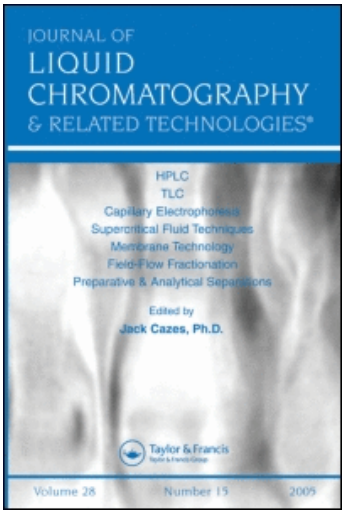

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High-Performance Liquid Chromatography for Analyses of Antibiotics in Biological Fluids

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR ANALYSES OF ANTIBIOTICS IN BIOLOGICAL FLUIDS.

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INTRODUCTION

The discovery of penicillin and subsequently other antibiotics represents one of the great progresses in modern medicine. Newer agents, such as the aminoglycosides and the cephalosporins, have also contributed significantly to man's struggle against bacterial and fungal infections, and antiviral agents are expected to emerge in the next decades. The evergrowing number of antibiotics have, however, also given rise to several problems. The emergence of bacterial strains resistant to antibiotics is a matter of increasing concern to the medical profession and necessitates the continuing search for new antimicrobial agents and judicious therapy with the ones already in use. Many antibiotics exert undesired toxic side effects when given in excessive doses: examples are the oto- and nephrotoxicity of aminoglycosides, the bone-marrow depressant action of

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chloramphenicol and the nephrotoxic effects of some of the early cephalosporins. Even a comparatively nontoxic drug such as penicillin causes coma and convulsions at high levels in the cerebrospinal fluid. To avoid such undesired effects it is of vital importance to the clinician to have a clear understanding of the pharmacological behaviour of an antibiotic. Usually, abundant data are available on the pharmacokinetics of these drugs in healthy, young volunteers but these do not represent the clinically difficult cases: the sick, often older, patient many times suffering from reduced renal or hepatic function and the very small child with immature function of liver and kidneys. When treating these patients, the clinician must be able to monitor serum levels of antibiotics to ensure safe and effective therapy. Therefore, when a new drug is introduced, it is necessary to perform extensive investigations of pharmacokinetic behaviour not only in healthy volunteers but also in patients with and without disturbances of metabolic pathways and elimination processes in order to make adequate dosage recommendations.

Assays for concentrations of antibiotics in body fluids have, by tradition, been performed in the microbiological laboratory by testing the samples against microbes. The capacity of the biological sample to inhibit growth of a susceptible bacterium is measured and

the results are compared to those obtained with standard samples of known concentrations (1,2). These assays require a rather long time; typically assay plates or tubes must be incubated for about 18 hours before results can be obtained. The demand on the technician for highly accurate laboratory work is great and standardization between different laboratories is difficult. These problems have been emphasized for some of the aminoglycosides (3) and are of continuous concern to the microbiologist and the clinician. A further disadvantage from the pharmacokinetic point of view is that the technique has no inherent potential for detection and quantitation of metabolic degradation products.

These considerations have prompted the development of other methods for assay of antibiotics. Several chemical procedures have been reported: spectrophotometric and fluorimetric measurements which, however, for specificity have to rely on complicated extraction procedures (4-10); gas-liquid chromatographic techniques which are also cumbersome because of the need for derivatization of the drugs to volatile compounds (11-15). A more attractive approach is the introduction of radioimmunoassays and radioenzymatic methods (16-24); these meet the requirements for specificity and sensitivity, but method development is quite demanding, relying on the production of specific antibodies of con-

TABLE 1. Published Assays for Analysis of Antibiotics in Biological Fluids

ANTIBIOTIC	PRETREATMENT	CHROMATOGRAPHIC MODE	DETECTION	REF.
β -lactam antibiotics				
ampicillin	perchloric acid	reverse-phase	UV absorption	26
"	trichloroacetic acid	"	postcol. deriv. UV absorption	27
mecillinam	trichloroacetic acid	"	"	27
"	dilution	"	"	28
amoxycillin	perchloric acid	"	"	26
"	a.dichloroethane+perchloric acid b.n-amylalcohol+dichloroethane	"	postcol. deriv. UV absorption	29
isoxazolyl penicillins*	a.methanol+perchloric acid b.methylene chloride	"	UV absorption	30
"	acetonitrile+methylene chloride	"	"	31
clavulanic acid	none	"	"	32

moxalactam	SEP-Pak	PIC	UV absorption	33
"	a.methanol b.dilution	reverse-phase PIC	"	34
"	ammonium sulphate	reverse-phase	"	35
cephalothin*	ion-pair extraction	ion exchange	"	36
" *	dilution	"	"	37
"	trichloroacetic acid	reverse-phase	"	38
" *	dimethyl formamide	"	"	39
"	chloroform+pentanol	"	"	40
cephazolin	trichloroacetic acid	"	"	38
"	"	"	"	41
"	chloroform+pentanol	"	"	40
cephalexin	a.dilution b.none	"	"	42

*assay includes determination of metabolite(s)

(continued)

TABLE 1, continued

ANTIBIOTIC	PRETREATMENT	CHROMATOGRAPHIC MODE	DETECTION	REF.
cephalexin	a.methanol b.none	reverse-phase	UV absorption	43
"	methanol	"	"	44
"	a.phosphoric acid+methanol b.trichloroacetic acid	"	"	45
cephaloridin	trichloroacetic acid	"	"	46
cephradine	a.dilution b.none	"	"	42
cephroxadin	trichloroacetic acid	"	"	45
cefatrizine	"	"	"	47
"	"	"	fluorimetry postcol. deriv.	48
cefuroxime	dimethyl formamide	"	UV absorption	49
"	perchloric acid	"	"	50
"	chloroform+pentanol	"	"	40

cefuroxime	phosphoric acid+methanol	reverse-phase	UV absorption	45
cefamandole	a.methanol b.dilution	"	"	51
"	chloroform+pentanol	"	"	40
cefotiam	a.phosphoric acid+methanol b.trichloroacetic acid	"	"	45
cefoxitin*	dilution	ion exchange	"	37
"	trichloroacetic acid +methanol	reverse-phase	"	52
"	chloroform+pentanol	"	"	40
"	acetonitrile	"	"	53
cefsulodin	phosphoric acid+methanol	"	"	45
cefotaxime*	acetonitrile	"	"	54
" *	a.acetonitrile b. dilution	PIC	"	55

*assay includes determination of metabolite(s)

(continued)

TABLE 1, *continued*

ANTIBIOTIC	PRETREATMENT	CHROMATOGRAPHIC MODE	DETECTION	REF.
cefotaxime*	trichloroacetic acid	reverse-phase	UV absorption	56
" *	chloroform+acetone	"	"	57
" *	perchloric acid	"	"	58
"	chloroform+pentanol	"	"	40
" *	acetonitrile	"	"	59
" *	phosphoric acid+methanol	"	"	45
cephaloglycin*	none	"	"	60
ceftizoxime	a.acetonitrile+buffer b. " +acetic acid	PIC reverse-phase	"	61
cefaclor	methanol	"	"	62
"	not stated	"	"	63
ceftriaxone	methanol	PIC	"	64
cefmenoxime	ultrafiltration	reverse-phase	"	65

Aminoglycosides

gentamicin	ion exchange chrom.	PIC	fluorimetry postcol. deriv.	66
"	adsorption chrom.	reverse-phase	fluorimetry precol. deriv.	67
"	acetonitrile+methylene chloride	"	"	68
"	ion exchange chrom.	PIC	fluorimetry postcol. deriv.	69
"	acetonitrile+methylene chloride+ethyl acetate	reverse-phase	fluorimetry precol. deriv.	70
"	acetonitrile+methylene chloride	"	UV absorption precol. deriv.	71
"	acetonitrile	"	"	72
"	acetonitrile-methylene chloride	ion exchange	fluorimetry postcol. deriv.	73

*assay includes determination of metabolite(s)

(continued)

TABLE 1, continued

ANTIBIOTIC	PRETREATMENT	CHROMATOGRAPHIC MODE	DETECTION	REF.
tobramycin	ion exchange chrom.	PIC	fluorimetry postcol. deriv.	69
"	adsorption chrom.	reverse-phase	fluorimetry precol. deriv.	74
"	acetonitrile+methylene chloride+ethyl acetate	"	"	70
"	acetonitrile+methylene chloride+2-propanol	"	"	75
netilmicin	acetonitrile+methylene achloride+ethyl acetate	"	"	76
"	"	"	"	70
amikacin	ion exchange chrom.	PIC	fluorimetry postcol. deriv.	69
"	adsorption chrom.	reverse-phase	fluorimetry precol. deriv.	77
"	a.methanol+chloroform b.methanol+diethyl ether	normal phase reverse-phase	"	78

sisomicin		acetonitrile	reverse-phase	UV absorption precol. deriv.	72
Chloramphenicol		ethyl acetate	"	UV absorption	79
"	**	methanol	"	"	80
"		acetonitrile	"	"	81
"		ether	"	"	82
"		ethyl acetate	"	"	83
"		acetonitrile	"	"	84
"		chloroform+isopropanol	"	"	85
"	**	ethyl acetate	"	"	86
"	**	trichloroacetic acid	"	"	87
"	**	diethyl ether	"	"	88
"		"	"	"	89

**assay includes determination of pro-drug

(continued)

TABLE 1, continued

ANTIBIOTIC	PRETREATMENT	CHROMATOGRAPHIC MODE	DETECTION	REF.
chloramphenicol**	acetonitrile	reverse-phase	UV absorption	90
" **	ethyl acetate	"	"	91
thiamphenicol	methanol	"	"	92
Sulphonamides				
trisulphapyrimidines sulphadiazine sulphamerazine sulphamethazine	trichloroacetic acid	"	"	93
salicylazosulphapyridine*	a.isoamylacetate b.4-methyl-2-pentane	"	"	94
sulphisoxazole*	methanol	"	"	95
sulphametrole*	perchloric acid	"	"	96
sulphamethoxazole*	"	"	"	97
" *	acetonitrile+ethyl acetate	"	"	98

sulphamethoxazole*	acetonitrile+ultracentrif.	reverse-phase	UV absorption	99
sulphadiazine*	ethyl acetate	normal phase	"	100
Trimethoprim	perchloric acid	reverse-phase	"	97
"	chloroform	normal phase	"	101
"	not stated	PIC	"	102
"	ethyl acetate	normal phase	"	100
"	acetonitrile+ultracentrif.	reverse-phase	"	99
Tetracyclines				
tetracycline	trichloroacetic acid +methanol	reverse-phase	"	103
"	ethyl acetate+phosphoric acid	"	"	104
"	"	"	"	105
" *	a.acetonitrile+phosphoric acid b.acetonitrile+ethyl acetate	"	"	106

*assay includes determination of metabolite(s)

(continued)

TABLE 1, continued

ANTIBIOTIC	PRETREATMENT	CHROMATOGRAPHIC MODE	DETECTION	REF.
oxytetracycline	ethyl acetate+phosphoric acid	reverse-phase	UV absorption	104
chlortetracycline	"	"	"	104
doxycycline	diethyl ether+ethyl acetate	"	"	106
"	a.phosphoric acid+acetonitrile b.acetonitrile+ethyl acetate	"	"	107
"	ethyl acetate	"	"	108
Nitroimidazoles				
metronidazole*	ammonium sulphate+methyl ethyl ketone	"	"	109
"	ethanol	"	"	110
" *	methanol+acetonitrile+ potassiumdihydrogen phosphate	"	"	111
"	ether+methylene chloride	"	"	112
" *	acetonitrile	"	"	113

metronidazole*	perchloric acid	reverse-phase	UV absorption	114
tinidazole	chloroform	normal phase	"	115
"	ether+methylene chloride	reverse-phase	"	112
"	perchloric acid	"	"	114
ketaconazole*	ethyl acetate	"	"	116.
"	SEP-Pak	"	"	117
"	diethyl ether	"	fluorimetry	118
econazole	"	"	UV absorption	119
thiabendazole*	acetonitrile	"	fluorimetry	120
Rifampicin*	chloroform	"	UV absorption	121
" *	isooctane+dichloromethane	normal phase	"	122
" *	ethyl acetate+n-heptane	reverse-phase	"	123
Nitrofurantoin	methanol	"	"	124

*assay includes determination of metabilite(s)

(continued)

TABLE 1, continued

ANTIBIOTIC	PRETREATMENT	CHROMATOGRAPHIC MODE	DETECTION	REF.
Nalidixic acid*	a.chloroform+sodium hydroxide b.sodium hydroxide+ion exchange	reverse-phase	UV absorption	125
Erythromycin**	diethyl ether	"	fluorimetry	126
Amphotericin B	methanol	"	UV absorption	127
5-fluorocytosine	none	ion exchange	"	128
"	ultrafiltration	"	"	129
"	trichloroacetic acid	reverse-phase	"	130
"	acetonitrile	PIC	"	131
Griseofulvin	dichloromethane	reverse-phase	"	132
"	acetonitrile	"	fluorimetry	133
"	diethyl ether	"	"	134
Acyclovir	a.aluminium sulphate+barium hydroxide b.dilution	PIC	UV absorption	135

Adenine arabinoside*	ultrafiltration	ion exchange	"	136
Antimalarial agents				
chloroquine*	a.ethylene dichloride b.trichloroacetic acid+ methanol	PIC	UV absorption or fluorimetry	137
"	hexane	"	UV absorption	138
" *	diethyl ether	normal phase	fluorimetry	139
" *	heptane	PIC	UV absorption	140
mefloquine	ethyl acetate	a.normal phase b.reverse-phase	"	141
dapsone	dichloroethane	normal phase	"	142
primaquine*	none	reverse-phase	"	143
pyrimethamine	dichloroethane	normal phase	UV absorption	142
"	butyl chloride+dichloro-methane	"	fluorimetry	144

*assay includes determination of metabolite(s)

(continued)

stant quality. Also, no potential for metabolite detection lies inherent in these techniques.

In the last decade high-performance liquid chromatography (HPLC) has undergone rapid technical progress and proved itself as a powerful analytical tool (25). The emergence of very small uniform particle packing materials with different physico-chemical properties, sophisticated high pressure pumps and very sensitive flow detectors has made this technique extremely versatile. The short time required for analysis and the obvious potential for separation and detection of metabolic products have held great appeal to investigators of the pharmacological aspects of antibiotics and, indeed, the literature is now, as shown in table I, replete with reports on techniques for determination of various antimicrobial drugs in biological fluids.

PRINCIPLES OF ANTIBIOTIC ASSAY BY HPLC

Basically, all reported methods for assay of antibiotics with use of HPLC employ the same methodological approach. The biological sample is treated with chemical procedures which usually aim at depleting the sample of proteins and/or extract the compound(s) of interest quantitatively. Subtances in the resulting solution are then separated by HPLC and eluting compounds are detected by spectrophotometry or fluorimetry. Recor-

ded peaks are then quantitated against known standards which have been chromatographed with the same separation system.

Sample Treatment

Samples of biological materials usually undergo pretreatment prior to chromatography. The objectives are mainly to rid the samples of protein (pertinent for samples of high protein concentration, such as plasma and serum) and to achieve a gross separation of substances not of interest in the assay while quantitatively solubilizing the compound under assay. Most assay procedures reported employ some pretreatment step (table I); the direct injection of biological samples, such as serum, with considerable protein content is not satisfactory since the analytical column will be obstructed and its performance will rapidly deteriorate. If such an approach is chosen, it is wise to use a guard column before the analytical column and to check column performance frequently (128). Samples of very low protein content can be injected directly or after dilution; however, with urine samples the many endogenous compounds present in high concentrations may produce such a multitude of chromatographic peaks that resolution of the compound(s) under analysis becomes a problem.

Protein removal

This approach is one of the two most commonly used procedures for treatment of samples prior to chromatography. Its objectives are two: first, to deproteinize the sample and second, to solubilize the antibiotic in order to ensure quantitative recovery from the sample. In general, there are four principle methods to precipitate proteins: acid and ammonium sulphate treatment, organic solvent and metal cation precipitation. As shown recently by Blanchard (145) these methods are all quite effective in precipitating proteins; the most popular in liquid chromatographic assays are acid precipitation and organic solvent treatment. Trichloroacetic and perchloric acid are very effective precipitants and are also, like solvents such as acetonitrile and methanol, quite compatible with subsequent chromatography in the reversed phase partition mode.

Another very simple and therefore attractive means of obtaining protein-free samples for chromatography is ultrafiltration (129,136) through filters with molecular cut-offs of 25,000 or 50,000 MW. However, one must remember that these filters let through only non-protein bound drug, giving a measure of the free fraction in serum. Also, there is a definite need for determining whether drug (or internal standard) is absorbed to the membrane material. If the losses in the filter are

small and constant, it is possible to correct for this in the final calculations. One way of getting around these problems is to use standards prepared in the same medium as the sample (for example, pooled serum); one must, in this case, be sure that protein binding is virtually identical in sample and serum pool. Ideally, such a procedure would use patient's sera obtained before therapy for the preparation of standards.

Extraction procedures

The second popular approach to cleaning the biological sample and recovering the drug for analysis with HPLC is extraction into organic solvents of low polarity. The most widely used are ethyl acetate, ethyl ether, methylene chloride and chloroform. These procedures work extremely well with lipofilic substances, for example chloramphenicol and nitroimidazoles (see table I). Generally, before chromatography, the organic extract is evaporated to dryness and the remaining residue redissolved in mobile phase; this adds another step in the procedure and, of course, some complexity and time. Sometimes, several extraction steps are necessary and the pretreatment procedure becomes quite timeconsuming and cumbersome (30,51,126,141).

In the case of aminoglycosides several reported methods use non-polar solvents to extract derivatives

of these compounds from an aqueous phase after attachement of chromophores to the molecules usually following initial protein precipitation and extraction of other non-polar substances (68,70,71,73,76,78). These antibiotics are extremely polar substances but the derivatives are of much lower polarity and readily dissolve in, for example, ethyl acetate. Therefore, when precolumn derivatization is used, this kind of pretreatment gives quantitative recovery and quite clean samples for chromatography. Another approach is the use of disposable small columns for initial separation by either absorption or ion exchange (66,67,69,74,77). The aminoglycosides are then eluted off the silica gel with methanol after derivatization or underivatized with an alkaline eluent from CM-Sephadex gel. The same method for initial separation of the biological sample is represented by commercially available disposable columns (Sep-Pak cartridges, Water's Ass.) which have been used for assay of moxalactam and a nitroimidazole (33,116).

One of the earliest methods for liquid chromatographic analysis of an antibiotic in body fluids was reported by Cooper et al (36). Cephalothin and desacetylcephalothin were extracted from serum into ethyl acetate as ion pairs with tetraheptyl ammonium ions. Although theoretically this is a quite feasible and rather elegant technique, no further reports on its use in this field have appeared.

Separation Systems

Since many antibiotics exist in solution as ions, a logical choice of separation mode would be ion exchange. Methods employing this approach have been reported (37,63,128,136) but most chromatographers are hesitant to use ion exchange due to the many problems with these systems: strict control of pH, ionic strength and temperature is necessary, column life is rather short since the matrix tends to bleed off from the solid support. From a practical point of view, bonded phase liquid partition is the most appealing chromatographic mode because of the stability and durability of the columns and the more predictable effects of changes in the mobile phase composition. The overwhelming majority of antibiotic assays described in the literature use reverse-phase partition on octadecylsilane chemically bound to a small particle silica matrix (table I); eluting solvents consist of mixtures of buffer and methanol or acetonitrile. In some cases, especially with compounds that are highly ionized or extremely polar, separations by this mode are not satisfactory. Badly tailing peaks may indicate ionization problems; difficulty to get any retention on a reverse-phase column may be encountered, as, for example, with the aminoglycosides. In these cases, paired ion chromatography (PIC) on reverse-phase columns is a very useful technique with

all the practical ease of bonded phase chromatography and none of the difficulties of ion exchange. For the analyses of aminoglycosides, another approach has also been feasible: derivatization of the primary amino groups with chromophores yields less polar compounds which then very readily are separated by reverse-phase partition (67,68,70-78). When post-column derivatization is preferred, however, the paired ion technique is the method of choice (66,69).

Normal phase separations have been successfully employed for determination of antimalarial agents (139, 141,144), trimethoprim (100,101), rifampicin (122) and tinidazole (115). Several of these compounds separate well in reverse-phase systems as well; tinidazole seems, indeed, to be better suited for reverse-phase separation, since published chromatograms in the normal phase mode show broad, tailing peaks.

Detection

Most antibiotics absorb ultraviolet or visible light with extinction coefficients large enough to allow spectrophotometric detection at therapeutic concentrations. The wavelength of detection is determined from the wavelenght of maximum absorption of the compound and, if need be, after consideration of potential interference by other compounds in the sample that may

co-elute from the chromatographic column. In the latter case, a second maximal absorption band for the antibiotic may be chosen where no interference is noted (103). Commercially available flow spectrophotometers for HPLC operate at either fixed or continuously variable wavelengths; naturally, greater flexibility is obtained with variable detectors, but for most of the methods reported, the fixed wavelength detectors are adequate. In some instances, spectrophotometry is not feasible for detection and quantitation. Sometimes, the sensitivity obtained does not suffice (48,133,134); fluorimetry can then be employed to increase the sensitivity of the assay. Another aspect is that fluorescence is a more specific phenomenon than light absorption requiring a certain wavelength for excitation and emitting at another. Therefore, it may be assumed, that less rigorous pretreatment procedures are necessary and specificity is still maintained (118).

Fluorimetry has been of special importance for the analysis of aminoglycosides. These clinically very important compounds show extremely little absorption in ultraviolet or visible light; spectophotometry cannot be used for detecting therapeutic concentrations in body fluids. It was realized by several investigators, that derivatization of these molecules was a necessary step in a chromatographic assay. The attachment of

fluorophores to the primary amino groups of the aminoglycosides was a logical and theoretically simple approach. Dansyl chloride (68,76), fluorescamine (65,66, 69) and orto-phtalaldehyde (67,70,74,75,77) can be used successfully and the reported assays with fluorimetric detection are sensitive enough for therapeutic monitoring and pharmacokinetic studies. More recently, three groups of investigators have reported precolumn derivatization with benzene sulphonyl chloride (71) and fluorodinitrobenzene (72,78) with subsequent spectrophotometric detection.

Derivatization with chromophores can be performed either as a precolumn (preceding chromatographic separation) or as a postcolumn procedure. While it cannot be stated that one method is superior to the other (146), there are some practical aspects to be considered. Postcolumn derivatization requires a more complicated chromatographic system involving a separate pump for the derivatizing reagent and a reaction coil. Precolumn derivatization is technically simpler and, when part of the sample pretreatment procedure, probably results in cleaner extracts of biological fluids. As mentioned above, this approach also facilitates the separation allowing the use of simple reverse-phase partition systems.

Quantitation

The concentration of antibiotic in a sample is calculated by comparing the size of the recorded peak for the specific compound with the peak size obtained from a standard sample of known concentration. There are two ways of measuring peak size: one is to determine the height of the peak, the other is to calculate the area of the peak (25).

Peak height measurement is usually done manually and is the simpler of the two methods. To obtain good accuracy with this approach, column performance must be stable and reproducible and peaks should be symmetrical. It is necessary to run standard samples quite often to check the column during a series of analyses.

Peak area is a more complicated measurement but has a definite advantage over height determinations: it is less influenced by changes in column performance or instrumental parameters. It is, furthermore, not so dependent on peak symmetry. Several methods are available for measurement of peak area (25); highest precision is obtained with use of an electronic digital integrator or a computer.

Another, perhaps more controversial, question is whether to use an internal standard for quantitation (25). In general, for assays with more complex pretreatment procedures or variable recovery of extraction

the addition of a suitable internal standard to the original sample will increase precision of the assay. However, for this statement to be valid, the internal standard must behave equivalently to the compound under analysis throughout the procedure. Furthermore, it must elute close to the substance under analysis but in a vacant spot in the chromatogram. These requirements often make it difficult to find a suitable internal standard; indeed, very often it is not clear in reported methods whether the demands are fulfilled. There exists a definite risk of increasing, instead of decreasing, the degree of imprecision with use of an improper internal standard. For assays employing simple pretreatment procedures, the method of external standard calibration is probably at least as precise, especially if sample injection is done with a sampling microvalve or an automatic injector, since the main source of assay imprecision is variation in the injected volume.

PHARMACOKINETIC IMPLICATIONS

As is evident from table I, many of the reported assays include the detection and quantitation of metabolites or precursors of several antibiotics. This is a unique feature of the HPLC technique as compared to microbiological and radioimmunological methods and opens up new possibilities for the study of pharmacoki-

netics of antibiotics. In some instances, unknown metabolites have been discovered (55,60,140), and it is feasible to isolate and by, for example, mass spectrometry identify such metabolites.

Several pharmacokinetic studies have been done on the cephalosporines; investigations with HPLC as assay method started already in 1973 when Cooper et al (36) showed that approximately one fifth of a cephalothin dose was excreted in urine of healthy volunteers as the metabolite desacetylcephalothin. A later study of the same drug in severely uremic patients gave evidence for an additional route of excretion or metabolism in these patients since only about 50% of a given dose was found in urine, most of it accounted for by the metabolite (147). Other cephalosporins have been studied in patients and volunteers: the kinetics of cefotaxime and its metabolites have been elucidated by several authors (55,56,59); Haginaka showed that extensive and complex metabolization occurs with cephaloglycin, only 0.5% of the dose being excreted in urine as the intact compound (60); a three-compartment pharmacokinetic model has been indicated for cefamandole (51); the perorally administered cephalexin was studied by Nakagawa et al and found to fit a two-compartment model with almost complete urinary recovery and no indication of metabolic degradation (43). Cefaclor, another peroral

cephalosporin, shows first-order absorption and evidence of a substantial non-renal clearance route (63). The excretion routes of some cephalosporins have been extensively investigated by Arvidsson who found that renal elimination of cephapirin and cephaloridin was dependent on drug concentration, that biliary excretion of ceftrixone was dependant on individual variability in the secretion of endogenous biliary lipids and correlated to effects of the drug on the intestinal microflora (148). Stoeckel et al (149) demonstrated concentration-dependant plasma protein binding of ceftriaxone and the kinetics of this drug in infants and young children have been investigated by Schaad et al (150). The plasma protein binding and kinetic behaviour of the two epimers of moxalaxtam in volunteers have been calculated (151) and a study of the clearance of this drug from serum of patients with varying degrees of renal insufficiency has led to a suggestion for dosage regimens with regard to renal function (35).

To date, published investigation of penicillins and their metabolites have been rather scarce; however, Thijssen et al have developed a method for analysis of isoxazolyl penicillins and their 5-hydroxymethyl metabolites and penicilloic acids and estimated the pharmacokinetic parameters of these compounds after oral administration of cloxacillin and flucloxacillin to a healthy volunteer (30).

The majority of HPLC assays for chloramphenicol includes the determination of its succinate ester, the substance used for intravenous administration of the drug. Great interindividual variability has been found in the renal clearance of the succinate ester both in children and adults, with no correlation to renal function (152-154). This variable kinetic behaviour of the pro-drug affects the levels of active chloramphenicol in serum, a factor which needs to be considered in clinical practice.

The pharmacokinetics of metronidazole, a drug which has come into frequent use owing to its excellent activity against anaerobic bacteria, has attracted increasing attention over the past years. The main metabolites of metronidazole can be easily detected and quantitated by HPLC (109,114). The parent drug undergoes extensive break-kown and only about 8% of administered metronidazole is excreted in urine as unchanged drug (114,155). Low concentrations of the hydroxy and acetic acid metabolites were found in healthy volunteers (114,155,156) whereas in patients with renal failure these metabolites reached considerably higher serum levels (157).

The kinetics of combinations of trimethoprim and different sulphonamides have been studied by the HPLC technique (97,100,102) as well as the conversion of

sulfametrole to the N_4-acetyl metabolite in man and the subsequent renal excretion of these two compounds (96).

The *in vivo* degradation of nalidixic acid and the behaviour of its two major metabolites have been studied by HPLC (125) and the hydrolysis of erythromycin ethyl succinate after peroral administration has been estimated by Tsuji who also detected metabolites of ethyl succinate and of erythromycin itself in serum by this technique (126).

Drugs used for therapy or prophylaxis of malaria exhibit complex pharmacokinetic patterns. It appears that HPLC will be a valuable tool for elucidation of the metabolism and kinetics of several of these compounds; to date, studies have appeared on dapsone with several metabolites (142), pyrimethamine (142) and primaquine (143). The latter study was performed with rats; the authors showed the existence of a new mammalian metabolite of primaquine and verified its presence and structuré by HPLC and mass spectrometry. The complex pharmacokinetics and metabolism of chloroquine are under study in several laboratories (137,139,140).

THE ROLE OF HPLC IN THE LABORATORY

Despite the abundant evidence that the HPLC technique is well suited for analysis of antimicrobial drugs in biological fluids and compares favorably with other

methods in current use, there exists one serious shortcoming of this technology: its capacity is limited. Separations usually take about 5-10 minutes and, with one chromatographic set-up, this means an assay capacity of 6-12 samples per hour. Furthermore, switching from one assay to another involves additional time spent in changing solvents and equilibrating the analytical column. Clearly, traditional microbiological assays and radioimmunological methods are superior with regard to the amount of samples that can be analyzed simultaneously, and, in large microbiological laboratories with large series of samples, routine clinical determinations of antibiotic concentrations are performed with these techniques. Some laboratories use both HPLC and microbiological or radioimmunological techniques routinely: the majority of routine analyses in microbiological laboratories consists of aminoglycoside determinations and with careful technique these can be carried out with bioassays in a satisfactory manner (3); analyses of other agents are done by HPLC (158).

Moreover, HPLC is an alternative method in special situations: interference in the biological technique caused by antimicrobial combination therapy is usually, because of the separative step involved, easily resolved with HPLC; assays of som agents, for example amphotericin B and 5-fluorocytosine, are more sensitively and

accurately done with HPLC; analyses of antibiotics which undergo metabolic breakdown are better performed with a technique that measures metabolites separately, especially in the problem cases represented by patients with hepatic and/or renal insufficiency.

For smaller hospitals without a microbiological laboratory HPLC provides an attractive alternative for routine assays by clinical chemists. Many hospitals use this method for analysis of other drugs and endogenous substances and are well suited to perform antibiotic assays also. It may well be clinically and economically advantageous to use HPLC for routine antibiotic assays in such instances instead of sending samples to a microbiological laboratory for analysis.

For laboratories engaged in pharmacokinetic research with antibiotics, the technique, as described above, offers definite advantages and is rapidly becoming indispensable.

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