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OPTIMIZATION OF GRADIENTS IN ANION-EXCHANGE SEPARATIONS OF OLIGONUCLEOTIDES USING COMPUTER-ASSISTED RETENTION PREDICTION AND A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SIMULATION SYSTEM

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SUMMARY

A computer-assisted retention prediction system which is an alternative to computer simulation is demonstrated to be an efficient tool for finding optimum gradients in anion-exchange separations of oligonucleotides. The effect of the gradient profile on the reslution is illustrated by computer simulation in the visualization of simulated chromatograms by changing the variables for the gradient profile. A convex gradient was found to be more effective for the separation of high-molecular-weight oligonucleotidas than a linear gradient which has been widely used. An increase in the gradient time also improved the resolution. Recommendations are presented for an approach to maximize the resolution and to minimize the analysis time in the anion-exchange high-performance liquid chromatography of olignonucleotides.

INTRODUCTION

The gradient elution technique is useful in the high-performance liquid chromatographic (HPLC) analysis of oligonucleotides[1–4]. Optimized gradients, however, are usually selected by a trial-and-error method, which is tedious and troublesome. During the past few years, this situation has rapidly changed with the development of computer-assisted retention prediction and HPLC computer simulation systems[5–16], which are applicable to finding optimum gradients.

Snyder and co-workers[9–16] have extensively studied the optimization of HPLC separations by computer simulation and demonstrated that HPLC computer simulation is an efficient tool for optimizing gradients of several gradient shapes, even in multi-step gradients[13].

We have reported a computer-assisted retention prediction system[5–8], which is alternative to computer simulation, for oligonucleotides and inorganic polyphosphates in gradient anion-exchange chromatography. The system predicted retention times for oligonucleotides within 8% error for a variety of gradient shapes, and visually presented simulated chromatograms[8].

In this study, we have tried to find optimized gradients in anion-exchange separations of oligonucleotides using a computer-assisted retention prediction (computer simulation) system[8].

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THEORY

The gradient profile used was expressed as the equation[5–8,17–19]

$$C = (C_i^{1/x} + Bt)^x \tag{1}$$

$$B = B'u = (C_f^{1/x} - C_i^{1/x})/t_f$$

where C is the eluent concentration at time t, C_i is the initial eluent concentration at the beginning of the gradient elution ($t = 0$ min), C_f is the final concentration at the end of gradient elution ($t = t_f$) and u is the flow-rate (ml/min). This function was chosen because of the possibility of describing a variety of gradients of any linear, convex, and concave shapes. The parameter B describes the gradient steepness. The parameter x characterizes the shape of the gradient profile: linear at $x = 1$; convex at $x < 1$; concave at $x > 1$. Typical gradient profiles are shown in Fig. 1.

Retention times, t_g, in gradient elution expressed by eqn. 1, can be predicted from the equation[5–8,17–19]

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

Constants a and b are obtainable from the relationship $k' = aC'^{-b}$ between the capacity factor, k', and the elution concentration, C', in isocratic elution. Eqn. 2 can be applied only to the ion-exchange mode and not to the reversed-phase mode.

The band width, w, in gradient-elution chromatography can be calculated from the equation[17–19]

$$w = (4t_0/N^{1/2})\{1 + a[C_i^{1/x} + B'(t_g - t_0 - t_D)]^{-xb}\} \tag{3}$$

where N is the plate number in isocratic elution chromatography and t_D is the system dwell time between the outlet of the gradient-generating device and the column inlet. The value of N was measured to be 2000.

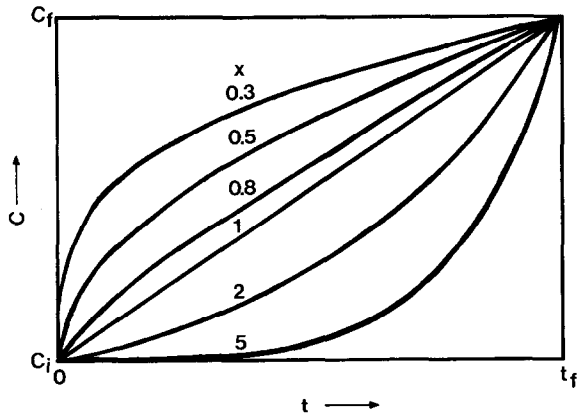

Fig. 1. Gradient profiles described by eqn. 1.

EXPERIMENTAL

Equipment and software

The HPLC system consisted of an L-6200 intelligent pump (Hitachi, Tokyo, Japan) equipped with a microcomputer-based gradient controller, a Rheodyne Model 7125 sample injector fitted with a 100-μl loop and a UVIDEC-100-IV spectrophotometric detector (Jasco, Tokyo, Japan) for detection at 260 nm. The separations were performed on a weakly basic anion-exchange column (Shim-pack WAX-1, 50 mm × 4 mm I.D., particle diameter 3 μm, pore size of 100 Å) (Shimadzu, Kyoto, Japan)[8,20]. The separation column was surrounded by a constant-temperature water-jacket maintained within ± 0.1°C. The sample solution (100 μl) was injected into the separation column and chromatographed at a flow-rate of 1.0 ml/min. Computer simulations were carried out using software developed by the authors[5,7,8]. The software runs on an NEC PC-9801 personal computer (Nippon Electric, Tokyo, Japan).

Chemicals

Unless stated otherwise, guaranteed reagents from Wako (Osaka, Japan) were used without further purification. Acetonitrile was of HPLC grade. Water was purified with a Milli-Q system (Millipore, Bedford, MA, U.S.A.).

All gradients were performed with a binary gradient elution technique. Buffer A was 0.01 *M* phosphate buffer (pH 6.8) containing 20% acetonitrile and buffer B 0.3 *M* phosphate buffer (pH 6.8) containing 20% acetonitrile.

Polyadenylate sodium salt [poly(A)] was obtained from Yamasa Shoyu (Chiba, Japan). Oligoadenylate fragments, A_n, from poly(A) were prepared by chemical or enzymatic hydrolysis of poly(A) with an alkaline solution[21], nuclease P1 (ref. 22) or nuclease SW (ref. 23). Nuclease P1 was purchased from Yamasa Shoyu and nuclease SW was a gift from Prof. Jun-Ichiro Mukai (Kyushu University).

RESULTS AND DICUSSION

To find the optimum gradients by the computer-assisted retention prediction system, all parameters in eqn. 1 were taken into consideration. First several kinds of x were selected, second the parameter B, which describes the steepness of the gradient, was changed, and third the number of columns was increased to improve the resolution.

Optimization of gradient shape

Ghrist and Snyder[15,16] proposed that samples to be separated in gradient elution could be classified into three groups (cases I–III). According to their classification, oligonucleotides correspond to case II, which usually contains homologous or oligomeric samples.

They concluded for such samples[16] that the resolution was improved by increasing in the gradient time, t_f, and volume fraction of organic solvent in the mobile phase at the beginning of the gradient. A convex gradient gave a modest improvement in resolution compared with a linear gradient. They suggested that a two-segment gradient plus adjustment of the starting mobile phase composition gives equivalent results to convex gradients.

Convex gradient elution generally gives better resolution than linear gradient elution for the analysis of homologous samples in ion-exchange HPLC. For example, such a situation was illustrated experimentally by the gradient elution HPLC of oligonucleotides[8,20] and inorganic polyphosphates[6,24], which also correspond to case II. Hence, we first considered the effect of gradient shape on the resolution of oligonucleotides in the ion-exchange mode.

Fig. 2 shows computer simulations obtained by varying the gradient shapes with x ranging from 0.3 to 5, other gradient conditions being kept constant. As clearly illustrated in Fig. 2, convex gradient elution gave an improved resolution, especially for the later eluted bands, compared with linear and concave gradients. For example, fifteen kinds of oligonucleotides were resolved almost completely using a convex gradient with $x = 0.3$ (Fig. 2a), whereas only ten kinds of oligonucleotides were resolved by linear gradient elution (Fig. 2d); concave gradient elution (Fig. 2e and f) gave the poorest resolution. The present results are similar to those obtained by Ghrist and Snyder[16].

As a result, we concluded that convex gradient HPLC, especially for $x = 0.3$, is more favourable for the separation of complex mixture of oligonucleotides than a linear, a concave or a convex gradient with $x = 0.8$.

Ghrist and Snyder[16] pointed out that the most effective strategy is to increase the

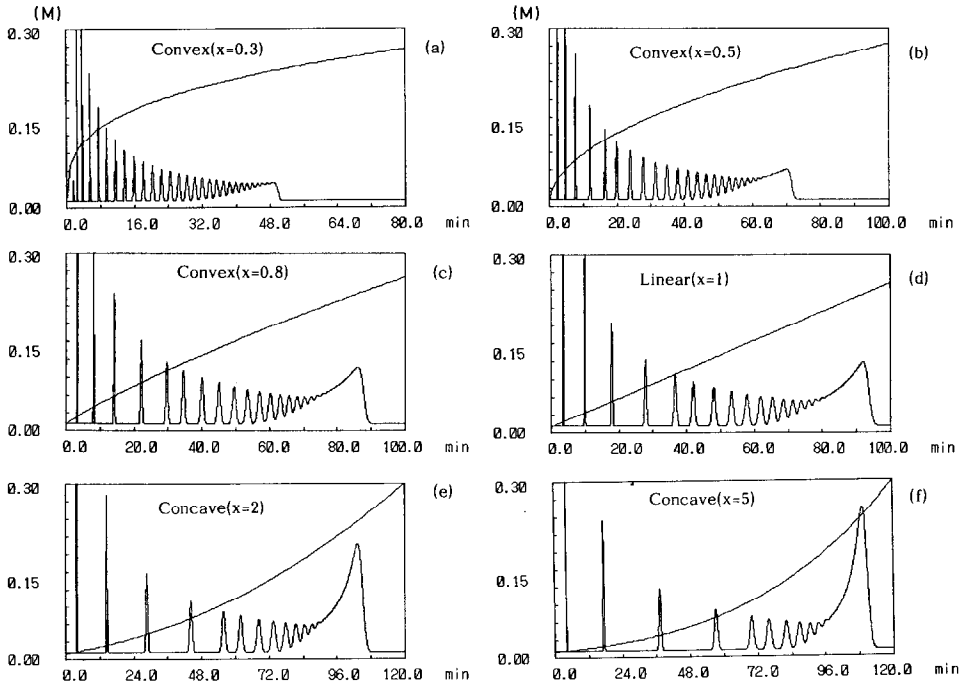

Fig. 2. Effect of gradient shape (x value) on the anion-exchange separation of oligonucleotides; computer simulation using the computer program developed by the authors[5,7,8]. Conditions: anion-exchange column (50 × 4.0 mm I.D.); flow-rate, 1.0 ml/min. Gradient profile: $C_i = 0.01$ M phosphate buffer, $C_f = 0.3$ M phosphate buffer, gradient time $t_f = 120$ min. The ordinate represents molar concentration of phosphate buffer.

volume fraction of organic solvent in the mobile phase at the beginning of the gradient. The initial concentration, C_i, of the gradient, however, did not affect the resolution in the present instance, as was revealed by computer simulations with C_i varying from 0.005 to 0.04 *M*. The discrepancy in these results is attributable to the difference in the nature of the samples used in the two studies. The samples used by Ghrist and Snyder were not eluted from the column at the initial portion of the gradient when separations were carried out using a gradient of the volume fraction of organic solvent (5–100%). On the other hand, oligonucleotides were eluted even in the initial portion of a concentration gradient of phosphate buffer, as shown in Fig. 2.

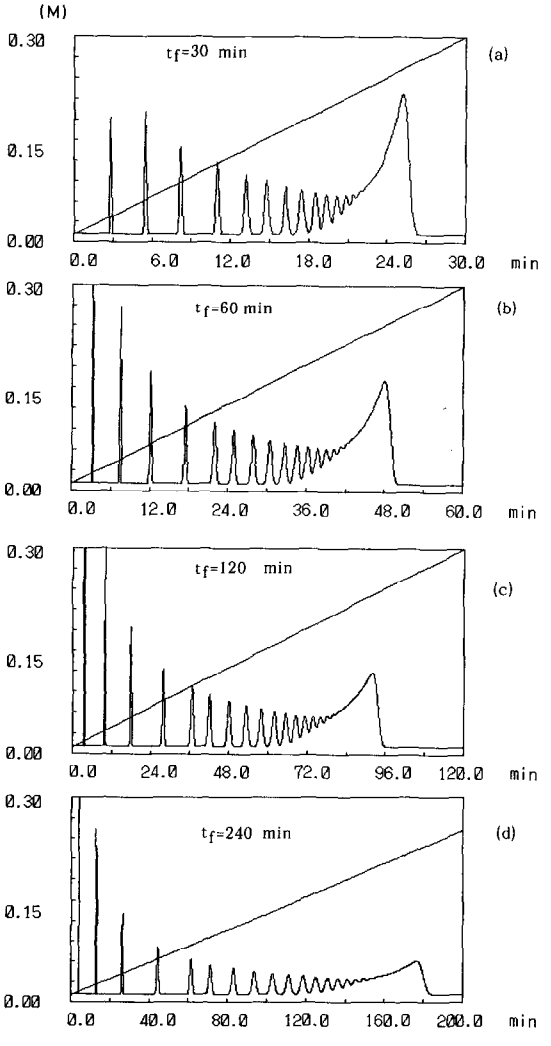

Fig. 3. Effect of gradient time (t_f) on the anion-exchange separation of oligonucleotides using linear gradien elution. Other conditions as in Fig. 2.

Optimization of gradient steepness

The effect of gradient steepness on the resolution was studied, the parameter B (eqn. 1) being varied by changing t_f and C_f.

An increase in t_f resulted in a continuous improvement in resolution, as shown in Figs. 3 and 4. For example, the number of samples to be resolved completely (baseline separation) increased from seven to ten with an increase in t_f from 30 to 240 min in linear gradient elution. Using a convex gradient with $x = 0.3$ a similar improvement in resolution was observed with increase in t_f. The analysis time, however, increased with increasing t_f. These results are the same as those obtained by Ghrist and Snyder[16]. We concluded that the separation of oligonucleotides should be carried out in the t_f range 120–240 min in order to maximize the resolution and minimize the analysis time.

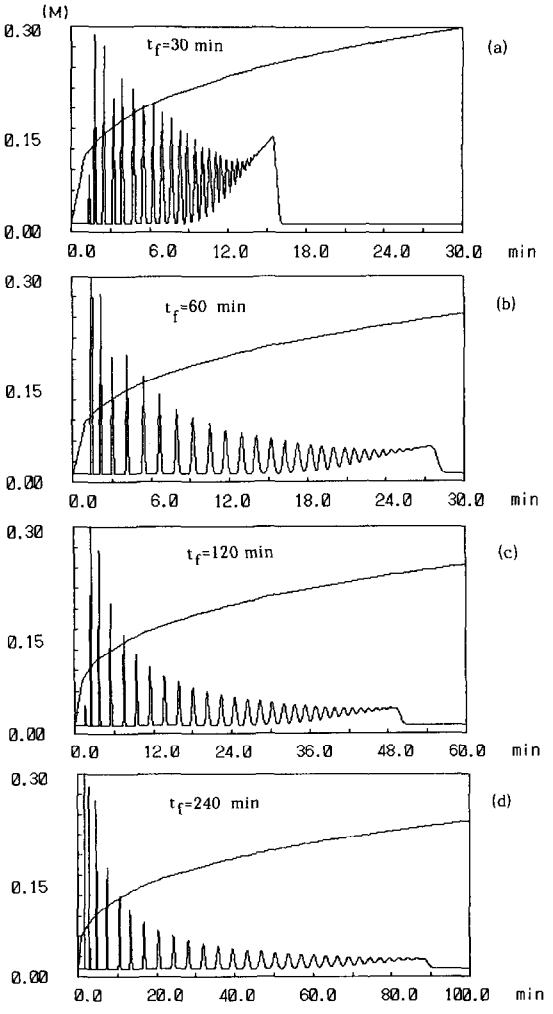

Fig. 4. Effect of gradient time (t_f) on the anion-exchange separation of oligonucleotides using convex ($x = 0.3$) gradient elution. Other conditions as in Fig. 2.

Simulated chromatograms were obtained by changing C_f, C_i and t_f being maintained constant. A change in C_f slightly improved the resolution under both linear and convex gradient conditions. About eight bands were resolved at $C_f = 0.5\ M$ compared with ten bands at $C_f = 0.3\ M$ with linear gradient elution. No improvements were observed even on changing C_f when the gradient steepness was maintained constant.

Effect of the number of columns on the resolution

As discussed above, the optimum gradient profile is a convex gradient with $x = 0.3$ using two buffer solution of 0.01 and 0.3 M phosphate and with t_f in the range 120–240 min. Nevertheless, the resolution of high-molecular-weight oligonucleotides is still poor under the optimum conditions, as shown in Fig. 5a. If one wants to obtain a better resolution using a Shim-pack column, several columns must be connected in series.

The resolution was improved by connecting two or ten columns, as demonstrated in Fig. 5. Fig. 5c illustrated that 30 kinds of oligonucleotides were resolved almost completely by using ten columns with convex gradient elution.

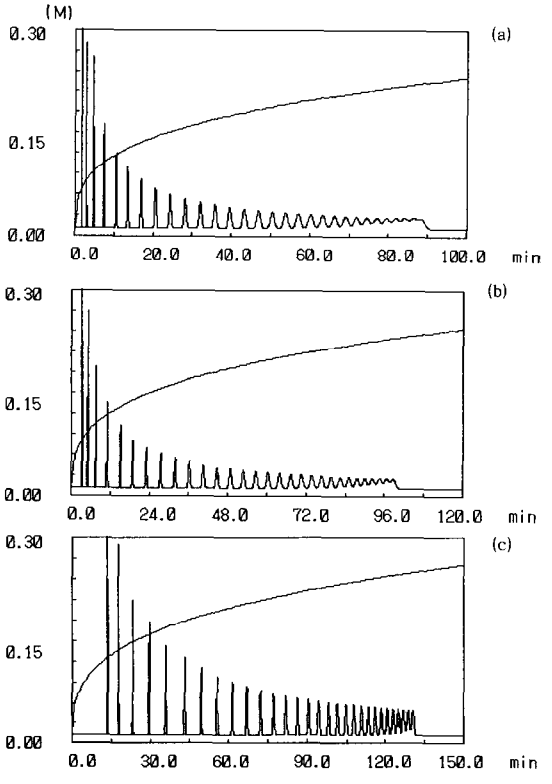

Fig. 5. Effect of the number of columns on the anion-exchange separation of oligonucleotides using convex gradient elution. Number of columns: (a)1; (b)2; (c)10. Gradient profile: $x = 0.3$, $t_f = 240$ min, $C_i = 0.01\ M$ and $C_f = 0.3\ M$.

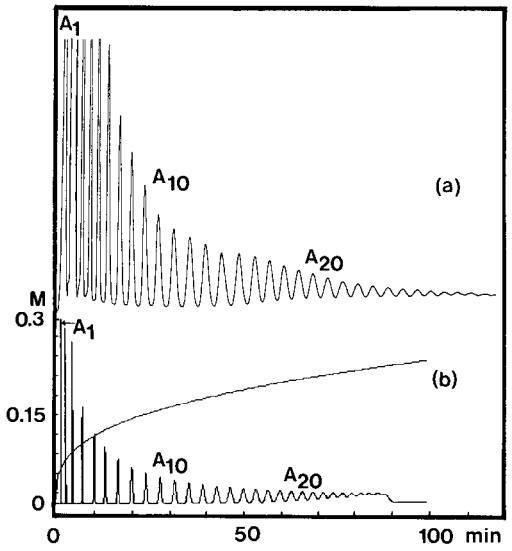

Fig. 6. (a) Observed and (b) simulated chromatograms for a polyadenylate partial hydrolysate (A_n) under the optimized gradient. Gradient profile: $x = 0.3$, $t_f = 240$ min, $C_i = 0.01$ *M* and $C_f = 0.3$ *M*. Column, Shim-pack WAX-1 (weak anion exchanger, 50 mm × 4.0 mm I.D.); flow-rate, 1.0 ml/min. Buffer: A, 0.01 *M* phosphate (pH 6.8) containing 20% acetonitrile; B, 0.3 *M* phosphate (pH 6.8) containing 20% acetonitrile. Column temperature, 40°C.

Recommendations for anion-exchange separations of oligonucleotides

We can propose guidelines for the anion-exchange separation of oligonucleotides from the simulations in Figs. 2–5, as follows. Convex gradient elution gives a favourable resolution, especially for $x = 0.3$. An increase in t_f improves the resolution, especially for later eluted bands. With series connection of several columns more than 30 oligonucleotides can be separated.

Fig. 6a demonstrates the anion-exchange separation of oligonucleotides under the optimum elution conditions determined by computer simulation. A simulated chromatogram is also illustrated in Fig. 6b. About twenty oligonucleotides were almost resolved within 70 min.

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