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DETERMINATION OF PHOSPHONOFORMATE (FOSCARNET) IN BIOLOGICAL FLUIDS BY ION-PAIR REVERSED-PHASE LIQUID CHROMATOGRAPHY

K.-J. PETTERSSON* and T. NORDGREN

Department of Bioanalysis, Research and Development Laboratories, Astra Research Centre, S-151 85 Södertalje (Sweden)

and

D. WESTERLUND

Department of Analytical Pharmaceutical Chemistry, Uppsala University Biomedical Centre, P.O. Box 574, S-751 23 Uppsala (Sweden)

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SUMMARY

Bioanalytical liquid chromatographic methods for the determination of phosphonoformate (foscarnet) have been developed. Biological fluids, after simple pre-treatment (ultrafiltration and/or treatment with charcoal), were injected into a reversed-phase liquid chromatographic system with electrochemical detection. Foscarnet was retained as an ion pair with tetrahexylammonium; addition of pyrophosphate was necessary in order to obtain an acceptable peak. This additive could also be used for the fine regulation of the retention to achieve the necessary selectivity.

INTRODUCTION

Trisodium phosphonoformate hexahydrate (INN: foscarnet sodium) (I) is an antiviral substance which inhibits the replication of several types of viruses [1]. It is currently undergoing clinical trials for the treatment of serious viral infections in patients with a deficient immune system. Today the patients receive the drug intravenously. The treatment consists of a bolus dose followed by continuous infusion, or by daily intermittent infusion for 1–4 weeks. Hence there is a need for a simple analytical method to monitor the levels of foscarnet in biological fluids from both therapeutic and pharmacokinetic points of view.

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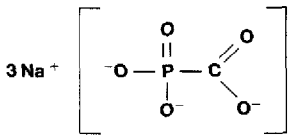

(I)

Foscarnet can be determined in plasma and bone by gas chromatography–mass spectrometry [2]. However, the sample workup is laborious and the method also requires access to expensive and sophisticated instrumentation. Further, urine can not be analysed with this technique.

The aim of this work was to develop a simple method that can be used in in-house and in hospital laboratories to determine levels of foscarnet in plasma and urine. As foscarnet is extremely hydrophilic and consequently difficult to extract into an organic phase, reversed-phase liquid chromatography was selected as a suitable analytical technique. It has the advantage of being easily automated and is, therefore, appropriate for routine analysis of a large number of samples.

EXPERIMENTAL

Chemicals and reagents

Pyrophosphoric acid was obtained from Riedel de Haën (Hannover, F.R.G.), tetrahexylammonium hydrogensulphate from Labkemi (Göteborg, Sweden) and trisodium phosphonoformate hexahydrate from Astra Pharmaceutical Production (Södertälje, Sweden). All other chemicals were of analytical-reagent grade.

Chromatographic system

The liquid chromatograph consisted of an LDC Constametric III solvent delivery system (Laboratory Data Control, Riviera Beach, FL, U.S.A.), a WISP 710A autosampler (Waters Assoc., Milford, MA, U.S.A.) and an ESA 5100 Coulochem electrochemical detector (Environmental Sciences, Bedford, MA, U.S.A.) with a 5020 guard cell and a 5010 analytical cell. The guard cell, which was placed after the injector, was operated at +0.75 V. The potentials on the analytical cell were set at +0.75 V and +0.90 V for channels 1 and 2, respectively. The signal from channel 2 was monitored on an SP4200 computing integrator (Spectra-Physics, San Jose, CA, U.S.A.).

The mobile phase was methanol–phosphate buffer (pH 5.8) (25:75, v/v) with the addition of 1 m*M* tetrahexylammonium hydrogensulphate and 0.2 m*M* pyrophosphoric acid. The total concentration of phosphate in the mobile phase was 0.043 *M*. A flow-rate of 1.0 ml/min was used in all experiments.

The analytical column (100 mm×4.6 mm I.D.) was an UltroPac C_{18} with 3-μm particles (LKB, Bromma, Sweden). It was operated at ambient temperature with a typical back-pressure of 200 bar. New columns were equilibrated with the

mobile phase for about 6 h before use. The volume injected was normally 20 μl.

Sample preparation

Plasma and urine samples were stored in polypropylene tubes at –20 °C or below until analysis.

Plasma

The proteins in plasma samples were removed by ultrafiltration, which was carried out with an MPS-1 ultrafiltration system with YMT membranes or Centricon 30 Microconcentrators (Amicon, Denvers, MA, U.S.A.). The samples were centrifuged at 1000–2000 *g* for 15 min with a CF 510-A centrifuge (Labsystem, Helsinki, Finland) with a 35° fixed-angle rotor.

Before and after the ultrafiltration, the plasma (80–100 μl) was diluted 10-fold with 1 m*M* pyrophosphoric acid solution (pH 5.8). The ultrafiltered and diluted samples were then transferred to an injection vial and injected into the chromatographic system. When the levels of foscarnet were low, plasma was treated for 30 s with charcoal (25 mg added to 100 μl of plasma diluted to 1 ml with 0.01 *M* pyrophosphoric acid solution).

Urine

Interfering compounds were removed by treatment with activated charcoal at pH 2. A 100-μl volume of urine was diluted with 900 μl of 0.01 *M* pyrophosphoric acid solution and mixed with about 25 mg of activated charcoal powder for 30 s on a Whirlimixer. The solution was sucked into a disposable syringe and filtered through a Millipore SJHVL04NS membrane filter (Nihon Millipore, Yonezawa, Japan). The filtrate was diluted 10-fold with 1 m*M* pyrophosphoric acid solution (pH 5.8) in the autosampler vial and injected (20 μl).

Cerebrospinal fluid

Cerebrospinal fluid could be analysed after dilution 100-fold with 1 m*M* pyrophosphoric acid solution (pH 5.8) as the only pre-treatment; 20 μl were injected.

Quantification

The quantification was based on the injection of standard solutions of foscarnet in water. During the development of the method, linear calibration graphs were always obtained up to a limiting concentration of 15 μ*M* in the solutions injected. In routine determinations, for every set of ten injections two standards containing the same concentration of foscarnet were injected and used to construct a calibration graph, which passed through the origin. The concentrations of the standards were chosen with relevance to the unknown samples.

In order to check the performance of the analytical system, control samples containing known concentrations of foscarnet were injected. These samples were prepared in plasma or urine and treated as described for sample preparation. Two levels were used: the concentration was either twice that of the standard solution or five times lower. One high or one low control sample was injected in every set

of ten injections. The quantification was based on peak-height measurements and the injections were normally made at 5-min intervals.

RESULTS AND DISCUSSION

Foscarnet is an extremely hydrophilic compound with pK_a values of 7.3, 3.4 and 0.5 [3]. During the development of the liquid chromatographic system, several problems arose related to the chemical properties of foscarnet.

Detection

UV detection could not be used as foscarnet has UV absorption only at wavelengths below 205 nm where endogenous compounds severely interfered. Instead, electrochemical detection by oxidation was used. The high potentials necessary, however, limited the choice of mobile phase components.

Composition of mobile phase

Methanol was used as an organic modifier as it gave a low background current compared with, e.g., acetonitrile. A disadvantage, however, was the high viscosity of water–methanol mixtures, which led to a high column pressure drop [4,5]. The UltroPac column was preferred owing to its superior efficiency, although it gave higher pressures than other C_{18} materials with 3-μm particles that were tested.

Because of its protolytic properties, foscarnet has to be chromatographed in ionized form. Several anion-exchange materials were tested but they all gave systems with insufficient selectivity towards endogenous compounds, mainly owing to tailing and low efficiency. Ionized compounds can also be retained on C_{18} materials as ion pairs [6,7]. A hydrophobic counter ion, tetrahexylammonium, was added to the mobile phase in order to retain the very hydrophilic foscarnet. The charge on foscarnet varies with the pH of the mobile phase, and it was found that optimum chromatographic conditions were obtained around pH 6 where the acid is divalent.

At pH below 4, the efficiency was found to be considerably lower. 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 [8]. Finally, pH 5.8 was chosen, being the best compromise between efficiency, stability and selectivity. Phosphate was used as the pH buffer component as it gives a low contribution to the background current.

Several parts of a liquid chromatographic system (tubing, column, etc.) are made of stainless steel, which can cause problems when the solute forms complexes with dissolved metal ions. This problem can be solved if a competing complexing agent is added to the mobile phase. Foscarnet forms complexes with several metal ions [9], and without the addition of an excess of another complexing agent the peaks were deformed. EDTA and citrate are the most common complex formers but neither of them could be used because they interfered at the high potentials used for the electrochemical detection. Pyrophosphate, like phosphate, gives a low background current and acts as a complexing agent. Fig. 1 illustrates the great improvement in peak performance obtained when pyrophosphate was

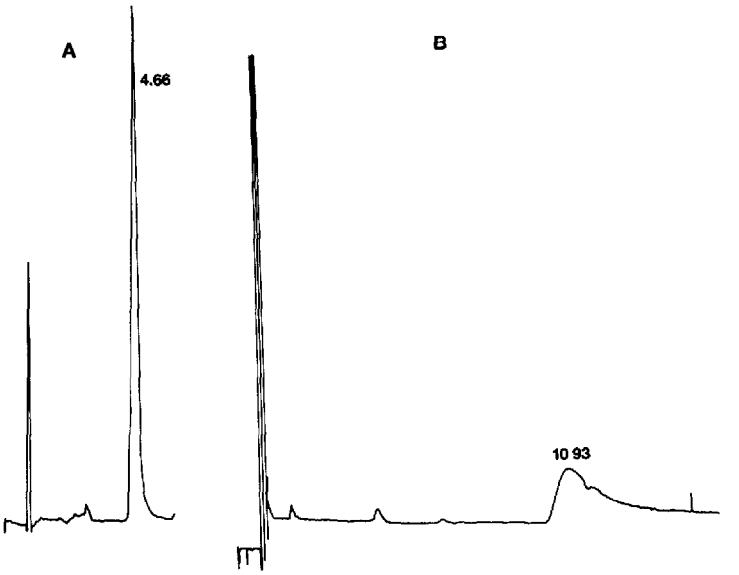

Fig. 1. Influence of pyrophosphate on chromatographic performance. Sample: 61 μM foscarnet in water. (A) Mobile phase according to Experimental. (B) same as (A), but without pyrophosphate.

added to the mobile phase. A large decrease in retention was also obtained. It was also important that pyrophosphate solutions were used to dilute samples, otherwise the peaks were deformed.

Regulation of retention

The effects on the capacity factor of foscarnet of different concentrations of methanol, tetrahexylammonium (THA), sulphate and pyrophosphate were studied. The pH of the mobile phase was always 5.8. The total concentration of phosphate was 0.043 M except when the concentration of sulphate was varied; in this instance, the ionic strength was kept constant at 0.05. Sodium and tetrahexylammonium were the only cations added to the mobile phase. Methanol concentrations higher than 25% were avoided because of too high a drop in pressure.

The effects of the additives on the retention of foscarnet were largely in qualitative agreement with the ion-pair adsorption retention mechanism [6,7]. However, a variation of THA concentration from 0.3 to 1 mmol/l did not change the retention significantly (capacity ratio, $k' \approx 4$), probably owing to saturation of the binding sites on the solid phase already at the lowest concentration by this hydrophobic counter ion. Additions of methanol (2.5–25%) and sulphate (0.1–0.8 mmol/l) decreased k' only slightly, from 6 to 4.3 and from 3 to 1.8, respectively. A larger effect was obtained with pyrophosphate (Fig. 2). If the chromatographic resolution is unsatisfactory using the mobile phase given under Experimental, a selective decrease in k' can be obtained by increasing the concentration of pyrophosphate whereas an increase in k' is achieved if the concentration of methanol is decreased. A concentration of pyrophosphate lower than 0.2 mM should be avoided as the peak performance then will decrease. The concentration

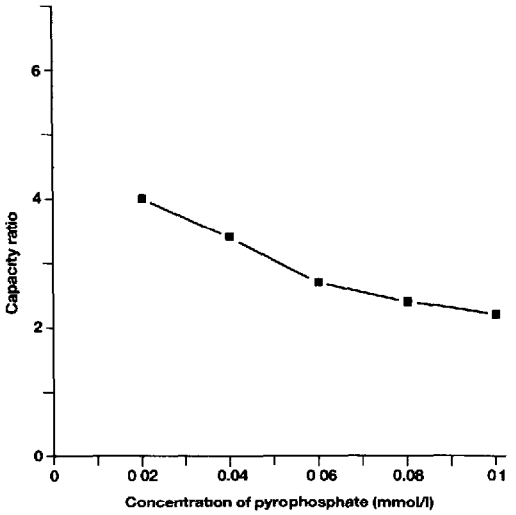

Fig. 2. Capacity ratio (k') at different concentrations of pyrophosphate. Mobile phase: 0.043 *M* phosphate buffer (pH 5.8)–methanol (75:25) containing 1 m*M* tetrahexylammonium and 1 m*M* sulphate.

Fig. 3. Typical chromatogram from plasma obtained by analysis according to the described method. The sample, collected 8 h after intravenous administration of 45 mmol of foscarnet, contained 79 μmol/l foscarnet (F).

Fig. 4. Typical chromatogram from urine obtained by analysis according to the described method. Foscarnet was administered as a continuous intravenous infusion of 42 μmol/min for 13 days. Urine was collected during 24 h, 4 days after the end of infusion. The total urine volume was about 5 l. The sample contained 61 μmol/l foscarnet (F).

of sulphate will be equivalent to that of THA as tetrahexylammonium hydrogensulphate is used as an additive.

Evaluation of the analytical procedure

Typical chromatograms for plasma and urine are shown in Figs. 3 and 4. With plasma, the sensitivity could be increased by a factor of about 100 if the samples, as with urine, were treated with activated charcoal at pH 2 before filtration. This step removed interfering endogenous compounds. The solution was ultrafiltered and the filtrate transferred to a vial and injected. Fig. 5 illustrates the effect of this treatment. Dilution with phosphate buffer (pH 2) instead of 0.01 *M* pyrophosphoric acid was also tested but gave a lower recovery and deformed peaks.

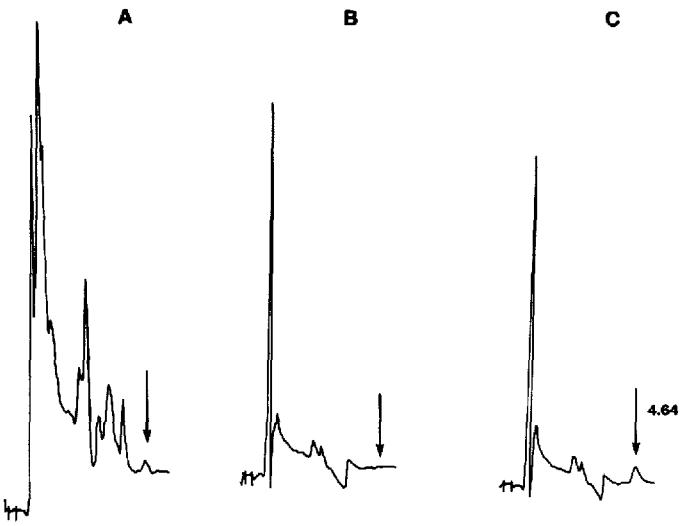

Fig. 5. Effect of treatment of plasma with activated charcoal. The same plasma was used in (A)–(C). The arrow indicates the retention time of the analyte. (A) Ultrafiltered blank plasma that was only diluted 10-fold with 0.01 *M* pyrophosphoric acid; (B) as in (A), but with treatment with activated charcoal before ultrafiltration; (C) as in (B), with addition of 0.6 μmol/l foscarnet.

TABLE I

ACCURACY AND INTER-ASSAY PRECISION

Medium	Concentration level (μmol/l)	Obtained (%)	Coefficient of variation (%)	*n*
Plasma	80	96	10	36
Plasma	900	96	4	32
Urine	60	114	14	60
Urine	900	94	6	60
Urine	1550	92	5	60

The recovery was determined from the ratio between the slopes of a calibration graph in plasma or urine and a calibration graph obtained after direct injection of foscarnet in water. Plasma and urine were processed according to Experimental. The recovery was higher than 90%, even if activated charcoal is added. The calibration graphs were linear up to 0.3 nmol of foscarnet injected.

The accuracy and the intra- and inter-assay precision were determined using spiked plasma and urine samples (Tables I and II). The inter-assay data were obtained from samples that were kept frozen at –20°C and analysed as control samples together with unknowns on different occasions.

The method has routinely been used at Astra Alab for 3 years and the only problems encountered have been that the column and the analytical cell for the detector had to be replaced once or twice a year.

TABLE II

ACCURACY AND INTRA-ASSAY PRECISION

Medium	Concentration level (μmol/l)	Obtained (%)	Coefficient of variation (%)	n
Plasma[a]	0.5	130	11	10
Plasma[a]	5	89	6	10
Plasma[a]	30	93	8	9
Plasma	50	103	16	10
Plasma	450	103	3	10
Plasma	900	99	3	10
Urine	30	116	5	10
Urine	250	102	2	10
Urine	750	102	2	10

[a]Treated with activated charcoal.

Stability

The stability of foscarnet in plasma and urine was determined at room temperature, +4°C and -20°C at three concentration levels. Foscarnet was found to be stable for more than 4 days in plasma and urine at room temperature and +4°C. At -20°C, foscarnet was stable for over 3 years.

In plasma ultrafiltrates, foscarnet was stable for 1 h at 100°C [10]. Subsequently, if plasma is ultrafiltered and heated for up to 60 min before delivery to the laboratory, the samples can be handled as non-infectious. This is of special importance when the samples originate from AIDS patients (treated for secondary viral infections).

In urine and low-level plasma samples, endogenous compounds are eliminated

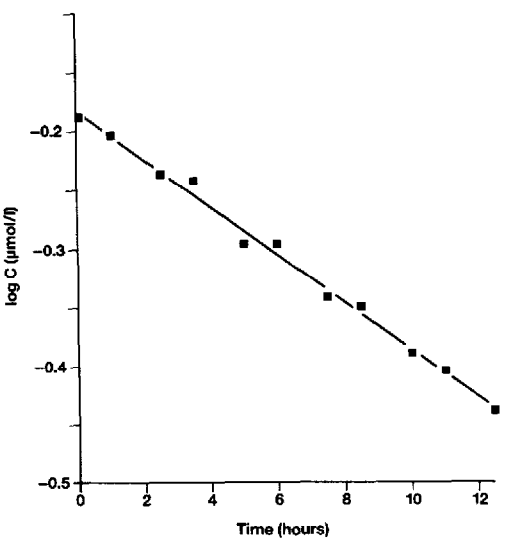

Fig. 6. Degradation of foscarnet at 25°C in 0.01 *M* pyrophosphate (pH 2). *C* = Foscarnet concentration.

by treatment with activated charcoal at pH 2. As foscarnet is known to be unstable at lower pH values [3], the stability at pH 2 was investigated (Fig. 6). If the sample workup and injection into the chromatographic system are done within 30 min, the decrease in concentration will be negligible. Treatment at higher pH values (where foscarnet is more stable) gave insufficient adsorption of interfering endogenous compounds.

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