CHROM. 5885

ANALYSIS OF DNA DERIVATIVES

II. HIGH-PRESSURE LIQUID ANION-EXCHANGE CHROMATOGRAPHY OF DEOXYRIBONUCLEOTIDES*

THOMAS F. GABRIEL AND JOSEPH MICHALEWSKY
Chemical Research Department, Hoffmann-La Roche Inc., Nutley, N.J. 07110 (U.S.A.)

(Received December 22nd, 1971)

SUMMARY

A procedure for assaying deoxyribomononucleotides released by diesterase digestion of oligomers is reported. The method is based on high-pressure anion-exchange chromatography on Zipax SAX at ambient temperature using a single buffer of low ultraviolet absorption. A simple procedure for determining peak overlap is included.

INTRODUCTION

In the course of an extensive synthetic program in the deoxyribonucleotide series[2-4], a sensitive analytical procedure for determining monomer composition on a micro scale was required. Previous procedures[5-7] involved paper chromatography (PC) for separating the mononucleotides released by phosphodiesterase degradation. Fairly large amounts of substrate are required for such methods, and PC is time-consuming. A faster and more sensitive method, based on ion-exchange chromatography was therefore sought. Several such systems have been reported; however, they involve gradient elution[8,9], concentrated buffers[10], hyperambient temperatures[11,12], or a combination of these[13-24].

We wish to report a procedure carried out at ambient temperature with a single buffer of low concentration.

EXPERIMENTAL

Materials

The resin used comes from a single batch of DuPont Zipax strong anion-exchange resin (SAX). Columns are prepared by dry-packing SAX into 1 mm × 1 m columns, 316 stainless steel, (SS), (Tube Sales Inc.) fitted with Swagelok or Gyrolok fittings. The bottom fitting contains a 2-μm porosity stainless steel fritted disk

* For part I see ref. 1.

(Applied Science Labs.). Dry-packing in layers with vertical tamping was found to give the best resolution. Connecting tubing is 0.4 mm I.D., 316 SS, with appropriate end fittings. A commercial HPLC (Nester Faust, Model 1200) and homemade units consisting of Milton Roy or Whitey pumps with Helicoid gauges have been used. The detector is either a Nester Faust or Laboratory Data Control UV monitor (254 nm) whose output passes into a Nester Faust Model 1504 integrator which drives a Photovolt Varicord 43 recorder. The Nester Faust unit has the advantage of constant flow pumps which give more reproducible retention times.

Buffers are prepared from a saturated stock solution (*ca.* 1.5 *M*) of KH_2PO_4, purified according to KENNEDY AND LEE[20], by dilution with quartz-distilled water to 0.008 *M*. The pH is adjusted to 4.40 ± 0.03 with phosphoric acid. Degassing is essential, and is carried out by stirring under vacuum at *ca.* 40 °C for 15 min.

A flow rate of 0.2–0.4 ml/min at pressures of 1000–2000 p.s.i. is standard, resulting in an elution time of 1 h. Samples are injected into the column with a Hamilton HP 305 syringe.

Procedure

Oligomers are digested with snake venom phosphodiesterase (Worthington) in 0.01 *M* Mg^{2+} and 0.1 *M* triethylammonium bicarbonate, pH *ca.* 9. After removal of buffer by evaporation, an aliquot is injected into the top of the column. A normal analysis requires about 10 nmoles of oligomer, of which 1/5 is injected for each run. Analyses are run in duplicate.

Peak areas, as printed by the integrator, are normalized by dividing the area by the ε_{254} pH 4.4, of the nucleotide being measured. The ε values were determined on carefully purified samples by a method to be reported elsewhere. The values used, expressed as 1 $mmole^{-1}$ cm^{-1}, are: d-pA, 14.26; d-pG, 13.86; d-pC, 4.88; and d-pT, 7.30. Nucleotides are identified by their retention times. Results are calculated as mole percent based on total normalized nucleotide area of the chromatograms. Some representative analyses of relatively pure materials are tabulated in Table I.

RESULTS AND DISCUSSION

Separation of the four common 5'-deoxyribonucleotides obtained from digestion with venom phosphodiesterase is shown in Fig. 1. They are well separated at

TABLE I

REPRESENTATIVE ANALYSES OF RELATIVELY PURE NUCLEOTIDES

Oligomer	*Mole %[a] found*				*Mole % theory*			
	d-pT	*d-pA*	*d-pC*	*d-pG*	*d-pT*	*d-pA*	*d-pC*	*d-pG*
A_3, T_3	50.9	49.2	—	—	50.0	50.0	—	—
A_4, G_2, C_2	—	49.8	24.8	25.3	—	50.0	25.0	25.0
A_3, T_4, C_2	45.2	31.5	23.3	—	44.4	33.3	22.2	—
A, T_5, C_2, G	55.3	12.8	21.6	10.3	55.6	11.1	22.2	11.1
A_4, T_5, C, G_2	41.7	33.3	8.9	16.2	41.7	33.3	8.3	16.7
A_6, T_2, C_2, G_2	17.8	49.2	17.0	15.8	16.7	50.0	16.7	16.7

[a] Based on total normalized nucleotide area.

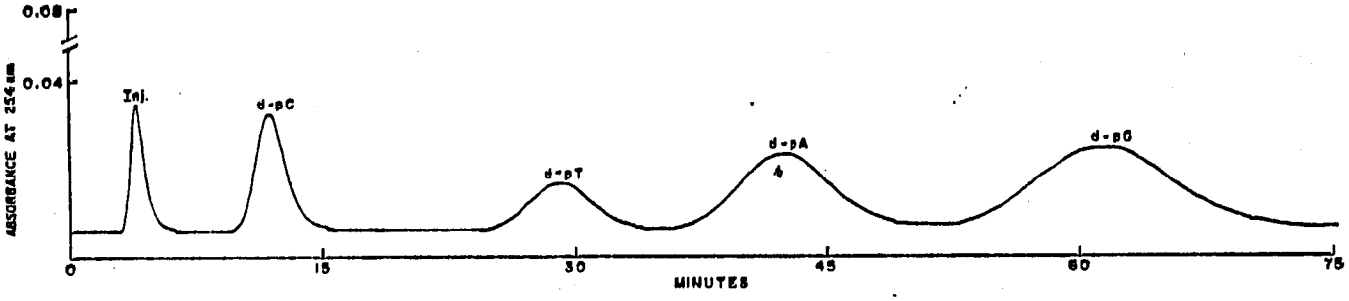

Fig. 1. Separation of 4 deoxyribonucleotides on 1 mm × 1 m SAX column using 0.008 *M* KH_2PO_4, pH 4.40, at 0.41 ml/min.

pH 4.40; any overlap is generated by a fluctuation in pH (see below). As can be seen from the table, values within 10% of theory are usually found with a precision of about 20 parts per thousand. Relative purity of the oligomers can be estimated from the molar ratios. The detector–integrator response is linear over a wide range

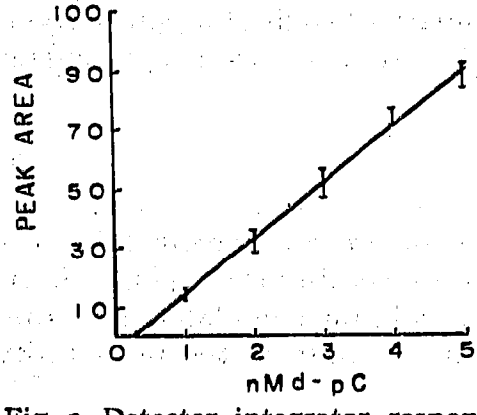

Fig. 2. Detector–integrator response for d-pC. Vertical bars represent range of experimental values, line is computer fit of data.

on each detector scale. When plotted as normalized area *vs.* nanomoles of nucleotide, a straight line results, as shown in Fig. 2 for d-pC. The fit was made by the least squares method as carried out by GE 635 computer. The other nucleotides give responses which follow lines with the same slope, but somewhat different intercepts.

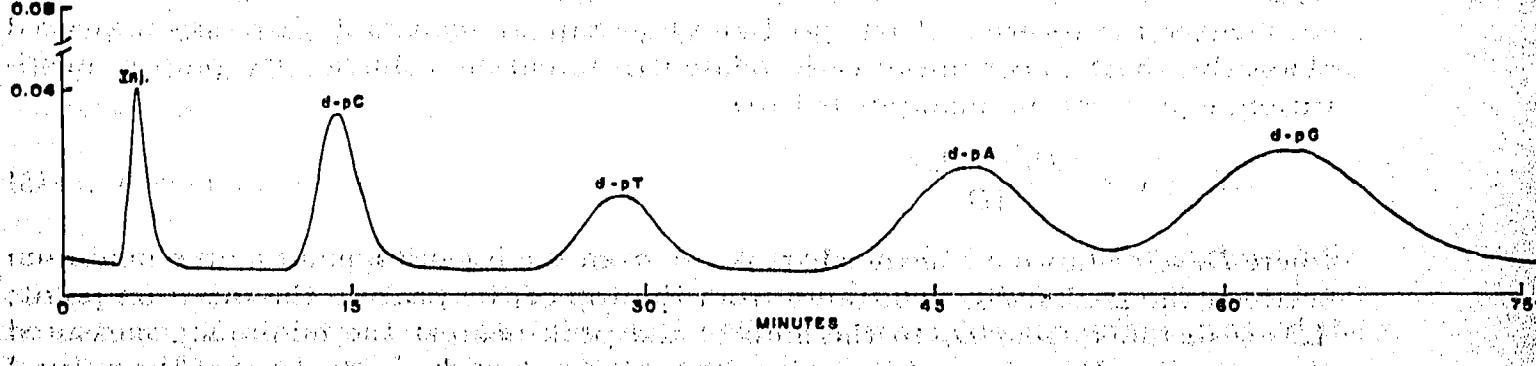

Fig. 3. Separation of 4 deoxyribonucleotides on 1 mm × 1 m SAX column using 0.008 *M* KH_2PO_4, pH 4.56, at 0.41 ml/min.

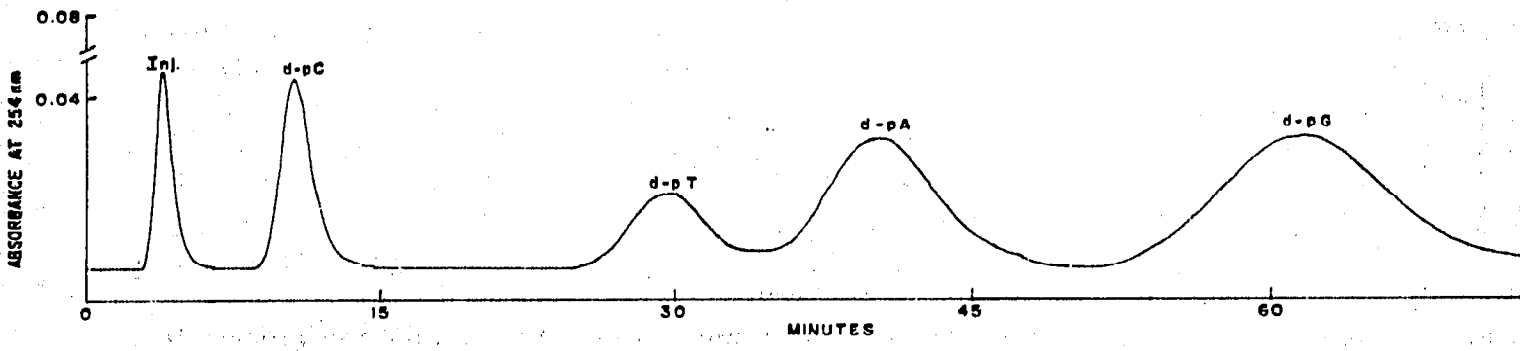

Fig. 4. Separation of 4 deoxyribonucleotides on 1 mm × 1 m SAX column using 0.008 *M* KH_2PO_4, pH 4.26, at 0.41 ml/min.

Precise pH control of the eluant is essential, since at a pH above 4.50, the d-pA and d-pG peaks overlap (Fig. 3) and below 4.30 the d-pT and d-pA peaks overlap (Fig. 4). Some change in buffer pH occurs upon storage. Corrections may be made in the calculations by making use of the approximately Gaussian shape of the peaks on the chromatogram. A general method for calculating the purity of each of a pair of overlapping peaks on a chromatogram makes use of the Gaussian function representing each peak:

$$X = \frac{U_i - U_1^*}{U_1^*} \left(\frac{P_1 + (1 + C_1)}{C_1}\right)^{\frac{1}{2}} \quad (1)$$

where U_1^* = elution volume at which the concentration of component 1 is at a maximum; U_i = elution volume at which the curves representing the 2 peaks intersect; P_1 = total number of theoretical plates in the column calculated for component 1; C_1 = distribution ratio for component 1; X = limit of the probability integral:

$$\int_{-\infty}^{X} \varphi(X)\mathrm{d}X = \tfrac{1}{2}(1 + \alpha) \quad (2)$$

where $\frac{1}{2}(1 + \alpha)$ is the area under the curve due to component 1, and is therefore the mole fraction of component 1 in the peak.

The value of X is then used to obtain $\frac{1}{2}(1 + \alpha)$ from a table of probability functions. A good treatment of the method can be found in RIEMAN AND WALTON's text[25].

Since the recorder chart speed and flow rate are constant, distances measured along the chart paper are directly related to retention volumes. By proper substitution, eqn. 1 can be manipulated to:

$$X = \sqrt{2}\,\frac{|D_i - D_e|}{\Delta D} \quad (3)$$

where D_i = distance along the chart paper from the injection point to the minimum between the peaks; D_e = distance from the top of the peak to the injection point; ΔD = distance from D_e to the limb of the peak nearest the minimum, measured along the line H/e where H is the height of the peak and e is the base of the natural logarithm.

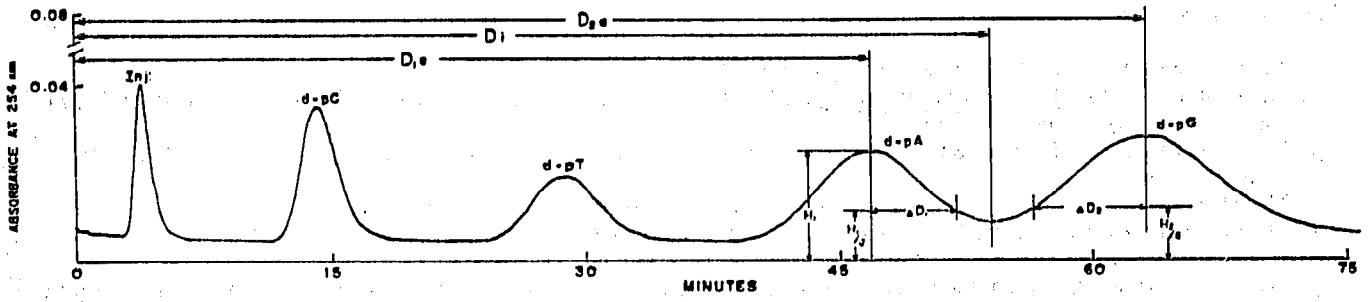

Fig. 5. Chromatography as in Fig. 3; illustration of method for calculating peak overlap. Distances measured on chart paper.

As an illustration, the purity, or overlap, of the d-pA and d-pG peaks in Fig. 5 may be calculated from the data in Table II. Substitution into eqn. 3 gives values of X of 2.46 for d-pA, and 1.91 for d-pG. From a probability function table, the corresponding values of $\frac{1}{2}(1 + \alpha)$ are 0.993 and 0.972, respectively. Thus, 0.7% of the area of the d-pA peak is due to d-pG contamination, and 2.8% of the area of the d-pG peak is due to d-pA contamination. Correction of the areas for this overlap allows greater precision in determining the molar ratios.

TABLE II

SEPARATION DATA FROM FIG. 5

Component	D_t *(cm)*	D_e *(cm)*	H *(cm)*	H/e *(cm)*	ΔD *(cm)*
d-pA	26.2	22.1	3.1	1.14	2.35
d-pG	26.2	30.4	4.2	1.52	3.10

Column life is greater than six months, with some eventual clogging and resultant increase in pressure required to maintain the proper flow rate. No degeneration in retention time or separation was noted. Removal of a few centimeters from the top end of the column restores the original flow rate and pressure. Examination of the resin from the cut-off portion shows that the presence of dirt and bits of rubber from the injection septum causes clogging.

If necessary, as little as 1 nmole of oligomer can be analyzed with good precision. Individual peaks corresponding to 100 pmoles of nucleotide give sufficient area for reproducible quantization. Below this level, stray electrical noise becomes a problem.

ACKNOWLEDGEMENTS

We wish to thank Dr. C. G. SCOTT of the Physical Chemistry Department for introducing us to the technique of high-pressure liquid chromatography, and for reviewing the manuscript; also Dr. A. L. NUSSBAUM for his encouragement of the work and assistance in preparing the manuscript.

REFERENCES

1 T. F. GABRIEL, *J .Chromatogr.*, 36 (1968) 518.
2 A. F. COOK, D. T. MAICHUK, M. J. HOLMAN, E. P. HEIMER AND A. L. NUSSBAUM, *J. Amer. Chem. Soc.*, 94 (1972) 1334.
3 E. P. HEIMER, M. AHMAD, S. ROY, A. RAMEL AND A. L. NUSSBAUM, *J. Amer. Chem. Soc.*, 94 (1972) 1707.
4 M. POONIAN, E. NOWOSWIAT AND A. L. NUSSBAUM, in preparation.
5 H. G. KHORANA, A. F. TURNER AND J. P. VIZSOLYI, *J. Amer. Chem. Soc.*, 83 (1961) 686.
6 S. A. NARANG AND S. K. DHEER, *Biochemistry*, 8 (1969) 3443.
7 K. L. AGARWAL, A. YAMAZAKI AND H. G. KHORANA, *J. Amer. Chem. Soc.*, 93 (1971) 2754.
8 E. W. BUSCH, *J. Chromatogr.*, 37 (1968) 518.
9 I. C. CALDWELL, *J. Chromatogr.*, 44 (1969) 331.
10 E. JUNOWISZ AND J. H. SPENCER, *J. Chromatogr.*, 44 (1969) 342.
11 G. BROOKER, *Anal. Chem.*, 42 (1970) 1108.
12 J. J. KIRKLAND, *J. Chromatogr. Sci.*, 8 (1970) 72.
13 J. G. GREEN, C. E. NUNLEY AND N. G. ANDERSON, *Nat. Cancer Inst. Monogr.*, 21 (1966) 431.
14 I. L. CAMERON AND W. D. FISHER, *Nat. Cancer Inst. Monogr.*, 21 (1966) 441.
15 L. POPA, A. CRACEANU AND R. PORTOCALA, *Rev. Roum. Biochim.*, 4 (1967) 297.
16 D. D. CHRISTIANSON, J. W. PAULIS AND J. S. WALL, *Anal. Biochem.*, 22 (1968) 53.
17 R. A. MILLER AND J. W. KIRKPATRICK, *Anal. Biochem.*, 27 (1969) 306.
18 A. CARISANO, M. RIVA AND A. BONECCHI, *J. Chromatogr.*, 40 (1969) 386.
19 A. C. BURTIS, M. N. MUNK AND F. R. MAC DONALD, *Clin. Chem.*, 16 (1970) 667.
20 W. P. KENNEDY AND J. C. LEE, *J. Chromatogr.*, 51 (1970) 203.
21 P. VIRKOLA, *J. Chromatogr.*, 51 (1970) 195.
22 P. R. BROWN, *J. Chromatogr.*, 52 (1970) 257.
23 H. W. SCHMUCKLER, *J. Chromatogr. Sci.*, 8 (1970) 653.
24 C. A. BURTIS AND D. R. GERE, *Varian Aerograph Pamphlet A-1011*, 1970.
25 W. RIEMAN AND H. F. WALTON, *Ion Exchange in Analytical Chemistry*, Pergamon Press, Oxford, 1970.