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Determination of phosphonoformate (foscarnet) in calf and human serum by automated solid-phase extraction and high-performance liquid chromatography with amperometric detection

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Abstract

An isocratic high-performance liquid chromatographic method with automated solid-phase extraction has been developed to determine foscarnet in calf and human serums. Extraction was performed with an anion exchanger, SAX, from which the analyte was eluted with a 50 m*M* potassium pyrophosphate buffer, pH 8.4. The mobile phase consisted of methanol–40 m*M* disodium hydrogenphosphate, pH 7.6 containing 0.25 m*M* tetrahexylammonium hydrogensulphate (25:75, v/v). The analyte was separated on a polyether ether ketone (PEEK) column 150×4.6 mm I.D. packed with Kromasil 100 C_{18}, 5 μm. Amperometric detection allowed a quantification limit of 15 μ*M*. The assay was linear from 15 to 240 μ*M*. The recovery of foscarnet from calf serum ranged from 60.65±1.89% for 15 μ*M* to 67.45±1.24% for 200 μ*M*. The coefficient of variation was ≤3.73% for intra-assay precision and ≤7.24% for inter-assay precision for calf serum concentrations ranged from 15 to 800 μ*M*. For the same samples, the deviation from the nominal value ranged from −8.97% to +5.40% for same day accuracy and from −4.50% to +2.77% for day-to-day accuracy. Selectivity was satisfactory towards potential co-medications. Replacement of human serum by calf serum for calibration standards and quality control samples was validated. Automation brought more protection against biohazards and increase in productivity for routine monitoring and pharmacokinetic studies.

Keywords: Foscarnet; Phosphonoformate

1. Introduction

Trisodium phosphonoformate (foscarnet) is an antiviral inhibiting selectively in vitro the DNA polymerase of human herpes viruses including cytomegalovirus (CMV) and the reverse transcriptase of human immunodeficiency virus (HIV) [1], which makes it an attractive drug for individuals infected with HIV-1, especially those with low CD4+ lymphocyte counts who are at risk for CMV disease [2,3]. There are wide inter-individual variations in plasma concentrations following infusion, possibly because of foscarnet deposition in bone and cartilage [1]. The most frequent adverse effect of foscarnet

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therapy was a two- to three-fold increase in serum creatinine levels [1]. Significantly better survival and better control of CMV retinitis progression were reported with maintenance therapy adjusted for renal function [4,5].

The development of techniques for measuring serum concentrations makes a major contribution to the rationalization of such a drug therapy. Few methods were proposed in the literature for the determination of foscarnet in drug formulations and biological samples.

Forsman et al. [6] described an ion-exchange chromatographic method for the separation of foscarnet and its degradation products in drug formulations. Detection was performed at 340 nm.

Woods et al. [7] proposed an ion pair chromatographic technique with UV detection at 254 nm.

The two mentioned methods were not validated for biological fluids. Otherwise, foscarnet shows an absorption maximum at 236 nm with a molar extinction coefficient of 160 M^{-1} cm^{-1} [8], leading us to predict a low sensitivity of UV detection.

Pettersson et al. [9] described an assay for plasma, urine and cerebrospinal fluid. After ultrafiltration and/or treatment with charcoal, the samples were injected into a reversed-phase liquid chromatography (LC) system with coulometric detection. Some modifications of this method, including the use of hydrochlorothiazide as internal standard, were proposed by Hassanzadeh et al. [10].

This paper describes a full automated high-performance liquid chromatography (HPLC) method comprising solid-phase extraction (SPE) of calf and human serum samples and ion pair chromatography of foscarnet with amperometric detection. As emphasized by McDowall [11], the automation of a process should produce greater increases in productivity (either in numbers of samples assayed per unit of time or speedier turn around time) but also reduce human contact with biohazards. Since foscarnet has been used successfully in the treatment of CMV retinitis in patients with acquired immunodeficiency syndrome (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.

Comparison of recovery from human and calf serums was done, validating the use of calf serum for the preparation of calibration and quality control (QC) samples.

2. Experimental

2.1. Chemical

Phosphonoformic acid trisodium salt hexahydrate was purchased from Sigma (Saint Quentin Fallavier, France). Compounds used for analytical interference studies, gifts from laboratories, were: didanosine (Bristol Myers Squibb, Paris, France), zidovudine, aciclovir, trimethoprim (Glaxo Wellcome, Paris, France), saquinavir, nelfinavir, ganciclovir, sulfamethoxazole (Produits Roche, Neuilly-sur-Seine, France), ritonavir (Abbott, Rungis, France), cidofovir (Pharmacia and Upjohn, Saint Quentin Yvelines, France), indinavir (Merck Sharp Dohme Chibret, Paris, France), pyrimethamine (Specia, Paris, France) and fluconazole (Pfizer, Orsay, France). Analytical grade (Normapur) potassium dihydrogenphosphate, disodium hydrogenphosphate dihydrate, potassium hydroxide, 85% orthophosphoric acid and methanol were provided by Prolabo (Fontenay-sous-Bois, France). Pyrophosphoric acid (purity=97%) and tetrahexylammonium hydrogensulphate ($THAHSO_4$) (purity=98%) were purchased from Fluka (Saint Quentin Fallavier, France). Calf serum and human serum were provided by Sigma. HPLC grade water was obtained with a Milli-Q water purification unit, Millipore (Saint Quentin Yvelines, France).

2.2. SPE and HPLC instrumentation and conditions

A fully automated SPE on 50 mg I.S.T. (International Sorbent Technology) strong anion exchanger Isolute SAX (Touzart and Matignon, Courtaboeuf, France) was performed using an ASPEC XLi system (Gilson Medical Electronics France, Villiers le Bel, France) equipped with an electrically actuated two positions switching valve including a 20-μl loop. This valve is connected to the injection port and to the analytical column, allowing injection of the extraction eluent into the LC system.

The LC system was composed of a Shimadzu LC10AT solvent delivery unit and a CBM-10A

communication bus module (Touzart and Matignon). Chromatographic separation was conducted on a Higgins Analytical polyether ether ketone (PEEK) column 150×4.6 mm I.D. packed with Kromasil 100 C_{18}, 5 μm (Touzart and Matignon).

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). The pH of the buffer was adjusted to 7.6 with orthophosphoric acid. The flow-rate of the mobile phase was 1.0 ml/min.

Electrochemical detection was performed in oxidation mode with a B.A.S. (Bioanalytical Systems) amperometric detector (Biochrom, Champiers, France) including a LC-17A module equipped with a glassy carbon flow cell and a LC-4B electronic module, set at a potential of +1.125 V and a sensitivity range of 100 nA.

Control of the LC system and data acquisition was performed with Shimadzu CLASS-LC10 software (Touzart and Matignon) on a Compak Deskpro 66M personal computer (Compak France, Issy-les-Moulineaux, France).

2.3. Stock solutions, working solutions and spiked serum samples

Foscarnet was made up as 10 mg (33333.33 μ*M*) per ml stock solution in water. Foscarnet was diluted with blank calf serum to make spiked samples of 960 μ*M* and 800 μ*M*, and with blank human serum to make a 800 μ*M* sample. Calibration standards of 3.75, 7.5, 15, 30, 60, 120, 240 and 480 μ*M* were prepared by successive dilution of the 960 μ*M* spiked calf serum with the corresponding blank matrix. Quality control samples of 15, 20, 100, 200 and 400 μ*M* were prepared from the 800 μ*M* spiked calf serum. For human serum, only QC samples were prepared and their concentrations ranged from 15 to 200 μ*M*.

As for clinical samples, HIV virus inactivation was simulated for calibration standards and quality control samples by heating 30 min at 56°C in a water-bath [12]. After cooling, portions of 1.0 ml were transferred to 5-ml glass tubes and stored at −20°C.

For analytical interference studies, stock solutions of 1.0 mg/ml were prepared by dissolution of reference compounds in water (didanosine, zidovudine, aciclovir, ganciclovir, cidofovir, sulfamethoxazole, trimethoprim, pyrimethamine, fluconazole) or methanol (saquinavir, ritonavir, nelfinavir, indinavir). Working solutions were prepared by successive dilution with water and the mixture water–methanol (90:10, v/v), respectively for aqueous and methanolic stock solutions, in order to achieve average therapeutic concentrations.

2.4. Preparation of buffers used for SPE

2.4.1. Potassium dihydrogenphosphate (20 mM, pH 5.0)

To 2.72 g of KH_2PO_4 was added 900 ml purified water. After total dissolution, the pH was adjusted to 5.0 with potassium hydroxide and the volume to 1000 ml with purified water.

2.4.2. Potassium pyrophosphate (50 mM, pH 8.4)

After dissolution of 9.17 g of pyrophosphoric acid in 900 ml purified water, the pH was adjusted to 8.4 by addition of potassium hydroxide. Then, the volume was adjusted to 1000 ml with purified water.

2.5. Sample processing

To 700 μl serum, 700 μl of 20 m*M* potassium dihydrogenphosphate buffer, pH 5.0 was added for sample pre-treatment in order to reduce viscosity and to increase the selectivity of retention on the ion-exchange sorbent. The sample was mixed by a multiple aspiration and dispensing technique. A 1000-μl sample volume was loaded onto the extraction cartridge previously activated, respectively, with 0.50 ml methanol and 0.50 ml purified water. The dispense flows were 1.0 ml/min for methanolic activation and sample loading, and 5.0 ml/min for aqueous solvation. After washing with 0.50 ml purified water at a flow of 3.0 ml/min, elution of foscarnet was performed with 2×0.50 ml 50 m*M* potassium pyrophosphate buffer, pH 8.4, at a dispensing flow of 1.0 ml/min. The eluent was mixed and then, a 20-μl volume was injected onto the HPLC column.

2.6. Calibration and calculation

The concentrations of unknown samples were calculated from a linear calibration curve. This

calibration curve was obtained by computing a $1/x$ weighted least-squares regression of the peak area y versus foscarnet concentration x from five standard samples within the linearity range.

2.7. Recovery

The recovery was established for calf serum by six analyses of four foscarnet concentrations, 15, 20, 100 and 200 μ*M*. The response of the worked-up sample was compared with that obtained by injection of foscarnet aqueous solution at equivalent concentration directly into the LC system.

3. Results and discussion

3.1. Determination of mobile phase pH and of detector working electrode potential

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 [9] and Hassanzadeh's [10] 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).

The detector is set at a given potential and the background current generated by the mobile phase and displayed in nA on the detector electronic module, is noted. Then, the current is set at zero by electronic compensation before injection of a constant amount (20 μl of a 120 μ*M* aqueous solution) of foscarnet. The peak height expressed as current displayed in nA is noted. The process is repeated with varying potentials from 0.850 to 1.250 V. Fig. 1 shows the plot of current versus applied potential both for mobile phase and foscarnet. Fig. 2 shows the plot of the difference between foscarnet and mobile phase currents versus applied potential. Phosphate buffer at pH 7.6 gave the highest maximum of difference corresponding to a potential of +1.125 V.

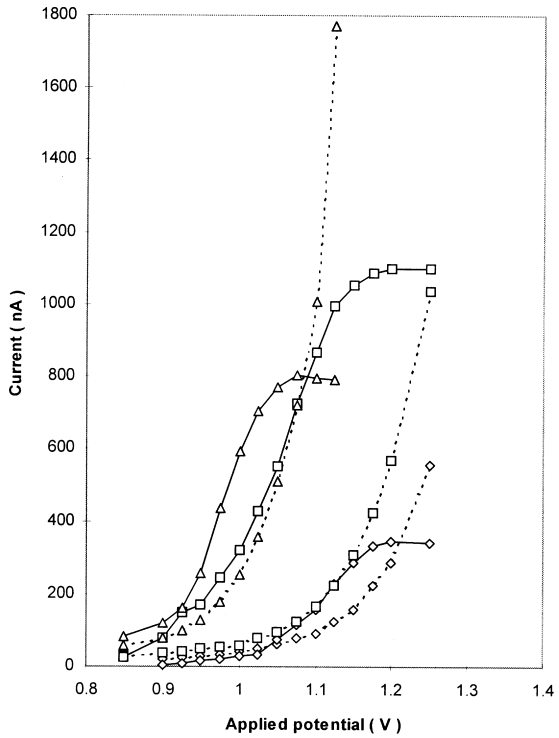

Fig. 1. Hydrodynamic voltammograms of mobile phase (- - -) and foscarnet aqueous solution (—) at mobile phase buffer pH of 5.8 (◊), 7.6 (□), and 8.8 (△).

This buffer and the value of +1.125 V were retained respectively as the mobile phase aqueous component and the working potential. The non-weighted least-squares regression of the half reaction potential y versus mobile phase pH x, led to the equation $y=-0.0458x+1.3798$, with a squared correlation coefficient r^2 of 0.9984. This equation fitted well with the Nerst equation, $E^{0\prime}=E^0-0.059(\text{pH})$, where $E^{0\prime}$ is the half reaction potential and E^0, the standard oxidation potential for the oxidation/reduction couple (25°C, 1 atm).

3.2. Selection of the ion-exchange sorbent – test of recovery

As foscarnet is extremely hydrophilic, the extraction mode retained for this acidic compound was anion-exchange. Two types of sorbent were tested, NH_2 sorbent with aminopropyl functional group (pK_a=9.8), and SAX sorbent with trimethyl amino-

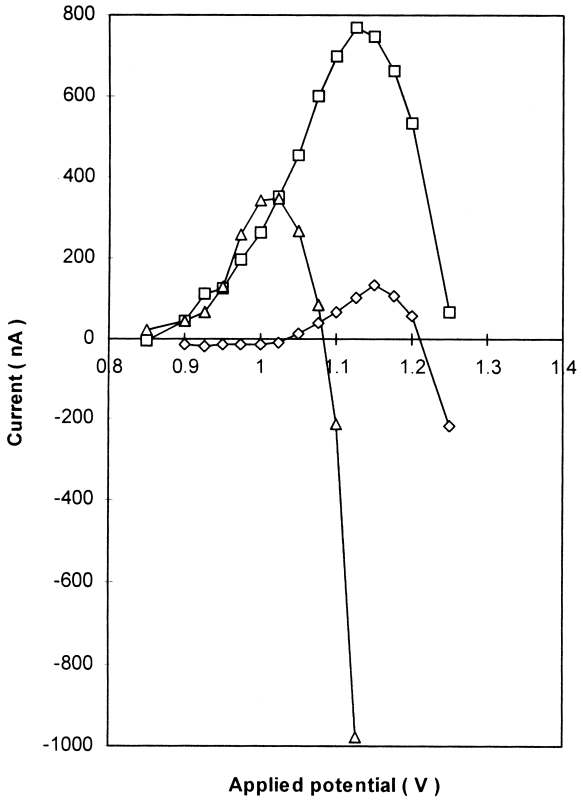

Fig. 2. Plot of the difference between currents generated respectively by foscarnet aqueous solution and mobile phase versus applied potential at mobile phase buffer pH of 5.8 (◊), 7.6 (□), and 8.8 (△).

propyl functional group. NH_2 is a very polar sorbent and is a weaker anion exchanger than SAX. Contrary to our expectation, recovery from the NH_2 sorbent (52.50±1.40–58.56±0.91%, n=6) was lower than that from SAX sorbent (60.65±1.89–67.45±1.24%, n=6) (Table 1). This result could be partly explained by the characteristic of NH_2 sorbent to exhibit all possible interactions, depending on the solvent–sample matrix environment. Therefore, SAX was retained as extraction sorbent.

Table 1
Extraction recovery of foscarnet from calf serum on NH_2 and SAX sorbents

Concentration (μ*M*)	Recovery (%) (mean±SD) (n=6)	
	NH_2	SAX
15	N.D.[a]	60.65±1.89
20	52.50±1.40	61.19±1.41
100	56.97±0.78	65.03±2.06
200	58.56±0.91	67.45±1.24

[a] N.D.=Not determined.

3.3. Selection of elution solvent

Because the fonctional group of SAX is a quaternary amine, the sorbent is always charged. Since SAX cannot be neutralized by changing the pH of the solvent, elution was accomplished by employing high selectivity counter-ions. Citrate could not be used because it interfered at the potential used for the electrochemical detection. Among the buffers tested (100 m*M* and 200 m*M* Na_2HPO_4, pH 7.6, 100 m*M* and 200 m*M* Na_2HPO_4, pH 8.4 containing or not 0.25 m*M* $THAHSO_4$, 50 m*M* potassium pyrophosphate, pH 8.4), the pyrophosphate was the most efficient. In addition, its pH insured the stability of foscarnet.

3.4. Analytical stationary phase and column selection

The stability of reversed-phase materials towards attack by various bases was tested by Wehrli et al. [13]. With strong bases such as sodium hydroxide and quaternary ammonium hydroxides, the silicate structure of these materials is attacked rapidly, rendering columns useless within one to three days. The authors used non endcapped stationary phases, LiChrosorb RP-8 and LiChrosorb RP-18, with a carbon content of 9.5 and 16.2%, respectively.

Horvath et al. [14] claimed that at pH>7, the life of the phase can be seriously reduced in the presence of aqueous salt solutions. The stationary phase used was Partisil ODS with a carbon content of 5%.

Since the aqueous component of our mobile phase was adjusted to pH 7.6, we avoided this drawback 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 longer period of time than Partisil ODS or LiChrosorb RP-8 and LiChrosorb RP-18 do.

All the validation tests, that is to say at least 450 samples, were analysed with the same column within

six weeks, without appreciable decrease of its efficiency, and, therefore, confirming its stability in the chromatographic conditions retained.

Foscarnet forms complexes with several metals ions [9] and, without addition of an excess of another agent, namely pyrophosphoric acid in the mobile phase and in biological samples, chromatographic system tubing and column made of stainless steel, can cause problems (deformation of peaks). The use of column and all tubing in PEEK, enabled us no addition of pyrophosphoric acid in the mobile phase and, even in aqueous solution samples before direct injection.

3.5. Attempt of internal standard selection

Errors in the analytical measurement are often reduced, since any loss of sample is compensated by the loss of an equivalent amount of internal standard [15]. Hydrochlorothiazide used by Hassanzadeh et al. [10] was not suitable for the selected extraction technique. Pipemidic acid interfered with foscarnet peak. Thiol containing compounds, sodium 2-mercapto-ethane sulphonate (mesna) and sodium 3-mercapto-1 propane sulphonate were also tested. The last one was promising concerning extraction recovery, peak shape and resolution. The formation of non electrochemically detectable disulphide was avoided by separated loading of serum sample and of internal standard solution. But, we noted, even with an aqueous solution, large variation of peak area in series of injections within a day. The plot of the voltammogram showed that the plateau was not attainable at usable potentials. Since assays without internal standard gave acceptable results, the validation was performed in this condition.

3.6. Linearity range, calibration curve and limit of quantification

Six samples of nine calf serum standards whose concentrations extended from 3.75 to 960 μ*M* were analysed. A graph was plotted with the response (peak area) on the *y*-axis and the corresponding concentration on the *x*-axis. The calibration curve was linear from 15 to 240 μ*M*. The correlation between foscarnet concentration (x) and peak area (y) was performed in the linear range using $1/x$ weighted least-squares regression. The resulting mean slope ($\pm$SD) was 1725.7 ($\pm$94.8) and the *y*-intercept ($\pm$SD) was 3630.8 ($\pm$1012.1) with an average correlation coefficient of 0.9995 ($n=6$).

With the 20 μl injection, the limit of quantification defined by the signal-to-noise level of ca. 10:1 was 15 μ*M*. The intra-assay coefficient of variation of spiked calf serum at this concentration was 3.73%, with a deviation from nominal value of −8.97%. The inter-assay precision and accuracy were 7.24% and −3.11%, respectively.

3.7. Precision and accuracy

The intra-assay and inter-assay precision (given by the relative standard deviation) and the accuracy (given as inaccuracy, i.e., the difference between found and added concentration) were checked using calf serum samples for concentrations of 15, 20, 100, 200, 400 and 800 μ*M*. A five-fold dilution was performed with the same blanck matrix for concentrations above 240 μ*M*. The intra-assay repeatability was determined by analysing six specimens of spiked serum on the same day. The inter-assay repeatability was obtained by analysing two specimens of spiked serum on six days over a period of two weeks. The results (Table 2) were acceptable within the concentration range checked.

3.8. Use of calf serum for calibration standard and quality control samples preparation

Citrated human plasma was usually provided from a blood bank. Since citrate interfered, the matrix selected for the determination of foscarnet was serum. Except for providing from laboratory suppliers but with a high cost, it is almost impossible to obtain a sufficient amount of human serum for quality control purposes. Therefore, we decided the replacement of human serum by non-human material. In judging whether human serum can be replaced by non-human serum, two important questions has to be answered: (1) does in serum drug determinations, non-human material respond in the same way as human material? (2) is non-human serum of the same grade of purity (or impurity) as human serum? (here, purity is defined as endogenous constituents in the normal range) [16].

Table 2
Intra-assay and inter-assay precision and accuracy of foscarnet determination in calf serum

Theoretical concentration (μM)	n	Concentration found (mean±SD) (μM)	RSD (%)	Accuracy (%)
Intra-assay				
15	6	13.65±0.50	3.73	−8.97
20	6	18.79±0.40	2.15	−6.04
100	6	105.40±1.28	1.21	+5.40
200	6	206.85±5.49	2.65	+3.42
400	6	391.86±11.31	2.88	−2.08
800	6	794.39±14.08	1.77	−0.75
Inter-assay				
15	12	14.53±1.05	7.24	−3.11
20	12	19.09±1.00	5.28	−4.50
100	12	102.77±4.01	3.90	+2.77
200	12	202.90±5.72	2.82	+1.45
400	12	408.16±16.59	4.06	+2.04
800	12	810.22±16.95	2.09	+1.27

We selected calf serum since this material could be obtained easily and its biochemical composition is close to that of human serum [16,17].

Cross-validation was performed as follows [18]: a calibration curve was prepared in the validated proxy matrix namely calf serum; replicate quality control samples (six for each concentration) including the limit of quantification (15, 20, 30, 100 and 200 μM) were prepared in both the calf serum and the human serum. All QC samples were back-calculated from the same calibration curve (Table 3). Table 4 shows the results from regression of human serum data on calf serum data. A slope of 1.01669 (not significantly different from 1, t-test, α level of significance=0.01) and a correlation coefficient of 0.9995 [superior to the theoretical limit (α=0.001, degree of freedom=22) of 0.6287] expressed a similar behaviour of the two biological fluids.

Table 3
Calculated values for calf and human serum QC samples using calf serum standards

Theoretical concentration (μM)	Concentration found (mean±SD) (n=6) (μM)	
	Calf serum	Human serum
15	14.56±0.17	14.10±0.22
20	18.83±0.27	20.32±1.16
100	103.44±0.70	108.06±1.84
200	207.87±3.08	211.25±1.48

3.9. Selectivity

Typical chromatograms of calf and human blank serums and calf and human serums spiked with foscarnet at concentrations of 15 and 200 μM are shown in Fig. 3. Foscarnet retention time was

Table 4
Regression of human serum concentrations on calf serum concentrations

Parameter	Estimate	Standard error	t value	Probability level
Intercept	0.819982	0.791405	1.03611	0.311406
Slope	1.01669	0.00678	149.941	0
Correlation coefficient	0.999511			
Standard error of estimate	2.61465			

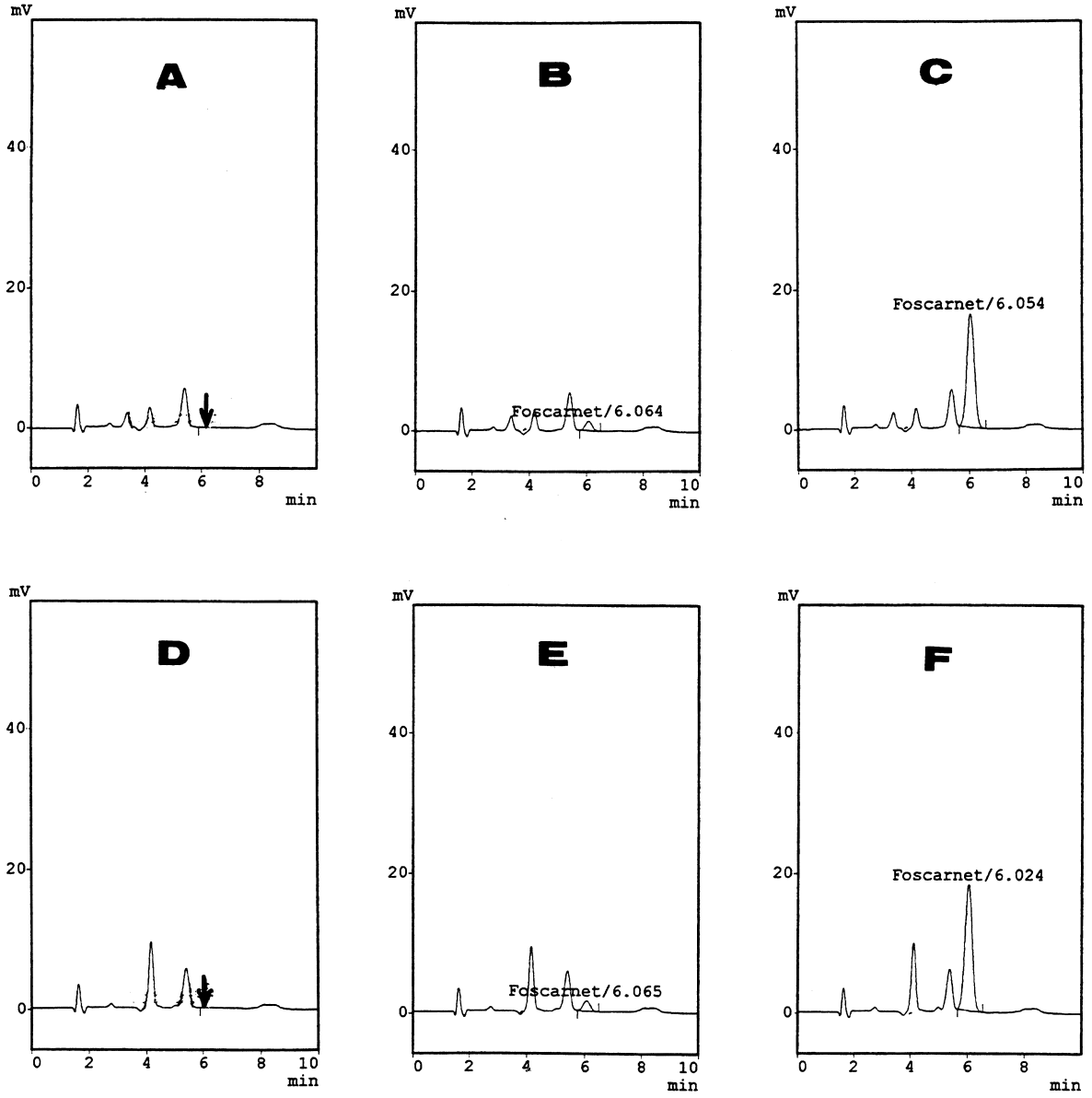

Fig. 3. Typical chromatograms of calf blank serum (A), spiked calf serum at 15 μ*M* (B) and 200 μ*M* (C), and human blank serum (D), spiked human serum at 15 μ*M* (E), and 200 μ*M* (F).

approximately 6.06 min. Separation from endogenous compounds was satisfactory.

Interference with other compounds used as synergistic co-medications or medications in patients with AIDS has been determined. Then, inhibitors of reverse transcriptase didanosine and zidovudine, an inhibitor of DNA polymerase, aciclovir, antiproteases saquinavir, ritonavir, nelfinavir and in-

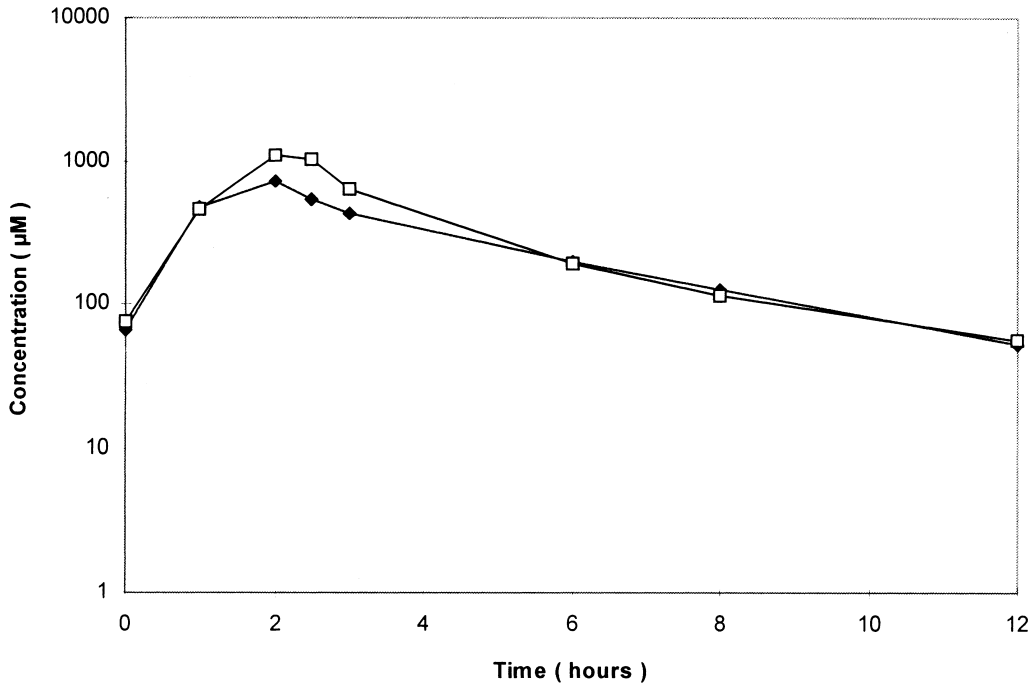

Fig. 4. Pharmacokinetic profile of foscarnet after 2-h infusion of 20 m*M* in a male (♦, subject 1) and a female (□, subject 2) AIDS patients.

dinavir, other antiviral agents for the treatment of CMV, ganciclovir and cidofovir, antibacterials sulfamethoxazol and trimethoprim, an antiprotozaol, pyrimethamine, and an antifungal, fluconazole, were injected as aqueous or hydroalcoholic solutions in the LC system. Only sulfamethoxazol showed a peak at 26.4 min.

4. Application

The method described was successfully applied to the analysis of serum samples from two patients with AIDS and CMV retinitis (one male, subject 1 and one female, subject 2) receiving a 2-h intravenous infusion of 6.0 g (20 m*M*) of foscarnet. Fig. 4

Table 5
Foscarnet pharmacokinetic parameters in AIDS patients

Parameters	Subject 1	Subject 2	Ref. [19] (n=11)
C_{max} (μM)	714.64	1073.71	581±161
C_{12h} (μM)	52.27	55.48	33±34
$T_{1/2}\alpha$ (h)	0.41	0.46	
$T_{1/2}\beta$ (h)	3.16	3.45	3.00±1.20
AUC_0^∞ (h μM)	3250.02	4111.03	
% Extrapolated AUC	7.34	6.72	
Cl (ml/min)	102.50	81.00	121±40
Vd (l/kg)	0.59	0.34	0.51±0.21
MRT (h)	4.90	4.43	

showed the pharmacokinetic profiles within 12 h and Table 5 shows the non-compartmental method pharmacokinetic parameters. The values obtained for elimination half life $T_{1/2}\beta$, total clearance Cl, and distribution volume Vd were close to those obtained by Taburet et al. [19] on day 1 in a pharmacokinetic study of 11 AIDS patients with CMV disease, after twice-daily infusion of 90 mg/kg of body mass for two weeks.

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