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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATIONS OF ISOMERIC PYRIMIDINE OLIGODEOXYNUCLEOTIDES

HERBERT SCHOTT and HEINER ECKSTEIN*

Institute of Organic Chemistry, University of Tübingen, Auf der Morgenstelle 18, D-7400 Tübingen-1 (F.R.G.)

SUMMARY

Mixtures of sequence-isomeric pyrimidine oligonucleotides up to pentamers were separated by anion-exchange and reversed-phase high-performance liquid chromatography. Of the 47 theoretically possible isomers in the investigated mixtures, more than 40 could be identified by degradation with snake venom phosphodiesterase or by the wandering spot method. This combination of anion-exchange and reversed-phase techniques proved to be very powerful. Sequence isomers not separated by ion exchange were separated on Nucleosil C_{18} and *vice versa*.

INTRODUCTION

Sequence isomeric pyrimidine and purine oligodeoxynucleotides can be obtained easily in preparative amounts according to a chemical degradation procedure starting from DNA. After several separation steps nine mixtures of sequence isomeric pyrimidine oligonucleotides of equal chain length are obtained from herring sperm DNA (see Table I and II). The separation and isolation of individual oligonucleotides from the mixtures can only be achieved using high-performance liquid chromatography[4-6]. The successful application of both anion-exchange and reversed-phase liquid chromatography is described in this paper.

MATERIALS AND METHODS

Materials

The mixtures of sequence-isomeric pyrimidine oligonucleotides were isolated from chemically degraded herring sperm DNA according to previously described methods[1-3]. Partisil 10 SAX, 10 μm (Chrompak, Berlin, F.R.G.); MicroPak AX-10, 10 μm (Varian, Walnut Creek, U.S.A.) and Nucleosil C_{18}, 7.5 μm (Macherey & Nagel, Düren, F.R.G.) were used as supplied. The other experimental details are described extensively elsewhere[4-6].

Characterization of the isolated sequence isomers

The isolated peak fractions from the ion-exchange column were freeze-dried

to remove triethylammonium acetate. An aliquot of the resulting lyophilizate was characterized by hydrolysis with snake venom phosphodiesterase, followed by high-performance liquid chromatographic (HPLC) analysis of the nucleotides released, as described previously[4,5]. The sequences of the oligonucleotides isolated by reversed-phase chromatography were determined after labelling with $[\gamma\text{-}^{32}P]ATP$ and T_4 polynucleotide kinase by the well-known wandering spot sequence analysis[7–11]. The autoradiograms (fingerprints) of the oligonucleotides investigated by this procedure are reported elsewhere[6].

Apparatus

HPLC was carried out using three chromatographs: Model 1010B (Hewlett-Packard, Böblingen, F.R.G.), Model 8500 (Varian, Walnut Creek, CA, U.S.A.) and Model SP8000 (Spectra Physics, Darmstadt, F.R.G.).

TABLE I

SEPARATION OF MIXTURES OF PYRIMIDINE OLIGONUCLEOTIDES (DI- TO TETRAMERS) BY ION-EXCHANGE AND REVERSED-PHASE HPLC

Chromatographed mixture of sequence isomers	Sequence isomers theoretically present	Isolation of a pure isomer possible by	
		Ion exchange chromatography*	Reversed-phase chromatography**
(dC, dT)	d(C-T)	−	+
	d(T-C)	−	+
(dC₂, dT)	d(C-T-C)	−	+
	d(C-C-T)	+	+
	d(T-C-C)	−	+
(dC, dT₂)	d(C-T-T)	+	+
	d(T-T-C)	+	+
	d(T-C-T)	+	+
(dC, dT₃)	d(T-T-T-C)	+	(+)***
	d(T-T-C-T)	+	(+)***
	d(T-C-T-T)	+	(+)***
	d(C-T-T-T)	+	+
(dC₂, dT₂)	d(T-T-C-C)	+	− ⎫ §
	d(T-C-C-T)	(+)***	− ⎬
	d(T-C-T-C)	(+)***	+
	d(C-T-T-C)	(+)***	+
	d(C-T-C-T)	+	(+)***
	d(C-C-T-T)	+	(+)***
(dC₃, T)	d(T-C-C-C)	+	+
	d(C-T-C-C)	(+)***	+
	d(C-C-T-C)	(+)***	− ⎫ §
	d(C-C-C-T)	+	− ⎬

* Partisil 10 SAX (10 μm), 50 × 0.3 cm I.D., at 60°C; flow-rate 1 ml/min; triethylammonium acetate buffer (for experimental details see ref. 4).

** Nucleosil C_{18} (7.5 μm), 25 × 0.4 cm I.D.; flow-rate, 1 ml/min; methanol–ammonium acetate buffer pH 6.5 (for experimental details see ref. 6).

*** Poor resolution.

§ Not resolved.

TABLE II

SEPARATION OF MIXTURES OF PYRIMIDINE OLIGONUCLEOTIDES (PENTAMERS) BY ION-EXCHANGE AND REVERSED-PHASE HPLC

Chromatographed mixture of sequence isomers	Sequence isomers theoretically present	Isolation of a pure isomer possible by	
		Ion exchange chromatography*	Reversed phase chromatography*
(dC, dT₄)	d(T-T-T-T-C)	(+)***	(+)***
	d(T-T-T-C-T)	(+)***	(+)***
	d(T-T-C-T-T)	+	(+)***
	d(T-C-T-T-T)	+	(+)***
	d(C-T-T-T-T)	+	+
(dC₂, dT₃)	d(T-T-T-C-C)	+	− } §
	d(T-T-C-T-C)	+	− }
	d(T-C-T-T-C)	− } §	+
	d(T-T-C-C-T)	− }	+
	d(T-C-T-C-T)	− } §	+
	d(C-T-T-T-C)	− }	+
	d(C-T-T-C-T)	+	− } §
	d(T-C-C-T-T)	(+)***	− }
	d(C-T-C-T-T)	(+)***	− }
	d(C-C-T-T-T)	+	− }
(dC₃, dT₂)	d(C-T-C-T-C)	−	− } §
	d(C-T-C-C-T)	−	− }
	d(C-C-T-T-C)	−	− } §
	d(C-C-T-C-T)	+	− }
	d(C-T-T-C-C)	−	− }
	d(T-C-C-T-C)	−	− } §
	d(T-C-T-C-C)	+	− }
	d(T-T-C-C-C)	+	−
	d(C-C-C-T-T)	+	−
	d(T-C-C-C-T)	−	−

* MicroPak AX-10 (10 μm), 30 × 0.4 cm I.D. at 70°C (dC, dT₄ and dC₂, dT₃) or 60°C (dC₃, dT₂); flow-rate, 1 ml/min; triethylammonium acetate buffer (for experimental details see ref. 5).
** See Table I.
*** Poor resolution.
§ Not resolved.

RESULTS

Chemically depurinated herring sperm DNA was partially hydrolysed and separated on DEAE-cellulose and QAE-Sephadex according to the previously described separation procedure[1–3]. From the resulting mixture of pyrimidine nucleotides nine mixtures of sequence-isomeric pyrimidine oligonucleotides (Tables I and II) were isolated in pure form and in preparative amounts. It is remarkable that in the series of the pentamers only three mixtures can be isolated: (dC, dT₄), (dC₂, dT₃) and (dC₃, dT₂). The fourth possible combination (dC₄, dT) cannot be found.

The sequence isomers can be only partially isolated by anion-exchange[4,5] or reversed-phase HPLC[6]. The elution profiles of the mixtures (dC, dT₃), (dC₂, dT₂), (dC₃, dT) and (dC₂, dT₃) obtained by both separation methods (Figs. 1–4) make the problem evident.
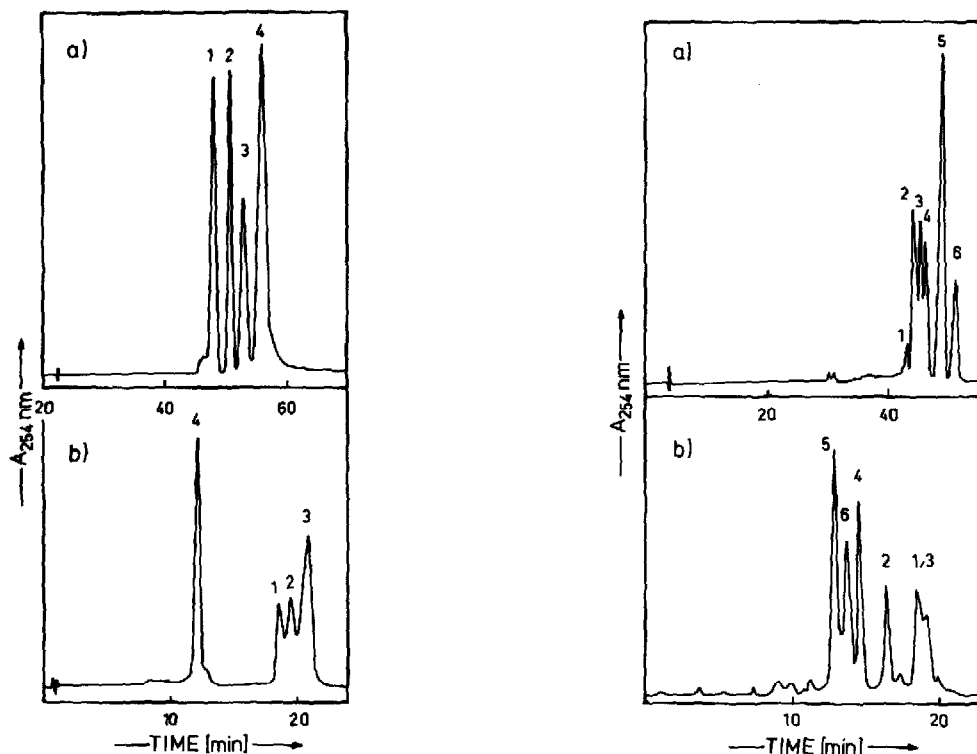
Fig. 1. Separation of the sequence isomers (dC, dT₃). (a) Column, Partisil 10 SAX (10 μm, 500 × 3 mm I.D.); temperature, 60°C; linear gradient from 0.15 M (pH 3.1) to 0.5 M (pH 3.4) of triethylammonium acetate within 70 min; flow-rate, 1 ml/min. (b) Column, Nucleosil C₁₈ (7.5 μm, 250 × 4.6 mm I.D.); room temperature; isocratic elution with 78% 0.1 M ammonium acetate and 26% methanol–water (60:40); flow-rate, 1 ml/min. The peaks were characterized by the fingerprint method as: 1 = d(T-T-T-C); 2 = d(T-T-C-T); 3 = d(T-C-T-T); 4 = d(C-T-T-T).

Fig. 2. Separation of the sequence isomers (dC₂, dT₂). (a) Column, Partisil 10 SAX (10 μm, 500 × 3 mm I.D.); temperature, 60°C; linear gradient from 0.05 M (pH 3.1) to 0.5 M (pH 3.4) of triethylammonium acetate within 70 min; flow-rate, 1 ml/min. (b) Column, Nucleosil C₁₈ (7.5 μm, 250 × 4.6 mm I.D.); room temperature; isocratic elution with 74% 0.1 M ammonium acetate and 22% methanol–water (60:40); flow-rate 1 ml/min. The peaks were characterized by the fingerprint method as: 1 = d(T-T-C-C); 2 = d(T-C-T-C); 3 = d(T-C-C-T); 4 = d(C-T-T-C); 5 = d(C-T-C-T); 6 = d(C-C-T-T).

In general, more isomers can be totally separated by anion-exchange chromatography, but some components are separated only poorly or not al all, *e.g.* peaks 2–4 in Fig. 2, peaks 2 and 3 in Fig. 3, and peaks 3–7 and 8 and 9 in Fig. 4. Reversed-phase chromatography (C₁₈) separates these isomers totally. However, some isomers separated by ion-exchange are not resolved by reversed-phase HPLC. Components 3 and 4 (Fig. 3a), for instance, are separated completely by ion-exchange chromatography but are eluted together in the first peak in reversed-phase HPLC (Fig. 3b).

By combination of reversed-phase and ion-exchange chromatography nearly all sequence isomers can be isolated from the hydrolysates of chemically depurinated herring sperm DNA up to the pentamers (Tables I and II).
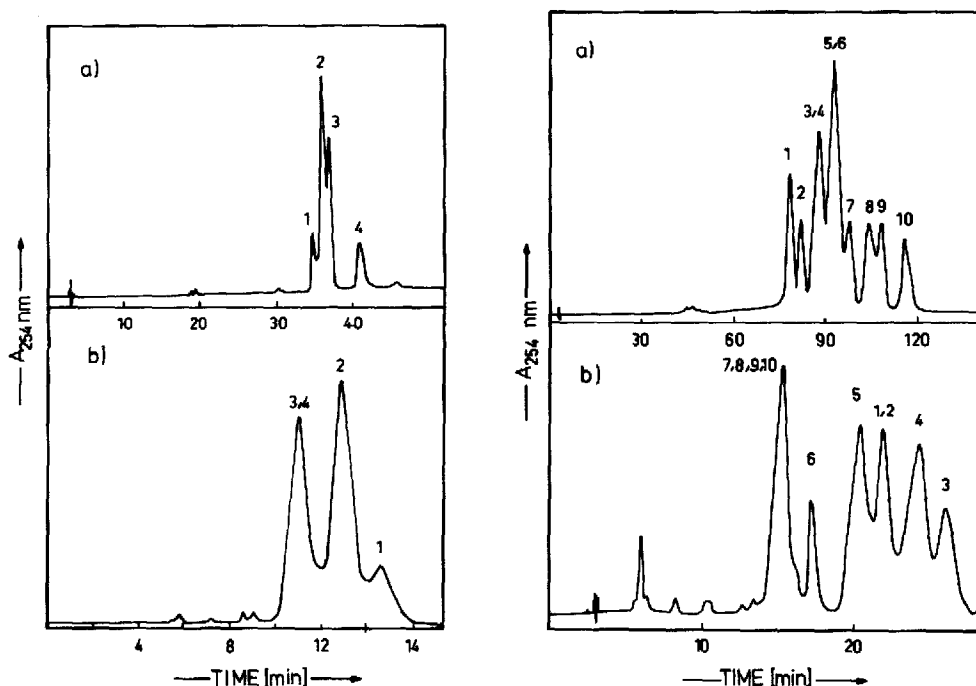
Fig. 3. Separation of the sequence isomers (dC₃, dT). (a) Column, Partisil 10 SAX (10 µm, 500 × 3 mm I.D.); temperature, 60°C; linear gradient from 0.03 M (pH 3.1) to 0.35 M (pH 3.4) of triethyl-ammonium acetate within 90 min; flow-rate, 1 ml/min. (b) Column, Nucleosil C₁₈ (7.5 µm, 250 × 4.6 mm I.D.); room temperature; isocratic elution with 66% 0.1 M ammoniumm acetate and 34% methanol–water (60:40); flow-rate, 1 ml/min. The peaks were characterized by the fingerprint method as: 1 = d(T-C-C-C); 2 = d(C-T-C-C); 3 = d(C-C-T-C); 4 = d(C-C-C-T).

Fig. 4. Separation of the sequence isomers (dC₂, dT₃). (a) Column, MicroPak AX-10 (10 µm, 300 × 4 mm I.D.); temperature, 70°C; linear gradient beginning with 30% A and 70% B and a 0.5% increase of B per minute (A = 0.01 M triethylammonium acetate, pH 3.1, and B = 0.5 M triethylammonium acetate, pH 3.4); flow-rate 1 ml/min. (b) Column, Nucleosil C₁₈ (7.5 µm, 250 × 4.6 mm I.D.); room temperature, isocratic elution with 73% 0.1 M ammonium acetate and 27% methanol–water (60:40); flow-rate, 1 ml/min. The peaks were characterized by the fingerprint method as: 1 = d(T-T-T-C-C); 2 = d(T-T-C-T-C); 3 = d(T-C-T-T-C); 4 = d(T-T-C-C-T); 5 = d(T-C-T-C-T); 6 = d(C-T-T-T-C); 7 = d(C-T-T-C-T); 8 = d(T-C-C-T-T); 9 = d(C-T-C-T-T); 10 = d(C-C-T-T-T).

DISCUSSION

The optimal procedure for separating sequence isomers starts with reversed-phase chromatography, which can be conducted under isocratic conditions at room temperature. This is an essential simplification for the separation of a substance in preparative amounts, because it leads to some advantages with respect to ion-exchange chromatography:

(1) Less equipment expense; no gradient-forming device is necessary

(2) Repetitive injections are possible without the need for re-equilibration after each chromatogram

(3) The lower temperature is favourable for the substances and the sorbent.

In case a peak contains more than one compound, the pooled and lyophilized

fraction must then be rechromatographed on an anion-exchange column. Following this order of chromatography is advantageous because all undesired products are eliminated in the first HPLC separation step. This chromatographic separation route permits isolation of oligonucleotides of defined sequences in a few chromatographic runs instead of the complicated chemical synthesis.

Beyond this, there is another interesting aspect. It can be assumed that the elution profiles of the mixtures of sequence isomers are characteristic and unique with respect to the origin of a certain DNA. It should therefore be possible to investigate DNA of various origins with respect to the relationship of their sequences by HPLC.

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