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Determination of impurities in a novel analogue of adenosine 5′-triphosphate by capillary electrophoresis

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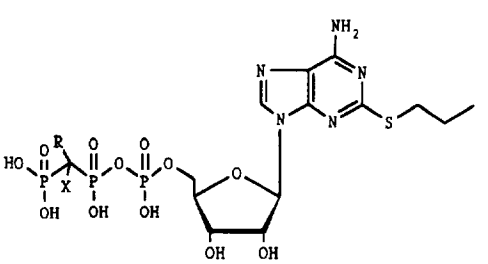
Abstract

A capillary electrophoretic method using an uncoated capillary was developed to resolve potential impurities in FPL 67085XX, a novel phosphonate analogue of adenosine 5′-triphosphate. The effects of buffers, buffer concentration, eluent pH and methanol were investigated. Optimum resolution of the impurities was achieved using an eluent consisting of 7% (v/v) methanol in 25 m*M* phosphate buffer–2 m*M* EDTA adjusted to pH 6.6.

1. Introduction

FPL 67085XX [1] (Fig. 1) is a novel phosphonate analogue of adenosine triphosphate and a potent antagonist at P_2T purinoreceptors. FPL 67085XX shows anti-thrombotic activity in animals and inhibits platelet aggregation ex vivo in man.



R = X = Cl, FPL 67085XX
R = Cl, X = H, FPL 69499XX
R = X = H, FPL 67933XX

Fig. 1. Structures of the compounds investigated.

FPL 69499XX and FPL 67933XX are potential impurities of FPL 67085XX. To date, it has not been possible to resolve the two impurities from FPL 67085XX using reversed-phase HPLC. Resolution was achieved using anion-exchange chromatography, with a Protein-Pak DEAE 5PW column and triethylammonium hydrogencarbonate eluent [2]. However, the peaks were too broad to allow accurate quantification of the impurities at low levels. The use of capillary electrophoresis (CE) was therefore investigated as an alternative means of resolving the two impurities from FPL 67085XX.

Although the analysis of nucleoside monophosphates by micellar electrokinetic capillary chromatography (MECC) [3–8] and by capillary zone electrophoresis (CZE) [4,9–11] is well established there are few references to the analyses of nucleoside 5′-triphosphates.

Dolnik et al. [12] and Takigiku and Schneider [13] used capillaries silylated with γ-methacryloxypropyl-trimethoxysilane to separate UTP, GTP, ATP, and CTP, and also UTP, dTTP, ITP, GTP, dGTP, dCTP, CTP, dATP and ATP from

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each other. However, these capillaries have the disadvantage of not being commercially available.

Liu et al. [8] used MECC with sodium dodecyl sulphate (SDS) to partially resolve CTP, UTP, GTP and ATP. Partial baseline resolution was achieved using the cationic surfactant dodecyltrimethylammonium bromide, though the polarity of the detector had to be changed.

There have been only two reports of CZE being used for the analysis of nucleoside 5′-triphosphates and neither study involved complex sample matrixes. Pentoney et al. [14] used a 0.2 *M* borate buffer, pH 8.1, to successfully separate ^{32}P-labelled ATP and GTP. De Bruijn et al. [15] demonstrated that UTP could be separated from 5′-fluorouridine 5′-triphosphate using a 10 m*M* phosphate buffer pH 4.8.

Investigations using MECC and a simple CE method for the separation of FPL 67085XX, FPL 69499XX and FPL 67933XX using an uncoated capillary are reported below. The method may be equally applicable to other nucleoside 5′-triphosphates.

2. Experimental

The optimized conditions for separation are as follows: 25 m*M* Phosphate, 2 m*M* EDTA, 7% (v/v) methanol adjusted to pH 6.6 with sodium hydroxide. Voltage 20 kV, 67 cm × 75 μm uncoated capillary. Temperature 25°C. Wavelength 280 nm. 10 s pressure injection.

2.1. Apparatus

The CE system used was a Beckman P/ACE system 2050, controlled with System Gold version 7 software (Beckmann Instruments, Fullerton, CA, USA). The uncoated fused silica capillary, 75 μm I.D. (60 cm from injector to detector, 67 cm total length or 50 cm from injector to detector, 57 cm total length) (FSA, Loughborough, UK) was fitted into a capillary cartridge and thermostatted at 25°C. The samples were injected by pressure for 10 s (0.5 p.s.i.; 1 p.s.i. = 6894.76 Pa) and detection was by on column UV absorbance at 280 nm. The voltage supplied was +20 kV.

Before use, capillaries were preconditioned with 1 *M* phosphoric acid (20 min, pressure) followed by purified water (5 min, pressure), 1 *M* sodium hydroxide solution (20 min, pressure) and purified water (5 min, pressure).

At the start of each day, the capillary was washed with 0.1 *M* sodium hydroxide solution (25 min, pressure) followed by purified water (5 min, pressure).

Before each injection the capillary was washed with 0.1 *M* sodium hydroxide solution (2 min, pressure) and buffer solution (1 min, pressure).

2.2. Chemicals

Purified water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). Phosphoric acid, EDTA disodium salt, boric acid, borax, tris, sodium hydroxide, methanol and SDS were purchased from FSA.

FPL 67085XX, FPL 69499XX and FPL 67933XX were manufactured by Fisons Pharmaceuticals, Loughborough, UK.

Buffer system 1

Buffer system 1 consisted of 30 m*M* borate–2 m*M* EDTA pH 9.0–variable SDS concentration. The required amount of SDS was added to a solution of 30 m*M* boric acid–2 m*M* EDTA adjusted to pH 9.0 with 3 *M* sodium hydroxide.

Buffer system 2

Buffer system 2 consisted of 10 m*M* phosphate–10 m*M* Tris–100 m*M* SDS (pH 7.05). SDS (1.44 g) in a 50-ml volumetric flask which was diluted to volume with 10 m*M* Tris–10 m*M* sodium dihydrogenorthophosphate which had been previously adjusted to pH 7.05 with orthophosphoric acid (specific gravity 1.69).

Buffer system 3

Buffer system 3 consisted of a solution of 30 m*M* boric acid and 2 m*M* EDTA adjusted to the required pH with sodium hydroxide.

Buffer system 4

Buffer system 4 consisted of a solution of borax (30 m*M*s sodium tetraborate decahydrate) and 2 m*M* EDTA adjusted to the required pH with orthophosphoric acid (specific gravity 1.69).

Buffer system 5

Buffer system 5 consisted of phosphoric acid and 2 m*M* EDTA adjusted to the required pH with 0.1 *M* sodium hydroxide and the required amount of methanol added.

3. Results and discussion

3.1. MECC

Using an MECC system of 30 m*M* sodium borate–2 m*M* EDTA–100 m*M* SDS (buffer system 1 with 100 m*M* SDS the resolution of a mixture of FPL 67085 XX, nucleoside 5′-monophosphate, nucleoside and base was achieved (Fig. 2). However, migration times were short, peaks were broad and also showed some splitting. At lower pH values (<9.0), migration times

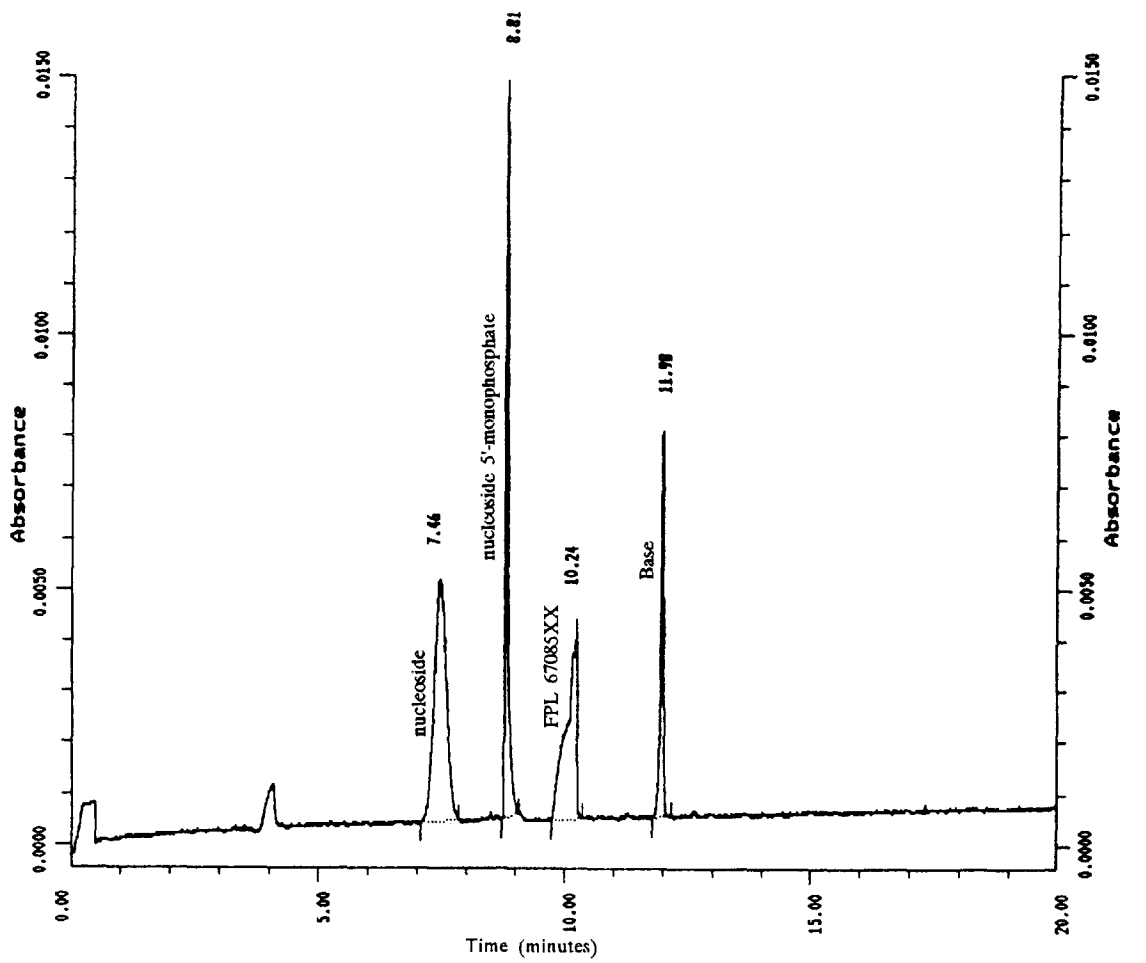

Fig. 2. Electropherogram of FPL 67085XX (0.06 mg/ml), nucleoside 5′-monophosphate (0.05 mg/ml), nucleoside (0.05 mg/ml) and base (0.03 mg/ml). 30 m*M* Borate–2 m*M* EDTA, adjusted to pH 9.0–100 m*M* SDS (system 1). Voltage 20 kV, 57 cm × 75 μm uncoated capillary. Temperature 25°C.

became very irreproducible. Different concentrations of SDS (25, 50, 75, 125, 150 m*M*) at pH 9.0 showed no significant improvement with respect to splitting of the FPL 67085XX peak. An injection of 1 mg/ml FPL 67085XX gave a very broad split peak and no resolution when a mixture of 1 mg/ml FPL 67085XX, 0.05 mg/ml FPL 69499XX and 0.16 mg/ml FPL 67933XX was chromatographed.

Using the system of Liu et al. [8] (buffer system 2), improved resolution of the components was achieved at pH 7.0 although some splitting of the FPL 67085XX peak was again observed (Fig. 3). At higher concentrations of FPL 67085XX, peak splitting increased and no resolution was achieved. Variable SDS concentrations (25, 50, 75, 125, 150 m*M*) again gave no improvement to the peak shape of FPL 67085XX, though as expected there was a dramatic change in relative migration times of the base and nucleoside to the nucleotides.

The results of the investigations suggested that

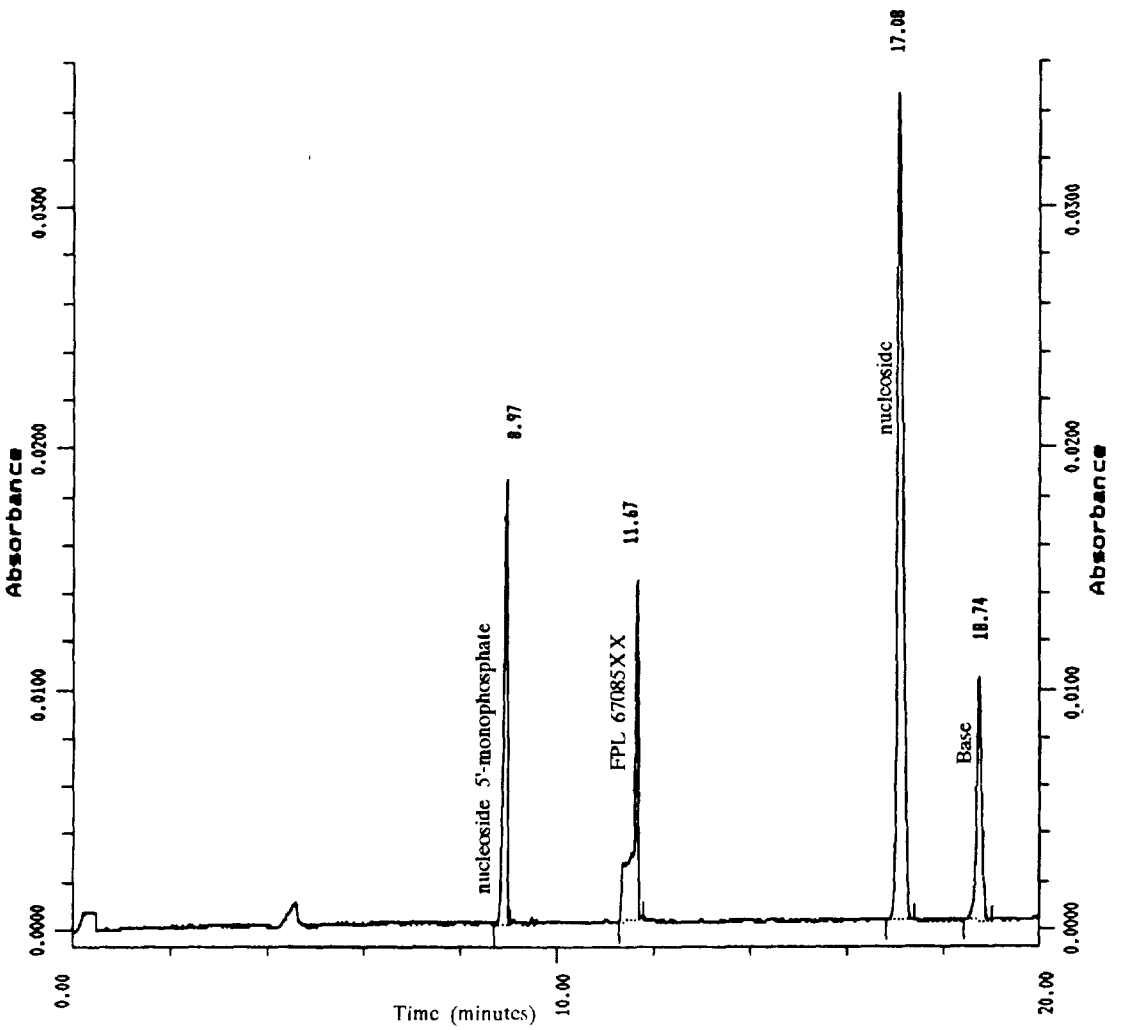

Fig. 3. Electropherogram of FPL 67085XX (0.06 mg/ml), nucleoside 5'-monophosphate (0.05 mg/ml), nucleoside (0.05 mg/ml) and base (0.03 mg/ml). 10 m*M* Tris–10 m*M* sodium dihydrogenorthophosphate, adjusted to pH 7.05–100 m*M* SDS (system 2). Voltage 20 kV, 57 cm × 75 μm uncoated capillary. Temperature 25°C.

eluents containing SDS are not suitable for the determination of the purity of FPL 67085XX. It is probable that similar problems will be encountered with other nucleoside triphosphates under similar conditions.

3.2. CZE

Borate buffer

A method similar to that of Pentoney et al. [14] was evaluated using a 30 m*M* sodium borate, 2 m*M* EDTA eluent, pH 8.0 to 9.0 (buffer system 3). The resolution of FPL 67085XX, nucleoside 5′-monophosphate, nucleoside and base was determined. Very broad and split peaks for FPL 67085XX were obtained at all pH conditions; peak shape and resolution of the other components were unaffected.

Borate–phosphate buffer

A 30 m*M* sodium borate–phosphate–2 m*M* EDTA (pH 6.0–7.8) eluent was studied (buffer system 4). In this system, the FPL 67085XX peak was sharp but the nucleoside 5′-monophosphate peak was split at eluent pHs greater than pH 6.6. The effect of pH upon the peak height and migration time of FPL 67085XX is shown in Fig. 4.

A dramatic change in peak height was observed with changes in eluent pH. The choice of pH was found to have a marked effect on sensitivity. At an eluent pH close to the pK_{a_4} of FPL 67085XX (7.1), the peak height was at a minimum. This is believed to be due to the increased electrophoretic mobility towards the anode as the charge increases from 3^- and 4^- thereby counteracting the electroosmotic flow.

The change in the migration time was explained as follows: as the pH increased from pH 6.0, the migration time decreased as the electroosmotic flow increases. However, this was counteracted by the migration time increasing as the ionisation of FPL 67085XX (pK_{a_4} 7.1) increased with increasing pH. This effectively increased the electrophoretic flow towards the anode.

The reasons for the splitting of the nucleoside 5′-monophosphate peak and not the FPL 67085XX peak are difficult to explain. Tadey and Purdey [10] suggested that nucleoside monophosphates complexed with borate which enhanced separation. It is possible that over the pH range studied, the complexation is causing split-

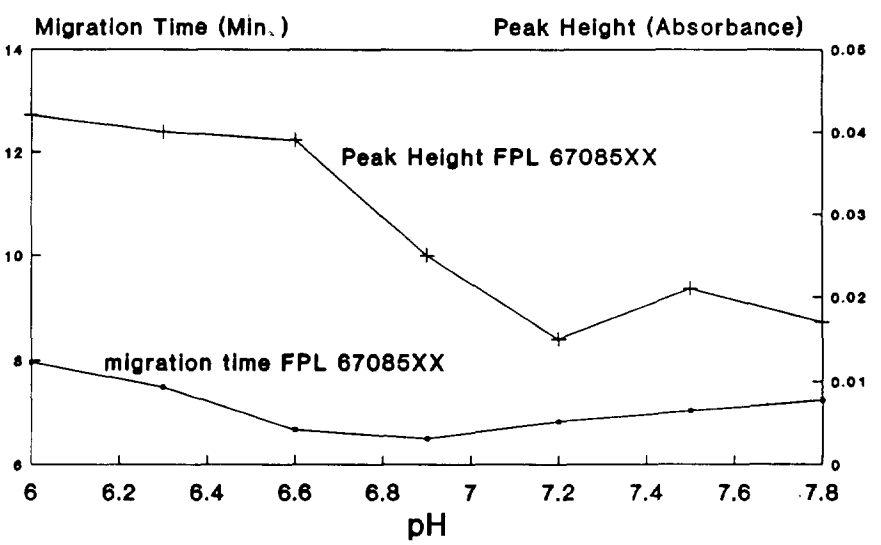

Fig. 4. The effect of pH on migration time (■) and peak height (+) of FPL 67085XX using a 30 m*M* borate–phosphate buffer–2 m*M* EDTA (system 4). Voltage 20 kV, 57 cm × 75 μm uncoated capillary. Temperature 25°C.

ting of the monophosphate peak but not of the triphosphate which carries more charge. At eluent pH values below 6.6, satisfactory peak shapes were obtained for both nucleotides. A compromise of pH 7.0 was chosen at which both the peak shape and migration times were considered to be acceptable.

Adjustment of the buffer concentration to 50 m*M* gave optimum resolution at pH 7.0. Although baseline resolution was achieved, the peak shapes were too broad to give high sensitivity (Fig. 5).

Phosphate buffer

Phosphate buffer having a pK_{a_2} of 7.1 was considered to be a potential eluent for the separation of phosphonate analogues of nucleoside triphosphates which have pK_{a_4} values of between 7.0 to 8.2 [16]. The use of phosphate

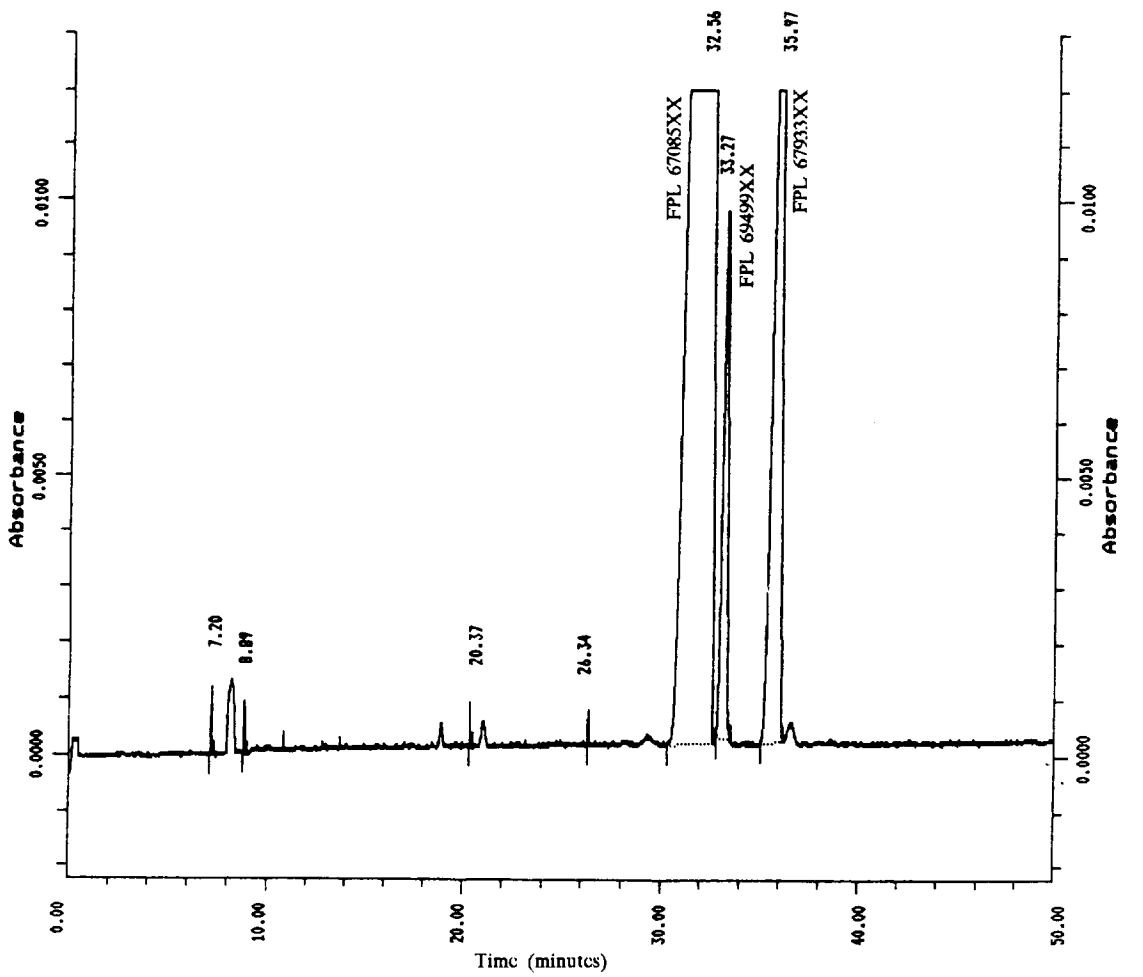

Fig. 5. Electropherogram of FPL 67085MX 1 mg/ml, FPL 69499X 0.05 mg/ml, and FPL 67933XX 0.16 mg/ml. 50 m*M* Sodium borate–2 m*M* EDTA, adjusted to pH 7.0 with phosphoric acid (system 4). Voltage 20 kV, 67 cm × 75 μm uncoated capillary. Temperature 25°C.

buffers for the resolution of the above compounds and as a means of eliminating peak splitting of the nucleoside 5'-monophosphate was thus investigated.

Effect of phosphate concentration

The effect of phosphate concentration on the migration time and peak height of FPL 67085XX at 0.25 mg/ml was studied over a sodium phosphate range of 10 to 80 m*M*. Buffers were prepared from phosphoric acid and titrated with sodium hydroxide to pH 7.0.

A dramatic change in peak height and therefore sensitivity was seen with changes in the phosphate buffer concentration. This was believed to be due to electro-dispersion caused by differences between the sample and running buffer conductivities at high and low phosphate concentrations. At high concentrations of phosphate buffer, Joule heating may also be causing band broadening. A concentration of about 30 m*M* phosphate was chosen as the optimum for further work.

Effect of pH

The effect of pH at 30 m*M* phosphate was investigated. The effect of pH on the migration times and the peak heights is shown in Fig. 6.

Major changes in the peak heights and resolution were found to occur with changes in eluent pH. Resolution between FPL 67085XX and FPL 69499XX was lost above pH 7 due to peak broadening. The poor peak shape of FPL 67085XX above pH 7 which resulted in low peak heights, cannot be attributed to a capillary wall interactions, as FPL 67085XX is highly ionised at high pH and will be repulsed from the wall.

Peak broadening may be a result of the increasing ionisation of FPL 67085XX from 3^- to 4^- state with increasing pH. This increases the electrophilic mobility towards the anode, counteracting the electroosmotic flow which will lead to an effective delay and cause band broadening. This effect may also explain why the migration times between pH 6.4 and 7.4 had remained relatively constant.

The resolution between FPL 67085XX and its analogues at eluent pH 5.8 to 6.8 was excellent.

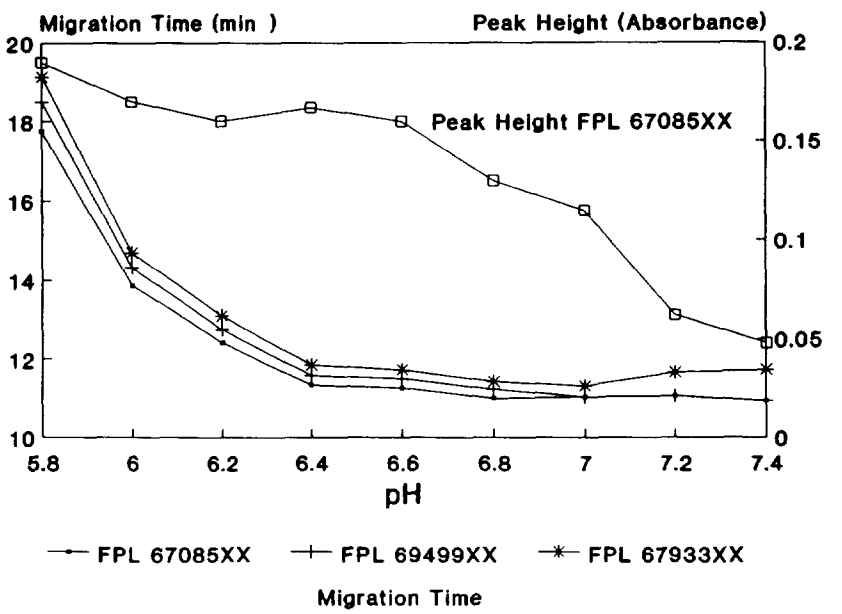

Fig. 6. The effect of pH on migration time and peak height. 30 m*M* phosphoric acid–2 m*M* EDTA, adjusted to the required pH with sodium hydroxide. Voltage 20 kV, 67 cm × 75 μm uncoated capillary. Temperature 25°C.

However, the reproducibility of the migration time was found to be very poor. This may be due to the relatively slow equilibration of the surface charge on the fused silica surface of the capillary at the above pH [17].

Effect of the organic modifier

Organic solvents are able to modify both mobility and electroosmotic force (EOF) [18], thereby leading to better resolution of components. The use of methanol was investigated as an organic modifier for the separation of FPL 67085XX and its related impurities.

Phosphate solutions, 25 m*M*, adjusted to pH 6.6 and containing between 0–12% (v/v) methanol were prepared. The effect on peak heights and variation of migration times of the compounds is shown in Fig. 7. The tailing factor and resolution between the peaks are shown in Table 1.

The migration time for all species was found to increase slightly as the methanol concentration was increased between 0 and 4% (v/v). This effect was anticipated as the EOF will have decreased. At higher methanol concentrations no further effect was seen on the migration time, however, the resolution and tailing factors showed subtle effects. Resolution of FPL 67085XX and FPL 69499XX was optimum at 4% (v/v) methanol and was effectively controlled by the width of the FPL 67085XX peak (expressed as the tailing factor). The resolution of FPL 69499XX and FPL 67933XX was optimum at 10% (v/v) methanol; this is controlled by the peak width of FPL 67933XX. A concentration of

Table 1
Effect of methanol concentration on resolution and peak tailing

Methanol (%, v/v)	Resolution FPL 67085XX/ FPL 69499XX	Resolution FPL 69499XX/ FPL 67933XX	Tailing factor
0	2.4	2.9	8.7
2	3.1	3.5	4.6
4	3.7	5.0	2.1
6	3.2	5.2	0.7
7	2.5	5.6	0.6
8	2.2	6.3	0.6
10	1.7	6.5	0.5
12	1.6	6.3	0.5

Conditions as in Fig. 7.

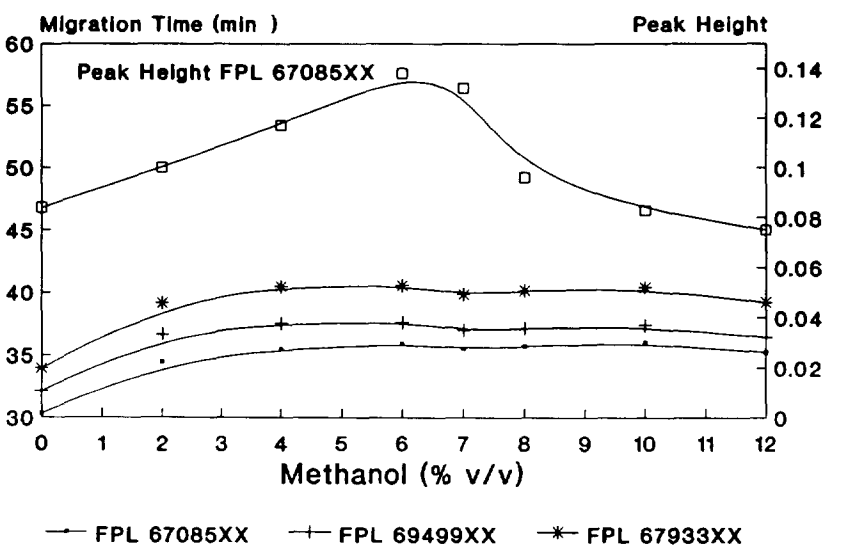

Fig. 7. The effect of methanol concentration on migration time and peak height, on FPL 67085XX using 25 m*M* sodium phosphate–2 m*M* EDTA pH 6.6–variable methanol concentration. Voltage 20 kV, 67 cm × 75 μm uncoated capillary. Temperature 25°C.

7% (v/v) methanol was chosen as optimum for the separation of all the components.

The reasons for the variation in the peak shape of FPL 67085XX with the methanol concentration are unknown. At low concentrations of methanol the peak had a tailing edge becoming symmetrical between 4 and 6% (v/v) methanol. At higher concentrations of methanol a leading edge is seen, hence tailing factors of less than 1.

An example of an electropherogram using the final conditions chosen is shown in Fig. 8. All three components are well resolved. The method gave a R.S.D. for the migration time of less than 1.0%. Using a 10 s injection of a FPL 67085XX solution the lowest detectable concentration was 0.0004 mg/ml. A linear relationship (correlation coefficient 0.9993) was established for the peak area and concentration between 0.0005 and 2.0 mg/ml (0.05–200% of the nominal concentra-

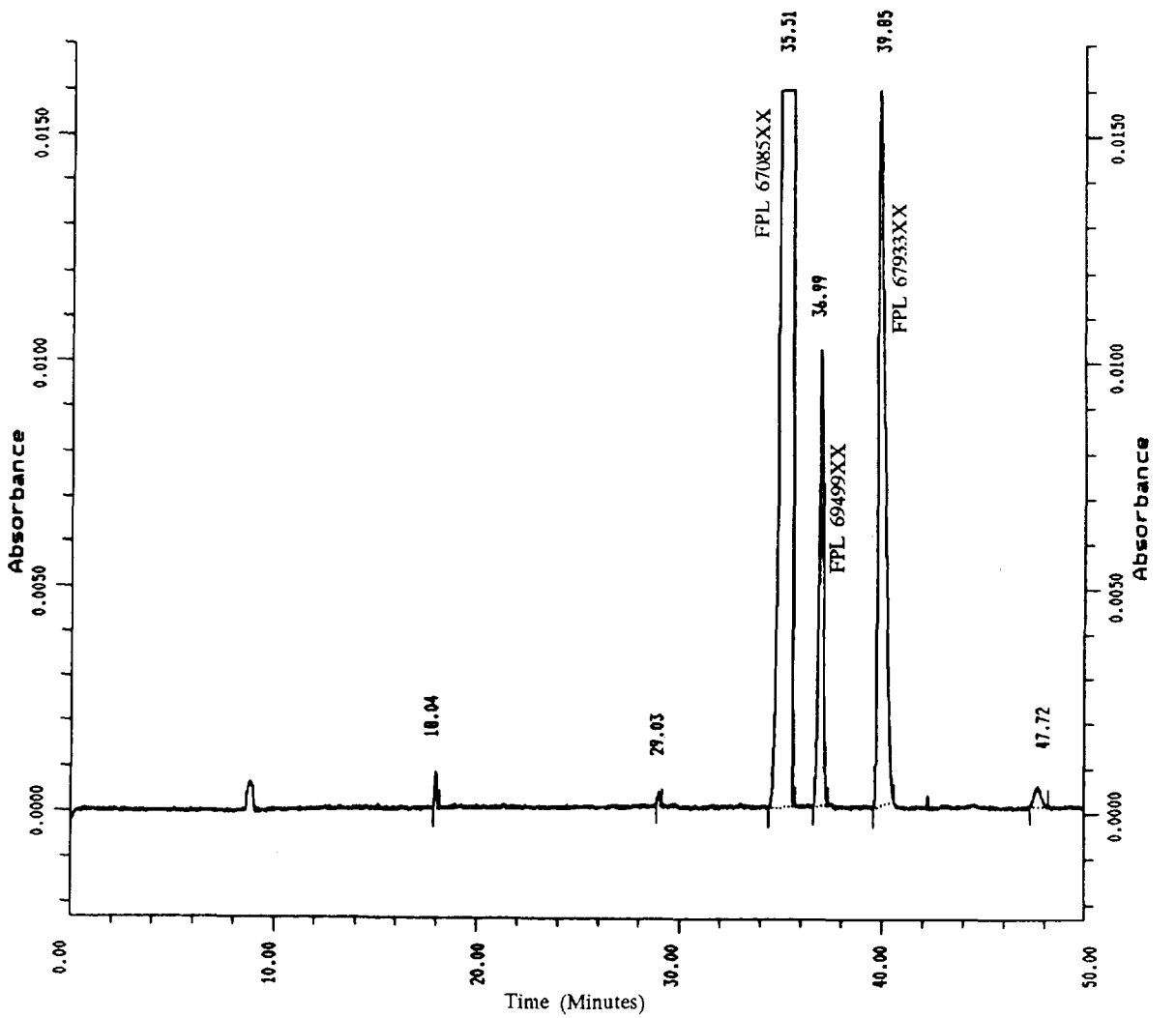

Fig. 8. Electropherogram of FPL 67085XX, FPL 69499XX and FPL 67933XX using 25 m*M* phosphate–2 m*M* EDTA–7% (v/v) methanol adjusted to pH 6.6 with sodium hydroxide. Voltage 20 kV, 67 cm × 75 μm uncoated capillary. Temperature 25°C, 10 s pressure injection.

Table 2
Reproducibility of migration time of FPL 67085MX in various buffer systems

	System 1	System 2	System 4	System 5
Number of injections	6	6	8	6
Mean migration time (min)	10.3	11.7	32.6	32.8
R.S.D. (%)	0.4	0.9	1.5	0.4

System 1: 30 m*M* borate–2 m*M* EDTA 100–m*M* SDS pH 9.0; system 2: 10 m*M* phosphate–10 m*M* Tris–100 m*M* SDS pH 7.05; system 4: 50 m*M* borax–2 m*M* EDTA adjusted to pH 7.0 with orthophosphoric acid; system 5: 25 m*M* orthophosphoric acid–2 m*M* EDTA–7% (v/v) methanol adjusted to pH 6.6 with sodium hydroxide.

tion); intercept values were less than 1% of the nominal concentration.

The reproducibility of migration times of FPL 67085XX in the above buffer systems was determined from multiple injections; data are presented in Table 2.

4. Conclusions

This work has demonstrated the major effects that buffer type, buffer concentration, pH and organic modifier can have on the resolution of a nucleoside phosphonate. By controlling all four parameters excellent resolution was obtained and variation in migration time was minimised. However, further investigations will be required to determine the robustness of the procedure, especially any day-to-day variation in the migration time.

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