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# Impurities at a level of 0.01% in foscarnet sodium determined by capillary zone electrophoresis with indirect UV detection and sample self-stacking

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**Abstract**

Low detection limits were obtained by the use of sample self-stacking in combination with careful selection of pH, co-ion and control of the baseline noise. The major component in the sample, foscarnet sodium, was used as a stacker. By choosing sulphanilic acid as co-ion, good peak shapes of the impurities were obtained in combination with low detection limits due to the strong absorption properties of this ion. To obtain a good peak shape for the slowest migrating impurity, a second stacker was added, partly to the sample and partly as a second injection. Rinsing the bare silica with Triton X-100 had a beneficial effect on the baseline noise and helped to produce a constant migration time. By means of this procedure a detection limit of 0.003% (w/w) was obtained for the impurities. The precision at a level of about 0.03% (w/w) in foscarnet was 3–4% (RSD). Linearity for the impurities was shown in the tested range 0.0085–0.085% (w/w). 

*Keywords:* Sample stacking; Isotachophoresis; Pharmaceutical analysis; Foscarnet sodium; Phosphate; Phosphite

## 1. Introduction

In indirect UV detection the non-absorbing sample ions displace the absorbing co-ion and zones with lower absorbance are therefore detected [1]. To be able to analyse low amounts of impurities, it is important that large amounts of sample can be injected without affecting the peak shapes of the impurities, which is often a problem in CE. However, sample self-stacking can be used if the major component has a mobility that is sufficiently different from that of the absorbing co-ion [2,3].

Foscarnet sodium (trisodium phosphonatoformate hexahydrate, see Fig. 1), in this report abbreviated as foscarnet, is a potent antiviral substance used for the treatment of cytomegalovirus infection. Its degradation products, phosphite and phosphate, cannot be detected by UV due to low absorption. The analyses of foscarnet, phosphite and phosphate can be performed by ion-exchange chromatography with a post-column reaction comprising the oxidation of foscarnet and phosphite to phosphate by the addition of bromine and, thereafter, the addition of a molybdovanadate reagent and, finally, sulphite to get rid of the excess bromine before the UV detection [4]. A method for the analysis of phosphate and phosphite in foscarnet by ion-exchange chromatography followed by indirect UV detection is given in Ref. [5]. According to this method, the detection limit for phosphate or phosphite is about 0.1% (w/w) (signal-to-noise ratio 3:1). The compounds disodium ethoxycarbonylphosphonate (I) and sodium ethyl ethoxycarbonylphosphonate (II), originating from the manufacturing process, can be analysed with ion-pair

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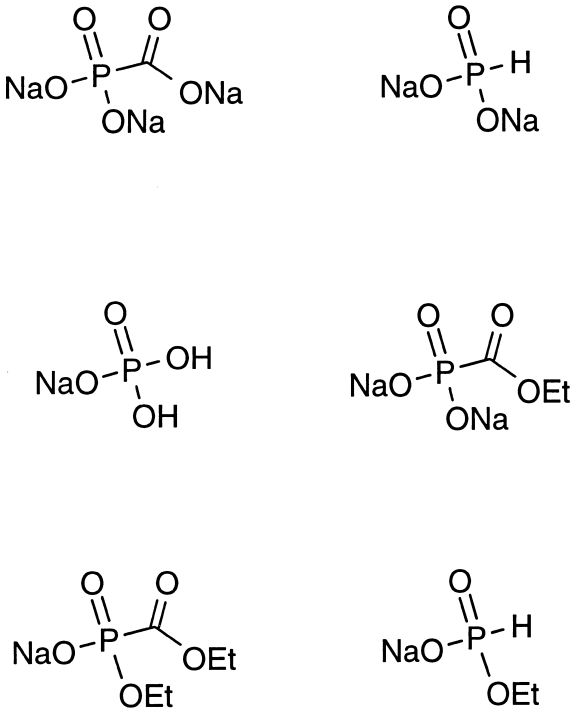


Fig. 1. Structures of foscarnet and the impurities. Upper left: foscarnet, upper right: sodium phosphite, middle left: sodium dihydrogenphosphate, middle right: compound (I), lower left: compound (II), lower right: sodium ethyl phosphite.

high-performance liquid chromatography (HPLC) on an ODS column and direct UV detection at a wavelength of 230 nm. An impurity level of about 0.1% (w/w) gave a relative standard deviation (RSD) of about 10%. The chromatographic procedure is described in Ref. [5]. Another potential impurity in foscarnet is sodium ethyl phosphite. Although a number of methods have been proposed, it was considered desirable to develop an alternative method with lower detection limits for the above-mentioned impurities.

Several factors must be taken into account to obtain low detection limits for impurities with indirect UV detection. In general, high concentrations in the background electrolyte (BGE) are preferred in capillary electrophoresis (CE) as this diminishes the electromigration dispersion [6]. However, to achieve a low detection limit, the UV-absorbing co-ion must have a high molar absorption coefficient [1]. This means that the concentration in the electrolyte must not be especially high if the detector is to operate in its linear range. Accordingly, it is very important that the mobility of the sample ion of interest matches that of the co-ion in order to get a good peak shape [1,7]. This reasoning holds true at least as far as strong electrolytes are concerned [8]. A large amount of sample can be injected if the major component has a higher mobility than the impurities and if an absorbing co-ion with a mobility much lower than the major component is chosen, as the sample self-stacking effect is then exploited [2], provided that the stacker concentration is sufficiently high [9]. If any of the impurities has a mobility lower than that of the absorbing co-ion, which results in a broad peak, the addition of a second matrix ion can improve the shape of the peak [3]. Another important factor to consider to obtain low detection limits is the baseline noise [10].

In this study foscarnet was used as a leading stacker and a second stacker was added as a terminator to obtain a good peak shape for one of the impurities. This procedure made it possible to detect impurities at a concentration of 0.003% (w/w). It was demonstrated that some part of the second matrix ion could be added as a separate second injection with very good results. Further, the effect of Triton X-100 solution on baseline noise and migration time was evaluated with a low concentration of tetradecyltrimethylammonium bromide (TTAB) in the running electrolyte.

## 2. Experimental

### 2.1. Chemicals

L-Glutamic acid, sulphanilic acid (4-aminobenzenesulphonic acid) and TTAB were supplied by Sigma (St. Louis, MO, USA); 6-aminocaproic acid (6-ACA) by Carl Roth (Karlsruhe, Germany); sodium dihydrogenphosphate monohydrate by Merck (Darmstadt, Germany); sodium phosphite pentahydrate by Riedel-de Haën (Seelze, Germany); mesaconic acid (*trans*-1-propene-1,2-dicarboxylic acid) and 1-hexanesulphonic acid, sodium salt by Aldrich (Steinheim, Germany), and Triton X-100 by Serva (Heidelberg, Germany). All the chemicals mentioned were of analytical reagent grade. Trisodium phosphonatoformate hexahydrate (foscarnet),

disodium ethoxycarbonylphosphonate (I), sodium ethyl ethoxycarbonylphosphonate (II) and sodium ethyl phosphite were obtained from Astra (Södertälje, Sweden). Ultrapure deionized water produced from a Milli-Q plus system (Millipore, MA, USA) was used for all solutions. The filtering was done with a Millex-HV 0.45-μm filter unit, Millipore, country of origin Ireland.

### 2.2. Equipment

A Beckman P/ACE System 2100, Beckman Instruments (Fullerton, CA, USA) was used. The electrophoretic separations were performed at 25°C in bare fused-silica capillaries, eCAP obtained from Beckman Instruments, 77 cm (effective length 70 cm)×100 μm I.D.×375 μm O.D. The aperture dimensions of the capillary cartridge were 800×100 μm. The detector was used at a wavelength of 254 nm, with rise time 0.5 s data collection 10 Hz, negative offset 90%, range 0.200 AU. The analyses were run in a constant current mode at 6.5 μA (voltage approximately 27 kV). The polarity was reversed, i.e., the anode was situated at the detector side. The injection was carried out by means of pressure for 3 s (3447 Pa) followed by a second injection of a glutamic solution with a concentration of 18.6 m*M* for 6 s (3447 Pa). Time until separation current is reached 0.17 min.

### 2.3. Procedures

Electrolyte without additives contained 3.56 m*M* sulphanilic acid and 6.98 m*M* 6-ACA. The electrolyte was prepared by simply weighing sulphanilic acid and 6-ACA and dissolving them in water, which gave the best precision as the pH is very near the p$K_a$ of 6-ACA. When the pH was measured, however, it was about 4.45. Stock solution with TTAB contains 1.29 mmol of TTAB per litre of electrolyte without additives. The solution containing Triton used for rinsing was prepared by adding 125 μl of Triton X-100 per litre of electrolyte without additives. The electrolyte without additives, the solution containing Triton and the stock solution containing TTAB were filtered and the initial portions were discarded. Running electrolyte was prepared by diluting 35 ml of the stock solution with TTAB to 500 ml with the electrolyte without additives (i.e., a final concentration of 0.09 m*M* TTAB). The solutions with sulphanilic acid should be stored in a refrigerator. On storage the solutions will be discoloured, but at least as long as the yellowish colour is faint, the results obtained so far indicate that there is no interference with the analysis.

Capillaries were flushed after dry storage with water for 4 min, 0.1 *M* hydrochloric acid for 10 min, water for 4 min, Triton solution for 5 min, electrolyte without additives for 1 min and running electrolyte for 3 min in sequence. (New capillaries were treated in the same way). Between runs the capillary was rinsed with electrolyte without additives for 1 min, Triton solution for 0.5 min, electrolyte without additives for 1 min and, finally, the running electrolyte for 3 min in sequence. The electrolyte in the outlet vial was exchanged after each run. Shut-down procedure: the capillary was rinsed with water for 5 min followed by nitrogen for 5 min.

The concentration of foscarnet in the standard/sample was about 8.2 mg/ml. Glutamic acid was added to the standard/sample, corresponding to about 362 mg/g trisodium phosphonatoformate hexahydrate. Sodium phosphite pentahydrate, sodium dihydrogenphosphate monohydrate and sodium ethyl phosphite, compounds I and II, were all added in varying amounts in the standards. The final concentration of mesaconic acid (I.S.) was 47 μ*M*.

## 3. Results and discussion

### 3.1. Electrolyte and sample

Sulphanilic acid (p$K_a$ 3.2) [11] was chosen as a probe as it has a high UV absorption at 254 nm (the equipment used has fixed wavelengths) and the concentration used was in the upper linear range. The mobility was approximately the same as that of the impurities at pH about 4.45; consequently, a low detection limit can be expected [7]. At this pH foscarnet has a much higher mobility than the impurites and it was therefore appropriate to reverse the polarity and thus necessary to reduce the electroosmotic flow (EOF) so that the slowest migrating ion could be detected in a reasonable time. This was done by the addition of a low concentration of TTAB

to the electrolyte. The number of visualisation agent molecules transferred by one analyte molecule, i.e., displacement ratio [10], depends not only on the mobilities of the analyte and probe, but also on the mobility of the counter-ion [12]. It should be noted that if two co-ions are present in the BGE, the signal can be reduced [13,14] and a system peak with a mobility between the two co-ions may interfere with the peaks [15]. This can be avoided by using TBAOH; however, this substance is, as far as we know, not commercially available and if an anion-exchange bed is used to transform TTAB into TTAOH, this involves extra work. If, however, the TTAB concentration can be kept at a low level, the reduction of the signal can be assumed to be small and the system peak will have a mobility near that of bromide and will therefore not cause interference.

The concentration of phosphonoformate in the injected sample was 27 m*M* (charge minus 2), as against that of the probe, 3.6 m*M* (charge minus 1). Phosphonoformate has a much higher mobility than sulphanilic acid and, consequently, its peak will be strongly fronting, although the peak shapes of the minor components migrating between this ion and the probe will not be adversely affected by the high sample concentration [2]. The impurities are first stacked and thereby concentrated at the rear of the zone of foscarnet by an isotachophoretic mechanism and thereafter gradually destack and migrate zone electrophoretically [2]. The conditions were chosen so that the zones would migrate by zone electrophoresis before reaching the detector window.

Glutamic acid was added to the sample solution. This had two effects: the pH of the sample solution was adjusted to a pH near the BGE (and could, therefore, be assumed to cause less disturbance to the EOF) and glutamic acid would also serve as a stacking terminator and give a better peak shape of compound (II), which otherwise would be tailing [3]. Foscarnet, however, is not stable in an acidic solution and if its pH is lowered too much with glutamic acid, some degradation to phosphite can be seen after a couple of hours. This problem was solved by adding some glutamic acid to the sample (but not more than enough to allow sufficient stability of foscarnet to be maintained) and the rest as a separate second injection. See Fig. 2a–d.

### *3.2. Effects of TTAB/Triton X-100 on baseline noise*

When indirect UV detection is used in CE, the baseline tends to be noisy. This has been reported to be due partly to Joule heating [1]. The relative fluctuation of voltage with the equipment used in this study was found to be lower when the current was constant than the relative fluctuation of current when the voltage was constant (the resistance was considered to be a constant). As Joule heat is the product of resistance and current raised to the second power or the product of the inverted value of resistance and voltage raised to the second power. The least variation was assumed to be in the constant current mode which was therefore chosen.

The baseline was found to be better if the TTAB concentration was relatively high, but at the price of less satisfactory separations as the mobility difference between the components relative to the mobility after correction for the EOF was decreased [16]. This can to some extent be compensated for by using a longer capillary; however, the run time will increase as well since the highest voltage available is 30 kV. The following approach was found to be a good alternative. If bare fused-silica was rinsed with electrolyte containing Triton, the baseline became much better. However, Triton could not be mixed with TTAB in the running electrolyte since this resulted in a poor baseline. With the conditions used in this study, it was sufficient to wash the capillary with a solution containing Triton during the starting-up procedure once a day to get a less noisy baseline without irreproducible long-term noise although the EOF was low which favours the separation. If the Triton-wash was not done the long-term noise often caused quite unacceptable electropherograms that had to be rejected. One run could be good but the next one could be very bad without any obvious reason.

If a low concentration of TTAB is used in the electrolyte, the capillary surface is not totally covered. The beneficial effect of the Triton wash on the baseline may be due to Triton being adsorbed to the surface and, together with TTAB, shielding the silica surface. Wang et al. obtained very low noise in a PEG-bonded capillary and suggested that interactions

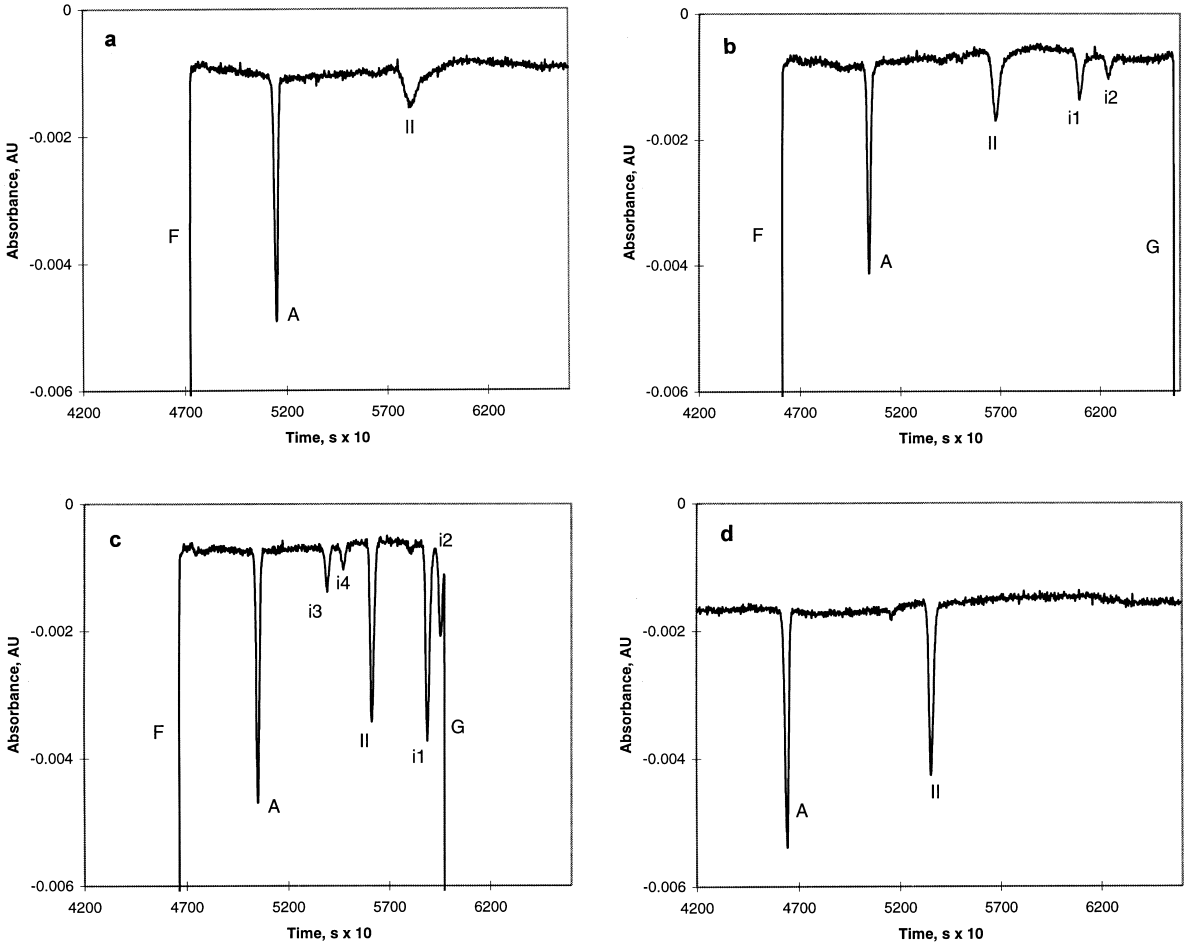


Fig. 2. (a) Sample: foscarnet (F) 8.2 mg/ml, phosphate (A) and II 3.6 and 3.7 μg/ml, respectively. Second injection: electrolyte. Other conditions are given in Section 2.3. (b). Sample: foscarnet (F) 8.2 mg/ml, phosphate (A) and II 3.6 and 3.7 μg/ml, respectively, and glutamic acid (G) 3 mg/ml. Second injection: electrolyte. Other conditions are given in Section 2.3. The peaks i1 and i2 are impurities in glutamic acid. (c) Sample: foscarnet (F) 8.2 mg/ml, phosphate (A) and II 3.6 and 3.7 g/ml, respectively, and glutamic acid (G) 3 mg/ml. Second injection: glutamic acid 2.8 mg/ml. Other conditions are given in Section 2.3. The peaks i1–i4 are impurities in glutamic acid. (d) Sample: phosphate (A) and II 3.6 and 3.7 μg/ml, respectively. Second injection: electrolyte. Other conditions are given in Section 2.3.

between the visualisation agent and the capillary surface could be responsible for the noise [10].

When the TTAB concentration in the electrolyte was kept fairly low (no large reversed EOF), there was a tendency for longer retention times during the day when the capillary was rinsed only with the running electrolyte. (The polarity was reversed, i.e. the anode was situated at the detector end.) The reason cannot be that the adsorption process is slow because the retention time should then tend to decrease. Even when the electrolyte was stored in the capillary (no voltage applied), a tendency for increasing retention times was seen, although this was not as large as if a voltage was applied. A possible reason for this may be that the silica surface is altered due to exposure to water – the electrolyte has a low ionic strength – and a ''silica gel'', mentioned by Israelachvili et al. [17] is formed. If, however, the capillary was washed for approximately half a minute with electrolyte containing Triton between

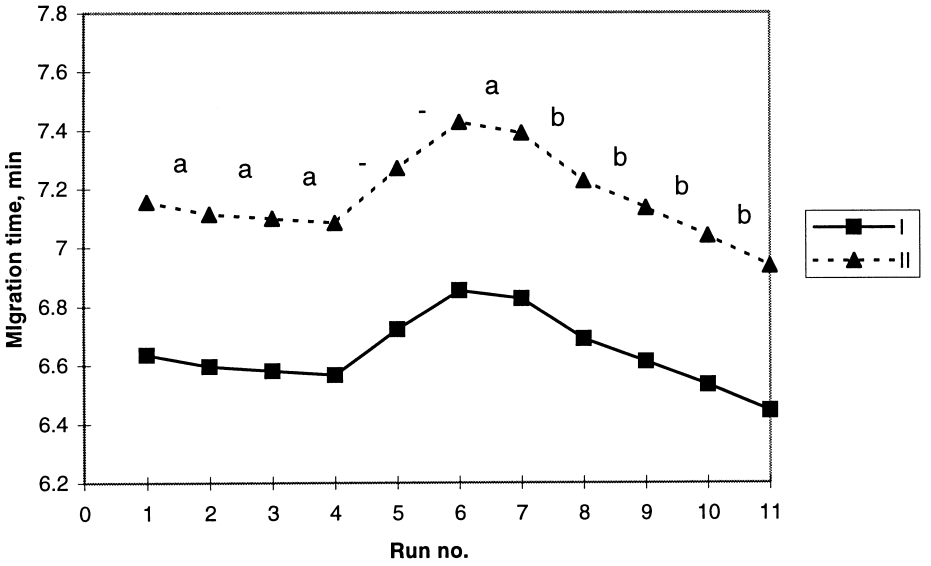


Fig. 3. Effect of Triton X-100 wash on the migration time for the two slowest migrating impurities. The new capillary was rinsed as described in Section 2.3, except for the last two steps which were changed to 0.5 min with water and 5 min with running electrolyte. The test solution used during the experiment contained 8 μg/ml of I and II. The rinse procedure between runs was varied (a)=0.5 min water, 0.5 min Triton solution, 0.5 min water, 5 min electrolyte with 0.1 m*M* TTAB; (–)=3 min electrolyte; (b)=0.5 min water, 1 min Triton solution, 0.5 min water, 3 min electrolyte. Other conditions as described in Section 2.3, but no second injection.

the runs, this tendency was counteracted. Fig. 3 shows that a rinse with Triton solution between the runs has an effect on the migration times (reversed polarity was used). If the Triton wash was too long, the retention times tended to be shorter and shorter. Thus, if suitable conditions are chosen, Triton could be used to reduce the variation in the migration times. Since a mixture of TTAB and Triton in the electrolyte gave a noisy baseline, the washing procedure between runs included rinsing steps with electrolyte without any additives.

### *3.3. Capillary dimension*

The dimension of the capillary was chosen to give a high sensitivity. A 50 μm capillary gave a linear response in a much lower absorbance range than a 75 μm capillary and the quotient of absorbance and concentration was about half of that for a 75 μm capillary (slit width 100 μm). A 100 μm capillary was chosen since it was even better than the 75 μm capillary and the low concentration used in the electrolyte was assumed to give low Joule heat and, thereby, cause comparatively few problems.

### *3.4. Slit dimensions and rise time*

Capillary cartridges are available with a slit height of 200 or 800 μm. The latter was chosen as it gave significantly lower noise (the absorbance is the same). A low rise time increases the noise, although if it is too high, the recorded peak height will be lowered [18].

### *3.5. Linearity for minor components*

Linearity has been determined for the impurities, see Fig. 4. In order to obtain comparable figures, the peak area was corrected by dividing by migration time. The corrected area of the impurity divided by the corresponding value of the internal standard (mesaconic acid) is here denoted as the response. This method of calculation is not strictly correct as the ions move in the isotachophoretic stack part of time [19]. However, as the amount of foscarnet and glutamic acid only varied to a minor extent and an internal standard was used, it was regarded as satisfactory for the intended use. The migration times relative to the internal standard were 0.984, 1.040,

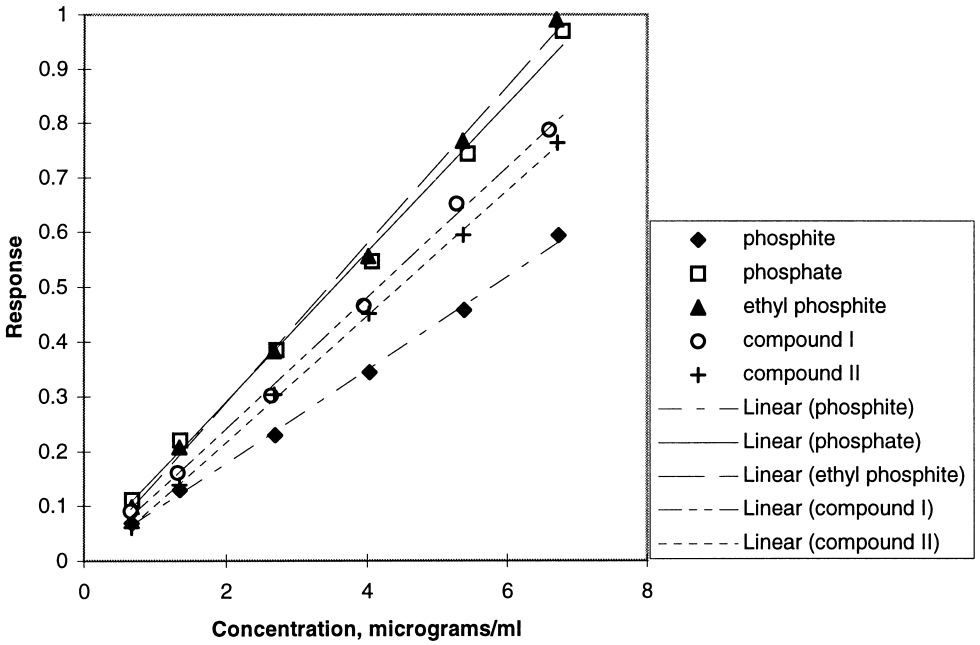


Fig. 4. Linear regression for impurities added to foscarnet (8.2 mg per ml), $n=6$. The concentrations of phosphite, phosphate and ethyl phosphite are calculated as sodium phosphite pentahydrate, sodium dihydrogenphosphate monohydrate and sodium ethyl phosphite, respectively. Compounds I and II are specified in Section 2.1. Other conditions are as in Section 2.3. For explanation of response, see text. The 95% confidence interval includes the origin for all five impurities. $R^2$ is in the range 0.9963 and 0.9995.

1.03, 1.077, 1.144 for phosphite, ethyl phosphite, phosphate, compound I and compound II with relative standard deviations (RSDs) of about 0.1%. The run time was 10 min and the total rinsing time between runs 5.5 min.

### *3.6. Precision for determination of minor components*

Precision was determined by injecting the same solution six times (see Table 1). Despite good precision, further validation will be needed before the method can be used routinely.

### *3.7. Detection limit*

Fig. 5 shows an electropherogram with concentrations of the added impurities corresponding to their detection limits. (None of the impurities were

Table 1
Precision[a]

| Compound | Concentration | | Response RSD (%) | Migration time relative I.S. RSD (%) |
|---|---|---|---|---|
| | μg/ml | % (w/w) | | |
| Foscarnet | 8190 | | – | – |
| Sodium phosphite pentahydrate | 2.694 | 0.033 | 4.4 | 0.04 |
| Sodium dihydrogenphosphate monohydrate | 2.718 | 0.033 | 2.8 | 0.11 |
| Sodium ethyl phosphite | 2.681 | 0.033 | 3.0 | 0.10 |
| I | 2.639 | 0.032 | 3.7 | 0.17 |
| II | 2.687 | 0.033 | 3.6 | 0.24 |

[a] Repeated injection, $n=6$, of a solution with the composition given in the table. Other conditions are given in Section 2.3. Response is defined in the text.

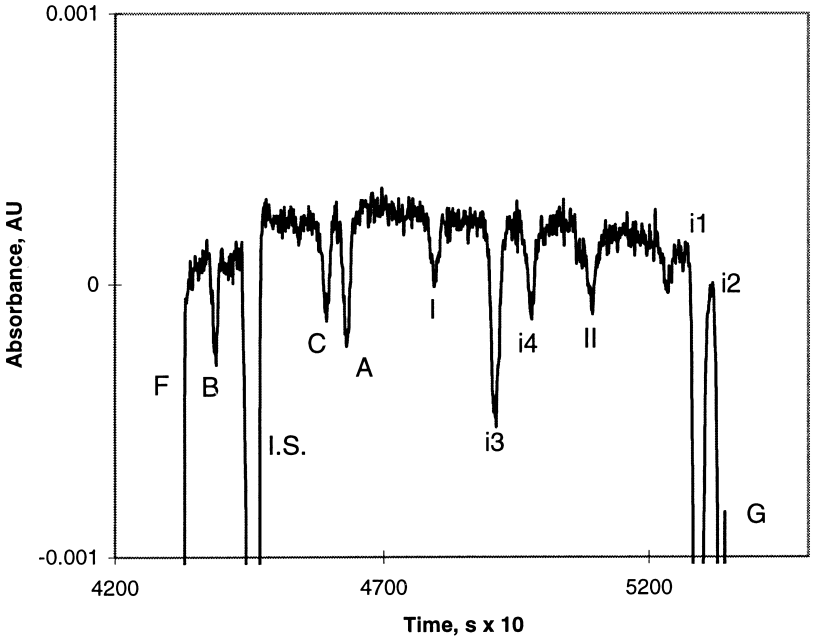


Fig. 5. Detection limit with conditions described in Section 2.3. A=Phosphate, B=phosphite, C=ethyl phosphite, F=foscarnet, G=glutamate, I and II are the compounds I and II, i1–i4=impurities in glutamic acid, I.S.=mesaconic acid. The concentrations of the impurities are 0.003% (w/w) of each (0.27 μg/ml), calculated as sodium phosphite pentahydrate, sodium dihydrogenphosphate monohydrate, sodium ethyl phosphite, I and II, respectively, in trisodium phosphonoformate hexahydrate 8.2 mg/ml.

detected in the foscarnet substance used in this study).

### *3.8. Unexpected small peaks with sample self-stacking*

Preliminary experiments performed with samples containing phosphate and hexanesulphonate (with intended use as an internal standard) in high concentrations of foscarnet and glutamic acid gave significantly lower detected amounts of hexanesulphonate compared to samples without foscarnet and glutamic acid (or with lower amounts). If, however, the same amount was injected in double the volume, the peak of hexanesulphonate became larger. These experiments were performed with an electrolyte containing a concentration of TTAB of 0.43 m*M*. The reason for this is not interactions with foscarnet, as this effect was small in a corresponding experiment with the electrolyte containing 0.09 m*M* TTAB described in Section 2.3. The effect may be due to the high ionic strength in the sample zone compared to that of the BGE. This has an impact on the critical micelle concentration, which will be lower in the sample zone. The reason for the better result with an electrolyte containing 0.09 m*M* TTAB might be that the micelle concentration, if any, is less in this case. It is at any rate reasonable to believe that hydrophobic ions can behave in this way. It is therefore important to compare the results obtained with sample self-stacking with those obtained under normal CE conditions or in another appropriate way.

## 4. Conclusions

An electrolyte containing sulphanilic acid as a UV absorbing co-ion with 6-aminocaproic acid as counter-ion makes it possible to use the major component, foscarnet, as a leading stacker. A terminating stacker, glutamate, can be used to improve the peak shape for one impurity with a mobility lower than that of sulphanilic acid. It is not necessary to add all the glutamic acid to the sample; however, part of it

can be added as a second injection, which is an advantage as foscarnet is not stable in an acidic solution. Good linearity for the impurities has been demonstrated in the range 0.0085–0.085% (w/w). Higher concentrations were of no interest and have not been tested. The precision on injecting a solution containing approximately 0.03% (w/w) of each impurity was 3–4% and the limit of detection was about 0.003% (w/w) of each impurity. Triton X-100 was found to have a beneficial effect on the baseline and was also helpful in keeping the migration time constant when a low concentration of TTAB was used.

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