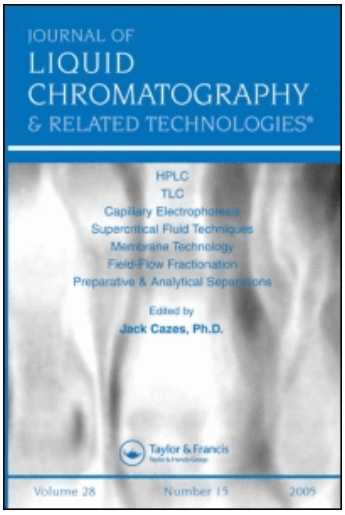

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Liquid Chromatographic Assays of Antimicrobial Agents

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LIQUID CHROMATOGRAPHIC ASSAYS OF ANTIMICROBIAL AGENTS

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ABSTRACT The increased use of antibiotics has prompted the need for rapid, sensitive and specific methods for monitoring concentrations in the biological samples. Methods available for detection and quantitation of antibiotics include microbiologic assays, radioimmunoassays, enzyme immunoassays, fluorescent immunoassays, fluorescence polarization immunoassays, latex agglutination and high performance liquid chromatography (HPLC). HPLC has played an increasing role in therapeutic monitoring of antibiotics since the 1970's. This paper provides a brief overview of the basic principles of HPLC as well as describes methods and procedures available for assaying many antimicrobial agents in use today.

In recent years there has been an increased awareness of the need to quantitate concentrations of antimicrobial agents in serum and other body fluids. This has been precipitated by a number of different factors. With most antimicrobial agents, potentially toxic concentrations are well above those needed for adequate therapy. A small number of antimicrobial agents (eg. aminoglyco-

sides, vancomycin) exhibit a narrow range between therapeutic and toxic concentrations. Serum assays should be performed periodically during the course of therapy to assure therapeutic concentrations are maintained while avoiding the nephrotoxic and ototoxic effects associated with accumulations of these agents. Patients with renal dysfunction who receive antibiotics excreted primarily by the kidney should also have concentrations carefully monitored. The increasing use of chloramphenicol in newborns and neonates for treatment of infections caused by beta-lactamase producing microorganisms. Since chloramphenicol is metabolized by the liver, the immature hepatic function of these patients may lead to toxic accumulations of the antibiotic. When oral therapy is being considered for the treatment of endocarditis and osteomyelitis, monitoring serum concentrations of the antibiotic will assess gastrointestinal absorption and patient compliance.

Assessment of antibiotic concentrations in fluids are influenced to varying degrees by such factors as the timing of the sample collection, processing of the specimen and methodology employed to measure concentrations. For most antibiotics, determination of either peak or trough concentrations will suffice for dosage adjustments and monitoring. Determination of both peak and trough measurements are important for aminoglycosides.

As a general rule, samples for measurement of peak concentrations are obtained 30 min. after completion of an intravenous infusion, 1 hr after an intramuscular injection and 1 - 3 hrs after an oral ingestion. Samples for determination of trough concentration are obtained immediately before the next dose is given. Serum samples may be stored at -20° C or lower until assayed without

affecting results. Some antibiotics and antibiotic combinations are known to be particularly unstable. The combination of an aminoglycoside and a B-lactam, usually carbenicillin or ticarcillin, is significantly unstable and may result in losses even when stored at -20° C.[1] This reaction can be avoided by the addition of a B-lactamase to the sample. Cephalosporins are very unstable in serum, probably due to the presence of lipoproteins.[2] Therefore, samples should be stored at -20° C and assayed as quickly as possible. Certain antimicrobial agents, such as imipenem, exhibit decreased stability upon freezing which greatly affects the reliability of routine assaying procedures.

The ideal antibiotic assay should be sensitive enough to detect low concentrations accurately, specific for the antibiotic under investigation, rapid to perform, technically simple and cost efficient. A variety of techniques have been utilized to measure antibiotic concentrations in clinical samples and include microbiological and non-microbiological methods.[Table 1] Because of the many disadvantages associated with both microbiologic and chemical assays, the trend is toward the use of high-performance liquid chromatography (HPLC) for determining antibiotic concentrations. HPLC can be most readily applied to the measurement of almost all antimicrobial agents.[3-5]

HPLC shares the basic principle of other chromatographic techniques, separating the various chemical species in a mixture based upon the affinity of these samples constituents to either the mobile phase or the stationary phase. In HPLC, the mobile phase is forced rapidly by pumps at high pressure (500-5,000 psi) transporting the sample constituents through the stationary phase (column) where the actual separation of sample components occurs. This separation

Table 1.

Comparison of various types of assays available for measuring antibiotic concentrations.

Assay Type	Advantages	Disadvantages
Microbiological (bioassays)	standard method; simplicity; low cost; minimum equipment; small sample cost.	slow; nonspecific in presence of multiple antibiotics.
Turbidimetric	simplicity; low cost; small sample size.	slow; nonspecific in presence of multiple antibiotics; specialized training and equipment required.
Immunofluorescence	small sample size; minimum training time; limited sample preparation; no reagent shelf life problem; rapid; sensitive; specific.	High reagent and equipment cost; one reagent source; sample pretreatment.
Radioimmunoassay	small sample size; rapid; sensitive; specific.	High reagent and equipment cost; specialized training and equipment required; limited reagent shelf life; biohazardous.
HPLC[a]	small sample size; relatively low reagent cost; sensitive; specific; moderate speed.	moderately high equipment cost; specific training required.

[a] high performance liquid chromatography.

of solutes depends upon solubility, ionization, molecular weight, volatility, structure and reactive sites. The success of HPLC depends upon the proper choice of both mobile phase and column matrix.

Basically, there are two types of column packings used in HPLC: porous and pellicular. The porous packings consist of large particles (silica, aluminum oxide) with a surface diameter of 40 um. The pellicular packings consist of a solid glass bead as the nucleus with a thin porous outer shell of a chromatographically active component. In the 1960's - 1970's, pellicular packings were the standard column packing with large porous particles being used mainly as packing for

precolumns or preparative columns. Recently, microparticulate packings have replaced pelliculars. These microparticles are 3 to 10 um in diameter and allow a greater column efficiency and speed of analysis.

HPLC uses a wide variety of modes for separating sample components. These modes are a) adsorption (liquid-solid) chromatography; b) partition (liquid-liquid) chromatography; c) ion exchange chromatography and d) exclusion chromatography.

Adsorption chromatography is derived from the oldest type of liquid chromatography, developed in 1906 by the Russian botanist M. Tswett.[6] The solid stationary phase is usually silica gel or alumina which contain active polar sites. These react with and reversibly absorb the polar solute molecules in the samples. The mobile phase is usually nonpolar (eg. hexane or chloroform).

The use of a non polar stationary phase (eg. charcoal or polymer beads) may also be employed together with a polar mobile phase such as water.[3] This is known as reversed-phase adsorption chromatography. Adsorption chromatography can be applied to compounds with a molecular weight of greater than 2,000 that are soluble in organic solvents. Adsorption chromatography is an excellent technique for the separation of compounds such as amines, alcohols, acids, lipids and steroids.

Partition chromatography utilizes a liquid stationary phase with the liquid held on the outside of a granular support or chemically bonded to support material such as silica (bonded phase chromatography).[7] Separation occurs by partitioning the sample between the mobile phase and the stationary phase by differential solute solubility. Generally, in liquid-liquid chromatography the mobile phase is nonpolar and the stationary phase is polar. However, the use of a nonpolar

stationary phase and a polar mobile phase has been employed. This is known as reversed-phase liquid chromatography (RPLC) - the most popular of techniques. RPLC has the advantage of minimal stripping of the stationary phase from the column due to dissolution in the mobile phase, a problem which is unavoidable with standard liquid-liquid chromatography. RPLC is dominating the application of HPLC for the following reasons:

1. The columns are reasonably reproducible and relatively stable, with their performance minimally affected by outside conditions (temperature, pH).

2. Stripping of the stationary phase is minimal.

3. Nonionic, ionic and ionizable compounds are often separated using a single column and mobile pnase with various reagents added.

4. Mobile phase reagents, predominately water, are inexpensive.

5. The elution order is often predicatable based on the degree of hydrophobicity of the solute molecule.

The silica-bonded stationary phase of reversed-phase liquid chromatography are as follows: a) Si-O-Si-CR_3, siloxane type; b) Si-CR_3, carbon type; c) Si-OR, ester type; d) Si-NR_2, amino type. The nature of the R substitution will determine the final property of the stationary phase. R substitutions commonly used are ethyl, octyl or octyldecyl (ODS) groups. In general, the greater the chain length of the R-group, the more the chromatographic retention increases.

The nonpolar phases have shown the highest stability among bonded phases toward aqueous mobile phases in a wide pH range which is another reason contributing to the popularity of RPLC. However, the Si-O-C bonds are not completely resistant to hydrolysis. Therefore, the recommended pH range for the mobile phase has been 2.0-7.5. Recent

improvements in silanizing technology have produced packings with a minimal amount of accessible free silanol groups at the surface so that such bonded phases can be used with mobile phases of pH 9.0 for extended periods of time.

Reversed phase liquid chromatography has the basic principle that polar solutes prefer polar mobile phases and therefore elute before and faster than nonpolar solutes. Any polar function on the solute opposes repulsion of the mobile phase and causes eventual elution from the column.

If ionic or ionizable substances such as amino acids, aminophenols and quaternary ammonium compounds can be made less ionic by suitable mobile phase additives, these may be separated by liquid-liquid chromatography. This special form is known as paired ion chromatography.

Ion exchange chromatography is a form of adsorption chromatography that utilizes a stationary phase consisting of resins or a non-porous silica matrix onto which ionic groups are attached or covalently bonded. The stationary phase surface carries a net charge. A positive charge (R^+) stationary phase interacts with and separates anions in the mobile phase. Samples containing anions (S^-) will compete and exchange with counter-ions (C^-) as follows:

$$R^+C^- + S^- \rightleftharpoons R^+S^- + C^-$$

This is known as anion exchange. If the stationary phase surface has a net negative charge (R^-) with positive counter ions (C^+) the balance is known as cation exchange:

$$R^-C^+ + S^+ \rightleftharpoons R^-S^+ + C^+$$

per selection of technique along with the correct choice of stationary phase. For the most part, analysis of antimicrobial

agents is performed utilizing reversed-phase chromatography. The column materials used most often are the nonpolar C_{18} (octadecylsilanyl) and C_8 (octylsilanyl) which have the advantages of versatility and durability.[8] With these nonpolar columns, a polar mobile phase is used. The more hydrophobic the stationary phase, the greater the attraction of the nonpolar molecule, therefore in terms of retention of a given solute, $C_{18} > C_8 > NH_2 > C_2$. Most antimicrobial agents are polar and their separation will occur within minutes.

Reverse phase ion-pair chromatography[9] has been used in separating highly ionizable compounds, such as the aminoglycosides [10,11], cephalothin [12] and 5-fluorocytosine[13,14] and is an attractive alternative to ion exchange chromatography.[15]

The mobile phase most often used with reverse-phase chromatography is an aqueous buffer (phosphate or acetate) or a mixture of water and an organic solvent such as methanol or acetonitrile. The mobile phase is pumped through the column at a flow of 1 - 3ml/min with the use of one of two types of pump: a constant volume pump or a constant pressure pump. The constant volume pump allows more reliable and reproducible results, higher resolution and a more stable baseline. This is accomplished by maintaining a constant flow rate in the face of changes in pressure.[7]

The sample components are retained by the column to different extents and will be carried to a detector at different times. These times (retention times) are reproducible and characteristic of a particular compound and are the basis for the identification of compounds under investigation. The detectors most commonly used in HPLC are UV spectrophotometers and fluorometers, which will produce a

measurable signal for each eluted compound. This signal is then converted into a graph peak by a linear recorder. Other techniques used to detect sample components include polarography, electrical conductivity, colorimetry and mass spectrometry. The differential refractometer is another type of optical detector widely used in HPLC having higher detection limits than seen with UV detectors. However, these detectors are generally less sensitive than UV dedectors and are not suitable for gradient elution.[16]

Most antimicrobial agents are detected and subsequently quantitated based on their high absorbance in UV light. Some antibiotics, such as the aminoglycosides, have low absorption potential and require another means of detection. For the aminoglycosides, this problem was solved by derivatization of the amino group with fluorogenic agents followed by detection by fluorometry. Derivatization may occur precolumn (prior to HPLC) or post column (after HPLC separation), with approximate sensitivity as low as 0.001 to 1.0ug/ml.

Solute concentration is lineraly proportional to peak height or area on the chromatogram. Because of variability that may occur with HPLC, the most accurate quantitation of an unknown peak is accomplished with the use of an internal standard, added to the sample prior to extraction or analysis.[17] The internal standard should be chemically similar to the compound under investigation but should elute at a time different than that compound. The internal standard acts as an automatic correction for losses due to error during processing by comparing the ratios between the peak of standard and unknown and then quantitating the unknown concentration. This monitors losses during the extraction process, due to nonspecific adsorption to glassware or variations in sample injection volume.

The extraction procedure utilized prior to the performance of HPLC is as important as chosing the proper column stationary phase and mobile phase. Direct injection of serum or plasma onto a column is not recommended since even traces of protein will obstruct column filters, increasing pressure and altering separation efficiency. Proteins may be removed by a variety of methods: a) protein precipitation, b) solvent extraction, c) precolumn adsorption and elution, d) ultrafiltration (Table 2).

The type of extraction procedure used depends upon the chemical nature of the antimicrobial agent(s) under investigation.

Separation may be readily accomplished by the use of organic solvents or acids. The most commonly used are methanol, acetonitrile, diethyl ether, dichloromethane, and trichloroacetic acid. The method can be further enhanced by preparing a solvent solution containing a known concentration of internal standard. Complete precipitation of the sample proteins is ensured by a 5-15 min period of centrifugation.

Extraction may also be accomplished by adsorption-chromatography with silica gel[18-20], ion-exchange chromatography with carboxymethyl-Sephadex (Pharmacia Fine Chemicals, Piscataway, N.J.),[11,12] or ultrafiltration. These methods are useful for preparing antimicrobial agents with low serum protein binding.

There are many advantages liquid chromatography has over other methods employed for the measurement of antimicrobial agents.
(1) HPLC has the specificity and selectivity to allow the quantitation of the particular antimicrobial agent under investigation. This is very important in cases where patients may be receiving more than one antibiotic for the treatment of an infection or where the metabolite of the agent in question has antibacterial activity. In the

TABLE 2. Methods available for HPLC sample preparation.

Method	Advantage	Disadvantage
protein precipitation	- simple to perform - high recovery of drug	- dilutes sample - removes few interfering substances
solvent extraction	- simple to perform - complements separation	- variable recovery - requires evaporation step
precolumn adsorption and elution	- complements separation - yields good recovery of drug	- requires care in performance - dilutes sample - may require evaporation step
ultrafiltration	- suitable for agents with low serum protein binding	- time consuming - requires centrifugation at high force of gravity to separate - variable recovery

later circumstance, bioassays would be inappropriate for measurement of the parent compound. (2) The reproducibility of HPLC is higher than that found with microbiological assays and comparable to immunoassay techniques with an average coefficient of variation of 5-15%. (3) The sensitivity of HPLC is adequate enough to detect milligram levels of antibiotic, in some cases nanogram concentrations. (4) HPLC assays have proven to be rapid to perform once pre-injection preparation is completed (15-30 min). This is far superior to the 4-24 hr completion time required for microbiological assays. (5) Although the initial cost of HPLC may be considered high, the daily operational costs of an HPLC assay is very cost effective. (6) HPLC can be applied for the separation and quantitation of virtually all antimicrobial agents. Limitations such as kit availability or type of sample matrix used are not encountered.

A selection of procedures useful in determining antimicrobial concentrations is found in Table 3. Below are more detailed de-

Table 3. Summary of available HPLC methods for quantitation of antimicrobial concentrations.

Antimicrobial agents	Ref.	Extraction Procedure	Stationary phase/ Mode of separation	Mobile Phase	Detector (wavelength)	Sensitivity (ug/ml)	Recovery (%)	Time (min
Aminoglycosides								
amikacin	19	silica gel adsorption	C_{18}/partition, RP	methanol-water acetonitrile	Fluoro.[a]	1.0	80-85	60
	11	CM-Sephadex-ion exchange	C_{18}/ion-pair RP	Na sulfate-Na pentane sulfonate-acetic acid-water	Fluoro. + int. std.[b]	2.0	93	--
gentamicin	18	silica gel adsorption	C_{18}/partition, RP	methanol-water	Fluoro	0.5	80-105	30
	22	CM-Sephadex-ion-exchange	C_{18}/ion-pair RP	Na sulfate-Na pentane sulfonate-acetic acid-water	Fluoro	1.0	95	--
	23	acetonitrile; methylene chloride	C_{18}/partition, RP	acetonitrile-water	Fluoro	1.0	--	--
	11	CM-Sephadex-ion exchange	C_{18}/ion-pair, RP	Na sulfate-Na pentane sulfonate-acetic acid-water	Fluoro + int. std.	2.0	$C_{1,2}$-89 C_2-93 C_1-100	--
	24	----	C_{18}/partition, RP	methanol-water diethylamine-acetonitrile	UV (365)	0.5	64	--
	25	acetonitrile/ dansyls	C_{18}/partition, RP	acetonitrile-water	Fluoro	0.2	--	--
	26	acetonitrile-phosphate buffer	C_{18}	pH 11 methanol K^+EDTA-water	Fluoro	0.5	92-100	--
netilmicin	26	acetonitrile-phosphate buffer	C_{18}	pH 11 Tris buffer triethylamine-H_2SO_4 methanol-water	Fluoro	0.5	92-100	--

	27	acetonitrile-methylene chloride	C_{18}/partition, RP	acetonitrile-water	Fluoro	0.5	--	20
tobramycin	11	CM-Sephadex-ion exchange	C_{18}/ion pair, RP	Na sulfate-Na pentane sulfonate-acetic acid-water	Fluoro + int. std.	2.0	93	--
	26	acetonitrile-phosphate buffer	C_{18}	pH 11 Tris buffer triethylamine-H_2SO_4 methanol-water	Fluoro	0.5	92-100	--
	24	--	C_{18}/partition, RP	methanol-water diethylamine-acetonitrile	UV (365)	0.5	64	--
	20	silica gel adsorption	C_{18}/partition, RP	methanol-water-EDTA	Fluoro	0.5	75	30
Bacitracin	28	--	C_{18}/partition, RP	methanol-acetonitrile phosphate buffer	UV (254)	---	--	--
Cephalosporins								
cephalothin	29	trichloroacetic acid	phenylsilane/partition, RP	methanol-ammonium acetate	UV	10.0	--	--
	30	dimethylformanide	C_{18}/partition, RP	methanol-acetic acid	UV	1.0	97-104	30
	31	tetraheptylammonium chloride-ethyl acetate-myristic acid-CCL_4	N^+R_3/ion exchange	sodium dihydrogen phosphate sodium nitrate	UV	1.0	81	60
	32	--	C_{18}/partition, RP	methanol-sodium hydrogen phosphate	UV (254)	0.3	--	25

[a] Fluorometry
[b] Internal standard

(continued)

Table 3 (continued)

Antimicrobial agents	Ref.	Extraction Procedure	Stationary phase/ Mode of separation	Mobile Phase	Detector (wavelength)	Sensitivity (ug/ml)	Recovery (%)	Time (min)
cefamandole	33	methanol-sodium acetate	C_{18}/partition, RP	methanol-sodium acetate	UV + int. std.	0.3	--	--
cefazolin	34	trichloracetic acid	Phenylsilane/ partition, RP	methanol-acetic acid	UV (254)	1.0	85-100	30
	32	--	C_{18}/partition, RP	methanol-ammonium carbonate	UV (254)	0.1	--	9
cephalexin	35	methanol	C_{18}/partition, RP	methanol-water	UV	0.5	2-100	--
cephaloridine	36	trichloroacetic acid	Phenylsilane/ partition, RP	methanol-ammonium acetate	UV (254)	2.0	98	30
cefoxitin	37	--	Cation/ion exchange	acetic acid buffer (pH 5.0)	UV (254)	2.0	--	30
cefotaxime/ desacetyl-cefotaxime	38	trichloracetic acid	C_{18}/partition, RP	methanol-phosphoric acid	UV (310)	0.3	--	20
	39	DEAE-Sephadex-anion exchange	C_{18}/partition, RP	acetonitrile-acetic buffer (pH 2.8)	UV	1.0	98	--
	40	acetonitrile	C_{18}/partition, RP	methanol-acetate buffer (pH 5.5)	UV	3.0	--	--
cefoperazone	41	methanol	phenyl/partition RP	acetonitrile-tetrabutyl ammonium buffer	UV	1.0	97-101	--
	42	chloroform pentanol	C_{18}/partition, RP	methanol-acetate buffer (ph 4.8)	UV	0.05	--	--
ceftazidime	43	perchloric acid	C_{18}/partition, RP	acetonitrile-formic acid-phosphate buffer	UV	0.6	99-100	--

ceftizoxime	45	trichloracetic acid	C_{18}/partition, RP	methanol-water H_2SO_4 buffer	UV	plasma:0.2 urine:25.0	89	--
	46	DEAE-Sephadex anion exchange	C_{18}/partition, RP	acetonitrile-acetic acid	UV	--	--	--
cefsulodin	47	ultrafiltration of plasma-phosphate buffer (pH 6.0)mixture	C_{18}/partition, RP	acetonitrile-acetate buffer (pH 4.2)	UV	0.2	99	--
ceftriaxone	48	acetonitrile	phenyl/partition, RP	acetonitrile-water-ammonium carbonate	UV	plasma:0.3 urine: 3.0	95-102	--
	49	acetonitrile	C_{18}/partition, RP	acetonitrile-phosphate buffer-ion pair	UV	--	--	--
cefmenoxime	47	ultrafiltration sodium dodecyl sulfate treated plasma	C_{18}/partition, RP	acetonitrile-acetate buffer (pH 5.3)	UV	0.05	--	--
cefotetan	50	trichloroacetic acid	C_{18}/partition, RP	acetonitrile-phosphate buffer (pH 3.0)	UV	0.7	---	--
cefuroxime	51	dimethylformanide	C_{18}/partition, RP	acetic acid-methanol water	UV (280)	1.0	95-100	30
	52	perchloric acid	C_{18}/partition, RP	KH_2PO_4-methanol	UV (278)	0.5	98	15
Chloramphenicol	53	methanol	C_{18}/partition, RP	acetic acid-methanol-water	UV	0.5	100	30
	54	ethyl acetate	C_{18}/partition, RP	acetonitrile-acetate buffer	UV + int. std. (270)	2.0	96	--

(continued)

Table 3 (continued)

Antimicrobial agents	Ref.	Extraction Procedure	Stationary phase/ Mode of separation	Mobile phase	Detector (wavelength)	Sensitivity (ug/ml)	Recovery (%)	Time (min)
	55	diethyl ether	C_{18}/partition, RP	methanol-water	UV (278)	0.1	94-100	20
	56	chloroform-isopropanol	C_{18}/partition, RP	acetonitrile-acetate buffe	UV + int. std. (280)	0.1 - 0.2	96	30
Clindamycin/ lincomycin + metabolites	38	--	TEA cellulose	boric acid (pH 8.8)	UV (254)	--	--	--
	57, 58	--	C_{18}/partition, RP	methanol-water	UV (214)	--	98-100	--
Erythromycin	59	diethyl ether	C_{18}/partition, RP	acetonitrile-ammonium-acetate water	Fluoro	0.1	96-100	--
	60	diethyl ether	C_{18}/partition, RP	acetonitrile-ammonium acetate-water-methanol	UV (245)	--	--	--
Imipenem	61	3-(N-morpholino)-propane sulfonic acid-ethylene glycol-methanol	C_{18}/partition, RP	borate buffer-sodium hydroxide	UV (313)	0.5	--	10
Metronidazole	62	methanol-acetonitrile-KH_2PO_4	C_{18}/partition RP	methanol-acetonitrile-KH_2PO_4	UV (313)	2.5	--	10
	63	ethanol	C_{18}/partition, RP	acetonitrile-phosphate buffer	UV (324)	0.5	--	--
	64	ammonium sulfate	C_{18}/partition, RP	diammonium-hydrogen phosphate-methanol	UV + int. std. (328)	--	93-100	15

	65	acetonitrile	C_{18}/partition, RP	acetonitrile-methanol-KH_2PO_4	UV + int. std. (312)	--	--	--
Nitrofurantoin	66	methanol	C_{18}/partition, RP	methanol-sodium acetate	UV + int. std. (365)	0.02	--	25
Penicillins								
ampicillin	67	perchloric acid	C_{18}/partition, RP	methanol-phosphate buffer	UV	0.5	--	15
	68[c]	trichloracetic acid	C_8/partition, RP	methanol-phosphate buffer	UV (310)	0.1	--	45
amoxicillin	67	perchloric acid	C_8/partition, RP	methanol-phosphate buffer	UV	0.5	--	15
	69	--	C_8/partition, RP	methanol-phosphate buffer	UV (254)	0.5	--	--
azlocillin	70	chloroform-tetrabutyl ammonium phosphate	C_{18}/partition, RP	acetonitrile-phosphate buffer	UV (210)	1.0	40-50	5
	71	Sep Pak C_{18}[d]	C_{18}/partition, RP	acetonitrile-phosphate buffer (pH 7.0)	UV	1.3 - 1.5	92-100	--
amdinocillin (mecillinam)	72	acetonitrile-diethyl ether	C_{18}/partition, RP	methanol-phosphate buffer (pH 7.0)	UV	0.5	74	--

[c] suitable derivatization procedure for other penicillins: cyclacillin, cecillinan, also, carbenicillin, benzylpenicillin, cloxocillin.

[d] Waters Associates, Milford, Mass.

(continued)

Table 3 (continued)

Antimicrobial agents	Ref.	Extraction Procedure	Stationary phase/ Mode of separation	Mobile phase	Detector (wavelength)	Sensitivity (ug/ml)	Recovery (%)	Time (min)
aztreonam	73	acetonitrile	C_{18}/partition, RP	acetonitrile-tetra butylammonium phosphate buffer	UV (280)	serum:1.0 urine:5.0	95-103	--
benzyl-penicillin	32	--	C_{18}/partition, RP	methanol-sodium dihydrogen phosphate	UV (225)	0.1	--	20
cloxacillin	74	acetonitrile-methylene chloride	C_{18}/partition, RP	acetonitrile-water ammonium acetate	UV (254)	0.5	97-98	--
mezlocillin	70	chloroform-tetra butyl ammonium phosphate	C_{18}/partition, RP	acetonitrile-phosphate buffer	UV (220)	0.5	40-50	5
	71	Sep-Pak C_{18}	C_{18}/partition, RP	acetonitrile-phosphate buffer	UV	1.3 - 1.5	92-102	--
piperacillin	75	methanol	C_{18}/partition, RP	acetonitrile-acetate buffer	UV	--	85	--
	76	HCL-chloroform pentanol phosphate buffer	C_{18}/partition, RP	methanol-acetate	UV	0.05	84	--
Rifampin/ metabolites	28	--	C_8/partition, RP	water-methanol gradient	UV (254)	0.05	96	10
	77	chloroform	dimethylsilane	ethyl acetate-piperidine-methanol	UV	0.1	105	--
	78	isooctane-dichloromethane	dimethylsilane	dichloromethane-isooctane-ethanol-water-acetic acid	UV	0.1	96	25

Sulfonamides

salicylazo sulfapyridine	79	isoamyl acetate	C_{18}/partition, RP	acetonitrile-phosphate buffer	UV (280)	0.005	44	--
sulfacetamide	80	perchloric acid	C_8/partition, RP	methanol-phosphate buffer	UV (260)	0.5	--	--
sulfadiazine	81	trichloracetic acid	C_{18}/partition, RP	acetonitrile-acetic acid	UV + int. std.	1.0	100	--
sulfadimidine	80	perchloric acid	C_8/partition, RP	methanol-phosphate buffer	UV (260)	0.5	--	--
sulfadozine	80	perchloric acid	C_8/partition, RP	methanol-phosphate buffer	UV (260)	0.5	--	--
sulfamerazene	81	trichloracetic acid	C_{18}/partition, RP	acetonitrile-acetic acid	UV + int. std.	1.0	100	--
	82	--	RNH^{+3}/ion exchange	ammonium hydroxide water-methanol-ethanol	UV (230)	--	--	--
sulfamethizole	81	trichloracetic acid	C_{18}/partition, RP	acetonitrile-acetic acid	UV + int. std.	1.0	100	--
sulfamethoxazole	80	perchloric acid	C_8/partition, RP	methanol-phosphate buffer	UV (260)	0.5	88	--
sulfanilamide	80	perchloric acid	C_8/partition, RP	methanol-phosphate buffer	UV (260)	0.5	--	--

(continued)

Table 3 (continued)

Antimicrobial agents	Ref.	Extraction Procedure	Stationary phase/ Mode of separation	Mobile Phase	Detector (wavelength)	Sensitivity (ug/ml)	Recovery (%)	Time (min
sulfapyridine	79	4-methyl 1-2-pentane	C_{18}/partition, RP	chloroform-methanol-ammonia	UV (280)	0.0007	--	--
sulfathiazole	80	perchloric acid	C_8/partition, RP	methanol-phosphate buffer	UV (260)	0.5	--	--
Tetracyclines								
tetracycline	83	--	cation exchange	EDTA	UV	--	99	--
	84, 85	methanol-trichloracetic acid	C_{18}/partition, RP	methanol-EDTA	UV (355)	0.3	95-100	25
	86[e]	--	C_8/partition, RP	methanol-ammonium carbonate	UV (254)	0.3	--	30
	87, 88	--	RNH^{+3}/ion exchange	acetonitrile-phosphoric acid	UV + int. std. (357)	--	--	--
Trimethoprin	80	perchloric acid	C_8/partition, RP	methanol-phosphate buffer	UV (225)	0.75	83	--
	82	--	RNH^{+3}/ion exchange	ammonium hydroxide-water-methanol-ethanol	UV (230)	--	--	--
Vancomycin	89	CM-Sephadex ion exchange	C_{18}/partition, RP	acetonitrile-phosphate buffer	UV (210)	0.5	--	--

Antiviral agents								
adenine arabinoside	90	ultrafiltration	N^+R_3/ion exchange	sodium borate-sodium acetate	UV + int. std. (254)	0.05	95-105	--
Antifungal agents								
amphotericin-B	84, 85	methanol	C_{18}/partition, RP	methanol-EDTA	UV (405)	0.02	98-100	25
econazole/ miconazole	92	diethyl ether	C_{18}/partition, RP	methanol-phosphate buffer	UV + int. std.	0.2	81-99	--
ketoconazole	93	diethyl ether (evaporation) methanol	C_{18}/partition, RP	acetonitrile-phosphate buffer	Fluoro + int. std.	0.1	74-79	--
5-Fluorocytosine	94	--	SO_3^-/ion exchange	ammonium phosphate	UV	1.0	--	15
	95	ultrafiltration	SO_3^-/ion exchange	ammonium phosphate	UV	1.0	76-81	30
griseofulvin	96	acetonitrile	C_{18}/partition, RP	acetonitrile-water	Fluoro	0.05	--	5-6
griseofulvin	97	dichloromethane	C_{18}/partition, RP	acetonitrile-phosphate buffer	UV + int. std.	0.2	94	--

[e] also applicable for assaying other tetracyclines: chlortetracycline, demeclocycline, doxycycline, methacycline, oxytetracycline

scriptions of procedures for some agents for which therapeutic monitoring is essential.

Aminoglycosides (24)

Extraction procedure

Combined equal portions (50ul) of serum and internal standard (20ug/ml in Tris buffer). Add 200ul of acetonitrile, vortex and then centrifuge 5 min at 2500 x g. A 200ul portion of the resultant supernatant is mixed with an additional 20ul of acetonitrile and incubated in a waterbath (85° C) for 45 min. A 150ul portion of the sample is injected onto the HPLC column.

Stationary phase

A uBondapak C_{18} column (30cm x 3.9mm, i.d.) is used at ambient temperature.

Mobile phase

The mobile phase consists of acetonitrile-water (70:30 v/v) to which 1.0ml acetic acid/liter is added. The solution is filtered and degassed prior to use. The flow rate is 3.0ml/min.

Detection

Absorption of UV light at 350nm is used.

Comments

This method was chosen because it utilized UV absorption rather than fluorometry to quantitate concentrations of aminoglycosides. The minimum sensitivity of the assay is 0.5ug/ml which is adequate enough to monitor trough concentrations.

Vancomycin (88)

Extraction procedure

Equal portions (400ul) of serum and serum diluent containing the internal standard, ristocetin (16ug/ml) are added together and mixed. This mixture is then added to a CM-Sephadex column and the

sample is allowed to drain completely. A 1.0 ml portion of sodium sulfate is then added and allowed to drain completely. The borate elution buffer (borax-sodium sulfate, pH 9.45) is then added to the column in two steps. The first 400µl is added and allowed to drain as all other solutions. The second 400µl of the buffer is then added and collected for injection onto the HPLC column. A 25µl portion of this is injected.

Stationary phase

A µBondapak C_{18} column (30 cm x 3.9nm, i.d.) at ambient temperature is used to separate vancomycin and the internal standard in each sample.

Mobile phase

The mobile phase is a mixture of 0.05 mole/l phosphate buffer and acetonitrile (91:9 v/v) which is filtered and degassed prior to use. The flow rate is maintained at 2ml/min.

Detection

The eluent is monitored using a UV spectrophotometer set at a wavelength of 210nm.

Comment

This procedure was fairly simple to perform and the CM-Sephadex columns were reusable and stable for 4 months when stored at 4° C. No interference occurs with other antibiotics that may be co-administered (eg. aminoglycosides).

Chloramphenicol (53)

Extraction procedure

A 0.5ml aliquot of sample is mixed with 1.5ml of methanol by vortex for 15 sec. The mixture is then centrifuged at 2000 x g for 10 min. The resulting supernatant is passed through a 0.6um filter.

Stationary phase

A uBondapak C_{18} column is used at ambient temperature.

Mobile phase

The mobile phase is a mixture of acetic acid-methanol-water (1:37:62 v/v/v) at a flow rate of 2.0ml/min.

Detection

The eluent is monitored at a wavelength of 280nm.

Comments

This procedure allows for the separation and quantitation of both chloramphenicol and its metabolite chloramphenicol succinate.

Amphotericin (83,90)

Extraction phase

A 1.0ml aliquot of sample is mixed vigorously with 3.0ml methanol by vortex for 15 min. The sample mixture is allowed to stand at room temperature for 10 min followed by centrifugation at 2,000 x g for 10 min. The resulting supernatant is passed through a 0.5um filter. A 300ul portion of this is injected onto the HPLC column.

Stationary phase

A uBondapak C_{18} reverse phase column at ambient temperature is used to detect amphotericin peaks.

Mobile phase

The mobile phase is a mixture of methanol and 0.005 mole/liter disodium EDTA (80:20 v/v) which is passed through a 0.5µm filter prior to use. The eluent is used at a flow rate of 2.5ml/min.

Detection

The eluent is monitored using a UV spectrophotometer set at 405nm.

Comments

The procedure allows for the separation and quantitation of both amphotericin and its metabolite with no interference from other antiviral or antibacterial agents.

Metronidazole[97]

Extraction procedure

A 1.0ml aliquot of sample is mixed vigorously with 4.0ml of acetonitrile for 15 min and then centrifuged at 2,000 x g for 15 min. A 20µl portion of the supernatant is then injected onto the HPLC column.

Stationary phase

A µBondapak C_{18} reverse phase column (30cm x 3.9mm i.d.) was used with a Co Pell ODS guard column inserted before the analytical column for protection.

Mobile phase

The mobile phase used is acetonitrile and 0.02 mole/filter acetate buffer (35:65 v/v) at a pH of 4.0. The eluent is pumped at a flow rate of 2.0ml/min.

Detection

The eluent is monitored using a UV spechtrophotometer at 313nm. Separation depends upon the strength of the interaction between sample ions and the exchange sites.

Ion exchange chromatography involves more variables than other types of chromatography. The exchange resin may be deactivated by preferential adsorption of retained ions. Separation is also affected by such variables as ionic strength, pH, temperature, solute charge, type of buffer ion present, type of solvent backbone of stationary

phase. Although it is a difficult technique, the number of variables makes ion exchange a versatile technique to allow optimal separation.

Besides being the predominant method for separation of metallic ions, ion exchange can be widely used to the separation of amino acids, nucleic acids, proteins, carboxylic acids, vitamins, glycosides and aromatic sulfonates.

Exclusion chromatography separates substances on the basis of molecular size and shape. The stationary phase used is a uniform, highly porous nonionic gel. The technique, introduced in the late 1950's utilized a beaded form of dextran. Further developments have resulted in the use of aerogels (eg. porous glass or silica), xerogels (crosslinked dextran or polyacrylamide) and a combination of aerogels and xerogels (eg. agarose, polystyrene and polyvinylacetate). Separation depends on the molecular size of the solute; large molecules are excluded from the gel pores and therefore pass quickly through the column where as smaller molecules are retained. The disadvantage with this technique is the inability to distinguish between solutes of similar molecular size. Exclusion chromatography is frequently used to characterize proteins or nucleic acids.

The development of a method of analysis depends upon the pro-

Comments

This procedure is utilized for the quantitation of both metronidazole and its major metabolites.

Conclusion

HPLC offers an excellent alternative to the more confining procedures available today for monitoring antimicrobial concentrations. The technique provides means of separating virtually all antibiotics

in clinical use or under development. The major advantage of HPLC is its ability to separate and quantitate several antibiotics that may be present in a sample due to concomitant administration, as well as separating parent compounds from bioactive metabolites. Other assays, particularly bioassays, are nonspecific in the presence of multiple antibiotics or bioactive metabolites and are, therefore, less desirable procedures. The major disadvantages of HPLC is its limited availability in the clinical laboratory. HPLC is used extensively in research, but because of high instrument cost and expertise required to develop/perform HPLC assays, has found limited use in the routine hospital laboratory. However, with increased refinement of methodology, HPLC is proving to be a highly versitile, rapid, sensitive technique, with an important role in therapeutic drug monitoring.

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