ELSEVIER

Journal of Chromatography A, 664 (1994) 111–116

JOURNAL OF CHROMATOGRAPHY A

Short Communication

High-performance liquid chromatographic characterization of monofluorophosphate, difluorophosphate and hexafluorophosphate

Norimasa Yoza*[,a], Sachiko Nakashima[a], Nobuyuki Ueda[a], Tohru Miyajima[a], Terumasa Nakamura[b], Pierre Vast[c]

[a]*Department of Chemistry, Faculty of Science, Kyushu University, Fukuoka 812, Japan*

[b]*School of Nursing and Medical Technology, University of Occupational and Environmental Health, Yahatanishi-ku, Kitakyushu 807, Japan*

[c]*Laboratory of Applied Chemistry, University of Science and Technology of Lille, 59655 Villeneuve d' Ascq Cedex, France*

(First received September 20th, 1993; revised manuscript received November 18th, 1993)

Abstract

The retention behaviour of fluorophosphates on an anion-exchange column was characterized and compared with that of phosphates. Postcolumn blue coloration of phosphorus with a molybdenum reagent allowed the sensitive detection of both fluorophosphates and phosphates. ^{31}P NMR measurements were also employed to provide complementary data for the identification of phosphorus compounds. The retention times of orthophosphate and its fluorine derivatives increased with increasing number of P–F bonds: $PO_4^{3-} < PO_3F^{2-} < PO_2F_2^- < PF_6^-$. Good separation among orthophosphate, monofluorophosphate and difluorophosphate was achieved, but hexafluorophosphate was adsorbed too strongly to be eluted. It is suggested that fluorine derivatives of diphosphate (pyrophosphate), $P_2O_6F^{3-}$ and $P_2O_5F_2^{2-}$, might be present as minor impurities in monofluorophosphate chemicals. A misleading suggestion as to the assignment of unknown peaks in a preliminary report was corrected. Retention times with elution using 0.18 *M* KCl of the authentic phosphates and fluorophosphates and the speculated diphosphate derivatives increased in the order $PO_4^{3-} < PO_3F^{2-} < P_2O_7^{4-} < P_2O_6F^{3-} < P_3O_{10}^{5-} < PO_2F_2^- < P_2O_5F_2^{2-} < P_3O_9^{3-} < PF_6^-$.

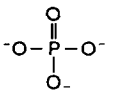
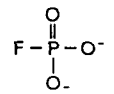
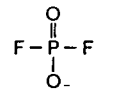
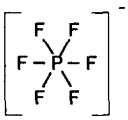
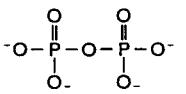
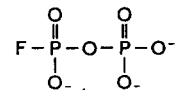
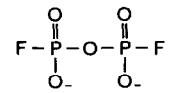
1. Introduction

Of the three fluorine derivatives of orthophosphate (P_1) [1] shown in Table 1, monofluorophosphate (MFP) has been widely used as a caries-preventive additive in dentifrices [2] and examined as a drug for the treatment of osteoporosis [3]. In connection with these important applications in biological systems, much attention has been focused on the bioavailability or enzyme-catalysed reaction of MFP. By employing high-performance liquid chromatography (HPLC), flow-injection analysis (FIA) and ^{31}P NMR spectrometry, we have demonstrated that alkaline phosphatase (EC 3.1.3.1) catalyses the hydrolysis of MFP with extremely high activity. The P–F bond cleavage to produce P_1 and fluoride ion at pH 7.2 is accelerated by a factor of as high as 10^{10} [4–6]. Such enzyme-catalysed reactions of inorganic phosphorus compounds,

* Corresponding author.

0021-9673/94/$07.00
SSDI 0021-9673(93)E0045-V

Table 1
Abbreviations for phosphorus compounds discussed in this paper

Abbreviation	Structural formula
P_1	
MFP	
DFP	
HFP	
P_2	
MFP_2	
DFP_2	

including the hydrolysis of diphosphate (pyrophosphate, P_2) by inorganic pyrophosphatase (EC 3.6.1.1) [7,8], have also been investigated to elucidate the role of inorganic phosphorus compounds in metabolic processes of organisms [9], prebiotic processes of evolution [10] and eutrophication processes of environmental water [11]. The results with MFP and P_2 stimulated our interest and studies were extended to the enzymatic degradation of other fluorophosphates [12] such as difluorophosphate (DFP), hexafluorophosphate (HFP) and derivatives of P_2 [13–15], monofluorodiphosphate (MFP_2) and difluorodiphosphate (DFP_2).

Advanced analytical techniques are needed in performing kinetic experiments on enzymatic reactions described above. This work was undertaken to establish an HPLC method for the determination of MFP, DFP and HFP. The successful separation of P_1, MFP and DFP and the extraordinarily strong adsorption of HFP on the column are described. ^{31}P NMR spectrometry was successfully employed as a complementary technique to HPLC for identifying phosphorus compounds for which authentic references were unavailable. The combined application of both HPLC and NMR methods to the fine characterization of several impurities in MFP chemicals is also described.

2. Experimental

Two samples of monofluorophosphate (Na_2PO_3F) from different sources were used: MFP-I was obtained from Aldrich (Milwaukee, WI, USA) and MFP-II was a gift from an industrial company. Sodium hexafluorophosphate ($NaPF_6$) was also available from Aldrich. Potassium difluorophosphate (KPO_2F_2) was prepared according to the literature [12].

The HPLC system consisted of a TSKgel SAX anion-exchange column (25 cm × 4 mm I.D.) and a postcolumn reaction detector as reported previously [4,5,7], except that a conventional spectrophotometric LC detector (Shimadzu SPD-6AV) was used instead of a photodiode-array detector. The conversion of phosphorus compounds into orthophosphate and the subsequent chromogenic reaction to form the so-called heteropoly blue was simultaneously achieved in a high-temperature reactor (140–150°C) using an acidic molybdenum(V)–molybdenum(VI) reagent. Retention times of anionic phosphorus compounds were effectively controlled by varying the potassium chloride concentrations of the eluents at a constant EDTA(4Na) concentration of 0.1% with a pH of *ca.* 10–10.5.

^{31}P NMR spectra were recorded at 25°C by using 10-mm tubes on a JEOL-GX-400 spectrometer operating at 162 MHz. Chemical shifts (δ values in ppm) are presented with respect to an external reference of 85% phosphoric acid, with positive values being downfield of the reference.

3. Results and discussion

The main objective of this work was to investigate the chromatographic retention behaviour of three fluorophosphates, MFP, DFP and HFP, on an anion-exchange separation column. Four phosphates, P_1, P_2, P_3 (triphosphate, $P_3O_{10}^{5-}$) and cP_3 (cyclotriphosphate, $P_3O_9^{3-}$), were used as reference samples. The reference samples are available commercially or can be prepared in the laboratory as sodium salts of high purity (>99%). HPLC data for these reference samples on the same anion-exchange separation column as used in this work have been reported previously [4,5,7]. A postcolumn chromogenic reaction system for phosphates and MFP using a molybdenum(V)–molybdenum(VI) reagent has been confirmed to be sensitive enough to permit the spectrophotometric detection of as low as $2 \cdot 10^{-7}$ *M* phosphorus(V) [4,7].

The successful separation of MFP from P_1, P_2 and P_3 has been reported in previous papers [4,5,7], but no chromatographic data were available for DFP and HFP. It was pointed out that MFP chemicals contain 6–13% of phosphorus as impurities. In addition to the peaks of P_1, P_2, and P_3 an unknown peak was observed between those of P_2 and P_3. We suggested that DFP, the closest homologue of MFP, might be one of the probable species assignable to this unknown peak. At that time DFP and HFP were not available as references. Further examination with additional use of authentic samples of DFP, HFP and cP_3 was attempted in this work. As will be mentioned later, neither DFP nor HFP was ascribable to the unknown peak.

3.1. ^{31}P NMR characteristics of fluorophosphate samples

As reliable standard methods for the purification and analysis of fluorophosphates have not been established, it seems difficult to obtain alkaline fluorophosphates of guaranteed high purity. Most MFP, DFP and HFP from commercial and industrial sources might contain considerable amounts of impurities that must often be analysed by users. Three fluorophosphates to be used for subsequent HPLC experiments were preliminarily analysed by ^{31}P NMR spectrometry to evaluate the approximate purity of each matrix compound and to identify the impurities. As shown in Figs. 1–3, MFP, DFP and HFP showed spectra with a doublet, triplet and septet, respectively, with $N + 1$ lines where N is the number of P–F bonds. The chemical shift (δ) and coupling constant ($^1J_{PF}$) of MFP were greatly affected by pH variations at pH <5, but remained unchanged in the pH range 6–11;

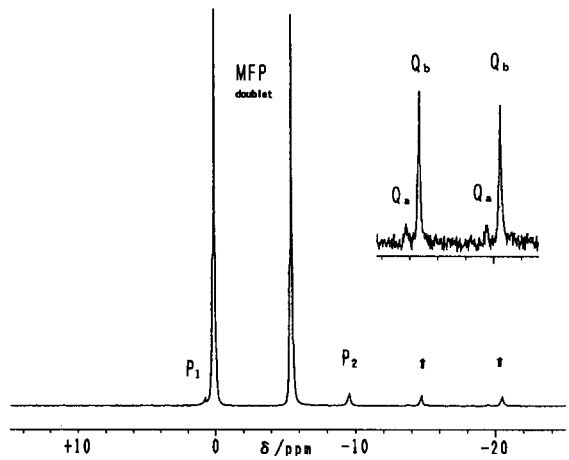


Fig. 1. ^{31}P NMR spectrum of monofluorophosphate. Sample, 0.1 *M* MFP-II (pH 4). P_1, P_2, Q_a and Q_b are impurities. Q_a and Q_b are discussed in Fig. 4. Small signals of P_3 may be included in the signal of P_2 and the upper field signal of Q_b.

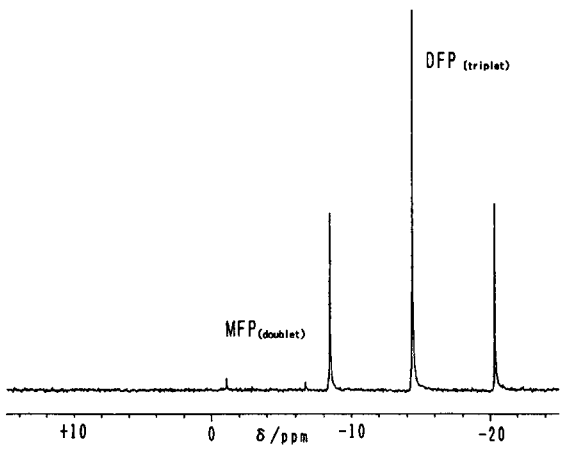


Fig. 2. ^{31}P NMR spectrum of difluorophosphate. Sample, 0.01 *M* DFP (pH 3.5). MFP is an impurity.

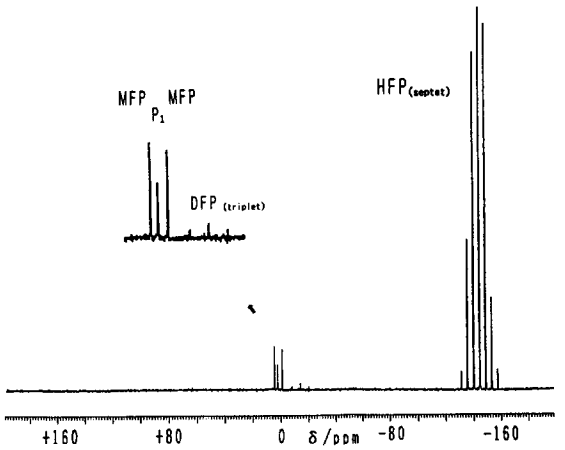


Fig. 3. ^{31}P NMR spectrum of hexafluorophosphate. Sample, 0.1 *M* HFP (pH 6.7). P_1, MFP and DFP are impurities.

$\delta = +1.3 \pm 0.2$ ppm and $^1J_{PF} = 869 \pm 2$ Hz [6]. On the other hand, the NMR parameters of DFP ($\delta = -14.2 \pm 0.1$ ppm, $^1J_{PF} = 962 \pm 1$ Hz) and HFP ($\delta = -144 \pm 1$ ppm, $^1J_{PF} = 712 \pm 1$ Hz) were independent of pH over the wide pH range of 3–10. Identification of most of the impurity signals was achieved as indicated in Figs. 1–3 by reference to published NMR data [1,6]. Detailed discussion as to the identification of Q_a and Q_b will be made late by cross-reference to both NMR and HPLC data.

3.2. HPLC profiles of reference samples

All chromatographic runs in this work were made by isocratic modes. Fig. 4 (top) shows an HPLC profile for a mixed solution of authentic samples, P_1, P_2, P_3 and MFP. The four components were well resolved with retention times (t_R) within 30 min. A cyclic reference sample, cP_3, appeared at 288 min far behind the linear phosphates. The HPLC profile will be referenced hereafter in assigning unknown peaks.

It was preliminarily confirmed by FIA [5,16] that DFP and HFP, in addition to MFP [4], were quantitatively detectable by the same post-column chromogenic reaction system as used for the above phosphates. HPLC of a freshly prepared aqueous solution of DFP gave a major

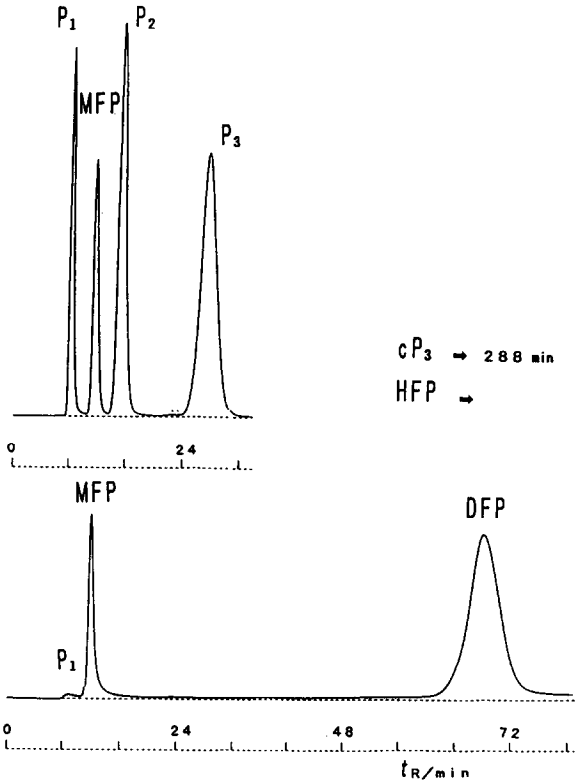


Fig. 4. HPLC profiles of an authentic mixture of monofluorophosphate, orthophosphate, diphosphate and triphosphate (top) and of difluorophosphate and its hydrolytic products (bottom). Eluent, 0.18 *M* KCl + 0.1% Na_4EDTA; sample, top, an equimolar mixture (0.1 m*M* each) of MFP, P_1, P_2, and P_3, and bottom, 0.22 m*M* DFP and its hydrolysate, MFP; detection, 690 nm, 0.16 AUFS (top) and 0.08 AUFS (bottom). cP_3 appeared at 288 min (not shown), but HFP was not eluted within 400 min.

peak of DFP at 68 min between the peaks of P_3 and cP_3. A small peak of MFP was also observed, indicating 98% purity of the DFP, as expected from the NMR data in Fig. 2. In order to elucidate the retention behaviour of DFP relative to that of MFP, the DFP sample solution including MFP, a hydrolysate produced during prolonged storage of DFP solution, was eluted. As shown in Fig. 4 (bottom) both peaks of MFP and DFP were well defined and separated. The retention behaviour of HFP was examined in a separate experiment. No positive appearance of

an HFP peak was observed within 400 min. The HFP might be strongly adsorbed on the column.

In order to shorten the retention times of DFP and HFP for practical purposes of routine analysis, the eluent concentration was increased from 0.18 *M* KCl to 0.35 and 0.40 *M* KCl. In both instances MFP, P_1, P_2 and P_3 were recorded as an unresolved muti-component peak at 8.0 min. DFP (40 min) appeared before cP_3 (45 min) with 0.35 *M* KCl, but the retention order was reversed with 0.40 *M* KCl; DFP (36 min) appeared after cP_3 (28 min). The effect of the eluent concentration on the retention time of DFP was smaller than that of cP_3 owing to the difference in the anionic charges of DFP and cP_3, −1 and −3, respectively [17]. Surprisingly, no peak of HFP was observed within 100 min, where most linear and cyclic oligophosphates were eluted [17,18]. Further attempts were made with stronger 1.0 *M* KCl but again without any positive appearance of HFP within 100 min.

3.3. Identification of impurities in MFP

Two MFP samples from different sources were eluted with the same eluent concentration as that in Fig. 4. As shown in the main profile (Fig. 5), MFP-II indicated five impurities; P_1, P_2 and P_3 were identified by reference to the standard profile (Fig. 4), but Q_a and Q_b are speculative. MFP-I (inset) contained a small amount of Q_a, but Q_b was not detected. In a previous paper [4] we carried out a similar experiment on MFP and observed unknown peaks of Q_a and Q_b. At that time DFP and HFP were not available as reference samples. It was speculated that Q_a and Q_b might be assignable to DFP and cP_3, respectively. The present re-examination with the additional use of DFP, HFP and cP_3, however, provided clear evidence that neither DFP, HFP nor cP_3 could be assigned to Q_a and Q_b (Fig. 5). A similar conclusion was also drawn from additional experiments with 0.35 *M* KCl eluent; Q_b

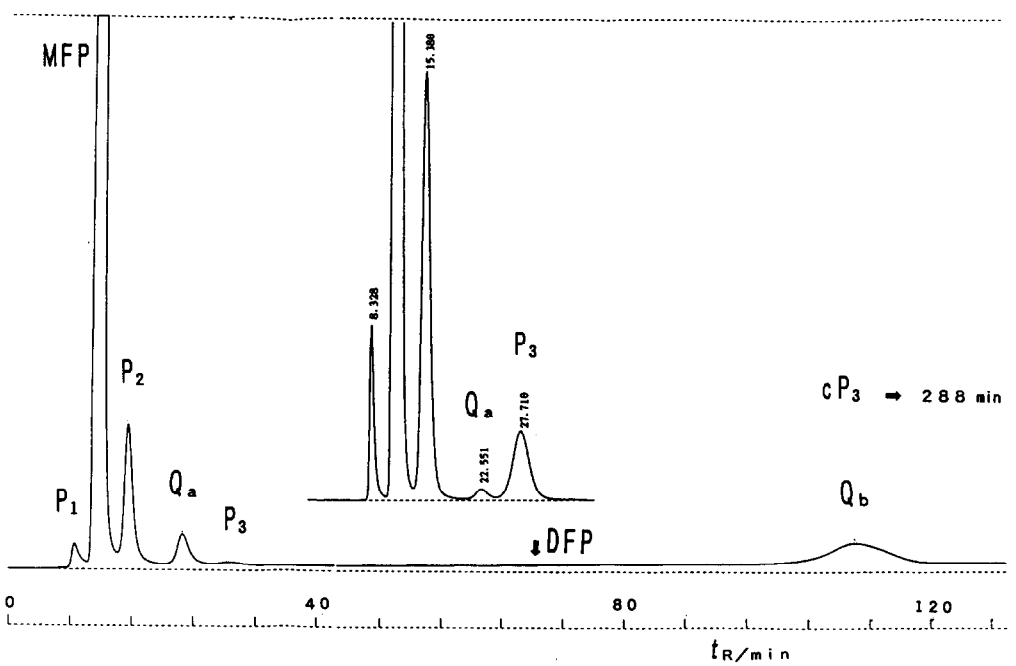


Fig. 5. HPLC profiles of two disodium monofluorophosphate samples from different sources and identification of impurities. Sample, 1 m*M* MFP-II and (inset) 1 m*M* MFP-I. Other conditions as in Fig. 4, except recording at 0.08 AUFS. Retention times of DFP and cP_3 from Fig. 4 are also indicated for comparison.

appeared at 36 min, *i.e.*, different from the retention times of cP_3 and DFP, with the order, $Q_b < cP_3 < DFP$.

4. Conclusions

None of the reference samples examined in this work is a candidate for Q_a and Q_b, and we must again speculate that some phosphorus compounds produced Q_a and Q_b. Fluorine derivatives of diphosphate (MFP_2 for Q_a and DFP_2 for Q_b) are considered to be the most probable candidates. Some reasons for suggesting such species are that (a) the relative amounts of Q_a and Q_b in the HPLC profile (Fig. 5) are in approximate agreement with those of the NMR signals of Q_a and Q_b in Fig. 1, (b) the anionic charges of Q_a and Q_b calculated from the effect of eluent concentration on retention time [17,18] were −3 and −2, respectively, and (c) a "doublet" peak of Q_b with $\delta = -17.7$ ppm and $J = 938$ Hz is similar to the doublet peak with $\delta = -17.8$ ppm and $^1J_{PF} = 943$ Hz of DFP_2 in the literature [14]. No information on the formation or NMR spectrum of MFP_2 is available, but the similarity between the large coupling constants of the Q_a "doublet" and Q_b "doublet" suggests that Q_a is assignable to MFP_2, although the small splitting of each signal of Q_a and Q_b has not been clarified.

The retention times in 0.18 *M* KCl (Fig. 4) of the seven authentic phosphates and fluorophosphates and the speculated derivatives of diphosphate decrease in the order $P_1 < MFP < P_2 < MFP_2 < P_3 < DFP < DFP_2 < cP_3 < HFP$. From the viewpoint of practical application it is worth noting that (1) the retention times of orthophosphate analogues increased with increasing number of P–F bonds, $P_1 < MFP < DFP < HFP$, and a good separation among P_1, MFP and DFP was achieved, whereas HFP was adsorbed too strongly to be eluted. Finally, we hope that MFP_2 and DFP_2 will become available as reference samples so that re-examination will become feasible to confirm the assignments of Q_a and Q_b speculated in this work.

5. References

[1] J.R. Van Wazer, *Phosphorus and Its Compounds*, Interscience, New York, 1958, Ch. 13.
[2] E.I.F. Pearce, *Caries Res.*, 17, Suppl. 1 (1983) 21.
[3] F. Liote, C. Bardin, A. Liou, A. Brouard, J.L. Terrien and D. Kuntz, *Calcif. Tissue Int.*, 50 (1992) 209.
[4] N. Yoza, S. Nakashima, T. Nakazato, N. Ueda, H. Kodama and A. Tateda, *Anal. Chem.*, 64 (1992) 1499.
[5] N. Yoza, S. Nakashima, I. Akazaki, N. Ueda, T. Nakazato, N. Tokushige and T. Yokota, *Mem. Fac. Sci. Kyushu Univ., Ser. C*, 18 (1992) 203.
[6] N. Yoza, N. Ueda and S. Nakashima, *Fresenius' J. Anal. Chem.*, in press.
[7] N. Yoza, I. Akazaki, T. Nakazato, N. Ueda, H. Kodama and A. Tateda, *Anal. Biochem.*, 199 (1991) 279.
[8] N. Yoza, H. Hirano, Y. Baba and S. Ohashi, *J. Chromatogr.*, 325 (1985) 385.
[9] H. Hirano, Y. Baba, N. Yoza and S. Ohashi, *Chem. Lett.*, (1986) 633.
[10] N. Yoza, N. Ueda, N. Tokushige, T. Miyajima, Y. Baba, M. Tsuhako and A. Tateda, *Inorg. Chim. Acta*, 202 (1992) 237.
[11] N. Yoza, S. Sagara, H. Morioka, T. Handa, H. Hirano, Y. Baba and S. Ohashi, *J. Flow Inject. Anal.*, 3 (1986) 37.
[12] P. Vast, G. Palavit and A. Semmoud, *Phosphorus Res. Bull.*, 1 (1991) 439.
[13] A. Addou, P. Vast and P. Legrand, *Bull. Soc. Chim. Fr.*, (1984) 55.
[14] H. Falius, *Angew. Chem., Int. Ed. Engl.*, 7 (1968) 622.
[15] U. Schulke and R. Kayser, *Z. Anorg. Allg. Chem.*, 600 (1991) 221.
[16] T. Nakazato and N. Yoza, *J. Chromatogr.*, 641 (1993) 221.
[17] Y. Baba, N. Yoza and S. Ohashi, *J. Chromatogr.*, 348 (1985) 27.
[18] G. Kura, E. Kitamura and Y. Baba, *J. Chromatogr.*, 628 (1993) 241.