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Computer-assisted retention prediction system for inorganic cyclic polyphosphates and its application to optimization of gradients in anion-exchange chromatography

YOSHINOBU BABA*
Kobe Women's College of Pharmacy, Kitamachi, Motoyama, Higashinada-ku, Kobe 658 (Japan)
and
GENICHIRO KURA
Department of Chemistry, Fukuoka University of Education, Akama, Munakata, Fukuoka 811-41 (Japan)

ABSTRACT

A computer-assisted technique for the retention prediction of inorganic cyclic polyphosphates under gradient elution conditions in anion-exchange chromatography is presented. A computer-assisted retention prediction system, which is an alternative to high-performance liquid chromatographic computer simulation systems, finds an optimum gradient easily in the anion-exchange separations of cyclic polyphosphates. The effect of gradient on the resolution and band spacing is illustrated by computer simulation. An increase in the initial salt concentration of the eluent is the most effective factor for the complete resolution of cyclic polyphosphates. A convex gradient is also essential for optimum resolution.

INTRODUCTION

Inorganic cyclic polyphosphates are interesting materials which have been used as multivalent electrolytes [1,2] and as phosphorylating agents for a starch and a protein [3,4]. Of these compounds, cyclo-triphosphate (P_{3m}, trimetaphosphate) has been recognized as an important material for use as a prebiotic condensing agent [5] and as an initial phosphorylating species in chemical synthesis of DNA [6]. Several authors [7–12] have investigated various chemical properties of cyclo-triphosphate as well as other cyclic polyphosphates, such as cyclo-tetra- (P_{4m}), hexa- (P_{6m}) and octa- (P_{8m}) phosphates (cyclo-penta- and cyclo-heptaphosphates are rarely available even now). However, few industrial applications for inorganic cyclic polyphosphates exists because of their difficulty of preparation and isolation.

The demand for purified synthetic cyclic polyphosphates has required the development of new methods which can rapidly purify these molecules. High-performance liquid chromatography (HPLC) provides an excellent means of rapidly and efficiently purifying synthetic cyclic polyphosphate from crude reaction mixtures. The availability of numerous anion-exchange columns makes HPLC an attractive alternative to classical methods for the purification of synthetic cyclic polyphosphates.

In 1980, the coupled system of HPLC and flow injection analysis (FIA) for the

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automatic separation and detection of inorganic polyphosphates was reported [13]. The authors applied it to the separation of inorganic cyclic polyphosphates [11,14–16] instead of classical liquid chromatography with a fraction collector and manual determination of phosphates in each fraction [17]. The HPLC–FIA system has also been successfully applied to the complete separation of complex mixtures of both linear and cyclic polyphosphates [11,18,19]. Although the HPLC–FIA system is the most suitable method of preparing and isolating cyclic polyphosphates, it remains a time-consuming and troublesome task to optimize the separation conditions of a complex mixture of cyclic polyphosphates.

In the present study, a computer-assisted retention prediction system was developed for the rapid optimization of separation conditions for inorganic cyclic polyphosphates under gradient elution conditions in anion-exchange chromatography. The authors have developed a prediction system for inorganic linear polyphosphates in isocratic [18] and gradient [19–21] anion-exchange chromatography. The system has been applied to the prediction of retention times for oligonucleotides [22] and to the optimization of isocratic [18] and gradient [19,23] elution conditions.

THEORY

Prediction of retention times, t_g, in gradient elution

The first step in the procedure for retention prediction is to determine constants a and b characteristic of each solute [20]. These constants are calculated from the relationship between the capacity factor, k', and mobile phase salt concentration, C', in isocratic ion-exchange chromatography [18,20,22]:

$$k' = aC'^{-b} \tag{1}$$

Retention time, t_g, in gradient elution is given as eqn. 2 [20–25]:

$$t_g = (1/u)\{(1/B')[(xb + 1)B'at_0u + C_i^{(xb+1)}]^{1/(xb+1)} - C_i^{1/x}/B'\} + t_0 \tag{2}$$

where u is the flow-rate (ml/min) and C_i, B', and x are adjustable parameters for the gradient profile. Constants a and b are estimated from eqn. 1.

The gradient profile [20–25] can be expressed as a function of the eluent concentration and the time:

$$\begin{aligned} C &= (C_i^{1/x} + Bt)^x \\ B &= B'u = (C_f^{1/x} - C_i^{1/x})/t_f \end{aligned} \tag{3}$$

where C is the mobile phase salt concentration at time t, C_i is the initial mobile phase salt concentration at the beginning of the gradient elution and C_f is the final salt concentration at the end of gradient elution ($t = t_f$); $B' = B/u$. The parameter x characterizes the shape of the gradient profile [20–25] as shown in Fig. 1.

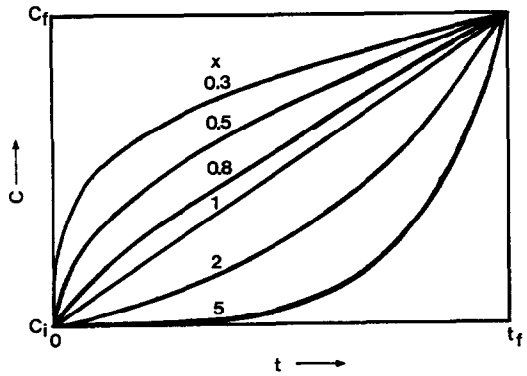

Fig. 1. Gradient profiles described by eqn. 3. (From ref. 23 with permission.)

Prediction of band widths, w_g, in gradient elution

The band width, w_g, in gradient-elution chromatography can be calculated from eqn. 4 [20–25]:

$$w_g = (4t_0/N^{1/2})\{1 + a[C_i^{1/x} + B(t_g - t_0 - t_D)]^{-xb}\} \quad (4)$$

where N is the plate number and t_D is the system dwell time between the outlet of the gradient-generating device and the column inlet. The mean value of N was measured to be 1900.

EXPERIMENTAL

Chemicals

Sodium salts of cyclo-triphosphate (P_{3m}), $Na_3P_3O_9 \cdot 6H_2O$, and cyclo-tetraphosphate (P_{4m}), $Na_4P_4O_{12} \cdot 4H_2O$, were prepared by the usual methods [26]. Sodium salts of cyclo-hexaphosphate (P_{6m}), $Na_6P_6O_{18} \cdot 6H_2O$, and cyclo-octaphosphate (P_{8m}), $Na_8P_8O_{24} \cdot 6H_2O$, were prepared according to the literature [8,9]. All other chemicals used were of reagent grade.

HPLC equipment

A Hitachi L-6200 HPLC system (Hitachi, Japan) coupled with an FIA system was used. Details of the HPLC–FIA system are described elsewhere [13,15,18].

The separations were performed on a column (500 × 4.0 mm I.D.) packed with a polystyrene-based quaternary ammonium anion exchanger (TSKgel SAX, 10 μm, Tosoh, Tokyo, Japan). The separation column was surrounded by a jacket containing circulating water at constant temperature within ± 0.1°C. The sample solution (0.5 ml) was injected into a separation column and chromatographed at a flow-rate of 1.0 ml/min. Concentrations of samples were $4 \cdot 10^{-5}$ M (P_{3m}), $3 \cdot 10^{-5}$ M (P_{4m}), $2 \cdot 10^{-5}$ M (P_{6m}), and $1.5 \cdot 10^{-5}$ M (P_{8m}). Eluent comprised appropriate concentrations of potassium chloride and 0.1% (w/v) Na_4EDTA buffered at pH 10.2.

Measurement of capacity factor

Prior to the elution of cyclic polyphosphates the column was washed with the eluent for 1 h. The column dead-time, t_0, was measured by injecting aqueous solution of $Co(NH_3)_6^{3+}$ and found to be 2.83 min. When an FIA system was used as a post-column reaction detector, extracolumn effects took place to some extent in HPLC [13,27]. The capacity factor (k'), therefore, was calculated from its retention time, allowing for the extracolumn effects.

Calculation

All predicted retention times and band widths were calculated with a PC-9801 personal computer (NEC, Tokyo, Japan). Computer simulations were carried out using software developed by the authors [20–23].

RESULTS AND DISCUSSION

Determination of constants, a and b, in isocratic elution

As described in the Theory section, constants a and b must be determined prior to the prediction of retention times. As shown in Fig. 2, the plot of log k' *vs.* log C' gave straight lines with correlation coefficients of 0.999 at a column temperature of 30°C. Constants a and b for cyclic polyphosphates were determined from the slope and intercept of the straight line in Fig. 2 and are compiled in Table I.

Fig. 2 shows that the plots for each solute are not parallel. Such irregular

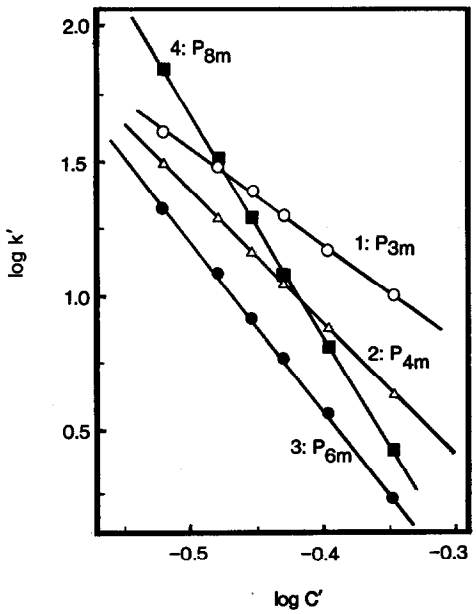

Fig. 2. Plot of log k' *vs.* log C' at pH 10.2 and a column temperature of 30°C. Eluent: potassium chloride solution with 0.1% Na_4 EDTA. Solutes are cyclo-tri- (P_{3m}), cyclo-tetra- (P_{4m}), cyclo-hexa- (P_{6m}), and cyclo-octa- (P_{8m}) phosphates.

TABLE I

VALUES OF CONSTANTS a AND b IN EQN. 1 AT A COLUMN TEMPERATURE OF 30°C

Solutes	b	a
P_{3m}	3.53	$5.91 \cdot 10^{-1}$
P_{4m}	4.88	$8.64 \cdot 10^{-2}$
P_{6m}	6.26	$1.16 \cdot 10^{-2}$
P_{8m}	8.11	$3.87 \cdot 10^{-3}$

tendencies have also been investigated by the authors through batch measurements of distribution ratio with varying salt concentration using ion-exchange resins [10].

The slope, b, corresponds to the ionic charge of cyclic polyphosphate in the exchanger phase [10]. The charge of each solute was expected to be 3 (P_{3m}), 4 (P_{4m}). 6 (P_{6m}), and 8 (P_{8m}). The b values in Table I were in rough agreement with the ionic charges of all solutes.

Prediction of retention times and band widths in gradient elution

The retention times and band widths for cyclic polyphosphates in gradient elution were predicted and compared with the observed values to test the performance of the present system. Tables II, III, and IV list the results with linear ($x = 1$), binary-convex ($x = 0.2$), and binary-convex ($x = 0.4$) gradients. The twelve observed retention times were predicted with an average error of $-2.8\% \pm 1$[relative standard deviation (R.S.D.)] and band widths with an average error of $-6.7\% \pm 7$(1 R.S.D.). Predictions reported in ion-exchange chromatography [20] were achieved in the range of errors from 1 to 10%. In comparison with the reported errors, accuracy of the retention prediction system presented here is good enough for the purpose of using computer simulations in optimizing gradients.

As an example, the simulated chromatogram displayed on the monitor screen was compared with a chromatogram observed under the gradient elution conditions in Table II, as shown in Fig. 3.

TABLE II

OBSERVED (Obs.) AND CALCULATED (Calc.) RETENTION TIMES AND BAND WIDTHS UNDER GRADIENT ELUTION CONDITIONS

$x = 1.0$; $C_i = 0.3$ M; $C_f = 0.4$ M; $t_f = 80$ min; $T = 30$°C. Error = 100 × (Calc. − Obs.)/Obs.

Solute	Retention time (min)			Band width (min)		
	Obs.	Calc.	Error (%)	Obs.	Calc.	Error (%)
P_{3m}	79.6	76.7	−3.6	6.46	6.80	5.3
P_{4m}	59.4	57.6	−3.0	5.19	5.09	− 1.9
P_{6m}	45.3	43.6	−3.8	4.51	3.79	−16
P_{8m}	71.4	68.7	−3.8	6.37	6.14	− 3.7

TABLE III

OBSERVED (Obs.) AND CALCULATED (Calc.) RETENTION TIMES AND BAND WIDTHS UNDER GRADIENT ELUTION CONDITIONS

$x = 0.2$; $C_i = 0.3$ M; $C_f = 0.4$ M; $t_f = 80$ min; $T = 30$°C. Error = 100 × (Calc. − Obs.)/Obs.

Solute	Retention time (min)			Band width (min)		
	Obs.	Calc.	Error (%)	Obs.	Calc.	Error (%)
P_{3m}	73.5	71.7	−2.3	6.37	6.33	0.10
P_{4m}	53.0	52.2	−1.5	5.01	4.59	− 8.5
P_{6m}	39.6	40.0	−2.3	4.04	3.35	−17
P_{8m}	61.7	61.3	−1.1	6.29	5.43	−14

TABLE IV

OBSERVED (Obs.) AND CALCULATED (Calc.) RETENTION TIMES AND BAND WIDTHS UNDER GRADIENT ELUTION CONDITIONS

$x = 0.4$; $C_i = 0.3$ M; $C_f = 0.5$ M; $t_f = 160$ min; $T = 30$°C. Error = 100 × (Calc. − Obs.)/Obs.

Solute	Retention time (min)			Band width (min)		
	Obs.	Calc.	Error (%)	Obs.	Calc.	Error (%)
P_{3m}	72.7	70.4	−3.2	5.95	6.26	5.2
P_{4m}	53.6	52.1	−2.8	4.55	4.59	0.88
P_{6m}	40.9	39.2	−4.2	3.99	3.40	−15
P_{8m}	61.2	59.7	−2.5	5.53	5.32	− 3.7

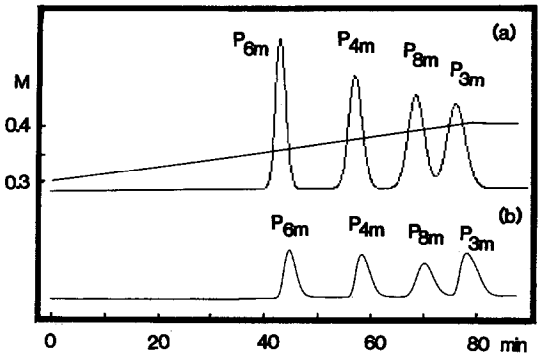

Fig. 3. Predicted (a) and observed (b) chromatograms for a mixture of four cyclic polyphosphates. (a) Gradient elution conditions as in Table II. (b) Column: TSKgel SAX (anion exchanger, 500 × 4.0 mm I.D.). Flow-rate: 1.0 ml/min. Eluents: A, 0.3 M potassium chloride +0.1% Na_4EDTA (pH 10.2); B, 0.4 M potassium chloride +0.1% Na_4EDTA (pH 10.2). Gradient elution conditions as in Table II. Column temperature: 30°C.

Optimization of gradients using computer simulation

In order to find an optimum gradient for cyclic polyphosphates, we examined effect of changes in the gradient parameters in eqn. 3 on band spacing and resolution. Gradient time, t_f, initial salt concentration, C_i, and gradient shape, x, were all taken into consideration. Gradient steepness was also considered.

Fig. 4 shows computer simulations of the separation for cyclic polyphosphates with a 0–0.5 M potassium chloride linear salt gradient and different gradient times, t_f, ranging from 50 to 400 min. An increase in gradient time corresponds to a decrease in gradient steepness, that is 0.01 M/min at 50 min (Fig. 4a), 0.005 M/min at 100 min (Fig. 4b), 0.0033 M/min at 150 min (Fig. 4c), 0.0025 M/min at 200 min (Fig. 4d), and 0.00125 M/min at 400 min (Fig. 4e). These simulations illustrated that all gradients failed to separate cyclic polyphosphates.

To improve resolution, we next examined the effect of changing initial salt concentration of eluent, C_i, from 0 to 0.4 M, while holding the gradient steepness constant. We have carried out computer simulations by varying C_i at each gradient steepness shown in Fig. 4a–e. As a result, simulations at 0.00125 M/min, which are shown in Fig. 5, gave better resolution of cyclic polyphosphates than those at any other gradient steepness.

Fig. 5 clearly demonstrates that an increase in C_i drastically improved resolution and band spacing of cyclic polyphosphates. A gradient with C_i of 0.3 M (Fig. 5c) or 0.4

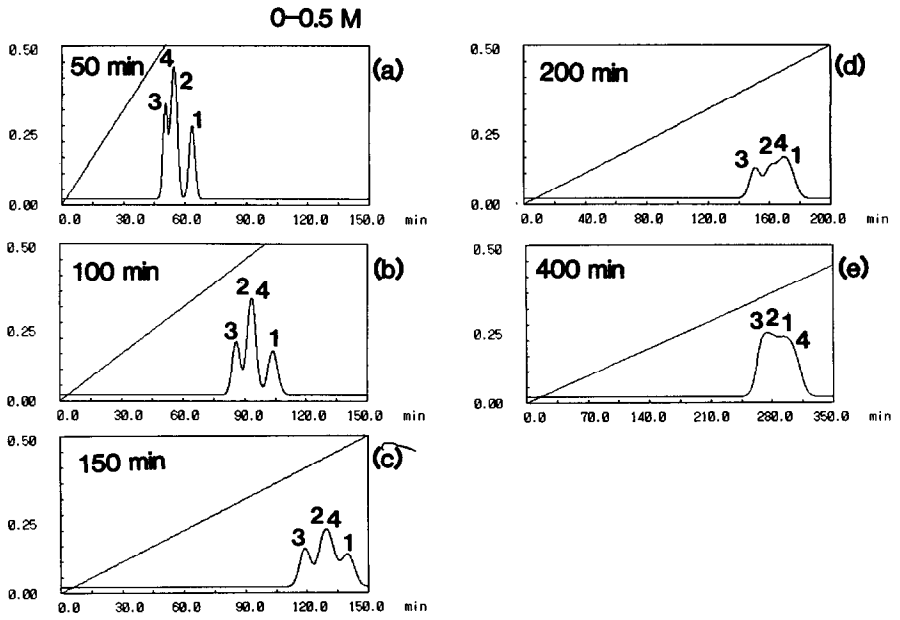

Fig. 4. Effect of gradient time, t_f, on the anion-exchange separation of cyclic polyphosphates demonstrated by computer simulations. Conditions: anion-exchange column (500 × 4.0 mm I.D.). Flow-rate: 1.0 ml/min. Gradient profile: C_i = 0.0 M potassium chloride, C_f = 0.5 M potassium chloride. Gradient shape: linear (x = 1). Gradient time: t_f = 50 min (a), 100 min (b), 150 min (c), 200 min (d), and 400 min (e). The ordinate represents molar concentration of potassium chloride solution. Peak numbers represent each cyclic polyphosphate as follows: 1, P_{3m}; 2, P_{4m}; 3, P_{6m}; and 4, P_{8m}.

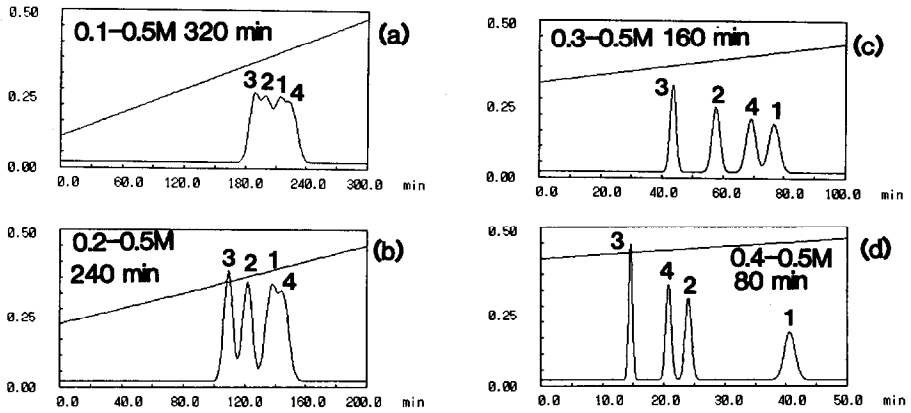

Fig. 5. Effect of initial salt concentration, C_i, on the anion-exchange separation of cyclic polyphosphates eluted with the same gradient slope as Fig. 4e demonstrated by computer simulations. Gradient profile: $C_i = 0.1\ M$ (a), $0.2\ M$ (b), $0.3\ M$ (c), and $0.4\ M$ (d), $C_f = 0.5\ M$ potassium chloride. Gradient shape: linear ($x = 1$). Gradient time: $t_f = 320$ min (a), 240 min (b), 160 min (c), and 80 min (d). Peak numbers as Fig. 4.

M (Fig. 5d) provides adequate resolution. Additionally, an increase in C_i resulted in a decrease in analysis time. For example, all bands are eluted within only 45 min in Fig. 5d, while the last eluted band is eluted at 230 min in Fig. 5a.

A change in C_i leads to a change in band spacing, as shown in Fig. 5, where the gradient steepness is held constant. Band 4 of cyclo-octaphosphate is seen to change its position in the chromatogram as C_i changes. Similarly, an increase in gradient time seems to change the position of band 4, as seen in Fig. 4. These variations in band spacing can be understood from the isocratic plots of Fig. 2. The isocratic plots predict

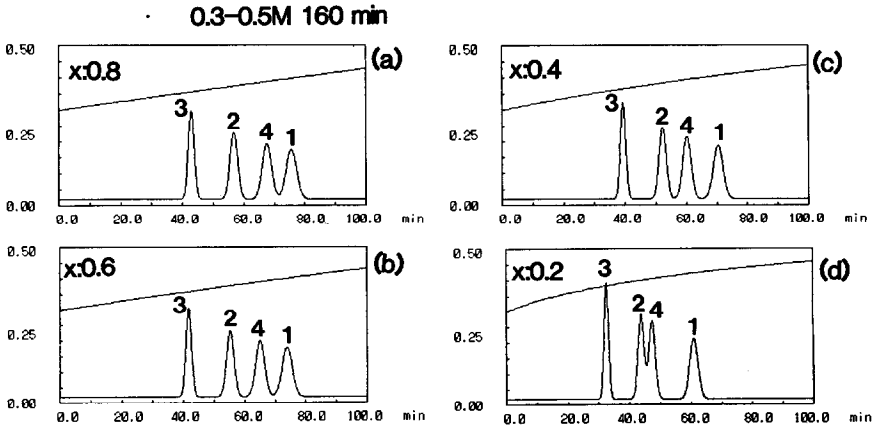

Fig. 6. Effect of gradient shape, x, on the anion-exchange separation of cyclic polyphosphates demonstrated by computer simulations. Gradient profile: $C_i = 0.3\ M$ potassium chloride, $C_f = 0.5\ M$ potassium chloride. Gradient shape: $x = 0.8$ (a), 0.6 (b), 0.4 (c), and 0.2 (d). Gradient time: $t_f = 160$ min. Peak numbers as Fig. 4.

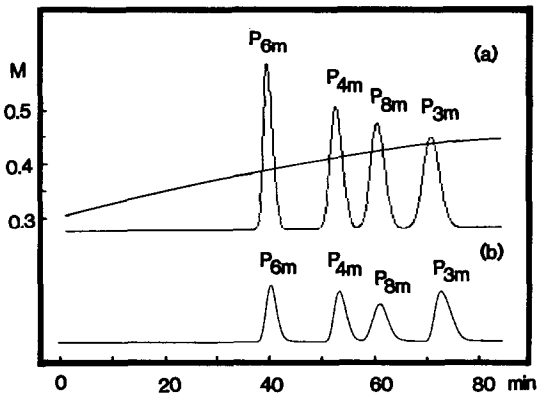

Fig. 7. Predicted (a) and observed (b) chromatograms for a mixture of four cyclic polyphosphates under an optimized gradient. (a) Gradient elution conditions as in Table IV. (b) Eluents: A, 0.3 *M* potassium chloride +0.1% Na_4EDTA (pH 10.2); B, 0.5 *M* potassium chloride +0.1% Na_4EDTA (pH 10.2). Gradient elution conditions as in Table IV.

that the relative retention of band 4 (cyclo-octaphosphate) should decrease with an increase in the salt concentration of the eluent, while the retention order of other solutes should be unchanged by changing salt concentration. Thus, an increase in C_i leads to a decrease in the relative retention of band 4, as shown in Fig. 5. On the other hand, an increase in gradient time leads to an increase in the relative retention of band 4, as shown in Fig. 4.

We further examined a fine-tuning of gradients by varying gradient shapes. Fig. 6 shows computer simulations by changing gradient shape, x, while the other gradient parameters are maintained similar to those in Fig. 5c. Fig. 6 demonstrates that a convex gradient with $x = 0.4$ (Fig. 6c) gives the best result in maximizing resolution and minimizing analysis time.

Consequently, the gradient listed in Table IV is the best choice for the rapid and complete separation of four cyclic polyphosphates. Fig. 7b demonstrates the anion-exchange separation of cyclic polyphosphates under the optimum gradient as listed in Table IV determined by computer simulations. A simulated chromatogram is also illustrated in Fig. 7a. All cyclic polyphosphates are completely resolved within 80 min. An HPLC technique using the optimum gradient presented here could become a new method of prepairing cyclic polyphosphates, such as cyclodecaphosphate, which have not yet been synthesized or prepared.

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