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Short communication

Combined pH gradient and anion-exchange high-performance liquid chromatographic separation of oligodeoxyribonucleotides

Tao Lu[1], Horace B. Gray, Jr.*

Department of Biochemical and Biophysical Sciences, University of Houston, Houston, TX 77204-5934, USA

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Abstract

A novel method of elution using a pH gradient to separate small thymidine- and guanosine-containing oligonucleotides on a Pharmacia Mono Q HR anion-exchange column is described. The method is based on the alkaline titration of ring protons of the thymine and guanine base moieties and results in excellent separations of di-, tri- and tetranucleotides that either are not resolved in salt gradients near neutral pH or require long elution times when salt gradient elution is used with strongly alkaline eluents.

1. Introduction

High-performance liquid chromatography (HPLC) has been applied to oligonucleotides, DNA restriction fragments and plasmid and phage DNAs using several techniques, including reversed-phase chromatography [1], ion-pair/reversed-phase methods [1–5], separations on ion-exchange resins eluted with organic solvent or salt gradients [1,4,6–10] and size-exclusion chromatography [11,12]. Both the ion-exchange and ion-pair methods can separate DNA polymers over a very wide range of sizes ranging from small single-stranded oligonucleotides [1–4,8,10] to large double-stranded restriction fragments and the intact form [5,6,8,9]. However, the separation of oligonucleotides by either of these techniques depends not only on the chain length but on base composition and sequence in a manner that is difficult to predict [1].

The Pharmacia–LKB Mono Q HR 5/5 anion-exchange column has been used to separate nucleotides and oligonucleotides using neutral [9], mildly alkaline (pH 9.7) [4] and strongly alkaline (10 m*M* NaOH) ([10], originally suggested by column supplier) salt gradients. Elution of this resin at pH 9.7 resulted in the co-elution of oligomers of a given chain length (isopliths) differing minimally in sequence while these species produced more than one peak when eluted at neutral pH, indicating that separation is based more on the chain length alone at mildly alkaline pH. Strongly alkaline salt gradients used with anion-exchange resins, however, are expected to result in the base composition-dependent separation of oligomers of a given chain length (isopliths) owing primarily to the titration at such pH values of the ring protons of the dT and dG residues, which

* Corresponding author.

[1] Present address: Department of Molecular Genetics, The University of Texas System Cancer Center, M.D. Anderson Hospital and Tumor Institute, Houston, TX 77030, USA.

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increases the negative charge on the oligomer. This presumably played a major role in the separation of complementary DNA strands in work with an earlier resin using alkaline elution [7].

Elution of a Mono Q column with an alkaline salt gradient (pH 12) was attempted to separate isopliths of small DNA oligonucleotides (up to the tetramer) containing dT residues which failed to resolve on elution near neutral pH. However, the time required to elute species as small as a tetramer containing titratable residues was prohibitively long. This paper reports the use of a pH gradient for elution, which results in reproducible baseline-to-baseline separations, in a predictable order, of dT- and dG-containing oligomers from isopliths not containing these residues and elution of all species in a reasonable time.

2. Experimental

2.1. Chemicals

Analytical-reagent grade inorganic chemicals were used. Mono- and dinucleotides were obtained from Sigma (St. Louis, MO, USA). The tri- and tetranucleotides were synthesized on a Biosearch (San Rafael, CA, USA) Model 8600 DNA synthesizer and were further purified by HPLC on a Mono Q HR 5/5 (Pharmacia Biotech, Piscataway, NJ, USA) 5 × 0.5 cm I.D. column (quaternary amine resin, particle size 10 μm) using an alkaline salt gradient elution as described [13] after the blocking groups had been removed. All species except mononucleotides lacked a 5′-phosphate group.

2.2. Chromatography

A Model 421 controller (Beckman Instruments, Palo Alto, CA, USA) with Model 110A pumps in conjunction with a Hitachi UV–visible monitor set at 260 nm and a Model 3390 integrator (Hewlett-Packard, Avondale, PA, USA) were used. The flow-rate through the Mono Q column (above) was 0.5 ml/min in all instances and all gradients were nominally linear. All experiments were carried out at room temperature.

Stock solutions of mono- and oligonucleotides were prepared by dissolving solid compounds in or diluting chromatographed compounds with 20 m*M* Tris–HCl–1 m*M* EDTA (pH 8). These were appropriately diluted, by a factor of at least ten, with the starting buffer for each elution prior to injection of 20-μl samples.

For elutions near neutral pH, the solvents were 20 m*M* Tris–HCl (pH 8.0)–0.1 *M* NaCl (buffer A) and 20 m*M* Tris–HCl (pH 8.0)–1.0 *M* NaCl (buffer B); for alkaline elution, 10 m*M* NaOH–0.1 *M* NaCl (solvent A) and 10 m*M* NaOH–1.0 *M* NaCl (solvent B); for pH gradient elution, 0.1 *M* NaCl–20 m*M* sodium phosphate (pH 11.0) (adjusted by titration with NaOH before dilution to the final volume) (buffer C) and 0.1 *M* NaCl–20 m*M* sodium phosphate (pH 8.0) (buffer D). Elution in a pH gradient was also carried out in 0.1 *M* NaCl containing 20 m*M* Tris base or Tris–HCl with the alkaline and neutral solutions at pH 12 (adjusted as above) (solvent C) and 8 (solvent D), respectively. For the near-neutral pH chromatography, the elution profiles were 100% buffer A for 5 min then buffer B to 8% in 15 min. Alkaline pH elution was carried out with 100% solvent A for 10 min followed by an increase to 50% solvent B in an additional 50 min. For the pH gradient elutions in phosphate buffer, pumping of buffer C for 2 min was followed by an increase to 100% buffer D in 5 min and elution with this solvent for 15.5 min, then a return to 100% buffer C over 3 min. Pumping of this solvent for ca. 20 min restored the pH to the initial value (ca. 10.8) and prepared the column for the next injection. Elution profiles for Tris-buffered pH gradients went from 100% solvent C to 30% solvent D in 20 min and then to 100% solvent D in 8 min. All buffers were filtered and degassed before use. The identities of peaks in all instances were confirmed by chromatographing the individual compounds.

The pH profile was determined for the phosphate-buffered solvent pair using a small combination electrode on 0.5-ml fractions from a

gradient programmed identically with those used in the elutions of the mixture and the individual compounds. Times presented are those corresponding to the collection of the mid-point of each fraction.

3. Results and discussion

Elution in a salt gradient near neutral pH shows increasing elution times with increasing oligomer length, as expected, but would not adequately resolve isopliths (see times for monomers, dimers and trimers in Table 1) as baseline-to-baseline separation requires at least 2 min of peak separation at the peak widths obtained here (those in Fig. 1 are representative for all four elution methods). Excellent resolution of isopliths based on the mole fraction of dT residues was observed in the alkaline salt gradient (Table 1) but d(TTA) required over 36 min to elute and d(AATT) eluted in a broad peak beginning at over 45 min. With pH gradient elutions, the order of elution is unchanged from that for the alkaline salt gradient (Table 1) but all species are eluted within 31 min. As the third ionization of orthophosphate lends phosphate solutions a very weak buffering capacity at pH 11, the studies of pH gradient elution, including determination of the pH of effluents, were initially done using the phosphate-buffered solvents. It was subsequently found that a Tris-buffered solvent pair, the alkaline member of which has no significant buffering capacity in that pH range, also gave reproducible separations (Table 1). Indeed, while there is some loss of resolution with the phosphate pH gradient compared with the alkaline gradient [the pairs pdA–$d(A)_2$ and pdT–d(ATT) are poorly resolved], the Tris pH gradient provided excellent resolution of all species in the mixture. The reproducibility does not

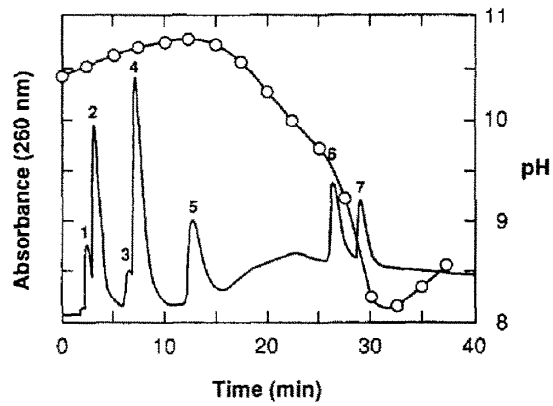

Fig. 1. Separation of a mixture of dA- and dT-containing mono- and oligonucleotides by pH gradient elution in phosphate buffer as described under Experimental. Peaks: 1 = $d(A)_2$; 2 = pdA; 3 = d(AAT); 4 = pdT; 5 = $d(T)_2$; 6 = d(TTA); 7 = d(AATT).

Table 1
Retention times and nominal electric charges of mono- and oligonucleotides under different chromatographic conditions

Compound	Charge		Uncorrected retention times (min)			
	pH 8	pH 12	pH 8[a]	pH 12[b]	pH gradient[c]	pH gradient[d]
$d(A)_2$	−1	−1	2.85	2.5	2.54 ± 0.00	2.91 ± 0.03
pdA	−2	−2	4.72	4.5	3.40 ± 0.02	5.20 ± 0.05
d(AAT)	−2	−3	3.74	13.3	6.53[e]	17.0 ± 0.2
pdT	−2	−3	3.97	15.4	7.38 ± 0.04	20.6 ± 0.2
$d(T)_2$	−1	−3	2.73	27.2	12.75 ± 0.04	24.3 ± 0.6
d(TTA)	−2	−4	4.24	36.5	26.8 ± 0.1	30.9 ± 0.5
d(AATT)	−3	−5	8.39	>45	29.4 ± 0.2	37.2 ± 0.2

[a] Salt gradient elution near neutral pH. Times are for the individually chromatographed species.
[b] Salt gradient elution of a mixture at pH 12.
[c] pH gradient elution of mixtures using phosphate buffer. Errors are ± half the range for two experiments.
[d] pH gradient elution of mixtures using Tris buffer. Errors are standard deviations for five experiments.
[e] Peak resolved well enough to be assigned a time by the integrator in only one experiment.

appear to be as good as for the phosphate-buffered gradient.

Fig. 1 shows the elution profile of a mixture of the oligodeoxynucleotides in Table 1 with the pH values (phosphate buffers) superimposed. The uncorrected (observed) retention times are used and the pH values represent those of the column effluent. The effluent pH gradient lags well behind the solvent gradient (compare the programme in the Experimental section with the profile in Fig. 1). It is expected, as noted by the manufactuer, that anionic buffers will bind to the quaternary amine resin, so that part of the fully doubly charged phosphate groups at pH 11 must be displaced/titrated by the pH 8 phosphates, which contain a fraction of singly ionized groups. The volume of buffer required to cause an appreciable pH change (ca. 8 ml after 100% buffer D is reached) is reasonable in the light of the ionic capacity of the column given by the supplier. This feature spreads out the pH gradient, which would nominally go through most of its range by the time 25% B is reached (1.2 min of pumping), to cover ca. 13 min of elution (Fig. 1).

As the solvent near the top of the column, where the oligonucleotides are adsorbed, changes pH well before pH changes are seen in the effluent, some of the compounds represented in peaks 3–7 of Fig. 1 (corresponding to species with titratable residues) should begin to migrate before the effluent pH changes significantly if such a change is required to desorb them. However, it appears that large changes in pH are needed only to elute the last two species. Apparently the species of peaks 1–5 are not tightly bound to the resin at pH near 11 and migrate according to different equilibrium constants for the ratio of species in the mobile and stationary phases. The progressive elution of the last two dT-containing compounds clearly depends on the protonation of titrated thymine moieties to reduce the net charge on the molecule. The good reproducibility of the elution times (Table 1) implies that the solvent changes involved are well defined.

The order of elution for the alkaline salt gradient and both types of pH gradient elutions follow the net charges on the various species at alkaline pH if it is assumed that internucleotide phosphates and thymine residues carry a charge of −1 and that a 5′-phosphate has a charge of −2 (Table 1). The elution times of isopliths should therefore increase with increasing mole fraction of dT and/or dG residues. There is no obvious rationale for the order of elution of the three species with nominal −3 charge (Table 1) where $d(T)_2$ is well separated from the other two (unresolved) −3 compounds in phosphate-buffered gradients and all three are well resolved in pH gradients containing Tris. At neutral pH, where only the phosphates are charged, species of a given charge are poorly separated, if at all. This leads to poor separation of the isoplith pairs $d(A)_2$–$d(T)_2$ and d(AAT)–d(TTA), which are well resolved in the alkaline salt and both types of pH gradients.

The role of titratable residues was confirmed in a single set of experiments with dG-containing compounds using the Tris-containing pH gradient. d(TG) eluted at 26.2 min, close to the time for $d(T)_2$, while d(GC) appeared at 9.6 min, well behind $d(A)_2$ as expected. pdG appeared, surprisingly, near 30.3 min, roughly 10 min after the other titratable mononucleotide pdT. pdC eluted at 4.3 min and would not have been resolved from pdA.

Elution at alkaline pH depends very strongly on the titratable residue content. The resulting long elution time for a species of nominal charge −5 suggests that alkaline salt gradient chromatography of DNA oligomers with this resin may fail to elute peaks corresponding to oligomers containing several titratable residues in reasonable times and caution with respect to this effect should be exercised even with higher final salt concentrations.

The pH gradient elution method known as chromatofocusing [14,15] is widely used for protein purification but has not, to our knowledge, been used to separate nucleic acids. The methods are analogous in that elution is facilitated by a pH-mediated reduction in net charge opposite to that of the resin. However, the elution buffers in chromatofocusing contain components that buffer over the entire pH range and titration of

the column resin itself is involved, neither of which is true of the simple procedure described here.

The present method extends the methods available for separating small oligonucleotides because it can resolve isopliths which fail to separate at neutral pH and also separate initially titrated species of the same nominal charge, but does not suffer from the long retention times/broad bands for oligomers containing titratable bases that can occur in elutions using alkaline gradients.

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