Separation of oligo-RNA by reverse-phase HPLC

Gloria D.McFarland and Philip N.Borer

Department of Chemistry, University of California, Irvine, CA 92717, USA

Received 22 June 1979

ABSTRACT

A rapid and highly reproducible chromatographic technique has been developed for analysis and purification of complex mixtures of oligoribonucleotides. The method utilizes a column of microparticulate porous silica beads fully derivatized with octadecylsilyl groups. The column is eluted with gradients in acetonitrile/water/ammonium acetate pumped at pressures of 1500-3000 psi. Most separations are completed in 5-15 min. with usually better than 1% reproducibility of absolute retention times and about 0.1% reproducibility of relative retention times. A single column accomplishes separations of mononucleosides, mononucleotides, and larger oligomers through at least 20-mers. The absolute detection limit is ∿1 pmole of base though most of the analytical separations described use ∿1 nmole. In favorable circumstances it is possible to use the analytical columns to purify ∿1 mg of an oligonucleotide in a single 10-30 min. elution.

INTRODUCTION

Most oligonucleotide syntheses have depended heavily on low-pressure liquid chromatography by ion-exchange.[1-5] Recent improvements in separation methods involve high-pressure ion-exchange,[6] RPC-5 (which combines ion-exchange and reverse-phase modes),[7-12] and reverse-phase chromatography on covalently bonded silica-octadecylsilane (ODS) columns where Khorana and his co-workers have made considerable progress in separating derivatized and underivatized DNA oligomers.[13] All but the latter suffer from the disadvantage that the long elution times (30 min. to several days) severely limit their analytical usefulness. A major objection to RPC-5 is the current lack of a stable commercial supply and severe irreproducibility between batches.[8] Additionally RPC-5 cannot be used to separate very short oligomers

because the non-covalently bound coating bleeds off the beads at the low salt concentrations required. This problem is not encountered with the bonded-phase ODS columns.

Prepacked microparticulate silica-ODS columns such as those used in our work are available from several manufacturers. We have used four different "Microbondapak C18" columns from Waters Corporation over a period of three years and find excellent reproducibility from column to column. A similarly formulated column from Altex Corporation provided different chromatographic patterns but would be suitable for separations like those described here. A stable supply of these columns is assured because the manufacturers sell thousands each year due to their wide use for separations in organic chemistry.

Reverse-phase separations are based on the exclusion of a solute containing lipophilic components from a polar mobile phase. When the mobile phase is made less polar partition no longer favors the stationary phase for the least lipophilic molecules; solutes elute from the column in order of decreasing polarity. In order to decrease separation time and band-broadening it is often useful to perform gradient elution where the solvent mixture is made progressively less polar. In our situation the acetonitrile content is increased in a solvent that is otherwise composed of 1% ammonium acetate in water. Methanol is also used in place of acetonitrile by many chemists; we prefer acetonitrile for two reasons: (1) its vapor pressure is much lower which reduces problems with bubble formation by cavitation at high pumping rates, and (2) the viscosity of acetonitrile-water mixtures is much lower than corresponding methanol-water mixtures allowing columns to be operated at lower pressures thus decreasing wear on pumps and columns. Acetonitrle is currently 3 times more expensive than methanol but one gallon of acetonitrile suffices for 300 hours of continous chromatography using our system. Thus solvent cost is a rather small factor.

MATERIALS AND METHODS

HPLC System. A modular HPLC system was purchased from Waters Corporation (Milford, Massachusetts) including two model 6000A high pressure pumps, a model 660 solvent programmer, a model 440

uv absorbance monitor, and a model U6K sample injector. The outlet of a 30 cm x 0.39 cm ID "Microbondapak C18" column was connected to the uv monitor and preceded by a guard column, cut from a length of a 2 mm ID "Corasil C18" column (also from Waters), which was connected to the injector. The guard column was cut to a length of 2 cm with a tubing cutter being careful to prevent any loss of packing material. The guard columns had to be replaced at 6-8 week intervals when the normal operating pressure at 3 ml/min rose to ∿3000 psi. A single 2 mm ID x 61 cm Corasil C18 column can provide at least 20 guard columns. All column connections were made with (0.009 in ID) stainless steel tubing and zero-dead-volume fittings (Parker-Hannifin, Irvine, California). The total void volume of the system was 1.24 ml from injector to uv detector.

Solvents. Solvents had to be maintained at a very high degree of purity. Deionized water was further purified through a "Milli-Q" (Millipore Corporation) system consisting of a prefilter, an activated charcoal filter, two ion-exchange cartridges, and a 0.22 micron final filter. To maintain high-purity water the system was allowed to recirculate at least 15 min before drawing off water; no more than 6ℓ should be removed before allowing another 15 min of recirculation. Because gradient elution is frequently performed over a range of only 2-3% in acetonitrile concentration it is absolutely necessary to prepare solvents in a uniform manner. 10% ammonium acetate (Mallinckrodt AR grade) was prepared by dissolving the entire contents of a ½ lb bottle in water, adjusting to pH = 5.9 with acetic acid and diluting to 1.11 ℓ, then filtering through a 0.45 micron filter (Millipore-HA). Acetonitrile (Baker HPLC grade) was filtered through a Fluoropore (Millipore-FH) filter. Solutions are prepared by diluting appropriate amounts of CH_3CN and 10% AmOAc to 9.00 ℓ according to the chart on the following page. Solution a (or a') is supplied to the low concentration pump, called pump A; likewise b (or b') is supplied to the high side, called pump B. Most elutions were performed with solutions a and b, covering a range from 2% to 12% CH_3CN. The model 660 programmer adjusts the pumping rates of pumps A and B to maintain a constant total flow rate and gives a readout of the portion of flow

Solution	%CH_3CN	CH_3CN(mℓ)	10% AmOAc(mℓ)
a	2	180	880
b	12	1080	790
a'	1	90	890
b'	11	990	800

provided by pump B as "%B". Selection of a narrow range in CH_3CN concentration allows greater accuracy in reproducing solvent composition and a 10% range between solutions a and b (or a' and b') allows easy computation of absolute CH_3CN concentration at any time during the elution by the formula %B/10 + A = CH_3CN concentration; A = 2% for solution a and 1% for a'. In the chromatograms shown in Figs. 1-3 solvent strength increased linearly with time from t = o until the end of the gradient program; thereafter solvent composition and flow rate remained constant until all peaks were eluted. In certain separations (not shown) convex or concave gradients were adopted to optimize resolution of components. Flasks containing ∿200 ml of pure CH_3CN and ∿1ℓ volumes of a and b were degassed in an ultrasonic bath at the beginning of each day. Solvent lines, pumps, and columns were stored in pure CH_3CN and air bubbles carefully cleared from solvent lines and pumps at the beginning of each day. Solvent switchover from pure CH_3CN to solutions a and b was accomplished by gradient programs of 5 min at 3 mℓ/min.

Sample Injection. Samples were always clarified by centrifugation if cloudiness was apparent. Syringes were purchased from Hamilton Corporation supplied with a 26S ga, 22° bent point needle designed to prevent damage to the U6K injector. Sample sizes smaller than 25 μℓ were injected without regard to chloride ion concentration but large samples and solvents con-

taining halide ions were avoided because of chemical attack on stainless steel. Following each run a 3 mℓ "bomb" of solution b (programmer set to 100%B) was run into the column to elute any strongly adsorbed species before returning to solvent conditions appropriate for the next run. The next sample cannot be loaded onto the U6K injector until the bomb has cleared the sample loop and the column restored to initial conditions for the next run.

Isolation of Purified Components. The analytical columns can be used for semi-preparative purifications in many instances. Acetonitrile and most of the water is removed from selected fractions in a flash evaporator. Samples are redissolved in water such that the ammonium acetate concentration is 10% or less, frozen, and lyophilized to remove ammonium acetate. Dialysis or desalting on polyacrylamide gels is usually not necessary.

Oligonucleotide Preparation. Nucleosides, nucleotides, dinucleoside monophosphates, poly U, and poly C were purchased from Sigma Chemical Corporation; poly A from Miles Biochemicals; alkaline phosphatase (calf intestine) from Boehringer-Mannheim. Primer-independent polynucleotide phosphorylase was purchased from P-L Biochemicals and made primer-dependent by limited trypsin digestion.[14]

Homologous series, oligo A_n, oligo C_n, and oligo U_n, were prepared by alkaline hydrolysis at 60°C (3.3 mM poly X, 1.0 M KOH, 100 µℓ total volume). Hydrolysis was stopped (at times indicated in the figure legends) by addition of 12 µℓ 10 M $HC\ell O_4$ and incubated at pH $\leq$2 to eliminate 2'-3'-cyclic phosphates (60° for 10 min). Terminal phosphates were removed by incubation overnight at 37°C with alkaline phosphatase (300 µg/mℓ) after adding 6 µℓ 2M HEPES buffer and adjusting to pH=7.5 with ~2µℓ 10 M KOH. Solid $KC\ell O_4$ was removed by centrifugation. Oligonucleotide preparations by primer-dependent polynucleotide phosphorylase followed in methods described earlier.[2-5]

RESULTS AND DISCUSSION

Dinucleoside monophosphates. Several general features of reverse-phase HPLC separations of oligo-RNA are shown in Fig. 1.

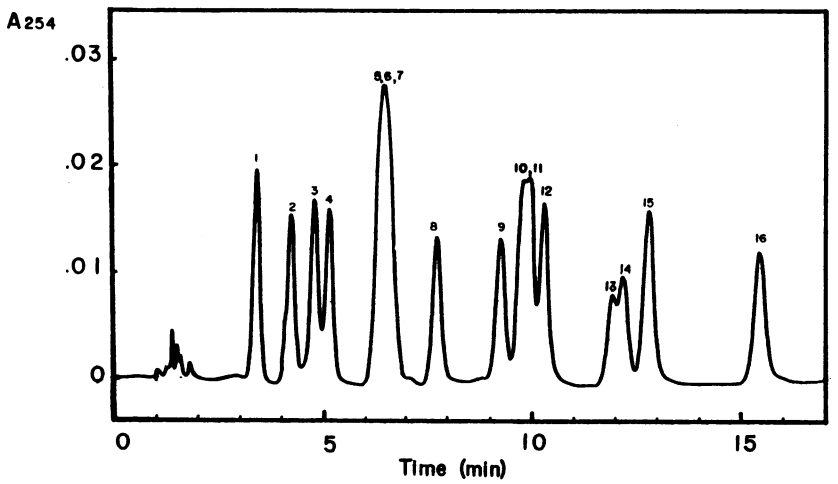


Figure 1. 16 dinucleoside monophosphates separated by a 15 minute linear CH_3CN gradient, from 2% to 8% (using solvents a and b), at 3 ml/min flow rate. The numbered peaks are identified in Table I.

All 16 of the dinucleoside monophosphates were loaded onto the column (~0.5 nmole each) and separated by a 15 minute linear gradient covering a range from 2% to 8% CH_3CN (Solvents a and b, gradient 0→60%B). Elution parameters for this separation; peak identities, retention times (t_R), and retention times relative to ApA are collected in Table I along with parameters for a similar 15 minute 2% to 11% CH_3CN program. The figure shows that most of the 16 components are clearly separated in this single 15 minute run; further illustrations of the tremendous resolving power of this system are found in comparing the isomeric molecules, eg. ApC and CpA which elute 2.5 min. apart. It is usually true that C-rich oligomers elute first, followed by U-rich, G-rich, then A-rich oligomers in that order; CpC elutes first and ApA last. The arrangement of data in Table I follows this order, the XpC group first (X is any of the four bases), followed by the XpU, XpG, and XpA groups. Within each group it is seen that as X changed through C, U, G, and A there is a 1.5 to 3.5 min. interval in t_R. This interval is fairly constant for a specific 5'-nucleotidyl base, eg. the difference in t_R between 5'-G and

TABLE I

Elution Parameters for 16 Dinucleoside Monophosphates Separated by Two Different 15 minute Linear Gradients, at 3 ml/min Flow Rate, Using Solvents a and b

			0→60% B (2% to 8% CH_3CN)		0→90% B (2% to 11% CH_3CN)	
	XpY	Number (Fig. 1)	t_R (min:sec)	t_R Relative to A_pA = 1.00	t_R (min:sec)	t_R Relative to ApA = 1.00
XpC	CpC	1	3:42	0.236	3:56	0.276
	UpC	4	5:26	0.347	5:37	0.394
	GpC	7	6:50	0.436	6:59	0.490
	ApC	12	10:32	0.672	9:55	0.696
XpU	CpU	2	4:30	0.287	4:48	0.337
	UpU	5	6:45	0.431	6:45	0.474
	GpU	9	9:28	0.604	8:40	0.608
	ApU	14	12:30	0.798	11:42	0.821
XpG	CpG	3	5:00	0.319	5:10	0.363
	UpG	6	6:45	0.431	6:48	0.501
	GpG	10	9:50	0.628	9:04	0.636
	ApG	15	13:00	0.830	11:58	0.840
XpA	CpA	8	8:01	0.512	7:50	0.550
	UpA	11	10:11	0.650	9:15	0.649
	GpA	13	12:10	0.777	11:16	0.791
	ApA	16	15:40	1.000	14:15	1.000

5'-A is 3:20±20 sec for each of the four groups. Such trends may be useful in adapting this analytical system to sequencing oligonucleotides.

There is some variation in absolute retention times from day to day but retention times relative to t_R = 1.000 for ApA are reproducible to ±0.020 or better (±20 sec). Within runs on a

single day these relative t_R values are usually reproducible to ±0.005 (±5 sec). Since ApA usually elutes later than anything else it is a convenient reference for expressing relative retention.

Nucleosides and nucleoside monophosphates. Table II shows the elution parameters for several monomeric constituents of RNA. The general trend of C, U, G, A in order of elution is again observed. We have examined the nucleosides (N), nucleoside-5'-phosphates (pN), nucleoside-3'-phosphates (Np), nucleoside-2':3'-cyclic phosphates (Np>), and nucleoside-3':5'-cyclic phosphates (cpN). We have not tabulated data on nucleoside-5'-diphosphates (ppN), triphosphates (pppN) and 2'-monophosphates (Np') but elution order follows this scheme: pppN, ppN, pN, Np, Np', Np>, N, cpN. We are usually unable to utilize the small difference in retention between pppN, ppN, and pN because of their close proximity to the void volume of the column; this is especially true for the pyrimidine nucleotides. The difference between the t_R values of N and cpN are also usually small. Retention of the pN, Np, and Np' species are quite sensitive to the pH of the solvent so care should be taken to reproducibly prepare solvents with pH = 5.9.

Homolgous series. Elution profiles of homooligonucleotide mixtures are presented in Figure 2. Panel a shows the separation of $(Cp)_nC$ oligomers, panel b $(Up)_n$ U oligomers, and $(Ap)_n$ A oligomers in panel c. The separations give excellent resolution of oligomers out through at least n = 10. In certain instances we have turned separation parameters to give resolution of n = 25 from n = 26 oligomers and see no reason why the resolution cannot be extended to longer oligomers. An oligomer with n = 10, eg. $(Ap)_{10}A$, is a polyanion with 10 negative charges; one might suppose that this would be such a highly polar molecule that it would not be retarded on a reverse-phase column. In fact, however, $(Ap)_{10}A$ is retarded more than $(Ap)_9A$, so the separation must be based upon the number of lipophilic substituents (bases);[15] there is no need to resort to ion-exchange or expensive ion-pairing reagents.

The amount of time required for a particular separation is directly related to the complexity of the mixture being analyzed. In the case of these homologous series, where up to twenty com-

TABLE II

Elution Parameters for Nucleosides and Nucleoside Monophosphates. The following conditions were used: 15 minute linear gradient, from 0 to 90% B, using solvents a and b (2% to 11% CH_3CN) at 3 ml/min flow rate.

		t_R (min:sec)	t_R Relative to ApA = 1.00	
pN	pC	1:28	0.122	
	pU	1:16	0.106	
	pG	1:32	0.128	
	pA	1:39	0.138	
Np	Cp	1:28	0.122	
	Up	1:34	0.130	
	Gp	2:04	0.161	
	Ap	3:50	0.296	
Np>	Cp>	1:22	0.113	
	Up>	1:37	0.132	
	Gp>	3:03	0.249	
	Ap>	5:37	0.460	
N	C	1:55	0.152	
	U	2:15	0.178	
	G	4:19	0.341	
	A	7:24	0.585	
cpN	cpC	2:31	0.206	
	cpU	2:59	0.243	
	cpG	4:07	0.335	
	cpA	7:10	0.581	

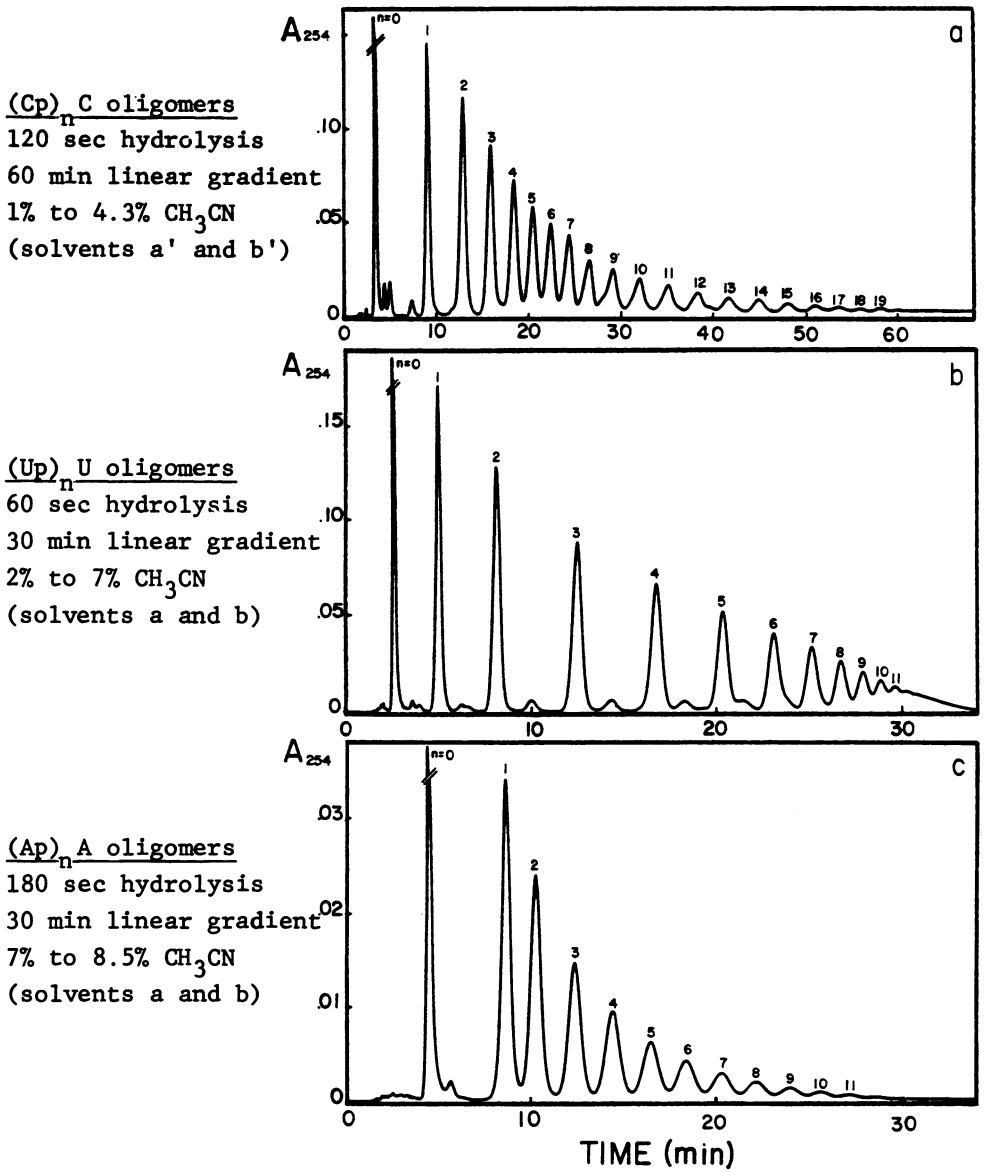


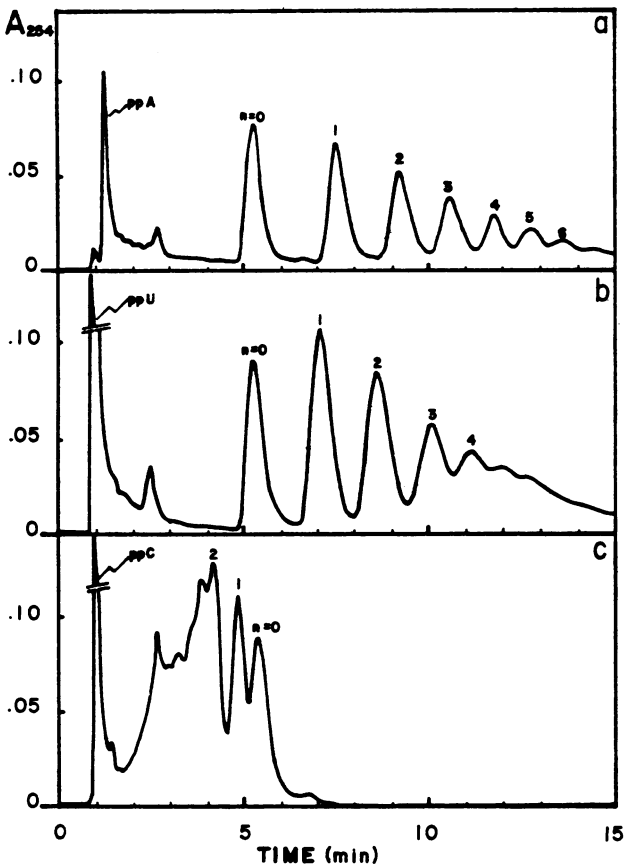
Figure 2. Elution profiles of homologous series. Each series was prepared by alkaline hydrolysis of the appropriate homopolymer. All elutions at 2 ml/min flow rate.

ponents have been separated in a single run (eg. $(Cp)_nC$ series), we have resorted to separations as long as 60 minutes in order to increase the resolving power of the system. However, in situations where only a few components are of interest, as it frequently occurs in practice, 10 to 15 minutes is sufficient; a 15 minute linear gradient from 1% to 3.8% CH_3CN at 3 ml/min flow rate will give baseline separation of C through $(Cp)_4C$ in the $(Cp)_nC$ series.

Block Copolymers. A common result of an oligonucleotide synthesis with primer dependent polynucleotide phosphorylase is a "block" addition product with a block of identical bases at the 3'-end and a primer molecule of defined sequence at the 5'-end of the molecule; the primer may be as short as a dinucleotide. Examples of the separation problems encountered are illustrated in Figure 3 with molecules of the type, $ApG(pX)_n$, where the primer is ApG. Separation of the $ApG(pA)_n$ (Figure 3a) and $ApG(pU)_n$ (Figure 3b) oligomers proceeds nicely with clear resolution of products with n = 1-3 (the usual range of interest) in 5-10 min. However, $ApG(pC)_n$ (Figure 3c) oligomers do not separate following the expected pattern, i.e. in order of increasing chain length. Instead, the reverse order is observed, in this case as well as in a number of other C righ oligomer series, under some separation conditions. The polyanion character of these molecules then must override the affinity of the lipophilic moities for the stationary phase under these solvent conditions and the least charged oligomers are retarded most.

Only at relatively low CH_3CN concentrations do $(Cp)_nC$ oligomers elute in order of increasing chain length as in Figure 2. To obtain these results it is necessary to use solvents a' and b' which cover a range of CH_3CN concentrations starting at 1%; the use of solvents a and b (2% and 12% respectively) results in separations where the order of elution is reversed, as in Figure 3c, or even some in which no simple pattern of elution is discernible.

Throughout all the separations described in this paper, pH has remained constant. However, it might prove useful in some difficult separations to lower the pH of the solvents to 3 in order to positively charge adenosine and cytidine residues



ApG(pA)$_n$ series
10 min linear gradient
6% to 8% CH_3CN

ApG(pU)$_n$ series
10 min linear gradient
6% to 7% CH_3CN

ApG(pC)$_n$ series
10 min linear gradient
6% to 7% CH_3CN

Figure 3. Block copolymers. Solvents a and b were used in each at a flow rate of 3 ml/min.

and thus change their elution properties. The use of pH above 6 is not recommended because silica slowly dissolves in water above pH 6.

The Microbondapak C18 column in combination with a single type of solvent, namely CH_3CN/water solutions, over a very narrow concentration range, has proved to be a very versatile tool in analyzing mixtures of oligoribonucleotides. These mixtures can be the result of the use of biosynthetic enzymes such as polynucleotide phosphorylase, RNA ligase, polynucleotide kinase and others in the synthesis of oligoribonucleotides of known sequence, for example, or they can be products of the activity of a variety

of nucleases on RNA, etc. The ability to analyze reaction mixtures in such short periods of time allows the study of the time course of these reactions in a quantitatively as well as qualitatively accurate manner. This could open the door to reaction mechanism studies which have been limited by the cumbersome and time consuming methods of product analysis previously available.

There are indications that the system can be adapted to other uses, for example, the analysis of longer nucleic acids, which would be of value in the purification and sequence analysis of RNA and DNA. A further application should be in the separation of alkylated mono- and oligonucleotide products resulting from the action of activated carcinogens or mutagens.

ACKNOWLEDGMENTS

This work was supported in part by grants from the National Science Foundation (PCM76-04992 A01), The National Institutes of Health (GM 24494), the Petroleum Research Fund (9196-G6), and the Research Corporation. We are grateful for helpful discussions with D. A. Brant, T. A. Posever, R. L. Boughton and applications personnel from Waters Corporation.

REFERENCES

1. Khorana, H. G. and Vizsolyi, J. P. (1961) J. Am. Chem. Soc., 83, 675.
2. Borer, P. N., Kan, L. S. and Ts'o, P.O.P (1975) Biochemistry, 14, 4847; appendix in microfilm edition contains details of synthesis and purification.
3. Borer, P. N. (1972) Ph.D. Thesis, University of California, Berkeley.
4. Martin, F. H. (1969) Ph.D. Thesis, Harvard University.
5. Uhlenbeck, O. C. (1969) Ph.D. Thesis, Harvard University.
6. Leutzinger, E. E., Miller, P. S. and Ts'o, P.O.P. (1978) in Nucleic Acid Chemistry, Improved and New Synthetic Procedures, Methods and Techniques, Townsend, L. B., and Tipson, R. S. (eds.), Wiley, (1978).
7. Walker, G. C., Uhlenbeck, O.C., Bedows, E., and Gumport, R. I. (1975) Proc. Nat. Acad. Sci. USA, 72, 122.
8. Shum. B. W., and Crothers, D. M. (1978) Nucleic Acids Res. 5, 2297.
9. Hardies, S. C. and Wells, R. D. (1976) Proc. Nat. Acad. Sci. USA, 73, 3117.
10. Egan, B. Z. (1973) Biochim. Biophys. Acta, 299, 245.

11. Pearson, R. L., WEiss, J. F., and Kelmers, A. D. (1971) Biochim. Biophys. Acta, 228, 770.
12. Kelmers, A. D., Weeren, H. O., Weiss, J. F., Pearson, R. L., Stulberg, M. P., and Novelli, G. D. (1971) Meth. Enzymology, XX Part C, 9.
13. Fritz, H. J., Belagafe, R., Brown, G. L., Fritz, R. H., Jones, R. A., Lees, R. G., and Khorana, H. G. (1978) Biochemistry, 17, 1257.
14. Klee, C. B. and Singer, M. F. (1967) Biochem. Biophys. Res. Comm., 27, 356.
15. Some exceptions have been observed, however, with C-rich oligomers (see text under "Block Copolymers").