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## DETERMINATION OF PHOSPHONOFORMIC ACID IN HUMAN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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### SUMMARY

Trisodium phosphonoformate (foscarnet) is used in the treatment of cytomegalovirus infections in immunocompromised patients, such as bone marrow and renal transplant recipients, as well as patients with the acquired immune deficiency syndrome. A simple high-performance liquid chromatographic assay is described using an electrochemical detector. The method is accurate, precise and reproducible. Hydrochlorothiazide is used as the internal standard. This assay allows measurement of foscarnet in biological fluids at concentrations as low as 33  $\mu\text{M}$ . This method is being used for the analysis of samples in clinical trials and is important in the evaluation of the pharmacokinetic disposition of the drug.

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### INTRODUCTION

Trisodium phosphonoformate (foscarnet) is an anti-viral agent active in vitro against a variety of RNA and DNA viruses including human herpes viruses, cytomegalovirus (CMV) and human immunodeficiency virus (HIV). Its antiviral effect is believed to be primarily due to selective inhibition of reverse transcriptase and viral DNA polymerase [1-3]. Clinical experience with this agent has shown it to provide some benefit for the treatment of CMV infections in immunocompromised patients, such as bone marrow and renal transplant recipients [4,5], as well as patients with the acquired immune deficiency syndrome (AIDS) [6,7]. In addition, research is currently underway to evaluate its effectiveness in treating HIV infection [8,9].

A method for the measurement of foscarnet in human plasma and urine was originally developed by Astra [10]. Following modification of this procedure, we describe a simple high-performance liquid chromatographic (HPLC) assay, including an internal standard, for the determination of this compound in plasma and urine. This method needs no extraction or other time-consuming procedures.

## EXPERIMENTAL

### *Instrumentation*

The high-performance liquid chromatograph consisted of a constant-flow, double-piston, Model 510 pump (Waters Assoc., Milford, MA, U.S.A.) equipped with a pulse dampener, an automatic injector (WISP 710B auto-injector, Waters Assoc.), a Coulchem Model 5100A electrochemical detector (ESA, Bedford, MA, U.S.A.) and a Model 3392A integrator (Hewlett Packard, Avondale, PA, U.S.A.). A stainless-steel Nova-Pak column (Waters Assoc.) packed with 4- $\mu\text{m}$  spherical particles of octadecylsilane ( $\text{C}_{18}$ ) was used. An Accumet<sup>®</sup> Model 810 pH meter (Allied, Fisher Scientific, Pittsburgh, PA, U.S.A.) equipped with a pencil combination polymer gel-filled body Fisher electrode was employed for the pH measurement of different solutions.

### *Reagents*

HPLC-grade methanol, mono- and dibasic sodium phosphate and anhydrous ethanol (Fisher Scientific) were used to prepare the mobile phase. Pyrophosphoric acid (Fluka, Ronkonkoma, NY, U.S.A.), sodium hydroxide pellets (VWR Scientific, San Francisco, CA, U.S.A.), tetrahexylammonium hydrogensulphate ( $\text{THAHSO}_4$ ) (Regis, Morton Grove, IL, U.S.A.), phosphoric acid (85%) (Aldrich, Milwaukee, WI, U.S.A.), phosphonoformic acid (PFA) (Astra, Sodertalje, Sweden) and hydrochlorothiazide (HCTZ) (Sigma, St. Louis, MO, U.S.A.) were supplied. All water used in this assay was deionized through a nanopure water filtration system (Barnstead, Newton, MA, U.S.A.) prior to use.

### *Mobile phase*

The mobile phase consisted of a 30:70 (v/v) mixture of methanol containing 0.001 M  $\text{THAHSO}_4$  as an ion pairing agent and 60 mM phosphate buffer, pH 5.8. To prevent tailing of the PFA peaks, 0.0002 M pyrophosphoric acid was added to the mobile phase and the final pH adjusted to 5.8. The mobile phase was prepared fresh daily and filtered through a 0.2- $\mu\text{m}$  membrane filter (Millipore, Bedford, MA, U.S.A.) and was then degassed.

### *Chromatographic conditions*

The mobile phase was pumped through the column at a flow-rate of 0.7 ml/min at a pressure of 11.7 MPa. The applied potentials to the detector for the guard cell, analytical cell I and analytical cell II were set at +0.99, +0.50 and +0.95 V, respectively. The sensitivity of the detector was set at 10 and the response factor to 4 s. In order to obtain a stable baseline the detector was left switched on overnight with a reduced solvent flow-rate (0.3 ml/min). The mobile phase was not recycled at any time since this caused an unstable baseline.

### *Standard preparation*

An initial 3300  $\mu\text{M}$  stock standard solution of foscarnet was prepared in water. An HCTZ stock solution of 671.8  $\mu\text{M}$  was prepared in a mixture of methanol–deionized water (50:50, v/v).

### *Plasma samples*

The standards (33–1100  $\mu\text{M}$ ) were prepared in pooled human plasma and stored at  $-20^{\circ}\text{C}$  in 1-ml aliquots. As needed, 1 ml of spiked plasma was transferred to the micropartition tube (Centricon-30<sup>®</sup>, Amicon, Division of W.R. Grace and Co., Danvers, MA, U.S.A.), and centrifuged at 1500 g for 20 min, to obtain at least 250  $\mu\text{l}$  of ultrafiltrate solution. This step was performed in order to remove proteins from biological samples. A 200- $\mu\text{l}$  volume of the ultrafiltrate was placed in 2-ml polypropylene plastic tubes (Sarstedt, Princeton, NJ, U.S.A.), which were capped and subsequently placed in a boiling water-bath for 20 min [10] to deactivate the HIV virus in the sample [11]. It is important to note that all materials used for sample processing and storage were selected to assure that foscarnet would not adhere to the material surface. After cooling the tubes, 100  $\mu\text{l}$  of ultrafiltrate-spiked plasma were transferred to 100 mm  $\times$  13 mm culture tubes, and 25–50  $\mu\text{l}$  of HCTZ (internal standard) solution were added. After mixing on a vortex for 10 s, 900  $\mu\text{l}$  of anhydrous ethanol were added and the mixture was again mixed for 20 s. The addition of ethanol was a second step for deactivation of HIV virus [12]. A 100- $\mu\text{l}$  volume of the ethanolic solution was transferred to another culture tube and diluted to 1 ml using 0.001 M pyrophosphoric acid solution. After mixing for 20 s, 20  $\mu\text{l}$  were injected into the column.

### *Urine samples*

The standards (33–1100  $\mu\text{M}$ ) were prepared in drug-free urine and stored at  $-20^{\circ}\text{C}$  as for plasma. As needed 1 ml spiked urine was diluted with 9.0 ml of 0.01 M pyrophosphoric acid solution and mixed with 0.25 g of activated charcoal. The mixture was shaken for 30 s using a vortex mixer. The activated charcoal was used to eliminate interfering endogenous compounds from the urine sample. A 2-ml volume of the charcoal suspension was transferred into a micropartition tube (Centricon-30) and centrifuged at 1500 g for 5 min. After

centrifugation, 200  $\mu\text{l}$  of the filtered urine were transferred to a 2-ml polypropylene plastic tube (Sarstedt). A 200- $\mu\text{l}$  volume of 0.02  $M$  sodium hydroxide solution and 400  $\mu\text{l}$  of anhydrous ethanol were added, the tube screw-capped and placed in a 56°C water-bath for 30 min [10]. After cooling the tubes, internal standard was added to the heated solution and mixed for 10 s. Finally 200  $\mu\text{l}$  of this solution were diluted ten times using 0.001  $M$  pyrophosphoric acid solution. After mixing for 20 s, 20–40  $\mu\text{l}$  were injected into the column.

### *Sample preparation*

The clinical plasma and urine samples were treated in the same way as the standard samples. The use of micropartition tubes (Centricon-30) for filtration was sufficient for standard samples which are not contaminated by contagious viruses. However, in processing standard samples and preparation of standard curves, identical steps were performed as for clinical samples for similarity and accuracy of the procedure.

## RESULTS

### *Separation*

Typical chromatograms for spiked human plasma samples are shown in Fig. 1, representing foscarnet plasma concentrations of 0 (Fig. 1A) and 95.7  $\mu\text{M}$  (Fig. 1B). The foscarnet eluted at approximately 12.0 min and the internal standard at 18.9 min. The several different peaks appearing in drug-free, spiked and clinical plasma sample chromatograms were mainly due to endogenous materials in plasma.

### *Linearity*

Standard curves were obtained by analysis of plasma and urine spiked with foscarnet (range 33–1100  $\mu\text{M}$ ). The standard curves were linear for both plasma ( $y=0.0033x+0.0047$ ;  $r^2=0.999$ ) and urine ( $y=0.0025x-0.066$ ,  $r^2=0.994$ ); however, for better precision it is more appropriate to use two different standard curves with low (0–230  $\mu\text{M}$ ) and high (230–1100  $\mu\text{M}$ ) concentration ranges. Using a method of least-squares analysis with a floating intercept and the origin as a data point, a coefficient of determination ( $r^2$ ) of 0.999 was obtained for plasma and urine standard curves. All other standard curves run in drug-free plasma and/or in drug-free urine yielded curves with  $r^2$  values of not less than 0.99.

### *Precision*

To evaluate the reproducibility of the system and method inter- and intra-assay precision studies were conducted. For inter-assay precision, on six separate occasions, different samples with three different concentrations were prepared and run. The coefficient of variation (C.V.) ranged from 2.4 to 3.3%

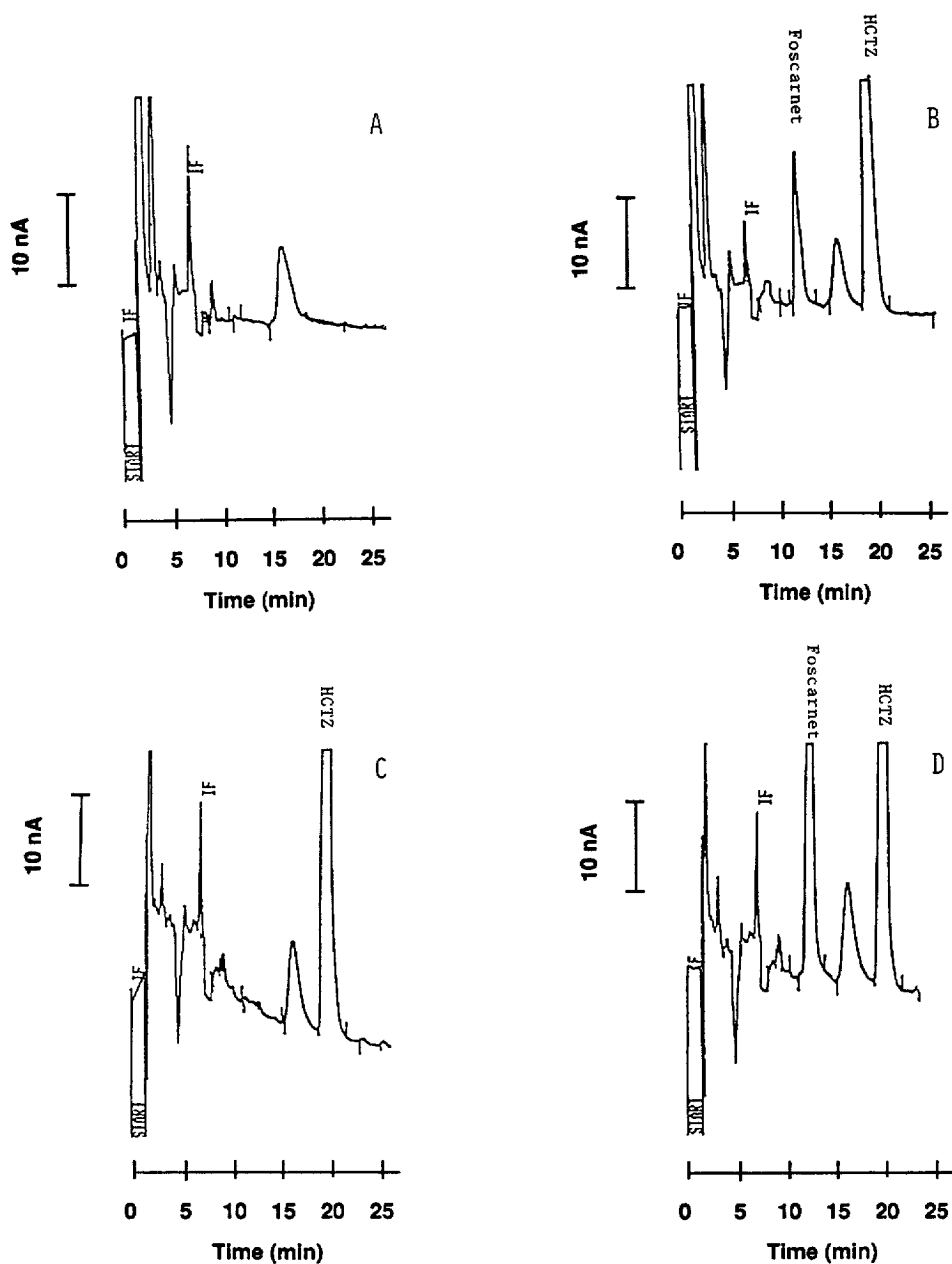


Fig. 1. Chromatograms of foscarnet in human plasma. (A) Drug-free plasma; (B) drug-free plasma spiked with 95.7  $\mu\text{M}$  foscarnet and internal standard (HCTZ); (C) patient trough sample and (D) peak plasma sample for a patient being treated with 30 mg/kg foscarnet every 8 h.

TABLE I

## INTER-ASSAY PRECISION FOR FOSCARNET IN PLASMA AND URINE

Sample	Concentration range	Concentration spiked ( $\mu\text{M}$ )	Concentration found (mean $\pm$ S.D.) ( $\mu\text{M}$ )	C.V. (%)
Plasma	High	551.1	559.1 $\pm$ 13.4	2.4
	Medium	231.0	231.1 $\pm$ 7.6	3.3
	Low	64.7	65.1 $\pm$ 1.7	2.6
Urine	High	551.1	537.0 $\pm$ 18.6	3.5
	Medium	231.0	224.3 $\pm$ 3.8	1.7
	Low	64.7	64.8 $\pm$ 2.1	3.2

TABLE II

## RECOVERY OF FOSCARNET FROM HUMAN PLASMA AND URINE

Concentration range	Concentration spiked ( $\mu\text{M}$ )	Foscarnet/HCTZ peak-height ratio		Recovery (%)	Foscarnet/HCTZ peak-height ratio		Recovery (%)
		Water	Plasma		Water	Urine	
Low	64.7	0.155	0.144	93.0	0.101	0.095	94.0
Medium	231	0.559	0.517	92.5	0.346	0.352	101.7
High	551	1.297	1.157	89.2	0.914	0.912	99.8

for plasma samples and from 1.7 to 3.5% for urine samples (Table I). To evaluate the intra-assay precision of the method, three different samples with high, medium and low concentrations of foscarnet were chosen and each was injected into the column six times. The C.V. of the six replicate injections for each sample ranged from 1.7 to 2.8% for plasma samples and from 0.4 to 6.3% for urine samples.

*Recovery*

The percentage recovery of foscarnet in plasma and urine compared to water is shown in Table II. Spiked plasma and urine were prepared and treated as described above except that the internal standard (HCTZ) was added just before the last dilution. The peak-height ratios in plasma, urine and water were compared as follows:

$$\text{Recovery (\%)} = \frac{\text{foscarnet/HCTZ peak-height ratio in plasma and/or urine}}{\text{foscarnet/HCTZ peak-height ratio in water}}$$

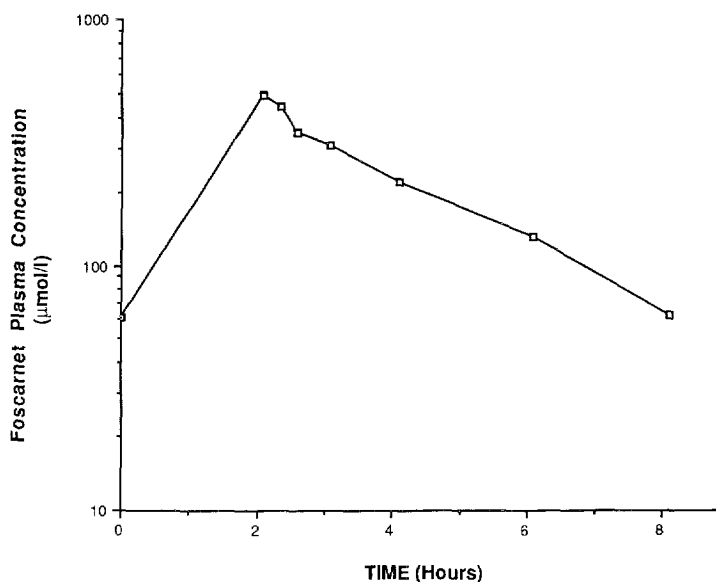


Fig. 2. Foscarnet plasma concentration versus time plot in a HIV-positive patient receiving a 30 mg/kg dose as a 1-h infusion.

The recovery ranged from 89.2 to 93.0% and from 94.0 to 101.7% for plasma and urine samples, respectively.

### *Clinical study*

Analysis of samples obtained from pharmacokinetic studies has been performed [13]. Fig. 1C and D represent chromatograms of trough and peak plasma samples, respectively, for a patient being treated with 30 mg/kg foscarnet every 8 h. Fig. 2 is a representative concentration versus time plot in one subject receiving a 30 mg/kg dose of foscarnet.

### DISCUSSION

Considering the chemical structure of foscarnet, it appears that an accurate way to analyze the compound in biological fluid samples is the use of an electrochemical detector in combination with a highly efficient  $C_{18}$  Nova-Pak column. This type of electrochemical detector and its guard and analytical cells are essential for the sensitivity and reproducibility of the method. Another brand of electrochemical detector was also tested (M460, Waters Assoc.) but failed to produce the same result as the ESA 5100A. Several different conditions were monitored to optimize a suitable potential in order to establish an acceptable and steady baseline. Because of partial temperature dependence, the column was kept constant at room temperature (22–25°C).

## CONCLUSION

In conclusion, the use of an ESA electrochemical detector, under optimal and constant conditions, allows for accurate, precise and reliable measurement of foscarnet in human plasma and urine at concentrations as low as  $33 \mu\text{M}$ . In situations where new analytical and guard cells are used, the limit of detection is  $14.0 \mu\text{M}$  (at a signal/noise of 3.0). This method for the analysis of foscarnet is important in the evaluation of the pharmacokinetics and therapeutic monitoring of this drug in the treatment of viral infections in immunocompromised patients.

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