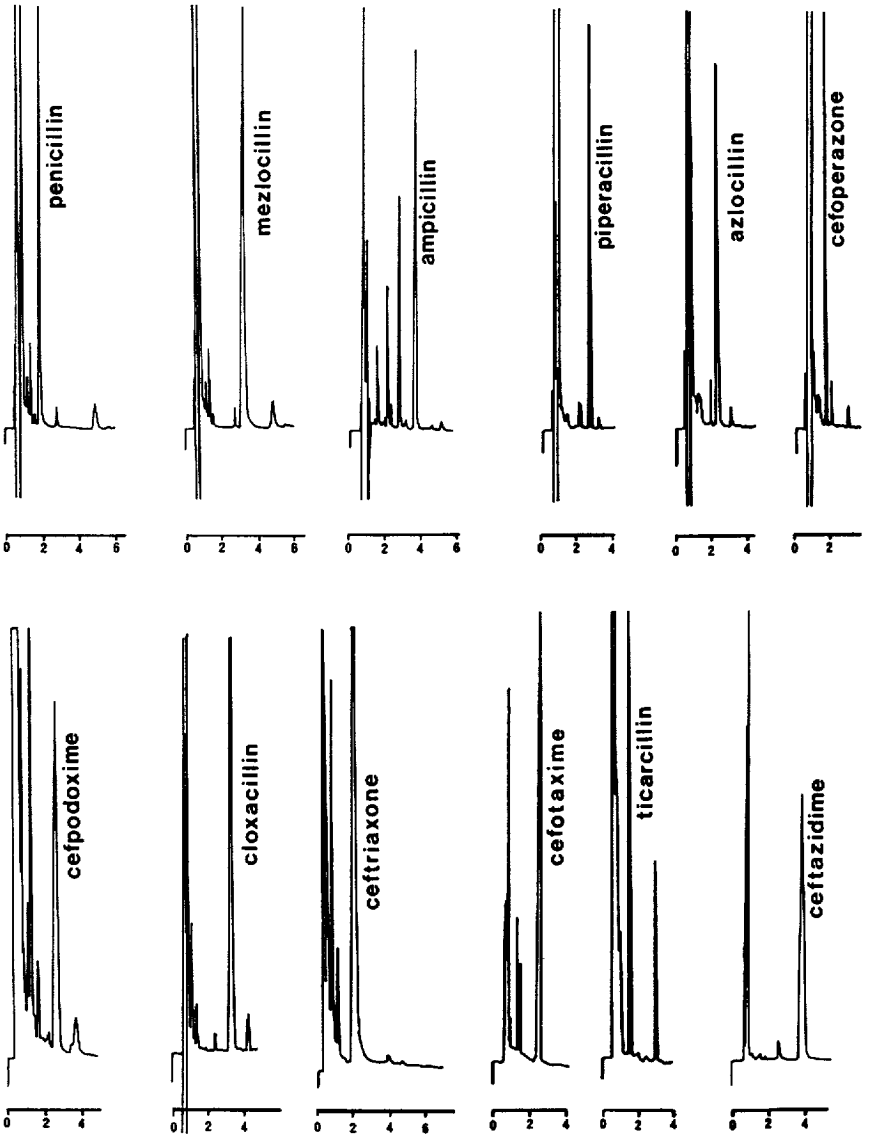

Fig 1 Representative chromatograms of some β-lactam antibiotics analysed with a high-speed column (as described in Table 1 and procedure 1) The concentration of each antibiotic was 50 μg/ml Time in min.

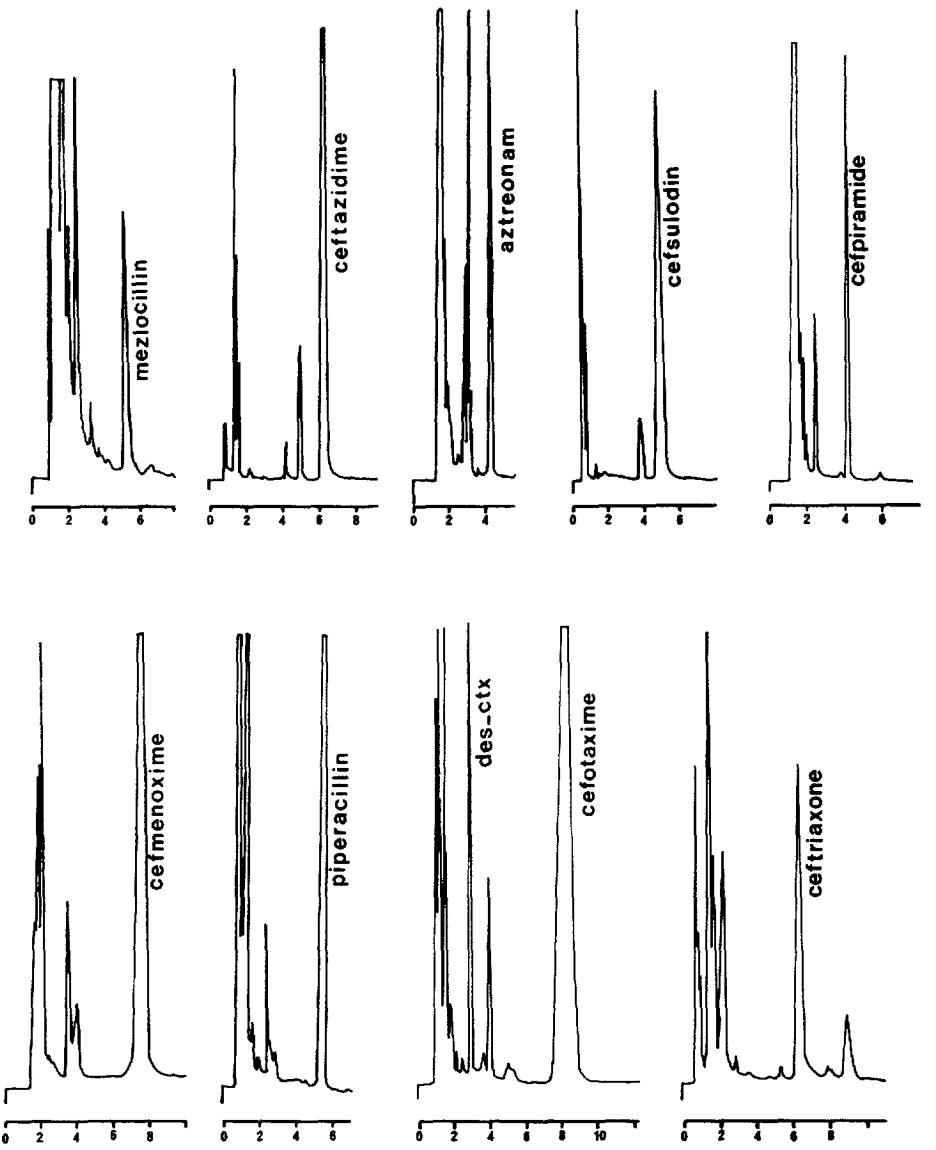

Fig 2 Representative chromatograms of some β-lactam antibiotics analysed with a 250 mm × 4 6 mm I D. column [cefotaxime, desacetylcefotaxime (des-ctx) and ceftriaxone] and a 150 mm × 4.6 mm I.D column (all other antibiotics) as described in Table 1 and procedure 1 Concentration range, from 20 μg/ml (cefpiramide) to 50 μg/ml (cefotaxime) Time in min

Quinolones. procedures 2–4. An aliquot of 500 μl of human serum is added to 3.2 ml of methylene chloride in a 6-ml screw-capped glass tube. After mixing on a vortex mixer, the tubes are gently shaken for 10 min by rotation (20 rpm) and

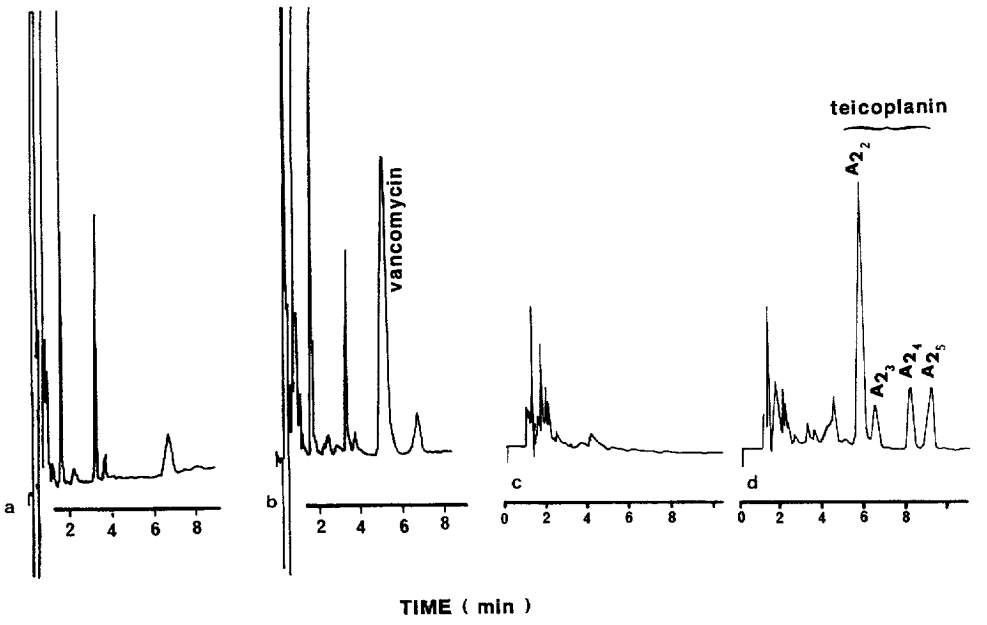

Fig 3. Chromatograms of (a and c) human serum extract free of antibiotics and (b and d) human serum extract supplemented with 25 μg/ml vancomycin and 25 μg/ml teicoplanin, respectively (Isocratic conditions for teicoplanin, as described in Table 9 and procedure 8)

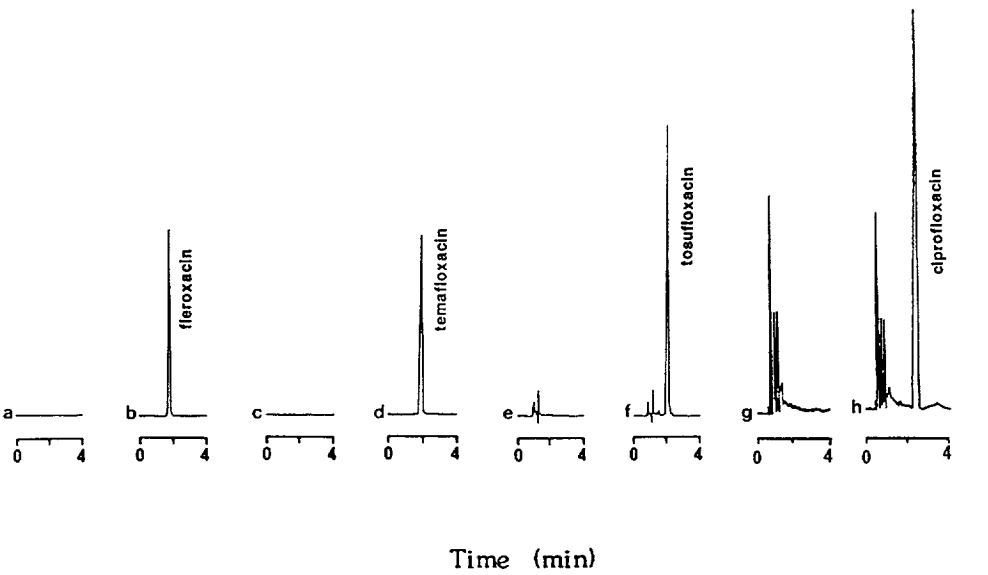

Fig 4 Chromatograms of extracts of control serum (a, c, e and g) and of serum supplemented with (b) 5 μg/ml fleroxacin, (d) 5 μg/ml temafloxacin, (f) 5 μg/ml tosufloxacin and (h) 1 μg/ml ciprofloxacin, respectively (as described in Table 2 and procedures 2–5)

then centrifuged for 10 min at 1000 *g*. The upper aqueous layer is aspirated off, and 3 ml of the lower organic phase are transferred to a second screw-capped glass tube. Antibiotics are back-extracted from the organic phase using 200 μl of 0.1 *M* sodium hydroxide (procedure 2), 200 μl of phosphoric acid at pH 2 (procedure 3) and 200 μl of acetic acid at pH 2.5 (procedure 4).

Centrifugation at 1000 *g* for 10 min results in phase separation. An aliquot of the upper aqueous layer is injected into the chromatograph.

Quinolones: procedure 5. The lower organic phase is evaporated to dryness under nitrogen at 37°C. The dry residue is diluted in 100 μl of phosphoric acid and an aliquot is injected into the chromatograph.

These procedures may be applied for all the quinolones listed in Table 2 (Fig. 4).

Roxithromycin· procedure 6. An aliquot of 2 ml of human serum is added to 3 ml of diethyl ether. After mixing on a vortex mixer, the tubes are centrifuged for 10 min at 1000 *g*. The upper organic phase is transferred to a second glass tube and evaporated to dryness under nitrogen at 37°C. The dry residue is diluted in 100 μl of methanol and an aliquot is injected into the chromatograph (Fig. 5).

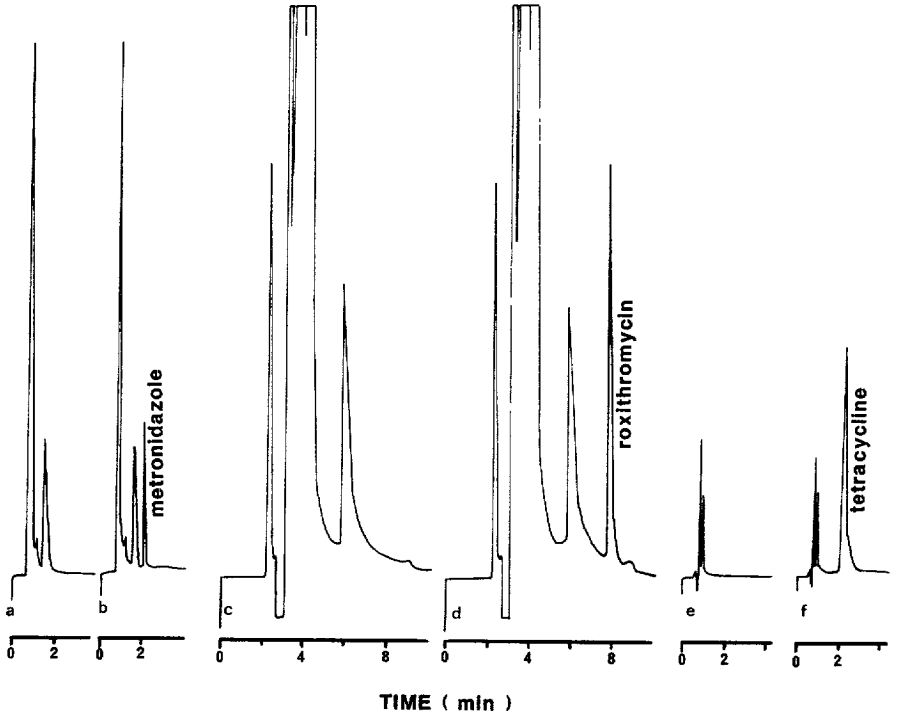

Fig 5 Chromatograms of (a, c and e) extracts of human serum free of antibiotics, and extracts of human serum supplemented with (b) 10 μg/ml metronidazole, (d) 2 5 μg/ml roxithromycin and (f) 10 μg/ml tetracycline, respectively.

Tetracycline. procedure 7. An aliquot of 500 μl of human serum is deproteinized with 50 μl of trifluoroacetic acid. After centrifugation, an aliquot of the supernatant is injected into the chromatograph (Fig. 5).

Teicoplanin: procedure 8. Teicoplanin is isolated and quantitatively recovered from biological samples (serum and urine) by pre-chromatography on a Sep-Pak cartridge (Waters) packed with C_{18} silica particles. Pre-chromatography eliminates most of the endogenous contaminants.

Each Sep-Pak cartridge is equilibrated with ultra-pure water (Milli-Ro, Milli-Q, Waters) before being loaded with 500 μl of the sample containing the internal standard. Then 4 ml of mixture 1 (water–acetonitrile, 90:10, v/v) are passed through the cartridge, and discarded. Then 500 μl of mixture 2 (water–acetonitrile, 50:50, v/v/) are added, and the eluate of 500 μl is also discarded. Finally, a further 500 μl of mixture 2 are loaded on the cartridge and allowed to elute selectively teicoplanin and piperacillin (internal standard) from the cartridge. This last eluate of 500 μl is kept, and a 50-μl volume of the extract is injected onto the chromatographic column for separation and quantitation of the major components of teicoplanin by gradient elution [7] (Fig. 3 and 6)

Tazobactam. procedure 9. An aliquot of 500 μl of human serum is stabilized with 10 μl of sodium bicarbonate, then deproteinized with 50 μl of trifluoroacetic acid. After mixing on a vortex mixing the tubes are centrifuged for 10 min at 1000 *g*. The supernatant is transferred to a second glass tube. then 1 ml of chloroform

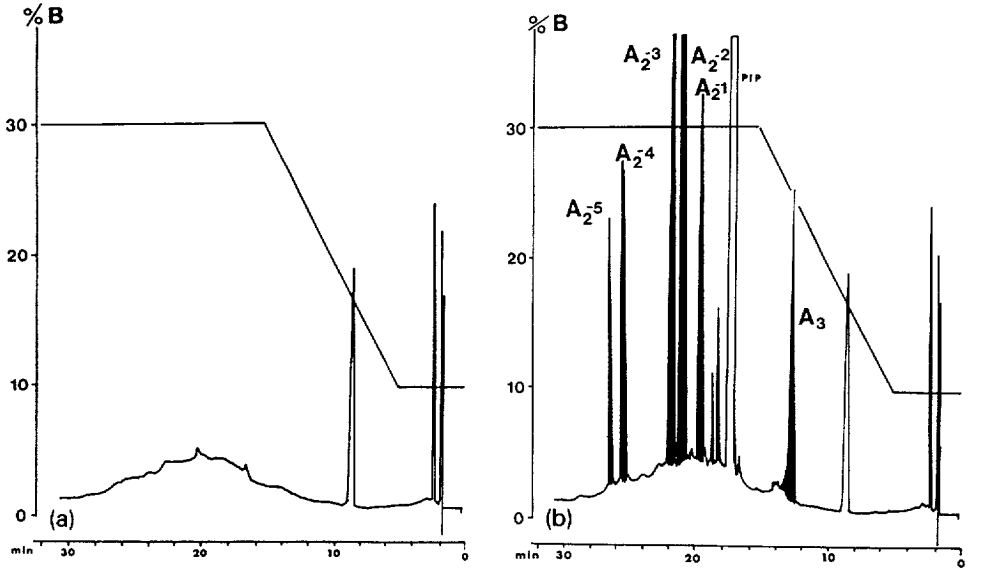

Fig 6 Chromatograms after cartridge extraction of (a) blank serum and (b) standard serum with 30 μg/ml teicoplanin and piperacillin (internal standard) (Gradient conditions as described in Table 9 and procedure 8)

is added. After mixing, the tubes are centrifuged for 15 min at 1000 *g*. An aliquot of the upper aqueous layer is injected into the chromatograph (Fig. 7)

5.2.2. Standardization of mobile phases

If two mobile phases consisting of different components are to be used consecutively on the same column to analyse two different antibiotics, the equilibrating period is often time-consuming. Thus, as often as possible, it is necessary to develop HPLC mobile phases consisting of the same components: organic solvents, buffer or counter-ion. This technique speeds the change from one antibiotic to another on the same analytical column, and consumes less chemicals. Table 1 lists the compositions of sixteen mobile phases all consisting of acetonitrile and ammonium acetate, and differing only in the percentage of acetonitrile. Notice that they correspond to sixteen antibiotics all extracted by the same procedure.

The same holds true for the mobile phases used for the analyses of quinolones (Table 2), which all consist of the same three components. Here again, there are no universal mobile phases and some antibiotics need their own elution system (Table 3).

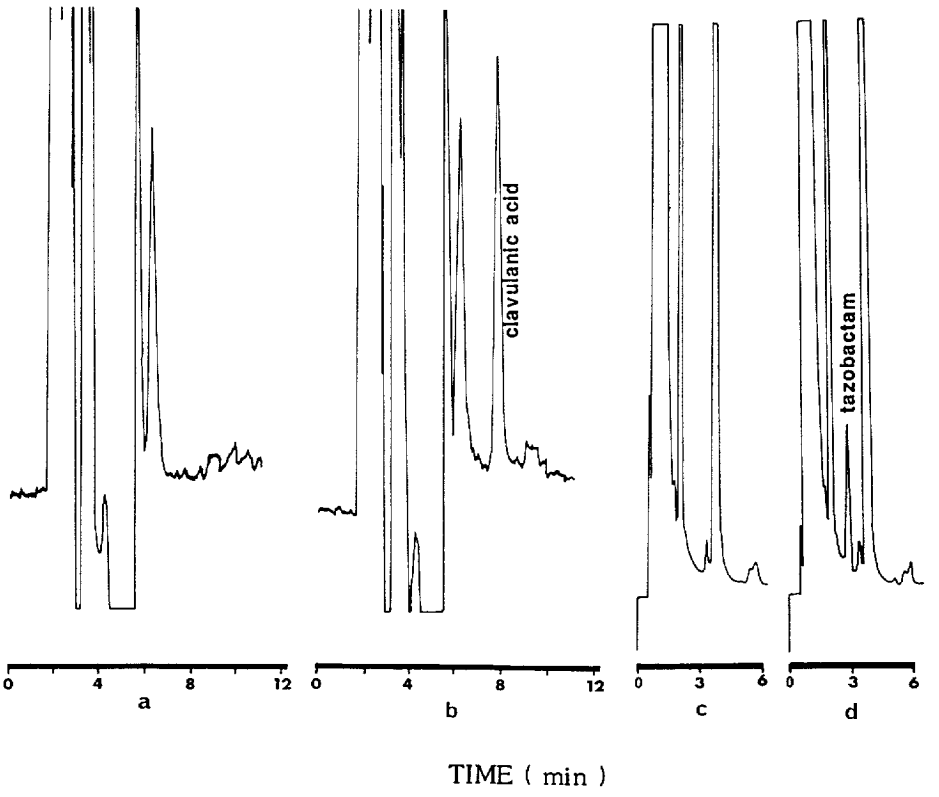

Fig 7 Typical chromatograms of two β-lactamase inhibitors (a and c) blank serum, (b and d) serum supplemented with 10 μg/ml clavulanic acid and 5 μg/ml tazobactam, respectively.

TABLE 3

EXAMPLES OF VARIOUS ANTIBIOTICS THAT COULD NOT BE INCLUDED IN A STANDARDIZED METHOD

Drug	Extraction procedure	Mobile phase			
		Acetonitrile (%)	Salt (m*M*)	TBAB (or HDTMAB*) (m*M*)	pH
Cefsulodin	1	22	–	10	3 7 (hydrochloric acid)
Aztreonam	1	33	Ammonium acetate (10)	5	6 8 (acetic acid)
Roxithromycin	6	60 (+20% methanol)	Ammonium acetate (8)	–	6 3
Clavulanic acid	1	10	Ammonium acetate (10)	1	5 3 (acetic acid)
Imipenem	1	–	Sodium dihydrogenphosphate (10)	–	7 7 (sodium hydroxide)
Tetracycline	7	20	Sodium dihydrogenphosphate (75)	–	2 (phosphoric acid)
Teicoplanin	8	27	Sodium dihydrogenphosphate (174)	–	2 (phosphoric acid)
Tazobactam	9	5	Phosphate buffer, pH 7 (80)	–	7
Ceftriaxone	1	50	Phosphate buffer, pH 7 (10)	11*	8

5.2.3. Isocratic HPLC versus gradient HPLC

Gradient HPLC may be an attractive alternative for difficult separations, or for the separation of all the constituents of the same drug, as is the case for gentamicin or teicoplanin [6,7]. Nevertheless, it is time-consuming as it is necessary to reequilibrate the column for every sample to be analysed, even for the same antibiotic. Thus, in routine analyses, gradient HPLC is less practicable than isocratic HPLC. For dosage adjustment of drugs consisting of a mixture of several components, it may be sufficient to monitor the major component of the mixture. For example, teicoplanin is a mixture of six constituents named A_3, A_{2-1}, A_{2-2}, A_{2-3}, A_{2-4} and A_{2-5} The constituent A_{2-2} represents *ca.* 54% of the mixture. We developed an isocratic methodology to measure A_{2-2} routinely (Table 9, Fig. 3), and gradient HPLC was used for the pharmacokinetic investigation of all six constituents (Table 9, Fig. 6).

5.2.4. Analytical column maintenance

An analytical column devoted to the analyses of antibiotics in biological samples should support *ca.* 800–1000 injections. The lifetime of a column is a function of several parameters (sample clean-up, pH of mobile phases, etc.). At the end of each day of work it is necessary to flush the column with a strongly elutive mobile phase to elute all the remaining material. For example, a reversed-phase analytical column should be washed with a mixture of organic solvent and water (50:50). It is advantageous to use the same non-polar solvent as in the mobile phases used on the column. Indeed, the following day, reequilibrating of the column is faster. The washing solvent should not contain salts. This allows an easier dissolution of mobile phase salts that may have crystallized in the system.

Sometimes, it is convenient to equilibrate the column at a low flow-rate during the night. Most modern HPLC systems may be programmed to wash and then reequilibrate the column with the appropriate mobile phase during the night. If not in use for a few days (weekend), the analytical column should be stored under the washing mixture (containing no salts).

5.3. Organization of the laboratory: how to save time?

Beside the standardization of the methodologies, there are numerous ways to save time in a laboratory devoted to the monitoring of antibiotics. The use of high-speed reversed-phase columns is one of the most convincing: these are short columns (50–75 mm) packed with 3 μm diameter coated silica particles. Beside an enhanced resolution (see Figs. 1 and 2, comparing conventional and high-speed reversed-phase HPLC of β-lactam antibiotics) and limit of detection, this type of column results in shorter analysis times (2–4 min). About fifteen samples per hour may be analysed by HPLC. The time required for column reequilibration when changing the mobile phase is also much shorter. These columns are really adapted to the analysis of numerous small series of different molecules.

When using ion-pairing chromatography, it may be useful to reserve one column for all the antibiotics needing this type of chromatography. Indeed, it often takes much time to impregnate an analytical column with a counter ion such as tetrabutylammonium salts (bromide, chloride or hydrogensulphate). Once the column is impregnated, it is then difficult to flush the counter ion out totally in order to work under conventional conditions.

One way to improve the practicability of HPLC is to dispose permanently of ready-to-use calibration serum for every antibiotic being analysed in the laboratory, and at each concentration entering in the corresponding calibration curves. Each set of standards should be regularly prepared every three of four months with pure titrated powder of antibiotic (Do not forget to correct for powder titer when weighing the antibiotic for the stock solutions.) Antibiotics are generally stable for this period when stored at −80°C. Nevertheless, studies on the stability at −80°C should be performed for every new antibiotic being analysed in the laboratory. It is convenient to keep a list of the calibration standards so as to know at all times the remaining amount of each standard (note the manufacture date). It is also important to point out every day or every two days the peak area or peak height of the standards when using one-point calibration curve. It is an easy way to detect any significant drift in the method.

Mobile phase should be stored at ambient temperature, not at 4°C, to be ready to use when necessary. A mobile phase that is too cold should be allowed to equilibrate at room temperature before use, because variations of retention time occur with varying temperature, owing to pH variation. Note that it is important to adjust the pH of the mobile phase when stabilized at room temperature, or to use a thermocompensator-equipped pH meter. Mobile phases are generally stable for three to four days at room temperature.

6 HPLC OF ANTIBIOTICS IN BIOMEDICAL RESEARCH

There are numerous applications of this technology applied to antibiotics in the pharmaceutical industry, the food industry and in general microbiology.

6.1. Drug monitoring

Whereas there is a need for fast analysis in clinical routine and therefore a preferential use of isocratic HPLC, in the field of biomedical research gradient HPLC is often preferred. Use of gradient HPLC is an easy way to determine the optimal isocratic analytical conditions for a new antibiotic with unknown chromatographic behaviour. After a few gradient runs with various slopes of non-polar solvent (*e.g.* from 5 to 100% in 30 min, then, as determined by the chromatograms from this first run, from 15 to 50% in 30 min), and as a function of the retention time of the antibiotic in each of these runs, it is possible to calculate the approximate non-polar solvent/water ratio to use under isocratic conditions.

6.2. Pharmacokinetic studies of antibiotics

The establishment of pharmacokinetic parameters of a newly developed antibiotic is an important step in antibiotic research. For this purpose, HPLC is the method of choice because it measures a chemical entity rather than a biological activity as in the case with bioassays.

Automated systems with a sample injector are particularly well adapted for the large series of samples involved in this kind of study. Pharmacokinetic studies imply investigations on the tissue distribution of a new antibiotic, and that means the determination of tissue concentrations. As tissue extracts are often complex mixtures, the use of gradient HPLC is particularly helpful.

6.3. Metabolism of newly introduced antibiotics

The use of HPLC is of great interest in the study of metabolism of a new drug. There are often very small differences between the molecular structures of antibiotics and their metabolites. Nevertheless, the great specificity of HPLC generally allows them to be separated under either isocratic or gradient conditions.

It is important to note that metabolites, even if they are microbiologically active, can be measured accurately by HPLC only in biological fluids. With this purpose in mind, it is essential to develop methodology with pure calibration material. Indeed, it is incorrect to suppose that metabolites give a detector response equivalent to that of the parent compounds, by UV absorbance or fluorescence. Sometimes it may also be necessary to develop different extraction procedures for metabolites and parent compounds

In the investigational steps of the metabolism of a new quinolone, for example, it may be of considerable interest to compare the results obtained by both microbiological and HPLC assays. Statistically significant differences in the amount of parent drug eliminated in bile or urine may be due to the presence of unknown active metabolites [10,11] (Figs. 8 and 9).

7 DETERMINATION OF ANTIBIOTICS BY HPLC

For the convenience of the reader, we will tabulate some HPLC methods available for different antibiotics in every major family. Only one or two procedures will be indicated for each antimicrobial agent despite the fact that for some molecules there are numerous papers.

7.1. β-Lactam antibiotics

7.1.1. Penicillins

The proposed methods for the measurement of penicillins in serum or plasma are shown in Table 4. In general, penicillins are easily quantified by UV absor-

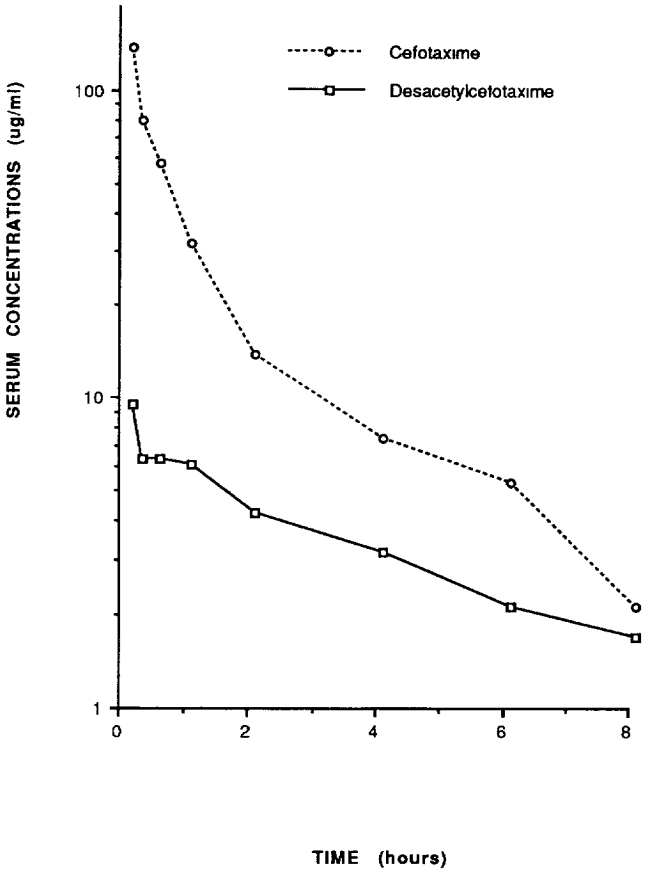

Fig 8 Concentration–time curves in serum of cefotaxime and desacetylcefotaxime after an intravenous dose of 15 mg/kg

bance at 214 nm. It is possible to work at longer wavelengths (254–280 nm), but this may result in lower detection limits. In these cases, derivatization is one way to enhance the detection limit, as is the case, for example, with clavulanic acid, a β-lactamase inhibitor [12]. One has to remember, however, that derivatization decreases the practicability of an analytical method The acylureidopenicillin apalcillin may be measured as described in ref. 16.

7.1.2. Cephalosporins

The different procedures for the HPLC of cephalosporins are presented in Table 5. Most of them produce a good absorbance at 254 nm, and some of these drugs may be quantified up to 280 nm. In almost all the cases, reversed-phase chromatography is used on both RP8 or RP18 stationary supports. Note that cefsulodin and ceftriaxon both need ion-pair chromatography with quaternary

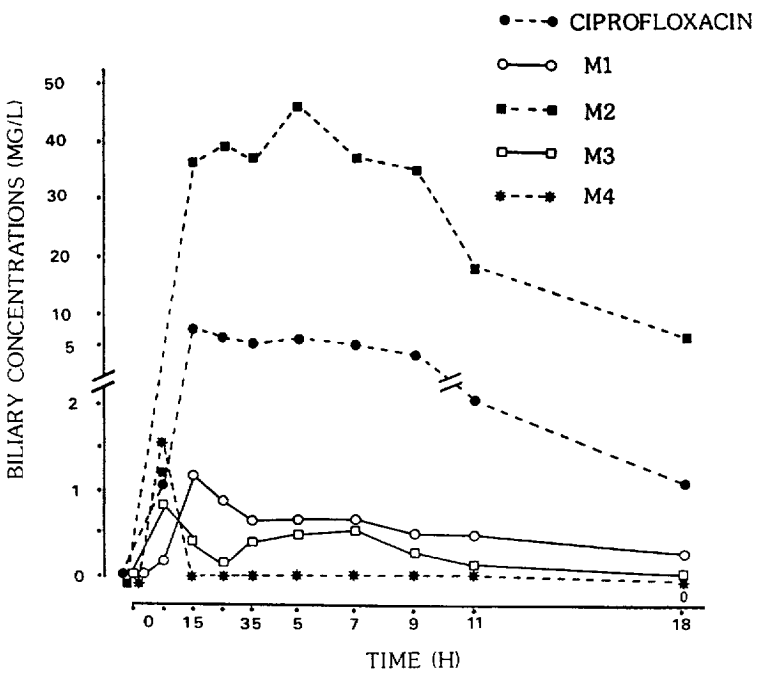

Fig 9 Biliary concentrations of ciprofloxacin and its four metabolites (M_1–M_4) after a single oral dose of 500 mg

ammonium as counter ion. Cefamandole, cefotiam and cefalexine may also be measured by ion-pair reversed-phase chromatography.

Owing to the good absorbance of cephalosporins, biological samples do not need extensive clean-up. Deproteinization by an organic solvent or an acid is the most often suitable. Three recently introduced cephalosporins, cefpirome, cefetamet and cefodixime, may be measured as described in refs 30–32.

7.1.3. Miscellaneous β-lactams

Clavulanic acid and sulbactam, which both are β-lactamase inhibitors, the monobactam aztreonam, the carbapenem imipenem and cilastatin are grouped in Table 6. Aztreonam, carumonam [36], sulbactam and clavulanic acid need ion-pair chromatography. For clavulanic acid we developed an ion-pair technique using tetrabutylammonium bromide as counter ion [4] This counter ion exhibits a bathochromic shift, rendering the derivatization step unnecessary.

7.2. Aminoglycosides

In most instances, aminoglycoside monitoring by HPLC necessitates derivatization (Table 7). This is generally done during the sample clean-up procedure rather than post-column.

TABLE 4

HPLC OF PENCILLINS

For abbreviations, see the list at the start of this review

Penicillin	Extraction[a]	Column	Mobile Phase		Detection
Penicillin G	D. S + CH_3CN (v/v)	Ultrasphere ODS,	H_2O	66%	UV 254 nm
Ref 1	O S1 + CH_2Cl_2M + C	150 × 4.6 mm,	CH_3CN	24%	
	S1	5-μm C_{18}	CH_3COONH_4 (0.2 *M*)	10%	
			pH	5	
Ref 2	*Idem*	Ultrasphere ODS, high-speed, 75 × 4 6 mm	*Idem*		UV 214 nm
Ampicillin	*Idem*	Ultrasphere ODS,	H_2O	82%	UV 254 nm
Ref 1		150 × 4 6 mm,	CH_3CN	8%	
		5-μm C_{18}	CH_3COONH_4 (0 2 *M*)	10%	
			pH	5	
Ref 2	*Idem*	Ultrasphere ODS, high-speed 3-μm C_{18}	*Idem*		UV 254 nm
Amoxicillin	*Idem*	Ultrasphere ODS,	H_2O	75%	UV 254 nm
(NPPM)		150 × 4 6 mm,	CH_3CN	15%	
		5-μm C_{18}	TBAB (0 1 *M*)	5%	
			CH_3COONH_4 (0 2 *M*)	5%	
			pH	8	
Ref 12	Ultrafiltration,	Bondapak,	H_2O	84%	UV 227 nm
	Amicon MPS 1 system	250 × 4.6 mm,	CH_3CN	6%	and
		C_{18}	KH_2PO_4 (1 *M*)	10%	derivatization
			pH	3.2	
Ref 13		Zorbax,		Gradient	UV 274 nm
		250 × 4 6 mm,		A B	
		7-μm C_{18},	H_2O	90% 45%	

		guard column	CH_3OH	5% 50%	
			KH_2PO_4 (0 2 *M*)	90% 45%	
			pH	7	
			5% to 65% B in 5 to 35 min		
Oxacillin	D S + CH_3CN (v/v)	Ultrasphere ODS,	H_2O	66%	UV 254 nm
Cloxacillin	O. S1 + CH_2Cl_2M + C,	150 × 4 6 mm,	CH_3CN	24%	
Ref 1	S1	5-μm C_{18}	CH_3COONH_4 (0 2 *M*)	10%	
			pH	5	
Ref 2	*Idem*	Ultrasphere ODS,	H_2O	62%	UV 214 nm
		high-speed	CH_3CN	28%	
			CH_3COONH_4 (0.2 *M*)	10%	
			pH	5	
Carbenicillin		Spherisorb ODS,	CH_3OH	36%	UV 254 nm
Ref 14		150 × 4 6 mm,	KH_2PO_4 (0.05 *M*)	64%	
		5 μm	TBAB	0 1% (w/v)	
			pH	3 35	
Ticarcillin	D S + CH_3CN (v/v)	Ultrasphere ODS,	CH_3CN	36%	UV 214 nm
Ref 1	O: S1 + CH_2Cl_2M + C	75 × 4.6 mm,	Citrate buffer (10 m*M*)	64%	
	S1	3-μm C_{18}	pH	2	
Mezlocillin	*Idem*	*Idem*	H_2O	66%	UV 214 nm
Ref 1			CH_3CN	24%	
			CH_3COONH_4 (0 2 *M*)	10%	
			pH	5	
Azlocillin	*Idem*	*Idem*	*Idem* but CH_3CN	20%	UV 214 nm
Ref 1					
Piperacillin	*Idem*	*Idem*	*Idem*		UV 214 nm
Ref. 1					
Mecillinam	D. S + CH_3CN (v/2v)	Bondapak,	H_2O	68%	UV 220 nm
Ref. 15	O S1 + diethyl ether,	300 × 4 mm,	CH_3OH	32%	
	C + N	10-μm phenyl	Phosphate buffer (1 *M*)	0 5%	
			pH	7	

[a] *Idem* same conditions as preceding entry.

TABLE 5

HPLC OF CEPHALOSPORINS

Cephalosporin	Extraction	Column	Mobile phase		Detection
Cephalotin Ref 17	D S + CH_3OH, CH_3COONa, S1 injected	Bondapak, 300 × 4 mm, 10-μm C_{18}	CH_3COONa (0 01 *M*) CH_3CN pH	80% 20% 5 2	UV 254 nm
Ref 18	O S + HCl + chloroform–pentanol, M + C, supernatant + PBS pH 7, M + C S1	Bondapak, 300 × 3 9 mm, 10-μm C_{18}	CH_3CN or CH_3OH CH_3COONa (0 01 *M*) pH	15% 85% 4 8	UV 240 nm
Cephalexin Ref 17	*cf* Cephalotin	*cf* Cephalotin	*cf* Cephalotin (55/12)		UV 254 nm
Ref. 19	D S + H_3PO_4 + CH_3OH or S + TCA 10% M + C, S1	LiChrosorb, 150 × 4 7 mm, 5-μm RP 18	CH_3OH THBS (0 02 *M*) K_3PO_4 (0 024 *M*) KH_2PO_4 (0 016 *M*)	23% 77%	UV 254 nm
Cefradin Ref 20	D S + TCA 6% (v/v), M + C, S1	Nucleosil SA, 200 × 4 6 mm, 5 μm	$NH_4PO_4H_2$ (0 6 *M*) CH_3OH CH_3CN	30% 35% 35%	UV 240 nm
Cefadroxil Ref 21	*Idem*	*Idem*	*Idem*		UV 240 nm
Cefazolin Ref 18	*cf* Cephalotin	*cf* Cephalotin	*cf* Cephalotin		UV 275 nm
Cefaclor Ref. 17	*cf* Cephalotin	*cf* Cephalotin	*cf* Cephalotin (91/9)		UV 254 nm
Cefapirin Ref. 17	*cf* Cephalotin	*cf* Cephalotin	*cf*. Cephalotin (81/19)		UV 254 nm
Cefamandole Ref. 18	*cf* Cephalotin	*cf* Cephalotin	*cf* Cephalotin		UV 270 nm
Cefuroxime	D S + TCA 6% (v/v),	Bondapak,	CH_3CN	11%	UV 254 nm

Ref 22	M+C, neutralization by $NaHCO_3$, S1	300 × 3 9 mm 10-μm C_{18}	Acetate (0 01 *M*) pH	89% 4 8	
Ref 18	*cf* Cephalotin	*cf* Cephalotin	*cf* Cephalotin		UV 254 nm
Cefoxitin Ref 18	*cf* Cephalotin	*cf* Cephalotin	*cf* Cephalotin		UV 245 nm
Cefotiam Ref. 19	*cf*. Cephalotin	Lichrosorb, 150 × 4 5 mm, 5-μm RP 18	*cf* Cephalotin		UV 254 nm
Cefsulodin Ref 19	*cf* Cephalexin	LiChrosorb RP 8	CH_3OH THBS (0.1 *M*) K_3PO_4 (0.09 *M*)	15% 85%	UV 254 nm
Ref 2	D S+CH_3CN (v/v) O S1+CH_2Cl_2 M+C, S1	Ultrasphere ODS, 150 × 4 6 mm, 3-μm C_{18}	CH_3CN TBAB (5 m*M*) pH	10% 90% 3 7	UV 254 nm
Cefoperazone Ref 2 Ref 23	*cf* Cefsulodin	Ultrasphere ODS, 75 × 4 6 mm, 3-μm C_{18}	H_2O CH_3CN CH_3COONH_4 (0 2 *M*)	70% 20% 10%	UV 254 nm
Cefroxadin Ref 19	*cf* Cephalexin	LiChrosorb, 100 × 7.5 mm, 5-μm RP 8	CH_3OH H_3PO_4 (0.002 *M*)	30% 70%	UV 254 nm
Cefotaxime + desacetyl-cefotaxime	*cf* Cefsulodin, ref 2	Ultrasphere ODS, 250 × 4.6 mm 5-μm C_{18}	CH_3CN H_2O CH_3COONH_4 (0.2 *M*) pH	8% 82% 10% 5	UV 254 nm
Cefotaxime Ref 2	*cf*. Cefsulodin, ref. 2	Ultrasphere high-speed, 3-μm C_{18}	CH_3CN H_2O CH_3COONH_4 (0 2 *M*) pH	12% 78% 10% 5	UV 254 nm
Ceftizoxime Ref 24	D. S+CH_3CN (v/v), injection S1	Bondapak, 300 × 4 mm, phenyl	13% CH_3CN in 0 02 *M* PBS pH	2 6	UV 254 nm
Cefmenoxime	D S+CH_3CN (v/v)	100 × 8 mm,	H_2O	69%	UV 254 nm

(Continued on p 538)

TABLE 5 *(continued)*

Cephalosporin	Extraction	Column	Mobile phase		Detection
Ref. 25	S1 + CH_3COOH (v/v)	10-μm C_{18}	CH_3CN	32%	
			CH_3COOH		
			pH	3.4	
Ref. 2	*cf.* Cefsulodin	*cf.* Cefsulodin	*cf.* Cefotaxime		UV 254 nm
Ceftriaxone	*cf.* Cefsulodin	*cf.* Cefperazone	50% CH_3CN		UV 254 nm
Ref. 2			in 11 m*M* HDTMAB		
			and 10 m*M* PBS		
			pH	8	
Ceftazidime	*cf.* Cefsulodin	*cf.* Cefsulodin	H_2O	81%	UV 254 nm
Ref. 2			CH_3CN	9%	
			CH_3COONH_4 (0.2 *M*)	10%	
			pH	5.4	
Latamoxef	D. S + CH_3OH (v/v),	HC-SIL-X ODS,	4% CH_3OH in 10 m*M*		UV 254 nm
Ref. 26	S1 + citrate (v/v)	250 × 4.6 mm,	$(NH_4)_3PO_4$		
		10-μm C_{18}	pH	6.5	
Cefpiramide	*cf.* Cefsulodin, ref. 2	*cf.* Cefsulodin, ref. 2	H_2O	75%	UV 254 nm
Ref. 27			CH_3CN	15%	
Ref. 28			CH_3COOH (0.2 *M*)	10%	
			pH	5	
Cefixime	D. S + TCA 6% (v/v),	RCM-Novapak,	H_2O	828 ml	UV 254 nm
Ref. 29	injection S1	100 × 8 mm,	CH_3CN	170 ml	
		5-μm C_{18}	NaH_2PO_4	1.36 g	
			H_3PO_4	2 ml	

TABLE 6

HPLC OF MISCELLANEOUS β-LACTAMS AND CILASTATIN

Drug	Extraction	Column	Mobile phase		Detection
Aztreonam [2]	*cf* Cefsulodin	*cf.* Cefsulodin	33% CH_3CN in 10 m*M* CH_3COONH_4 and 5 m*M* TBAB pH 7		UV 254 nm
Clavulanic acid [4]	*cf* Cefsulodin	Ultrasphere ODS, 250 × 4 6 mm, 5-μm C_{18}	10% CH_3CN in 1 m*M* TBAB and 20 m*M* CH_3COONH_4 pH 5		UV 214 nm
Sulbactam [33]	Derivatization, then *cf* cefsulodin, ref. 2	Ultrasphere ODS, 150 × 4 6 mm, 5-μm C_{18}	PBS (0 1 *M*), pH 6 1 CH_3CN TBAB 40%	89% 11% 2%	UV 313 nm
Imipenem [34]	Ultrafiltration Amicon-CF 50 A	Hypersil ODS, 100 × 5 mm, 5 μm	Boric acid (0 2 *M*) pH 7 2		UV 313 nm
Cilastatin [35]	Derivatization, then cartridge (Sep-Pak or Baker)	Biosil ODS, 250 × 4 mm	11% isopropanol in 0 2% H_3PO_4 pH 3		Fluorimetry· exc. 335 nm em. 455 nm

TABLE 7

HPLC OF AMINOGLYCOSIDES

Aminoglycoside	Extraction	Column	Mobile phase		Detection
Gentamicin [6]	Chromatography on SM-Sephadex	Bondapak, 300 × 3 9 mm C_{18}	3% CH_3OH in Na_2SO_4 (0 2 *M*) PSS (0.02 *M*) and 0 1% CH_3COOH		Fluorimetry exc. 340 nm em. 418 nm
Tobramycin [37]	D S + CH_2CN, O S1 + CH_2Cl_2, then derivatization	Bondapak, 300 × 3 9 mm, cyano	H_2O CH_3OH CH_3CN EDTA	35% 62% 3% 2 5 g	Fluorimetry exc 340 nm em 418 nm
Dibekacine [38]	P S + perchloric acid, S1 + derivatization	Radialpak, 10-μm C_{18}	20% CH_3CN in 37 m*M* ESD and 5 m*M* OSS pH	3 5	Fluorimetry exc 351 nm em 440 nm
Netilmicin [39]	D S + CH_2CN, O S1 + CH_2Cl_2 + derivatization	C_{18}	H_2O CH_3CN	5% 95%	Fluorimetry exc 220 nm
Amikacin [6]	*cf* Gentamicin	*cf* Gentamicin	*cf.* Gentamicin		Fluorimetry exc 340 nm
Kanamycin [38]	*cf* Dibekacin	*cf* Dibekacin	*cf* Dibekacin		*cf* Dibekacin
Neomycin [40]	Derivatization (DNFB), O $C_2H_2Cl_2$	Zorbax SIL, 250 × 4 6 mm	$C_2H_2Cl_2$ Heptane CH_3OH H_2O Diethylamine	790 ml 150 ml 55 ml 3.6 ml 1.5 ml	UV 350 nm
Novobiocin [41]		LiChrosorb S1-100, 250 × 4 6 mm, 5 μm	Butyl chloride THF CH_3OH CH_3COOH	58 vol. 5 vol. 4 vol 3 vol	UV 340 nm or 254 nm

Fluorimetry is the commonest quantitation method for aminoglycosides, except for neomycin and novobiocin, which are quantitated by UV absorbance.

7.3. Quinolones

HPLC is particularly suitable for the measurement of quinolones in biological fluids in clinical routine or during pharmacokinetic studies [11]. Indeed, some of them are biotransformed in one or more metabolites, with varying antimicrobial activity [2,11,42,43]. Some methodologies are described in Table 8. It is important to note that quinolones are often well resolved only by ion-pair reversed-phase chromatography. Contrary to a widespread idea, fluorimetry is not necessarily the best quantitation method, as has been proven by UV absorbance sometimes resulting in lower detection limits.

The assays of quinolones by HPLC are quite easy to standardize [9,52–54].

7.4. Macrolides and glycopeptides

Some assays for these antibiotics are summarized in Table 9. Whereas pristinamycin and glycopeptides show good absorbance in the UV region, erythromycin is more easily quantified by electrochemical detection [56]. The selectivity of HPLC is well illustrated in this group of molecules, as it allows the separate measurement of the two main constituents PI_A and PII_A of pristinamycin [42] and the six main constituents of teicoplanin (Fig. 9). For the clinical routine use of teicoplanin, we developed an isocratic method to measure $A_{2\text{-}2}$, the main constituent (Table 9, Fig. 3).

7.5. Trimethoprim, sulfamethoxazole, tetracyclines and chloramphenicol

The methods used for these antibiotics are shown in Table 10. The detection mode is generally UV absorbance

7.6. Others

The methods listed in Table 11 are also suitable for the assay of the metabolites of isoniazid, metronidazole and tinidazole.

8 FUTURE PROSPECTS AND CONCLUSIONS

The HPLC of antibiotics was first applied to pharmacokinetic studies. Its suitability for routine hospital use where numerous different antibiotics are to be assayed is greatly dependent on the development of standardized clean-up procedures and standardized chromatographic conditions. The use of high-speed columns represents undeniable progress. In our hospital, we have been monitoring

TABLE 8

HPLC OF QUINOLONES

Quinolone	Extraction	Column	Mobile phase		Detection
Nalidixic acid [44]	S + NaOH + HCl, O + $CHCl_3$, M + C, $CHCl_3$ + NaOH, M + C, injection NaOH	Zipax SAX anion exchange 500 × 2.1 mm	0.05 *M* Na_2SO_4 0.02 *M* boric acid pH 9		UV 254 nm
Pipemidic acid [45]		Amino SIL-X-1 250 × 2.6 mm	DMF $CHCl_3$ acetic acid	50 ml 50 ml 1 ml	UV 254 nm
Flumequine [46]	S + HCl + $CHCl_3$, M + C, $CHCl_3$ + NaOH, M + C, injection NaOH	Zipax SAX anion exchange 610 × 2.3 mm	*cf* Nalidixic acid		UV 254 nm
Pefloxacin [20]	S + PBS 7.4 (v/v) + CH_2Cl_2, M + C $CHCl_2$ evaporation mobile phase	Nucleosil 250 × 4.6 mm 5-μm C_{18}	H_2O CH_3OH H_3PO_4 TBAI	700 ml 300 ml 1.9 ml 1.4 g	UV 278 nm
Pefloxacin [47]	*Idem* + ammonia 1%	LiChrosorb RP 18 100 × 4.6 mm 10 μm	Gradient CH_3CN in H_2O + NaH_2PO_4 + TBAI + TEA		UV 270 nm
Norfloxacin [20]	*cf* Pefloxacin	*cf* Pefloxacin	*cf* Pefloxacin		UV 278 nm

Norfloxacin [48]	S + NaOH + CH_2Cl_2 + PBS, M + C CH_2Cl_2 + NaOH, M + C, injection NaOH	Vydac 250 × 4 6 mm anion exchange 10 μm	CH_3CN PBS (0 05 *M*) pH	20% 80% 7	UV 273 nm
Ofloxacin [20,47,49]	*cf* Pefloxacin	*cf* Pefloxacin	*cf* Pefloxacin		UV 294 nm
Enoxacin [50]	D S + TCA 3%, injection S1	LiChrosorb RP2 150 × 4 6 mm 5 μm	H_3PO_4 (7 m*M*) DMF ethanol	77% 20% 3%	UV 342 nm
Ciprofloxacin [8]	S + CH_2Cl_2, M + C, CH_2Cl_2 + H_3PO_4 pH 2, M + C, injection H_3PO_4	Ultrasphere ODS 150 × 4.6 mm 5-μm C_{18}	10% CH_3CN in 5 m*M* TBAB pH 2		UV 254 nm
Ciprofloxacin [20]	*cf.* Pefloxacin	*cf.* Pefloxacin	*cf* Pefloxacin		UV 278 nm
Fleroxacin [51]	S + chloroform, isopropanol, M + C, evaporation, then mobile phase	Toyo-Soda ODS, 120 T 250 × 4 6 mm 5 μm	TBABS (5 m*M*) CH_3OH	18 vol 7 vol	Fluorimetry exc 290 nm em 450 nm or UV 290 nm
Difloxacin [20]	Ultrafiltration system micropartition, Centrifree TM or S + buffer + CH_2Cl_2, M + C, evaporation CH_2Cl_2, then mobile phase	Adsorbosphere HS 250 × 4 6 mm 7-μm C_{18}	50% CH_3CN PBS (0 05 *M*) 0 2% SDS		Fluorimetry exc 280 nm em 390 nm or UV 280 nm

TABLE 9

HPLC OF ERYTHROMYCIN, PRISTINAMYCIN AND GLYCOPEPTIDES

Antibiotic	Extraction	Column	Mobile phase		Detection
Erythromycin [55]	Plasma + ether evaporation, residue in CH_3CN	Novapak C_{18}	CH_3COONa (56 m*M*) CH_3CN CH_3OH pH	56% 40% 4% 7	Electrochemical
Pristinamycin ($PI_A + PII_A$) [42]	Plasma + hexane M + C, aqueous phase + CH_2Cl_2, M + C, CH_2Cl_2 evaporated, residue in 60 μl	S G E, 250 × 4 mm 5-μm C_{18}	CH_3CN Sodium phosphate (43 m*M*) pH	53 5% 46 5% 6	UV 254 nm
Vancomycin [3–5]	D S + (CH_3CN–2-propanol), M + C, S1 + CH_2Cl_2, injection S	Ultrasphere ODS, 150 × 4.6 mm, 5-μm C_{18}	CH_3CN CH_3COONH_4 (0 2 *M*) H_2O pH	9% 10% 81% 5 4	UV 214 nm
Teicoplanin [7] (A_3, A_{2-1}, A_{2-2}, A_{2-3}, A_{2-4}, A_{2-5})	Cartridge Sep-Pak	Ultrasphere ODS, 150 × 4 6 mm, 5-μm C_{18}	Gradient of acetonitrile in NaH_2PO_4 (15 4 m*M*)		UV 214 nm
Teicoplanin (A_{2-2}) (NPPM)	*Idem*	*Idem*	27% CH_3CN in NaH_2PO_4 (17 4 m*M*) pH 2		UV 214 nm

TABLE 10

HPLC OF CHLORAMPHENICOL, TRIMETHOPRIM-SULFAMETHOXAZOLE, TETRACYCLINE AND DOXYCYCLINE

Antibiotic	Extraction	Column	Mobile phase		Detection
Chloramphenicol [57]	Direct injection of serum	Radial Pak, 100 × 8 mm, 10-μm C_{18}	Acetic acid (pH 3) CH_3CN	75% 25%	UV 280 nm
Trimethoprim and sulfamethoxazole [58]	D S+CH_3CN (v/v), M+C, S1 diluted 50 50 with H_2O, injection	LiChrosorb RP 8, 150 × 4 6 mm, 5 μm	KH_2PO_4 (1/15 *M*) Na_2HPO_4 (1/15 *M*) CH_3CN	977 ml 23 ml 5 7 ml	UV 254 nm
Tetracycline [59]	S+TBAB (v/v) + buffer + chloroform–heptanol, M+C, chloroform–heptanol + PA, M+C add hexane injection of aqueous phase	LiChrosorb RP2, 5 μm	35% CH_3CN in 0 01 *M* H_3PO_4		UV 357 nm
Doxycycline [60]		Hamilton PRP1, 250 × 4 6 mm, 10 μm	THF PBS (0 2 *M*) (pH 8) TBAS (0 2 *M*) (pH 8) EDTA (pH 8) H_2O	6% 10% 5% 1% 78%	UV 254 nm

TABLE 11

HPLC OF ISONIAZID AND IMIDAZOLE DERIVATIVES

Antibiotic	Extraction	Column	Mobile phase		Detection
Isoniazid [61]	Plasma + $(NH_4)_2SO_4$ + H_2O–chloroform–butanol, M + C, organic phase + H_2SO_4, M + C, injection H_2SO_4	μBondapak, 115 × 80 mm, 5-μm C_{18}	1 m*M* diocethylsulphosuccinate in H_2O–ethanol (55 45), pH 2.5		UV 254 nm
Metronidazole [62]	D plasma + ethanol + $ZnSO_4$ (0.1 *M*), injection S1	μBondapak, 300 × 3 9 mm, 10-μm C_{18}	KH_2PO_4 (0 005 *M*), pH 4 5 CH_3OH THF	82 6% 16 5% 0 9%	UV 324 nm
Metronidazole [63]	D S + perchloric acid, injection S1	Nucleosil, 300 × 4 mm, 10-μm C_{18}	CH_3OH CH_3CN KH_2PO_4 (0 005 *M*), pH 4	4% 3% 93%	UV 320 nm
Tinidazole [63]	*Idem*	*Idem*	CH_3OH KH_2PO_4 (0 005 *M*), pH 4	20% 80%	UV 320 nm

almost all available antibiotics in clinical routine for seven years with only three isocratic chromatographs and six high-speed analytical columns. All results are generally obtained within a day thanks to the standardized methods that we have developed for β-lactam antibiotics and quinolones.

9 SUMMARY

High-performance liquid chromatographic (HPLC) monitoring of antimicrobial agents has recently become more widely used, and represents an interesting alternative to other methods. The methodology is characterized by good specificity and accuracy, and it is applicable to almost all antibiotics. This review first describes the successive steps to investigate for the development of an HPLC method for a new antibiotic, and how to make use of it. Particular emphasis is put on the problems related to the standardization of sample preparation and to the development of mobile phases for use with different molecules belonging to the same class. The second part of the review describes one or more HPLC techniques for a representative antibiotic of each major class.

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