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COMPARISON OF SEVERAL HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY TECHNIQUES FOR THE SEPARATION OF OLIGODEOXYNUCLEOTIDES ACCORDING TO THEIR CHAIN LENGTHS

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SUMMARY

The use of reversed-phase, reversed-phase–ion-pair and anion-exchange high-performance liquid chromatography (HPLC) was investigated for the analytical and preparative separation of oligodeoxynucleotides according to their chain length. The data obtained with homooligonucleotides and oligonucleotides of defined sequence show that reversed-phase–ion-pair and anion-exchange, but not reversed-phase, HPLC can be used reliably to separate oligodeoxynucleotides according to their chain length, largely irrespective of their base composition. The chain length limits for complete separation within 30 min by reversed-phase, reversed-phase–ion-pair and anion-exchange HPLC are approximately 10, 15 and 20 nucleotides, respectively.

INTRODUCTION

High-performance liquid chromatography (HPLC), because of its high resolution, reproducibility and ease of operation, has become an indispensible method for the separation of oligodeoxynucleotides, for both analytical and preparative purposes. A variety of different techniques have been developed, based on reversed-phase HPLC[1–3], reversed-phase–ion-pair HPLC[4,5], strong anion-exchange HPLC[6–9] and size exclusion HPLC[10], and have been used so far mainly for the separation of short oligonucleotides. The separation of oligodeoxynucleotides by these techniques is determined by the base composition and chain length of the oligodeoxynucleotides to be separated, which in turn determine their polarity and size. These properties, albeit to different extents in the various chromatographic systems, control the degree of retention of a particular oligodeoxynucleotide. The retention mechanisms involved are not fully understood.

Reversed-phase HPLC of oligodeoxynucleotides is dominated by hydrophobic interactions between the solute and the bonded phase[1]; it is more sensitive to the base composition than reversed-phase–ion-pair, anion-exchange and size exclusion HPLC. Nevertheless the retention sequence of oligonucleotides with similar base composition is controlled by the charge of the oligonucleotide.

In reversed-phase–ion-pair HPLC, negative charges on the oligodeoxynucleo-

tide are neutralized by positively charged alkylammonium ions. It is not clear, how-
ever, whether this process occurs in the mobile phase[11] or in the stationary phase[12],
leading to dynamic ion exchange. The retention is determined mainly by the charge
of the oligodeoxynucleotide; in addition to these dominating electrostatic interac-
tions, hydrophobic interactions between the oligodeoxynucleotides and the reversed
phase also play a role in the chromatographic process[5].

The separation of oligodeoxynucleotides by anion-exchange HPLC is depen-
dent on differences in charge; as currently used anion exchangers are based on a silica
gel matrix, in which the tertiary ammonium moiety is connected to the resin via an
aliphatic spacer, there are hydrophobic interactions in addition to the ionic interac-
tions. The inclusion of non-polar solvents in the mobile phase diminishes the effect
of the reversed phase on the chromatography[13,14].

Recently, silica gel bonded polyol phases have become commercially available
for the separation of oligodeoxynucleotides by size exclusion HPLC. The separation
can be carried out with a variety of mobile phases. The resolution depends on the
mobile phase, indicating that size is not the only determinant for the chromatogra-
phy. For small and medium sized oligodeoxynucleotides the resolution is not as good
as with the above-mentioned chromatographic systems[10].

One characteristic feature of a particular oligodeoxynucleotide, and one that
can be used conveniently for the purpose of analysis or preparative purification, is
its size. It is therefore desirable to have chromatographic systems that allow sepa-
rations according to size, *i.e.*, chain length. A separation in such a chromatographic
system ideally should be unaffected by base composition and should allow separation
with high resolution over a wide range of chain lengths. For this purpose we inves-
tigated the suitability of two standard and one new chromatographic system for chain
length-dependent separations of oligodeoxynucleotides, namely (1) reversed-phase
HPLC on Zorbax ODS columns, (2) reversed-phase–ion-pair HPLC on LiChrosorb
RP-8 columns and (3) anion-exchange HPLC on Partisil SAX columns. We used
commercial homooligodeoxynucleotides of specified chain lengths as well as chemi-
cally or enzymatically synthesized oligodeoxynucleotides of defined sequence to study
the limits of resolution of these chromatographic systems.

The results show that both reversed-phase–ion-pair HPLC and anion-exchange
HPLC, but not reversed-phase HPLC, can be used reliably to separate oligodeoxy-
nucleotides according to their chain length, with only slight interference by base
composition. Whereas, reversed-phase–ion-pair HPLC has a cut-off for an acceptable
resolution at a chain length of around 15–20 nucleotides, anion-exchange HPLC
allows even longer oligodeoxynucleotides to be separated.

EXPERIMENTAL

Instrumentation

Two chromatographs were used: (1) a DuPont 850 liquid chromatograph, con-
sisting of a gradient controller, a three-head pump, a temperature-controlled column
compartment, a UV spectrophotometer and a Rheodyne 7125 injection valve, and
(2) a Pye Unicam liquid chromatograph, consisting of an LC-XP gradient program-
mer, an Altex Model 100A pump, an LC-UV detector and a Rheodyne 7125 injection
valve, to which a Merck LiChrocart-Autofix pneumatic mounting device was at-
tached.

Chemicals and oligonucleotides

All homodeoxyoligonucleotides were obtained from P.L. Biochemicals (St. Goar, G.F.R.). d(GGAATTCC) and d(CGAATTCG) were kindly given by Dr. H. Bloecker and Dr. R. Frank (GBF, Stöckheim, G.F.R.) and by Dr. M. Zabeau (EMBL, Heidelberg, G.F.R.), respectively. Tetrabutylammonium hydrogen sulphate (TBAHSO$_4$), triethylamine and formamide were products of Fluka (Neu-Ulm, G.F.R.) and were used without further purification. HPLC-grade acetonitrile was a product of Burdick & Jackson Labs. (Muskegon, MI, U.S.A.) and was provided by Macherey, Nagel & Co. (Düren, G.F.R.). T4 DNA ligase was obtained from BRL (Neu-Isenberg, G.F.R.). All other chemicals used were of pro analysi grade from Merck (Darmstadt, G.F.R.).

Columns and chromatographic conditions

All eluents were filtered through a Whatman GF/F glass-microfibre-filter (Vetter, Wiesloch, G.F.R.) and degassed prior to chromatography.

For reversed-phase separations Zorbax ODS columns (25 cm × 4.6 mm I.D., 5 µm) from DuPont (Bad Nauheim, G.F.R.) were used. The triethylammonium acetate (TEAA) buffer was prepared as described elsewhere[1]. Solvent A was 0.1 M TEAA (pH 7.0)–1% acetonitrile and solvent B was 0.1 M TEAA (pH 7.0)–50% acetonitrile. If not stated otherwise, a linear gradient from 15 to 30% B in 30 min was applied. The flow-rate was 1.0 ml/min. Chromatography was carried out at ambient temperature.

Reversed-phase–ion-pair separations were performed on Hibar LiChrocart cartridges (25 cm × 4 mm I.D.) packed with LiChrosorb RP-8, 10 µm (Merck). Solvent A was 50 mM potassium phosphate (pH 5.9)–2 mM TBAHSO$_4$ and solvent B was 50 mM potassium phosphate (pH 6.5)–2 mM TBAHSO$_4$–60% acetonitrile. The standard linear gradient used was from 15 to 80% B in 60 min. The flow-rate was 1.0 ml/min. Chromatography was carried out at ambient temperature.

Whatman Partisil 10 SAX columns (25 × 4.6 mm I.D., 10 µm) obtained from IC-Chemikalien (Munich, G.F.R.) were used for anion-exchange HPLC. Solvent A was 1 mM potassium phosphate (pH 6.3) in formamide–water (6:4) and solvent B was 0.3 M potassium phosphate (pH 6.3) in formamide–water (6:4). The linear gradient used was from 0 to 80% B in 60 min. The flow-rate was 1.0 ml/min. Chromatography was carried out at 45°C.

RESULTS

Figs. 1 and 2 show chromatograms of mixtures of oligo-(dA)s and oligo-(dT)s separated by reversed-phase chromatography. Oligonucleotides of different chain length are well resolved within the oligo-(dA) and oligo-(dT) series up to a chain length of at least ten nucleotides under the conditions given.

Oligo-(dT)s are considerably more retarded than oligo-(dA)s of the same chain length. Thus, for example, p(dA)$_{10}$ is eluted before p(dT)$_6$. This demonstrates that the base composition of the oligonucleotide has a considerable effect on the retention of the oligonucleotides. This effect, of course, can be of advantage when oligomers of similar chain length or even sequence isomers have to be separated. This is illustrated in Fig. 3, where a separation of d(GGAATTCC) from its sequence isomer
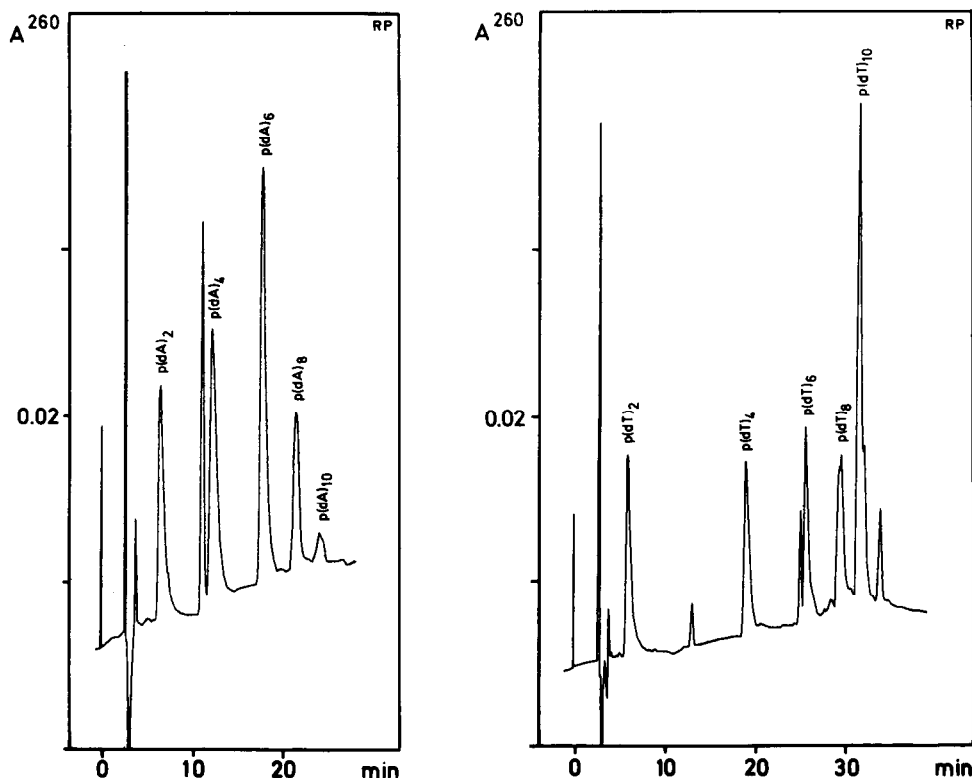
Fig. 1. Reversed-phase HPLC of oligo-(dA)s. Column: Zorbax ODS (25 cm × 3.6 mm I.D.). Eluents: A, 0.1 *M* TEAA (pH) 7.0–1% acetonitrile; B, 0.1 *M* TEAA (pH 7.0)–50% acetonitrile; linear gradient from 15 to 30% B in 30 min at ambient temperature; flow-rate, 1.0 ml/min. Unidentified peaks are due to contaminants in the individual samples.

Fig. 2. Reversed-phase HPLC of oligo-(dT)s. Chromatographic conditions as in Fig. 1.

d(CGAATTCG) is shown. In fact, our analysis of commercially available oligo-(dT)s and in particular of oligo-(dA)s has shown that these preparations contain consider-able amounts of impurities (*cf.*, Figs. 1 and 2), which are not detected by polyacryl-amide gel electrophoresis under denaturing conditions, by homochromatography after labelling the 5′-end with [^{32}P]phosphate or by anion-exchange HPLC (see be-low), suggesting that they consist of chemical derivatives of these oligonucleotides with the same chain length or charge.

Figs. 4 and 5 show separations of mixtures of oligo-(dA)s and oligo-(dT)s in a reversed-phase–ion-pair system. The individual oligonucleotides are extremely well resolved up to a chain length of at least 16 nucleotides, the peaks being sharper and more symmetrical than in the reversed-phase system (compare Figs. 4 and 1 or Figs. 5 and 2). Oligo-(dA)s are slightly more retained than oligo-(dT)s, *e.g.*, p(dA)$_8$ is eluted after p(dT)$_{10}$. The influence of base composition is less pronounced in the reversed-phase system. Accordingly, reversed-phase–ion-pair HPLC is not as useful as reversed-phase HPLC for the separation of oligonucleotides of different base com-position but similar chain length. This is also apparent from Fig. 6, which shows the
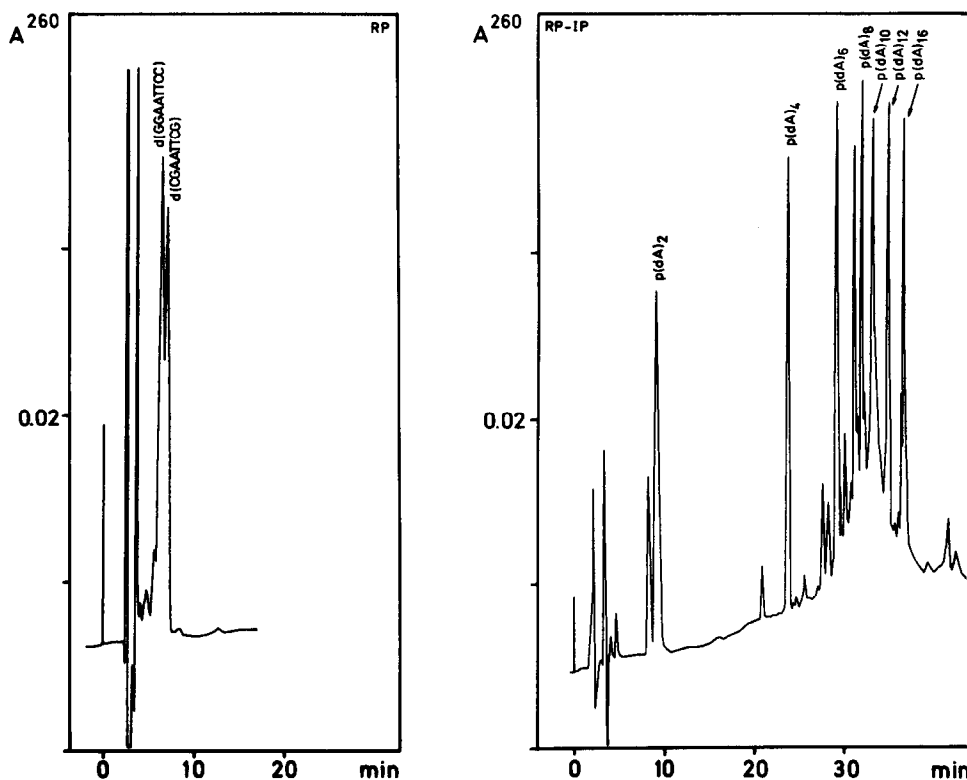
Fig. 3. Reversed-phase HPLC of the sequence isomers d(GGAATTCC) and d(CGAATTCG). Column: Zorbax ODS (25 cm × 4.6 mm I.D.). Eluents: A, 0.1 M TEEA (pH 7.0)–1% acetonitrile; B, 0.1 M TEAA (pH 7.0)–50% acetonitrile; linear gradient from 20 to 25% B in 20 min at ambient temperature; flow-rate, 1 ml/min.

Fig. 4. Reversed-phase–ion-pair HPLC of oligo-(dA)s. Column: LiChrocart cartridge (25 cm × 4.6 mm I.D.) filled with LiChrosorb RP-8. Eluents: A, 50 mM potassium phosphate (pH 5.9)–2 mM TBAHSO$_4$; B, 50 mM potassium phosphate (pH 6.5)–2 mM TBAHSO$_4$–60% acetonitrile; linear gradient from 15 to 80% B in 60 min at ambient temperature; flow-rate, 1.0 ml/min.

reversed-phase–ion-pair chromatogram of a mixture of d(GGAATTCC) and d(CGAATTCG). Although the retention time is longer than in the reversed-phase chromatography shown in Fig. 3, the separation is not as good.

Figs. 7 and 8 show chromatograms of mixtures of oligo-(dA)s and oligo-(dT)s separated by strong anion-exchange HPLC. The retention times under the chosen conditions are nearly constant with respect to base composition, e.g., p(dA)$_{10}$ is eluted at the same position as p(dT)$_{10}$. The resolution of oligonucleotides of increasing chain length is excellent up to a chain length of about 20. The high resolution is also demonstrated in Fig. 9, which shows the chromatogram of the separation of commercial oligo-(dA)$_n$. More than 30 discrete peaks can be seen. The usefulness of ion-exchange HPLC for the separation of longer oligonucleotides is particularly apparent from Fig. 10. In the HPLC run shown the mixture of products resulting from the T4 DNA ligase catalysed oligomerization of pd(GGAATTCC) was separ-
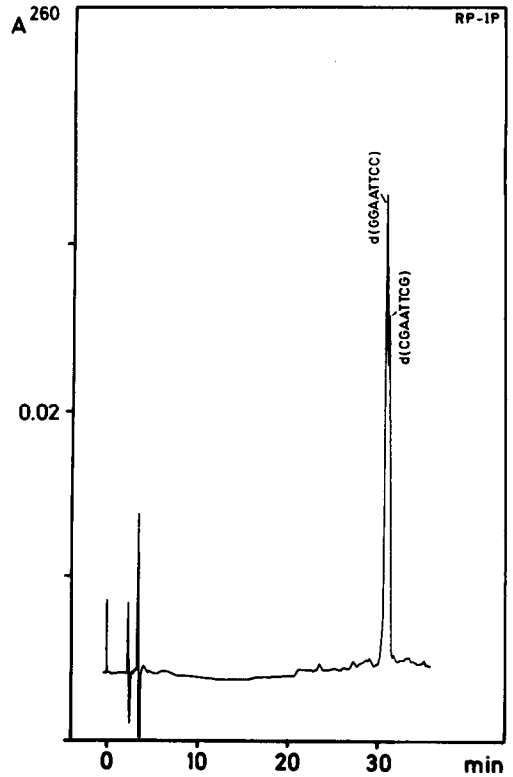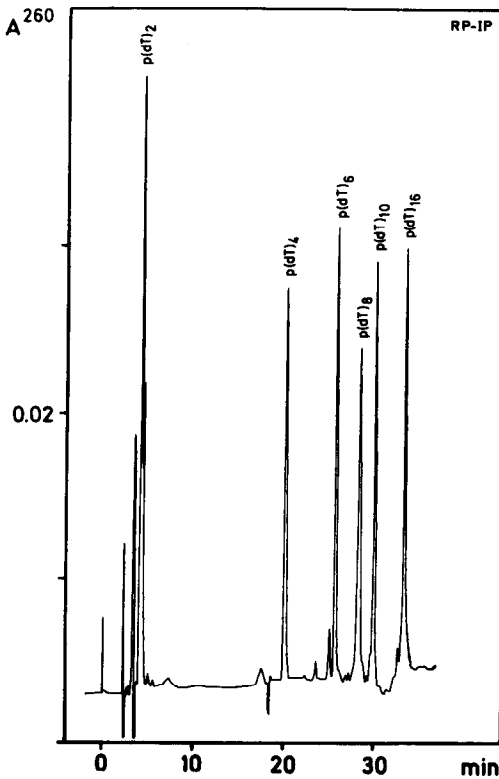
Fig. 5. Reversed-phase–ion-pair HPLC of oligo-(dT)s. Chromatographic conditions as in Fig. 4.

Fig. 6. Reversed-phase–ion-pair HPLC of the sequence isomers d(GGAATTCC) and d(CGAATTCG). Chromatographic conditions as in Fig. 4.

ated by anion-exchange HPLC. Resolution was achieved up to the "octamer", *i.e.*, up to a chain length of 64 nucleotides.

We have also used commercial preparations of oligodeoxynucleotides of the (dG) and (dC) series in reversed-phase, reversed-phase–ion-pair and anion-exchange HPLC. In general, the oligonucleotides of defined chain length as stated on the label proved to be much less homogeneous than the corresponding (dA)- and (dT)-oligodeoxynucleotides. Nevertheless, similar results were also obtained with the oligonucleotides of the (dG) and (dC) series compared with those of the (dA) and (dT) series (data not shown).

DISCUSSION

We have compared the utility of three different chromatographic systems for the separation of oligodeoxynucleotides by HPLC according to chain length. It was not our aim to improve previously developed chromatographic system, but rather to use them, with minor modifications, in order to establish their limits of resolution with respect to size and base composition. Our results show that within a series of homooligonucleotides all three systems reliably lead to separation according to
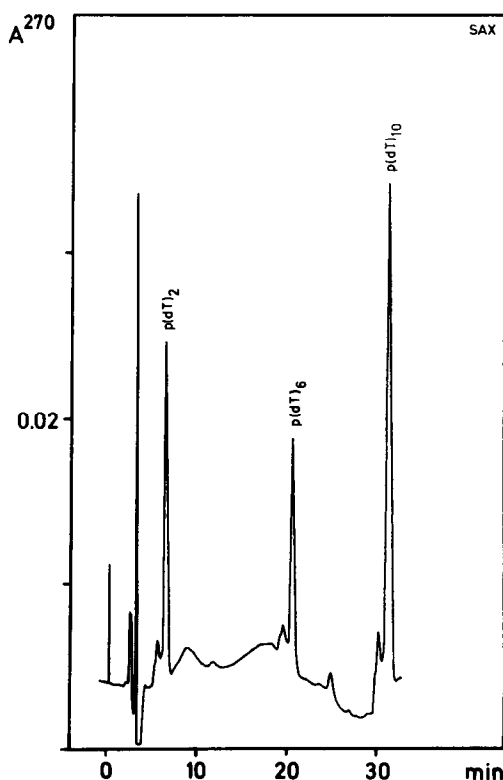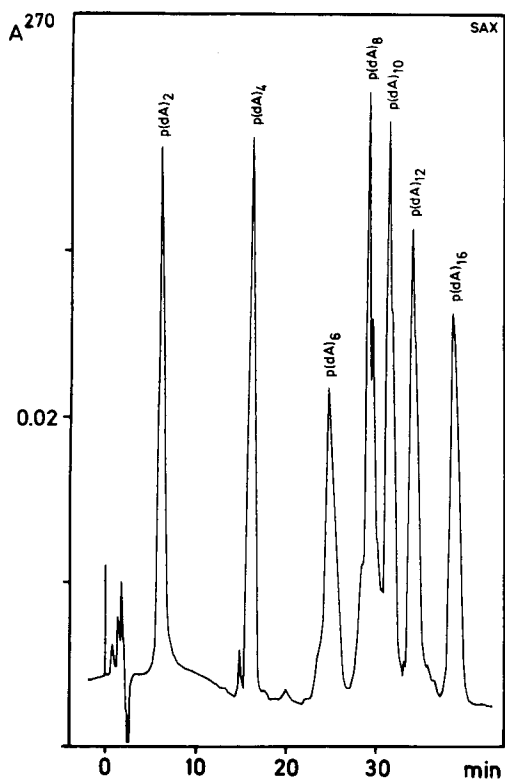
Fig. 7. Anion-exchange HPLC of oligo-(dA)s. Column: Whatman 1 SAX (25 cm × 4.6 mm I.D.). Eluents: A, 1 m*M* potassium phosphate (Ph 6.3) in formamide–water (6:4); B, .3 *M* potassium phosphate (Ph 6.3) in formamide–water (6:4); linear gradient from to 8% in 6 min at 45°c; flow-rate, 1. ml/min.

Fig. 8. Anion-exchange HPLC of oligo-(dT)s. Chromatographic conditions as in Fig. 7.

chain length. The chain length limits for separation by reversed-phase, reversed-phase–ion-pair and strong anion-exchange HPLC are *ca.* 10, 15 and 20 nucleotides, respectively. These limitis apply for the complete separation of homologous oligo-deoxynucleotides within *ca.* 30 min. With suitable modifications, *e.g.*, by using different gradients, these limits may well be extended towards longer chain lengths.It is known from the separation of short oligonucleotides that the retention of oligo-deoxynucleotides in reversed-phase HPLC is very sensitive to the base composition[1]. Our results show that oligo-(dT)s are much more strongly retained on Zorbax ODS (this work) and LiChrosorb RP-8 columns (data not shown) than oligo-(dA)s. The retention of oligodeoxynucleotides in reversed-phase–ion-pair HPLC, on the other hand, is only slightly influenced by the base composition. Interestingly, our results indicate that with this chromatographic system oligo-(dA)s are more strongly retained than oligo-(dT)s, probably a consequence of the different base stacking[15], which might affect the accessibility of the phosphate residues of the oligodeoxynucleotides for the ion-pairing agent.

Under the conditions applied the retention of oligodeoxynucleotides is least affected by the base composition of the sample in anion-exchange HPLC. It has been
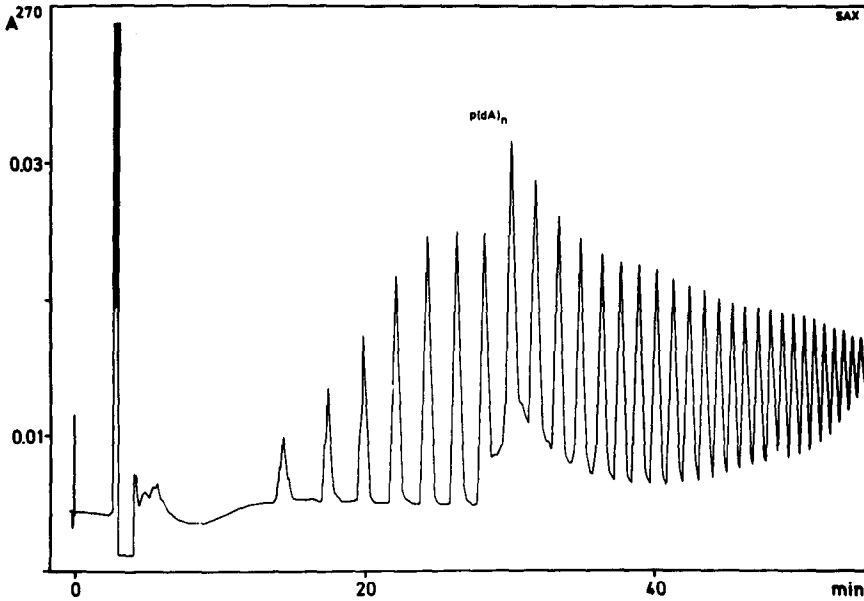
Fig. 9. Anion-exchange HPLC of a material that was designated by the supplier as $p(dA)_{25-30}$. Column and eluent as in Fig. 7; linear gradient from 0 to 100% B in 75 min at 45°C; flow-rate, 1 ml/min.
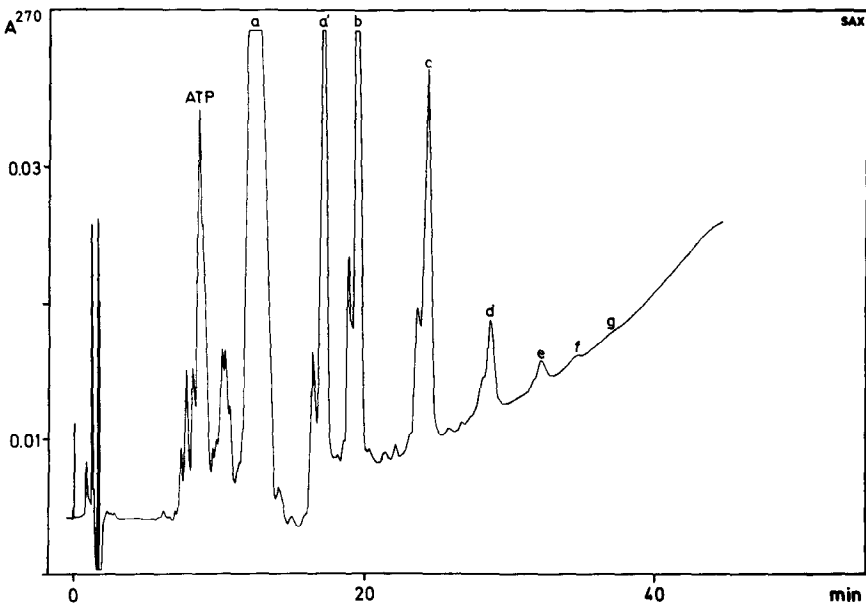

Fig. 10. Anion-exchange HPLC of the product mixture of the T4 DNA ligase catalysed oligomerization of pd(GGAATTCC) that was obtained by incubating 6 μM [^{32}P]pd(GGAATTCC) and 24 μM d(GGAATTCC) at 22°C for 16 h with 48 units/ml of T4 DNA ligase in a mixture of 40 mM Tris HCl (pH 7.6), 8 mM magnesium chloride, 1 mM dithioerythritole (DTE) and 470 μm ATP. The separated peaks were identified by polyacrylamide gel electrophoresis[15]: a = d(GGAATTCC) monomer; b = dimer; c = trimer; d = tetramer; e = pentamer; f = hexamer; g = heptamer; a' = AMP-pd(GGAATTCC) ligation intermediate. Analysis of the fractions by Cerenkov radiation in a scintillation counter indicated that oligonucleotides beyond the octamer were resolved. The small satellite peaks of peaks a, a', b, c, d and e probably are due to slight heterogeneity in the starting material.

reported, however, that under different conditions, in particular in the absence of organic solvents in the mobile phase, anion-exchange HPLC can by used to separate sequence isomers of small oligonucleotides[8,17]. This demonstrates that the inclusion of organic solvents in the mobile phase is necessary to suppress hydrophobic interactions between the sample and the stationary phase[13,14].

Taken together, our results suggest that for short to medium sized oligonucleotides reversed-phase–ion-pair and anion-exchange HPLC are equally suitable for separations according to chain length (cf. also ref. 18), whereas for longer oligonucleotides anion-exchange HPLC is superior to reversed-phase–ion-pair HPLC. It must be mentioned in this context, however, that anion-exchange HPLC columns seem to suffer from shorter lifetimes[4] than reversed-phase HPLC columns. Thus, for analytical or preparative work with small to medium sized oligonucleotides reversed-phase and reversed-phase–ion-pair HPLC is the combination of choice, one system separating according to polarity and size, the other predominantly according to size. For separations of larger oligonucleotides by HPLC there is as yet no alternative to anion-exchange or size exclusion HPLC.

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