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Review

Separation methods for acyclovir and related antiviral compounds

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Abstract

Acyclovir (ACV) is an antiviral drug, which selectively inhibits replication of members of the herpes group of DNA viruses with low cell toxicity. Valaciclovir (VACV), a prodrug of ACV is usually preferred in the oral treatment of viral infections, mainly herpes simplex virus (HSV). Also other analogues such as ganciclovir and penciclovir are discussed here. The former acts against cytomegalovirus (CMV) in general and the latter against CMV retinitis. The action mechanism of these antiviral drugs is presented briefly here, mainly via phosphorylation and inhibition of the viral DNA polymerase. The therapeutic use and the pharmacokinetics are also outlined. The measurement of the concentration of acyclovir and related compounds in biological samples poses a particularly significant challenge because these drugs tend to be structurally similar to endogenous substances. The analysis requires the use of highly selective analytical techniques and chromatography methods are a first choice to determine drug content in pharmaceuticals and to measure them in body fluids. Chromatography can be considered the procedure of choice for the bio-analysis of this class of antiviral compounds, as this methodology is characterised by good specificity and accuracy and it is particularly useful when metabolites need to be monitored. Among chromatographic techniques, the reversed-phase (RP) HPLC is widely used for the analysis. C_{18} Silica columns from 7.5 to 30 cm in length are used, the separation is carried out mainly at room temperature and less than 10 min is sufficient for the analysis at 1.0–1.5 ml/min of flow-rate. The separation methods require an isocratic system, and various authors have proposed a variety of mobile phases. The detection requires absorbance or fluorescence measurements carried out at 250–254 nm and at λ_{ex}=260–285 nm, λ_{em}=375–380 nm, respectively. The detection limit is about 0.3–10 ng/ml but the most important aspect is related to the sample treatment, mainly when body fluids are under examination. The plasma samples obtained from human blood are pre-treated with an acid or acetonitrile deproteinization and the supernatant after centrifugation is successively extracted before RP-HPLC injection. Capillary Electrophoresis methods are also discussed. This new analytical approach might be the expected evolution, in fact the analyses are improved with regard to time and performance, in particular coated capillary as well as addition of stabilisers have been employed. The time of analysis is shortened arriving at less than half a minute. Furthermore by using an electrochemical detection, and having a calibration linearity in the range of 0.2–20.0 ng/ml, the detection limit is 0.15 μg/ml. The measurements of acyclovir and penciclovir have been presented but in the future other related drugs will probably be available using CE methods.

Keywords: Reviews; Acyclovir; Valaciclovir; Ganciclovir; Penciclovir

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Contents

1. Introduction

Acyclovir (9-[2-hydroxyethoxymethyl]-9H-guanine, ACV) is an acyclic analog of the natural nucleoside 2′-deoxyguanosine (Fig. 1A), which selectively inhibits replication of members of the herpes group of DNA viruses [1,2].

Shortly after it had been described as a potent and selective inhibitor of the replication of herpes simplex virus (HSV) and, to a lesser extent, of varicella-zoster virus (VZV), ACV became the drug of choice for the treatment of HSV and VZV infections, particularly primary and recurrent genital herpes and mucocutaneous HSV and VZV infections in immunosuppressed patients [3].

Because of its limited oral bioavailability (only 20%), acyclovir has, in turn, been replaced by its prodrug, valaciclovir (VACV, the L-valyl ester of acyclovir), in the oral treatment of HSV and VZV infections [4].

The remarkable potency of acyclovir has prompted the development of several structural analogs (Fig. 1B) [5]. Foremost among these acyclovir congeners are ganciclovir (GCV, 9-[1,3-dihydroxypropoxymethyl]guanine) and penciclovir (PCV, 9-[4-hydroxy-3-hydroxymethyl-but-1-yl]guanine). Ganciclovir has a more pronounced activity against cytomegalovirus (CMV) than acyclovir and became the drug of choice for the treatment of CMV infections in immunosuppressed patients [6]. Penciclovir has a similar activity spectrum and mechanism of action to acyclovir and has been used under its oral prodrug form, famciclovir (FCV, the diacetyl ester of 6-deoxypenciclovir), for the same indications as valaciclovir [7,8].

1.1. Mechanism of action and antiviral activity in vitro

As described in the review of Richards et al. [9], acyclovir exhibits a selective inhibition of herpes virus replication, with extremely low toxicity towards uninfected host cells. This selectivity derives from the specific, sequential phosphorylation of acyclovir to acyclovir triphosphate into infected cells.

The initial phosphorylation to acyclovir monophosphate is catalysed by herpes virus-coded thymidine kinase (Fig. 2). Acyclovir monophosphate is subsequently converted to the diphosphate via cel-

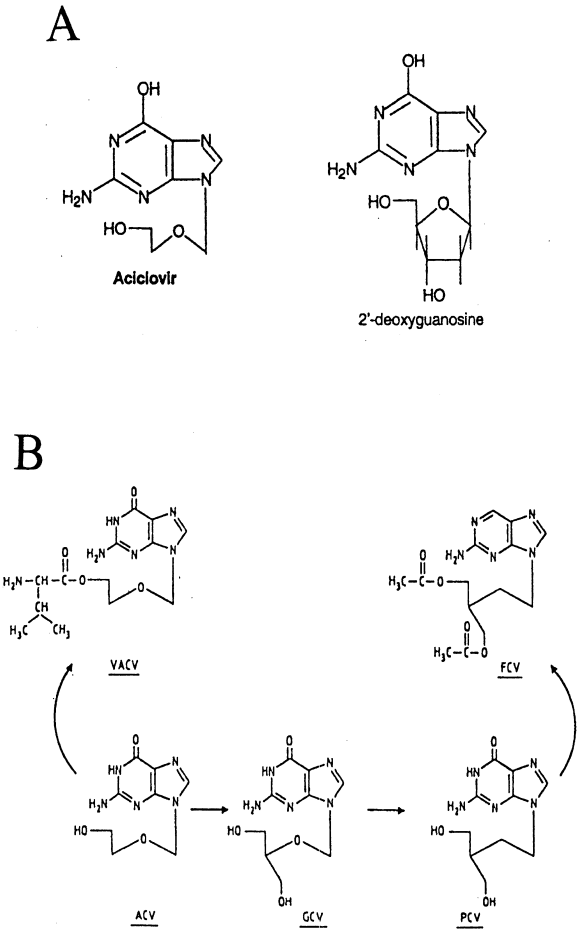

Fig. 1. (A) Chemical structure of acyclovir and 2′-deoxyguanosine; (B) Structural analogs of acyclovir. ACV, acyclovir, 9-(2-hydroxyethoxymethyl)guanine; GCV, ganciclovir, 9-(1,3-dihydroxypropoxymethyl)guanine; PCV, penciclovir, 9-(4-hydroxy-3-hydroxymethyl-but-1-yl)guanine; VACV, valaciclovir, L-valyl ester of acyclovir; FCV, famciclovir, diacetyl ester of 6-deoxypenciclovir.

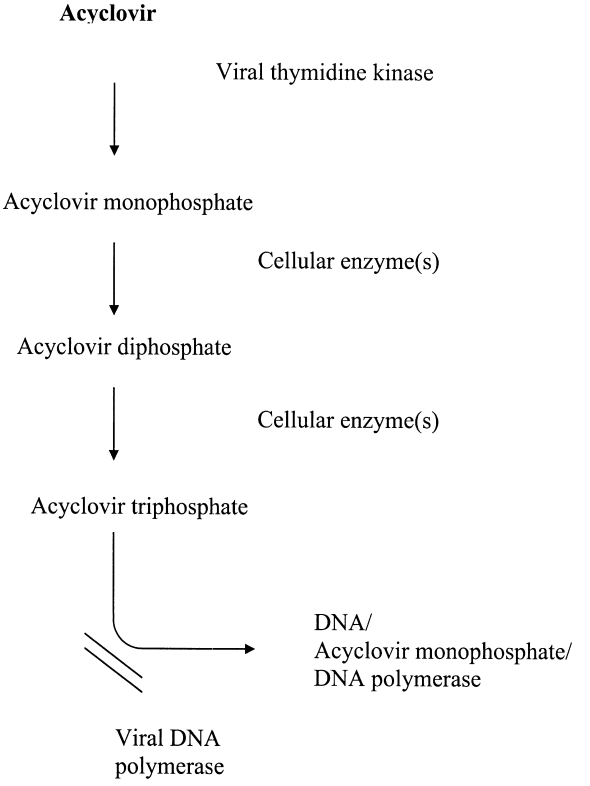

Fig. 2. Acyclovir inhibition of viral DNA synthesis. Acyclovir competes with deoxyribonucleosides for viral thymidine kinase or cellular kinases. In addition to competitively inhibiting the association of deoxyribonucleoside triphosphates with viral DNA polymerase, acyclovir triphosphate incorporates in the growing viral DNA chain, leading to the termination of DNA synthesis because of its lack of a 3′-hydroxyl moiety. Acyclovir monophosphate is not excised from the primer template by the 3′,5′ exonuclease activity of viral DNA polymerase, but binds strongly to inactivate the polymerase [12].

lular guanylate kinase, and then to the triphosphate via other host cell enzymes. Acyclovir triphosphate is the active metabolite and functions as both a substrate for and preferential inhibitor of viral DNA polymerase. In competition with the natural nucleoside, deoxyguanosine triphosphate, acyclovir triphosphate binds to herpes simplex virus DNA polymerase and is incorporated into a DNA primer template, thus preventing further elongation of the DNA chain [10,11]. Of the human herpes viruses, herpes simplex types 1 and 2 (HSV-1 and HSV-2) are the most susceptible to acyclovir in cell culture, followed in descending order of general susceptibility by varicella-zoster virus (VZV), Epstein–Barr virus (EBV), human herpes virus 6 (HHV-6) and cytomegalovirus (CMV) (see Table 1) [13]. The activity of acyclovir is generally similar to or greater than that of most other antiviral agents tested against HSV and VZV, greater than that of penciclovir against EBV, less than that of ganciclovir, idoxuridine and vidarabine against CMV and less than that of ganciclovir and foscarnet against HHV-6. Acyclovir does not appear to exhibit in vitro antiviral activity against viruses outside the herpes group [14].

Table 1
Summary of in vitro antiviral activity of acyclovir

Virus	IC_{50}[a] (mg/l)	Comparative activity								Refs.
		Brivudine	FIAC	Foscarnet	Ganciclovir	Idoxiridine	Interferon-α	Penciclovir	Vidarabine	
HSV-1	0.01–2.7	=	−/=	+	−/=	+	+	+/=	+	[9]
HSV-2	0.01–4.4	+	+/=	+	−/=	+	+	+/=	+	
VZV	0.17–26	−		+	+	=		=	+/=	[12]
EBV	1.5–8.8							+		
HHV-6	3–25			−	−					[13]
CMV	1.82–68			+	−	−		+	−	

Abbreviations and symbols: CMV=cytomegalovirus; EBV=Epstein–Barr virus; FIAC=2′-fluoro-5-iodoarabinosylcytosine; HHV-6=human herpes virus-6; HSV=herpes simplex virus; VZV=varicella-zoster virus; + indicates antiviral activity of acyclovir greater than that of comparative antiviral; − indicates antiviral activity of acyclovir less than that of comparative antiviral; = indicates antiviral activity of acyclovir similar to that of comparative antiviral. (Reproduced from Ref. [13], with permission).

[a] Concentration of acyclovir inhibiting viral-induced cytopathogenicity of viral plaques by 50%.

Valaciclovir, the L-valyl ester of acyclovir, is an oral prodrug that undergoes rapid and extensive metabolism to yield acyclovir and the essential amino acid L-valine [15,16]. Thus, the antiviral activity of valaciclovir is the same as that of acyclovir. Ganciclovir has demonstrated good in vitro activity against human cytomegalovirus and is considerably more potent (about 26 times) than acyclovir against this organism [17]. As well as acyclovir, ganciclovir is phosphorylated (by the viral UL97 protein) to form an active nucleotide [18,19]. After its formation, ganciclovir 5′-monophosphate is further phosphorylated by cellular kinases to the di- and tri-phosphate forms. Ganciclovir triphosphate competitively inhibits the incorporation of dGTP into DNA and is also directly incorporated into viral DNA, impeding and prematurely terminating its elongation [20,21].

Famciclovir, a synthetic acyclic guanine analog, is a prodrug, which after oral administration, is rapidly metabolised to the highly bioavailable antiviral compound penciclovir [22]. Similarly to acyclovir and ganciclovir, penciclovir is selectively phosphorylated (initially by thymidine kinase) in herpes virus-infected cells to yield high intracellular concentration of penciclovir triphosphate which inhibits viral replication [23]. Penciclovir is active in vitro against HSV-1, HSV-2, VZV and EBV [24]. Like acyclovir, famciclovir has limited activity against CMV [25,26].

1.2. Therapeutic use

Intravenous, oral and to a lesser extent topical acyclovir is well established in the treatment of herpes simplex infection, with significant therapeutic benefit in genital herpes simplex and recurrent orofacial herpes simplex [12,13]. Intravenous acyclovir is the treatment of choice in herpes simplex encephalitis in adults, and has also shown benefit in the treatment of severe complications of HSV infection in pregnancy and neonatal HSV infections [27–29]. Ophthalmic application of acyclovir ointment rapidly heals herpetic dendritic corneal ulcers and superficial herpetic keratitis [30]. Use of acyclovir is effective but controversial in the treatment of otherwise healthy individuals with varicella (chickenpox) [31–35], whereas it has been recommended for the treatment of herpes zoster (shingles) [36–38].

Acyclovir also appears to offer partial protection from invasive CMV disease in CMV-seropositive bone marrow transplant recipients [13,39,40].

Valaciclovir is an effective treatment for herpes zoster in immunocompetent adults [41]. Importantly, valaciclovir was reported to be significantly more effective than acyclovir in reducing the duration of zoster-associated pain [42]. Results of several studies indicate that valaciclovir is as effective as acyclovir in the treatment of genital herpes [15,16] and significantly prolonged the time to a recurrent episode

of infection compared with placebo [43]. Thus, valaciclovir may ultimately succeed acyclovir as a first-line treatment for genital herpes or herpes zoster.

The analogue ganciclovir effectively treats infection of human cytomegalovirus in various immunocompromised groups of patients [44], for example in patients with acquired immune deficiency syndrome (AIDS)-related cytomegalovirus retinitis [45,46]. AIDS-related gastrointestinal and, to a lesser extent, pulmonary cytomegalovirus infection also respond to treatment with ganciclovir [47–49]. Ganciclovir is also useful against cytomegalovirus infection in organ transplant recipients [44]. The drug is most effective when given prophylactically or as early treatment for asymptomatic infection in bone marrow transplant recipients [50,51]. However, established infection in solid organ transplant recipients also appears to respond to treatment with ganciclovir [52,53]. In conclusion, ganciclovir has an important role in the prevention of CMV disease after bone marrow and liver transplantation and is likely to gain wider clinical use in heart, lung and kidney transplant recipients [54–56].

Famciclovir is an effective treatment of immunocompetent patients with acute herpes zoster (shingles) caused by VZV [57], with a therapeutic efficacy similar to that of oral acyclovir [22]. Moreover, famciclovir is reported to be the first antiviral agent to significantly reduce symptoms associated with multiple genital herpes lesions [58,59]. Thus, famciclovir is now established as an effective treatment of immunocompetent patients with herpes zoster and genital herpes infection [60].

1.3. Pharmacokinetic profile

The pharmacokinetics of intravenously administered acyclovir are best described by a 2-compartment open model [61]. The pharmacokinetic disposition of the drug is not affected by dose, duration or frequency of administration. Mean plasma acyclovir concentrations at steady state (6.7–20.6 mg/l) after intravenous administration in immunocompromised patients (2.5–15 mg/Kg every 8 h) are similar to the peak plasma concentrations obtained with equivalent single doses [62]. Absorption of oral acyclovir across the small intestine appears to be passive [63,64] and is incomplete, resulting in 15–30% bioavailability and mean peak plasma concentrations (C_{max}) 1.5–2.5 h post dose (Table 2).

There is no systemic absorption of topical acyclovir from the ointment, but 30–50% of the drug reaches the basal epidermis in cutaneous infections treated with the cream formulation [65–67]. Substantial intraocular penetration is evidenced by a mean acyclovir concentration of 1.7 mg/l in aqueous humour with multidose application of the 3% ointment every 5 h [68].

Plasma protein binding occurs in a range of 9–33%, irrespective of plasma acyclovir concentration [69]. Acyclovir appears to be distributed to a wide range of tissues and fluids in humans after oral and intravenous administration (Table 2), as it was detected at autopsy in the kidney, lung, nervous tissue, liver and heart, cerebrospinal fluid, saliva and tear fluid [70–72].

Although acyclovir appears to accumulate in breast milk, exposure of the nursing child is estimated to be less than 1 mg/day, presenting a low theoretical risk [73,74]. The elimination half-life ($t_{1/2}$) of acyclovir after intravenous administration is 2–3 h, and the mean total body clearance (CL) is 15.6 l/h/1.73 m^2 (Table 2). The main metabolite of acyclovir, 9-carboxymethoxymethyl guanine, is pharmacologically inactive and accounts for up to 14% of an acyclovir dose in recipients with normal renal function. A minor metabolite, 8-hydroxy-9-[2-(hydroxyethoxy)methyl]guanine, represents less than 0.2% of a dose [9]. The main route of elimination of acyclovir is via renal excretion, with 45–79% of an intravenous dose recovered unchanged in the urine. In neonates, $t_{1/2}$ is slightly longer (2.5–5 h), whereas in infants aged $\geq$1 year the pharmacokinetics of acyclovir are generally comparable with those of adults [75].

As the kidneys are the principal route of acyclovir elimination, renal impairment affects the plasma concentrations, extent of metabolism and rate of elimination of the drug. In patients with end-stage renal failure who are administered acyclovir, mean C_{max} values are nearly doubled, mean $t_{1/2}$ is increased 10-fold to approximately 20 h, and mean CL

Table 2
Pharmacokinetic profile of acyclovir

Parameter/medium	Acyclovir route and dosage	Value	Refs.
Absorption			
C_{max}	IV, 5 mg/Kg	8.8 mg/l, varies with IV dose	[9]
	Oral, 200–800 mg	in approximate linear fashion 0.35–1.6 mg/l	[12]
t_{max}	Oral	1.5–2.5 h	
C^{SS}	IV 2.5–15.0 mg/Kg q8h	6.7–20.6 mg/l	[13]
	Oral 200–400 mg q4h	0.52–1.22 mg/l	
Time to C^{SS}	Oral	1–2 d	
Bioavailability	Oral	15–30%	
Topical absorption	3% ointment q5h (4–6 doses)	1.7 mg/l in aqueous humour	[61]
Distribution			
Kidney	IV 400–1200 mg/m^2 q8h	1000% of plasma values	[62]
Lung, liver, heart		Approx. 130% of plasma values	
Brain, spinal cord		25–70% of plasma values	[63]
CSF	IV infusion	50% of plasma values	
	Oral 800 mg q8h	13–52% of plasma values	[64]
Saliva	Oral 200 or 400 mg	13% of plasma values	
Tears	Oral 2000 mg/d	18% of plasma values	[65]
Aqueous humour	Oral 400 mg (5 doses in 24 h)	30–50% of plasma values	
Placental cord blood	Oral 200 or 400 mg q8h	0.1–0.7 mg/l (60–99% of maternal plasma values)	[66]
Amniotic fluid		0.1–2.6 mg/l (300–600% of maternal plasma values)	[67]
Breast milk		Up to 324% of maternal plasma values	[68]
	Oral 1000 mg/d		
Vd		48 (range 22.5–101) l/1.73 m^2	[69]
Plasma protein binding		9–33% independent of plasma acyclovir concentration	[70]
	IV, resulting in plasma acyclovir concentrations of 0.4–5.1 mg/l		[71–75]
Metabolism and elimination			
$t_{1/2}$	IV 0.5–15.0 mg/Kg	2–3 h	
$t_{1/2}$ Neonates		2.5–5.0 h independent of dose	
$t_{1/2}$ CSF	IV infusion 50 mg/Kg	28 h	
Renal excretion	IV	45–79% of dose	
CL		15.6 (range 5.5–30.2) l/h/1.73 m^2	
CL neonates		3.5–10.1 l/h/1.73 m^2	
CL_R		75–80% of CL	
Mean % of dose recovered unchanged in urine		45–79%, decreasing with decreased creatinine clearance	

Abbreviations: C_{max}=peak plasma acyclovir concentration; C^{SS}=steady-state plasma concentration; CL=total body clearance; CL_R=renal clearance; CSF=cerebrospinal fluid; d=days; h=hours; IV=intravenous; q=every; t_{max}=time to peak plasma concentration; $t_{1/2}$=elimination half-life; Vd=volume of distribution.

is decreased 10-fold compared with patients with normal renal function [9].

Valaciclovir is readily absorbed after oral administration and the bioavailability of acyclovir following valaciclovir administration is greater than after oral aciclovir administration (54 vs. 12–20%) [15]. After single 100–1000 mg doses of valaciclovir, the area under the plasma concentration–time curve (AUC) for acyclovir ranged from 2.28 to 19.5 mg/l×h [16]. The mean plasma elimination half-life ($t_{1/2}$) of

acyclovir after administration of single oral valaciclovir doses (100–1000 mg) to healthy volunteers was 2.62–3.13 h [15]. In patients with end-stage renal diseases the $t_{1/2}$ of acyclovir after a single 1000 mg dose of valaciclovir was prolonged to 14 h. After absorption from gastrointestinal tract, valaciclovir undergoes rapid first-pass intestinal or hepatic hydrolysis, giving acyclovir and L-valine as the main products of hydrolysis [76]. Two other compounds, 9-[(carboxymethoxy)methyl]guanine and 8-hydroxy-9-[2-(hydroxyethoxy)methyl]guanine, are the results of further partial metabolism of acyclovir and are both pharmacologically inactive [77]. The kidneys are the main route of elimination of valaciclovir and its metabolites, although valaciclovir, as acyclovir, is also excreted in the faeces. After single or multiple doses (100–2000 mg) of valaciclovir, acyclovir accounted for 80–85% of the recovered drug in urine, 7–12% was recovered as metabolites and <1% as valaciclovir.

The pharmacokinetic properties of intravenous ganciclovir are again best described by a 2-compartment open model with mean peak drug concentrations increasing in a linear fashion over a 1–5 mg/Kg dosage range [17,44]. A single oral 10 mg/Kg dose of ganciclovir solution produces peak plasma concentrations of between 0.23 and 0.35 mg/l approximately 1.5 h after administration [78]. The bioavailabilty of orally administered ganciclovir is approximately 6%. Ganciclovir is minimally (1–2%) bound to plasma proteins with a steady-state volume of distribution after intravenous administration of 32–45 l/1.73 m^2 [6]. Other than its phosphorylation in infected cells, ganciclovir is not metabolised. The drug is excreted almost exclusively via glomerular filtration. Elimination is biphasic, with a half-life ($t_{1/2}$) of 2–4 h after intravenous administration of 5 mg/Kg [6] and about 4.5 h after oral administration (3000 mg/day) [79]. Renal dysfunction increases plasma concentrations of ganciclovir and reduces elimination of the drug ($t_{1/2}$ was 9–30 h in patients with decreased creatinine clearance) and dosage reductions are therefore required [6,80].

After oral administration, famciclovir is rapidly and extensively absorbed in the upper intestine [81,82]. Thereafter, famciclovir undergoes substantial presystemic metabolism in the intestinal wall and liver (deacetylation and oxidation) to yield penciclovir [81]. Penciclovir is highly bioavailable (77%) and has a linear dose-proportional pharmacokinetic profile over the 125–750 mg dose range [83]. In healthy volunteers or patients with uncomplicated herpes zoster infection, C_{max} of penciclovir ranged from 2.73 to 3.97 mg/l within 1 h of a single 500 mg dose of famciclovir [84]. Penciclovir is excreted primarily by the renal route, and elimination of famciclovir was found to decrease in patients with varying degrees of renal impairment. Following administration of single doses of famciclovir 125, 500 and 750 mg to healthy volunteers, $t_{1/2}$ for penciclovir ranged from 2.06 to 2.66 h [22].

Relevant pharmacokinetic parameters of valaciclovir, ganciclovir and famciclovir/penciclovir are summarised in Table 3, in comparison with those of acyclovir.

1.4. Drug monitoring

The quantitation of acyclovir and related compounds in biological samples poses a particularly significant challenge because these drugs tend to be structurally similar to endogenous substances. This fact makes analysis complicated and requires the use of highly selective analytical methodology. Moreover, acyclovir and some related drugs tend to be metabolised to products that can coelute with the original compound. In particular, acyclovir is metabolised mainly to 9-carboxymethoxymethyl guanine (14%) and to a lesser extent to 8-hydroxy-9-[2-(hydroxyethoxy)methyl]guanine (<0.2%), which are pharmacologically inactive [9]. The main products of hydrolysis of the prodrug valaciclovir are L-valine and acyclovir [77], which is further metabolised as described above. Penciclovir is the principal metabolite found in plasma and urine after administration of famciclovir; minor metabolites include monoacetylated penciclovir and monoacetylated 6-deoxypenciclovir [81]. Therefore, chromatographic methods for the analysis of these antiviral drugs in biological fluids must be capable of separating and quantitating the metabolites as well as parent compounds.

Therapeutic drug monitoring of acyclovir and related compounds is useful mainly in situations where toxic events are experienced at clinically

Table 3
Comparison of pharmacokinetic parameters of acyclovir, valaciclovir, ganciclovir and famciclovir/penciclovir in healthy adult volunteers

Parameter	Acyclovir	Valaciclovir	Ganciclovir	Famciclovir/ Penciclovir	Refs.
C_{max}	8.8 mg/l (with IV 5 mg/Kg dose) 0.35–1.6 mg/l (with oral 200–800 mg dose)	0.83–6.65 mg/l, depending on dosage	7.0 mg/l (with IV 1–5 mb/Kg dose) 0.23–0.74 mg/l (with oral 10–20 mg/Kg dose)	2.73–3.97 mg/l	[15] [16]
t_{max}	1.5–2.5 h	0.88–2.29 h	1.5 h	1 h	[76]
AUC	88.6 mg/l×h	2.28–19.5 mg/l×h		2.2–18.7 mg/l×h	[77]
Bioavailability	15–30%	54%	5.4–7.1% (mean 6%)	77%	[17]
Vd	22.5–101 l/1.73 m^2	49.7 l/1.73 m^2	32–45 l/1.73 m^2		
Plasma protein binding	9–33%	13.5–17.9%	1–2%		[44] [6,78–80]
$t_{1/2}$	2–3 h	2.62–3.13 h	2–4.5 h	2.06–2.66 h	[81]
Renal excretion	45–79% of dose	<1% valaciclovir 80–85% acyclovir	100% of dose	72% of dose	[82]
CL	15.6 l/h/1.73 m^2		12 l/h/1.73 m^2		[81]
CL_R	15.3 l/h	As acyclovir		21.0–31.9 l/h	[83]
Mean % of dose recovered unchanged in urine	45–79%	<1%	100%	50.9–64.6%	[84] [22]

Abbreviations: AUC=area under the plasma concentration–time curve; C_{max}=peak plasma concentration; CL=total body clearance; CL_R=renal clearance; h=hours; IV=intravenous; t_{max}=time to peak plasma concentration; $t_{1/2}$=elimination half-life; Vd=volume of distribution.

relevant dosages. Acyclovir has a large therapeutic index and after more than a decade of therapeutic use, it has been shown that it is well tolerated in a wide variety of diseases, population types and age groups. However, recent reports of possible neurotoxic effects of acyclovir in patients with renal disease has renewed interest in identifying reasons behind the inter-patient variations in the kinetics of the drug [85–87]. Regarding related compounds, ganciclovir has been associated with serious toxic side effects, such as haematological toxicity [17]. Elimination of ganciclovir is again mainly renal, thus monitoring plasma level is useful in renal failure. With regard to famciclovir, because it requires deacetylation and oxidation in the liver to form penciclovir and because hepatic insufficiency could impair this conversion process, it might be useful to study the metabolism of famciclovir in patients with hepatic diseases [88].

Monitoring of acyclovir and structural analogs is also used in case of therapeutic failure to differentiate how much is due to individual pharmacokinetics and how much to viral resistance [89]. In fact, all antiviral drugs, including acyclovir and related compounds, exhibit substantial intra- and inter-subject variability in their absorption, distribution, metabolism and elimination leading to wide variability in plasma and tissue concentrations. Because of this variability, the range of concentrations that need to be measured by analytical methods is quite wide.

Moreover, since acyclovir and related compounds are often co-administered with other drugs, there is a strong interest in the ability to determine their concentrations in plasma and serum samples collected from patients in clinical studies, particularly in drug interaction studies. For this reason, the investigations into the pharmacokinetic profile of these compounds need an assay method that must be not only simple, rapid, precise and sensitive, but also capable of being applied to analyse plasma and tissues in the presence of other drugs.

Finally, besides pharmacokinetics studies, determination of antiviral drugs in pharmaceutical formulations is also important concerning standardisation and monitoring of stability of such formulations [90,91].

2. Methods of analysis

2.1. General considerations

Various analytical studies have been carried out to determine drug content in pharmaceuticals, pharmacokinetics and optimal dosing of acyclovir, and related compounds. Drug concentrations were measured either by immunological techniques or reversed-phase high-performance liquid chromatography.

Radioimmunoassay (RIA) [92,93] and enzyme-linked immunoabsorbent assays [94] are very sensitive, but the costs, the large number of slow steps in the experimental procedure and the need to develop antiserum or monoclonal antibodies make these methods disadvantageous.

Furthermore, a highly selective analytical methodology is required because it is quite difficult to measure the amount of antiviral agents in the biological medium showing these drugs a chemical structure quite similar to a number of endogenous substances. Chromatographic techniques have been widely used for the analysis of acyclovir and related compounds in pharmacokinetic investigation as well as for therapeutic drug monitoring purposes in biological samples.

2.2. Chromatographic methods

2.2.1. Sample preparation

Plasma, serum, urine, bulk drug are the samples analysed in relation to the antiviral drug (i.e. acyclovir and ganciclovir) measurements. The assay of antiviral drugs in plasma or serum by reversed-phase high-performance liquid chromatography (RP-HPLC) currently requires sample pre-treatment, but direct sample injection has also been described [95].

Pre-treatment of acyclovir plasma sample can be achieved in different ways: deproteinization with perchloric acid [96–98] and solid–phase extraction (SPE) [99–101].

The injection by HPLC of the acid supernatant after perchloric acid deproteinization contributes significantly to the reduction of the lifetime of the analytical column even when the volume injection is low. In any case, even when a low volume was injected, only 600 samples could be analysed without deterioration of the performance of the column [97,101]. Moreover, the injection of acid supernatants leads to numerous late-eluting peaks. The analysis is disturbed and it is necessary to pass from isocratic to gradient elution to remove the compounds causing these late peaks [102].

Solid-phase extraction (SPE) cartridges were used [99–101] to overcome the above mentioned problems, Svensson [100] used Sep-Pak Light C_{18} cartridge, conditioned by 1 ml of methanol and 1 ml of water. The sample for the extraction was obtained by mixing 500 μl of serum or 100 μl of urine with 500 or 900 μl of saturated sodium chloride solution in water, respectively. The sample solution passed through the cartridge, undertaking a complete absorption of the acyclovir and its metabolite 9-carboxy-methoxy-methyl-guanine. After loading in the cartridge and suitable washing, the elution was carried out with 1 ml of 3% acetonitrile solution in 38 m*M* phosphoric acid. The eluate was collected and 20 μl were injected into the HPLC column. Because of the hydrophobic nature of C_{18} bonded phase, the sorbent must not be allowed to dry out before loading the sample. If it does dry out, the obtained recoveries are absolutely variable. This limitation significantly reduces the number of samples that can be manually processed. Poirier et al. [101] have developed SPE procedure with Oasis HLB extraction cartridges (Waters) containing a polymeric reversed-phase sorbent that exhibited both hydrophilic and lipophilic characteristics. A 250-μl plasma sample was loaded into SPE conditioned cartridges. After washing with 1 ml of water, the drug (acyclovir) was eluted with 750 μl of HPLC mobile phase (see Table 4) and 100 μl aliquot was injected into the HPLC (Fig. 3).

The hydrophilic properties of these new cartridges obviated the difficulty of maintaining moisture encountered with the C_{18} packing and reproducibility was obtained even when the cartridges had run dry [107].

Ganciclovir plasma sample pre-treatment can be achieved with the following methods: acid deproteinization [108,109], acetonitrile deproteinization and chloroform extraction [110,111], ultrafiltration [108].

Perchloric acid (0.8 *M*) was added to serum

Table 4
Acyclovir chromatographic conditions

Guard column	Column (dimension, particle size)	Temperature (°C)	Flow-rate and retention time	Mobile phase	Detection (UV or fluorescence) (nm)	Injection volume (μl)	Detection limit	Quantification limit (ng/ml)	Refs.
	C_{18} Silica column (300×4.6 mm, 10 μm)	R.T.	1.5 ml/min; 6.7–6.9 min	8:92 (v/v) Methanol and 0.05 *M* octane sulfonic acid buffer (pH 2.5)	254	100		20	[98]
	Spherisorb S5-ODS2 μm (250×4.6 mm)	40	1.3 ml/min; 1.7 min	5:95 (v/v) Methanol, and 5 m*M* monopotassium phosphate (pH 3.0)+ 7 m*M* hexylamine	254	10		1000	[103]
C_8 (1 cm, 5 μm)	C_8 Column (150×4.6 mm, 5 μm)		1.0 ml/min; 5.3 min	0.1 *M* Acetate/citrate buffer (pH 3.0)+3.7 m*M* octane sulfonic acid/ methanol (92:8, v/v)	250	100	10 ng/ml	62	[101]
	Supercosil LC_{18} (75 mm, 3 μm)		1.0 ml/min; 1.7–2.0 min	3:97 (v/v) Acetonitrile/ 100 m*M* glycine buffer (pH 2.3)	λ_{ex}=260 λ_{em}=375	20		25	[104]
	Hypersil ODS (150×4.6 mm, 3 μm)	R.T.	1.5 ml/min; 9.8 min	0.02 mol/l Potassium dihydrogenphosphate (pH 3.5)	254	20		100	[97]
	Ultrasphere ODS RP (75×4.6 mm, 3 μm	R.T.	1.5 ml/min; 1.4 min	30 m*M* Phosphate buffer (pH 2.1)+5 m*M* duodecyl sulphate+ 18% acetronitrile)	λ_{ex}=285 λ_{em}=380	20	0.12 μ*M* in serum 0.60 μ*M* in urine		[100]
	C_{18} (250×4.6 mm, 5 μm)	R.T.	1.2 ml/min; 9.0 min	Distilled water	254	50		8 in skin samples	[105]
Perisorb RP-18 (30–40 μm)	LiChrosorb RP-8 (250×4 mm, 7 μm)		1.2 ml/min; 9.4–9.6 min.	1% Acetronitrile+0.02 M disodium hydrogen orthophosphate (pH 2.5)	λ_{ex}=270 λ_{em}=380	50	30 ng/ml		[96]
	Techsphere 5 C_8 (100×4 mm)	R.T.	1.0 ml/min; 14.9 min	1% Ortho-phosphoric acid+octane sulphonic acid 10 g/l	254	20	500 ng/ml		[106]

R.T.: Room temperature.

sample at 1:2 volume ratio. The supernatant was neutralised with phosphate buffer (0.2 *M*), pH 8.0 [108]. This deproteinization, such as that carried out with trichloroacetic acid (50%), was followed by an extraction with chloroform. This extraction procedure eliminates the endogenous interferences which are frequently present in trichloroacetic and perchloric extracts of plasma [109].

Acetonitrile deproteinization was followed by the extraction of the acetonitrile from the supernatant with chloroform [110]. Chloroform was preferred because of its lower polarity and higher density [111]. Ultrafiltration of the sample was carried out with a Centrifree filter (30 000 M_r cut-off) [108].

2.2.2. Sample stability

The analysis of acyclovir stability in plasma samples showed that it was stable when stored at −20°C over a period of 4 weeks. However, the blood sample should be collected and centrifuged without delay at low temperature (4°C) [97,98].

No changes in ganciclovir concentrations were detected in working standard solutions after 1 month of storage at 4°C. Ganciclovir resulted stable in

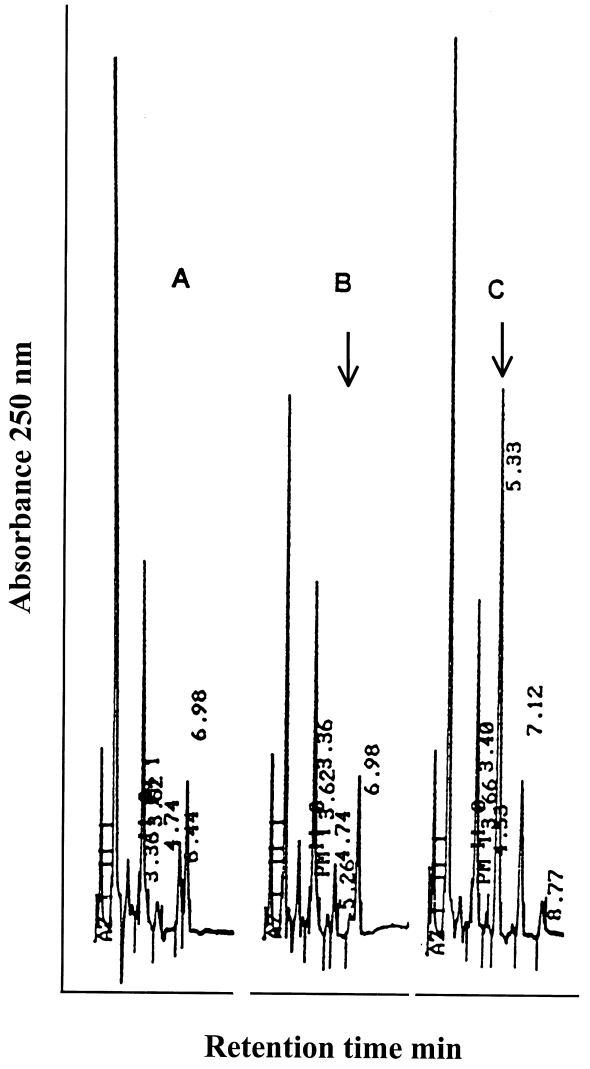

Fig. 3. Chromatograms of 250 μl plasma extracts. (A) Drug-free sample; (B) Drug-free sample spiked with 125 ng/ml of acyclovir; (C) Sample obtained from a patient 8 h after administration of an oral dose of valacyclovir (1000 mg/thrice daily) containing 960 ng/ml of acyclovir. Retention time of acyclovir was 5.3 min [101].

biological fluids when stored at −20°C for at least 6 months. The supernatant (pH 6.5–6.8) after precipitation by perchloric acid must be stored for no more than 48 h at 4°C prior to analysis [101,108,109,112].

Both acyclovir and ganciclovir can be used in patients with acquired immunodeficiency syndrome (AIDS). The effect of heat treatment to inactivate HIV was investigated by heating samples to 56–70°C for 40–70 min. No significant differences were observed between pretreated samples and those kept at room temperature [101,108].

2.2.3. Acyclovir chromatographic conditions

The main chromatographic technological aspects such as column characteristics, elution conditions and detectors are reported in Table 4. RP C_{18} or C_8 columns were mainly utilised for acyclovir determination either with or without a pre-column system.

The analysis was usually carried out at room temperature, but a higher temperature (40°C) was utilised in the analysis of drugs in liposomal formulation. The mobile phase in these methods was composed of a high percent of buffer at pH 2.5–3.5 (see Table 4). The flow-rate averaged from 1.0 to 1.5 ml/min. Spectrophotometric or fluorimetric detectors, working at 254–250 nm or at λ_{ex} 260–285 nm and λ_{em} 375–380 nm, respectively, were usually employed.

The pH of the mobile phase in HPLC influences the analysis performance. At pH 3.0 the acyclovir retention time was 5.3 min; when the mobile phase was adjusted at pH 2.75 or 3.25 a 10% increase or 12% decrease, of retention time of the drug, respectively, was observed [101]. The pH also influences the quantification limit. The value reached by Poirier's method at pH 3.0 was 62.5 ng/ml and the detection limit based on a signal-to-noise ratio of 2:1 was 10 ng/ml. A higher sensitivity can be achieved using spectrofluorimetric detection instead of UV detection, but acyclovir fluorescence is heavily pH dependent, in fact the fluorescence increases dramatically with increasing acidity of the solution below pH 2 [113]. Therefore only a highly acid mobile phase can improve the detection limit of the assay, but extreme pH condition (pH 1.5) may lead to a rapid deterioration of the analytical column [102]. Peh et al. [96] used a mobile phase adjusted to pH 2.5 with 60–62% perchloric acid and obtained a detection limit of 30 ng/ml at a signal-to-noise of 3:1 with fluorimetric detection. This value gave a satisfactory result for pharmacokinetic studies (Fig. 4).

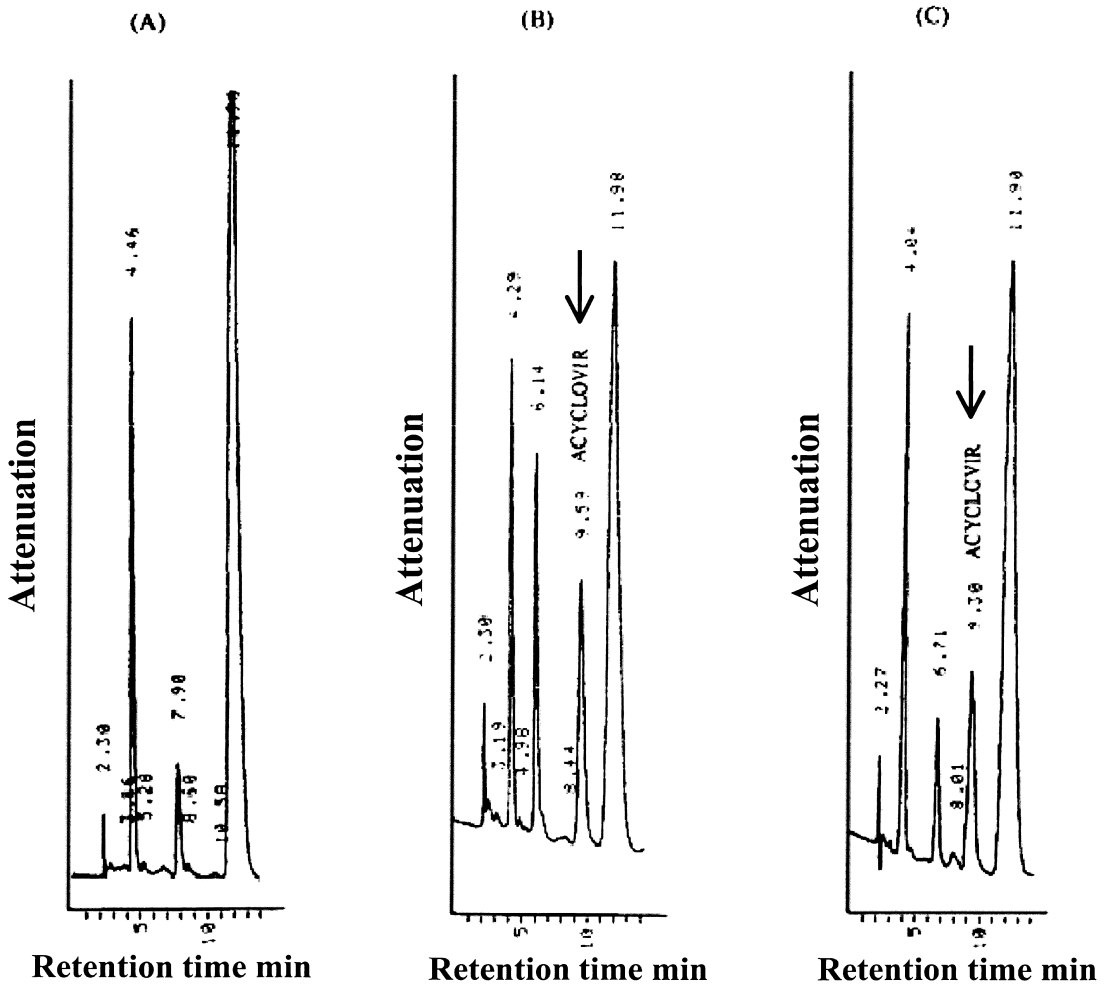

Fig. 4. Chromatograms for the analysis of acyclovir in plasma. (A) Blank plasma; (B) Plasma spiked with 1000 ng/ml acyclovir; (C) A volunteer plasma containing 470.54 ng/ml acyclovir 1 h after oral administration of 400 mg of acyclovir (*y*-axis: attenuation, 5; *x*-axis: chart speed, 2.5 mm/min) [96].

Jankowski et al. [104], using a fluorimetric detector and a mobile phase with pH 2.3, reached a detection limit of 10 ng/ml. Jankowski's method can be utilised in pharmacokinetic studies of various forms of the drug.

Linearity of the assay procedures was usually determined by calculation of regression lines and the results were linear and cover a whole range of bio-analysis parameters. This range comprehends the circulating levels reached during drug treatment including the higher and lower doses [97,98,103,104].

Precision of the assay in the Jankowski method, calculated as a coefficient of variation (C.V.%) for within-day variability, ranged from 4.5% for 200 ng/ml to 13.0% for 50 ng/ml. Precision in between-day tests ranged from 7.1% for 400 ng/ml to 11.3% for 100 ng/ml. The within and between day precision using values concentrated ten fold were 3.7 and 4.7% for 2 μg/ml and 0.9 respectively and 1.2% for 10 μg/ml respectively. The accuracy, calculated as the average of the recoveries for three different concentrations of acyclovir, was higher than 80% when the concentrations were in the ng/ml order.

Furthermore, the recovery was 96±6% and 94±4% at the concentration of 2.0 and 10.0 μg/ml, respectively [97]. Bangaru's method precision over the concentration range of 0.02–5.0 μg/ml was 7.5 and 0.52%, the accuracy was 102.2 and 100.0%, respectively [98]. The assay in these works was carried out in plasma matrix. When the assay was carried out to analyse the acyclovir in liposomal formulations, the validations in the range 1–150 μg/ml in relation to the within day and between day variability ranged from 0.6 to 20.5%, and from 9.9 to 4.2%, respectively. The accuracy ranged from 99.4 to 79.5% [103].

Most HPLC procedures for acyclovir quantification did not employ an internal standard, thereby reducing the precision and the reproducibility of the analyses [97]. Poirier et al. [101] suggested the use of guanosine, a compound structurally related to acyclovir, as an internal standard (retention time 7.8 min instead of 5.3 min for acyclovir). In fact, endogenous guanine was not at a detectable level (10 ng/ml), neither in blank nor patient plasma samples.

Among the various methods described here, Bangaru's method [98] appears to be simple and the most economical for the measuring of acyclovir. It was applied to detect acyclovir in healthy human volunteer plasma samples after oral administration of a single dose of a 400-mg tablet. The low quantitation limit of about 20 ng/ml allowed the pharmacokinetic parameter investigation of absorption until the last sampling point. The pharmacokinetic parameters were in agreement with other corresponding values in literature. The method was linear and reproducible over the concentration range of 0.02 to 5.0 μg/ml for acyclovir.

Drug formulation analysis. When acyclovir is administered in dosage forms, its low bioavailability and brief $t_{1/2}$ in the plasma (2.3 h) are the main analysis problems [114]. These two problems make acyclovir a candidate for the encapsulation in a sustained release system such as liposomal formulations. M.M. Caamaño et al. [103], studied a method to measure drugs in liposomal formulations. Acyclovir and guanine, the major impurity of the drug synthesis and one of the compounds found in the chemical degradation process of acyclovir, were analysed.

The high pK_a of guanine caused problems for RP-HPLC analysis with silica based columns, particularly when the usual technique of ionisation suppression was performed at the pH range supported by this type of column. In order to avoid these problems, there are four basic strategies. (1) Ionic pair reagents is a good choice when the manufacturing process is set, but when samples are unknown, it is difficult to adjust the amount of ionic pair reagent in the mobile phase. (2) Working at neutral pH with deactivated silica columns is the second choice, however the cost of the analyses increases because it is necessary to use a higher percentage of organic modifier in the mobile phase. (3) Another strategy is the use of polymeric based columns. (4) Silanol masking agents that bind the stationary phase matrix are suggested, rather than the silanol groups of the stationary phase. The silanol-masking agent used in Caamaño's method [103] was hexylamine, which increased the hydrophobic character of the stationary phase. The surfactant agent Sodium Deoxy-Cholato (DOC), used to solubilise the phospholipid bilayer, and release the encapsulate drug, did not interfere in the analysis, since they do not elute under these analytical conditions.

2.2.4. Valaciclovir chromatographic conditions

The HPLC methods for acyclovir analysis do not usually measure its prodrug valaciclovir, however in literature this measurement has been described. Weller's HPLC method [115], using a gradient mobile phase, measured valaciclovir and acyclovir simultaneously. Recently Pham-Huy et al. [116] has developed a simple and specific rapid HPLC assay and an isocratic elution was proposed for the simultaneous quantification of these two drugs in biological fluids. Serum sample deproteinization was carried out with perchloric acid and 1-methylguanosine was used as an internal standard. Urine samples were diluted with the mobile phase, obtained by mixing (2:98, v/v) acetonitrile and 0.025 *M* mono-ammonium phosphate buffer adjusted at pH 4.0 with 10% diluted phosphoric acid. Symmetry Shield RP-C_8 column (5 μm, 250×4.6 mm I.D.) and a guard column (20×3.9 mm I.D.), packed with the same bonded phase, were used. Flow-rate was 1.0 ml/min. Injection volume of samples and standards was 50 μl. The detector was set at 254 nm.

In Pham-Huy's method [116] the detection limit (setting signal-to-noise ratio >3), after serum de-

proteinization, was 50 ng/ml for acyclovir and 70 ng/ml for valaciclovir. The lower limit of quantification was about 0.20 μg/ml for acyclovir and 0.25 μg/ml for valaciclovir. Standard calibration curves prepared with serum, urine or liquid of dialysis exhibited linearity for both valaciclovir and acyclovir over the range of concentrations (from 0.5 to 20.0 μg/ml). Furthermore the recovery measured with the internal standard was about 94–95%. The precision of this method, measuring acyclovir, in within and between-runs, was respectively: 0.75 and 2.76% for serum and 1.07 and 1.69% for urine. The precision, measuring valaciclovir, was respectively: 1.17 and 3.17% for serum and 1.03 and 3.03% for urine.

2.2.5. Ganciclovir chromatographic conditions

RP C_{18} or C_8 columns were utilised for the ganciclovir determination and a pre-column system was suggested. The main chromatographic technological aspects such as column characteristics, elution conditions and detectors are reported in Table 5.

The analysis was usually carried out either at room temperature or at 30–40°C. The mobile phase was composed of a high percent of buffer at pH 2.1–6.6. Flow-rate averaged from 1.0 to 1.75 ml/min. Spectrophotometric or fluorimetric detectors settled at 254 nm or $\lambda_{ex}=278$, $\lambda_{em}=380$ nm, respectively, were employed.

The HPLC analysis of ganciclovir is difficult, since the chemical structure of this molecule, being a nucleoside analogue of guanine, is quite similar to endogenous substances. Moreover, the polarity of the ganciclovir is high; therefore it is slightly retained by apolar stationary phases such as C_{18} or C_8 RP columns. Addition of ion pair agents or organic modifiers (i.e. triethylamine) in the mobile phase was employed to increase the selectivity, when biological samples are analysed. These additives decreased ganciclovir polarity, but caused a rapid deterioration

Table 5
Ganciclovir chromatographic conditions

Guard column	Column (dimension, particle size)	Temperature (°C)	Flow-rate and retention time	Mobile phase	Detection (UV or fluorescence)(nm)	Injection volume (μl)	Detection limit (ng/ml)	Quantification limit (ng/ml)	Refs.
4×4 mm with the same material of column	C_8 LiChrospher Select B column (250×4.6 mm, 5 μm)	40	1.0 ml/min; 5.5–5.8 min	2% Acetonitrile in 0.05 M ammonium acetate (pH 6.5)	254	10	10	50	[117]
Supelcosil LC-8 (50×4.6 mm, 5 μm)	HAIsil 120, BD, C_{18} (250×4.6 mm, 5 μm)	30	1.0 ml/min; serum=7.2 min, plasma=10.2 min	3:97 (v/v) Acetonitrile-sodium sulfate/H_2SO_4 (0.025 *M*, pH 2.4, containing 8 m*M* 1-heptanesulfonic acid)	$\lambda_{ex}=278$ $\lambda_{em}=380$	40		40	[118]
10×4.6 mm	Hypersil ODS (100×4.6 mm, 3 μm)	40	1.0 ml/min; 3.97–4.0 min	0.1 *M* Sodium dihydrogen phosphate monohydrate+0.04 *M* triethylamine, pH 6.6	254	80	10	50	[109]
	Supelcosil ABZ	R.T.	1.0 ml/min; 6 min	2:98 (v/v) Acetonitrile-ammonium acetate buffer (10 m*M*, pH 5.0)	254	30	3	10	[110]
10×4.6 mm	Ultraspher C_{18} (250×4.6 mm, 5 μm)	R.T.	1.75 ml/min; 9.05 min	15 m*M* Potassium dihydrogenphosphate pH 2.5–2.9+0.25% acetonitrile	254	100 (serum) 10 (urine)		50	[108]
	Techsphere 5 C_8 (100×4 mm)	R.T.	1 ml/mm; 9.7 min.	1% ortho-phosphoric+octane sulphonic acid 10 g/l	254	20	300		[106]

R.T.: Room temperature.

of the column, ghost peaks, baseline disturbances, aggregation and precipitation of polymers or macromolecules usually employed in the preparation of nanoparticulates dosage forms. Acyclovir as an internal standard was also used in some methods [109,110,117] (Fig. 5).

In order to solve these problems, Merodio et al. [117] included ammonium acetate in the mobile phase, avoiding the use of an ion agent or other organic modifier. Furthermore, a column with a slightly more polar endcapped stationary phase (Li-Chrospher C_8) replaced the RP-C_{18}. On the contrary, Campanero et al. [109] used triethylamine as a component of the mobile phase, enhancing chromatographic peak shape (Fig. 6).

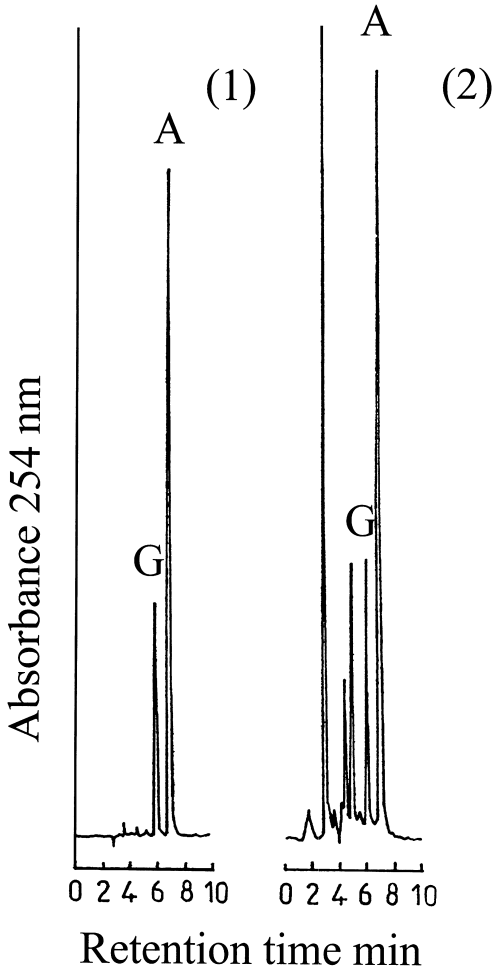

Fig. 5. Chromatograms of ganciclovir samples of median concentration (0.55 mg/l). (1) Water sample; (2) plasma sample. G: Ganciclovir; A: acyclovir used as an internal standard [110].

Triethylamine, an organic modifier, competed with free silanol groups of the column stationary phase, inactivating them and therefore avoiding the development of tailing peaks. These authors utilised a mobile phase at pH 6.6; in these conditions ganciclovir was in its neutral form and could be analysed by RP-HPLC. Chu et al. [118] and Page et al. [108] used a buffer at pH 2.5–2.9 and 2.4, respectively. In this case more than 3000 analyses could be performed without loss of quality (Fig. 7).

The experimental conditions, summarised in Table 5, allowed the assay of ganciclovir in both pharmaceuticals (i.e. albumin nanoparticles) and biological samples [108,110,117,118]. Run time for ganciclovir HPLC ranged from 12.5 to 22 min in various methods, which are summarised in the same Table. The retention time is also in relation to mobile phase characteristics, and some of them depend on the percentage of acetonitrile. In fact, Merodio's method [117] demonstrated retention time of 3.5 and 5.2 min with 5 and 1% of acetonitrile respectively. Other methods, as outlined in Table 5, showed retention time of ganciclovir changing from approximately 9 to 4 min. Changes in the retention time depend also on separation procedure as well as on flow-rate.

The limit of detection ranged from 3 to 300 ng/ml and the limit of quantification from 10 to 50 ng/ml (Table 5). The lower quantification limit could be achieved in plasma by increasing the injection volume, or by increasing the sample volume in the extraction and isolation procedures. Campanero's method [109] can be applied to assay ganciclovir in plasma monitoring therapeutic drugs and studying pharmacokinetics in normal patients, in patients with severely impaired renal function, and in solid organ transplant patients. The working range was 1–200 μg/ml, the calibration was linear from 0.05 to 10 μg/ml and the detection was spectrophotometric. The recovery was 95±3.26% and the intra- and inter-assay (C.V.) ranged from 2.40 to 5.85% and from 2.09 to 6.65%, respectively.

The range of detection of Chu's method [118] varied from 0.04 to 4.00 μg/ml, using fluorescence detection. The detection limit of this method was sensitive enough for pharmacokinetic studies (≤ 0.05 μg/ml). Furthermore, it used volumes adequate

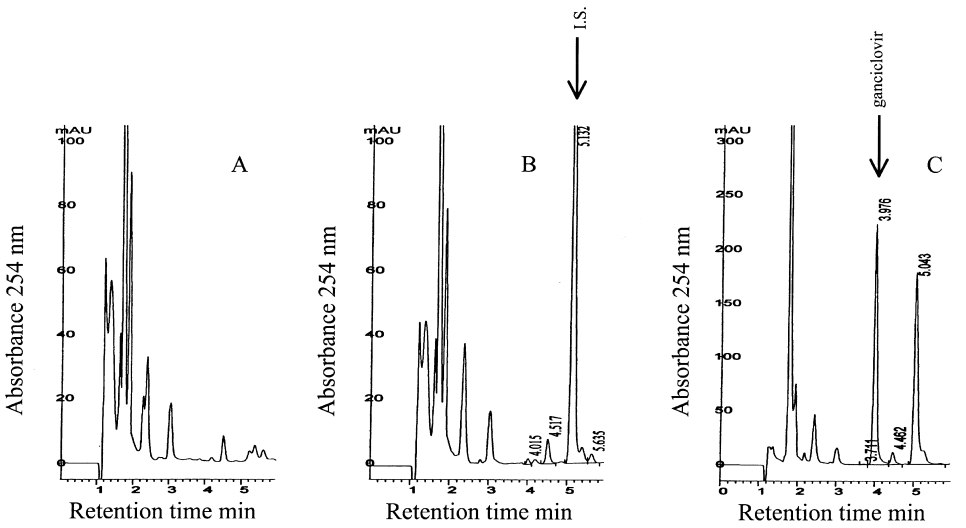

Fig. 6. Chromatograms resulting from the analysis of blank human plasma (A), human plasma spiked with 50 ng/ml of Internal Standard (I.S.); (B), and the plasma sample (6.7 μg/ml) obtained at 2 min post-infusion from a subject who received a single 5 mg/kg intravenous infusion dose of ganciclovir (C), respectively. Retention times: ganciclovir=3.97–4.0 min, acyclovir (I.S.)=5.0–5.1 min [109].

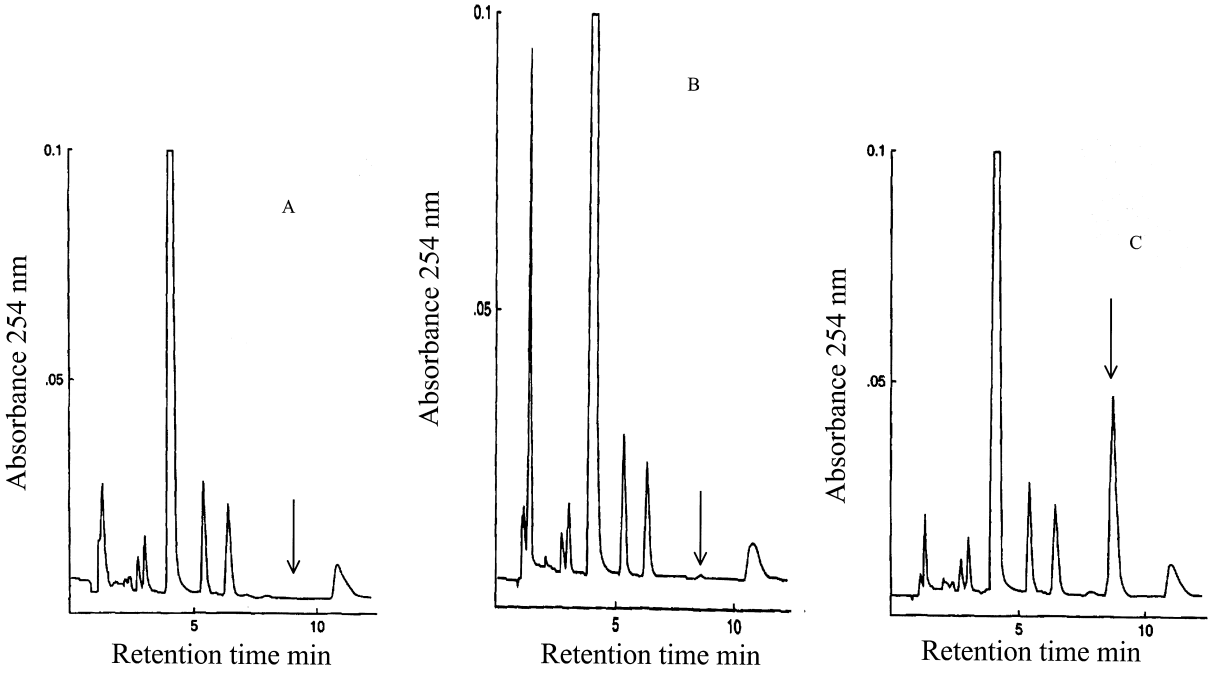

Fig. 7. Chromatograms of serum blank (A) and ganciclovir at 0.05 μg/ml (B) and 4 μg/ml (C). Ganciclovir peak is indicated by the arrow at 9.05 min [108].

for paediatric studies (25 μl). The recovery ranged from 94.0 to 110% and the intra- and inter-assay variation (C.V.) ranged from 3.17 to 9.52% and from 2.32 to 6.55%, respectively.

Merodio's method [117] was used to assay the ganciclovir in albumin nanoparticles and can be applied in the estimation of the drug uptake by cultured human corneal fibroblasts. The recovery demonstrated by this method ranged from 97.9%±1.79 to 102.1%±2.62, and the C.V.% of intra-day and inter-day ranged from 0.24 to 3.14% and from 0.04 to 2.55%, respectively. The recoveries of Page's method [108], assaying ganciclovir in serum and urine, varied from 91 to 107% and the intra-and inter-assay variation ranged from 0.63 to 1.2% and from 1.1 to 3.2%, respectively.

2.2.6. Simultaneous acyclovir and ganciclovir analysis

The simultaneous separation and detection of acyclovir and ganciclovir has also been proposed. In fact, McMullin et al. [106] developed a HPLC method that used a Techsphere 5 C_8 column (100×4 mm). The mobile phase was constituted by 1% ortho-phosphoric acid containing 10 g/l octane sulphonic acid. The flow-rate was 1 ml/min and the detection was carried out at 254 nm. The serum samples were deproteinised with 7% perchloric acid and 20 μl of the supernatant was injected.

The detection limits of this method for acyclovir and ganciclovir were 500 and 300 ng/ml, respectively. Serum recovery was about 100% for both antiviral drugs, their concentrations ranging from 0 to 100 μg/ml. The coefficients of correlation of drug concentration vs. peak height, for acyclovir and ganciclovir in standard and serum samples, were $r=0.994$ and 0.999, respectively. The accuracy was investigated by measuring serum samples containing acyclovir and ganciclovir at different concentrations, ranging from about 1 to 9 μg/ml. The accuracy for acyclovir and ganciclovir, expressed as percentage of error, ranged from 0 to 3.2% and from 1.1 to 6.7%, respectively.

2.2.7. RS-79070-004 ganciclovir prodrug method

A new prodrug has been proposed and tests and studies are upgrading. To increase the limited oral bioavailability (6–10%) of ganciclovir, RS-79070-004, the hydrochloride salt of the mono-L-valylester of ganciclovir, is under evaluation as a prodrug to increase the bioavailability of ganciclovir. The measurement of this prodrug has also been proposed.

A column switching HPLC method was developed and validated [119]. This system was constituted by a pre-column solvent filter (2.0 μm frit), a capture column (C_{18} BDS Hypersil column 5 μm, 20×4.6 mm) and the analytical column (C_{18} BDS Hypersil 5 μm, 250×4.6 mm). An 80-μl volume of sample or standard, both deproteinised by a cold 15% TCA solution, was injected. Flow-rate was 1.3 ml/min. Elution time of the prodrug was 2.47 min and the detection was carried out at 254 nm.

The method was validated for RS-79070-004 plasma concentrations in the range 0.04–4.00 μg/ml. The calibration curve range was 0.04–0.8 μg/ml and an extrapolation to 4.00 μg/ml was verified by the use of standards in each run. The signal-to-noise as the limit of quantification (0.04 μg/ml) was 10. Mean recoveries ranged from 99.3 to 106% and the intra- and inter-runs C.V.% values were less than 10.9%. RS-79070-004 resulted in two adjacent but distinct peaks and the total concentration was achieved by integration of the chromatographic window that contained both peaks.

One problem in the analysis of RS-79070-004 in the plasma was related to the hydrolysis of its ester group, furthermore the presence of two diastereoisomers also created inconveniences. In any case the separation of plasma from blood at 4°C and its immediate freezing and storage at −80°C were an adequate procedure to avoid decomposition of RS-79070-004. No degradation of RS-79070-004 was observed when the plasma sample was extracted with TCA and stored at room or at low temperatures (−80°C) for an extended time.

2.2.8. Analytic interference

There is no interference of acyclovir, valaciclovir and ganciclovir in the chromatographic region of the investigated molecules with related endogenous compounds such as uric acid, hypoxanthine, xanthine, guanine, guanosine [97,101,116]. With regard to co-administered drugs, Poirier's method reported that no drug has been found to interfere with acyclovir. Analogous data was reported by others with regards to ganciclovir [97,100,101,104,106,109,118]. A

method using RP-HPLC coupled with electrospray ionization and selected reaction monitoring mass spectrometry has been developed for the quantitative analysis of ganciclovir in rat plasma. The use of liquid chromatography selected reaction monitoring/mass spectrometry eliminated potential interference from endogenous constituents in plasma [120].

2.2.9. Famciclovir chromatographic conditions

Penciclovir is the principal and active metabolite found in plasma and urine after famciclovir administration. Minor metabolites include mono-acetylate penciclovir and mono-acetylated 6-deoxy-penciclovir.

Penciclovir and 6-deoxy-penciclovir concentrations in plasma and urine samples were measured by RP-HPLC [121,122]. These methods were utilised to study pharmacokinetic of famciclovir. Penciclovir and 6-deoxypenciclovir quantitation limit in plasma sample was 0.2 and 0.4 μg/ml, respectively, and in urine sample was 10 and 20 μg/ml, respectively. Linearity of each assay was established over the concentration ranging from 1 to 80 μg/ml. Literature methods demonstrated intra - and inter-assay precision and accuracy values better than 15% across the linear range in both plasma and urine [82,88,123]. It is interesting to point out that famciclovir originates penciclovir, and it is measured as a marker of the active drug which gives an idea of the difficulty in understanding the significance of the analysis of drugs after their metabolism in the body. On decomposition it generates a compound co-eluting with the penciclovir form so that it is difficult to measure the active drug in the circulation.

To solve this problem, however, Hsu et al. [124] developed a HPLC method by YMC AQ reversed-phase C_{18} columns (250×4.6 mm, 5 μm particle-size) that provided sufficient accuracy and precision. One limitation was that the use of a weak strength solvent, required for good separation, resulted in a long separation time (22 min) and in peaks broader than desired (Fig. 8).

The analytes were eluted isocratically with methanol–water (5:95, v/v) containing 23 m*M* potassium phosphate buffer (pH 7.0). The flow-rate was 1.0 ml/min and the column temperature 30°C. The injection volume was 20 μl and the detection limit was estimated to be 0.5 μg/ml.

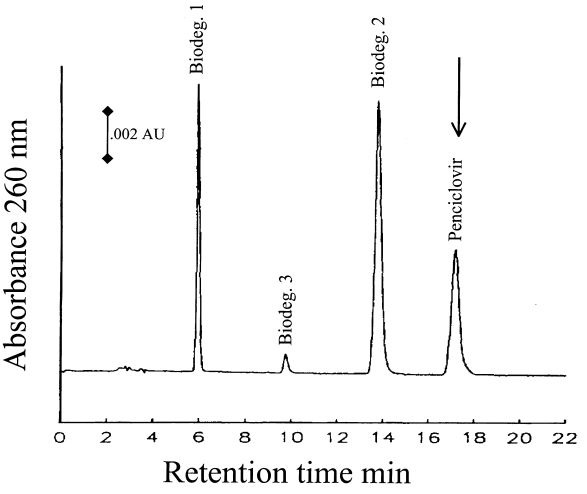

Fig. 8. HPLC chromatogram of penciclovir degradation. Three degradants were observed and are labelled as biodeg. 1, biodeg. 2 and biodeg. 3 [124].

2.3. Capillary electrophoresis methods

Capillary electrophoresis (CE) can be also proposed for the analysis of these antiviral drugs. In fact, both drug and prodrug molecules are quantitated using CE methods by UV visible detectors, but also fluorimetric and electrochemical detectors. Only a few authors have already described CE methods, which are briefly outlined.

2.3.1. Acyclovir assay

High-performance capillary electrophoresis (HPCE) techniques have been published for the analysis of ACV and related compounds both in pharmaceutical and in biological fluids (such as serum and urine). These methods have been proposed only after removing the difficulties of using fused-silica capillary in the assay of basic drugs. The use of coated capillary as well as the addition of stabilisers has been proposed. Assi and co-workers employed polyamine-coated capillary [125] (Fig. 9), but recently Zhang et al. [126] used fused-silica capillaries and Neubert et al. [127] applied micellar electrokinetic chromatography (MECK).

Length of polyamine-coated capillary ranged from 57 to 27 cm (50 μm I.D.); the operating buffer was 50 m*M* sodium acetate pH 4.2. The experimental operating parameters were: field strength 20 kV,

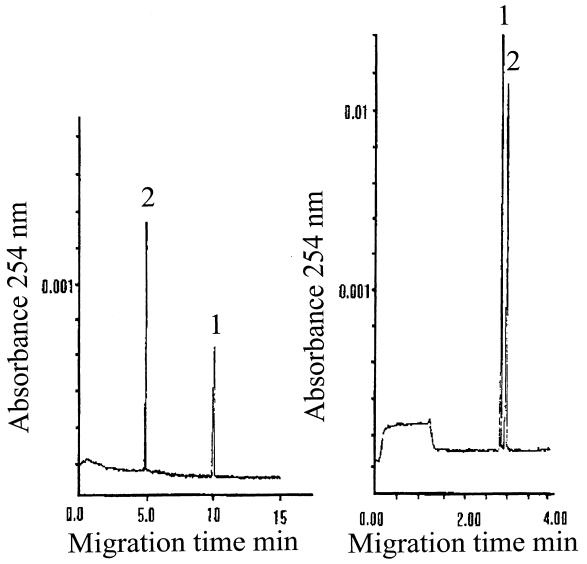

Fig. 9. The separation of the antiviral drug, acyclovir (peak 1) and its major degradation product, guanine (peak 2) on: (a) conventional capillary (57 cm (50 cm)×50 μm), (Beckman P/ACE 2210) and operating parameters: buffer=20 m*M* sodium citrate pH 2.5; voltage=30 kV; wavelength=25 nm; temperature −25°C; (b) the polyamine coated eCAP^{tm} (57 cm (50 cm)×50 μm I.D.), (Beckman P/ACE 2210) and operating parameters for both were: buffer=50 m*M* sodium acetate (pH 4.2); voltage=30 kV; wavelength=254 nm; temperature=25°C [125].

detection at 254 nm and temperature 30°C. A longer capillary (37 cm) gave more reproducible assay results in terms of peak area than a shorter one (27 cm length). The ranges of separation times of acyclovir, in dependence of the capillary length, varied from 0.4 to 1 min (27 cm) to 0.5–2.5 min (37 cm) and to 3–6 min (57 cm), respectively. The stability was tested over 6 months on these drugs and the C.V.% values were less than 3% on migration time and less than 4% on the peak area. This research studied a rapid assay of drug mixtures, and biologic fluids were not analysed. Linearity was described for a 50-μg/ml drug, but other analytical parameters were not investigated.

Zhang and co-workers [126] examined urine samples using HPCE with electrochemical detection. This research compared UV detection at 280 nm with amperometric detection. Using HPCE with UV detection, the calibration curve was linear in the range of 10–300 μg/ml and the detection limit of ACV was 8.5 μg/ml. Using HPCE with amperometric detection a calibration linearity in the range of 0.2–20.0 μg/l was demonstrated and the detection limit was 0.15 μg/ml. Bare fused-silica capillaries were utilised (50 μm I.D. and 42 cm length). The running buffer was 40 m*M* borax, pH 9.2. The instrumentation system operated with electrokinetic injection (20 kV for 5 s) and a field strength of 20 kV was applied for about 10 min. The working temperature was 20°C. Moreover, α-amino-5-mercapto-3, 4-dithiazole (AMD) was used as an internal standard. Crude ACV pharmaceuticals and human urine samples were analysed. The ACV purity was investigated; both UV and amperometric detection were acceptable. Sample solutions of ACV pharmaceuticals at about 200 μg/ml and 10 μg/ml were utilised. In a healthy volunteer, administered with 80 mg of ACV, an ACV concentration of 8.35±0.26 μg/ml was measured when a urine sample was collected within 0–2.5 h and of 10.53±0.19 μg/ml within 2.5–5.5 h (Fig. 10).

Neubert and co-workers applied micellar electrokinetic chromatography (MEKC) for the determination of acyclovir and together with the antiviral drug brivudin (BV) [127]. The CE apparatus system used fused-silica capillaries (length 64.5 cm, Ø 50 μm). The separation was carried out in field strength of 30 kV, using 20 m*M* borate buffer, pH 10. The running buffer contained additional surfactants: 5 m*M* dodecyltrimethyl ammonium bromide (DTAB) and 10 m*M* sodium dodecyl sulphate (SDS). Ace-

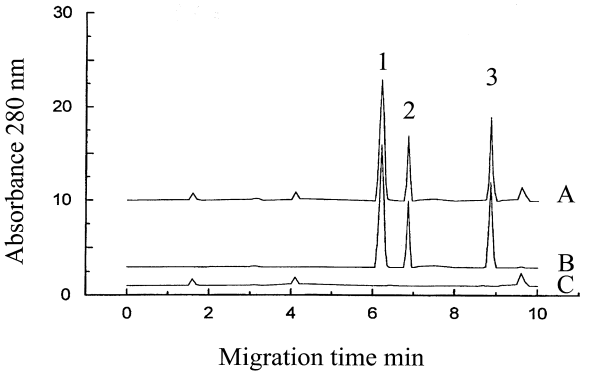

Fig. 10. Electropherograms of standard, blank and urine samples of guanine (G), ACV and AMD by HPCE with UV detection. (A) Urine sample including standard of ACV, G and AMD; (B) standard of ACV, G and AMD; (C) blank urine. Peaks: 1, G 50 mg/l; 2, AMD 4 g/l; 3, ACV 50 mg/l [130].

tone was used as a marker substance for the determination of the electro-osmotic flow (EOF). A pressure injection was used. Calibration curve was linear from 5 to 500 μg/ml and the detection limit was about 5 μg/ml. The use of this method for determining antiviral drugs both in hydrophilic and in lipophilic ointment was suggested.

2.3.2. Penciclovir assay

Hsu et al. [124] demonstrated that the long separation time was the limitation of Penciclovir HPLC method and this author, obtained a faster separation by CE. In this method the running buffer was 23 m*M* potassium phosphate, pH 7, and fused-silica capillaries with 50 μm I.D. and 64.5 cm length (56.5 cm to detector) were used. The sample injection was carried out applying a pressure of 50 mbar for 15s. The injection volume was about 17 ml, a voltage of 30 kV was applied, and a temperature of 30°C was maintained. Detection was at 260 nm. The advantages of this CE method in comparison with the HPLC method were high speed (7 vs. 22 min) without significant loss in resolution, and without the use of organic solvents (Fig. 11).

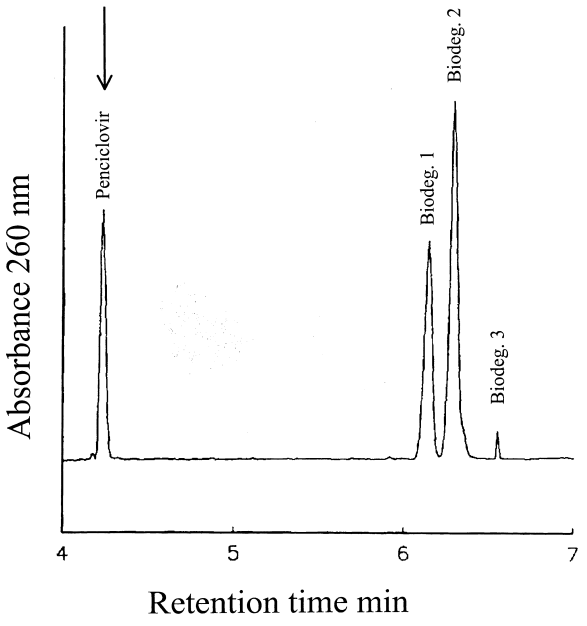

Fig. 11. Electrophoresis of penciclovir degradation: three degradants were observed and are labelled as biodeg. 1, biodeg. 2 and biodeg. 3 [124].

Therefore CE is more economical and environmentally safer than conventional HPLC. Sensitivity in CE was limited compared to HPLC. However, by increasing the injected sample to about 17 ml, the sensitivity was comparable to that of HPLC and the detection limit was estimated at 0.5 μg/ml. Migration-time precision for CE was 0.42% C.V. and was better than that of HPLC (0.84% C.V.). CE might be useful in those cases, i.e. penciclovir biodegradation studies, in which fast, highly efficient separation is required.

In conclusion, the methods using capillary electrophoresis seem useful, but further investigations must be carried out, particularly for body fluid analysis.

3. Future prospects and conclusions

Antiviral chemotherapy came of age with the advent in 1977 of acyclovir as the first truly specific antiviral agent [1,2]. After more than two decades, the place of acyclovir as an effective agent in the therapy of herpes virus infections in both immunocompetent and immunocompromised patients remains firmly established. Acyclovir is still a first-line option for treatment and prophylaxis of HSV and VZV infection. Moreover, therapy with acyclovir and related compounds has become increasingly important as the immunocompromised population grows with the rapid and global spread of AIDS, and the more frequent use of bone marrow and organ transplant procedures.

Like many other antiviral drugs, acyclovir and related compounds require therapeutic drug monitoring to achieve the optimum therapeutic effect and to minimise adverse reactions. The need for therapeutic drug monitoring is particularly important in patients who have acute or chronic renal impairment and may therefore be susceptible to adverse effects due to renal accumulation.

Chromatography can be considered the technique of choice for the bioanalysis of this class of antiviral compounds, as this methodology is characterised by good specificity and accuracy and it is particularly useful when metabolites need to be monitored. Other methods for the assay of acyclovir in serum or plasma have been proposed including radioimmunoassay (RIA) [92,93], enzyme-linked immuno-

sorbent assay (ELISA) [94], and scintillation proximity radioimmunoassay [128]. However, immunological techniques have a number of significant disadvantages, including the length of time to obtain final quantitative results, the large number of steps in the procedure, and the need to develop antiserum and/or monoclonal antibodies.

Much has been published on the chromatographic analysis of acyclovir and related compounds in biological samples since this subject was last reviewed [129]. Whereas most of the previously published methods involved time-consuming and costly extraction procedures and had a low sensitivity, recently reported HPLC methods appear to be more simple, specific and sensitive enough for therapeutic monitoring and pharmacokinetic investigations. Moreover, methods allowing for the simultaneous analysis of acyclovir and related compounds (i.e. valacyclovir and ganciclovir) [106,116] or other drugs (i.e. teicoplanine) [110] have been developed.

In the future, new efforts could be focused on the use of capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC). With larger numbers of theoretical plates, shorter separation times, adequate detection limits and essentially no organic mobile phase consumption, capillary electrophoresis (CE) can be an analytical tool for routine use that replaces conventional reversed-phase HPLC. However, up to date only a few CE methods for determination of acyclovir and related compounds in biological fluids and pharmaceuticals have been described [124,125,127,130].

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