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Review

Chromatographic methods for the bioanalysis of antiviral agents

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CONTENTS

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1 INTRODUCTION

Viral infections remain the most common cause of disease in man. Fortunately, most viral infections are self-limiting and mass immunizations have drastically reduced the number of complications and deaths from serious viral diseases such as poliomyelitis, mumps, measles, rabies, smallpox and influenza. Great strides have been made in antiviral therapy and research, but ever present is the morbidity and mortality caused by hepatitis viruses, herpes viruses and cytomegalovirus in immunocompromised patients However, the impact of the human immunodeficiency viruses (HIV-1 and HIV-2), the causative agent of acquired immunodeficiency syndrome (AIDS), in stimulating research is unequalled. AIDS is invariably fatal and the presently available treatments are extremely expensive; therefore, the need for the discovery of new and improved antiviral agents has never been greater [1–4] and, clearly, new bioanalytical technology will be required for newly developed antiviral drugs. The purpose of this paper is to review present methodologies for existing antiviral drugs and to provide guidelines for the development of bioanalytical methods for new antiviral drugs.

1.1. Nomenclature and classifications

The vast majority of antiviral compounds (Table 1) that are used clinically, or are being considered for use, are the nucleoside analogues [1,3] (Fig. 1), which, for the purpose of this review, have been further divided into five groups: the arabinosides (**1,2**); ribavirin (**3**) and riboxamide (**4**); the acyclic guanosine derivatives (**5–7**); the 2′,3′-dideoxynucleosides (**8–13**); and the 5-halo-2′-deoxynucleosides (**14–17**). This list of nucleoside analogues does not include the large number of related compounds which are under pre-clinical or early clinical investigations [2] and has been restricted, for the purposes of this review, to those compounds for which there is a reasonable body of bioanalytical literature. The non-nucleoside drugs (Fig. 2) mostly represent a diverse class of structurally unrelated compounds and it is convenient to discuss their analysis individually in a section separate form the nucleoside analogues This group of drugs includes arildone

TABLE 1

NOMENCLATURE OF THE ANTIVIRAL DRUGS

Drug name	Chemical names	Common trade names	Common abbreviation
Acyclovir (**5**)	9-[(2-Hydroxyethoxy)methyl]-guanine, 2-amino-1,9-dihydro-9-[2-hydroxyethoxy]6*H*-purin-6-one; acycloguanosine	Zovirax	ACV
Amantidine (**19**)	Tricyclo[3.3 1 $1^{3,7}$]decan-1-amine, 1-adamantanamine; 1-aminoadamantane	Amazolon, Mantadix, Mantadan; Midantan, Mydantane; Symmetrel	
Ampligen (**24**)	Mismatched double-stranded RNA		
Arildone (**18**)	4-[6-(2-Chloro-4-methoxyphenoxy)hexyl]3,5-heptanedione		
Bromovinyldeoxyuridine (**15**)	5-(2-Bromovinyl)-2′-deoxyuridine		BVDU
Carbovir (**8**)	Carbocyclic 2′,3′-didehydro-2′,3′-dideoxyguanosine		
Cytarabine (**2**)	4-Amino-1-β-D-arabinofuranosyl-2(1*H*)-pyrimidinone, 1-β-D-arabinofuranosylcytosine, 1-β-cytosine arabinoside	Alexan, Arabitin, Aracytidine; Aracytine, Ara-C, Cytosar, Erpalfa, Iretin, Udicil	Ara-C
Desciclovir (**7**)	2-[(2-Amino-9*H*-purin-9-yl)-methoxy]ethanol, deoxyacyclovir		DCV
2′,3′-Didehydro-2′,3′-deoxythymidine (**13**)	1-(2,3-didehydro-3-deoxy-β-D-*erythro*-pentofuranosyl)thymine		d4T
2′,3′-Dideoxyadenosine (**9**)	2′,3′-Dideoxy-9-β-ribofuranosyl-9*H*-purin-6-amine		DDA
2′,3′-Dideoxycytidine (**10**)	2′,3′-Dideoxy-4-amino-1-β-D-ribofuranosyl]-2-(1*H*)-pyrimidinone		DDC
2′,3′-Dideoxyinosine (**12**)	2′,3′-Dideoxy-9-β-ribofuranosyl-1,9-dihydro-6*H*-purin-6-one		DDI
Enviroxime (**22**)	6-[(Hydroxyimino)phenylmethyl]-1-[(1-methylethyl)sulfonyl]-(1*H*)-benzimidazol-2-amine		
2′-Fluoro-5-iodoaracytosine (**16**)	2′-Fluoro-5-iodo-4-amino-1-β-D-arabinofuranosyl-2(1*H*)-pyrimidinone; 2′-fluoro-5-iodo-1-β-D-arabinofuranosylcytosine		FIAC

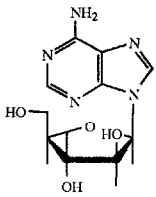
(Continued on p. 298)

TABLE 1 (*continued*)

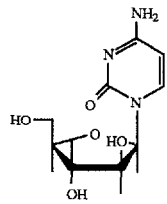
Drug name	Chemical names	Common trade names	Common abbreviation
Foscarnet sodium (**23**)	Dihydroxyphosphinecarboxylic acid oxide trisodium salt, trisodium phosphonoformate		
Ganciclovir (**6**)	9-(1,3-Dihydroxy-2-propoxymethyl)guanine, 2-amino-1,9-dihydro-9-(1,3-dihydroxy-2-propoxymethyl)-6*H*-purin-6-one		DHPG
Idoxuridine (**14**)	1-(2-Deoxy-β-D-ribofuranosyl)-5-iodouracil, 5-iodo-2′-deoxyuridine	Dendrid, Emanil, Herpid, Herplex, Idexur, Idoxene, Idulea, Idu Oculos, Iduridin, Kerecid, Ophthalmidine, Stoxil	IDU; IDUR, IUDR
5-Iodo-2′-deoxycytidine (**17**)	2′-Deoxy-5-iodo-4-amino-1-β-D-ribofuranosyl]-2-(1*H*)-pyrimidinone		IDC
Moroxydine (**21**)	N-(Aminoiminomethyl)-4-morpholinecarboximidamide, abitylguanide	Bioxine, Vironil, Virusmin, Virustat	ABOB
Ribavirin (**3**)	1-β-D-Ribofuranosyl-1*H*-1,2,4-triazole-3-carboxamide	Viramid, Virazole	RTCA
Riboxamide (**4**)	2-β-D-Ribofuranosyl-4-thiazolcarboxamide, tiazofurin		TCAR
Rimantidine (**20**)	α-Methyltricyclo[3 3 1 $1^{3,7}$]decan-1-methanamine, α-methyl-1-adamantanmethylamine	Meradan	
Vidarabine (**1**)	9-β-D-arabinofuranosyl-9*H*-purin-6-amine, 9-β-D-arabinofuranosyladenine, adenine arabinoside	Vira-A	Ara-A
Zidovudine (**11**)	1-(3-Azido-2,3-dideoxy-β-D-*erythro*-pentofuranosyl)thymine, 3′-azido-3′-deoxythymidine	Retrovir	AZT

(**18**), amantidine (**19**), rimantidine (**20**), moroxydine (**21**), enviroxime (**22**), foscarnet (**23**) and ampligen (**24**, structure not shown).

In addition to these drugs (**1–24**), immunomodulators are currently under intense investigation for the treatment of viral disease but because they represent a chemically and biochemically diverse group of compounds they were consid-

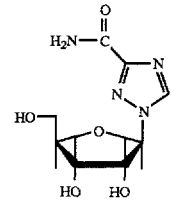


Vidarabine (1)

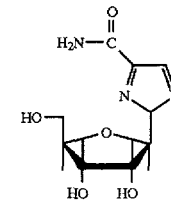


Cytarabine (2)

Arabinosides

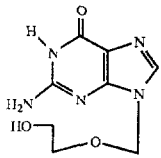


Ribavirin (3)

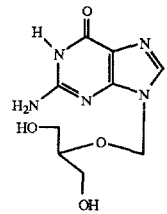


Riboxamide (4)

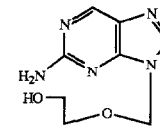
Ribavirin and Riboxamide



Acyclovir (5)

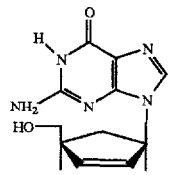


Ganciclovir (6)

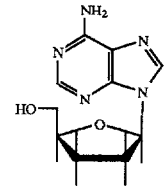


Desciclovir (7)

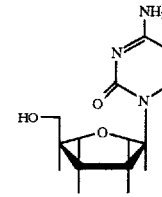
Acyclic guanosine derivatives



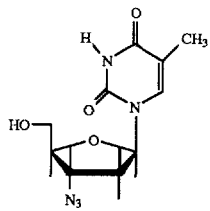
Carbovir (8)



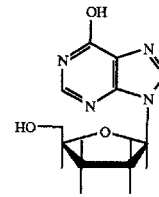
2',3'-Dideoxyadenosine (9)



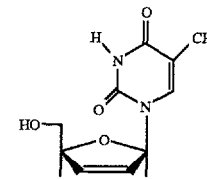
2'3'-Dideoxycytidine (10)



Zidovudine (11)



2'3'-Dideoxyinosine (12)



2,'3'-Didehydro-3'-deoxy-thymidine (13)

Deoxynucleotides

Fig 1

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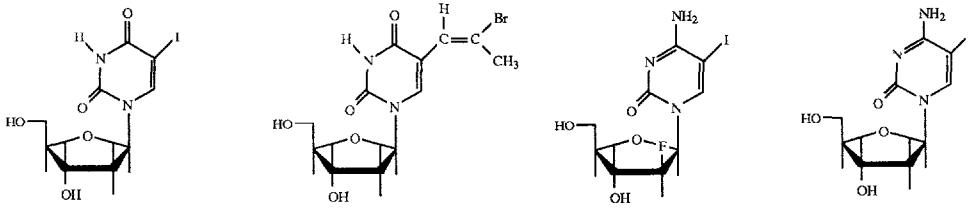


Fig. 1 Structures of the nucleoside analogues with antiviral activity

ered to be beyond the scope of this publication. The interferons have also been used as antiviral drugs; however, the use of chromatographic methods for the determination in biological fluids has been very limited.

1.2. The need for bioanalysis of antiviral agents

The bioanalysis of antiviral agents poses a particularly significant challenge because they tend to be structurally similar to endogenous substances and therefore require highly selective bioanalytical methodology. The need for analytical selecitivity is particularly critical for the nucleoside analogues (**1–17**, Fig. 1) because, in addition to their similarity with endogenous substances, they tend to be extensively metabolized to products that often have significant biological activity

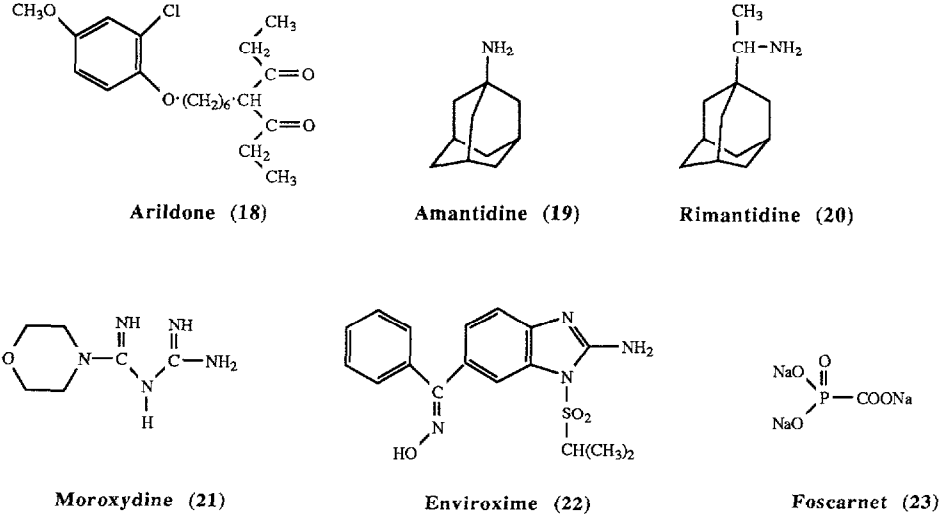


Fig 2 Structures of the non-nucleoside antiviral agents.

[1–4]. Therefore, chromatographic methods [5] for the analysis of antiviral drugs in biological fluids must be capable of separating and quantitating the metabolites as well as the parent compounds. Antiviral drugs also exhibit substantial intra- and inter-subject variability in their absorption, distribution, metabolism and elimination leading to wide variability in plasma and tissue concentrations. This variability in systemic concentrations probably helps to explain the range of response and toxicity that is generally observed with antiviral drugs, and can lead to modification of dosage regimens or withdrawal of treatment. Because of first-pass metabolism, the problem of variability in response is particularly acute with drugs such as zidovudine (**11**) that are administered orally. Although the rationale for therapeutic drug monitoring of the newer orally administered antivirals has yet to be firmly established, recent reports on the variability in the pharmacokinetics of zidovudine (**11**) [6–16] indicate that it may be generally necessary.

1.3. Handling of infectious samples

1.3.1. Potential sources of infection

AIDS is invariably fatal and the contraction of other virally transmitted diseases such as hepatitis B can have serious and often fatal consequences. Therefore, even if the origin of a sample of a biological fluid or tissue from an animal, a patient or a volunteer is known, that sample must be treated with extreme caution. All laboratories handling such potentially infectious samples must establish standard operating procedures based on published recommendations [17–38]. These procedures are designed to eliminate direct contact with the specimens and are subject to frequent review. Skin, mucous membranes and the respiratory tract should be considered possible routes of entry for infectious materials. Gloves, masks and eye protection should be worn at all times. Hands should be washed with soap and water, even if gloves have been worn. Precautions must be taken to avoid contact with sharp or broken objects, to avoid splashing and spilling of contaminated materials and to ensure proper disposal of all contaminated materials, especially syringes and needles. Personnel should not be allowed to handle biological samples without prior instruction and they should not be allowed to work independently until they have demonstrated competency. For all materials and equipment that have been in direct contact with potentially infectious samples, viruses should be assumed to be viable. More detailed guidelines on avoiding personnel contamination by infective agents in research laboratories that use human tissues are to be found in a recent review by Grizzle and Polt [36].

1.3.2. Heat inactivation of viruses

It has been recommended that biological samples contaminated with viruses be heated to 56–60°C for 10–30 min, conditions that are known to prevent the transmission of bloodborne infections, including hepatitis B [23–26]. However, the Centers for Disease Control (CDC) have stated: "it is not certain how effec-

tive 56–60°C heat is in destroying HIV in serum ([19,20,27]), but heating small volumes of serum for 30 minutes at 56°C before serological testing reduces residual infectivity to below detectable levels. Such treatment causes false-positive results in HIV enzyme immunoassays ([28–31]) and may also affect some biochemical assays performed on serum ([28,32,33])". Uncertainty over the effectiveness of heating at 56°C for 30 min has prompted the American Society of Hospital Pharmacists to recommend [17] heating HIV-infected samples to 56°C and maintaining that temperature for at least 5 h. It is known [6,22] that heating to 56°C for 60 min does not compromise the chemical stability of the most commonly used anti-AIDS drug, zidovudine (**11**); however, future validation procedures for the bioanalysis of any anti-AIDS drug and its metabolites should include a stability study in the relevant biological fluids. These stability studies should be conducted at a temperature of 60°C for at least 5 h because these are more excessive conditions than any of those presently used to inactivate HIVs. Recent work in this laboratory [16] has shown that heating plasma to temperatures greater than 60°C results in denaturation of proteins and a coincidental reduction in the extraction efficiency of zidovudine (**11**).

1.3.3. Direct injection of biological fluids

The procedures described to date have been designed to minimize exposure during routine treatment of potentially infectious samples prior to chromatography or other bioanalytical methods. The chance of accidental bodily contact with virus-infected samples is highest in routine clinical laboratories that handle large numbers of samples. In a recent review on therapeutic drug monitoring, Wong [38] has advocated the use of automatic methods of analysis including robotics, direct injection and column switching as possible ways of reducing the handling of biological fluids to an absolute minimum.

2 NUCLEOSIDES

Details of the various methods that have been described for the analysis of the nucleoside antiviral drugs are summarized in Table 2.

2.1. Liquid chromatography

Liquid chromatography (LC) with detection by ultraviolet (UV) absorption is the method of choice for the routine determination of nucleosides in biological fluids. Fluorescence, mass spectroscopic (MS) and electrochemical detection (ED) are other options in LC for more specialized applications.

2.1.1. Detection systems

2.1.1.1. Absorbance. The majority of LC methods for the analysis of the nucleosidic antiviral agents in biological samples (Table 2) use UV absorbance [6–12, 14-16,39–99] for detection. In solutions of neutral to mildly acidic pH, the nat-

urally occurring nucleosides (adenosine, cytidine, guanosine, thymidine and uridine) have absorption maxima between 260 and 280 nm and molar absorptivities at those maxima of between 10 000 and 15 000 (Table 3). The intense absorption of UV light arises from the hereocyclic ring systems of the nucleosides and generally allows LC detection limits of 5–50 ng/ml to be achieved in plasma and serum when UV detection is employed Because of interferences from structurally related endogenous substances, detection limits in urine tend to be somewhat higher and are generally in the range 20–200 ng/ml. The absorption spectra of the nucleosides exhibit shifts in intensity and wavelength maxima with changes in pH of the solvent; however, the changes in intensity with change in pH are not generally sufficient to have any significant effect on the sensitivity of LC assays.

Because of the similarity of the nucleosidic antiviral drugs and endogenous compounds, peak identification and peak homogeneity are important issues to be addressed when a non-specific method of detection, such as UV absorbance, is employed. Interestingly, Shrecker and Urshel [39] in one of the earliest papers written on the LC of nucleosides and antiviral drugs described the value of the ratio of the peak heights measured at two wavelengths (typically 260 and 280 nm) for the determination of peak purity. With the advent of diode-array detectors, multi-channel UV detection of nucleosides is ideally suited for peak-purity testing [39,80,100–102]. Peak identification in the chromatography of the nucleoside antiviral drugs can be further complicated by extensive metabolism of the parent compound, which can give rise to numerous metabolites. Consequently, metabolism studies are frequently conducted *in vivo* and *in vitro* with radiolabeled material to aid the identification of chromatographic peaks. For example, Feinberg *et al* [93] have used both UV detection and on-line radioactivity monitoring for the identification of several metabolites of 2′-fluoro-5-iodoaracytosine (FIAC) (**16**) (Fig. 3) in the urine of patients infected with Herpes virus following intravenous administration of drug spiked with 190 μCi of [2-^{14}C]FIAC.

2.1.1.2. Fluorescence. Fluorescence has not been as widely used as UV absorbance for the detection of nucleosides or nucleosidic antiviral agents in biological samples because the purine and pyrimidine ring systems are only significantly fluorescent in their protonated forms. For example, it has been observed [103–113] that the guanine and adenine derivatives are fluorescent in aqueous solutions of low pH and this observation has been exploited [114–117] for the detection of nucleosides and their bases in LC eluents. Because of the instability of the silica-based bonded phases available at the time, Assenza and Brown [114] used a post-column reactor to lower the mobile phase pH prior to the fluorescence detection of purines. Subsequently, Borak and Smrz [115] and Jandera *et al.* [116] have detected nucleosides and bases by fluorescence following their separation on stable, polymeric stationary phases eluted with acidic mobile phases. This approach [114–117] to nucleoside analysis was later adapted by Šalamoun *et al.* [117] for the determination of acyclovir (**5**) in plasma. Fig 4 shows that the fluorescence of acyclovir (**5**) increases dramatically with increasing acidity of the solution below

TABLE 2

CHROMATOGRAPHIC METHODS FOR THE DETERMINATION OF THE NUCLEOSIDE ANTIVIRAL DRUGS IN BIOLOGICAL FLUIDS

N S = not stated or could not be determined from information in the reference

Drug	Method[a]	Detection[b]	Chromatographic conditions		Limit of detection[d]	Sample matrix	Comments	References
			Stationary Phase[c]	Mobile phase or carrier gas				
Acyclovir (**5**)	LC	UV (254 nm)	Zorbax ODS (5 μm, 250 × 4 6 mm)	Sodium acetate (5 m*M*) containing sodium heptane sulfonate (2 5 m*M*)	1-10 μM	Plasma and urine	Interference in some patient samples limited detection to 10 μM	Land and Bye [66]
		UV (254 nm)	μBondapak C_{18} (10 μm, 300 × 3 9 mm)	Acetonitrile–potassium dihydrogenphosphate (0 01 *M*, pH 2 3) containing heptanesulfonic acid (5 m*M*) (0 25 999 75)	250 ng/ml	Plasma		Bouquet *et al* [67]
	LC	UV (254 nm)	PRP-1 (10 μm, 150 × 4 2 mm)	Methanol–hydrochloric acid (0 1 *M*)–sodium heptanesulfonate (0 02 *M*)–sodium chloride (0 025 *M*) (10 10 20 60)	1 2 μg/ml	Serum	PRP-1 columns had better stability than Zorbax ODS Variable recoveries (R S D >20%) at concentrations below 1 2 μg/ml	Smith and Walker [68]
	LC	UV (254 nm)	YWG-C_{18}	Methanol -water (7·93 for plasma or 3·97 for urine)	500 ng/ml	Plasma and urine	Clinical drug monitoring	Guan *et al* [69]
	LC	UV (250 nm)	Nucleosil C_{18} (5 μm, 200 × 4 mm)	Methanol–water (5 95, v/v)	300 ng/ml	Serum and plasma		Cronqvist and Nilsson-Ehle [70]

	LC	FL (λ_{ex} 285 nm, λ_{em} 370 nm cut-off filter)	Guard column, Spheron Micro 300 (16 μm, 150 × 3 2 mm), analytical column, Spheron Micro 300 (12.5 μm, 150 × 3.2 mm)	Phosphoric acid (0.1 *M*) and sodium sulfate (0.1 *M*) (pH 1 8)	1 ng/ml	Plasma	Fluorescence at low pH	Salamoun *et al.* [117]
	LC	ED	Partisil 10 SCX (10 μm, 250 × 4 2 mm)	Sodium perchlorate (0 1 *M*) in ammonium phosphate (0 01 *M*) (pH 3)–methanol (97 3, v/v)	150 pg	None	Stability studies	Visor *et al* [132]
	TLC	FL	Silica	Butanol–acetic acid–water (60.20 30, v/v)	80 ng	Plasma and urine	Fluorescamine derivatization	Ray [128]
BVDU (**15**)	LC	(UV (254 nm)	Guard column, Corasil (37 μm, 20 × 3 9 mm), analytical column, μBondapak C_{18} (10 μm, 300 × 3 9 mm)	Acetonitrile–phosphate buffer (7 m*M*) (pH 7) (12 5:87 5, v/v)	25 ng/ml	Plasma	Oral bioavailability in man and the rat	Robinson *et al.* [94]
	LC	UV (292 nm)	Spherisorb ODS 2 (5 μm, 125 × 4.6 mm)	Gradient solvent A, methanol–tetrabutyl ammonium bromide (12.5 m*M*) and sodium	0 175 μ*M* (serum), 3 μ*M* (urine)	Serum, urine and Herpes simplex cells	Cellular uptake and metabolism *in vitro*	Ayisi *et al* [95]

(Continued on p. 306)

TABLE 2 (*continued*)

Drug	Method[a]	Detection[b]	Chromatographic conditions		Limit of detection[d]	Sample matrix	Comments	References
			Stationary phase[c]	Mobile phase or carrier gas				
				dihydrogenphosphate (50 m*M*) (25 75, v/v); solvent B, methanol–tetrabutylammonium bromide (12.5 m*M*) and sodium dihydrogenphosphate (50 m*M*) (50 50, v/v)				
	LC–LC	UV (292 nm)	(a) PRP-1 (10 μm, 100 × 3 mm) or (b) Nucleosil 100 C_8 (5 μm, 100 × 3 mm)	(a) Hexadecyltrimethylammonium bromide (5 m*M*) in Tris buffer (pH 8.2, 50 m*M*), (b) methanol–perchloric acid (pH 2, 10 m*M*) (12.5 87 5, v/v)	6 ng/ml	Plasma and urine	Clinical pharmacokinetics	Reeuwijk *et al* [96]
	LC	RA	Partisil PXS 10/25 SAX (10 μm, 250 × 4 6 mm)	Gradient: solvent A, ammonium hydrogenphosphate (5 m*M*, pH 2 8); solvent B, ammonium hydrogenphosphate (750 m*M*, pH 2 8)	N S	Herpes simplex cells	Cellular uptake	Suzutani *et al* [97]
Carbovir (**8**)	LC	UV (252 nm)	Adsorbosphere HS C_{18} (5 μm, 250 × 4 6 mm)	Methanol–ammonium phosphate (10 m*M*) containing triethylamine (5 m*M*)(pH 7) (22·78)	50 ng/ml	Plasma	Pharmacokinetics in the rat	Remmel and co-workers [98,99]

Cytarabine (**2**)	LC	UV (265 nm)	Dowex (AG) 1-X2	Ammonium hydroxide (0 01 *M*)	N S	Cells	Uptake by tumor cells, *in vitro*	Shrecker and Urshel [39]
	LC	UV (254 nm)	Aminex A-7 (130 mm)	Potassium phosphate–potassium chloride (0 2 *M*, pH 4 3)	100 ng/ml	Plasma and urine	Clinical pharmacokinetics	Wan *et al* [40]
	(a) LC or (b) LC (LC)	UV (254 nm) or RA	(a) μBondapak NH_2 (10 μm, 300 × 6 4 mm) or (b) two ABX Permaphase (10 μm, 600 × 2 7 mm)	Gradient solvent A, potassium dihydrogenphosphate (pH 3 0, 5 m*M*), solvent B, potassium dihydrogenphosphate (pH 3 0, 4 *M*)	N S (UV), 100 pmol (RA)	Cells	Tumor cells and ascites fluids	Kreis *et al* [41]
	LC	UV (254 nm)	(a) Partisil ODS (10 μm) or (b) Partisil PXS 10/25 (10 μm, 250 × 4.6 mm)	Ammonium formate (pH 4 8)	20 ng/ml	Plasma	Leukemia patients	Bury and Keary [42]
	LC	UV (260 nm)	Aminex A-6 (17 μm)	Boric acid (0 1 *M*)	N S	None	Complexation with boric acid	Pal [43]
	LC	UV (254 nm)	Zorbax ODS (5 μm, 250 × 4 6 mm)	Methanol–phosphate (2 5 m*M*, pH 3 or 9) or borate (50 m*M*, pH 9) (2 5 97 5, v/v)	500 pg	Plasma	Normal levels in rat plasma	Rustum [44]
	LC(LC)	UV (254 and 280 nm) or RA	Two Dupont ABX (1 m × 2 mm)	Gradient solvent A, potassium dihydrogenphosphate (pH 3 0, 2 5	N.S.	Cells	Clinical pharmacology	Rustum and Preisler [45]

(Continued on p 308)

TABLE 2 (*continued*)

Drug	Method[a]	Detection[b]	Chromatographic conditions		Limit of detection[d]	Sample matrix	Comments	References
			Stationary phase[c]	Mobile phase or carrier gas				
				mM); solvent B, potassium dihydrogenphosphate (pH 4.4, 500 mM)				
	LC	UV (270 nm)	(a) Aminex A-27 (13 5 μm, 500 × 4 mm) or (b) A-29 (9 0 μm, 200 × 4 mm)	Sodium citrate (25 mM) + sodium tetraborate (80 mM) (pH 9.3)	2.5 ng	Cells	Uptake by tumor cell implants in the rat Clean-up with boronate column	Pallavicini and Mazrimas [46]
	LC	UV (270 nm)	μBondapak C_{18} (10 μm, 300 × 3 9 mm)	Phosphate (10 mM, pH 5 6)	500 pg	Plasma	Rat plasma	Pallavicini and Mazrimas [46]
	LC	UV (254 and 280 nm) or RA	Partisil SAX 10 (10 μm, 25 × 4.6 mm)	Sodium dihydrogen-phosphate (pH 3 3, 0 4 M)	N S	Cells	Uptake by L1210 cells in mice	Grant *et al* [47]
	LC	UV (254, 280 nm)	Partisil SAX 10 (10 μm, 25 × 4 6 mm)	(a) Ammonium phosphate (0.5 M, pH 3.5)–methanol (98:2, v/v) or (b) gradient: solvent A, ammonium phosphate (0 5 M, pH 3 5); solvent B, ammonium phosphate (0 5 M, pH 3 5)–methanol (95 5, v/v)	N S	Cells	Human leukemia cells	Plunket *et al* [48], Danks [52]

LC	UV (270 nm)	Spherisorb ODS (5 μm, 300 × 4 mm)	Phosphate (50 m*M*, pH 7 0)	50 ng/ml	Plasma and CSF	Clinical pharmacokinetics	Breithaupt and Schick [49]
LC	UV (280 nm)	Nucleosil 10 C_{18} (10 μm, 250 × 4.6 mm)	Phosphate (0.2 *M*, pH 2)	2 ng/ml	Plasma	Clinical pharmacokinetics	Linssen *et al* [50]
LC	UV (254 nm)	LiChrosorb SI (5 × 10 μm, 250 × 4 6 mm)	Various combinations of dichloroethane, methanol and formic acid	None	None	Normal-phase chromatographic study	Ryba and Beranek [51]
LC–LC	UV (254 nm)	Pre-column Ranin OD-MP RP-18 (5 μm, 30 × 4 6 mm), columns 1 and 2, Ranin OD-MP RP-18 (5 μm, 100 × 4 6 mm)	Complex column-switching system (see ref 53)	125 ng/ml	Plasma	Simultaneous analysis of ara-C, ara-U and salicylate Pharmacokinetics in the dog	Liversidge *et al* [53]
LC(LC)	UV (280 nm)	Ultrasphere ODS (5 μm, 150 × 4.6 mm) + Partisil PXS 10/25 (10 μm, 250 × 4 6 mm)	Phosphate (2.5 m*M*, pH 3.2)–methanol (97.5·2 5, v/v)	20 ng/ml	Blood and CSF	Clinical pharmacokinetics	Sinkule and Evans [54]

(Continued on p 310)

TABLE 2 (*continued*)

Drug	Method[a]	Detection[b]	Chromatographic conditions		Limit of detection[d]	Sample matrix	Comments	References
			Stationary phase[c]	Mobile phase or carrier gas				
	LC	UV (280 nm)	Radial-Pak C_{18} (5 μm)	Phosphate (0.1 *M*) + tetrabutylammonium-phosphate (5 m*M*)(pH 4 5)	5 pmol	Plasma and cells	Uptake by human breast cancer cells	Schilsky and Ordway [55]
	LC	N S	(a) Silasorb C_{18} or (b) μBondapak C_{18} (10 μm, 300 × 3 9 mm)	Ammonium sulfate (0 1–2 5 *M*, pH 4 8–5 3)	N S.	None	None	Galushko *et al.* [56,57]
	LC	UV (254 nm)	N S	Methanol–water–acetate (2 5 95 2 5, v/v)	200 ng/ml	Plasma	Rabbit pharmacokinetics	Chen *et al.* [58]
	LC	UV (280 nm)	Pre-column, Corasil II, analytical column, μBondapak C_{18} (10 μm, 150 × 3 9 mm)	Ammonium acetate (0 5 *M*, adjusted to pH 6 5 with 6 *M* acetic acid)	2 μg/ml	Plasma and serum	Clinical pharmacokinetics Interference from uridine and hypoxanthine limits sensitivity	Wermeling *et al* [59]
	PC	RA	N S	*n*-Propanol–tetrahydro-furfuryl alcohol–citrate buffer (340 85 72, v/v)	N.S.	Tumor cells	Cellular uptake in tumor-bearing mice	Uchida and Kries [134]

	GC	MS (CI,EI)	3% OV-17 on Chrom-Q (230°C)	He(EI), CH_4(CI)	10 ng	Serum	Pharmacokinetics	Pantarotto *et al.* [138]
	GC	NPD, FID or MS	3% SE-30 or OV-17 (280°C)	Helium	50–500 pg	None	TMS and other silyl derivatives	Boutagy and Harvey [141]
	GC	NPD, FID or MS	3% SE-30 or OV-17 (230°C)	Helium	1 ng/ml (MS); 70 ng/ml (NFID)	Plasma	Clinical pharmacokinetics Acetylation and methylation	Boutagy and Harvey [142]
	GC	MS	3% SE-30 (250°C)	N.S.	N.S.	Urine		Makino *et al.* [143]
DDA (**9**)	LC	UV (254 nm) or RA	μBondapak C_{18} (10 μm, 150 × 3.9 m)	Methanol–KH_2PO_4 (10:90, v/v, pH 4.5)	100 n*M* (UV)	Plasma	Clinical pharmacology	Koeller *et al.* [78]
	LC	UV (254 nm) or RA	(a) Ultrasphere ODS (5 μm, 250 × 4.6 mm) or (b) Utremex C18IP (5 μm, 250 × 4.6 mm)	(a) ammonium dihydrogenphosphate (10 m*M*, pH 6.8)–N,N-dimethylformamide (92.5:7.5, v/v) or (b) gradient elution, solvent A, ammonium dihydrogenphosphate (20 m*M*, pH 6.8) and tetrabutylammonium phosphate (5 m*M*); solvent B, solvent A–N,N-dimethylformamide (90:10, v/v)	200 ng/ml (UV)	Plasma	Pharmacokinetics in the cat	Kalin and Hill [79]

(Continued on p. 312)

TABLE 2 (*continued*)

Drug	Method[a]	Detection[b]	Chromatographic conditions		Limit of detection[d]	Sample matrix	Comments	References
			Stationary phase[c]	Mobile phase or carrier gas				
	LC	UV or MS (TS)	Guard column, Brownlee RP-8 (5 μm, 30 × 4 6 mm), analytical column Zorbax C_8 (6 μm, 250 × 4.6 mm)	UV acetonitrile–water (10 90, v/v) containing ammonium dihydrogenphosphate (50 m*M*), MS acetonitrile–water (5·95, v/v) containing ammonium dihydrogenphosphate (100 m*M*)	Low ng/ml	Plasma	Pharmacokinetics and metabolism in the mouse	Blau *et al* [133]
DDC (**10**)	LC	(a) UV (273 nm) (b) RA	Guard column, Bondapak C_{18}/Corasil (37–50 μm); analytical column: (a) Ultrasphere ODS (5 μm, 250 × 4.6 mm) or (b) Partisil 10 SAX (10 μm, 300 × 3 9 mm)	(a) Acetonitrile–phosphate buffer (pH 6 8) (3.97, v/v) (b) Gradient: solvent A, ammonium-phosphate (pH 3 6, 0 01 *M*); solvent B, ammonium phosphate (pH 3 8, 0 6 *M*)	50 ng/ml	Plasma and cells	Pharmacokinetics and analysis of intracellular levels of anabolites in mice and monkeys Method (a) for parent drug and catabolites, method (b) for anabolites	Kelley *et al* [80]
	LC	UV (273 nm)	Radial-Pak C_{18} (5 μm, Z-module)	Acetonitrile–ammonium acetate buffer (3 97, v/v)	21 ng/ml	Plasma	Clinical pharmacokinetics	Klecker *et al* [81]

	LC	UV (254 nm) or RA	(a) Ultrasphere ODS (5 μm, 250 × 4 6 mm) or (b) Ultremex C18IP (5 μm, 250 × 4.6 mm)	Ammonium dihydrogenphosphate (10 m*M*, pH 6 8)–N,N-dimethylformamide (92 5 7 5, v/v) or gradient elution solvent A, ammonium dihydrogenphosphate (20 m*M*, pH 6 8) and tetrabutylammonium phosphate (5 m*M*), solvent B, solvent A–N,N-dimethylformamide (90·10, v/v)	200 ng/ml (UV)	Plasma	Pharmacokinetics in the cat	Kalin and Hill [79]
	LC–LC (LC)	UV (272 nm)	Column 1, Pinkerton ISRP (5 μm, 150 × 4 6 mm), column 2, two Cyclobond I (5 μm, 100 × 4 6 mm and 5 μm, 250 × 4 6 mm)	Complex solvent programming, see ref 10	312 ng/ml	Plasma	Pharmacokinetics in the cat	Mathes *et al* [10]
	GC	MS	3% OV-17 on Gas Chrom Q (100–120 mesh, 5 ft × 2 mm)	Methane	2 ng/ml	Plasma	Silylation of 5′-OH-pentafluorobenzoylation of NH_2 group and methylation of ring nitrogen; clinical pharmacology	Rubio *et al* [144]
DDI(12)	LC	(a) UV or (b) MS (TS)	Guard column, Brownlee RP-8 (5 μm, 30 × 4 6	(a) Acetonitrile–water (10 90, v/v) containing ammonium dihydrogenphosphate (50 m*M*) or	Low ng/ml	Plasma	Pharmacokinetics and metabolism in the mouse	Blau *et al.* [133]

(Continued on p. 314)

TABLE 2 (*continued*)

Drug	Method[a]	Detection[b]	Chromatographic conditions		Limit of detection[d]	Sample matrix	Comments	References
			Stationary phase[c]	Mobile phase or carrier gas				
			mm); analytical column, Zorbax C_8 (6 μm, 250 × 4 6 mm)	(b) Acetonitrile–water (5 95, v/v) containing ammonium dihydrogenphosphate (100 m*M*)				
	LC	UV (254 nm) or RA	(a) Ultrasphere ODS (5 μm, 250 × 4 6 mm) or (b) Utremex C18IP (5 μm, 250 × 4 6 mm)	(a) ammonium dihydrogenphosphate (10 m*M*, pH 6 8)–N,N-dimethylformamide (92.5.7.5, v/v) or gradient elution solvent A, ammonium dihydrogenphosphate (20 m*M*, pH 6 8) and tetrabutylammonium phosphate (5 m*M*), solvent B; solvent A–N,N-dimethylformamide (90 10, v/v)	200 ng/ml	Plasma	Pharmacokinetics in the cat	Kalin and Hill [79]
Desciclovir (7)	LC	UV (300 nm)	Microsorb C_{18} (5 μm, 250 × 4 6 mm)	Gradient solvent A, ammonium formate (pH 3 5, 25 m*M*), solvent B, acetonitrile–ammonium phosphate (pH 7 2, 25 m*M*) (60 40, v/v)		Plasma, Urine	Metabolism and pharmacokinetics in man	Krasny and Petty [76], Petty *et al.* [77]

d4T (**13**)	LV	UV (254 nm)	Guard column, Pellicular ODS (37–53 μm), analytical column, Apex C_8 (5 μm, 250 × 4 6 mm)	Methanol–phosphate buffer (50 m*M*) (20 80)	50 ng/ml	Plasma	Pharmacokinetics in the rat and the monkey	Kaul *et al* [82]
FIAC (**16**)	LC	UV (273 nm) or RA	Guard column, CO Pell, analytical column Partisil ODS-3 (5 μm, 250 × 4 6 mm)	Gradient methanol–phosphate buffer (0 02 *M*, pH 3) (*x*.100−x, v/v) Different gradient used depending on detection method and analytes to be determined	N.S. (UV), 0.1 n*M* (RA)	Plasma, urine and tissues	Metabolism and distribution studies in rat and mouse	Philips *et al* [92], Feinberg *et al*. [93]
Ganciclovir (**6**)	LC LC	UV (254 nm)	Guard column, CO Pell ODS (30 × 4.6 mm); analytical columns, Partisil 10 SCX (10 μm, 50 × 4 6 mm) + Partisil 10 ODS-3 (10 μm, 50 × 4 6 mm)	Methanol- ammonium phosphate (5 m*M*, pH 2 5) (x·100 − x, v/v, where *x* varied between 10 and 60 depending on the analytical problem)	N S	Liver and intestinal homogenates	*In vitro* metabolism of the diesters of ganciclovir	Benjamin *et al* [71]
	LC	UV (254 nm)	μBondapak C_{18} (10 μm, 300 × 3 9 mm)	Gradient solvent A, ammonium phosphate (25 m*M*, pH 7.2), solvent B, acetonitrile–ammonium phosphate (25 m*M*, pH 7 2) (60·40, v/v)	250 ng/ml	Plasma, urine and CSF	Clinical pharmacokinetics	Fletcher *et al* [72]

(Continued on p 316)

TABLE 2 (*continued*)

Drug	Method[a]	Detection[b]	Chromatographic conditions		Limit of detection[d]	Sample matrix	Comments	References
			Stationary phase[c]	Mobile phase or carrier gas				
	LC	UV (254 nm)	IBM RP-18 (5 μm, 250 × 4 mm)	Heptanesulfonic acid (5 m*M*) in potassium phosphate buffer (20 m*M*, pH 2.6)–acetonitrile (97 5 2 5, v/v)	100 ng/ml	Plasma	Clinical pharmacokinetics	Sommadossi and Bevan [73,75]
	LC	UV (254 nm)	Supelcosil (5 μm, 150 × 4 6 mm)	Sodium acetate (5 m*M*) and sodium pentanesulfonate (5 m*M*) (pH 6.5 with NaOH)	50 ng/ml (serum)	Serum and urine	Clinical pharmacokinetics	Wiltink *et al* [74]
	LC	(a) ED (+1 2 V *vs* Ag/AgCl) or (b) UV (254 nm)	(a) Partisil 1025 ODS-3 (5 μm, 250 × 4 2 mm) or (b) Partisil-1025 SCX ODS-3 (5 μm, 250 × 4 2 mm)	(a) Sodium perchlorate (0 1 *M*) in ammoniumphosphate (0 01 *M*) (pH 3 with phosphoric acid)–methanol (97 3, v/v) or (b) ammoniumphosphate (0 01 *M*)–methanol (72 28, v/v)	150 pg (ED); 750 pg (UV)			Visor *et al* [132]
Idoxuridine (**14**)	LC	UV (254 nm)	(a) μPorasil (10 μm, 300 × 4 mm) or (b) μBondapak C_{18} (10 μm, 300 × 3 9 mm)	(a)Dichloromethane–methanol (95 5, v/v) or (b) Methanol–water (13.87, v/v)	N S	Eye drops	Quality control	Carr [89]

	LC	UV (262 nm)	μBondapak C_{18} (10 μm, 300 × 3.9 mm)	Methanol–water (10.90, v/v)	N.S.	None	Hydrolysis, *in vitro*	Szepesi *et al* [90]
Idoxuridine (**14**) and IDC (**17**)	LC	UV (290 nm)	Aquapore RP300	Acetic acid–water (1·99, v/v)	N.S.	Aqueous humor	Ocular penetration and metabolism	Sincholle *et al* [91]
Ribavirin (**3**)	LC	UV (207 nm)	Guard column, RP-18 Guard-Pak, analytical column, LiChrosorb C_{18} (7 μm, 250 × 5 mm)	Water	100 ng/ml	Serum	Clinical pharmacokinetics	Paroni *et al.* [83]
	LV	UV (210 nm)	LiChrosorb 5 RP-18 (5 μm, 250 × 4.6 mm)	Water	10 ng/ml	Plasma	N.S.	Shlyankevich *et al* [84]
	LC	UV (207 nm)	Microsorb C_{18} (5 μm, 250 × 4 6 mm)	Ammonium phosphate (20 m*M*, pH 5 5 with ammonium hydroxide)–methanol (99 1, v/v)	100 ng/ml	Serum, lung tissue and tracheal aspirates	Phenylboronate clean-up column	Smith and Gilber [85]
	LC	UV (235 nm)	μBondapak C_{18} (10 μm, 2x 300 × 3 9 mm)	Ammoniumphosphate (pH 2 5, 10 m*M*, pH 2 5 with phosphoric acid)	4 n*M*	Serum, urine and CSF	Comparison with RIA method, phenylboronate clean-up column	Granich *et al.* [86]

(Continued on p. 318)

TABLE 2 (*continued*)

Drug	Method[a]	Detection[b]	Chromatographic conditions		Limit of detection[d]	Sample matrix	Comments	References
			Stationary phase[c]	Mobile phase or carrier gas				
	GC	CI-MS	SE 30 on Chromosorb W HP (80–100 mesh, 1 m × 2 mm)	Methane (serum 175–240°C, urine 230°C)	10 ng/ml (serum), 500 ng/ml (urine)	Serum and urine	Silylation; clinincal pharmacokinetics	Roboz and Suzuki [140]
Riboxamide (**4**)	LC–LC	UV (254 nm)	Column 1, ODS Hypersil (5 μm, 50 × 4 6 mm) or Hypersil (5 μm, 50 × 4 6 mm), column 2, μBondapak C_{18} (10 μm, 2x 300 × 3 9 mm), Partisil 10 SAX (10 μm, 250 × 5 mm) or ODS Hypersil (5 μm, 50 × 4 6 mm)	Complex column switching system (see refs 85 and 86)	40 ng/ml	Plasma	Pharmacokinetics in man and the dog	Riley *et al* [87], Meltzer and Sternson [88]
Vidarabine (**1**)	LC	UV (254 nm)	Partisil 10 SAX (10 μm, 250 × 4 6 mm)	Gradient solvent A, ammonium dihydrogenphosphate (pH 2 8, 5 m*M*), solvent B ammonium dihydrogenphosphate (pH 3.7, 750 m*M*)	N S	Cancer cells	Human leukemia cells implanted into mice	Rose and Brockman [60]

LC	UV (254 nm)	Aminex A-28 (150 × 3.7 mm)	Sodium borate (5–20 m*M*, pH 5–9)	2 ng	Plasma and aqueous humor	Human opthalmologic studies	Schneider and Glazko [61]
LC	UV (254 nm)	Ultrasphere ODS (5 μm, 150 × 4 6 mm)	Gradient solvent A, potassium dihydrogen-phosphate (10 m*M*, solvent B, methanol–solvent A (3.7, v/v)	10 pmol	Plasma, urine and CSF	Clinical studies	Agarwal *eta al* [62]
LC	UV (250 nm)	LiChrosorb RP-8 (5 μm, 250 × 4 6 mm)	Acetonitrile–sodium pentanesulfonic acid (pH 7.2, 5 m*M*) (4·96, v/v)	500 ng/ml (Plasma and CSF) 2 5 μg/ml (urine)	Plasma, CSF and urine	Clinical samples	Bowman and Kauffman [63]
LC	UV (254 nm)	Partisil PXS 10/25 C_8 or ODS (10 μm, 250 × 3 mm)	Sodium tetraborate (75 m*M*)–ammonium dihydrogenphosphate (10 m*M*) (8 2, v/v, pH 6 03) plus various amounts of methanol or acetonitrile	N S	None		Delia *et al* [64]
LC	UV (254 nm)	Browlee C_{18}* and Partisil 10 ODS (10 μm, 250 × 4 6 mm)	Potassium dihydrogen-phosphate (10 m*M*, pH 3 25)	8 pmol	None	Drug purity testing	Schroeder *et al* [65]
LC	FL (λ_{ex} 315 nm, λ_{em} 415 nm	Spherisorb 5 ODS (5 μm, 250 × 4 6 mm)	Gradient: solvent A, sodium tetraborate (20 m*M*); solvent B, solvent A–methanol (35 15, v/v)	1 5 ng/ml	Plasma and urine	Clinical pharmacokinetics	McCann *et al* [124]
GC	FID	1% STAP (Carbowax 20 reacted with succinic acid)	Nitrogen (200–220°C)	N S	None	Per-methylation	De Leenheer and Gelijkens [139]

(Continued on p. 320)

TABLE 2 (*continued*)

Drug	Method[a]	Detection[b]	Chromatographic conditions		Limit of detection[d]	Sample matrix	Comments	References
			Stationary phase[c]	Mobile phase or carrier gas				
	GC	NPD or FID	30% SE-30 (260–265°C)	Helium	50 ng/ml	Plasma	Acetylation	Boutagy and Harvey [141,142] and Boutagy [145]
Zidovudine (**11**)	LC	UV (267 nm)	Guard column, Adsorbosphere C_{18} (5 μm, 10 × 4 6 mm), analytical column, Adsorbosphere C_{18} (5 μm, 250 × 4 6 mm)	Acetonitrile–ammonium phosphate (0 1% phosphoric acid, pH 2 7 with ammonium hydroxide), (15 85, v/v)	100 n*M*	Serum	Clinical pharmacokinetics	Good *et al* [6]
	LC	UV (266 nm)	Guard column, Co Pell ODS (30–38 μm, 100 × 4 6 mm), analytical column, Econosil C_{18} (10 μm, 250 × 4 6 mm)	Acetonitrile–potassium dihydrogenphosphate (20 80, v/v) (pH 2.2)	12 5 ng/ml	Plasma and urine	Pharmacokinetics in the rat	Unadkat *et al*

LC	UV (266 nm)	C_{18} (3 μm, 100 × 3.2 mm)	Acetonitrile–phosphate (pH 2.2, 30 m*M*) (9:91, v/v)	11 ng/ml	Serum		Solomon [8]
LC	UV (265 nm)	LiChrosorb RP8 (10 μm, 300 × 3.9 mm)	Acetic acid (1%, pH 5)–methanol (82:8, v/v)	20 ng/ml (plasma) and 200 ng/ml (urine)	Plasma and urine	Clinical pharmacokinetics	Underberg *et al.* [9]
LC–LC	UV (267 nm)	Column 1, Pinkerton ISRP (5 μm, 150 × 4.6 mm); column 2; two Cyclobond I (5 μm, 100 × 4.6 mm and 5 μm, 250 × 4.6 mm)	Complex solvent programming (see ref. 10)	312 ng/ml	Plasma	Pharmacokinetics in the cat	Mathes *et al.* [10]
LC	UV (266 nm)	Supelcosil LC-18 (5 μm, 150 × 4.6 mm)	Acetontrile–ammonium dihydrogenphosphate (10 m*M*) (9:91)	15 ng/ml	Plasma and urine	Two internal standards to account for range of concentrations encountered	Hedaya and Sawchuk [11]
LC	UV	(a) Partisil ODS-2 (10 μm, 250 × 4.6 mm) or (b) Partisil M9 (10 μm, 250 × 9 mm)	(a) Gradient: solvent A, phosphate buffer (pH 3, 10 m*M*)–methanol (95:5, v/v); solvent B, phosphate buffer (pH 3, 10 m*M*)–methanol (55:45, v/v) or (b) gradient: solvent A, methanol–chloroform (1:99, v/v); solvent B: methanol–chloroform (9:91, v/v)	N.S.	None	Degradation, *in vitro*	Brubaker and Prusoff [12]

(Continued on p. 322)

TABLE 2 (*continued*)

Drug	Method[a]	Detection[b]	Chromatographic conditions		Limit of detection[d]	Sample matrix	Comments	References
			Stationary phase[c]	Mobile phase or carrier gas				
	LC	UV (267 nm)	Partisil-5 ODS-3 (5 μm, 200 × 4 mm)	Acetonitrile–phosphate buffer (120 m*M*, pH 6.2) (13.87, v/v)	10 ng/ml	Plasma	Clinical pharmacokinetics, semi-automated method	Kuperschmidt and Schmid [14]
	LC	UV (266 nm)	Guard column Co Pell (30–38 μm); analytical column, Econosil C_{18} (10 μm, 100 × 4.6 mm)	Acetonitrile–potassium dihydrogenphosphate (25 m*M*, pH 2.2) (20:80, v/v)	12.5 ng/ml	Plasma	Pharmacokinetics in the rat	Unadkat *et al* [15]
	LC	UV (267 nm)	ODS Hypersil (5 μm, 150 × 4.6 mm)	Methanol–potassium dihydrogen phosphate (pH 3.1) (10:90, v/v)	10 ng/ml	Plasma	Clinical pharmacokinetics	Klutman *et al* [16]

[a] LC = Liquid chromatography, LC LC = column-switching, LC(LC) = coupled-column method, TLC = thin-layer chromatography, GC = gas chromatography, PC = paper chromatography

[b] UV = ultraviolet absorption with detection wavelength in parentheses, FL = fluorescence with excitation (λ_{ex}) and emission (λ_{em}) wavelengths given in parentheses, ED = electrochemical with operating potential given in parentheses, RA = radioactivity, MS = mass spectrometry, CI = chemical ionization, EI = electron impact, TS = thermospray, FID = flame ionization, NPD = nitrogen-phosphorus, ECD = electron capture

[c] Brand name of stationary phase material with particle diameter (in μm), length (in mm) × internal diameter (in mm) given in parentheses

[d] Where possible, this has been expressed as the detection limit in the relevant biological sample.

TABLE 3

ABSORBANCE MAXIMA (λ_{max}) AND MOLAR ABSORPTIVITIES (ε) OF SELECTED NUCLEOSIDES

Source M Windholz, S Budavari, R F Blumetti and E S Otterbein (Editors), *The Merck Index*, Merck, Rahway, NJ, 1983.

Nucleoside	Solvent	λ_{max} (nm)	ε (M^{-1} cm^{-1})
Adenosine	Water	260	15,100
Cytidine	Water (pH 8 2)	271	9,100
	Water (pH 2 2)	280	13,400
Cytarabine (**2**)	Water (pH 2 2)	212 5	10,230
		281	13,170
	Water (pH 12 0)	272 5	9,259
Idoxuridine (**14**)	Water	288	7,410
Guanosine	Water (pH 5 5)	188	26,800
		252 5	13,000
Thymidine	Water (pH 7 2)	206 5	9,800
		267	9,700
Uridine	Water (pH 7 3)	205	9,800
		261	10,100
Vidarabine (**1**)	Water (pH 1.0)	257 5	12,700
	Water (pH 7.0)	259	13,400
	Water (pH 13 0)	259	14,000

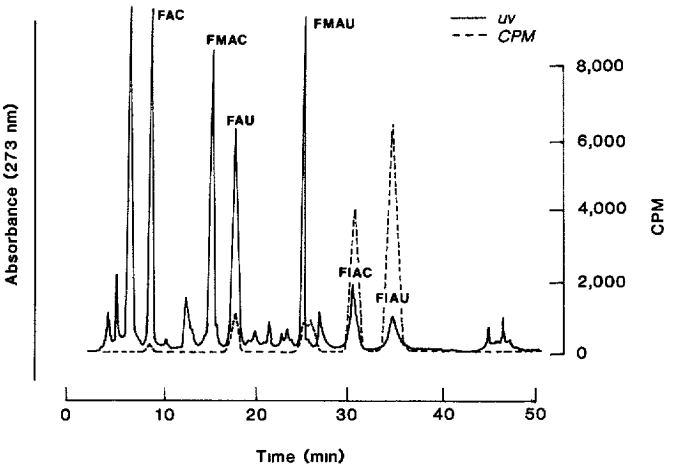


Fig 3 LC of urine collected during the first 6 h after intravenous administration of 100 mg/m^2 FIAC (**16**) spiked with 190 μCi of [2-^{14}C]FIAC LC conditions see Table 2. (Reproduced from ref 93 with permission)

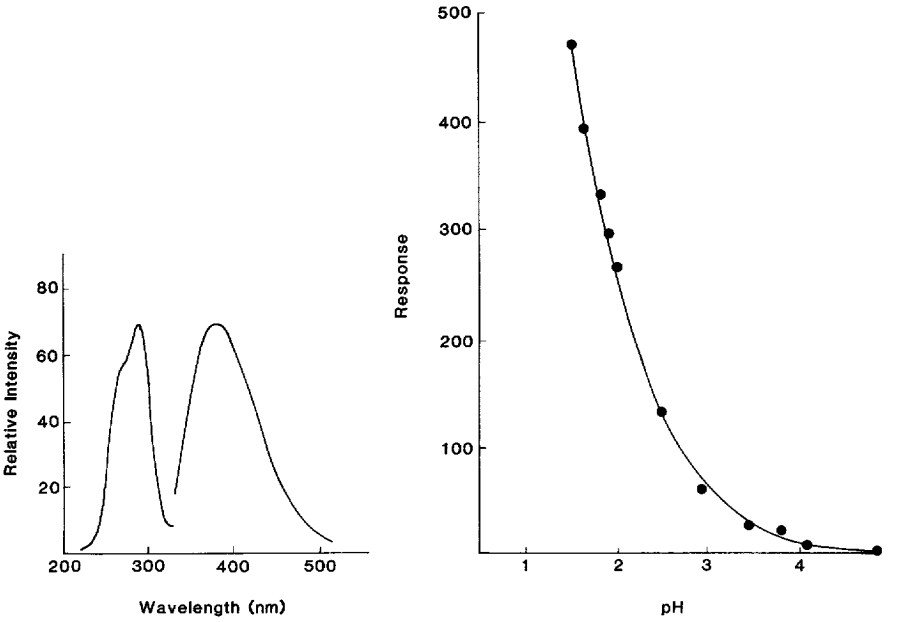


Fig 4 Fluorescence excitation and emission spectra of acyclovir (**5**) at pH 2 0 and the effects of mobile phase pH on the relative fluorescence intensity (Reproduced from ref 117 with permission)

pH 2. Šalamoun *et al.* [117] also showed that the fluorescence intensity was independent of the presence of organic solvents, oxygen and sodium sulfate (up to 0.1 *M*) The fluorescence intensity of acyclovir (**5**) was found to increase with decreasing temperature [117] and maximum sensitivity was obtained by maintaining the detector flow cell at 2°C. Using this approach [117], detection limits of 1 ng/ml were achieved for acyclovir (**5**) after separation from plasma on a hydroxyethyl methacrylate column (Spheron Micro 300) with a mobile phase of phosphoric acid (0.1 *M*) in sodium sulfate (0.1 *M*) (pH 1.8). The method [114] was applied to a single-dose (5 mg/kg, intravenously) pharmacokinetic study in the dog It should be noted that the concentration of acyclovir (**5**) found 12 h after administration of the dose was about 300 ng/ml, which is much greater than the detection limit for the method and also could have been achieved by other detection methods (Table 2). Nevertheless, LC–ED for acyclovir (**5**) [117] could prove very useful if high sensitivity is required or when the available sample size is small.

While derivatization is essential for the analysis of the nucleosidic antiviral drugs by gas chromatography (GC), pre- or post-column derivatizations have rarely been used for their analysis by LC Chemically, the nucleosides and their bases are relatively unreactive compounds and they are not generally suitable for pre- or post-column chemical modification by the reagents commonly used in LC.

In addition to their lack of reactivity, the compounds in question tend to contain more than one functional group available for derivatization and steps must be taken to prevent incomplete reaction as well as the production of multiple-reaction products.

Notwithstanding these undesirable chemical properties, reactions have been described for the pre-column fluorogenic derivatization of adenine and cytosine analogues [118–126]. Adenine, cytosine and their derivatives react with chloroacetaldehyde to give the corresponding 1,N^6-etheno derivatives (Fig. 5) which are highly fluorescent [118–126]. The reaction (Fig. 5) has been applied to the pre-column derivatization of adenine derivatives [117,121] for LC applications and Leonard *et al.* [122] have described the use of chloroacetaldehyde as a spray reagent in thin-layer chromatography (TLC). Although pre-column derivatization with chloroacetaldehyde (Fig. 5) has not been widely used in the determination of antiviral drugs by LC, McCann and co-workers [121,124] have adapted this reaction (Fig 5) to the sensitive and selective determination of vidarabine (**1**) and its metabolite ara-AMP in plasma and urine. Being chemically unstable,

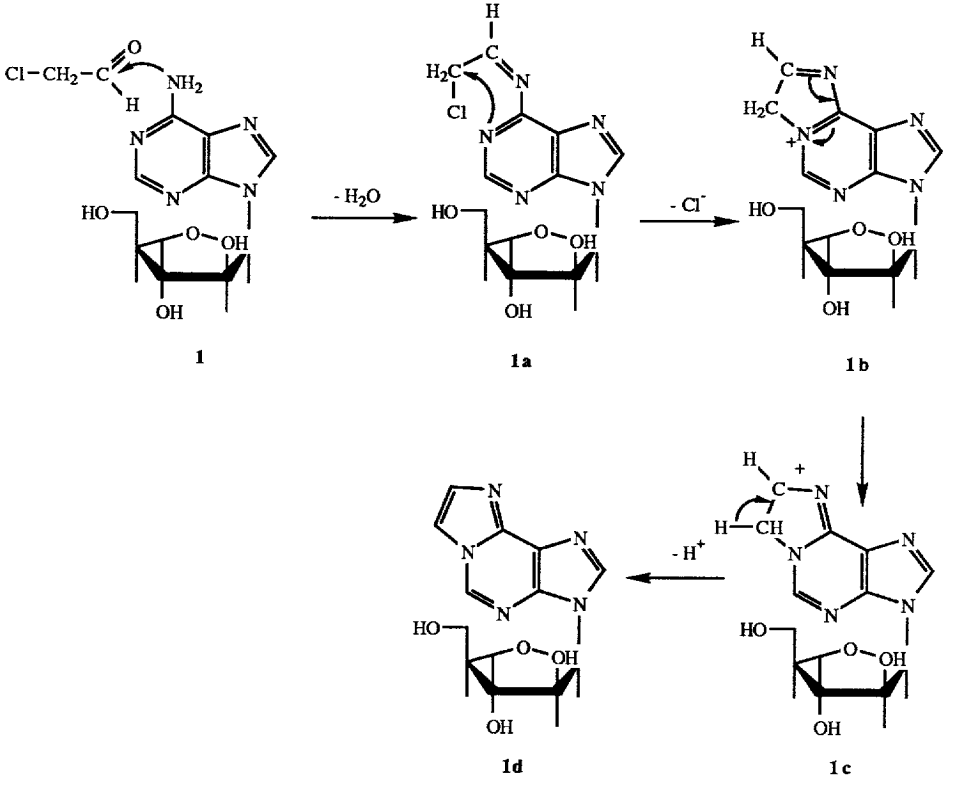


Fig. 5. Fluorogenic derivatization of vidarabine (ara-A) (**1**) with chloroacetaldehyde [118–126]

chloroacetaldehyde has to be prepared *in situ* from its dimethyl acetal [124] and then titrated with bisulfite to determine its concentration. Additionally, both chloroacetaldehyde and its dimethyl acetal are potent lacrimators Consequently, chloroacetaldehyde is not a convenient reagent, which probably explains why it has not been widely used for the routine bioanalysis of drugs. Another similar reaction, potentially useful in the pre-column derivatization of nucleosides, is that based on the fluorometric determination of adenine and its derivatives by reaction with glyoxal hydrate trimer [127].

Ray [128] has described the determination of acyclovir (**5**) in human plasma and urine by a quantitative TLC procedure that used fluorescamine as a derivatization reagent. It was claimed that the TLC method gave results equivalent to those obtained by LC. Unfortunately, the author did not propose a structure for the fluorescent derivative and gave no indication of its stability.

2.1.1.3. Electrochemical detection. Whereas adenine derivatives are easily reduced at mercury electrodes [129], the other purines and pyrimidines can be oxidized at carbon electrodes [130]. Unfortunately, the electrode potentials required for oxidation are high (>1.0 V *vs.* Ag/AgCl) and, for that reason, ED has not been widely used for the LC analysis of nucleosidic antiviral drugs. Chatten and Amankwa [131] have described the electrochemical properties of idoxuridine (**14**) and bromovinyldeoxyuridine (BVDU) (**15**) and showed that both compounds exhibited well defined dc and differential-pulse polarographic waves with half-wave potentials of -0.97 and -1.8 V (*vs.* saturated calomel electrode) for idoxuridine (**14**) and BVDU (**15**), respectively. From these observations, they were able to develop a differential-pulse method for the determination of idoxuridine (**14**) in pharmaceutical formulations [131]. The electro-reduction of these two antiviral drugs was attributed to the fission of carbon–halogen bonds, indicating that ED in the reduction mode may be a viable option for the LC analysis of halogenated nucleosidic antivirals.

Visor *et al.* [132] have described the amperometric detection of acyclovir (**5**) and ganciclovir (**6**) in LC eluents (Fig. 6). They have reported a detection limit of 200 pg for a 50-μl injection and showed that the method was useful for monitoring the chemical degradation of ganciclovir (**6**) *in vitro*. The detection limit for ganciclovir (**6**) by LC–ED was claimed to be about five times lower than that achievable by UV detection; however, the high oxidation potentials required for detection of ganciclovir (**6**) ($+1.2$ V *vs.* Ag/AgCl) limit the usefulness of LC–EC for determinations *in vivo*.

2.1.1.4. Mass spectroscopic detection. The nucleosides and related compounds as a whole are relatively non-volatile and the analysis of these compounds by GC–MS is generally preferred to analysis by LC–MS as an aid to structural identification. Nevertheless, Blau *et al.* [133] have demonstrated the value of thermospray LC–MS in the positive-ion mode as an aid to the confirmation of 2′,3′-dideoxyinosine (DDI) (**12**) as a major metabolite of the antiviral drug 2′,3′-dideoxyadenosine (DDA) (**9**) in the mouse.

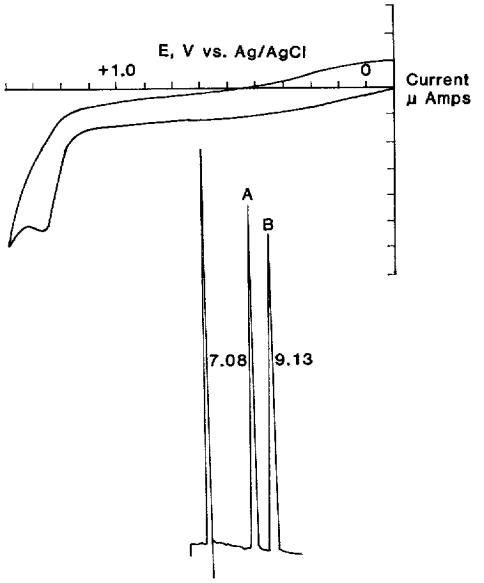


Fig 6 Cyclic voltammogram and LC–ED of acyclovir (**5**) ED conditions sample, 10^{-3} *M* acyclovir (**5**) in 0.1 *M* sodium perchlorate (pH 3), glassy carbon working electrode, Ag/AgCl reference electrode, Pt auxiliary electrode, scan rate, 100 mV s^{-1} Key (A) acyclovir (**5**), (B) tyrosine (internal standard). LC–ED conditions see Table 2 (Reproduced from ref 132 with permission)

2.1.2. Separation systems

The nucleosides are characteristically very polar organic compounds that are relatively insoluble in both aqueous and non-aqueous solvents. This has important implications in the bioanalysis of nucleoside-related antiviral drugs because it can mean that they are difficult to isolate from biological fluids by liquid–liquid extraction. Additionally, the high polarity of these antiviral drugs largely precludes the use of normal-phase systems [89,128,134] for their determination in biological fluids and most methods have used either reversed-phase or, to a lesser extent, ion-exchange systems (Table 2).

2.1.2.1. Reversed-phase systems The majority of reversed-phase methods for the analysis of the nucleoside-related antiviral drugs require hydrocarbonaceous (typically ODS) supports of moderately high carbon load to achieve significant retention of these highly polar compounds (Table 2). Relatively acidic mobile phases are preferred (pH 2–4, with phosphoric or acetic acid-based buffers) and retention has generally been manipulated by adjustment of the pH of the buffer or the concentration of organic modifier (typically methanol). The retention of these nucleosides can be related to the hydrophobicity, which in turn determines the concentration of methanol (or acetonitrile) required to elute the compound of interest from a hydrocarbonaceous support Fig. 7 shows the approximate concentrations of methanol that have been used to elute various nucleosidic antiviral drugs from ODS phases with reasonable analysis times.

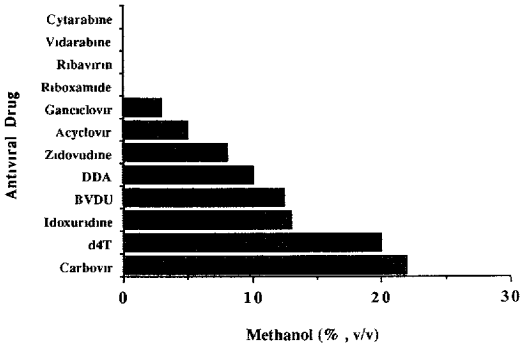


Fig. 7 Approximate concentrations of methanol used to elute various nucleosidic antiviral drugs from ODS stationary phases. The figure was generated from the data in Table 2 This graph is intended to be a guide to methods development and to provide a measure of solute hydrophobicity.

The LC of the very polar antiviral agents vidarabine (**1**), cytarabine (**2**), ribavirin (**3**) and riboxamide (**4**) is particularly troublesome because in general they can only be retained on reversed-phase supports with purely aqueous mobile phases. This limits the options available in separation optimization and can also result in long analysis times arising from late-eluting endogenous substances. Fortunately, the chemical modifications made to alter the pharmacological properties of the antiviral drugs have resulted in compounds with increased hydrophobicity. The more hydrophobic nucleoside analogues are better retained by reversed-phase supports and therefore easier to analyze by LC. Fig. 7 shows that the removal of the 2′- and 3′-hydroxyl groups from the sugar moiety results in substantial increase in the affinity of DDA (**9**) for ODS phases compared with vidarabine (**1**). The introduction of a $C^{2'}$–$C^{3'}$ double bond in combination with the removal of the 2′- and 3′-hydroxyl groups further enhances hydrophobicity such that the antiviral drugs 2′,3′-didehydro-2′,3′-deoxythymidine (d4T) (**13**) and carbovir (**8**) require greater than 20% methanol in the mobile phase for elution from a reversed-phase column. The other substitution which appears to increase hydrophobicity substantially and, hence, retention on reversed-phase supports is the introduction of halogenated functional groups in the 5-position such as iodo (in idoxuridine, **14**) or 2-bromovinyl (in BVDU, **15**).

The most widely used support for the reversed-phase LC of these compounds as a whole has been μBondapak C_{18}, 300 mm x 3.9 mm) (Table 2) [46,56,57,67,72,78,80,86,87,89,90,94]. However, ODS-based 5-μm materials of similar carbon load (10–15%) such as Ultrasphere ODS [54] permit similar separations to be achieved with shorter columns (*e.g.* 150 mm) and shorter analysis times. Whereas various ODS phases have been employed for the bioanalysis of these agents, the mobile phases reported by different groups for the same compound have been remarkably similar. This is probably because the options available for the manipulation of retention of these compounds, being very polar, are

limited. For example, all the nucleosidic drugs with the exception of d4T (**13**) and carbovir (**8**) have very poor retention on hydrocarbonaceous phases if the concentration of methanol (or acetonitrile) is greater than about 12%.

Nucleosides and their synthetic derivatives contain both acidic and basic functional groups. In principle, the manipulation of retention and selectivity of these compounds on reversed-phase materials is possible, and knowledge of the pK_a values may be useful in separation optimization (Table 4). However, because of the pH constraints of most silica-based stationary phases, the only pK_a that is relevant to consider when optimizing the separation of nucleosides is that associated with the protonation of a nitrogen in adenine, guanine and cytosine. The pK_a of this group ranges from 1.6 in guanosine to 4.6 in cytosine (Table 4) Retention of the nucleosides is generally greatest when the solutes are neutral, and protonation of an amino group generally leads to reduced retention. However, the use of an acidic mobile phase in which the solutes are ionized does introduce the possibility of ion-pair chromatography. For example, the advantages of adding alkylsulfonates to the mobile phase have been demonstrated in the reversed-phase LC of the acyclic guanosine derivatives acyclovir (**5**) [66–68] and ganciclovir (**6**) [73,74].

The nucleosidic antiviral drugs can be expected to be neutral or cationic within the pH range (2.5–7.5) in which silica-based bonded phases are stable and the observation of poor peak shape because of interactions of polar functional groups with residual silanols is rare. Therefore, there is little theoretical basis for expecting the retention of these compounds themselves to be influenced by the addition of hydrophobic cations such as tetrabutylammonium. Nevertheless,

TABLE 4

pK_a VALUES OF VARIOUS PURINES, PYRIMIDINES, NUCLEOTIDES AND NUCLEOSIDES

Solute	pK_a[a]		
	NH_3^+	–NH	$-PO_3H$
Uracil	–	9 45	–
Thymine	–	9.94	–
Cytosine	4 60	12 2	–
Adenine	4 15	9.80	–
Guanine	3 30	9.20, 12.3	–
Adenosine	3.30	12 5	–
Guanosine	1 6	9.2, 12 3	
AMP	3 74		6 05
ADP	3 95		6 26
ATP	4 00		6 48

[a] Taken from Edsall and Wyman [135] A more complete discussion of the ionic equilibria of purines, pyrimidines, nucleotides and nucleosides is to be found in the review of Izatt *et al* [136]

there have been several reports of the value of adding hydrophobic cations to the mobile phases of reversed-phase systems developed for the bioanalysis of nucleoside-related antivirals [55,79,95,96,99]. These types of cations may be used to enhance the retention of oppositely charged metabolites (phosphates) and to manipulate the retention of potentially interfering ionic substances present in biological fluids. The nucleoside analogue riboxamide (**4**) is a very polar compound and is poorly retained on most reversed-phase supports. Riley *et al.* [87] have shown that even though riboxamide (**4**) is unionized in mildly acidic mobile phases, its retention may be enhanced on silica and several ODS columns by the addition of hexadecyltrimethylammonium bromide (HTAB) to an aqueous mobile phase. The mechanism by which HTAB enhances the retention of riboxamide on silica gel or reversed-phase columns is unclear but it probably arises from interactions with HTAB adsorbed onto the stationary phase rather than as a result of interactions in the mobile phase.

The pK_a values (Table 4) associated with dissociation of either an enolic group or a hydroxylic group of the sugars are between 9 and 12, and dissociation of these groups is unlikely to affect the retention of nucleosides on silica-based phases. However, dissociation of these acidic groups may be important in the chromatography of these solutes on alkaline-stable phases such as polystyrene–divinylbenzene co-polymer columns (*e.g.*, PRP-1). Smith and Walker [68] and Reeuwijk *et al.* [96] have described the use of PRP-1 columns for the analysis of acyclovir (**5**) and BVDU (**15**), respectively, in plasma, and both groups obtained adequate resolution and good chromatography in mildly acidic mobile phases. Furthermore, Smith and Walker [68] claimed better stability of PRP-1 columns compared with Zorbax ODS in acidic mobile phases (pH 2.3) for the bioanalysis of acyclovir (**5**) and Reeuwijk *et al.* [96] have claimed better peak shape on the same polymer-based column for the chromatography of BVDU (**15**).

2.1.2.2. Ion-exchange systems and borate complexation. Both anion- and cation-exchange chromatography have found applications in the analysis of nucleosidic antiviral drugs in biological fluids and other media. Whereas cation exchange is potentially useful for the chromatography of fully protonated nucleosides at low pH and has been used for the analysis of acyclovir (**5**) [130] and cytarabine (**2**) [54] (Fig 8), anion-exchange chromatography has been found to be particularly useful for the analysis of negatively phophorylated metabolites. In particular, several methods based on anion-exchange chromatography have been described for the separation of cytarabine (**2**) and vidarabine (**1**) and their numerous metabolites. Indeed, one of the first column LC applications described in the literature [39] was the application of ion-exchange chromatography to the metabolism of cytarabine (**2**) in leukemia cells. It is interesting to compare the broad peaks obtained in very early attempts at the LC of nucleosides and related compounds on Dowex 1-X2 (approximately 150–200 μm, Fig. 8d) [39] with what can be achieved on 5- or 10-μm silica-based ion exchangers [54,60] or reversed-phase [55] columns (Fig. 8 a, b and c).

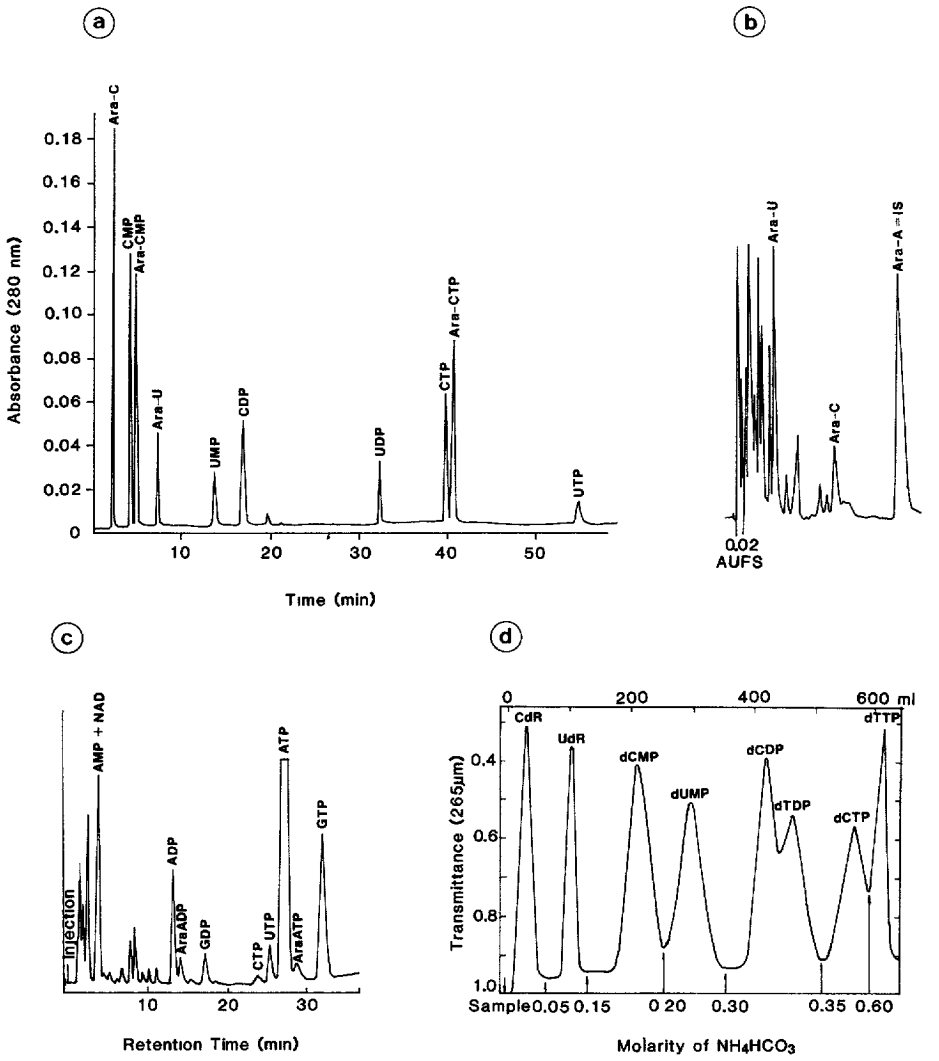
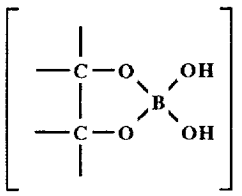


Fig. 8 Separation of nucleosides and nucleotides by (a) reversed-phase ion-pair chromatography, (b) cation-exchange chromatography and (c and d) anion-exchange chromatography. Conditions: see Table 2 (Reproduced from refs 55, 60, 54 and 39 with permission)

Borate ion reacts with *cis*-1,2-glycol structures and, to a much lesser extent, with the corresponding *trans* structures, to form a rigid five-membered ring system (**24**) (Fig. 9). Therefore, the borate ion reacts strongly with ribonucleosides and much less strongly with similar nucleosides in which one or both of the 2'- and 3'-hydroxyl groups have been removed or they are in the less reactive *trans* configuration. The stereospecific nature of this reaction has been used widely in nucleic acid chemistry and forms the basis of the Böeseken test [137]. The com-



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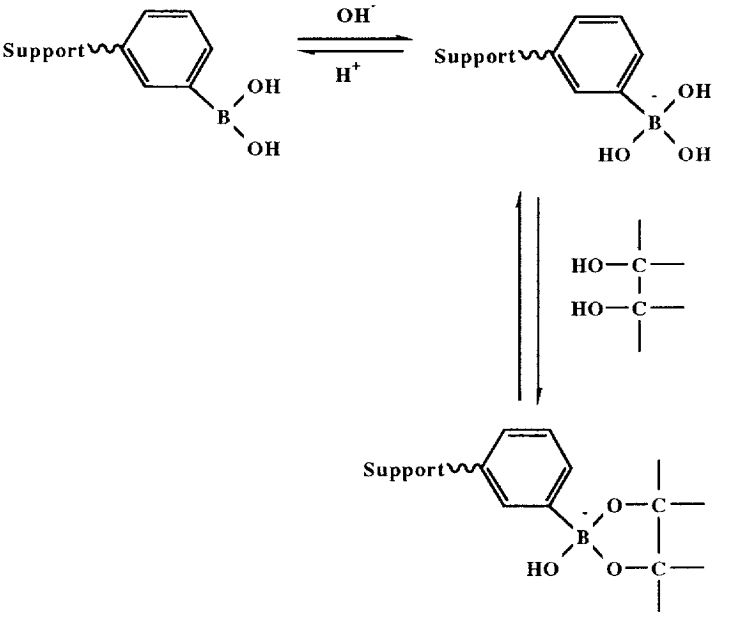


Fig 9 Product of the reaction of borate ion with a 1,2-*cis*-diol structure (**24**) and a scheme for the reaction of 1,2-*cis*-diols with phenylboronate affinity gels

plexation by borate ion of nucleosides in the mobile phase imparts a negative charge to an otherwise neutral molecule allowing the compound to be retained on an anion exchanger. Alternatively, if a cation-exchange column is employed then the negatively charged complexes are excluded from the negatively charged stationary phase. Complexation with borate can be particularly helpful in improving the separation of arabinonucleosides [*e g.* cytarabine (**2**) and vidarabine (**1**)] from the corresponding isomeric ribonucleosides. Fig. 8, which shows the partial resolution of ara-ADP from ADP and ara-ATP from ATP, exemplifies the problem of separating ribonucleosides from the corresponding arabinonucleosides. Pal [43] has demonstrated the value of borate complexation in the resolution of arabi-

nonucleosides, ribonucleosides and 2′-deoxyribonucleosides on anion exchangers. With borate in the mobile phase the elution order is ribonucleosides, arabinonucleosides and 2′-deoxyribonucleosides, reflecting the extent of complexation with borate and the resultant amount of negative charge carried by the complexes.

The stereospecific complexation of nucleosides with borate has also been applied in sample clean-up methods with boronate affinity gels [46,85,87]. Pallavicini and Mazrimas [46] have used this technique (Fig. 9) for the selective isolation of arabinose-containing compounds in biological fluids, making use of both the difference in affinities of arabinose- and ribose-containing compounds and the dependency of the reaction on pH. After loading the biological sample, the arabinose-containing compounds were eluted in ammonium acetate (pH 8.8) and the ribose-containing compounds were eluted in formic acid.

2.1.2.3. Column-switching systems. The majority of compounds in this class of drugs are highly polar which leads to poor retention on reversed-phase supports and the need for weak chromatographic eluents (*i.e.* low concentrations of organic modifiers). If the compound of interest is also neutral then ion-pair or ion-exchange techniques for the enhancement of retention are not viable options. This problem is illustrated in the analysis of riboxamide (**4**), which is an analogue of ribavirin (**3**) Riley *et al.* [87] found that the retention of riboxamide (**4**) on most reversed-phase supports was poor but could be increased by coating ODS columns with HTAB Nevertheless, purely aqueous mobile phases were still required for retention and this led to excessively long retention times for a number of endogenous compounds. The problem was solved [87] by a column-switching technique in which two ODS columns, both coated with HTAB, were linked together by an automatic switching valve. Separation from biological fluids was then accomplished (Figs. 10 and 11) by transferring the fraction of mobile phase containing the riboxamide (**4**) to column 2 as it eluted from column 1. This method was initially applied to the analysis of riboxamide (**4**) in plasma. Subsequently, Meltzer and Sternson [88] showed that by modification of the extraction procedure, the method was also applicable to the determination of the drug in urine, which is a more substantial analytical challenge than plasma because of the very high concentration of polar interferences in urine.

Liversidge *et al.* [53] have described an interesting column-switching procedure for the simultaneous analysis of cytarabine (**2**), its metabolite ara-U and salicylate ion [a formulatory adjuvant used to enhance rectal absorption of cytarabine (**2**)]. In this method, a short (3 cm) ODS pre-column was connected to two longer (10 cm) ODS columns which were arranged in parallel. The mobile phase conditions were such that the salicylate was switched from the pre-column to the first analytical column in one fraction and the cytarabine and the ara-U were switched to the second analytical column in a second fraction.

Concerns for the safety of laboratory workers handling biological fluids contaminated with the HIV virus have prompted Mathes *et al.* [10] and Kupfer-

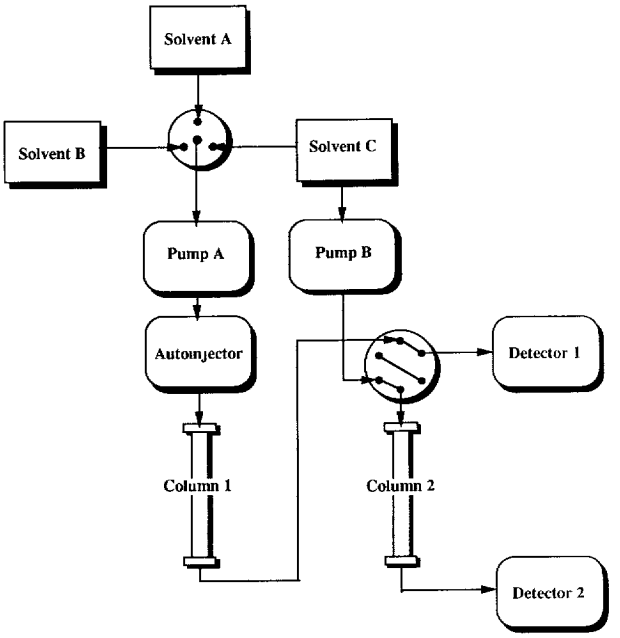


Fig 10 Column-switching arrangement that has been described for the bioanalysis of riboxamide (**4**) and zidovudine (**11**). Conditions see Table 2

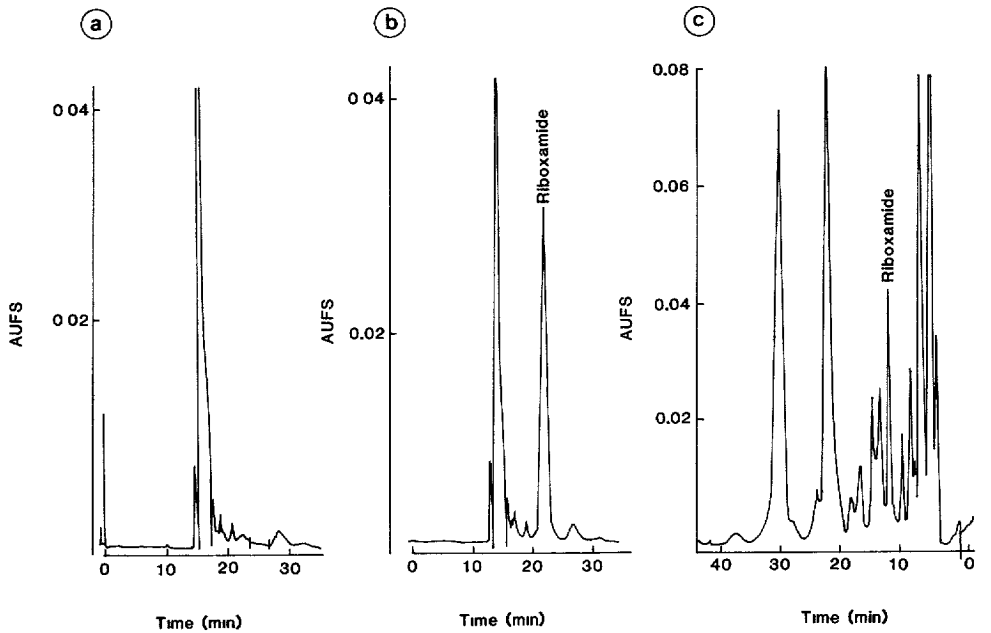


Fig 11 Column-switching separation of riboxamide (**4**) from urine Conditions: see Table 2 and refs. 87 and 88 Key (a) blank urine analyzed by column switching; (b) urine containing 500 ng/ml riboxamide (**4**) analyzed by column switching, (c) urine analyzed by a single-column method (Reproduced from ref. 88 with permission.)

schmidt and Schmid [14] to develop a column-switching procedure for the analysis of the anti-AIDS drug zidovudine (**11**) in biological fluids. Mathes *et al.* [10] chose to employ a column-switching arrangement similar to that shown in Fig. 10 in which the plasma was injected directly into a Pinkerton internal-surface reversed-phase column where zidovudine and the internal standard 2′,3′-dideoxycytidine (DDC) (**10**) were separated from the plasma proteins. The zidovudine (**11**) and DDC (**10**) were then separated from each other on two cyclodextrin-bonded phases (100 mm x 4.6 mm and 250 mm x 4.6 mm) linked in series. A different approach to the automated analysis of zidovudine (**11**) has been described by Kupferschmidt and Schmid [14]. To minimize sample handling in this method the plasma was first loaded directly onto an ODS solid-phase extraction cartridge. The zidovudine (**11**) was then eluted from the ODS cartridge onto on AASP cartridge for subsequent on-line injection onto an analytical LC column (PartiSphere 5 C_8 or C_{18} or Partisil 5 ODS-3).

2.2. Gas chromatography

LC with UV detection is the method of choice for the determination of the nucleosidic antiviral drugs because they are non-volatile compounds often containing several polar functional groups, a situation not well suited to analysis by GC. Nevertheless, GC [138–145] can offer advantages over LC for the determination of nucleosidic antiviral drugs if higher sensitivity is required. Furthermore, GC–MS [138–145] is particularly useful if higher selectivity is required for the structural determination of metabolites.

Becaus of their polarity, derivatization of nucleosides is essential prior to their analysis by GC, and several standard procedures have been adapted to the GC analysis of nucleosidic antiviral drugs. Derivatization procedures that have been used [138–145] for the GC of nucleosides include silylation, alkylation, acylation and N,O-permethylation. Trimethylsilylation of the hydroxyl groups successfully reduces the polarity of the sugar moiety, but trimethylsilyl (TMS) derivatives of nucleosides containing a primary amino group are difficult to form [142] and derivatives of cytidine and adenosine have to be reacted in two steps Acylation is a viable alternative to trimethylsilylation of the hydroxyl groups but this approach also results in poor chromatography of nucleosides containing a primary amino group.

Boutagy and Harvey [141,142] have described a GC method for the determination of cytarabine (**2**) and its deaminated metabolite Ara-U. Ara-U could be determined in biological fluids following acetylation with acetic anhydride; however, methylation of the amino group with diazomethane was also required for the simultaneous determination of cytarabine (**2**) (Fig. 12). Detection limits of 40–70 ng/ml for cytarabine (**2**) in plasma are possible with nitrogen-specific detection and detection limits of 1 ng/ml are possible by GC–MS. A similar method involving acetylation and methylation has been described subsequently by Maki-

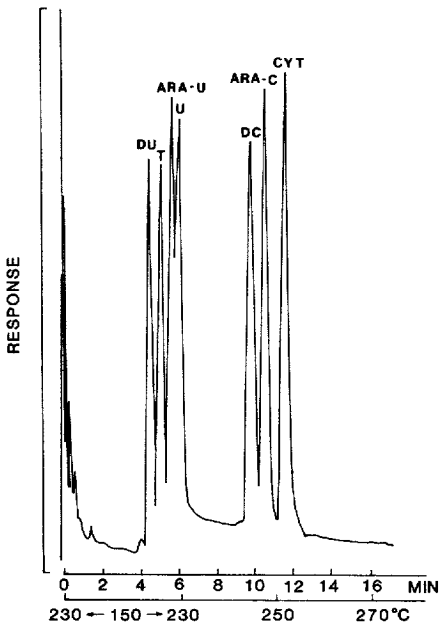


Fig 12 GC separation of acetyl methyl derivatives cytarabine (Ara-C) (**2**), Ara-U (**29**) and several related nucleosides Conditions see Table 2 (Reproduced from ref 142 with permission)

no *et al.* [143] for the determination of the metabolites of N^4-behenoyl-1-β-D-arabinofuranosylcytosine, namely, cytarabine (**2**) and Ara-U. Roboz and Suzuki [140] have described the determination of ribavirin (**3**) in plasma and urine by GC–MS. In this case derivatization of both the hydroxyl and the amino group of the carboxamide moiety could be achieved in a single step by reaction with a mixture of N,O-(bis)trimethylsilyltrifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS).

2.3. Specific methods for the bioanalysis of nucleoside antiviral drugs

The conditions for the bioanalysis of the nucleoside antiviral drugs are summarized in Table 2. The important issues that have been raised in the chromatographic determination of these compounds are discussed in details in this section. These issues include sample pretreatment, special considerations in the separation from endogenous compounds, metabolites and other drugs, applicability of the methodology to metabolism studies, pharmacology and pharmacokinetics, and comparison with other non-chromatographic methods.

2.3.1. Arabinosides

2.3.1.1. Sample pretreatment. The arabinosides are highly polar compounds that are not easily extracted from biological fluids and tissues into organic sol-

vent. Consequently, urine, cerebrospinal fluid (CSF) and other protein-free samples can be analyzed by direct injection [49,54]. Whereas direct injection of plasma has also been advocated [54], most methods for the analysis of plasma or serum involve precipitation of protein with trichloroacetic acid, perchloric acid and acetonitrile [42,44,50,53,54], followed by centrifugation and injection of the supernatant. Precipitation of protein with acetonitrile has been claimed to be superior to precipitation with acid but broad peaks result unless the solvent is evaporated to dryness and the residues are reconstituted with mobile phase [59]. This is also the general method of choice for the pre-treatment of cells [39,40,46,47,55,60]. Ultrafiltration has been advocated as an alternative to precipitation for the deproteination of plasma [61,62]

Deamination of vidarabine (**1**) and cytarabine (**2**), which may continue in biological fluids after collection of the samples, should be prevented [53,54] by the addition of enzyme inhibitors (tetrahydrouridine or pentostatin). The use of phenylboronate affinity columns for the selective isolation of arabinonucleosides in the presence of the ribonucleosides was discussed in Section 2.1.2.2. An alternative approach for the removal of ribonucleosides that might otherwise interfere with the analysis of arabinonucleosides involves the selective oxidation with periodate [52,146].

2.3.1.2. Chromatographic methodology Although vidarabine (**1**) and cytarabine (**2**) possess both antiviral and antineoplastic acitivities, the former has been developed predominantly as an antiviral agent and the latter mainly as an antitumor agent. However, it was considered helpful to discuss the analysis of both compounds because of their similar structure and properties. Being isomers of adenosine and cytidine, the antimetabolites vidarabine (ara-A) (**1**) and cytarabine (ara-C) (**2**) are extensively metabolized by the same pathways as the naturally occurring nucleosides. Both cytarabine (**2**) and vidarabine (**1**) are phosphorylated in mammalian cells as well as being deaminated to 9-β-D-arabinofuranosyluracil (ara-U) (**29**) and 9-β-D-arabinofuranosylhypoxanthine (ara-Hx) (**25**), respectively (Fig. 13) [1].

Because of the high polarity of vidarabine (**1**), cytarabine (**2**) and their metabolites, the methods of choice for their analysis in biological samples are LC in the reversed-phase, reversed-phase ion-pair or anion-exchange modes (Table 2). The high polarity of these compounds generally limits the usefulness of GC for routine quantitative analysis in biological fluids; however, GC–MS is invaluable for the identification of metabolites [138,139,141–143].

The extensive metabolism of these compounds (Fig. 13) presents a significant challenge to the bioanalytical chemist concerned with development of methodology for the determination of these drugs and their metabolites because the compounds of interest contain different charges, which may lead in turn to a general elution problem. Although they are highly polar compounds, the nucleosides can be retained on reversed-phase supports in their unionized forms and they are generally well separated from each other. The structure–retention relationship for

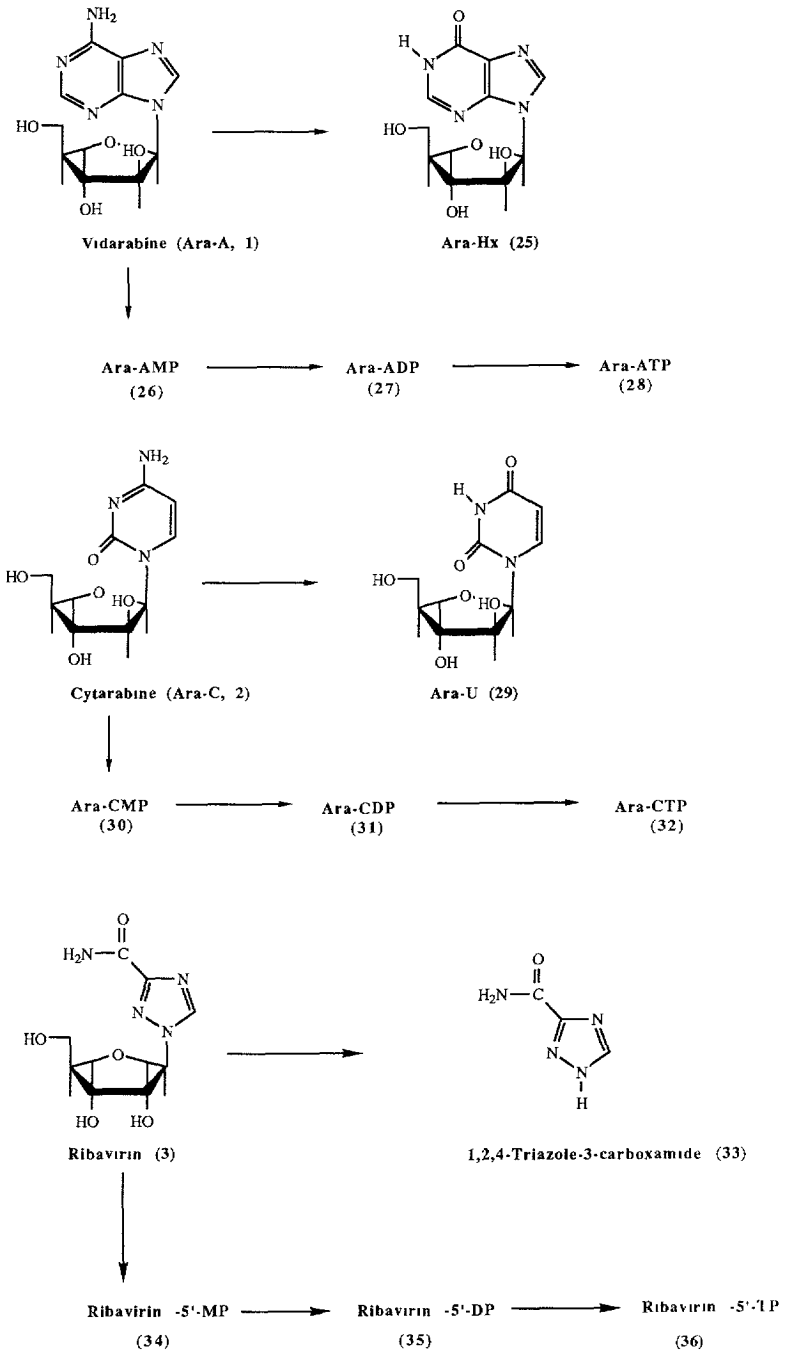


Fig. 13 Metabolism of vidarabine (ara-A) (**1**), cytarabine (ara-C) (**2**), and ribavirin (**3**)

nucleosides on hydrocarbonaceous supports has been described by Pompon *et al.* [147] who found that the order of elution was C < U < G < T < A and that the arabinonucleosides were slightly more retained than the corresponding ribonucleosides. Accordingly, numerous reversed-phase LC methods have been described for the determination of cytarabine (**2**) and its metabolite ara-U (**29**) [40,42,44,46,48–50,53,54,55,58,59] and vidarabine (**1**) and its metabolite [60–63,118] ara-Hx (**25**) (Fig. 13) in plasma [40,42,44,46,49,50,53,55,58,59,62,63,118], whole blood [54], urine [40,62,63,118], aqueous humor [61] and CSF [49,62,63]. Vidarabine (**1**) and cytarabine (**2**) are only retained on hydrocarbonaceous supports with very weak, predominantly aqueous mobile phases This generally results in long retention times for many of the polar endogenous components present in biological fluids, which in turn leads to overall chromatographic run times of 30–60 min. Bowman and Kauffman [63] have shown that the total chromatographic run time for the analysis of vidarabine (**1**) and ara-Hx (**25**) can be reduced to 8 min if the late-eluting components are extracted with chloroform.

The major extracellular metabolites of vidarabine (**1**) and cytarabine (**2**) are ara-Hx (**25**) and ara-U (**29**), respectively, and these are the relevant analytes for analytical methodology developed for pharmacokinetic investigations of these drugs. In contrast, the major intracellular route of metabolism is phosphorylation leading to accumulation of mainly the phosphate esters (**26–28** and **30–32**), with the triphosphate esters (**28** and **32**) being the predominant intracellular species The nucleotides typically elute at the solvent front under reversed-phase conditions that are optimal for the analysis of the corresponding nucleoside. Therefore, LC methodology developed for the study of the cellular pharmacology of vidarabine (**1**) [60] and cytarabine (**2**) [39,41,45–48,52,55] generally involves anion-exchange [39,41,45–48,52,60] or reversed-phase ion-pair [55] chromatography (Fig. 8 c and d) in which the retention of the negatively charged phosphates (**26–28** and **30–32**) is enhanced *via* electrostatic interactions.

2.3.2. Ribavirin and riboxamide

Although the antiviral drugs ribavirin (**3**) and riboxamide (**4**) do not contain a purine or a pyrimidine nucleus, their chromatographic properties are similar to those of the nucleosides and it is convenient to discuss them in this section. Ribavirin (**3**) and riboxamide (**4**) are electronically neutral but extremely polar compounds that can only be retained on hydrocarbonaceous support with completely aqueous mobile phases. Therefore, the options for the manipulation of their retention on reversed-phase supports are limited and the weak eluents required for adequate retention of **3** and **4** result in long retention times for the endogenous components. Two different multi-dimensional approaches have been described to solve the problem of late-eluting peaks in the reversed-phase LC analysis of **3** and **4**. Riley *et al* [87] have described an automated column-switching procedure (Figs. 10 and 11; Section 2.1.2.4.) in which riboxamide (**4**) is separated from plasma. This method was later adapted to the analysis of urine by

Meltzer and Sternson [88]. An alternative approach has been described by Smith and Gilbert [85] who used an off-line phenylboronate affinity column (Section 2.1.2.3.) to isolate ribavirin selectively from plasma and other biological fluids and tissues. Ribavirin (**3**) was then separated from the other ribonucleosides and related compounds that were co-eluted from the boronate affinity column by reversed-phase LC with an overall chromatographic run time of 10 min.

Granich *et al.* [86] have described the advantages of LC methodology over GC–MS [140] and bioassay [148] for the analysis of ribavirin in biological fluids. They concluded that LC was superior to the other two methods in terms of speed of analysis and ease of performance. They also performed a quantitative comparison of the LC methodology of Smith and Gilbert [85] with the radioimmunoassay (RIA) technique described by Austin *et al.* [149]. They concluded that the RIA method was more sensitive but the two methods gave equivalent results for the quantitative determination of ribavirin (**3**) in plasma within the clinically relevant range of concentrations. Furthermore, the RIA method was found to be more suitable for the processing of large numbers of samples because the boronate affinity column clean-up necessary for LC analysis was not easily automated. However, RIA requires specific anitserum, which is not commercially available, and many laboratories prefer to avoid the use of radioisotopes.

Ribavirin (**3**) is extensively metabolized (Fig. 13) with 1,2,4-triazole-3-carboxamide (**33**), ribavirin-5′-monophosphate (**34**) and ribavirin-5′-triphosphate (**36**) being the major metabolites. The phosphorylated derivatives are not retained by hydrocarbonaceous supports and, although ion pairing has been suggested [85] as a potential method of retaining the phosphate esters of ribavirin (**33–35**) on reversed-phase supports, this has yet to be demonstrated. It is of interest to note that despite lacking the ribose function, 1,2,4-triazole-3-caboxamide (**33**) appears to be more polar than the parent drug and is therefore less retained on an ODS column. Nevertheless, Paroni *et al.* [83] have identified suitable conditions for the simultaneous analysis of ribavirin and 1,2,4-triazole-3-carboxamide (**33**) in biological samples. Of course the boronate affinity clean-up procedure is not applicable to the simultaneous extraction of 1,2,4-triazole-3-caboxamide (**33**) and ribavirin (**3**), which can only be accomplished by a complex procedure involving ultrafiltration and solid-phase extraction [83].

2.3.3. Acyclic 2′-deoxyguanosine analogues

The determination of acyclovir (**5**) and the related antiviral drug ganciclovir (**6**) by reversed-phase [69–71,117], reversed-phase ion-pair [66–68,71–75] or ion-exchange [132] LC is relatively straightforward (Table 2) and UV detection generally provides adequate sensitivity for plasma or serum [66,67,70–73,117], urine [66] and CSF [72] analyses. The opportunities for electrochemical [132] or fluorescence [117,128] detection of **5** and **6** in applications requiring greater sensitivity or where the sample size is limited have been described earlier in this review (see Sections 2.1.1.2. and 2.1.1.3.). Neither of these drugs are readily extracted from

biological fluids into an organic solvent and the preferred method for the deproteination of samples such as plasma, serum or CSF is by precipitation with acetonitrile [67], aluminum sulfate [66,74], perchloric acid [70] or trichloroacetic acid [73,75].

Deproteination produces a clear supernatant which is generally reported to be free of chromatographic interferences. However, Wiltink *et al.* [74] have indicated that plasma samples from seriously ill patients may produce several chromatographic peaks, probably arising from other drugs, that can disturb the assay of ganciclovir (**6**). Because of the structural similarity of ganciclovir (**6**) and acyclovir (**5**), it is reasonable to assume that similar interferences can also arise in the LC assay of acyclovir (**5**) from the plasma of patients receiving multiple-drug therapy. Wiltink *et al.* [74] have shown that potential interferences in plasma can be removed by extraction with chloroform.

Desciclovir (**7**) is a prodrug of acyclovir (**5**) that has been developed in an attempt to increase the blood levels of acyclovir (**5**) following oral dosing. Desciclovir (**7**) itself has no antiviral activity but is converted to acyclovir by the action of xanthine oxidase [76] (Fig. 14). Petty and co-workers [76,77] have described an LC method for the study of metabolism [77] and pharmacokinetics of desciclovir (**7**) in man. Because their methodology was capable of separating acyclovir (**5**) from its major metabolites, carboxyacyclovir (**37**) and 8-hydroxyacyclovir (**38**), it should also be useful for pharmacokinetic investigations of the active drug, acyclovir (**5**). Although only partial separation of carboxydesciclovir (**39**) and 8-hydroxyacyclovir (**38**) was obtained with that method [76,77], this did not compromise the usefulness of the method because 8-hydroxyacyclovir (**38**) was not detected in the plasma or urine of patients receiving desciclovir (**7**).

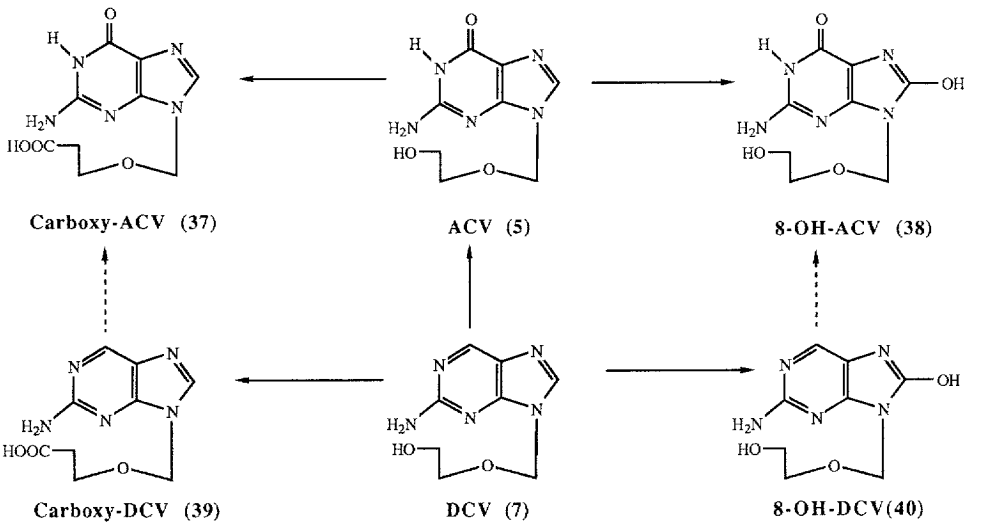


Fig. 14 Metabolism of acyclovir (**5**) and its pro-drug desciclovir (**7**)

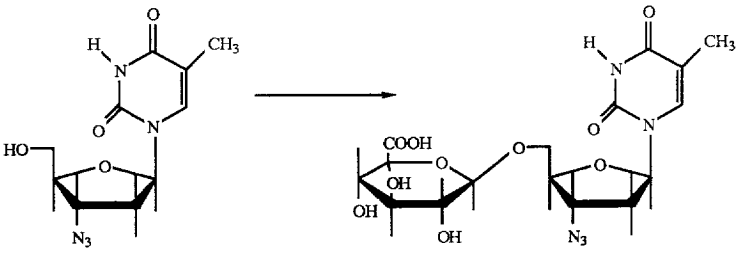
RIA [150–152] and enzyme-linked immunosorbent assay (ELISA) [153] are viable alternatives to LC for the bioanalysis of acyclovir (**5**) and ganciclovir (**6**) in the clinical setting Although cross-reactivity of antibodies is always a concern in immunoassays, Sommadossi and Bevan [73] have shown that RIA [151] and LC [73] methodologies are equivalent for the quantitative determination of ganciclovir (**6**) in plasma. Furthermore, they also concluded that LC was superior to immunological techniques when rapid results were needed. Other disadvantages of immunological assays include the need to develop antisera and/or monoclonal antibodies.

2.3.4. 2′,3′-Dideoxynucleosides

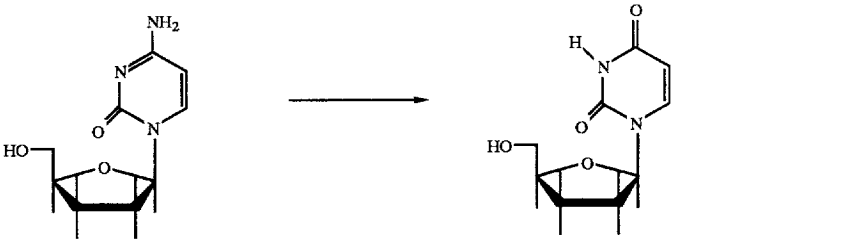
Changes in the substituents at the 2′- and 3′-carbon atoms to the nucleosides have resulted in several compounds with significant antiviral activity, a number of which (**8**–**13**) are in various stages of clinical development. Within this class of drugs, zidovudine (AZT) (**11**) has received the most attention because it was the first drug found to be effective in reducing the morbidity and mortality associated with severe HIV infection. Carbovir (**8**), 2′,3′-dideoxyadenosine (**9**), DDC (**10**), DDI (**12**) (a metabolite of **9**) and d4T (**13**) are in various stages of clinical or pre-clinical development. They are expected to have significant impact on the treatment of various viral infections and HIV infections in particular.

The 2′,3′-dideoxynucleosides are substantially more lipophilic than the corresponding naturally occurring nucleosides and as a result they tend to be more retained by hydrocarbonaceous supports. Consequently, isocratic or gradient elution reversed-phase LC are the methods of choice for the determination of these drugs and their metabolites in biological fluids and tissues. Furthermore, the increased lipophilicity facilitates sample preparation for the determination of the 2′,3′-dideoxynucleosides in biological fluids, which generally involves a simple liquid–liquid extraction [7,9,11,15,78], liquid–solid extraction [6,13,81,82,98,133] or direct injection [10,14] (Section 2 1.2 3.).

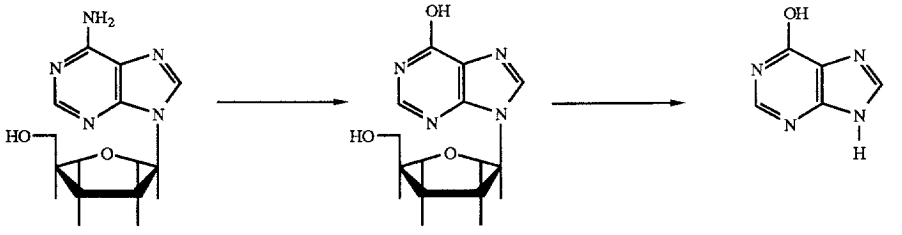
Because of the widespread clinical interest in zidovudine (**11**), several LC methods have been described [6–16] for the determination of this drug in biological fluids such as plasma, serum, [6–11,13–16] or urine [7,9,11]. Following intravenous injection into human subjects, zidovudine (**11**) is cleared mainly by metabolism (60%) to the 5′-O-glucuronide (**41**) (Fig 15) or by excretion of the unchanged drug (25%). Because of first-pass metabolism, the oral bioavailability in man of zidovudine (**11**) is only 60% and significant inter-subject variability has been reported [6–16]. When studying the pharmacokinetics of zidovudine (**11**) in detail, LC methodology [6,9,16] which is capable of determining both zidovudine (**11**) and its major metabolite (**41**) should be used (*e g.* Fig. 16). On the other hand, when measurement of the glucuronide is of no interest for example in therapeutic drug monitoring, then Unadkat *et al.* [7] have suggested that a rapid and simple LC method for zidovudine (**11**) alone may be sufficient. Alternatively, Granich *et al* [13] have recently described a fluorescence polarization immunoas-



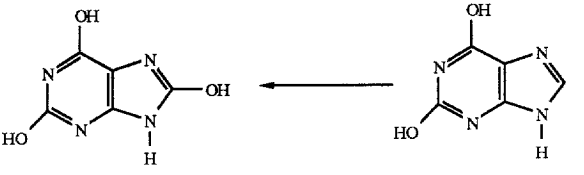
Zidovudine (11) Zidovudine-5'-O-glucuronide (41)



2',3'-Dideoxycytidine (10) 2'3'-Dideoxyuridine (42)



2',3'-Dideoxyadenosine (9) 2',3'-Dideoxyinosine (12) Hypoxanthine (43)



Uric acid (45) Xanthine (44)

Fig 15. Metabolism of zidovudine (AZT) (**11**), 2',3'-dideoxycytidine (**10**) and 2',3'-dideoxyadenosine (**9**) The metabolic steps leading to the mono-, di- and triphosphates have been omitted for clarity

say (FPIA) for zidovudine (**11**) which is claimed to be more sensitive than LC. The FPIA requires only 50–60 μl of plasma compared with 200–500 μl for a typical LC method, and interference from **41** and other related compounds is insignificant. Additionally 45 samples per hour can be analyzed by FPIA compared with 3 per hour by LC, making the former particularly attractive for the therapeutic drug monitoring.

The metabolism of 2',3'-dideoxyadenosine (**9**) and 2',3'-dideoxycytidine (**10**) proceeds *via* phosphorylation and deamination in the same manner as **1** and **2** (Fig. 13), with the former reaction resulting in the major intracellular products and the latter being the major mechanism of clearance from the body. Ion-exchange LC with radioactivity detection [80] has been described for the determination of **9** and **10** and their phosphorylated metabolites. Reversed-phase [80,81] or reversed-phase ion-pair [79] LC with UV detection represent more convenient methodologies for pharmacokinetic investigations in which determination of the DDI (**12**) and 2',3'-dideoxyuridine (DDU) (**42**), metabolites of **10** and **11**, respectively, are more relevant. Anticipating the further metabolism of DDI (**12**) to hypoxanthine (**43**), xanthine (**44**) and uric acid (**45**), Kalin and Hill [79] have developed a reversed-phase method for the simultaneous determination of all the

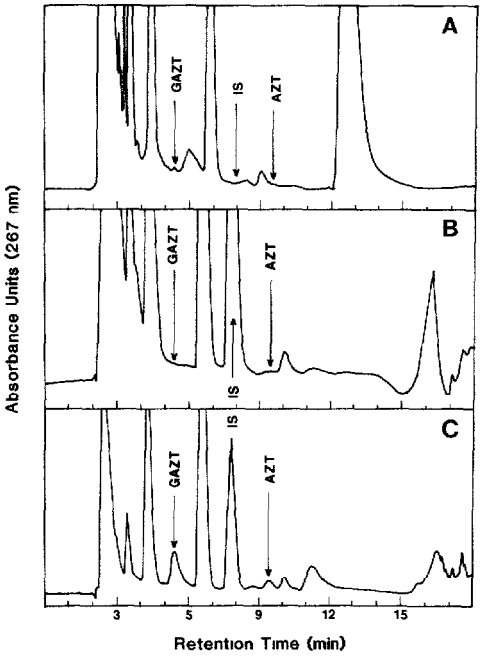


Fig 16. Determination of zidovudine (AZT) (**11**) and its major metabolite (GAZT) (**41**) in human serum (A) Normal serum from five donors, (B) serum from an AIDS patient prior to receiving **11**; (C) serum obtained 2.5 h after oral administration of **11** Conditions. see Table 2 (Reproduced from ref 6 with permission)

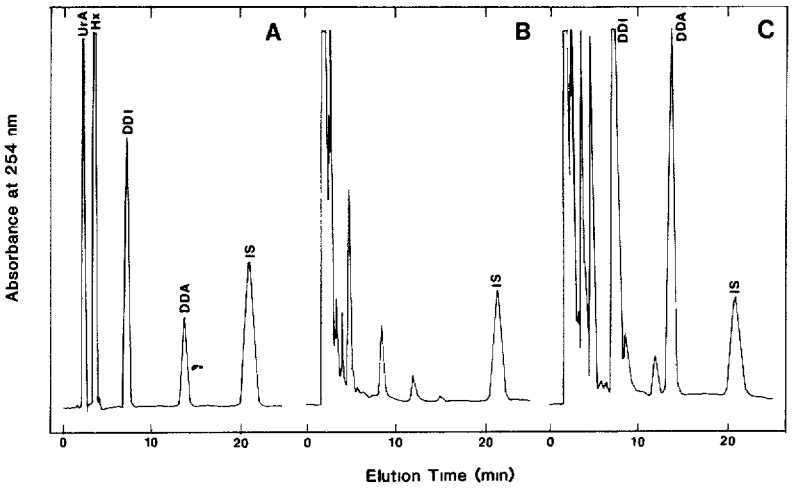


Fig. 17 Separation of (A) a mixture containing 500 ng each of the standards of DDA (**9**), DDI (**12**), hypoxanthine (Hx) (**43**) and uric acid (UrA) (**45**), (B) blank mouse plasma and (C) plasma from a mouse 5 min following intraperitoneal administration of DDA (478 mg/kg) The internal standard was N-methyl-2′-deoxyadenosine Conditions see Table 2 (Reproduced from ref 79 with permission)

possible catabolites of DDA (**9**) (Fig. 17). By administering tritiated **10** they [79] were able to show that deamination is the major mechanism of clearance of the drug. Previously, Blau *et al.* [133] had shown using LC–MS that deamination of **9** can occur, *in vitro*, in RPMI-1640 growth medium as well as in human and mouse plasma.

2.3.5. 5-Halo-2′-deoxynucleosides

5-Iodo-2′-deoxyuridine (idoxuridine) (**14**) was the first clinically useful antiviral drug. Systemic toxicity, however, has limited its application to the treatment of infections of the eye and skin caused by the Herpes viruses. Therefore, most of the LC methodology developed for the analysis of idoxuridine (**14**) has concentrated on applications such as quality control [89,131] and stability testing [90]. Sincholle *et al.* [91] have used LC with UV detection to study the corneal metabolism and penetration of idoxuridine (**14**) and the related antiherpetic drug 5-iodo-2′-deoxycytidine (**17**). Because the two drugs (**14** and **17**) were separated on an Aquapore RP 300 column with a mobile phase of acetic acid–water (1:99, v/v), one drug could be used as an internal standard for the determination of the other in the aqueous humor of the rabbit.

The clinical effectiveness of idoxuridine (**14**) against Herpes simplex virus and its structural similarity to the potent antimetabolite 5-fluorouracil has resulted in the synthesis of a number of biologically active 5-halo-2′-deoxynucleosides [1]. Out of this group of potential antiviral drugs, (**15**) and FIAC (**16**) have emerged and have shown sufficient promise in animal and tissue culture models to warrant further clinical investigation.

The analysis, pharmacokinetics and metabolism of FIAC (**16**) have been studied extensively by Philips and co-workes [92,93] using reversed-phase LC By administering [2-^{14}C[FIAC to mice, they were able to show that the major metabolite of FIAC (**16**) arises from deamination to 2′-deoxy-2′-fluoro-5-iodoarauri-

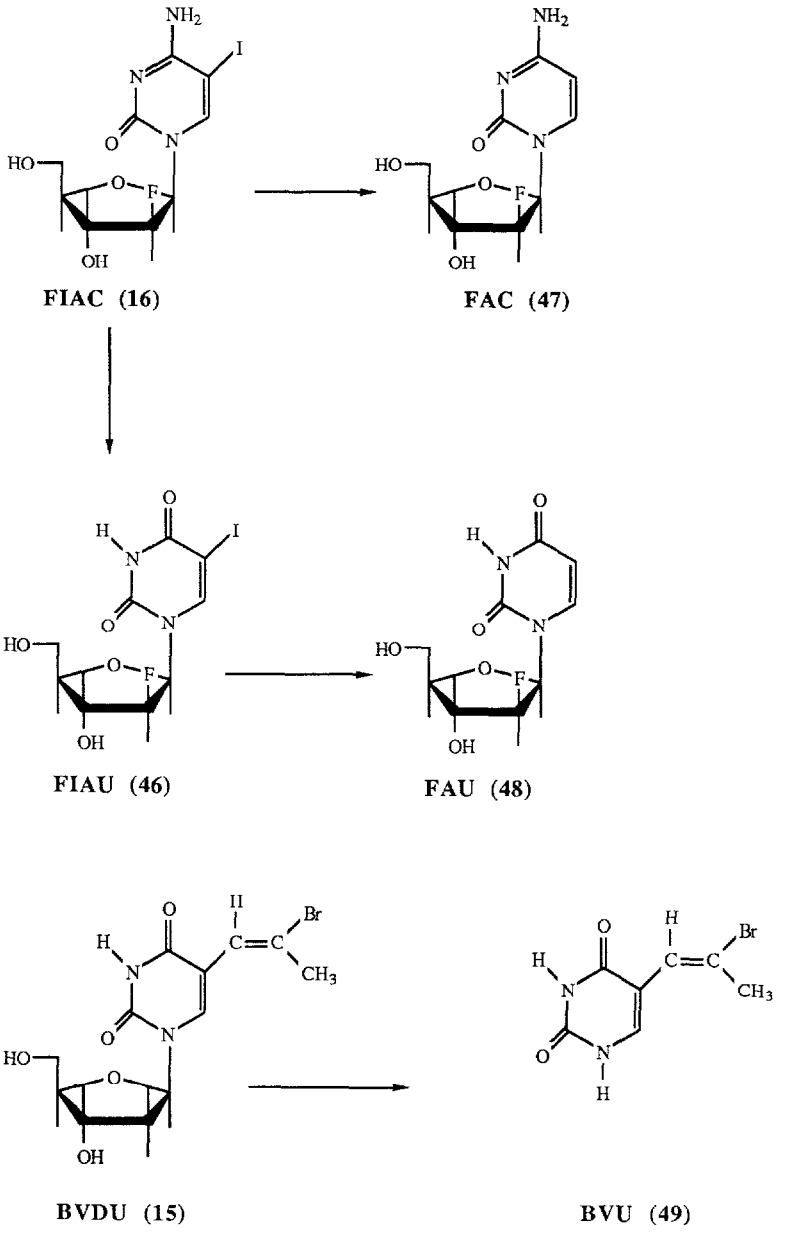


Fig. 18 Metabolism of FIAC (**16**) and BVDU (**15**) The metabolic steps leading to the glucuronides of FIAC (**16**) and the mono-, di- and triphosphates of BVDU (**15**) have been omitted for clarity

dine (FIAU) (**46**) (Fig. 18) with deiodination to 2′-fluoroaracytosine (FAC) (**47**) and 2′-fluoroarauridine (FAU) (**48**) and glucuronidation representing less important metabolic pathways (Fig. 17). In contrast, after administration of [2-^{14}C]FIAC to dogs, the 24-h urinary radioactivity was mainly composed of unchanged drug and the deiodinated products (**47** and **48**). The initial studies on the metabolism of FIAC (**16**) in mice and dogs involved precipitation of proteins with perchloric or trichloroacetic acid, followed by separation on an ODS column with a linear gradient of 0 to 30% methanol in a sodium phosphate buffer (0.02 *M*, pH 3.0). Subsequently, this group [93] used the same analytical methodology to study the metabolism and pharmacokinetics of FIAC (**15**) following intravenous administration of the drug to immunosuppressed patients with Herpes virus infections In man, FIAC (**16**) was found to be cleared primarily by deamination to FIAU (**46**), which is an active metabolite and was cleared much more slowly than the parent compound. Therapeutic levels of FIAU (**46**) were maintained for more than 12 h, leading to the conclusion [93] that the antiviral activity of FIAC (**16**) observed *in vivo* is due to its primary metabolite rather than the drug itself.

Several LC methods have been described [94–97] for the determination of the antimetabolite BVDU (**15**), its major catabolite 5-bromovinyluracil (BVU) (**49**) [92,93] and its major intracellular anabolites, the mono-, di- and triphosphate esters [93,95]. Because they are neutral molecules, BVDU (**15**) and its primary excretory metabolite BVU (**49**) are most easily separated by reversed-phase LC, which permits separations on the basis of hydrophobicity. On the other hand, anion-exchange chromatography represents the most convenient method for the separation of BVDU and its phosphate esters [93,95].

Reeuwijk *et al.* [96] have criticized the early methods [94,95] for the determination of BVDU (*15*) for having complex and time-consuming sample preparation steps. For example, the method of Ayisi *et al.* [95] involves deproteination with perchloric acid followed by lyophilization of the supernatant obtained after centrifugation. The method of Robinson *et al* [94] is less complex but still involves extraction of the drug from plasma into ethyl acetate followed by phase separation, evaporation of the solvent and reconstitution of the final residue in mobile phase Reeuwijk *et al.* [96] have developed a simpler method for the analysis of BVDU (**15**) in plasma, which involves deproteination with perchloric acid followed by direct injection of the supernatant onto an ODS column and detection at 307 nm. They also showed that the extent of plasma–protein binding of BVDU (**15**) could be determined by ultrafiltration through YMT membranes (Amicon, Danvers, MA, U.S.A.) (cut-off 30 000 daltons) and direct injection of the filtrate. Because of the low concentration of BVDU (**15**) excreted unchanged into human urine, solvent extraction was still necessary for the analysis of the drug in urine. Reeuwijk *et al.* [96] did not discuss the analysis of BVU, the major metabolite of BVDU, and it may be concluded that the simpler method described by this group would be useful for routine pharmacokinetic investigations (*i.e.* in therapeutic drug monitoring) where information on the metabolism of the drug is not required.

TABLE 5

CHROMATOGRAPHIC METHODS FOR THE DETERMINATION OF THE NON-NUCLEOSIDE ANTIVIRAL DRUGS IN BIOLOGICAL FLUIDS

See Table 2 for notes and abbreviations

Drug	Method	Detection	Chromatographic conditions		Limit of detection	Sample matrix	Comments	References
			Stationary Phase	Mobile phase or carrier gas				
Amantidine (**129**)	GC	ECD	10% OV-1 on Chromosorb W (80–100 mesh, 1 8 m × 4 mm, 95°C)	Helium	10 ng/ml	Plasma	Correlation of plasma levels with side effects, derivatization with pentafluorobenzyl chloride	Hayden *et al.* [158,159]
	GC	FID	20% Carbowax 20 M on Chromosorb W (4 ft, 95°C)	Helium	100 ng	Blood, urine and tissue samples	Analysis of mouse, monkey, dog and human samples	Bleidner *et al*, [160]
	GC	ED	1 5 SE-30 on Anakrom AS (90–100 mesh)	Nitrogen (220°C)	10 ng	Plasma	No application stated	Tsubouchi *et al* [161]
	GC	ECD or MS	3% OV-17 on Chromosorb Q (100–120 mesh, 2 m × 4 mm, 220°C)	Nitrogen/helium	25 ng/ml	Plasma	Toluene extraction, derivatization with trichloroacetyl chloride; clinical pharmacokinetics	Biandrate *et al* [162]
	GC	FID	15% Carbowax 4000 on Celite 545 (1 5 m × 4 mm, 165°C)	Nitrogen	N.S.	Urine	Clinical pharmacokinetics	Montanari *et al.* [163]

GC	FID or MS	2% Dexsil 300 on Gas Chrom Q (80–100 mesh, 1.5 m × 2 mm, 180–260°C, 10°C/min)	Helium	N S	Urine and feces	Metabolism studies	Wesemann *et al* [164]
GC	MS	3% OV-225 on Gas Chrom Q (190°C, 180 mm × 3 mm) 3% OV-17 on Chromosorb W-HP (190°C, 180 mm × 3 mm) 3% OV-101 on Gas Chrom Q (150°C, 180 mm × 3 mm)	Helium	20 ng/ml	Plasma and dialysate	Derivatization with isothiocyanate, clinical samples	Narasimhachari *et al* [165]
GC	ECD	5% SE-30 on Chromosorb W-HP (80–100 mesh, 2 m × 3 mm, 200°C)	Argon–methane (90·10)	10 ng/ml	Plasma and urine	Derivatization with trichloroacetyl chloride, stability in plasma; clinical pharmacokinetics	Sioufi and Pommier [166]

(Continued on p 350)

TABLE 5 (*continued*)

Drug	Method	Detection	Chromatographic conditions		Limit of detection	Sample matrix	Comments	References
			Stationary phase	Mobile phase or carrier gas				
	GC	FID or MS	5% Apiezon L on Gas Chrom Q (100–120 mesh, 1.2 m × 4 mm, 160°C)	Nitrogen	N.S.	Urine, plasma	Oral pharmacokinetics in man	Belanger and Grech-Belanger [167]
	GC	FID	10% Apiezon L and 2% potassium hydroxide on Chromosorb W AW (80–100 mesh, 145°C)	Nitrogen	0.5 ng/ml	Urine	β-Phenylethylamine as internal standard	Stumph *et al.* [168]
Ampligen (**24**)	LC	UV (260 nm)	μBondapak C_{18} (10 μm, 300 × 3.9 mm)	Sodium acetate (pH 6.5, 0.5 *M*)	100 ng/ml	Plasma	Clinical pharmacokinetics; analyte degraded enzymatically to inosine and cytidine	Rosenblum and Cheung [188]
Arildone (**18**)	LC	RA	LiChrosorb RP-8 (25 cm × 3.2 mm)	Acetonitrile, water, and formic acid step gradient	6 ng/ml	Blood	Analysis and isolation of metabolites; metabolic pathway proposed; comparison of metabolism in rats, mice and dogs	Benziger *et al.* [154]

	TLC	RA	Silica gel 60 F254	Chloroform–methanol (99.1, v/v)	N S	Blood	None	Benziger *et al.* [154]
	GC	MS	3% OV-1 (91 4 cm × 2 mm)	Helium	N S	Urine and feces	Dog urine and rat feces	Benziger *et al* [154]
	GC	ECD	3% OV-1 on Gas Chrom Q (100–120 mesh, 275°C, 285°C feces)	7% Methane in argon	6.0 ng/ml (plasma); 1 4 ng/ml (urine), 15 ng/g (feces)	Plasma, urine and feces	Derivatization with *o*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine or 3-nitrogenphenylhydrazine (feces)	Park *et al* [155, Bosso *et al* [156]
Enviroxime (**22**)	LC	ED	Zorbax C_8 (6 μm, 250 mm × 4 6 mm)	Methanol–sodium acetate (0 14 *M*) (65 35) containing EDTA (3 mg/l)	4 ng/ml (plasma)	Plasma, urine, nasal washing, bile and tissue homogenate	Distinguishes enviroxime from zinviroxime	Bopp and Miner [178]
	LC	ED or UV (272 nm)	Zorbax C_8 (6 μm, 250 mm × 4 6 mm)	Methanol–sodium acetate (0 14 *M*) (65 35) containing 3 mg/l disodium edetate	> 10 μg (UV), 400 ng (ED)	Plasma and urine		Miner *et al* [180]
Foscarnet (**23**)	LC	ED (0.90 V)	Ultropac C_{18} (3 μm, 100 × 4 6 mm)	Methanol–phosphate buffer (pH 5.8) (25 75) containing tetrahexylammonium hydrogensulfate (1 m*M*) and sodium pyrophosphate (0 2 m*M*)	70 ng/ml	Plasma, CSF and urine	Clinical pharmacokinetics, stability demonstrated at +4 and −20°C, plasma and urine treated with activated charcoal to remove endogenous compounds	Pettersson *et al* [183], Sjovall *et al* [184]
Moroxydine (**21**)	GC	MS	10% SE/ Chromosorb W HP (80–100 mesh, 3 mm)	Helium	0 02 μg/ml	Serum and urine	Clinical pharmacokinetics	Doi *et al* [175]

(Continued on p. 352)

TABLE 5 (*continued*)

Drug	Method	Detection	Chromatographic conditions		Limit of detection	Sample matrix	Comments	References
			Stationary phase	Mobile phase or carrier gas				
	GC	ECD	2% OV-225 on Chromosorb G (1.5 m × 1 8 mm, 220°C)	Nitrogen	17 pg (derivative)	Plasma and urine	Time course study in serum, ion-pair extraction and derivatization with chlorodifluoroacetic anhydride	Vessman *et al* [176]
	LC	Colorimetric (520 nm)	Amberlite CG-50, Type 1 (100–200 mesh)	0 01 *M* and 0.2 *M* sodium hydroxide	10 μg	Serum	Fractions collected and detected by colorimetric assay involving reaction of moroxydine with 3,5-dihydroxybenzoic acid–diacetyl solution	Tanabe and Sakaguchi [174]
Rimantidine (**20**)	GC	ECD	10% OV-1 on Chromosorb W (80–100 mesh, 1 8 m × 4 mm, 95°C)	Helium	10 ng/ml	Plasma	Clinical pharmacokinetics, assessment of correlation of plasma levels and side effects, derivatization with pentafluorobenzyl chloride	Hayden *et al* [158,159]
	GC	MS	Chrompak CP Sil 8 CB (25 m × 0.33 mm, 1 25 μm film thickness, 265°C)	Methane	4 2 ng/ml (plasma); 20 7 ng/ml (urine)	Plasma and urine	Stability studies at room temperature and −20°C, derivatization with pentafluorobenzoyl chloride	Fukuda *et al.* [170]

GC	MS	DB-1 (30 m × 0.25 mm, 0.25 μm film thickness, 50–300°C, 15°C/min)	Argon	10 μg/l	Plasma	Deuterium labeled rimantidine as an internal standard; extraction with Bond Elut CN solid-phase columns; analysis of plasma samples	Herold *et al.* [171]
GC	MS	DB-1 (15 m × 0.25 mm, 200–260°C, 10°C/min)	N.S.	N.S.	Plasma	Mouse	Hoffman *et al.* [172]
GC	ECD	10% OV-1 on Chromosorb W (80–100 mesh, 1.8 m × 4 mm, 95°C)	Helium	N.S.	Plasma, urine, feces and tissue	Plasma levels determined in the mouse and dog of rimantidine and metabolites; derivatization with pentafluorobenzyl chloride; modification of Hayden *et al.* assay	Hoffman *et al.* [172]
GC	MS	DB-1 (30 m × 0.32 mm, 0.25 μm film thickness, 164–264°C, 10°C/min)	Hydrogen	2.5 ng/ml	Plasma and urine	Derivatization with (+)-α-methyl-α-methoxy(pentafluorophenyl)acetic acid; patient samples analyzed; separation of rimantidine enantiomers	Miwa *et al.* [173]

3 OTHER ANTIVIRAL DRUGS

The non-nucleoside antiviral drugs (Fig. 2) represent a diverse group of compounds that, with the exception of amantidine (**19**) and rimantidine (**20**), are structurally unrelated. It is convenient to discuss the chromatographic analysis of these compounds individually. The details of the analysis of these compounds are summarized in Table 5.

3.1. Arildone

Arildone, 4-[6-(2-chloro-4-hydroxy)phenoxy]hexyl-3,5-heptanedione (**18**) (Fig. 2) is a compound with activity against both DNA and RNA viruses. It is particularly active against Herpes virus types 1 and 2. Benzinger *et al.* [154] have used various LC, TLC, GC and column chromatography systems to isolate and identify several metabolites of arildone (**18**) in the plasma, feces and urine of laboratory animals, following administration of [^{14}C]-arildone. They found that the drug was extensively metabolized by O-demethylation, loss of the hexyl-3,5-heptanedione side-chain and conjugation with sulfate (Fig. 19). In the dog, mouse and rat, the drug is mainly excreted in the feces as the unchanged drug and as O-desmethylarildone (DMA) (**50**). All the metabolites of **18** shown in Fig. 19 were found in the body fluids of experimental animals; however, Benzinger *et al.* [154] have indicated that the 2-chlorohydroquinone (CHQ) (**51**) found in urine was probably an artifact arising from the degradation of its sulfated conjugate (**52**) during isolation by ion-exchange chromatography

Whereas LC with radioactivity detection can be used for the identification of metabolites in experimental subjects, it is not suitable for routine clinical investigations. Furthermore, LC with UV detection does not provide adequate sensitivity because concentrations of arildone (**18**) in body fluids and tissues are in the range 1–250 ng/ml (or ng/g). Park *et al* [155] have described GC methodology with electron-capture detection (ECD) for determination of arildone (**18**) in plasma, feces and urine. This method was used by Bosso *et al.* [156] to study the percutaneous absorption of arildone from various topical formulations. The methods for the analysis of plasma and urine involved extraction with hexane, evaporation of the solvent and derivatization of the residue with O-(2,3,4,5,6-pentafluorobenzylhydroxylamine (PFBHA). The derivative of arildone (**18**) was chromatographed on 3% OV-1 supported by Gas-Chrom Q at 275°C with a carrier gas of methane–argon (7:93, v/v). Derivatization of the β-diketone groups gave rise to *E*- and *Z*-isomers which were resolved by GC. Quantification of **18** was simplified by the use of a short column which allowed the two peaks arising from the isomers to merge. Park *et al.* [155] have also reported on the binding of arildone to glass and GC columns. This binding was aggravated by silanization but could be minimized by injecting high concentrations of the derivatives prior to the analysis of the samples Derivatization of fecal extracts gave rise to numer-

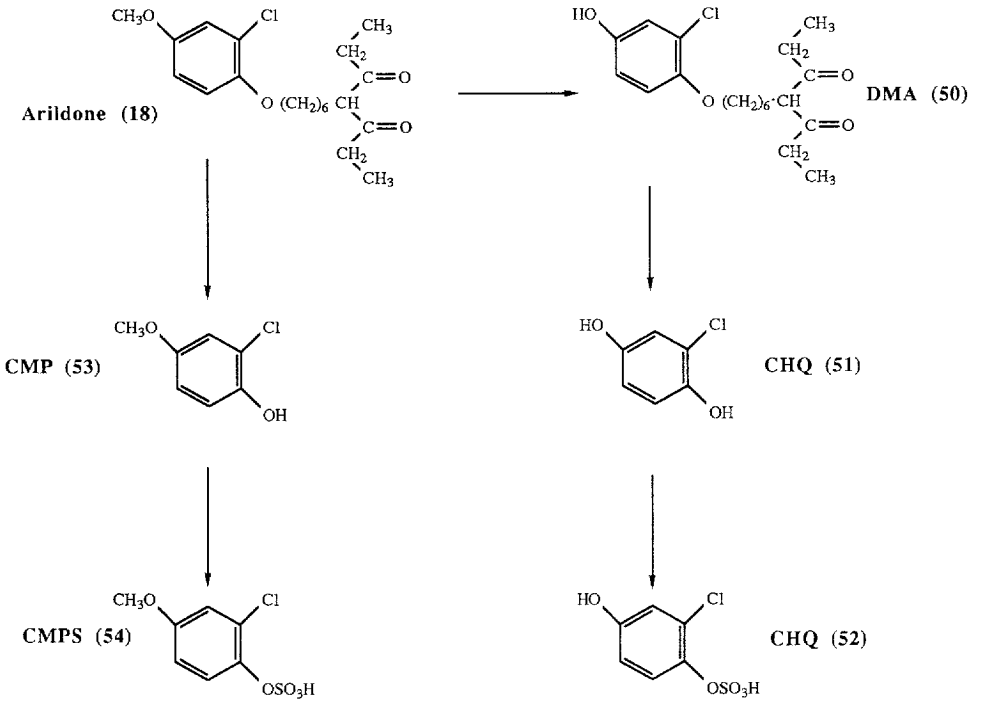


Fig. 19 Metabolism of arildone (**18**).

ous peaks which interfered with the determination of **18** and could not be removed by extraction. This problem was solved by derivatization of **18** in fecal extracts with 3-nitrophenylhydrazine.

3.2. Amantidine and rimantidine

Amantidine (**19**) is a symmetrical C-10 primary amine with significant activity against various strains of influenza. Amantidine (**19**) is also used widely for the treatment of Parkinson's disease. Rimantidine (**20**) is a close structural analogue of **19**, which is reported [157,158] to be better tolerated than **19** in man. Hayden and co-workers [158,159] have reported that the reduced toxicity of **20** compared with **19** is a result of the lower plasma concentrations of **20** that are achieved for a comparable dose

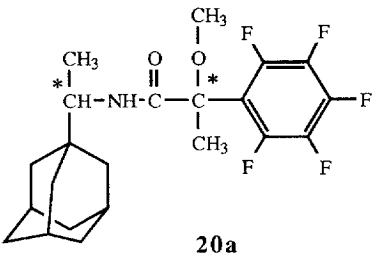
Because they lack a suitable chromophore or electrochemically active functional group, amantidine (**19**) and rimantidine (**20**) are generally unsuitable for direct analysis by LC. The method of choice for the determination of these two drugs in biological fluids has been GC [157–172]. Pre- or post-column derivatization of the primary amino group would be necessary for the determination of **19** and **20** in biological fluids by LC The fact that this has not been reported in

the literature probably reflects the adequate nature of existing GC methodology rather than any particular difficulty in the development of an LC method. Although several metabolites of **19** such as N-hydroxy-1-amino adamantane, 1-nitrosoadamantane, 1-nitroadamantane, 1-acetamidoadamantane and 1-amino-3-hydroxyadamantane have been isolated from the urine of experimental animals [161,167,169], no such metabolites have been reported in man and the GC methodology developed for clinical studies has concentrated on the determination of the parent compound. Recently Hoffman *et al.* [172] have shown that the main metabolites of rimantidine (**20**) in the mouse are ring-substituted isomers of hydroxyrimantidine.

Amantidine (**19**) is an unsaturated aliphatic amine (pK_a approximately 9.0 [160]) that can be extracted efficiently at high pH from biological fluids into a variety of organic solvents such as benzene [160], toluene [162,163,166,168,169] or diethyl ether [165,167]. Solid-phase extraction onto cyano-bonded solid-phase extraction cartridges (*e.g.* Bond Elut CN) has generally been employed for the pre-treatment of urine and plasma samples containing rimantidine (**20**) despite being structurally similar to **19**. Early GC methods [160,163,167,168] for **19** relied on flame-ionization detection (FID) of the underivatized compound and allowed the determination of **19** in plasma and urine with detection limits of approximately 100 ng/ml and 4 μg/ml, respectively. Belanger and Grech-Belanger [169] have suggested that these detection limits are adequate for most clinical investigations. However, there is considerable inter-patient variability in the plasma concentration of **19** following oral dosing and, for a given dose, the plasma concentrations of **20** are lower than those for **19** and the sensitivity of FID may be inadequate for certain clinical applications For example, Hayden *et al* [158] have found that the mean (± S.E) plasma concentrations of **19** and **20** in human subjects 4 h after 100 mg oral doses were 300 ± 98 and 140 ± 68 ng/ml, respectively. Detection limits of 5–25 ng/ml have been reported for both **19** and **20** with ECD or MS, following derivatization of the analytes with pentafluorobenzoyl chloride [158,159,170, 172], acetyl chloride [162], trichloroacetyl chloride [162,166], isothiocyanate [165] or N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide [171].

Several internal standards have been claimed as being suitable for the determination of **19** in biological samples. These include amphetamine [162,166], 2-aminoadamantane [163], β-phenethylamine [167] and chlorphentermine [168]. Belanger and Grech-Belanger [169] have criticized the use of β-phenethylamine [167] as internal standard in the bioanalysis of amantidine (**19**) because it is an endogenous compound that is excreted in appreciable amounts in urine. Fukuda *et al* [170] and Herold *et al.* [171] have described d_3-rimantidine and d_4-rimantidine, respectively, as being suitable internal standards in stable-isotope dilution assays involving capillary GS–MS for the determination of rimantidine (**20**) in biological fluids.

Unlike **19**, **20** contains a chiral carbon atom and the drug is administered as the racemic mixture. Recognizing the possibility for the stereoselective disposi-



20a

Fig 20 Structure (**20a**) of the diastereomeric derivative of rimantidine (**20**) The chiral centers are indicated by an asterisk

tion, Miwa *et al.* [173] have developed a GC–MS method for the determination of the two enantiomers of **20** in plasma. The method for the determination of the enantiomers of **20** involved solid-phase extraction, derivatization with a chiral reagent to form diastereomers (**20a**, Fig 20), negative-ion chemical ionization (NICI), selective-ion monitoring and stable-isotope dilution. The derivatization reaction of **20** with (+)-α-methyl-α-methoxy(pentafluorophenyl)acetic acid was

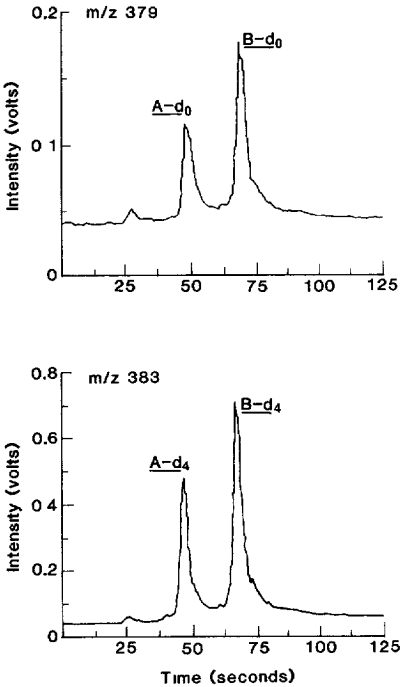


Fig 21 Selected-ion current profiles from a 72-h post-infusion plasma sample from a subject given 200 mg oral dose of rimantidine (**20**). The 1 0-ml plasma sample was spiked with 100 ng of tetradeuterated rimantidine The upper chromatograms of the rimantidine-d_0 diastereomers are labelled A-d_0 and B-d_0 and from the rimantidine-d_4 diastereomers (internal standards) are labelled A-d_4 and B-d_4 (Reproduced from ref 173 with permission)

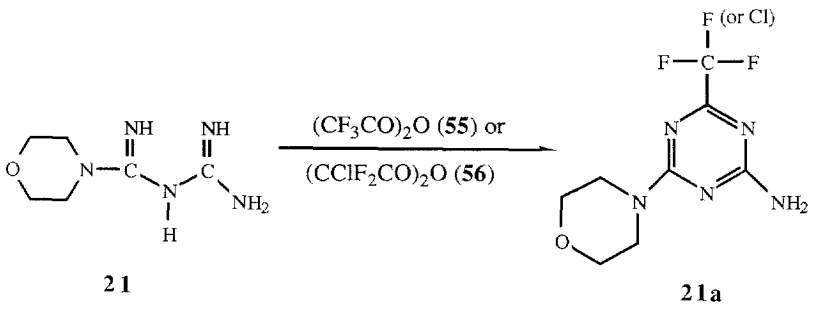
catalyzed by 1,3-dicyclohexylcarbodiimide and 1-hydroxybenzotriazole hydrate, which are known to couple amino acids and peptides without racemization. The actual ions utilized for quantitative measurements were $[M-HF-CH_3OH]^{\cdot-}$ at *m/z* 379 and 383 for the derivatives of rimantidine (**20a**) and the internal standard, d_4-rimantidine, respectively. The two diastereomers were formed in unequal amounts and the intensity ratio of the peaks A and B (Fig 21) varied between 1:2 and 1:1 but was generally about 2:3. This variability did not affect the quantification because it was compensated for by the variation in the derivatization of the enantiomers of the internal standard. Interestingly, when the method was applied to a pharmacokinetic study in healthy volunteers, they found no differences in the disposition of the two enantiomers of **20** in the plasma and urine; however, only one enantiomer was generated following treatment of urine with glusulase (Du Pont Pharmaceuticals, Wilmington, DE, U.S.A.). It was not known whether the observed stereospecificity was the result of *in vivo* formation or the failure of the glusulase to hydrolyze one enantiomer.

3.3. Moroxydine

Moroxydine (**21**) is an orally active biguanide antiviral agent. Because it is a highly polar compound and lacks a suitable chromophore or electrochemically active functional group, moroxydine (**21**) must be derivatized prior to its analysis in biological samples by GC or LC. Tanabe and Sakaguchi [174] have described an ion-exchange method for the analysis of moroxydine (**21**) in serum, in which the drug is separated from endogenous biguanides on an Amberlite GC-50 column. This method is time-consuming because quantification involves collection of fractions which then have to be analyzed colorimetrically at 520 nm after reaction with diacetyl (2,3-butanedione) and 3,5-dihydroxybenzoic acid. Furthermore, this method is of limited usefulness for the determination of moroxydine (**21**) in biological fluids because the limit of detection in serum is 1 μg/ml and it has been shown [175] that the peak to trough plasma concentrations of **21** in man range between 20 and 1000 ng/ml following chronic oral administration of 500 mg moroxydine hydrochloride.

Doi *et al.* [175] and Vessman *et al.* [176] have described similar GC methods for the determination of moroxydine (**21**) in human plasma and urine. Both these methods involve reaction of the biguanide (Fig. 22) with either trifluoroacetic anhydride (**55**) [175] or chlorodifluoroacetic anhydride (**56**) [176] to give a 1,3,5-*s*-triazine. The original method of Doi *et al.* [175] utilized MS detection with single-ion monitoring. The molecular ions of the moroxydine and buformin (internal standard) triazine derivatives were recorded at *m/z* 249 and 235, respectively.

The limit of detection for the GC–MS method for **21** is 20 ng/ml, which is generally adequate for the 24-h monitoring of moroxydine (**21**) following oral administration of 500 mg of the drug. Vessman *et al.* [176] have described an improved GC procedure for moroxydine (**21**) in plasma and serum which in-



21 21a

Fig 22 Derivatization of moroxydine (**21**) with trifluoroacetic anhydride (**53**) or chlorodifluoroacetic anhydride (**53**) to give the corresponding 1,3,5-*s*-triazine derivative (**21a**).

volves deproteination with trichloroacetic acid followed by extraction of the moroxydine–bromothymol blue ion-pair into methylene chloride. The absolute recovery of moroxydine was 70%. This could be improved by a second extraction with methylene chloride; however, samples analyzed with only one extraction gave the same results as those samples extracted twice and the more practical single-extraction procedure was preferred [176]. Vessman *et al.* [176] have reported a lower detection limit of about 5 ng/ml for moroxydine with ED compared with MS; however, both GC–MS and GC-ED methods [175,176] are adequate for clinical pharmacokinetic studies and the choice of detector will probably be based on the availability of equipment.

3.4. Enviroxime

Enviroxime (**22**) has been shown to be a highly specific inhibitor of the multiplication of the rhinovirus in tissue culture [177] and the pharmacokinetics of this potentially useful antiviral drug have been studied in dogs an rats [178]. Unfortunately, the potential of this drug for the treatment of the common cold [179] is severely limited by its poor oral bioavailability in man. Nevertheless, Bopp and co-workers [178,180] have provided a great deal of interesting information on the analysis of **22** in biological samples. They emphasized the importance of being able to to measure enviroxime (**22**) in the presence of its *syn*-oximer isomer, zinviroxime (**22a**, structure not shown), which is less biologically active. Initial studies aimed at developing a GC assay for **22** and **22a** were unsuccessful. Derivatization of the oxime groups of **22** and **22a** was necessary to make the analytes volatile and this resulted in products that could not be separated by GC. Whereas the two isomers could be separated by LC, UV detection did not produce detection limits in the low ng/ml range that were needed for pharmacokinetic investigations. Fortunately, cyclic voltammetric analysis of **22** showed a nonreversible oxidation with a maximum at about +0.8 V *vs.* Ag/AgCl, suggesting that ED would be a viable alternative to UV. Accordingly, an LC–ED method for **22** was developed that had limits of detection of 4, 15 and 20 ng/ml in plasma,

nasal washings and urine, respectively. It was indicated that the developed method for **22** could be used to study the toxicology, pharmacology, formulation, dose ranging and clinical aspects of enviroxime (**22**), and its pharmacokinetic application was demonstrated in the dog following oral dosing. Interestingly, the amount of zinviroxime (**22a**) found in plasma was only 5–10% that of enviroxime (**22**) following oral dosing with enviroxime (**22**). However, when zinviroxime (**22a**) was administered orally, the major component in plasma was enviroxime (**22**), suggesting that an equilibrium mixture is formed by either metabolism or acid-catalyzed isomerization in the intestine.

3.5. Foscarnet

Forscarnet (trisodium phosphonoformate, hexahydrate) (**23**) is a compound with demonstrated activity against a variety of human viruses *in vitro* [181]. Recently, foscarnet (**23**) has been tested clinically in bone-marrow transplant patients suffering from servere herpes and cytamegalovirus infections [182] and for the treatment of AIDS [183,184]. From a bioanalytical perspective, foscarnet (**23**) has several undesirable physical–chemical properties. In particular, it is an extremely hydrophilic salt of a triprotic acid with pK_a values of 0.5, 3.4 and 7.3. Therefore, it is difficult to retain on hydrocarbonaceous supports and impossible to extract from biological fluids into organic fluids. Additionally, it has no UV absorption above wavelengths of 205 nm and is therefore difficult to detect in LC eluates.

GC–MS was utilized by Ringden *et al.* [182] to study the pharmacokinetics of foscarnet (**23**) in bone marrow-transplant patients While they claimed a detection limit of 50 ng/ml for foscarnet (**23**) by GC–MS, no experimental details were provided and Pettersson *et al.* [183] have criticized the laborious sample work-up. Pettersson *et al.* [183] have described an alternative LC assay for foscarnet (**23**) in biological fluids that employs a reversed-phase ion-pair separation and ED. This method has a detection limit of about 70 ng/ml which is adequate for pharmacokinetic investigations following intravenous administration of foscarnet (**23**) [184]. However, Sjövall *et al.* [184] found that the plasma concentrations were consistently below the limit of detection of the LC–ED method in four of six patients receiving 4 g of foscarnet by mouth. For the patients in which drug was detected after oral dosing, the bioavailability was estimated at 12–22%.

Anion-exchange or reversed-phase ion-pair chromatography would be logical choices for the separation of foscarnet (**23**) from biological samples; however, both these techniques gave very poor peak shape (Fig. 23). Pettersson *et al.* [183] have shown that foscarnet (**23**) can be retained on an UltroPac C_{18} column by the addition of the very hydrophobic ion-pairing agent tetrahexylammonium (THA) hydrogensulfate to the mobile phase. They also demonstrated that excellent peak shape could be obtained (Fig. 23) by the addition of pyrophosphate to the mobile phase at concentrations between 0 02 and 0.20 m*M*. Retention decreased with

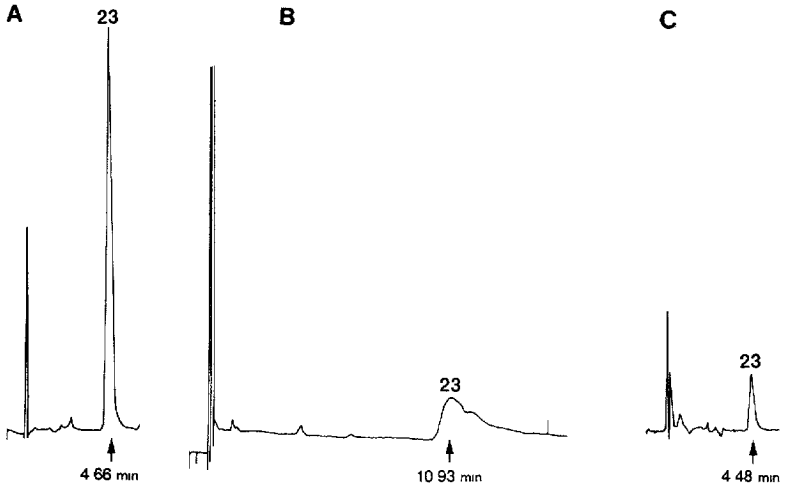


Fig. 23 Influence of pyrophosphate on reversed-phase ion-pair chromatography of foscarnet (**23**) (A and B) and a chromatogram (C) of plasma extract from a human subject 8 h after administration of foscarnet (intravenously, 45 mmol) The calculated concentration of foscarnet in the plasma sample was 79 μmol. Key (A) mobile phase without pyrophosphate (B and C) mobile phase with pyrophosphate. Other chromatographic conditions as Table 2 (Reproduced from ref 183 with permission)

increasing concentration of pyrophosphate, presumably because of competition with the analyte for the oppositely charged pairing ion. Retention and separation from biological material could also be regulated by the pH of the mobile phase and the concentrations of methanol, THA and sodium sulfate. The plasma samples were prepared by ultrafiltration and the recovery was 90%, indicating low binding of foscarnet (**23**) to plasma proteins. Chromatographic interferences by endogenous compounds that were evident at low concentrations of foscarnet (**23**) were removed by shaking the plasma ultrafiltrates or diluted urine samples with charcoal. Dual-cell coulometric detection (ESA) was utilized with the first cell set at +0.75 V and the second cell set at +0 90 V.

Generally, the detection limit of this LC–ED method (70 ng/ml) for foscarnet (**23**) in plasma is inadequate for pharmacokinetic studies following oral administration of the drug, thus development of a more sensitive detection system for foscarnet (**23**) would seem desirable. Forsman *et al.* [185] have described a post-column derivatization procedure for the detection of foscarnet (**23**) in aqueous LC eluents. This detection system involved the sequential oxidation of **23** to phosphate with bromine followed by reaction of the phosphate with molybdovanadate to produce a species which was monitored at 340 nm. The excess bromine, which would have otherwise given an unacceptable background signal, was reduced with sulfite. Unfortunately, the detection limit of the post-column derivatization procedure for **23** is 20 μM which is considerably higher than that obtainable by ED [184]. Nevertheless, the post-column derivatization method [185]

may be useful in non-biological applications and it could also be useful as a detection technique for flow-injection analysis.

3.6. Ampligen

Ampligen (**24**) is a biological response modifier with demonstrated activity *in vitro* agaist three genetic strains of HIV in different lymphoid cell targets [186]. Carter *et al.* [187] have shown progressive reductions of HIV load in ten patients with AIDS, AIDS-related complex (ARC) or lymphadenopathy syndrome (LAS) following twice weekly 1-h infusions of ampligen (200–250 mg/m^2). Chemically, ampligen (**24**) is a synthetic mismatched double-stranded RNA.

Rosenblum and Cheung [188] have shown that ampligen (**24**) can be determined in the plasma of AIDS patients by LC. In this method the drug was separated from plasma on small (5 ml) polypropylene columns packed with DE-52 ion-exchange resin and then degraded to its primary components cytidine and inosine by nuclease and alkaline phosphatase Inosine and cytidine derived from ampligen (**24**) were then determined by reversed phase LC on μBondapak C_{18} with a mobile phase of aqueous sodium acetate (0.5 *M*, pH 6.5). The limit of detection of the method for ampligen (**24**) in plasma was 100 ng/ml, which was more than sufficient to study the pharmacokinetics of **24** in AIDS patients. Following a 1-h intravenous infusion of 640 mg/m^2 ampligen (**24**), the peak concentration in plasma was about 60 μg/ml and the concentration had decreased to about 2 μg/ml after 6 h. Analysis of patient samples containing ampligen (**24**) prior to *in vitro* enzymatic degradation showed no inosine or cytidine, which would have been indicative of metabolism of ampligen (**24**) *in vivo*. Of course, the method did not distinguish between ampligen (**24**) and smaller fragments of ampligen (**24**) arising from partial degradation *in vivo*.

4 CONCLUSIONS

Over the last ten years, significant strides have been made in the diagnosis and treatment of viral diseases. Several effective drugs are available for the treatment of many viral diseases and new diagnostic tests enable the identification of specific viral etiologies. The mandatory testing of new drugs, in experimental animals and man, necessitates the development of sensitive and specific methods of analysis. Many new antiviral drugs will require therapeutic drug monitoring to achieve the optimum therapeutic effect and to minimize adverse reactions. The need for therapeutic drug monitoring will be particularly important in patients who are seriously ill and may therefore be especially susceptible to the adverse effects of potent antiviral drugs. Chromatography has been the technique of choice for the bioanalysis of antiviral drugs and it is particularly useful when the analysis of metabolites is necessary. Much has been published on the chromatographic analysis of antiviral drugs in biological samples since this subject was last reviewed [5].

This growth in the literature will continue in parallel with the development of new antiviral agents and their eventual therapeutic application.

5 SUMMARY

The present review has concentrated on chromatographic techniques for the quantitative determination of antiviral drugs in biological samples. Special attention has been paid to the elements of chromatographic assays that are essential to ensure selectivity, sensitivity, accuracy and precision of the various methods. Wherever possible, attempts have been made to determine the suitability of the methods for application to investigations in pharmacokinetics in man and experimental animals, biopharmaceutics, therapeutic drug monitoring, metabolism and pharmacology Because of the serious consequences of infection from material contaminated with viruses, special consideration has been given to the handling of contaminated samples. It was convenient to divide the antiviral drugs for the purpose of this review into two groups, the nucleoside and the non-nucleoside antiviral drugs The nucleosides discussed are vidarabine, cytarabine, ribavirin, riboxamide, acyclovir, ganciclovir, desciclovir, carbovir, 2′,3′-dideoxyadenosine, 2′,3′-dideoxycytidine, zidovudine, 2′,3′-dideoxyinosine, 2′,3′-didehydro-3′-deoxythymidine, idoxuridine, 5-(2-bromovinyl)-2′-deoxyuridine, 2′-fluoro-5-iodoaracytidine and 5-iodo-2′-deoxycytidine. The non-nucleoside antiviral drugs discussed are arildone, amantidine, rimantidine, moroxydine, enviroxime, foscarnet and ampligen.

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