CHROM. 24 848

Resolution of diastereomeric phosphoramidate bridged unnatural oligonucleotides by micellar electrokinetic chromatography

C. D. Bevan* and I. M. Mutton

Structural Chemistry Department, Glaxo Group Research Ltd., Greenford Road, Greenford, Middlesex UB6 0HE (UK)

A. J. Pipe

Medicinal Chemistry Department, Glaxo Group Research Ltd., Greenford Road, Greenford, Middlesex UB6 0HE (UK)

ABSTRACT

Micellar electrokinetic chromatography was used to resolve diastereomers of oligonucleotides possessing several chiral phosphoramidate bridges. These materials were not resolved by conventional liquid chromatographic techniques. Parameters that were found to affect electrophoretic resolution included the nature and concentration of surfactant, presence of organic modifiers such as propan-2-ol and urea, use of deuterium oxide-based buffers and temperature control. Practical optimization was achieved by employing Terabe's resolution principles.

INTRODUCTION

Antisense oligonucleotides have attracted much attention as potential drugs for the treatment of cancer and viral infections. Synthesis of a specific cellular protein can be inhibited by introduction of a synthetic oligonucleotide (ODN) complementary to a region of the appropriate mRNA. The interaction is based on specific Watson–Crick base-pairing between the mRNA sense strand and the anti-sense ODN, thereby inhibiting translation to the protein. However, small ODNs normally do not penetrate well into cells, and additionally are readily degraded by intra- and extra-cellular enzymes.

Modification of the inter-nucleotide phosphate bridge to remove negative charge can lead both to improved stability and better cell penetration, as well as increased binding affinity. The phosphoramidate-linked oligonucleotides used here are known

to be stable to nuclease P1 and SVP [1,2], and were synthesised [3] as part of an investigation in this area.

Introduction of a substituted amine into the phosphate bridge gives rise to a new chiral centre at phosphorus. As the synthesis is not stereoselective, a mixture of diastereomers results, which in the case of dimers can readily be separated [1]. However, with longer ODNs the number of diastereomers arising is 2^n (where n is the number of new chiral centres), of which only a few may possess the necessary ability to base-pair in a similar manner to the unmodified oligonucleotide. Clearly, where n is greater than one or two, this provides a considerable analytical challenge.

Analysis of unmodified oligonucleotides has been accomplished using a variety of separation technologies, including reversed-phase [4], ion-pair [5] and ion-exchange [4,6] liquid chromatography, free-solution capillary electrophoresis [7], micellar electrokinetic chromatography (MEKC) [7,8] and capillary

---

* Corresponding author.

gel electrophoresis [9–13]. Separations are obtained on the basis of factors such as ionic character, lipophilicity and size. Since we were interested in the analysis of a number of modified oligonucleotides that contained no ionizable functions, we selected the technique of MEKC for further study, after first briefly assessing the utility of some liquid chromatographic approaches.

This paper demonstrates the ability of MEKC to resolve complex mixtures of ODN diastereomers and reports some effects of various experimental parameters on the separations obtained.

EXPERIMENTAL

Apparatus

Liquid chromatography was performed on a Gilson liquid chromatograph (Anachem, Luton, UK) comprised of twin No. 303 pumps and a Model 115 variable-wavelength UV absorption detector operated at 260 nm.

MEKC was performed using an Applied Biosystems (ABI, Warrington, UK) Model 270HT capillary electrophoresis system. Fused-silica capillaries (720 × 0.05 mm I.D.) were obtained from ABI. The effective length of the capillary from injector to detector (small length of external polyimide coating removed) was 500 mm. The spectrophotometric detector was employed at 260 nm and the column was normally air thermostatted at 30°C. Operation at 10°C was achieved by cooling the column compartment with carbon dioxide gas from a cylinder of the compressed liquid. The instrument was operated at various voltages between 10 and 30 kV, and sampling was achieved by applying to the detector end of the column a vacuum of 127.0 mmHg (1 mmHg = 133.322 Pa) for 1.5 s.
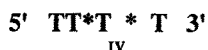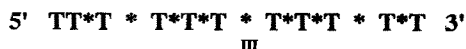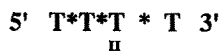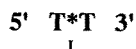
Materials

Reagents used in preparation of buffers were of analytical-reagent or electrophoresis-purity grades with the exception of dodecyltrimethylammonium bromide (DTAB) (99 + %; Aldrich, Gillingham, UK), sodium cholate, sodium taurocholate and sodium deoxycholate which were all purchased from Sigma (Poole, UK). Water was purified by passage through an Elgastat Spectrum system (Elga, High Wycombe, UK), and deuterium oxide (99.9% ^{2}H) was obtained from Aldrich. These reagents and the

electrophoretic markers [mesityl oxide (Aldrich) and Sudan III (Sigma)] were used without further purification. Test compounds analyzed (I–V) are listed in Fig. 1 and were synthesised within Glaxo Group Research.

Methods

Reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on an Aquapore C_8 5 μm particle diameter, 300 Å pore diameter column (250 × 4.6 mm I.D.) (Brownlee Aquapore RP-300; obtained from ABI). Mobile phases consisted of aqueous 0.1 M triethylammonium acetate (TEAA) at pH 7.0–acetonitrile (95:5, v/v). and pure acetonitrile. Various acetonitrile–buffer gradients were selected to optimize each particular separation. A flow-rate of 1.5 ml/min was adopted.

Ion-exchange liquid chromatography was performed on a Nucleopac PA-100 column (250 × 4 mm I.D.) obtained from Dionex (UK) (Camberley, UK). A mobile phase mixture of aqueous 25 mM tris(hydroxymethyl)aminomethane (Tris) buffer adjusted to pH 8.0 with dilute hydrochloric acid was adopted with a salt gradient of 0 to 1.0 M potassium chloride over 30 min at 1.5 ml/min.

5' T*T 3'
   I

5' T*T*T * T 3'
   II

5' TT*T * T*T*T * T*T*T * T*T 3'
   III

5' TT*T * T 3'
   IV

5' TTT-TTT-TTT-TT 3'
   V

* denotes a 3',5' chiral phosphoramidate bridge:

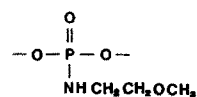$$\begin{array}{c} O \\ \parallel \\ -O-P-O- \\ | \\ NH\,CH_2CH_2OCH_3 \end{array}$$

Fig. 1. Structures of compounds studied.

Electrophoresis buffers were prepared by dissolving appropriate amounts of surfactants and urea in a solution obtained by adjusting the pH of 50 mM Tris to 7.0 with 50 mM sodium hydrogenphosphate. Citrate buffer, pH 2.5, was obtained from ABI. New capillaries were conditioned by flushing for 30 min with 1.0 M sodium hydroxide, rinsing with deionized water for 10 min, 0.1 M sodium hydroxide for 30 min and the running buffer for 30 min. Prior to each run, the column was rinsed for 5 min with the running buffer.

RESULTS AND DISCUSSION

Compounds I–IV (Fig. 1) were available as a result of non-stereoselective synthesis; introduction of chiral centres at phosphorus atoms gives compounds I–IV that can exist respectively in 2, 8, 512 and 4 diastereomeric forms, whereas the phosphate bridged compound V exists as a single isomer.

The RP-HPLC method was unable to discriminate between the diastereomers of compounds I–IV. Similarly, ion-exchange HPLC failed to resolve the diastereomers of III and IV, although V was resolved from its T deletion analogues with ease. Where there are no phosphate bridges present there is no anionic charge and therefore little retention by ion-exchange. In the case of compound IV there is a single ionic phosphate bridge present whilst all the remaining bridges are phosphoramidate. Consequently, retention of this compound is meagre with no resolution of diastereomers. Standard liquid chromatographic techniques therefore appeared unlikely to be able to distinguish between the very similar lipophilicities and ionic characters of diastereomeric phosphoramidate bridged ODNs I–IV, although it is noted that protected ODNs possessing a single terminal N-alkylphosphoramidate linkage have been resolved by RP-HPLC [1,2].

The high efficiencies and selectivities offered by MEKC are well known and so an assessment was made of the potential of the technique to address our novel analytical problem. A concurrent requirement involved analysis of conventional oligonucleotides that had the potential for duplex formation arising from self-complementarity. In order to inhibit this we had added 7 M urea to the usual sodium dodecyl sulphate (SDS) micellar solution. These urea–SDS-containing buffers were then also used for our initial experiments with compounds I–III, although these compounds are not self-complementary. Fig. 2a–c demonstrates the excellent potential that MEKC offers for the resolution of compounds I–III. Not only were the expected high plate counts obtained (370 000 for II), but the electropherograms demonstrated a degree of selectivity beyond that anticipated from consideration of RP-HPLC. The combination of efficiency and selectivity resulted in complete resolution of I and partial resolution of the eight diastereomers of II. About 35 peak maxima can be counted in the envelope obtained from III; the chemical purity and integrity of this material was confirmed by mass spectrometry [14], and further evidence that the resolution was not artefactual was obtained by subjecting the corresponding conventionally bridged, non-diastereomeric analogue V to an identical analysis, when only one peak was obtained (Fig. 2d). These results emphasise the need to examine such compounds with a variety of techniques since the isomeric nature of the materials is revealed only by examination with MEKC. The latter technique is clearly an extremely powerful tool for the analysis of phosphoramidate bridged ODN diastereomers, and so a brief examination was undertaken of the effects on resolution of some of the available experimental parameters.

Capacity factors k' of neutral ODNs I and II were obtained in the conventional manner using the equation derived by Terabe et al. [15]:

$$k' = \frac{t_{\mathrm{r}} - t_{\mathrm{eo}}}{t_{\mathrm{eo}}(1 - t_{\mathrm{r}}/t_{\mathrm{mc}})} \tag{1}$$

where t_{r}, t_{eo} and t_{mc} are the migration times of analyte, electroosmotic marker (mesityl oxide) and micelle marker (Sudan III), respectively. For anionic ODNs III and IV containing a conventional phosphate bridge, eqn. 1 was modified [16]:

$$k' = \frac{t_{\mathrm{r}} - t_0}{t_0(1 - t_{\mathrm{r}}/t_{\mathrm{mc}})} \tag{2}$$

where t_0 is the migraton time of the analyte in the absence of micelles.

Maximum resolution between two closely resolving peaks having $k'_1 \approx k'_2 = k'$ occurs when the function $f(k')$ is maximized [15]:

$$f(k') = \left(\frac{k'}{1 + k'}\right)\left(\frac{1 - t_{\mathrm{eo}}/t_{\mathrm{mc}}}{1 + (t_{\mathrm{eo}}/t_{\mathrm{mc}})k'}\right) \tag{3}$$
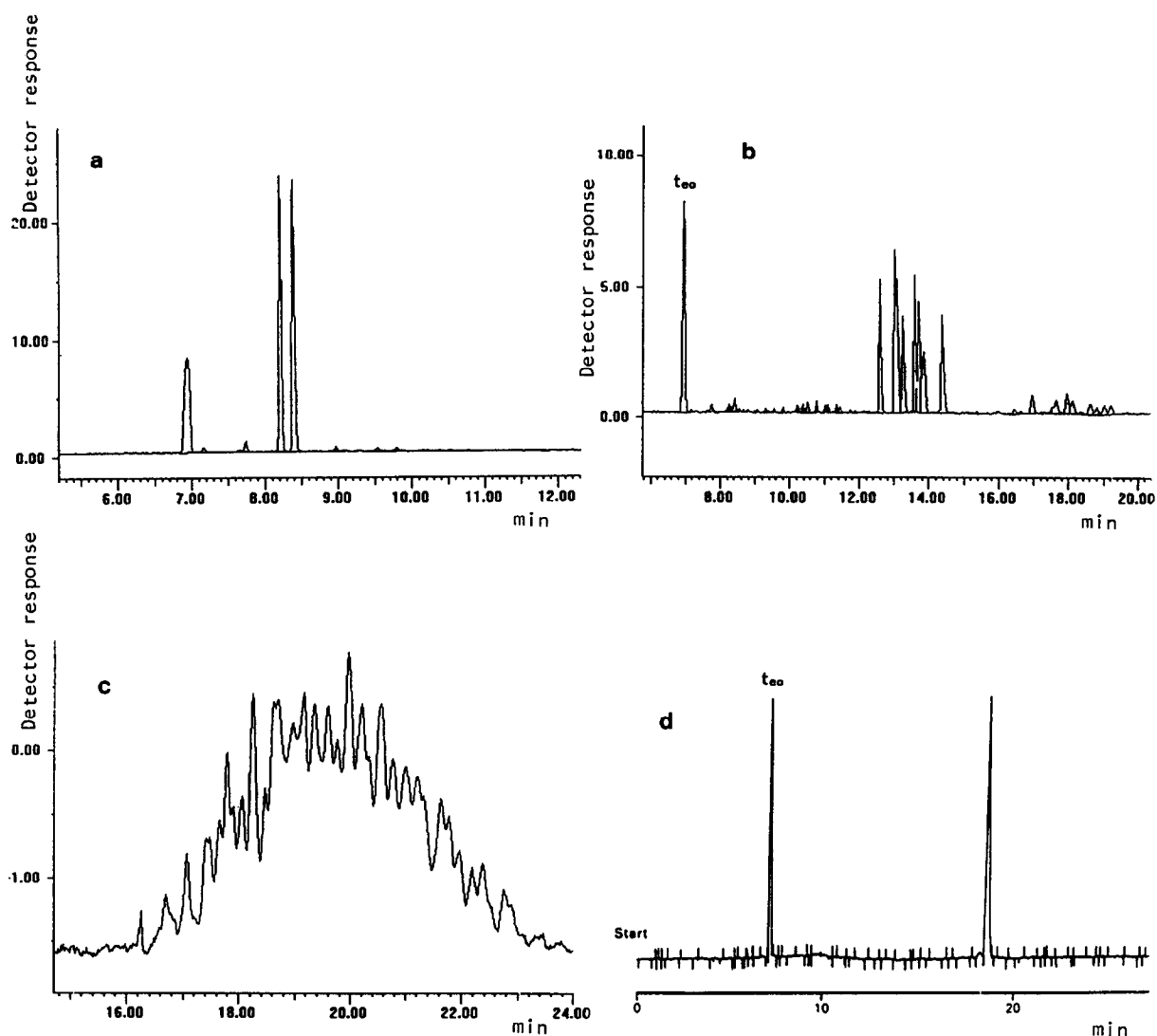
Fig. 2. Separations of phosphoramidate and phosphate bridged oligonucleotides by MEKC. Compounds: (a) **I**, (b) **II**, (c) **III**, (d) **V**. Buffer: 50 mM Tris phosphate containing 50 mM SDS and 7 M urea. Capillary: 720 mm (500 mm to detector) × 0.05 mm I.D., 20 kV, 30°C, detection at 260 nm. Migration times are shown in minutes, and detector response in arbitrary units. The apparent resolution shown by these figures is limited by the printer resolution. The actual resolution is 16 000 data points per electropherogram.

For typical t_{eo}/t_{mc} values of 0.1 to 0.3, optimal resolution is obtained when the capacity factor is about 2; more specifically, differentiation with respect to k' shows that maximum resolution occurs at $k' = (t_{mc}/t_{eo})^{1/2}$ [17].

Guided by these results, we tested the effect of SDS concentration and the presence or absence of urea on the resolution of the diastereomers of compounds **I**, **II** and **III**. (Compound **IV** provided an interesting digression and will be considered separately.) A general trend shown by the results of these experiments (summarised in Tables I–III and in Figs. 3–5) is the increase of k' with increasing SDS concentration. Deviations from linearity may reflect

TABLE I

CAPACITY FACTORS, PLATE NUMBERS (N), SEPARATION FACTORS AND RESOLUTION (R_s) OF COMPOUND I IN MEKC

Background electrolyte: 50 mM Tris phosphate (pH 7.0) containing indicated amounts of additives; capillary 720 mm (500 mm to detector) × 0.05 mm I.D., 20 kV, 30°C.

Additive		I (Peak 1)		I (Peak 2)		α^a	R_s^b
SDS (mM)	Urea (M)	k'_1	$N \cdot 10^{-3}$	k'_2	$N \cdot 10^{-3}$		
0	0	0	ND^c	0	ND^c	—	0
5	0	0.04	ND^c	0.04	ND^c	1.00	0
10	0	0.14	78	0.16	35	1.14	0.9
25	0	0.34	69	0.40	47	1.18	1.9
50	0	0.86	49	0.99	38	1.15	2.4
100	0	3.4	69	4.2	59	1.24	3.6
0	7	0	ND^c	0	ND^c	—	0
50	7	0.21	370	0.24	270	1.14	3.0
100	7	0.31	430	0.35	340	1.13	4.5

^{a} α = Separation factor, k'_2/k'_1.
^{b} R_s = Resolution, defined by $R_s = 1.177 \ (t_2 - t_1)/(w_1 + w_2)$, where t_1 and t_2 are the respective migration times of diastereomers of compound I and w_1 and w_2 the corresponding peak widths at half height.
^{c} ND = Not determined (insufficient resolution).

inaccuracies in the precise location of t_{eo}, distortion of peak maxima when efficiencies were low, and subjective assessment of the migration time of the apex of the envelope of peaks obtained from III. Addition of urea greatly decreased the capacity factors of the analytes and increased the micellar window t_{mc}/t_{eo}. Thus t_{eo}/t_{mc} was 0.14 in the presence of 50 mM SDS and 7 M urea, but almost doubled to

0.27 in the absence of urea. These effects are in close agreement with data reported by Terabe et al. [18]. An additional effect of added urea was to greatly

TABLE III

CAPACITY FACTORS OF APEX (k'_a) AND SIMPLE RESOLUTION ASSESSMENT OF MEKC OF COMPOUND IV

Conditions as for Table I.

SDS (mM)	Urea (M)	k'_a	Resolution^a
0	0	0	1
5	0	3.1	51
10	0	4.9	50
25	0	9.1	26
50	0	25	20
0	7	0	1
10	7	0.17	1
15	7	1.2	44
20	7	1.9	39
25	7	2.3	54
30	7	2.8	49
35	7	3.2	49
40	7	3.7	47
50	7	2.8	41

^{a} Resolution of the complex envelope comprised of a theoretical 512 components was evaluated by simply counting the number of peak maxima that appeared within the total envelope.
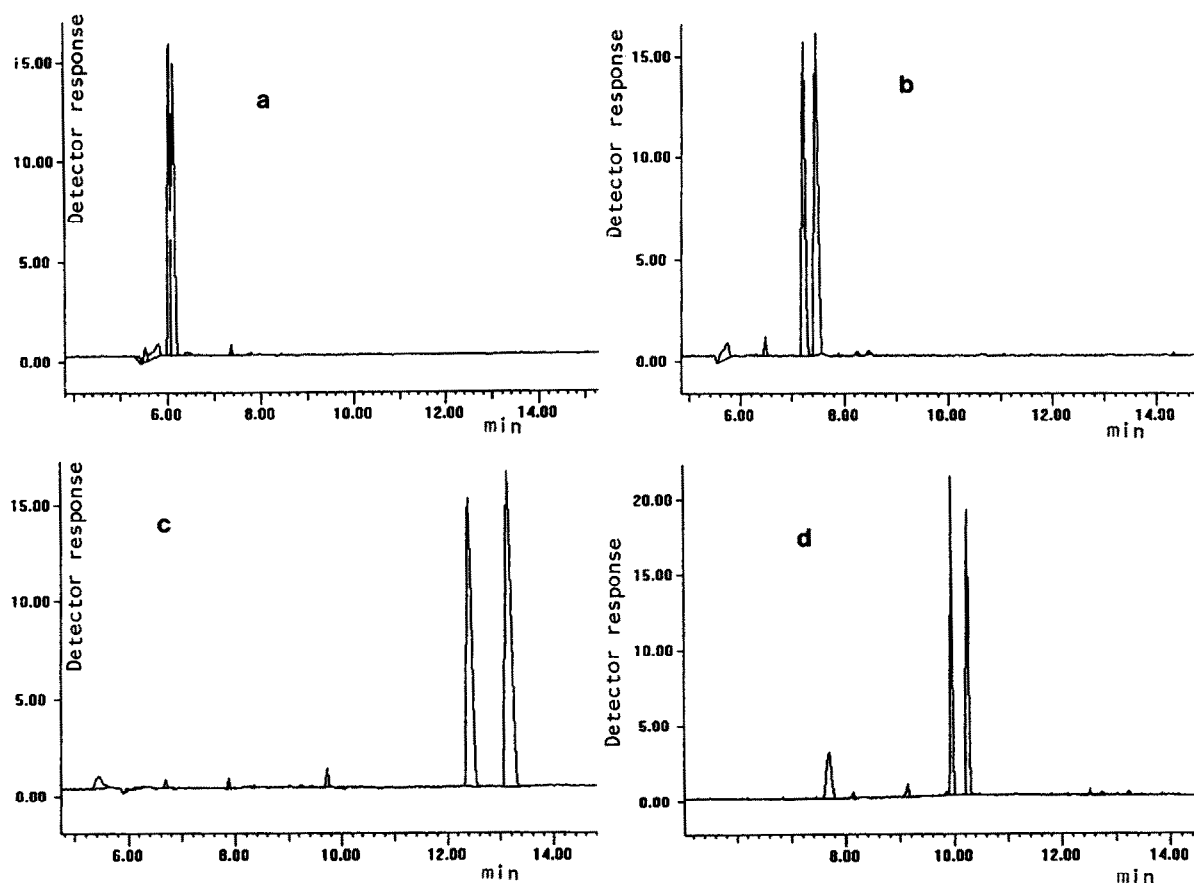
TABLE II

CAPACITY FACTORS^a, PLATE NUMBERS (N)^a AND NUMBER OF PEAKS RESOLVED (NR) BY MEKC OF COMPOUND II

Conditions as for Table I.

SDS (mM)	Urea (M)	k'_a	$N \cdot 10^{-3\,a}$	NR
0	0	0	ND^b	1
5	0	0.37	ND^b	1
10	0	1.1	ND^b	5
25	0	3.3	100	7
50	0	14	200	7
0	7	0	ND^b	1
10	7	0.03	ND^b	1
50	7	1.2	200	5

^{a} Measured on fourth peak of anticipated octet.
^{b} ND = Not determined (insufficient resolution).

Fig. 3. Effects of SDS concentration and urea on the separation of diastereomers of I. (a) 10 m*M* SDS, (b) 25 m*M* SDS, (c) 100 m*M* SDS, (d) 100 m*M* SDS + 7 *M* urea. Other conditions as in Fig. 2.

enhance the plate count of the least lipophilic analyte I; although this was not observed for II and III.

Figs. 3–5 show not only the practical use of manipulating k' into the optimal range for maximum resolution (*e.g.*, $k' > 20$ in Fig. 5b and c, but < 10 in Fig. 5a and d), but also that the beneficial effect of increased efficiency caused in certain cases by addition of urea can be dominant. Thus although best resolution of II and III diastereomers are obtained within the k' range of approximately 1 to 10, the high efficiencies obtained by using urea concentrations that depress k' of I to 0.3 [*i.e.*, to low values of f(k')], resulted in improved diastereomer separation for this analyte.

Addition of organic modifiers is a well established means of modifying the migration and selectivity characteristics of an MEKC system [19,20]. The addition of 5% (v/v) propan-2-ol to a running buffer containing 50 m*M* SDS and no urea impaired selectivity and reduced values of k' by a factor of approximately 10, destroying all resolution of the isomers of compounds I and III. However, despite some concomitant loss of resolution, 6 peaks of the octet from II remained resolved, due to modifier-specific selectivity changes and to the very high plate counts generated. ($N \approx 530\,000$ for compound with migration time 11.66 min in Fig. 6.) Higher levels of propan-2-ol and modifiers such as methanol and acetonitrile caused considerable reductions of both
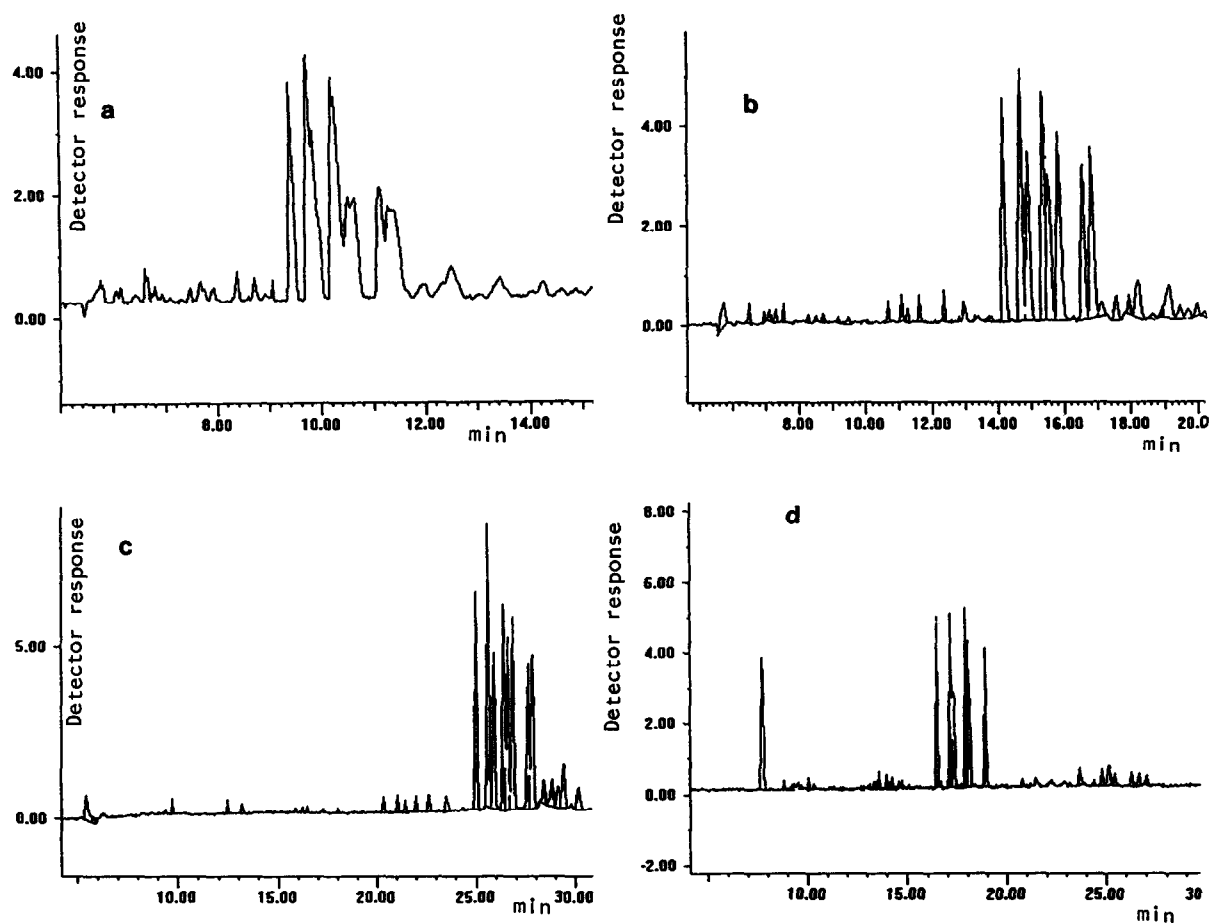
Fig. 4. Effects of SDS concentration and urea on the separation of diastereomers of II. (a) 10 mM SDS, (b) 25 mM SDS, (c) 100 mM SDS, (d) 100 mM SDS + 7 M urea. Other conditions as in Fig. 2.

capacity factors and selectivity values. Lower levels of modifiers would appear to warrant further study.

Micelles comprised of bile salts have been used to facilitate MEKC of hydrophobic analytes [21] and to promote selectivity toward chiral substrates [22, 23]. Buffer solutions containing 25 mM cholic, taurocholic or deoxycholic acid sodium salts, both with and without 7 M urea, were prepared and screened for selectivity towards I–III. Generally, low values of k' and resolution were obtained. Only with deoxycholate in the absence of urea was there any evidence of significant interaction with the micelle. Capacity factors were ca. 4 × lower than with SDS, and selectivity was also much reduced. Clearly the

nature of the SDS micelle contributes substantially towards the selectivities evident in Figs. 2–6. Cole et al. [21] have ascribed differences in behaviour of SDS and bile salts in MEKC to the "inverted micelle" formed by bile salts.

Attempts were made to optimize resolutions of I–III by using a deuterium oxide-based buffer. This approach has been shown for example to improve resolutions of certain nucleosides and dansylated amino acids, due in part to a reduction in electro-osmotic flow arising from the increased viscosity of deuterium oxide solutions over their aqueous counterparts [24]. Despite a significant reduction in electroosmosis, there was little change in selectivity
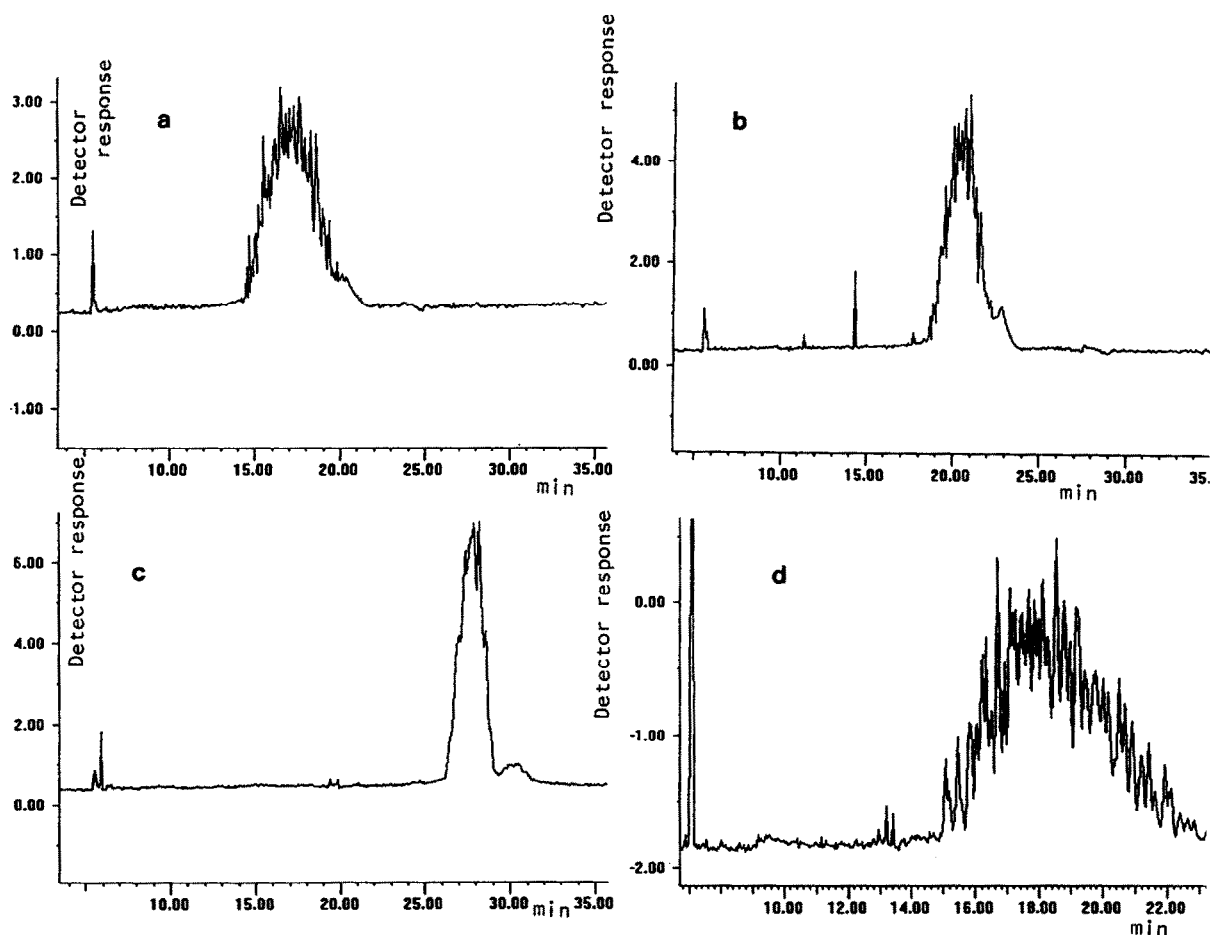
Fig. 5. Effects of SDS concentration and urea on the separation of diastereomers of III. (a) 10 mM SDS, (b) 25 mM SDS, (c) 100 mM SDS, (d) 25 mM SDS + 7 M urea. Other conditions as in Fig. 2.

or resolution of I–III. An increase of about 35% in the efficiency obtained by the use of deuterium oxide was offset by a reduction in capacity factors of I by about 10% to lower values of f(k').

Optimal resolutions of compounds II and III were obtained by reduction of Joule heating by running at 10°C and 15 kV (Fig. 7), when all eight diastereomers of II were resolved completely. Here $N \approx 230\ 000$ and the main beneficial effect of temperature control is evident in selectivity changes. Furthermore, the peak envelope derived from III shows evidence for at least 45 components.

Compound IV differs from II in that a covalent chiral bridge has been replaced by an ionic achiral

phosphate bridge, and from III in that it possesses a significantly shorter covalently bridged chain. However, even in the absence of urea, a capacity factor k' of only 0.94 was obtained for IV at [SDS] of 100 mM. There was no resolution of diastereomers (Fig. 8a). Retention and selectivity were both greatly diminished in comparison to compound II (k' = 14 at [SDS] = 50 mM, Table II), and it is clear that electrostatic repulsion excludes II from the negatively charged micelle. Note that although similar considerations must apply to compound III, the greater lipophilic character of this molecule evidently overcomes the electrostatic repulsion, enabling significant interaction with the micelle. Therefore, in
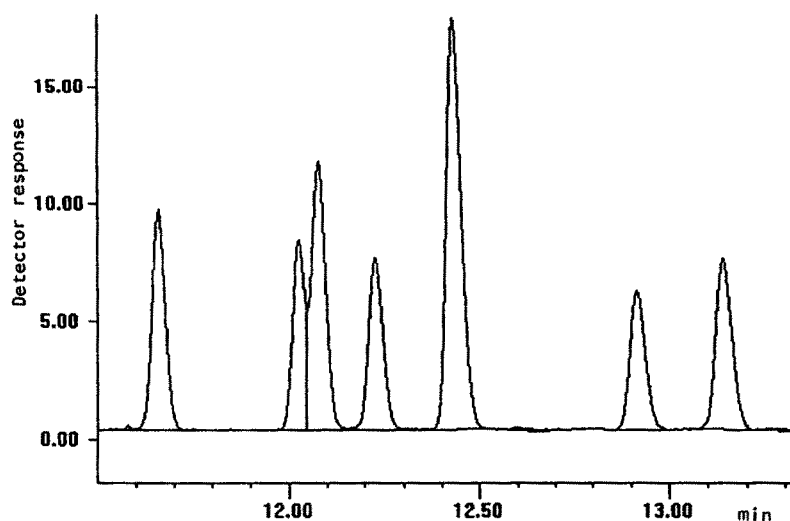
Fig. 6. Effect of propan-2-ol on the separation of the diastereomers of II. 50 mM SDS, 5% (v/v) propan-2-ol, no added urea. Other conditions as in Fig. 2.

order to facilitate interaction of IV with a micellar system, we selected cationic micelles of dodecyltrimethylammonium bromide (DTAB). Polarity was reversed to accommodate anodic electroosmotic flow [25,26]. Ion pairing and dispersive interactions combined to give excellent resolution of the quartet of diastereomers of IV (Fig. 8b). The DTAB system could also be used for the resolution of isomers of compounds I–III, although for the latter two the resolution was somewhat inferior to that obtainable with SDS micelles.

Finally, some comment is appropriate in regard to the remarkable differences observed between the selectivities towards modified ODN diastereomers of alkylsilyl reversed-phase and long-chain alkyl micellar electrokinetic systems. These two separation modes derive their generally similar selectivities primarily from dispersive interactions. Diastereomers of molecules such as I–IV have tertiary shapes and dispositions of hydrophobic surfaces that are determined by the chirality at the phosphorus atom(s). These structures, whilst not being restricted in interactions with the widely spaced fixed alkyl strands of an RP-HPLC selector, may exhibit differential abilities to penetrate the more intimate dynamic internal environment of a micelle. Further

data and detailed molecular modelling studies would be necessary to substantiate this argument.

MEKC using cationic or anionic micelles as appropriate is a highly selective method for the analysis of diastereomeric oligonucleotides containing phosphoramidate bridges and could reasonably be expected to be applicable to the stereochemical analysis of oligonucleotides possessing alternative bridging chemistries such as phosphate esters and phosphorothioates that have been summarised by Uhlmann and Peyman [27].

CONCLUSIONS

MEKC has been shown to be an extremely powerful means to obtain information about the distribution of diastereomers in samples of synthetic phosphoramidate bridged oligonucleotides. Both anionic and cationic micellar systems are effective, but there appears to be little interaction with bile salt micelles. Capacity factors can be controlled by variation of micelle concentration and by addition of modifiers such as propan-2-ol and urea. In certain instances, efficiencies are substantially improved by addition of modifiers, and plate counts in excess of one million per metre have been observed. Resolu-
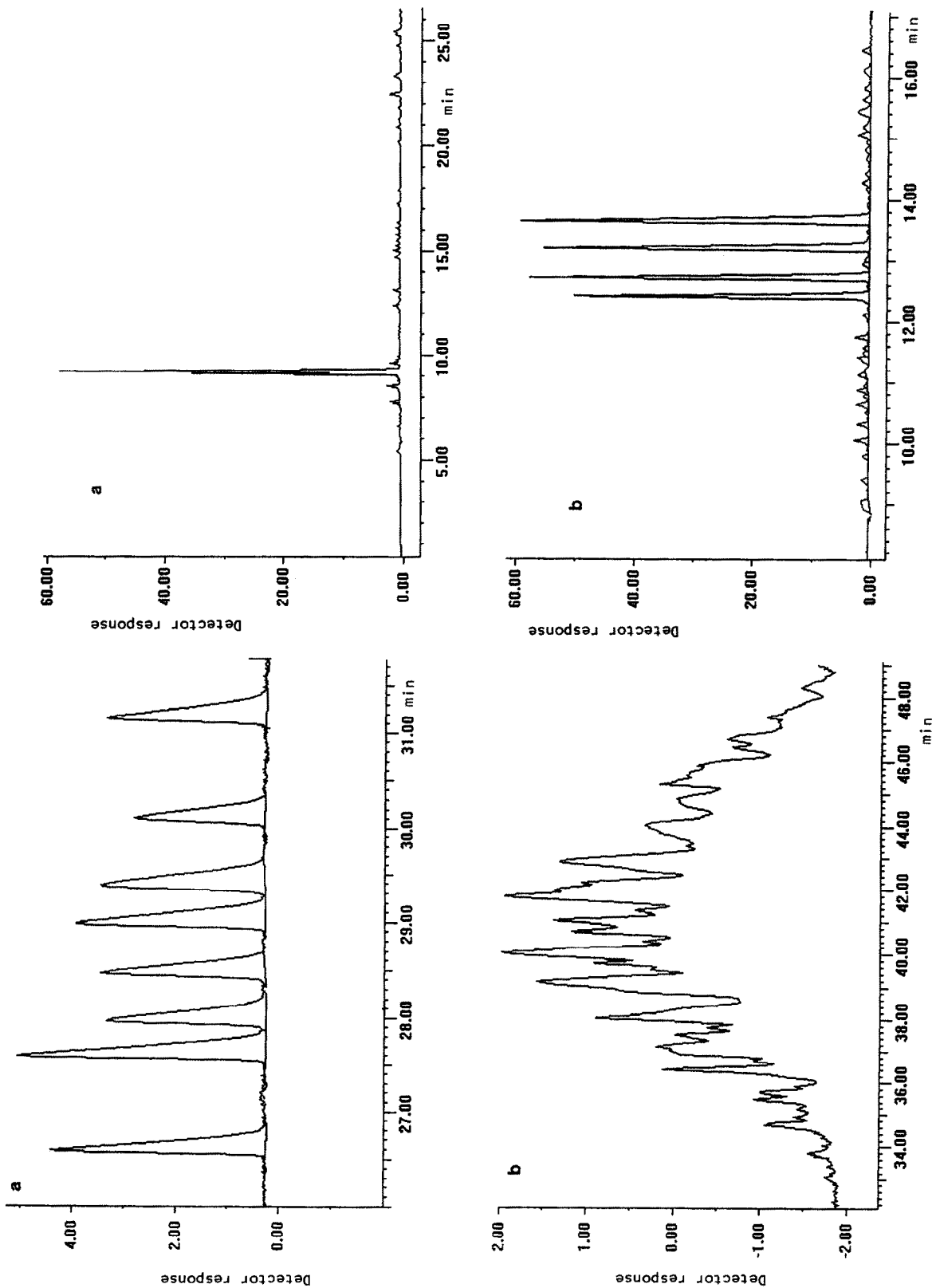
Fig. 7. Effects of control of Joule heating and sub-ambient operation on the separation of diastereomers of (a) II and (b) III. 40 mM SDS, 7 M urea, 15 kV, 10°C. Other conditions as in Fig. 2.

Fig. 8. Separations of the diastereomers of IV using (a) SDS and (b) DTAB micelles. (a) Conditions as in Fig. 2a. (b) 100 mM DTAB in pH 2.5 citrate, voltage −15 kV (i.e., polarity reversed); other conditions as in Fig. 2.

tions are controllable and can be refined if required by minimizing Joule heating and operating at sub-ambient temperatures. The role of concentrations of propan-2-ol below 5% (v/v) and of urea below 7 M would appear to merit investigation.

REFERENCES

1 A. Jager, M. J. Levy and S. M. Hecht, *Biochemistry*, 27 (1988) 7237–7246.
2 R. L. Letsinger, S. A. Bach and J. S. Eadie, *Nucleic Acids Res.*, 14 (1986) 3487–3499.
3 B. C. Froehler, *Tetrahedron Lett.*, 27 (1986) 5575–5578.
4 S. Agrawal, J. Y. Tang and D. M. Brown, *J. Chromatogr.*, 509 (1990) 396–399.
5 J. C. Bigelow, L. R. Chrin, L. A. Mathews and J. J. McCormak, *J. Chromatogr.*, 533 (1990) 133–140.
6 Y.-F. Maa, S.-C. Lin, Cs. Horváth, U.-C. Yang and D. M. Cruthers, *J. Chromatogr.*, 508 (1990) 61–73.
7 V. Dolnik, J. Liu, J. F. Banks, Jr., M. Novotny and P. Boček, *J. Chromatogr.*, 480 (1989) 321–330.
8 A. S. Cohen, S. Terabe, J. A. Smith and B. L. Karger, *Anal. Chem.*, 59 (1987) 1021–1027.
9 A. S. Cohen, D. R. Nagarian, A. Paulus, A. Guttman, J. A. Smith and B. L. Karger, *Proc. Natl. Acad. Sci. U.S.A.*, 85 (1988) 9660–9663.
10 A. Guttman, A. S. Cohen, D. N. Heiger and B. L. Karger, *Anal. Chem.*, 62 (1990) 137–141.
11 A. Paulus and J. I. Ohms, *J. Chromatogr.*, 507 (1990) 113–123.
12 H.-F. Yin, J. A. Lux and G. Schomburg, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 13 (1990) 624–627.
13 D. Demorest and R. Dubrow, *J. Chromatogr.*, 559 (1991) 43–56.
14 C. D. Bevan, W. P. Blackstock, K. Brinded, R. J. Dennis, T. Haley, H. Muenster and E. Schroeder, in R. M. Caprioli (Editor), *Proceedings of the 39th ASMS Conference on Mass Spectrometry and Allied Topics, Nashville, TN, May 1991*, ASMS, East Lansing, MI.
15 S. Terabe, K. Otsuka and T. Ando, *Anal. Chem.*, 57 (1985) 834–841.
16 M. G. Khaledi, S. C. Smith and J. K. Strasters, *Anal. Chem.*, 63 (1991) 1820–1830.
17 J. P. Folcy, *Anal. Chem.*, 62 (1990) 1302–1308.
18 S. Terabe, Y. Ishihama, H. Nishi, T. Fukuyama and K. Otsuka, *J. Chromatogr.*, 545 (1991) 359–368.
19 A. T. Balchunas and M. J. Sepaniak, *Anal. Chem.*, 59 (1987) 1466–1470.
20 J. Gorse, A. T. Balchunas, D. F. Swaile and M. J. Sepaniak, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 11 (1988) 554–559.
21 R. O. Cole, M. J. Sepaniak, W. L. Hinze, J. Gorse and K. Oldiges, *J. Chromatogr.*, 557 (1991) 113–123.
22 H. Nishi, T. Fukuyama, M. Matsuo and S. Terabe, *J. Microcol. Sep.*, 1 (1989) 234–241.
23 S. Terabe, M. Shibata and Y. Miyashita, *J. Chromatogr.*, 480 (1989) 403–411.
24 P. Camilleri and G. Okafo, *J. Chromatogr.*, 541 (1991) 489–495.
25 T. Tsuda, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 10 (1987) 622–624.
26 J. Liu, F. Banks, Jr. and M. Novotny, *J. Microcol. Sep.*, 1 (1989) 136–141.
27 E. Uhlmann and A. Peyman, *Chem. Rev.*, 90 (1990) 543–584.