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Review

Separation methods for antiviral phosphorus-containing drugs

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Abstract

Among antiviral drugs, phosphorus-containing compounds, foscarnet and cidofovir, present adverse effects including renal toxicity. Since their main therapeutic target is the treatment of CMV retinitis, which needs lifelong maintenance therapy, accurate analytical methods are required for drug monitoring. According to the high hydrophilic property of the two compounds, ion pair reversed-phase HPLC methods were proposed for their separation in drug formulations and biological samples. Their lack of UV absorption at wavelengths above 205 nm does not allow the use of this detection technique for biological fluids. Electrochemical detection methods (coulometry and amperometry) led to a quantification limit of 15 μM for foscarnet. Fluorescent derivatives obtained by modification of cidofovir cytosine nucleus with α-haloketones offered advantage over UV detection and allowed to reach a detection limit of 5 ng/ml, making possible investigations on the drug time-course in biological fluids.

Keywords: Reviews; Foscarnet; Cidofovir

Contents

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1. Introduction

1.1. Place in antiviral therapeutics

Among antiviral drugs, the two phosphorylated compounds, namely foscarnet and cidofovir, belong to the therapeutic arsenal against cytomegalovirus (CMV). Although primary infection with CMV is usually benign, the virus remains latently present within the host thereafter, a property this virus shares with all other herpes viruses [1]. Under conditions of immune compromise, especially impairment of cell-mediated immunity, latent virus may reactivate to produce a variety of clinical syndromes including chorioretinitis, esophagitis, colitis, pneumonia, encephalitis, and adrenalitis [2]. The survival of patients infected with the human immunodeficiency virus (HIV) has improved over the past decade due to the use of antiretroviral therapy and better management of acquired immunodeficiency syndrome (AIDS)-related opportunistic infections [3]. However, as patients live longer with very low CD4 counts, it is not surprising that the incidence of opportunistic infections has increased. Retinitis is the presenting manifestation of CMV disease in approximately 85% of persons with HIV infection [3]. It is plausible that the immunological benefits of highly active antiretroviral treatment (HAART) regimens consisting of an HIV protease inhibitor combined with one or two dideonucleoside agents may be sufficient to prevent CMV retinitis in patients with very advanced HIV disease [4]. Patients with suspected or confirmed CMV chorioretinitis should be treated with ganciclovir, foscarnet or cidofovir. It has become clear that the approach to treatment of CMV retinitis in patients with AIDS is analogous to the therapy for a responsive, but not curable, malignancy. Thus, it has become customary to have an initial period of ''induction'' therapy followed by lifelong maintenance therapy [1]. After continuous ganciclovir therapy, some patients excrete resistant strains of CMV [1,5] which appear to remain sensitive to foscarnet [6]. Foscarnet blocks the pyrophosphate binding site of the viral DNA polymerase, preventing cleavage of pyrophosphate from deoxyadenosine triphosphates [1,7]. Excretion of foscarnet is entirely renal without a hepatic component. Its adverse effects include renal impairment, anemia, hypocalcemia and hypophosphatemia [1]. Cidofovir diphosphate, the active metabolite of cidofovir exerts its antiviral effect by functioning as a competitive inhibitor and alternative substrate, with respect to deoxycytosine triphosphate, of human CMV DNA polymerase [8]. Nephrotoxicity is also the dose-limiting toxicity of cidofovir [9] which is excreted unchanged at more than 80% in urine [10]. It is therefore important to measure renal function frequently and adjust dosage of those compounds accordingly in order to minimize toxicity [1]. Accurate analytical methods are required to perform such drug monitoring.

1.2. Nomenclature and physical–chemical properties

1.2.1. Foscarnet

Foscarnet (Fig. 1) is trisodium phosphonoformate hexahydrate. From the bioanalytical point of view, foscarnet has several undesirable physical–chemical properties. It is an extremely hydrophilic salt of a triprotic acid with pK_a of 0.49 and 7.27 (P–OH), and 3.41 (CO_2H) [11,12]. Therefore, it is difficult to retain on hydrocarbonaceous supports and impossible to extract from biological fluids into organic solvents [11]. Additionally, it has no UV absorption above wavelengths of 205 nm and is therefore difficult to detect in LC eluates.

Studies of pharmacokinetics of foscarnet have shown that the drug is poorly and variably absorbed following oral administration [13]. Evidence was furthermore presented to suggest that the absorption of foscarnet occurs mainly by a carrier-mediated process via the phosphate transport system existing in the intestinal brush-border membrane. A limited capacity or saturation of this carrier system may be a major cause for the poor absorption of foscarnet in man [13,14]. Foscarnet is known to decompose in acidic solution to form carbon dioxide and phosphorous acid [13]. Therefore, an additional factor contri-

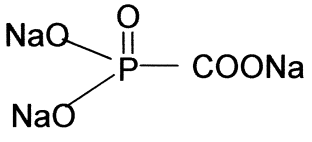


Fig. 1. Chemical structure of foscarnet.

buting to the incomplete and variable absorption pattern could be acid catalysed degradation of the drug in stomach [13]. This factor was put forward by Ritschel et al. [15] to explain the much higher absorption of foscarnet observed in rabbits (which have high gastric pH) than in dogs. The kinetics and mechanism of decarboxylation of foscarnet have been studied by Warren and Williams [12] in strongly acidic solutions (0.4–3 *M* perchloric acid). To assess the potential impact of degradation of foscarnet in the stomach on its bioavailability, Bungaard and Mørk [13] have investigated the kinetics of decarboxylation of the drug under conditions similar to those found during oral absorption. It appears that intragastric degradation of foscarnet may be of significance for the absorption of the drug following peroral administration, especially at low gastric pH and/or long gastric emptying times. The unreactivity of any ionized species of foscarnet makes the compound stable in weakly acidic, neutral and alkaline solutions where only a minor fraction of the reactive undissociated acid form occurs [12].

Foscarnet forms complexes with several metal ions [16] and several parts of a liquid chromatographic system (tubing, column, injection needle, etc.) in contact with samples are made of stainless steel. The resulting compound retention leads to the deformation of foscarnet peak.

1.2.2. Cidofovir

Cidofovir ((*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine, HPMPC) is an acyclic cytidine monophosphate analog with pK_a of 1.7 and 6.9 (phosphonic acid), and 4.7 (cytosine). Its solubility and stability depend on the medium pH. The maximum solubility is obtained between pH 5.9 and 7.8 and the maximum stability of the aqueous solution is obtained between pH 8.5 and 10.5 [17]. Deamination (Fig. 2) is the primary degradation pathway for cidofovir in aqueous solutions to form the corresponding uracil analogue 1-[(*S*)-3-hydroxy-2-(phosphonomethoxy)-propyl]uracil (HPMPU) [18]. Cidofovir undergoes intracellular phosphorylation by host enzymes to form mainly cidofovir phosphate and cidofovir diphosphate (the purported active form) (Fig. 3) [19]. Two additional metabolites have been observed in rat tissues and in the urine of rabbits, rats and monkeys dosed with [^{14}C]cidofovir

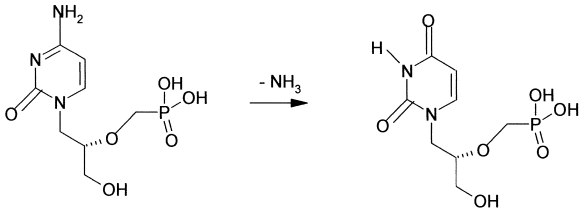


Fig. 2. Reaction scheme for deamination of cidofovir.

[20–22]. One metabolite has been identified in vitro as an adduct of cidofovir with phosphocholine. The other metabolite coeluted with a cyclic analog of cidofovir, cyclic HPMPC (cHPMPC) {1-[((*S*)-2-hydroxy-2-oxo-1,4,2-dioxaphosphorinan-5-yl)methyl]-cytosine} on several different HPLC systems [19]. The observed presence of cHPMPC results from internal cyclization of the phosphorylated species ex vivo. Cyclic HPMPC is easily formed in aqueous solutions of authentic, chemically synthesized cidofovir diphosphate and cidofovir–phosphocholine as a result of their chemical hydrolysis at low pH [19]. The long intracellular half-lives of these phosphorylated metabolites are responsible for the extended duration of action of the drug. Cyclic HPMPC is also a prodrug of cidofovir which is stable in plasma but is readily converted to cidofovir in cells [8,23].

2. Liquid chromatography

2.1. Foscarnet

Few LC methods were proposed in the literature for the determination of foscarnet in drug formulations and biological fluids (Table 1). Forsman et al. [24] described an ion-exchange chromatographic method for the separation of foscarnet and its degradation products, phosphite and phosphate, in drug formulations. The separation was performed on a silica-based DEAE anion-exchanger within 6 min. The mobile phase consisted of a mixture of 0.1 *M* acetic acid, 0.015 m*M* sulphate as elution ion and 1 m*M* citric acid. The presence of citric acid avoided severe tailing of the foscarnet peak. Foscarnet and phosphite were initially oxidized to phosphate by post-column addition of bromine. Thereafter, a

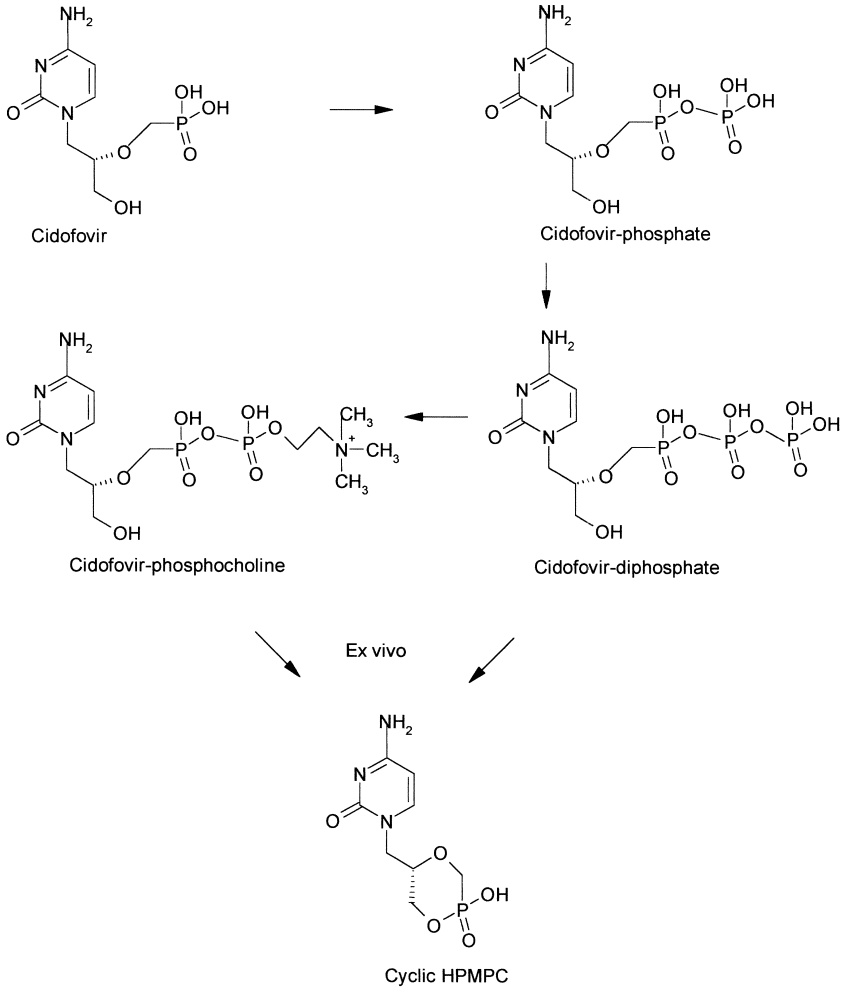


Fig. 3. Intracellular metabolic scheme for cidofovir and ex vivo transformation.

molybdovanadate reagent was added to form a yellow product detected at 340 nm. Sulphite was added to reduce the excess of bromine which otherwise would disturb detection of the compound of interest. The assay was linear from 0.03 to 2.7 m*M* for phosphate and phosphite peak area or phosphite peak height, and from 0.03 to 1.5 m*M* for phosphate peak height. The detection limit for phosphate and phosphite was about 4 ng calculated as phosphorus, and for foscarnet was about 12 ng, for an injection volume of 20 μl. The reaction system proposed by Forsman et al. was rapid and did not require elevated temperature in comparison to the similar technique proposed by Hirai et al. [25] which required a reaction coil with a hold up time of 2.5 min and heating to 140°C. However, the general method reveals some drawbacks. The sodium sulphite solution was freshly prepared each day. With increasing concentration of citric acid in the mobile phase, the peak tailing is reduced. Citric acid did however interfere in the detection system and caused a decrease in response when the concentration reached a critical level. It was also found that a good quality peristaltic pump was necessary for the reagents delivery or else pulsation in combination with the reagents background gave a noisy baseline. Otherwise, previously unused columns were preconditioned for 1 to 2 days. Without this pre-treatment,

Table 1
Chromatographic methods for the determination of antiviral phosphorus-containing drugs

Drug	Method	Chromatographic conditions		Detection	Limit of quantification Linearity range	Biological fluids or other samples	Sample handling	Comments	References
		Stationary phase	Mobile phase or carrier gas flow						
Foscarnet	Ion-exchange HPLC	DEAE–Si 100 Polyol, 3 μm 125 mm× 4.6 mm I.D. (Serva)	0.1 *M* acetic acid +1 m*M* citric acid +0.015 *M* sulphate (sodium sulphate+ sodium hydrogen sulphate) pH=2.7 1 ml/min	UV 340 nm (1) Oxidation of foscarnet to phosphate by bromine; (2) Reaction of phosphate with molybdovanadate reagent	0.03 m*M* 0.03–2.7 m*M*	Pharmaceutical formulations		Not validated for biological fluids	Forsman et al. [24]
Foscarnet	Isocratic ion-pair reversed-phase HPLC	NovaPak C_{18} 150 mm× 3.9 mm I.D. (Waters) at 25°C	5% Methanol; 95% 5 m*M* H_2SO_4 containing 0.904 g/l $THAHSO_4$ 1.5 ml/min	UV 254 nm		Pharmaceutical formulations		Not validated for biological fluids	Woods et al. [27]
Foscarnet	Isocratic ion-pair reversed-phase HPLC	UltroPac C_{18}, 3 μm, 100 mm× 4.6 mm I.D. (LBK)	25% Methanol; 75% 43 m*M* phosphate with 1 m*M* $THAHSO_4$ and 0.2 m*M* PPA pH=5.8 1.0 ml/min	Coulometric ECD Guard cell: +0.75 V; Analytical cells: +0.75 V, +0.90 V	15 m*M*	Plasma; Urine; CSF	Dilution with 1 m*M* PPA (pH 5.8) before and after ultrafiltration +activated charcoal for samples with low levels		Pettersson et al. [16]
Foscarnet	Isocratic ion-pair reversed-phase HPLC	NovaPak C_{18}, 4 μm (Waters) at 22–25°C	30% Methanol; 70% phosphate with 1 m*M* $THAHSO_4$ and 0.2 m*M* PPA pH=5.8 0.7 ml/min	Coulometric ECD Guard cell: +0.99 V, Analytical cells: +0.50 V, +0.95 V	33 μ*M* 33–1100 μ*M*	Plasma, urine	– Plasma: ultrafiltration +dilution with 1 m*M* PPA; – Urine: Dilution with 1 m*M* PPA+ activated charcoal	Use of hydrochlorothiazide as internal standard	Hassanzadeh et al. [29]
Foscarnet	Isocratic ion-pair reversed-phase HPLC	PEEK column 150 mm× 4.6 mm I.D. packed with Kromasil 100 C_{18}, 5 μm (Higgins Analytical)	25% Methanol; 75% 40 m*M* phosphate with 0.25 m*M* $THASO_4$ pH=7.6 1.0 ml/min	Amperometric ECD Flow cell: +1.125 V	15 μ*M* 15–240 μ*M*	Human serum, Calf serum	SPE on SAX anion exchanger; Elution with 50 m*M* potassium pyro-phosphate buffer, pH 8.4		Ba et al. [42]

Table 1. Continued

Drug	Method	Chromatographic conditions		Detection	Limit of quantification Linearity range	Biological fluids or other samples	Sample handling	Comments	References
		Stationary phase	Mobile phase or carrier gas flow						
Foscarnet	GC–MS			MS	0.06 mg/l 0.05–50 mg/l	Human plasma			Rindgen et al. [52]
Cidofovir	Isocratic ion-pair reversed-phase HPLC	Analytical column: Hypersil ODS C_{18}, 5 μm, 150 mm× 4.6 mm I.D. Guard column: Hypersil ODS C_{18}, 5 μm, 10 mm× 4.6 mm I.D. (Alltech)	3.5 m*M* phosphate with 5.0 m*M* $THAHPO_4$ pH 6.0 2.0 ml/min	UV 280 nm	2 μg/ml 2–400 μg/ml	Pharmaceutical formulations		Not validated for biological fluids	Yaan et al. [18]
Cidofovir	Gradient (serum) and isocratic (urine) ion-pair reversed-phase HPLC	– Serum: Analytical column: Zorbax C_8, 5 μm 250 mm× 4.6 mm I.D.; Guard column: Zorbax C_8, 6.0 mm×4.0 mm I.D. – Urine: Analytical column: Ultrasphere ODS IP 150 mm× 4.6 mm I.D. (Alltech); Guard column: Brownlee RP 18 15 mm× 3.2 mm	– Serum: Aceto-Nitrile A: 5% B: 15% 100 m*M* phosphate buffer pH 7.2 containing 5 m*M* $TBAHPO_4$ A: 95% B: 85% Linear gradient from 100% A to 100% B over 9 min; 1.4 ml/min – Urine: 5% acetonitrile 5% methanol 90% water containing 5 m*M* Q8 2.0 ml/min	UV 274 nm	– Serum: 0.22 μg/ml 0.22–2.19 μg/ml – Urine: 1.0 μg/ml 1.0–99 μg/ml	Serum, urine	– Serum: SPE on SAX anion-exchange cartridge; – Urine: SPE on C_{18} cartridge	Use of PMEG as internal standard	Cundy et al. [45]

Table 1. Continued

Drug	Method	Chromatographic conditions		Detection	Limit of quantification / Linearity range	Biological fluids or other samples	Sample handling	Comments	References
		Stationary phase	Mobile phase or carrier gas flow						
Cidofovir	Isocratic ion-pair reversed-phase HPLC	ODS-2, 5 μm 100 mm× 4.6 mm I.D. (Phenomenex)	30% acetonitrile, 70% 2 m*M* phosphoric acid containing 6 m*M* Q12 pH 3.0	Fluorescence detection: Precolumn derivatization with phenacyl bromide; Excitation: 305 nm, Emission: 370 nm	10 ng/ml 10–320 ng/ml	Plasma	Protein precipitation with Acetonitrile–Water–acetic acid (80:19:1) Derivatization on the supernatant	Use of 5′-CMP as internal standard	Eisenberg and Cundy [51]

the columns gave broad and asymmetrical foscarnet peaks. In addition, the retention of foscarnet was highly dependent on the pH of the mobile phase. This method is not suitable for biological fluids because of the presence of endogenous phosphate.

An ion-exchange liquid chromatography with conductivity detection was proposed by den Hartigh et al. [26] for analysis of mono- or biphosphonates in pharmaceutical preparations. The separation was performed on a Waters IC–PAK anion column using 2 m*M* nitric acid or 25 m*M* succinic acid as the mobile phase. This technique has only moderate sensitivity which makes the proposed assay less suitable for bioanalysis at least for biphosphonates e.g., pamidronate and dimethylpamidronate. However, bioanalysis of foscarnet by this method should cause few problems as the drug is administered in much higher dosages.

Woods et al. [27] described an ion-pair chromatographic technique with UV detection for the study of foscarnet stability in 0.9% sodium chloride injection. A sample volume of 20 μl was injected onto a 150 mm×3.9 mm C_{18} reversed-phase NovaPak column (Waters) at 25°C. The mobile phase (5% methanol, 0.005 *M* sulfuric acid, and 0.904 g of tetrahexylammonium hydrogen sulphate ($THAHSO_4$) in a total volume of 1000 ml) was used at a flow-rate of 1.5 ml/min. The eluent was monitored at 254 nm. This technique was used by Bundgaard and Mørk [13] for the study of decarboxylation kinetics of foscarnet in acidic aqueous solution but the effluent was monitored at 236 nm. At this wavelength, foscarnet shows a molar extinction coefficient of 160 $M^{-1}cm^{-1}$, leading us to predict a low sensitivity of UV detection. The method was not validated for biological fluids.

Pettersson et al. [16] described an assay for plasma, urine and cerebrospinal fluid (CSF). Before and after the ultrafiltration, the biological fluids were diluted with 1 m*M* pyrophosphoric acid solution (pH 5.8) as competing complexing agent avoiding foscarnet retention by metal ion of the LC system and its peak deformation. Samples with low levels of foscarnet were treated with activated charcoal in order to remove endogenous interfering compounds. EDTA and citrate are the most common complex formers but neither of them could be used because they interfered at high potentials used for electrochemical detection (ECD). Indeed, UV detection could not be used as foscarnet has sensitive UV absorption at wavelengths where endogenous compounds severely interfered. Instead, ECD by oxidation was used. The high potentials necessary (+0.75 V for guard cell and +0.90 V for analytical cell), also limited the choice of mobile phase components. Methanol, despite the high viscosity of its mixture with water, was used as an organic modifier as it gave a low background current compared with e.g., acetonitrile. Because of its protolytic properties, foscarnet has to be chromatographed in ionized form. A hydrophobic counter ion, $THAHSO_4$, was added to the mobile phase in order to retain foscarnet on a LKB UltroPac C_{18} column as ion pairs. Phosphate buffer pH of 5.8 was chosen, being the best compromise between

column efficiency, stability and selectivity. According to the investigation performed by Wehrli et al. [28], a pH above 7 with a lipophilic quaternary ammonium ion added to the mobile phase gave short column lifetimes owing to destruction of the silica support. Linear calibration graphs were obtained up to a limiting concentration of 15 μ*M*. As foscarnet is known to be unstable at lower pH values [12], the stability at pH 2 was investigated. If sample workup and injection into the LC system are done within 30 min, the decrease in concentration will be negligible. Some modifications of this method, including the use of hydrochlorothiazide as an internal standard, were proposed by Hassanzadeh et al. [29]. Their technique allowed reliable measurement of foscarnet in human plasma and urine at concentrations as low as 33 μ*M*. The limit of detection was 14.0 μ*M*. The last two methods were fully used for pharmacokinetic and/or pharmacodynamic studies of foscarnet after intravenous and/or oral administration [14,30–33], investigations of its penetration into CSF [32,34,35] or eye's vitreous humor [36], studies of pharmacokinetic interaction between foscarnet and zalcitabine [37], ganciclovir [38] or zidovudine [39], and the evaluation of its disposition during peritoneal dialysis [40]. After oral administration of 4000 mg in solution every 6 h for 3 days in six patients [14], plasma concentrations were less than 33 μmol (the limit of quantification of Hassanzadeh's method) in four patients, two had occasional concentrations of 35 to 50 μ*M*. During intravenous administration of 16,000 mg/24 h over 72 h in the same subjects, plasma concentrations ranged between 33 to 50 μ*M*. The method proposed by Pettersson [16] which allows to reach a limit of quantification of 15 μ*M* was applied successfully. The mean intravitreous foscarnet concentrations in AIDS patients receiving maintenance dosage of 120 mg/kg/day was 163±167 μ*M* (n=4) [36]. The foscarnet vitreous–plasma concentration ratio averaged 1.43. After repeated 2-h infusion of 90 mg per kg of body weight in 26 patients [34], mean foscarnet levels in plasma at steady state were 464±219 μg/ml (1553 μ*M*) and mean levels in CSF were 308±155 μg/ml (1023 μ*M*). The CSF penetration coefficient was 0.66±0.11.

As foscarnet has been used successfully in the treatment of CMV retinitis in patients with AIDS, handling of biological samples for drug monitoring or clinical pharmacokinetic studies must be reduced to its simplest form, in spite of a previous inactivation by heating. As emphasized by McDowall [41], the automation of sample treatment reduces human contact with biohazards. Therefore, we proposed a fully automated HPLC method comprising solid-phase extraction (SPE) of calf and human serum samples and ion pair chromatography of foscarnet with amperometric detection [42]. SPE on a 50 mg strong anion-exchanger was performed using a Gilson ASPEC XLi system connected to the LC system via a switching valve. The analyte was eluted with a 50 m*M* potassium pyrophosphate buffer, pH 8.4. Its pH insured the stability of foscarnet. The use of column and all tubing in PEEK, allowed no addition of pyrophosphoric acid in the mobile phase. The mobile phase consisted of methanol–40 m*M* disodium phosphate buffer, pH 7.6 containing 0.25 m*M* $THAHSO_4$ (25:75, v/v). Since the aqueous component of our mobile phase was adjusted to pH 7.6, we avoided the rapid attack of the stationary phase by selecting Kromasil C_{18}, a full endcapped octadecyl silica stationary phase with a carbon content of 19%. It is expected that octadecyl silicas which have a high carbon content would withstand eluent having higher pH and salt concentration over a significantly long period of time. The successful use of electrochemical detection requires knowledge of the appropriate potential to effect the desired electrochemical reaction (i.e., oxidation) of the species of interest. This potential is dependent on a large number of factors including the pH of the mobile phase. A plot of hydrodynamic voltammogram was performed with the buffer component of the mobile phase at different pH, 5.8 (pH proposed in Pettersson's [16] and Hassanzadeh's [29] methods), 7.6 and 8.8 (pH values greater than 7.3, the highest pK_a value of foscarnet and at which the compound is mostly under its completely ionized form). Fig. 4 shows the plot of current versus applied potential both for mobile phase and foscarnet. Fig. 5 shows the plot of the difference between foscarnet and the mobile phase currents versus applied potential. Phosphate buffer at pH 7.6 gave the highest maximum difference corresponding to a potential of +1.125 V. This buffer and the value of +1.125 V were therefore retained respectively as the mobile

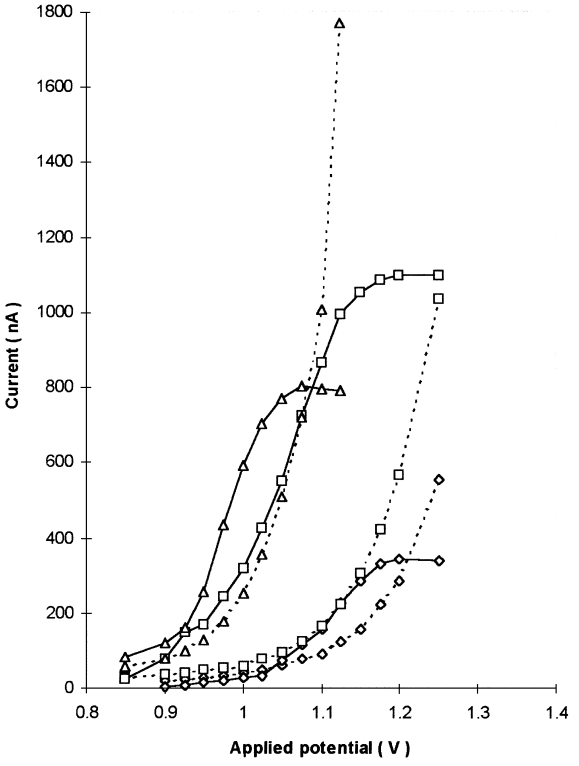


Fig. 4. Hydrodynamic voltammograms of mobile phase (— — —) and foscarnet aqueous solution (———) at mobile phase buffer pH of 5.8 (◇), 7.6 (□) and 8.8 (△).

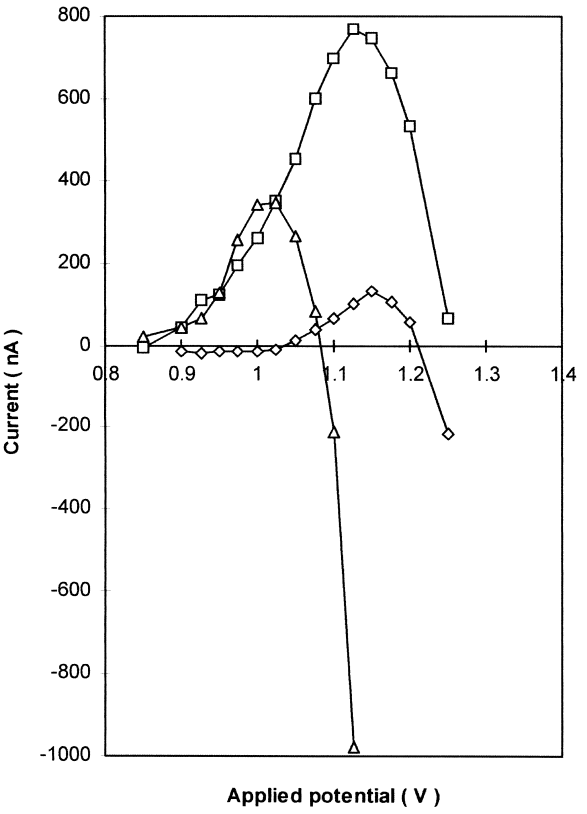


Fig. 5. Plot of the difference between currents generated respectively by foscarnet aqueous solution and mobile phase versus applied potential at mobile phase buffer pH of 5.8 (◇), 7.6 (□) and 8.8 (△).

phase aqueous component and the working potential. Amperometric detection allowed a quantification limit of 15 μ*M*. The assay was linear from 15 to 240 μ*M*. In routine analysis, quality control samples are usually prepared with citrated human plasma provided from blood banks. Since citrate interfered, the matrix selected for the determination of foscarnet was serum. Except for providing from laboratory suppliers but with high cost, it is almost impossible to obtain a sufficient amount of human serum for quality control purposes. Therefore the replacement by calf serum was validated since this matrix could be obtained easily and its biochemical composition is close to that of human serum [43,44]. Typical chromatograms of calf and human blank serums and calf and human serums spiked with foscarnet at concentrations of 15 and 200 μ*M* are shown in Fig. 6. The method was successfully applied to the analysis of serum samples from two patients with AIDS and CMV retinitis receiving a 2-h intravenous infusion of 6.0 g (20 m*M*) of foscarnet. Maximal concentrations of 714.64 μ*M* and 1073.71 μ*M* and $C_{12\ h}$ of 52.27 μ*M* and 55.48 μ*M* were obtained.

2.2. Cidofovir

Analytical methods that employ reversed-phase HPLC coupled with detection by UV absorbance were proposed for the determination of cidofovir in 0.9% sodium chloride and 5% dextrose injections [18] or in biological samples [45] and for the analysis of other cytosine-based compounds, i.e., 2′,3′-dideoxycytidine (ddc) in biological fluids [46]. Yuan et al. [18], used an Hypersil octadecyl silane (ODS) C_{18} column for the separation of cidofovir and its deaminated degradation by-product, HPMPU.

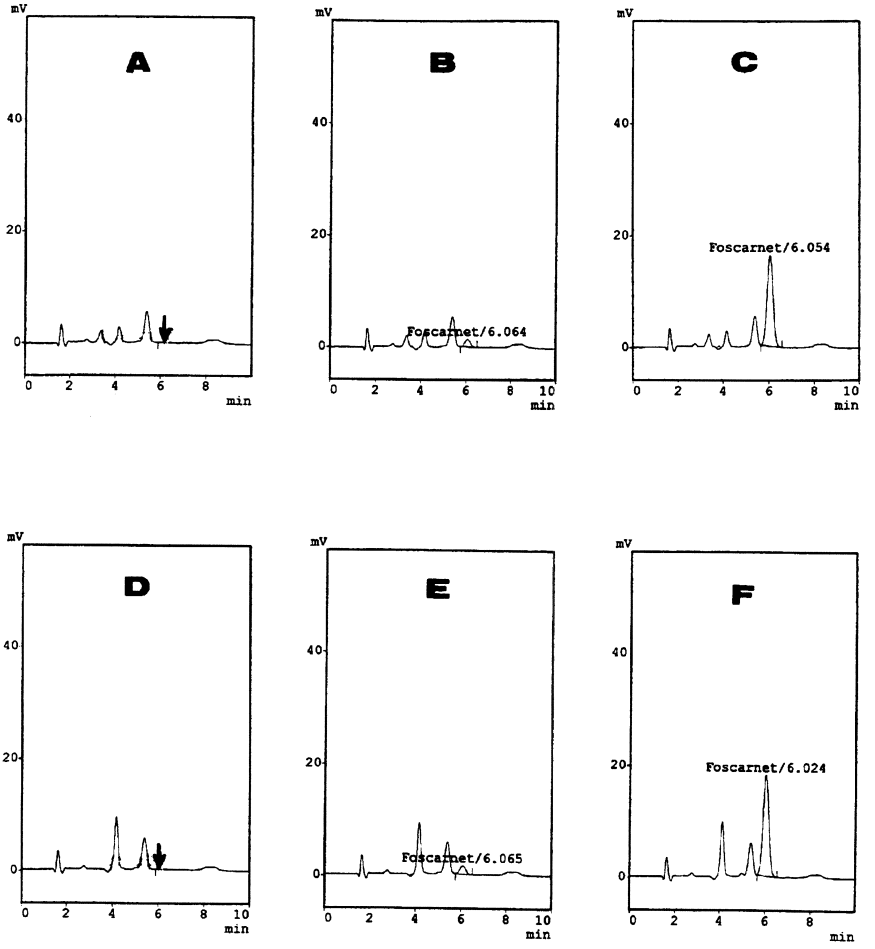
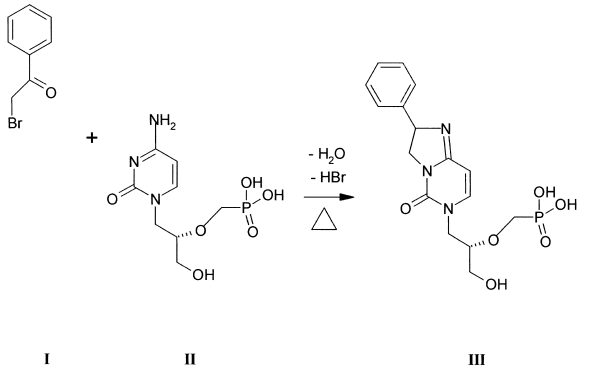


Fig. 6. Typical chromatograms of calf blank serum (A), spiked calf serum at 15 μ*M* (B) and 200 μ*M* (C), and human blank serum (D), spiked human serum at 15 μ*M* (E), and 200 μ*M* (F).

The mobile phase consisted of 3.5 m*M* dibasic sodium phosphate heptahydrate with 5.0 m*M* tetrahexylammonium dihydrogen phosphate ($THAHPO_4$) adjusted to pH 6.0. The eluent was monitored at 280 nm. The linearity of the assay was validated for the concentration range of 2–400 μg/ml. Cundy et al. [45] proposed a gradient elution reversed-phase ion pairing HPLC method with internal standardization. Serum or urine (0.5 ml) was added to 9-(2-phosphonylmethoxyethyl)guanine (PMEG), the internal standard and the mixture was incubated at 63°C for 25 min to inactivate HIV. Then a SAX ion-exchange solid-phase extraction column and a C_{18} solid-phase extraction column were used for serum and urine, respectively. The analytical separation for serum samples was performed with a Zorbax C_8 column equipped with a C_8 guard column and a binary linear gradient using mixtures of acetonitrile and 100 m*M* phosphate buffer (pH 7.2) containing 5 m*M* tetrabutylammonium hydrogen phosphate ($TBAHPO_4$). The column used for urine samples was an Ultrasphere ODS IP equipped with a RP-18 guard column and the isocratic elution was performed with a mixture acetonitrile–methanol–water containing 5 m*M* Q8 (octyltriethylammonium phosphate) (5:5:90, v/v). Peaks were detected by determining the UV absorbance at 274 nm. The method was linear over 0.22 to 2.19 μg/ml and over 1.0 to 99 μg/ml for

serum and urine samples, respectively. The method was used by the authors to monitor cidofovir levels in serum and urine during 12 h in 42 HIV-infected patients receiving 1-h infusion of 1.0 to 10 mg/kg of body weight. Following intravenous administration, the pharmacokinetics of cidofovir were characterized by an apparent biexponential decline in concentrations in serum, with an elimination half-life of approximately 2.5 h. The majority of the administered drug was recovered unchanged in the urine, and no metabolites of cidofovir were detected in clinical urine or serum samples. The mean C_{max} values ranged from 3.12±0.67 μg/ml (n=5) to 23.56±4.88 μg/ml (n=8) for a dose range 1–10 mg/kg of body weight. Even for the maximal dose, $C_{12\ h}$ was close to 1.0 μg/ml. Since the standard cidofovir dosage regimen was 5 mg/kg every week for 2 weeks (induction dosage) followed by a maintenance dosage of 5 mg/kg every 2 weeks [47], the proposed method allows at the very most to follow serum concentrations within the first 12 h post-infusion. A similar dose ranging study performed by Wachsman et al. [48] and using the same analytical method led to data close to those of the previous investigation. The method described by Kalin and Hill [46] for the measurement of ddc in plasma had a limit of quantification in the same order of size, namely, 0.20 μg/ml. Since UV detection might not be sufficient for the detection of cidofovir and other cytosine-containing compounds in biological fluids, the search for suitable techniques turned towards fluorescent derivatives. A promising approach to the synthesis of fluorescent nucleotide derivatives consisted in fusion of an additional ring onto a purine or pyrimidine system of common nucleotides [49]. Reaction of chloroacetaldehyde with adenine and cytosine derivatives was the first example of this approach. The ethenoadenine derivatives formed in purine series were found to be highly fluorescent. At the same time, the ethenocytosine derivatives obtained from cytosine derivatives exhibited rather weak fluorescence [49]. The reaction may be improved by using bromoacetaldehyde in place of chloroacetaldehyde, because the bromo-compound is expected to be more reactive than the chloro-compound [50]. The derivatization of cytosine-containing compounds with haloacetaldehydes offered no advantage over direct UV detection [51]. Useful fluorescent cytosine derivatives were obtained by modification of cytosine nucleus with α-haloketones. It has been reported that 2-phenyl-substituted 3,N^4-ethenocytosine derivatives possess much stronger fluorescence than the unsubstituted derivatives and a reaction with ω-bromoacetophenone (phenacyl bromide) (Fig. 7) has been suggested [49,51]. Eisenberg and Cundy [51] described the application of precolumn derivatization with phenacyl bromide to HPLC detection of various cytosine-containing compounds, including cidofovir. The 2-phenyl-3,N^4-ethenocytosine derivatives are significantly more lipophilic than cytosine-containing compounds which generally are poorly retained on reversed-phase sorbents. One of the most difficult problems in detection of cidofovir in biological fluids is the presence of a large number of endogenous bases and their nucleotides and nucleosides. These endogenous compounds possess very similar chromatographic and detection characteristics to those of cidofovir. The combination of derivatization of the drug and ion-pair reversed-phase HPLC separation greatly improves selectivity of detection [51]. For demonstration of the general applicability of the method for derivatization and detection of various cytosine-containing compound including (−)-*cis*-5-(4-amino-1,2-dihydro-2-oxo-1-pyrimidinyl)-1,3-oxothiolane-2-methanol (lamivudine, 3TC) and ddc, an isocratic reversed-phase HPLC method was used. The mobile phase contained 0.1% (v/v) trifluoroacetic acid in acetonitrile–water (16:84, v/v). The column (ODS-2) was maintained at 45°C. The



I II III

Fig. 7. Schematic reaction of phenacyl bromide with cidofovir (I, phenacyl bromide; II, cidofovir; III, fluorescent 2-phenyl-3,N^4-ethenocytosine derivative of cidofovir).

fluorescence detector was set at the excitation wavelength of 305 nm and the emission wavelength of 370 nm. The most efficient separation of the derivatives of cidofovir and cytidine-5′-monophosphate (5′-CMP) (internal standard) from endogenous compounds in plasma was achieved using an isocratic ion-pair reversed-phase HPLC method. The mobile phase consisted of Q12 (dodecyltriethylammonium phosphate) (6 m*M*) and phosphoric acid (12 m*M*) in acetonitrile–water (30:70, v/v). The pH of the aqueous component was adjusted to 3.0. Protein precipitation of plasma samples was performed with a mixture acetonitrile–water acetic acid (80:19:1, v/v) containing the internal standard. To find the optimum conditions for the derivatization reaction, several parameters of the reaction were investigated, including the concentration of phenacyl bromide, pH, temperature and organic solvent. The response remained constant within the pH range of 3.5–5 but decreased outside that range (at pH 2 the fluorescence responses were about 50% of the maximum value and at pH 7, they were about 25% of the maximum). A plateau in the response curve was generally reached at about 100 m*M* phenacyl bromide for the aqueous standards and 350 m*M* phenacyl bromide for plasma sample. The difference is probably due to the presence of high concentrations of reactive endogenous compounds competing with cidofovir for the reagent [51]. Since phenacyl bromide itself is not fluorescent, the high concentration of the unreacted reagent did not interfere with chromatography. Acetonitrile produced a better yield than methanol, methyl cellosolve or dimethylsulfoxide (DMSO). A reaction temperature of 80°C and reaction time of 45 min provided the optimal combination of the highest yields in the shortest period of time [51]. Standard curves were linear from 10 to 320 ng/ml and the method detection limit, determined at the signal-to-noise ratio of 3, was 5 ng/ml for cidofovir. These results express a notable enhancement of the sensitivity in comparison to the UV detection method and allow drug monitoring within longer a post-infusion period.

3. Gas chromatography

Rindgen et al. [52] used a gas chromatography–mass spectroscopy method to study the pharmacokinetics of foscarnet in bone marrow and renal transplant patients with CMV infections. No details about this method were provided except the linearity range (0.05–50 mg/l), the reproducibility [±3% (intra-) and ±7% (inter-assay)] and the sensitivity limit of 0.06 mg/l in plasma. Nevertheless, the laborious sample work-up of this method was criticized by Pettersson et al. [16]. As far as we are aware, no GC method for determination of cidofovir in biological fluids or pharmaceutical formulations has been published.

4. Conclusion

Since the two phosphorus-containing antiviral drugs, foscarnet and cidofovir were highly nephrotoxic, sensible and selective analytical methods were needed for accurate drug monitoring. Taking into account the great hydrophilicity of the two compounds, ion-pair reversed-phase HPLC was essential as a predilection technique. Otherwise, if their low UV absorption was not a nuisance for the determination in pharmaceutical formulations, it became a drawback when the analysis had to be performed on biological fluids. The alternatives were the use of ECD for foscarnet and the development of fluorescent derivatives for cidofovir, allowing to reach sensitivity and selectivity targets.

5. Nomenclature

AIDS	acquired immunodeficiency syndrome
cHPMP	cyclic HPMPC 1-[((*S*)-2-hydroxy-2-oxo-1,4,2-dioxaphosphorinan-5-yl)methyl] cytosine
5′-CMP	cytidine-5′-monophosphate
CMV	cytomegalovirus
CSF	cerebrospinal fluid
ddc	2′,3′-dideoxycytidine
DEAE	diethylamino ethyl
DNA	deoxyribonucleic acid
ECD	electrochemical detection
EDTA	ethylenediaminetetra-acetic acid
GC	gas chromatography
HAART	high active antiretroviral treatment
HIV	human immunodeficiency virus

HPLC	high-performance liquid chromatography
HPMPC	(*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl) cytosine
HPMPU	1-[(*S*)-3-hydroxy-2-(phosphonomethoxy)propyl] uracil
LC	liquid chromatography
ODS	octadecyl silane
PEEK	polyether ether ketone
PMEG	9-(2-phosphonyl methoxyethyl) guanine
PPA	pyrophosphoric acid
Q8	octyl triethylammonium phosphate
Q12	dodecyl triethylammonium phosphate
SPE	solid-phase extraction
$TBAHPO_4$	tetrabutylammonium hydrogen phosphate
$THAHPO_4$	tetrahexylammonium hydrogen phosphate
$THAHSO_4$	tetrahexylammonium hydrogen sulphate
UV	ultraviolet

References

[1] W.L. Drew, Clin. Infect. Dis. 14 (1992) 608.
[2] D. Armstrong, J.W.M. Gold, J. Dryjanski, E. Whimbey, B. Polsky, C. Hawkins, A.E. Brown, E. Bernard, T.E. Kiehn, Ann. Intern. Med. 103 (1985) 738.
[3] M.A. Polis, H. Masur, J. Am. Med. Assoc. 273 (1995) 1457.
[4] M.A. Jacobson, M. Zegans, P.R. Pavan, J.J. O′Donnell, F. Sattler, N. Rao, S. Owens, R. Pollard, Lancet 349 (1997) 1443.
[5] W.L. Drew, R.C. Miner, D.F. Busch, S.E. Follansbee, J. Gullett, S.G. Mehalko, S.M. Gordon, W.F. Owen Jr., T.R. Matthews, W.C. Buhles, B. De Armond, J. Infect. Dis. 163 (1991) 716.
[6] M.A. Jacobson, W.L. Drew, J. Feinberg, J.J. O'Donnell, P.V. Whitmore, R.D. Miner, D. Parenti, J. Infect. Dis. 163 (1991) 1348.
[7] P. Chrisp, S.P. Clissold, Drugs 41 (1991) 104.
[8] D.B. Mendel, T. Cihlar, K. Moon, M.S. Chen, Antimicrob. Agents Chemother. 41 (1997) 641.
[9] M.J.M. Hitchcock, S.A. Lacy, J.R. Lindsey, E.R. Kern, Antiviral Res. 26 (1995) A358.
[10] A.P. Lea, H.M. Bryson, Drugs 52 (1996) 225.
[11] C.M. Riley, J.M. Ault Jr., N.E. Klutman, J. Chromatogr. 531 (1990) 295.
[12] S. Warren, M.R. Williams, J. Chem. Soc. B (1971) 618.
[13] H. Bundgaard, N. Mørk, Int. J. Pharm. 63 (1990) 213.
[14] J. Sjövall, A. Karlsson, S. Ogenstad, E. Sandström, M. Saarimäki, Clin. Pharmacol. Ther. 44 (1988) 65.
[15] W.A. Ritschel, K.W. Grummich, S.A. Hussain, Methods Find. Exp. Clin. Pharmacol. 7 (1985) 41.
[16] K.J. Pettersson, T. Nordgren, D. Westerlund, J. Chromatogr. 488 (1989) 447.
[17] Vistide®, Cidofovir–Monographie, Pharmacia and Upjohn, 1997.
[18] L.-C. Yuan, G.J. Samuels, G.C. Visor, Am. J. Health Syst. Pharm. 53 (1996) 1939.
[19] E.J. Eisenberg, G.R. Lynch, A.M. Bidgood, K. Krishnamurty, K.C. Cundy, J. Pharm. Biomed. Anal. 16 (1998) 1349.
[20] K.C. Cundy, G. Lynch, P.-P. Shaw, M.J.M. Hitchcock, W.A. Lee, Curr. Eye Res. 15 (1996) 569.
[21] K.C. Cundy, Z.H. Li, W.A. Lee, Drug Metab. Dispos. 24 (1996) 315.
[22] K.C. Cundy, Z.-H. Li, M.J.M. Hitchcock, W.A. Lee, Drug Metab. Dispos. 24 (1996) 738.
[23] N. Bischofberger, M.J.M. Hitchcock, M.S. Chen, D.B. Barkhimer, K.C. Cundy, K.M. Kent, S.A. Lacy, W.A. Lee, Z.-H. Li, D.B. Mendel, D.F. Smee, J.L. Smith, Antimicrob. Agents Chemother. 38 (1994) 2387.
[24] U. Forsman, M. Andersson, H. Törnros, J. Chromatogr. 369 (1986) 151.
[25] Y. Hirai, N. Yoza, S. Ohashi, J. Chromatogr. 206 (1981) 501.
[26] J. den Hartigh, R. Langebroek, P. Vermeij, J. Pharm. Biomed. Anal. 11 (1993) 977.
[27] K. Woods, W. Steinmann, L. Bruns, J.T. Neels, Am. J. Hosp. Pharm. 51 (1994) 88.
[28] A. Wehrli, J.C. Hildenbrand, H.P. Keller, R. Stampfli, R.W. Frei, J. Chromatogr. 149 (1978) 199.
[29] M.K. Hassanzadeh, F.T. Aweeka, S. Wu, M.A. Jacobson, J.G. Gambertoglio, J. Chromatogr. 525 (1990) 133.
[30] F. Castelli, L. Tomasoni, C. Zeroli, F. Suter, G. Trespi, G. Carosi, A. Hedman, Eur. J. Clin. Pharmacol. 52 (1997) 397.
[31] A.-M. Taburet, C. Katlama, C. Blanshard, G. Zorza, D. Gazzard, E. Dohin, B.G. Gazzard, C. Frostegard, E. Singlas, Antimicrob. Agents Chemother. 36 (1992) 1821.
[32] J. Sjövall, S. Bergdahl, G. Movin, S. Ogenstad, M. Saarimäki, Antimicrob. Agents Chemother. 33 (1989) 1023.
[33] M.A. Jacobson, B. Polsky, D. Causey, R. Davis, W. Tong, J.J. O'Donnell, B.D. Kuppermann, M.H. Heinemann, J. Feinberg, P. Lizak, J.G. Gambertoglio, F.T. Aweeka, Antimicrob. Agents Chemother 38 (1994) 1190.
[34] U.R. Hengge, N.H. Brockmeyer, R. Malessa, U. Ravens, M. Goos, Antimicrob. Agents Chemother. 37 (1993) 1010.
[35] F. Raffi, A.-M. Taburet, B. Ghaleh, A. Huart, E. Singlas, Antimicrob. Agents Chemother. 37 (1993) 1777.
[36] J. Fernando Arevalo, C. Gonzalez, E.V. Capparelli, L.S. Kirsch, R.F. Garcia, J.I. Quiceno, J.D. Connor, J. Gambertoglio, G. Bergeron-Lynn, W.R. Freeman, J. Infect. Dis. 172 (1995) 951.
[37] F.T. Aweeka, S.R. Brody, M. Jacobson, K. Botwin, S. Martin-Munley, Clin. Ther. 20 (1998) 232.
[38] F.T. Aweeka, J.G. Gambertoglio, F. Kramer, C. van der Horst, B. Polsky, A. Jayewardene, P. Lizak, L. Emrick, W. Tong, M.A. Jacobson, Clin. Pharmacol. Ther. 57 (1995) 403.
[39] F.T. Aweeka, J.G. Gambertoglio, C. van der Horst, R. Raasch, M.A. Jacobson, Antimicrob. Agents Chemother. 36 (1992) 1773.
[40] A.C.M. Alexander, A. Akers, G.R. Matzke, F.T. Aweeka, D.S. Fraley, Ann. Pharmacother. 30 (1996) 1106.
[41] R.D. Mc Dowall, J. Chromatogr. 492 (1989) 3.

[42] B.B. Ba, A.-G. Corniot, D. Ducint, D. Breilh, J. Grellet, M.C. Saux, J. Chromatogr. B 724 (1999) 127.
[43] I.C. Dijkhuis, Ph D. Thesis, University of Leiden, 1979.
[44] S. Touratier, P. Prognon, E. Postaire, J.C. Darbord, H. Cerceau, D. Pradeau, B. Lages, K. Gelinier, J. Pharm. Clin. 5 (1986) 287.
[45] K.C. Cundy, B.G. Petty, J. Flaherty, P.E. Fisher, M.A. Polis, M. Wachsman, P.S. Lietman, J.P. Lalezari, M.J.M. Hitchcock, H.S. Jaffe, Antimicrob. Agents Chemother. 39 (1995) 1247.
[46] J.R. Kalin, D.L. Hill, J. Chromatogr. 431 (1988) 184.
[47] G.L. Plosker, S. Noble, Drugs 58 (1999) 325.
[48] M. Wachsman, B.G. Petty, K.C. Cundy, H.S. Jaffe, P.E. Fisher, A. Pastelak, P.S. Lietman, Antiviral Res. 29 (1996) 153.
[49] N.K. Kochetkov, V.N. Shibaev, A.A. Kost, A.P. Razjivin, A.Yu. Borisov, Nucleic Acids Res. 3 (1976) 1341.
[50] K. Kayasuga-Mikado, T. Hashimoto, T. Negishi, K. Negishi, H. Hayatsu, Chem. Pharm. Bull. 28 (1980) 932.
[51] E.J. Eisenberg, K.C. Cundy, J. Chromatogr. B 679 (1996) 119.
[52] O. Ringdén, B. Lönnqvist, T. Paulin, J. Ahlmén, G. Klintmalm, B. Wahren, J.-O. Lernestedt, J. Antimicrob. Chemother. 17 (1986) 373.