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Optimization of the separation of the Rp and Sp diastereomers of phosphate-methylated DNA and RNA dinucleotides

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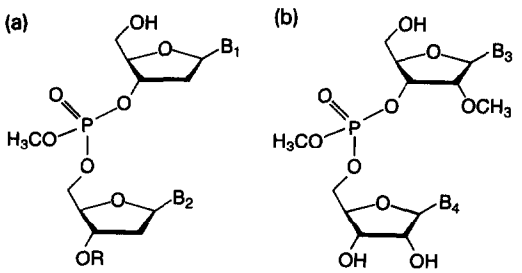
ABSTRACT

The separation by reversed-phase high-performance liquid chromatography of Rp and Sp diastereomers of phosphate-methylated DNA and RNA dinucleotides was studied with respect to pH, organic modifier type and concentration and reversed-phase packing material. Drylab G was used to deduce optimum conditions. On the basis of the observed discrepancies between the computer predictions and experimental results, the gradient operation procedure with volatile buffers was improved. By repetitive chromatography on a 250 × 22 mm I.D. reversed-phase column, fourteen diastereomeric pairs were obtained in at least 97% purity and 60% yield, in amounts of 10–100 mg.

INTRODUCTION

Phosphate-methylated 2′-deoxyribodinucleotides and 2′-O-methylphosphate-methylated ribodinucleotides (Fig. 1) show interesting structural properties [1–4]. Study of these structures using high-field proton NMR requires separation of the two diastereomeric forms present in the synthesis product. Several groups have analysed similar compounds. Cadet and Voituriez [5] used Nucleosil C_{18} and methanol–water (30:70) to separate dTp(OC_2H_5CN)T diastereomers. The separation of some O-isopropyl phosphate triesters and methyl phosphonates was performed by Stec *et al.* [6] with a volatile acetonitrile–triethylammonium acetate buffer system (pH 7). Recently Weinfeld *et al.* [7] purified O-ethylphosphate triesters on a reversed-phase system with methanol–water as an unbuffered mobile phase. These investigations all involved the isolation of the particular compounds and no optimization of the high-performance liquid chromatography (HPLC) system was carried out. Stec *et al.* [6] cautioned against predicting even the order of elution in these types of separations, let alone the relative separation factors. This means that each separation has to be optimized empirically.



R = H or $COCH_3$

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Naturally, it is of practical value if one can limit the number of phase systems to those with the largest orthogonality in selectivity. We therefore investigated the effect of the type and concentration of the organic modifier, the pH and the stationary phase to expedite the isolation of 0.01–0.1-g amounts of the Rp and Sp diastereomers of phosphate-methylated dinucleosides for study by high-resolution proton and phosphorus NMR.

EXPERIMENTAL

The isolation of 10–100-mg amounts of the diastereomers was effected in essentially three stages: (1) determination of suitable isocratic conditions using an analytical gradient system; (2) repetitive preparative separations on 10–20 mg of the analytes and collection of fractions; and (3) analysis of each fraction to assess its Rp and Sp diastereomer content. By repeating steps 2 and 3 on part of the fractions all diastereomers of d(CpG), d(GpC), d(ApC), d(ApA)ac, d(ApT)ac, d(CpC)ac, d(TpC), d(TpT), r(ApC), r(ApG), r(CpG), r(CpC), r(CpU) and r(ApU) were obtained in at least 97% purity.

The separation of the diastereomers was developed on an HP 1090 or HP 1050 (Hewlett-Packard, Waldbronn, Germany) gradient HPLC system using a built-in diode-array detector to locate the phosphate-methylated dinucleotides between highly absorbing N-(9-fluorenyl methoxycarbonyl) (Fmoc) derivatives and other reaction impurities, or using a Linear (Reno, NV, USA) Model 204 absorbance detector set at 270 nm. Preparative chromatography was performed on a high-performance liquid chromatograph consisting of a Waters (Milford, MA, USA) Model M590 solvent delivery system equipped with a solvent-selection valve module for sample introduction, an RSil C_{18} (10 μm particle size) column (250 × 22 mm I.D.) (Alltech, Deerfield, IL, USA) and a Waters Model 480 absorbance detector. Fractions were collected with an LKB (Bromma, Sweden) Model 2211 Superrac fraction collector and checked for purity on an analytical HPLC system consisting of a Spark (Emmen, Netherlands) SPH125 autosampler, an HP 1050 (Hewlett-Packard) or a Gilson (Villiers-le-Bel, France) Model 302 pump, a 250 × 4 mm I.D. Nucleosil 120-3 C_{18} reversed-phase column (Macherey–Nagel, Düren, Germany) and a linear UV-203 absorbance detector that monitored the eluate at 260 nm.

Other reversed-phase columns used for development were a 125 × 4 mm I.D. LiChrospher C_{18} (5 μm) (Merck, Darmstadt, Germany) and a 100 × 4.6 mm I.D. Microspher C_{18} (3 μm) column (Chrompack, Middelburg, Netherlands). The mobile phases were acetonitrile (gradient grade, Merck) or methanol (LiChrosolv, Merck) as organic modifiers and 0.1% (v/v) formic or acetic acid (analytical-reagent grade, Merck), 100–200 μl/l triethylamine (zur Synthese, Merck) in water purified with a Milli-Q water purification system, adjusted to the desired pH with ammonia solution (Baker Analysed Reagent, 25%, aqueous; Baker, Deventer, Netherlands).

RESULTS AND DISCUSSION

Phase system selectivity

Ribonucleotide derivatives of C and A with C, G and U were run in shallow acetonitrile and methanol gradients at pH 2.7 and 5.4 (see Table I). These are the extremes of a pH range encompassing the expected pK_a values of C and A nucleotides of 4.3 and 3.8, respectively, and well within the range of stability of reversed-phase silica-based columns, a prerequisite for preparative HPLC.

From Table I, isocratic phase systems can be calculated with the help of DryLab to compare the selectivity in a 15-min separation for the diastereomers with respect to modifier and pH (see Table II). For the r(CpG) sample a 10-min separation time was chosen, as the selectivity trend observed in Fig. 6 (and discussed below) decreases ($\alpha - 1$) rapidly on lowering the modifer concentration to obtain a 15-min separation.

It is possible that the optimum pH for separation is near the pK_a of a base owing to a slight difference in pK_a between the diastereomers, the more so if the capacity factor changes dramatically at the pK_a [6]. The variation of retention and selectivity with pH is shown in Figs. 2a and b, respectively, for some analyte pairs.

The retention of the Rp diastereomer is generally more sensitive to increase in pH and decrease in modifier concentration than that of the Sp diastereomer, so that in general the Rp diastereomer is eluted last at neutral pH and at low acetonitrile con-

TABLE I

RETENTION TIMES OF RIBONUCLEOTIDE DERIVATIVES UNDER GRADIENT CONDITIONS

Column: Microspher C_{18} (100 × 4.6 mm I.D.). Mobile phase: (A1) 0.1% formic acid–0.01% triethylamine (pH 2.7); (A2) 0.1% formic acid–0.2% triethylamine (pH 5.4); (B) acetonitrile. Gradient I: 5 → 15% B, 2 ml/min, temperature 25°C. Gradient II: 20 → 40% methanol in A1, 1 ml/min. Gradient III: 15 → 25% methanol in A2, 1 ml/min.

Compound	Gradient I								Gradient II				Gradient III			
	pH 2.7, 20 min		pH 2.7, 40 min		pH 5.4, 10 min		pH 5.4, 20 min		pH 2.7, 20 min		pH 2.7, 40 min		pH 5.4, 20 min		pH 5.4, 40 min	
	1[a]	2[a]	1	2	1	2	1	2	1	2	1	2	1	2	1	2
r(CpC)	7.00	7.40	8.59	9.17	4.09	4.29	5.43	5.75	7.70	7.78	8.20	8.32	8.75	9.50	9.97	10.95
r(CpG)	7.37	7.83	9.90	10.48	3.88	4.06	5.33	5.68	7.13	7.74	7.18	7.91	8.44	9.26	9.44	10.69
r(CpU)	8.37	8.62	11.35	11.86	4.45	4.70	5.95	6.51	7.86	7.86	8.54	8.54	8.94	10.06	10.09	11.80
r(ApC)	9.90	10.39	13.37	14.35	7.00	7.00	10.62	10.78	10.72	11.45	12.07	13.02	18.61	19.42	25.51	27.07
r(ApG)	9.19	10.33	13.70	15.07	6.85	7.26	10.90	11.65	8.92	10.00	9.85	11.39	20.17	21.59	28.52	31.04

[a] 1 and 2 denote the first and last eluting diastereomer, respectively.

TABLE II

DRYLAB PREDICTIONS FOR ISOCRATIC SEPARATIONS, BASED ON DATA IN TABLE I

Compound	Acetonitrile modifier						Methanol modifier					
	pH 2.7			pH 5.4			pH 2.7			pH 5.4		
	Modifier (%)	k'_2	$\alpha - 1$	Modifier (%)	k'_2	$\alpha - 1$	Modifier (%)	k'_2	$\alpha - 1$	Modifier (%)	k'_2	$\alpha - 1$
r(CpC)	4.5	29.8	0.09[a]	4.5	32.3	0.09	12	13.7	0.05[a]	14.5	13.6	0.13
r(CpG)	6	21.9	0.09[a]	5	32.9	0.22	5[b]	9.7	0.41[a]	14	15.1	0.26
r(CpU)	6	28.0	0.10	5	31.6	0.36				15	13.7	0.28
r(ApC)	6.5	30.4	0.15	7	35.5	0.13	20	14.2	0.10	21	13.5	0.11
r(ApG)	7	27.8	0.24	8	28.7	0.31	18	14.9	0.27	22	14.2	0.21

[a] Elution order: Rp, Sp.

[b] Separation is better at smaller k' than with a 15-min separation time (see Discussion of Fig. 6).

centrations (*i.e.*, $k' > 5$). The elution order of r(CpC) reverses at pH 3.1, below which the Rp diastereomer elutes first (see Fig. 3). Also, the elution order of d(CpG) and r(CpG) changes with pH of the mobile phase (Fig. 4a and b); similar chromatograms to those in Fig. 4a and b can be obtained with 15% methanol instead of 4–6% acetonitrile (*e.g.*, compare Fig. 4b and c). For d(CpC)ac, a maximum in resolution is found at pH 3.5 with a Nucleosil 120-3 C_{18} column and small capacity factors, and for r(CpC) at pH 4.0 with a Microspher C_{18} column (see Fig. 2b).

Overall, acetonitrile as a modifier and neutral pH appear to be a good choice in terms of selectively. We prefer acetronitrile to methanol owing to comparable selectivity, comparable expense at the modifier concentration used, the relatively low UV background at 200 nm and the low back-pressure generated. A disadvantage of the use of pH 2.7 is that the peaks of positively charged analytes show

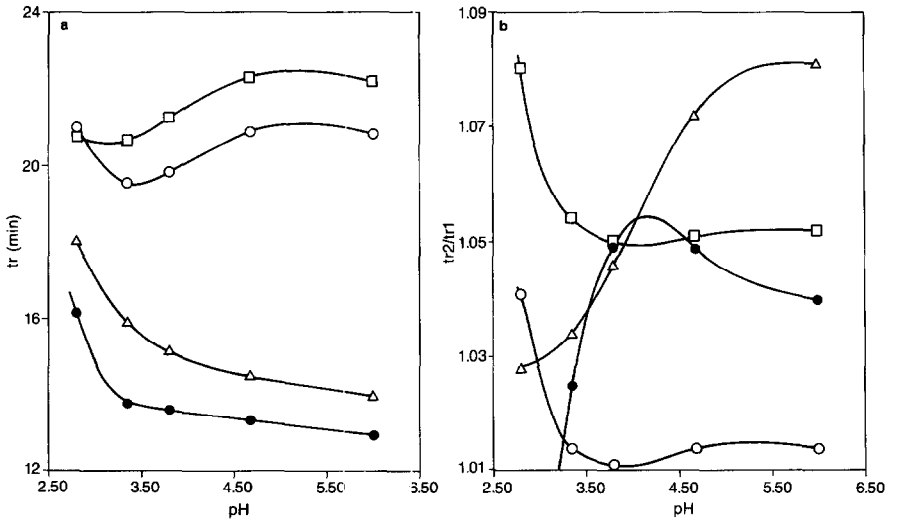

Fig. 2. Dependence of (a) retention time (tr) and (b) retention ratio of diastereomers on the pH of the mobile phase for some phosphate-methylated ribodinucleotides. Column, Microspher C_{18} (100 × 4.6 mm I.D.). Mobile phase: (A) 0.1% (v/v) formic acid–100 µl/l triethylamine adjusted to the required pH with 25% ammonia solution; (B) acetonitrile; gradient, 2–15% B in 33 min; flow-rate, 2 ml/min. Analytes: ● = r(CpC); △ = r(CpU); ○ = r(ApC); □ = (ApG).

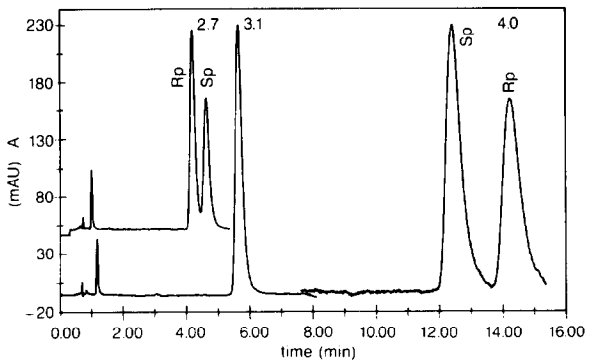

Fig. 3. Elution order reversal of r(CpC) with pH (2.7, 3.1, 4.0). Column, Microspher C_{18}; mobile phase, 6% acetronitrile–0.1% formic acid–60 μl/l triethylamine, adjusted to required pH; flow-rate, 2 ml/min.

tailing. Tailing can be decreased by increasing the ionic strength and the concentration of triethylamine as a competitive base. As shown in Fig. 5b, the capacity factors of the Sp diastereomers tend to decrease more than those of the Rp diastereomers with increasing triethylamine concentration, so that the diastereomeric selectivity at pH 2.7 increases for r(CpU) and decreases for r(CpC) and r(CpG) because at pH 2.7 the Rp diastereomers of r(CpC) and r(CpG) elute before the Sp diastereomers, and the Rp diastereomer of r(CpU) later than its Sp diastereomer (see Table II).

The selectivity of three types of reversed-phase columns was tested. On Nucleosil 120-5 C_{18} and LiChrospher 100 RP-18 the Rp diastereomers are more retained relative to the Sp diastereomers than on Microspher C_{18}, so the elution order reversal of r(CpC), r(CpG) and d(CpG) and the maximum in selectivity for r(CpC) occur at a pH that is lower (by *ca.* 0.4) on LiChrospher and Nucleosil than on Microspher. Apparently the last packing creates a more acidic environment for the analytes than the others; little effect on stereoselectivity was observed on testing packing materials having a different pore size, surface area, coverage or end-capping.

The most difficult separation, r(ApU), can be performed on a short Nucleosil 120-5 C_{18} column with a low acetonitrile concentration and at pH 4.5–6. The selectivity for r(ApU) on Nucleosil is then slightly better than that on LiChrospher and superior to that on Microspher.

There is a general trend to less retention of the Rp

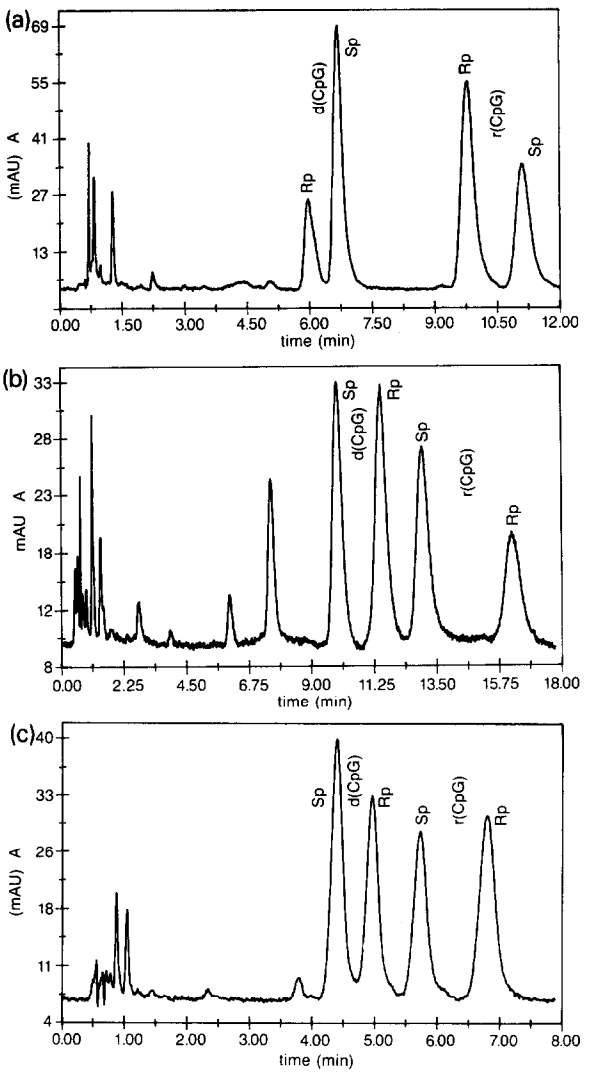

Fig. 4. Separation of d(CpG) and r(CpG) diastereomers. Column, Microspher C_{18}; flow-rate, 2 ml/min. Mobile phase: (a) 6% acetonitrile–0.1% formic acid–60 μl/l triethylamine (pH 2.7); (b) 4% acetonitrile–0.1% acetic acid–60μl/l triethylamine, adjusted to pH 5.2; (c) 15% methanol–0.1% acetic acid–60μl/l triethylamine, adjusted to pH 5.2

diastereomer relative to that of the Sp diastereomer at higher modifier concentrations. This is illustrated in Fig. 6, where for r(CpG) the selectivity factor increases with acetonitrile concentration when the Rp diastereomer is eluting first. The replacement of acetonitrile with methanol at concentration giving the same retention times for a given column and

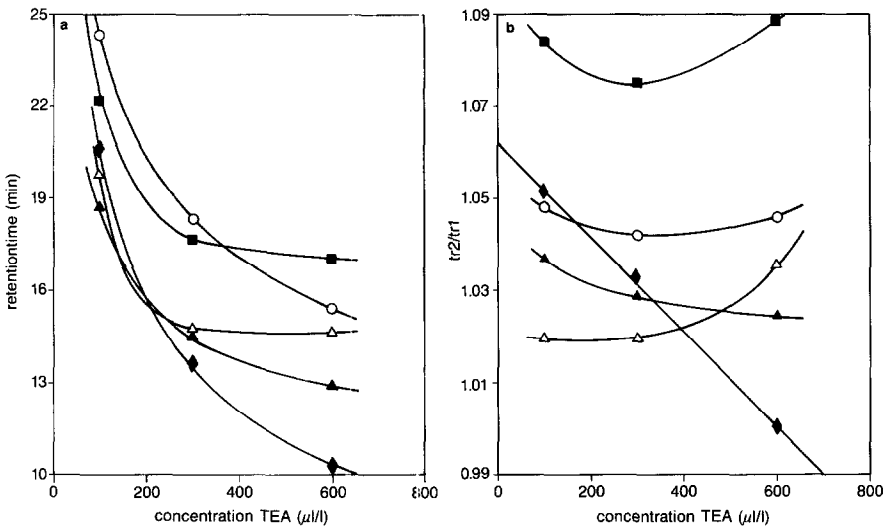

Fig. 5. Dependence of (a) retention time and (b) retention time ratio of diastereomers of phosphate-methylated ribodinucleotides on triethylamine (TEA) concentration. Column, Microspher C_{18}; flow-rate, 2 ml/min. Mobile phase: (A) 0.1–0.2% formic acid–100–600µl/l triethylamine; (B) acetonitrile; gradient, 2–14% B in 33 min. Analytes: ◆ = r(CpC); △ = r(CpU); ○ = r(ApC); ■ = r(ApG); ▲ = r(CpG).

back-pressure usually means that a much higher methanol concentration is needed. A general neat effect of the type of modifier in the experiments performed cannot be observed and often an acetonitrile concentration and pH can be found that give the same selectivity as is observed when using methanol (*cf.*, Table II and Fig. 4b and c). It can be predicted, however, that r(ApU) will be best separated in the order Rp–Sp on a Microspher C_{18} column at a low pH using methanol as a modifier (experimentally: $k' = 9.0$, $\alpha = 1.09$).

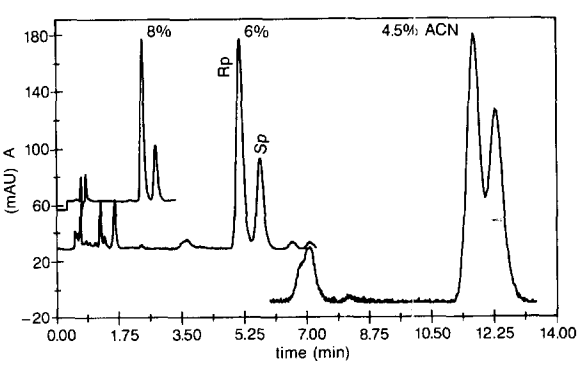

Fig. 6. Increasing resolution with modifier concentration for the separation of r(CpG). Column, Microspher C_{18}; flow-rate, 2 ml/min. Mobile phase: (a) 8% acetonitrile (ACN)–0.2% formic acid–60 µl/l triethylamine (pH 2.7); (b) 6% acetonitrile–0.2% formic acid–60 µl/l triethylamine (pH 2.7); (c) 4.5% acetonitrile–0.2% formic acid–60 µl/l triethylamine (pH 2.7).

Gradient operation

The data in Table I can be used for calculation of an optimum gradient system for a mixture that contains more than one pair of diastereomers; this is not the case with the present dimers, but more complex mixtures are obtained for larger oligomers. We used Drylab G to calculate such a gradient for the mixture of r(CpC), r(CpG), r(CpU), r(ApC) and r(ApG). The retention times of the experimental run were within 0–3% of the calculated values for the optimum gradient (see Table III). The authors of Drylab pointed out several factors that can contribute to errors in computer simulation amounting to 1–3% of the retention time [8]. This would mean additional fine tuning of the final gradient, but can be considered to be still acceptable for difficult separations. In the present case the major cause of discrepancy was not among the causes found by Dolan *et al.* [8]. Our studies were performed with a volatile buffer system to facilitate the transfer to preparative scale HPLC. The largest error turns out to be a drift

TABLE III

COMPARISON OF A CALCULATED AND EXPERIMENTAL OPTIMUM GRADIENT RESULTS FOR THE SEPARATION OF A COMPLEX TEST MIXTURE

Column: Microspher C_{18} (100 × 4.6 mm I.D.). Mobile phase: (A) 0.1% formic acid–0.01% triethylamine (pH 2.7). Gradient: 2 → 14% acetonitrile in A in 33 min, 2 ml/min.

Compound	Retention time (t_r) (min)		Δt_r (%)	ΔpH[a]
	Experimental	Calculated		
r(CpC)	14.45	13.97	3.4	0.05
	15.04	14.55	3.4	0.05
r(CpG)	15.91	15.97		
	16.39	16.38		
r(CpU)	17.13	17.13		
	17.61	17.60		
r(ApC)	18.80	18.66	0.8	0.05
	19.87	19.55	1.7	0.05
r(ApG)	19.03	19.11		
	20.29	20.21		

[a] Difference in experimental and calculated pH based on Fig. 2a. Only calculated for r(CpC) and r (ApC) owing to their strong dependence.

of 0.05 in the pH of the mobile phase over a period of 1 day. This meant that in order to use the computer program in such a way as to obtain even more accurate results we had to improve our operation with volatile buffers.

HPLC systems are to some extent sensitive to out-gassing. To eliminate bubble formation problems, the solvents are usually degassed with helium prior to use and sparged with helium during operation. From the solubility curves of gases in several solvents it seems clear that the high concentration of dissolved carbon dioxide in the organic solvent causes the problem [9]. By using three reservoirs, two with water containing different concentrations of volatile buffer, without helium sparging, and the other with pure organic modifier with helium degassing, the pH of an ammonium formate buffer of pH 4.3 (capacity = 0.005 *M* [10]) could be maintained within 0.02 over a 16-h period of use, keeping the discrepancy between calculated and experimental retention times within 1.4% (*cf.*, Fig. 2a). The two aqueous buffer solutions are very convenient for adjusting the absorbance when working at lower wavelengths (for maximum sensitivity) or for applying a slight pH gradient.

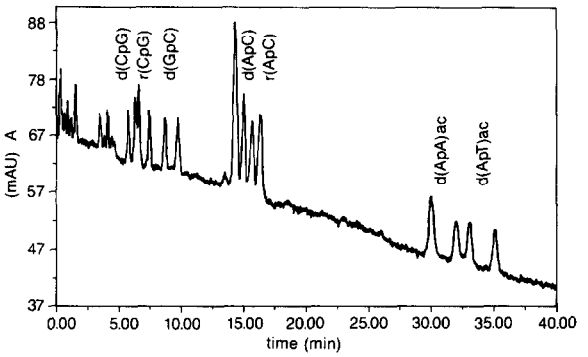

Fig. 7. Separation of a mixture of phosphate-methylated dinucleotides. Column, LiChrospher 100 RP-18 (125 × 4 mm I.D.). Mobile phase: (A) 0.1% formic acid–100 μl/l triethylamine adjusted to pH 5.3; (B) acetonitrile; gradient, 4–14% B in 40 min; flow-rate, 5 ml/min; starting pressure, 320 atm.

To demonstrate the overall separation capabilities of HPLC for the phosphate-methylated dinucleotides, a mixed organic modifier gradient separation is shown in Fig. 7.

Considering the complex dependence of selectivity on the combination of type and concentration of modifier, pH, ionic strength and competing base and stationary phase, the only sensible way to optimize such a separation is by a chemometric method with the help of a computer. Such a method is at present not available in our laboratories but it is expected to be commercially available soon and may prove invaluable for the separation of the more complex tetranucleotides that will be the next generation of modified oligonucleotides to be separated.

Preparative HPLC

After method development on an analytical scale to maximize the selectivity factor for the diastereomers, 10–100 mg amounts of the analyte mixtures were diluted with 2 ml of a solution consisting of the aqueous part of the mobile phase, containing at most half of the percentage of organic modifier to be used in preparative chromatography. By on-column concentration, volumes of 250 ml can be introduced on a 250 × 22 mm I.D. preparative column with the aid of a solvent-selection valve without much detrimental effect on the separation due to volume overloading. Mass overloading and, in particular, solubility in the injection solvent appear to be the limiting conditions.

TABLE IV

CONDITIONS AND RESULTS FOR ANALYTICAL AND SEMI-PREPARATIVE HPLC OF PHOSPHATE-METHYLATED RIBODINUCLEOTIDES

Analytical HPLC						Semi-preparative HPLC					
Compound	Mobile phase		k'_{Rp}	$\alpha_{Sp,Rp}$	$R_{Sp,Rp}$	Sample	Mobile phase		Purity (%)	Yield (%)
	Acetonitrile (%)	pH					Acetonitrile (%)	pH		
r(ApC)	10	4.5	0.0	1.10	2.1	r(ApC) 1			99.5	85
r(ApG)	12	2.5	1.9	1.33	3.8	2	8	4.5	97.5	77
r(CpG)	7	2.5	1.8	1.19	2.9	r(CpG) 1	4	2.5	99.8	86
r(CpC)	8	5.0	4.1	1.15	3.0	2	3	5.8	99.7	93
r(CpU)	8	5.0	3.4	1.12	2.0	r(CpC) 1	4	4.0	99.4	60
r(ApU)	12	4.7	3.0	1.07	1.1	2			99.5	75

The 4,4′-dimethoxytrityl-d( )methylphosphonate-( )-3′-menthoxycarbonates [where ( ) are the respective bases] were separated by Katti and Agarwal [11] using silica by virtue of the 3′-menthyl group. These derivatives are even less soluble in aqueous media, so normal-phase HPLC has to be used for isolation. Employing normal-phase HPLC we obtained recoveries of less than 50% for similar compounds, although the selectivities (but not efficiency) were much higher than with reversed-phase HPLC.

The type of reversed-phase column used for preparative HPLC (RSil-C_{18} HL, 10 μm) was different from that used in the analytical separations as it was not available at that time. The optimum conditions for analysis were therefore not always optimum for preparative HPLC [1]. The purities and yields of a few methylated ribodinucleotide pairs achieved by using 2–4% less acetonitrile and overlapping band fractionation are given in Table IV. Optimum conditions for most of the phosphate-methylated deoxyribunocleotides are given in Table I in ref. 1.

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