Journal of Chromatography, 369 (1986) 151–157
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 911

ION-EXCHANGE CHROMATOGRAPHY WITH POST-COLUMN REACTION FOR THE ANALYSIS OF PHOSPHONOFORMATE, PHOSPHITE AND PHOSPHATE

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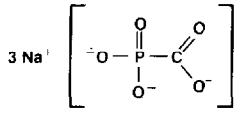
(Received July 2nd, 1986)

SUMMARY

Phosphonoformate, phosphite and phosphate are baseline separated within 6 min on a silica-based DEAE anion exchanger. The mobile phase consists of acetic acid, sulphate and a small amount of citric acid. Without citric acid, phosphonoformate is eluted with severe tailing. The presence of citric acid sharpens the peak dramatically. The detection system comprises an initial oxidation to phosphate by post-column addition of bromine. Thereafter a molybdovanadate reagent is added. Finally sulphite is added to reduce the excess of bromine which otherwise would disturb the subsequent detection at 340 nm. The detection limit for phosphate and phosphite is about 4 ng calculated as phosphorus, and for phosphonoformate is about 12 ng. The relative standard deviation for the peak area of 3.3 m*M* phosphonoformate is about 0.4%.

INTRODUCTION

Phosphonoformate (PFA) (I) is a potent antivirus substance with activity against several types of viruses[1,2]. Its use in different drug formulations requires methods of analysis of PFA and of its degradation products phosphite and phosphate. An ion-pair chromatography system with amperometric detection for PFA has been presented[3]. In that work a phosphate buffer was used. Furthermore, pyrophosphate was added to the mobile phase in order to obtain sharp peaks for PFA, which otherwise gave tailing peaks. However, this system is unable to determine phosphate and phosphite. Detection of these two ions in chromatography is complicated due to the lack of physical properties utilizable by, *e.g.*, UV and amperometric detectors. A conductometric detector could of course be employed. However,



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0021-9673/86/$03.50

the choice of mobile phase is limited with this detector. Only phases with a sufficiently low background conductivity could be employed. Consequently, there is little possibility of finding a chromatographic system for the three title compounds which has sufficiently high stability, efficiency and resolution for drug formulation analysis.

In the present work, ion-exchange chromatography is explored for the separation of these three components. The column used is a silica-based DEAE anion exchanger. Detection is accomplished by employing a post-column reaction system based on the reaction between phosphate and a molybdovanadate reagent to form a yellow product[4,5]. PFA and phosphite are initially oxidized to phosphate by addition of bromine. A post-column reaction system for phosphate, phosphite and hypophosphite, based on oxidation by sulphite to phosphate and a subsequent reaction with molybdenum, has been presented[6]. However, it requires a reaction coil with a hold-up time of 2.5 min, and heating to 140°C. The present system is rapid and does not require elevated temperature.

The mobile phase used must be compatible with the detection system. The eluting ion employed here is sulphate. Citric acid is also added, otherwise PFA shows severe tailing. However, citric acid interferes in the detection system. The optimization of the citric acid content in the mobile phase, as well as other factors influencing the chromatography and detection, are discussed.

EXPERIMENTAL

The chromatographic system

An LKB 2150 high-pressure pump (LKB, Bromma, Sweden) was used with a flow-rate of 1.0 ml/min. The column contained DEAE Si100 Polyol, 3 μm (125 mm $\times$ 4.6 mm) (Serva, Heidelberg). Injections of 20 μl sample were performed with a Waters WISP autoinjector. Peak evaluations were made with a Spectra-Physics 4270 integrator.

The mobile phase consisted of 0.1 *M* acetic acid, 1 m*M* citric acid and 0.015 *M* sulphate. The sulphate was added as a mixture of sodium sulphate and sodium hydrogensulphate so that the final pH of the mobile phase was 2.7. When the effect of pH on retention was investigated, pH values below 3 were adjusted with appropriate amounts of the sulphate salts; above pH 3, 0.1 *M* sodium acetate was employed. When the effect of the sulphate concentration was investigated, the pH was kept constant at 2.9.

Previously unused columns were pre-conditioned at a flow-rate of 1 ml/min for 1–2 days with a mobile phase consisting of 8 m*M* citric acid, 0.015 *M* sulphate and 0.1 *M* acetic acid, pH 2.7.

The post-column detection system

Fig. 1 shows the detection system. First the bromine solution is added to the column effluent, the PFA and phosphite being oxidized to phosphate. Secondly, the molybdovanadate reagent and finally the sulphite solutions are added. The sulphite reduces the excess of bromine which otherwise would give rise to a background absorbance in the final detection. The detector was an LDC Spectromonitor III used at a wavelength of 340 nm. The reference cell of the detector was used due to its slightly larger dimensions and thus lower back-pressure. This was found advanta-

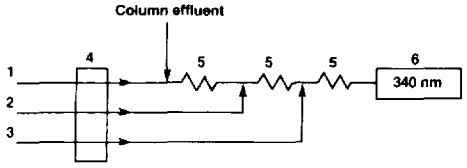


Fig. 1. The post-column detection system. 1 = Bromine; 2 = molybdovanadate reagent; 3 = sodium sulphite; 4 = peristaltic pump; 5 = PTFE mixing coil; 6 = detector.

geous for minimizing the risk of leakage. The mixing block was a Bifok Chemifold Type III (Tecator, Sweden) and the mixing coils were PTFE tubings (600 mm × 0.5 mm I.D.). The peristaltic pump used was an Alitea C-4V (Ventur AB, Uttran, Sweden). The success of the detection system was found to depend largely on the choice of peristaltic pump. Other pumps examined gave poor results due to large pulsations in the flow. The pump speed and choice of pump tubings were adjusted so that the bromine and sulphite solutions were added at 0.4 ml/min, and the molybdovanadate reagent at 0.8 ml/min.

Reagent and standard solutions

The bromine was prepared by the reaction between bromide and bromate in acidic solution. A 2.9-g amount of potassium bromide and 0.64 g of sodium bromate were dissolved in 25 ml of 1 *M* hydrochloric acid. The reaction was allowed to proceed for at least 2 h. This solution was then diluted to 500 ml in 0.1 *M* sodium acetate in order to obtain a slightly alkaline pH. Prior to use, the stock solution was diluted four times in deaerated 0.1 *M* sodium acetate. The concentration of the bromine solution was determined by titration with sodium sulphite to decolouration.

The molybdovanadate solution consisted of 1.6 m*M* ammonium molybdate and ammonium monovanadate in 0.15 *M* deaerated hydrochloric acid.

The sodium sulphite solution was made 0.03 *M* in deaerated water. This solution was freshly prepared each day.

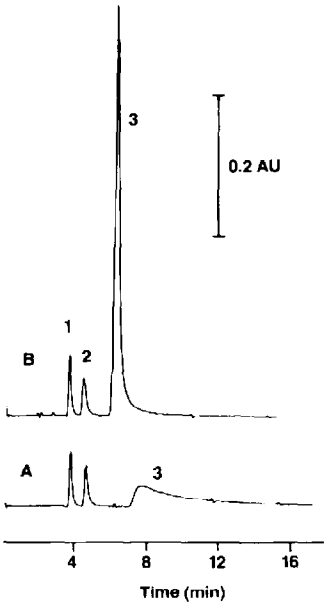
Trisodium phosphonoformate hexahydrate (Astra, Södertälje, Sweden), sodium dihydrogenphosphate (Merck) and sodium phosphite pentahydrate (Riedel-DeHaen) were used for preparation of standard solutions in water. All chemicals used were of analytical grade.

RESULTS AND DISCUSSION

Influence of citric acid

The dramatic influence of citric acid in the mobile phase on the PFA peak shape is illustrated in Fig. 2. Without citric acid, PFA is eluted with severe tailing. A pronounced sharpening of the peak is obtained at a citric acid concentration of 0.5 m*M*. With increasing concentration of citric acid, the peak tailing is reduced, though only minute changes can be seen by increasing the concentration from 1 to 5 m*M*.

Citric acid does however interfere in the detection system, and causes a decrease in response when the concentration reaches a critical level compared to the concentration of molybdovanadate reagent added. Under the conditions stated in



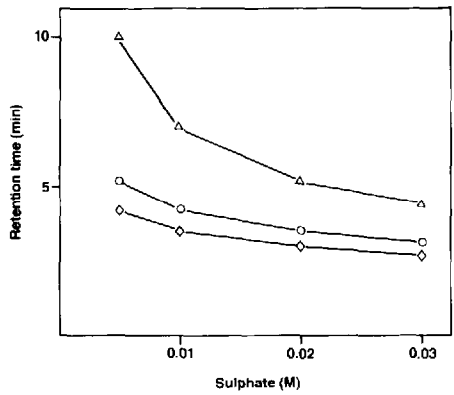


Fig. 2. Influence of citric acid on the peak shape of PFA. Sample: 0.23 m*M* phosphate (1), 0.23 m*M* phosphite (2) and 3.3 m*M* PFA (3). (A) Mobile phase: 0.1 *M* acetic acid and 0.01 *M* sulphate, pH 2.9. (B) As (A) but 1 m*M* citric acid added.

Fig. 3. Retention time at different sulphate concentrations. Mobile phase: 0.1 *M* acetic acid and 1 m*M* citric acid, pH 2.9. △—△, 3.3 m*M* PFA; ◇—◇, 0.25 m*M* phosphate; ○—○, 0.25 m*M* phosphite.

the Experimental section, the response decreases rapidly when the citric acid concentration exceeds 5 m*M*. In order to minimize this effect a citric acid concentration of 1 m*M* was chosen.

The pre-conditioning of new columns (see Experimental) with 8 m*M* citric acid was found necessary for good chromatography of PFA. Without this pre-treatment, the columns gave broad and asymmetrical PFA peaks. It should be noted that the investigation of the influence of citric acid concentration on the peak shape, as shown in Fig. 2, was performed on a column pre-conditioned as described. Shorter conditioning times than the one stated in the Experimental section were not tested, but may well be sufficient.

Effect of sulphate and pH

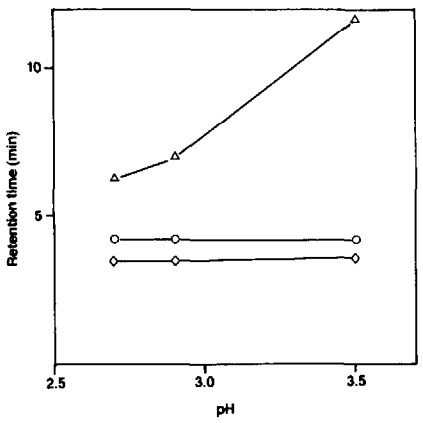
The effect of the sulphate concentration on the elution of the three ions is illustrated in Fig. 3. PFA shows the most pronounced reduction in retention time with increasing sulphate concentration. With the sample concentrations used (see Fig. 3), baseline separation between PFA and phosphite was achieved at all sulphate concentrations examined. Phosphate and phosphite were baseline separated at sulphate concentrations up to 0.02 *M*.

The retention of PFA is highly dependent on the pH of the mobile phase (Fig. 4). Phosphite and phosphate are less influenced, though the resolution between them increases slightly with decreasing pH. A representative chromatogram obtained with the mobile phase finally chosen (see Experimental) is shown in Fig. 5.

The detection system

The detection system consists of two consecutive reactions. First, the oxidation of PFA and phosphite to phosphate by bromine. Secondly, the reaction between phosphate and the molybdovanadate reagent. It is desirable that the oxidation to phosphate proceeds to completion, though this is not a requirement. The effectiveness of the oxidation was investigated by measuring the peak areas of PFA and phosphite compared to that of phosphate. Since PFA and phosphite are converted into phosphate prior to detection, all three components should give the same detector response calculated on a molar basis. By injecting three solutions containing 3.333 m*M* PFA, 1.638 m*M* phosphite and 1.699 m*M* phosphate respectively, the following responses were obtained: PFA, 255 area units/m*M*; phosphite, 254 area units/m*M*; phosphate, 258 area units/m*M*. It can thus be concluded that the oxidation is approximately complete. The responses of PFA and phosphite were constant when the bromine concentration was varied between 2 and 8 m*M*. However, below 2 m*M*, the responses decreased due to incomplete oxidation. The bromine concentration used in the response test above, as well as in all subsequent work, was about 4 m*M*.

The influence of different molybdovanadate concentrations on the response of PFA is shown in Fig. 6. Between 1.6 and 2.7 m*M* the response differs only by 1.5%. With lower concentrations, however, the response decreases rapidly. It should be noted that the citric acid concentration in the mobile phase will influence the relationship between the reagent concentration and response due to the interference discussed above. If the citric acid concentration is higher than 1 m*M*, the response will decrease at a higher molybdovanadate concentration compared to that shown in Fig. 6. Too



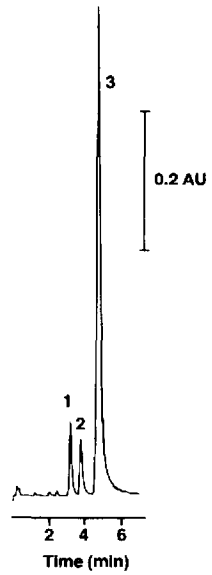


Fig. 4. Influence of pH on retention time. Mobile phase: 0.1 *M* acetic acid, 0.01 *M* sulphate and 1 m*M* citric acid. Sample concentrations and symbols as in Fig. 3.

Fig. 5. Chromatogram of 0.26 m*M* phosphate (1), 0.26 m*M* phosphite (2) and 3.3 m*M* PFA (3). Mobile phase: 0.1 *M* acetic acid, 0.015 *M* sulphate and 1 m*M* citric acid, pH 2.7.

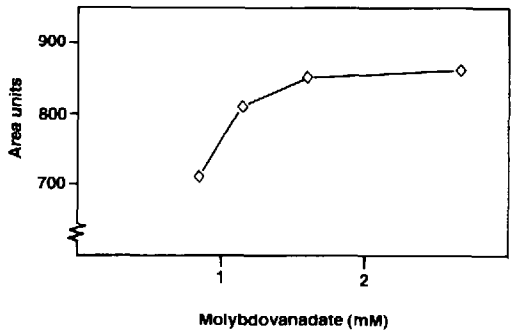


Fig. 6. Influence of molybdovanadate reagent concentration on the peak area of 3.3 mM PFA. Mobile phase as in Fig. 5.

high reagent concentrations are unfavorouble due to the generation of a high background absorbance and thus increased noise. The reagent concentration chosen for subsequent work with 1 mM citric acid was 1.6 mM.

The final reduction of the excess of bromine by addition of sulphite is not critical, and requires only an excess of sulphite. Since bromine is eliminated before the final detection, only the molybdovanadate reagent contributes to the background absorbance. It was found that a good quality peristaltic pump must be used for the reagent delivery or else pulsation in combination with the reagent background gave a noisy baseline.

The total residence time in the reaction detection system is only about 18 s. PFA shows UV absorbance at 230 nm, and can thus be detected directly without the reaction detection system. By comparing the PFA peak obtained by direct UV detection and that obtained with the reagent detection system, the band broadening in the detection system was found to be too small to be measurable.

Linearity

The peak areas of phosphate and phosphite are linearly related to concentration at least between $3 \cdot 10^{-5}$ and $2.7 \cdot 10^{-3}$ M; $r > 0.9995$. The peak height of phosphite is linear within the same interval, while the phosphate peak height is only linear up to about 1.5 mM. The PFA peak area is linear with concentration within the interval investigated, 1–4 mM; $r > 0.9998$. A plot of peak height against concentration, however, is slightly concave at low concentrations. This is probably due to the tailing tendency of PFA, which may result in relatively broader peaks at lower concentrations.

A typical value for the relative standard deviation (R.S.D.) of the peak area for ten injections of 3.3 mM PFA is 0.4%. For $1.5 \cdot 10^{-4}$ M phosphate and phosphite, the R.S.D. was 2%.

Detection limits

The detection limit was calculated as the amount giving a peak height three times the amplitude of the background noise. This peak height was found for phosphite and phosphate after injecting 20 μl of a $6 \cdot 10^{-6}$ M solution of the two components. The detection limit is then about 4 ng calculated as phosphorus, for both

substances. For PFA, 20 μl of a $2 \cdot 10^{-5}$ M solution, and thus about 12 ng phosphorus, could be detected.

CONCLUSIONS

The chromatography and detection system presented shows sufficient reproducibility, separation efficiency and sensitivity for drug formulation analysis of the three phosphorus compounds discussed. So far, three columns of the same packing material, but of different batches, have been tested, with similar results concerning the peak shape and retention times. One column has been in continuous use for about 6 months, without any changes in chromatographic behaviour. Undisturbed performance of the detection system can be achieved, provided pulsations in the delivery of the molybdovanadate reagent are kept low, and deaerated solutions are used to avoid air-bubbles in the detection system.

ACKNOWLEDGEMENTS

The authors thank Dr. Per-Olof Edlund for valuable information concerning the column used, and Dr. Jörgen Lindquist for discussions concerning the bromine oxidation.

REFERENCES

1 B. Öberg, *Pharmacol. Ther.*, 19 (1983) 387.
2 E. Helgstrand, B. Eriksson, N. G. Johansson, B. Lannerö, A. Larsson, A. Misiorny, J. O. Norén, B. Sjöberg, K. Stenberg, G. Stening, S. Stridh, B. Öberg, S. Alenius and L. Philipson, *Science (Washington, D.C.)*, 201 (1978) 819.
3 J. Pettersson, T. Nordgren and D. Westerlund, in preparation.
4 W. D. Basson, J. F. van Staden and P. M. Cattin, *Fresenius' Z. Anal. Chem.*, 307 (1981) 373.
5 G. Misson, *Chem.-Ztg.*, 32 (1908) 633.
6 Y. Hirai, N. Yoza and S. Ohashi, *J. Chromatogr.*, 206 (1981) 501.