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Review

Prediction of the behaviour of oligonucleotides in high-performance liquid chromatography and capillary electrophoresis

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ABSTRACT

The present state of studies on prediction of the chromatographic and electrophoretic behaviour of oligonucleotides is reviewed; particular emphasis is given to high-performance liquid chromatographic and capillary electrophoretic separations. Attention is paid to fundamental theory for the prediction of retention and migration times, and bandwidths. The article also deals with the applicability of the theory to the computer-assisted prediction and the computer simulation for these two types of separation of oligonucleotides. Optimization of separation conditions using the computer simulation system is briefly described.

CONTENTS

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LIST OF ABBREVIATIONS

CE	Capillary electrophoresis
CGE	Capillary gel electrophoresis
CZE	Capillary zone electrophoresis
HPLC	High-performance liquid chromatography
PC	Personal computer

LIST OF SYMBOLS

B, B'	Gradient steepness parameters
C	Eluent salt concentration at time t in gradient elution
C_i, C_f	Initial and final eluent salt concentrations in gradient elution
C'	Eluent salt concentration in isocratic elution
C_1	Constant
E	Applied field
E_a	Activation energy for the viscous flow
K_R	Retardation coefficient
N	Plate number
Q	Net charge of polynucleotides
R	Universal gas constant
S	Volume fraction of organic solvent in eluent at time t in gradient elution
S_i, S_f	Initial and final volume fraction of organic solvent in gradient elution
S'	Volume fraction of organic solvent in eluent in isocratic elution
T	Absolute temperature
T'	Gel concentration (g/100 ml)
a, b	Constants in eqn. 3
d	Constant in eqn. 10
k'	Solute capacity factor
l	Effective length (cm) of the capillary up to the detection point
m, n	Constants in eqn. 7
p	Total chain length of the polynucleotide expressed in nucleotide units
p_t, p_a, p_c, p_g	The number of each type of base (T, A, C and G) in the polynucleotide
t	Time (min)
t_D	Dwell time (min) of gradient equipment between the outlet of the gradient-generating device and column inlet
t_f	Gradient time (min)
t_g	Retention time (min) in gradient elution
t_0	Column dead time (min)
t', t'_0	Migration time (min) of solute in the presence and the absence of gel
$t'(T_p)$	Migration time of T_p
$t'(A_p)$	Migration time of A_p
$t'(C_p)$	Migration time of C_p
$t'(G_p)$	Migration time of G_p
u	Flow-rate (ml/min)
w_g	Bandwidth in gradient elution
x	Parameter characterizing gradient shape in eqn. 1
η	Viscosity of the surrounding gel–buffer medium
μ, μ_0	Mobility of solute in the presence and the absence of gel
σ_T^2	Total variance of the band broadening in CGE separation
σ_{Inj}^2	Partial variance arised from the electrokinetic injection
σ_{Det}^2	Partial variance arised from the finite detection volume
$\sigma_{\Delta T}^2$	Partial variance arised from thermal gradients across the capillary
σ_{Diff}^2	Partial variance arised from diffusion of bands in the gel

1. INTRODUCTION

There is a great deal of interest in the separation and characterization of oligonucleotides and polynucleotides. Rapid separations and significant resolving power would result in important potential advances for molecular biology and biotechnology, including gene mapping, DNA sequencing and purification of DNA probes. Chromatography [1] and electrophoresis [2] have been recognized as the main techniques for the separation of oligonucleotides, polynucleotides, RNA, and DNA. More recently, high-performance liquid chromatography (HPLC) [3,4] and capillary electrophoresis (CE) [5,6] have been developed rapidly. In HPLC separations, several novel packing materials, including ion-exchange, reversed-phase and mixed-mode, have been developed for the separation of oligonucleotides and polynucleotides [7–27]. Ion exchangers based on non-porous polymers [12–14] have led to high resolving powers for polynucleotides. Mixed-mode packing materials [18,19], which have both ionic and hydrophobic functional groups and allow two modes of interaction with the solutes, showed high resolution for polynucleotides via mixed interactions. Reversed-phase packing materials [15–17] have provided an excellent means of rapidly and efficiently purifying DNA probes. The gradient elution technique is essential for the separation of complex mixtures of oligonucleotides when using these packing materials. In CE separations, capillary zone electrophoresis (CZE) was used for the separation of oligonucleotides [28–30], and high-speed single-base resolution of polynucleotides was demonstrated with capillary gel electrophoresis (CGE) [31–51]. CGE has been successfully applied to the rapid DNA sequencer [52–57].

Although HPLC and CE methods are the most suitable techniques for separating oligonucleotides and polynucleotides, it remains a time-consuming and troublesome task to optimize the separation conditions. Most routine HPLC and CE methods are still being developed by trial-and-error methods. This situation is rapidly changing with the increasing availability of powerful personal computers (PC) for HPLC and CE method development [20–22,58–60]. A computer-assisted prediction system for retention and migration times has been developed, which enables researchers easily to find optimum separation conditions [20–27,55]. Recently a more efficient system, the so-called HPLC and CE computer simulation system, which can simulate visually HPLC and CE separations, was developed by combining a computer-assisted prediction system with computer graphics.

This review deals with advances in the prediction of the chromatographic and electrophoretic behaviour of oligonucleotides and polynucleotides. Fundamental theories for the prediction of retention times and bandwidths under gradient elution will be described, because the gradient elution technique is essential for the separation of oligonucleotides. Theories for the prediction of migration times and bandwidths for CGE separations will be described, because CGE has been mainly used for the separation of oligonucleotides and polynucleotides. Application of the theories to the computer-assisted prediction and computer simulation system and to the optimization of the separation conditions are described.

2. OVERVIEW OF COMPUTER-ASSISTED PREDICTION AND COMPUTER SIMULATION

This section gives a practical overview of the general procedures. A flow diagram is given in Fig. 1 to illustrate the specific steps that should be taken in computer-assisted prediction and computer simulation, and these can be explained as follows.

(A) Store experimental results on a floppy disk

The theory for *ab initio* (non-empirical) prediction, which simulates an HPLC and a CE separation before the actual run, has never been established. Most computer-assisted prediction and computer simulation systems operate in a semi-empirical mode. After the minimum number of actual HPLC or CE runs under specific separation conditions, it is possible to predict the separations with further changes in conditions.

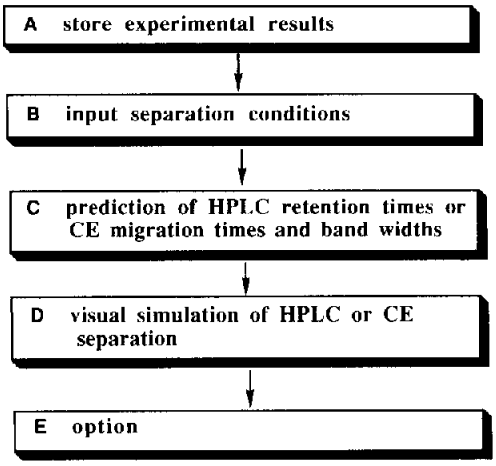

Fig. 1. Flow diagram of the computer-assisted prediction and computer simulation system.

Experimental results, therefore, should be obtained prior to prediction, and then one must input them into the system or store them on a floppy disk or a hard disk for later re-use. Details of the method for obtaining experimental results are described in Sections 3.1 and 4.1.

(B) Input separation conditions

To predict HPLC retention times or CE migration times and bandwidths, the following parameters are input into the system: (1) column or capillary conditions; (2) separation conditions; and (3) extra-column or extra-capillary effects.

Examples of parameters input into the system for HPLC separations are as follows. The column conditions, which include the type of column (ion-exchange, reversed-phase or mixed-mode), column diameter, column length, particle size and mobile phase flow-rate, affect the resolution, analysis speed, column pressure, peak height and solvent consumption per run. The theoretical plate number and column dead time can be predicted from the column conditions, otherwise one must measure them for the specific analytical column and input them into the system. Elution conditions are the composition of the mobile phase and the gradient profile. Extra-column effects include the extra-column residence time and band broadening.

Examples of parameters for CE separations are as follows. The capillary conditions, which include the type of capillary (open or gel-filled), capillary length (total and effective) and the gel composition. The theoretical plate number can be estimated from the capillary conditions, otherwise one should measure it and input it into the system. The electrophoretic conditions are the composition of the buffer and the electric field strength. Extra-capillary effects include the sample injection conditions, because extra-capillary band broadening is mainly caused by the sample injection, as will be described in Section 4.1.

(C) Prediction of HPLC retention times or CE migration times and bandwidths

After inputting of several of the parameters listed above, the prediction system estimates the retention and migration times, and bandwidths. Details of these steps are described in Sections 3.1 and 4.1.

(D) Visual presentation of chromatogram and electropherogram

Simulated chromatograms or electropherograms are displayed on the monitor screen using predicted retention times or migration times and bandwidths. Simulated ones are given ideal Gaussian profiles with arbitrary units of area for each peak. At this stage, the PC exhibits its power of graphical presentation, *i.e.* visual simulation. Such computer graphics help the chromatographer to understand how to change the chromatographic separation by varying the elution conditions.

(E) Other options

To help in optimizing the procedure and method development, other functions are available in the prediction system, such as the presentation of a relative resolution map.

3. PREDICTION OF OLIGONUCLEOTIDE BEHAVIOUR IN HPLC SYSTEMS

3.1. Theoretical aspects

Two research groups derived general theories for the prediction of chromatographic behaviour

under gradient elution: (1) the approach of Jandera and Churacek [59,60] and (2) the approach of Dolan *et al.* [58]. The former theory has been principally applied to the computer-assisted retention prediction and computer simulation of HPLC separations for oligonucleotides [20 26]. Emphasis will therefore be placed on the theory of Jandera and Churacek [59,60].

3.1.1. Ion-exchange HPLC

Jandera and Churacek [59,60] introduced a new function for gradient profiles. The function was chosen because of the possibility of describing a variety of gradients of any linear, convex or concave shape. Fig. 2 shows typical gradient profiles obtained from the following equation:

$$C = (C_i^{1/x} + Bt)^x \quad (1)$$
$$B = B'u = (C_f^{1/x} - C_i^{1/x})t_f$$

where C is the eluent salt concentration at time t, C_i is the initial salt concentration of eluent at the beginning of the gradient elution ($t = 0$ min), C_f is the final one at the end of gradient elution ($t = t_f$), t_f is gradient time and u is the flow-rate (ml/min). The parameter B describes the gradient steepness. The parameter x characterizes the shape of the gradient profile: linear, $x = 1$; convex, $x < 1$; and concave $x > 1$.

The retention time, t_g, under the gradient profile expressed as eqn. 1, can be predicted from eqn. 2 [20–27,59,60]:

$$t_g = (1/u)\{(1/B')[(xb + 1)B'at_0u + C_i^{(xb+1)/x}]^{1/(xb+1)} - C_i^{1/x}/B'\} + t_0 \quad (2)$$

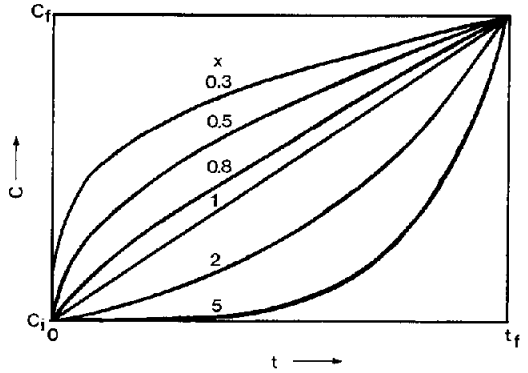

Fig. 2. Gradient profiles expressed as eqn. 1 by changing x values. (From ref. 26 with permission.)

where a and b are constants characteristic of each oligonucleotide and t_0 is the column dead time (min). Prior to the prediction of retention times using eqn. 2, it is necessary to obtain constants a and b and save them to a floppy disk as described in Section 2A. These constants are obtainable from the relationship (eqn. 3) between the isocratic eluent salt concentration, C', and the capacity factor, k', measured in isocratic elution ion-exchange HPLC [25,26]:

$$k' = a\,C'^{-b}$$
$$\log k' = -b \log C' + \log a \quad (3)$$

The bandwidth, w_g, with gradient elution can be calculated from eqn. 4 [20–27,59,60]:

$$w_g = (4t_0/N^{1/2})\{1 + a[C_i^{1/x} + B'(t_g - t_0 - t_D)^{-xb}\} \quad (4)$$

where N is the plate number in isocratic elution chromatography and t_D is the system dwell time between the outlet of the gradient-generating device and the column inlet.

3.1.2. Reversed-phase HPLC

The retention time, t_g, under the gradient profile expressed as eqn. 5 can be predicted by eqn. 6 [21,59,60]:

$$S = S_i + Bt \quad (5)$$
$$B = B'u = (S_f - S_i)/t_f$$

$$t_g = (1/u)(1/nB')[\log(2.3 \ln (B'mt_0u + 10^{nS_i}))] - S_i/B'u + t_0 \quad (6)$$

where S is the volume fraction of organic solvent in eluent at time t, S_i is the initial volume fraction of organic solvent at the beginning of the gradient elution ($t = 0$ min), S_f is the final one at the end of gradient elution ($t = t_f$), and m and n are constants characteristic of each oligonucleotide. Prior to the prediction of retention times using eqn. 6, it is necessary to obtain constants m and n and save them to a floppy disk as described in Section 2A. These constants are obtainable from the relationship (eqn. 7) between the volume fraction of organic solvent, S', and the capacity factor, k', measured in isocratic elution reversed-phase HPLC [21,59,60]:

$$k' = m\,10^{-nS'}$$
$$\log k' = -nS' + \log m \quad (7)$$

The bandwidth, w_g, in gradient elution can be calculated from eqn. 8 [21,59,60]:

$$w_g = (4t_0/N^{1/2})\{m\ 10^{-m[S_i + B'(t_g - t_0 - t_D)]} + 1\} \quad (8)$$

Eqns. 6 and 8 can be used for linear gradient elution, and further applied to the prediction of retention times and bandwidths with gradient elution using steps with different slopes, such as multi-linear gradients.

3.1.3. Mixed-mode HPLC

General theory for mixed-mode HPLC has never been established. Eqns. 2 and 4 are applicable to the prediction of retention times and bandwidths when a salt gradient is used. Eqns. 6 and 8 can be applied when a gradient of the volume fraction of organic solvent is used.

3.2. Retention prediction and computer simulation of HPLC separations

Baba and co-workers [20–26] developed a computer-assisted retention prediction system that is an alternative to computer simulation for the separation of oligonucleotides by ion-exchange, reversed-phase and mixed-mode HPLC. The system, which is based on Jandera and Churacek's approach [59,60], predicted the retention times and bandwidths of oligonucleotides and simulated the chromatogram.

The ion-exchange chromatographic behaviour of oligonucleotides was predicted by eqns. 2 and 4. Comparison of the simulated chromatogram with the observed one shown in Fig. 3 illustrates that the simulated chromatogram of oligouridylates under binary-linear salt gradient elution conditions was very similar to the observed one with respect to the retention times, bandwidths, and resolution.

The retention times of oligonucleotides under several elution conditions were predicted and compared with the observed t_g values. Tables 1 and 2 list the predicted and observed retention times of oligoadenylates under the binary-linear and the binary-convex gradient, respectively. The 60 observed t_g values were predicted with an average error of 7.7% [25]. Fig. 4 shows the computer simulation of an ion-exchange chromatographic separation of oligoadenylates under a binary-convex gradient and illustrates that eqns. 2 and 4 are successfully applied to the prediction of the chromatographic behaviour of oligonucleotides of a wide range of chain lengths.

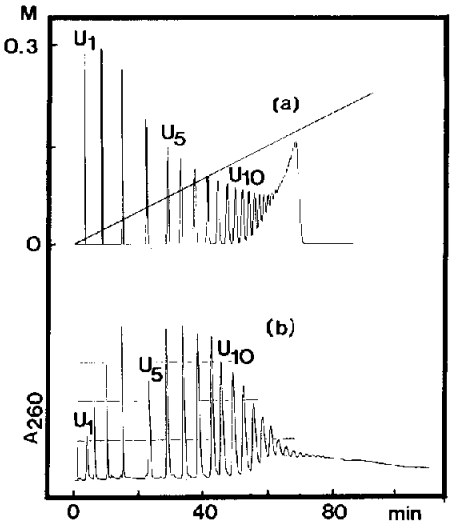

Fig. 3. (a) Simulated and (b) observed chromatograms for oligouridylates, U_n, in ion-exchange chromatography. Gradient profile expressed as eqn. 1 using the following parameters: x = 1.0, t_t = 120 min, C_i = 0.01 M and C_f = 0.3 M. Column, Shim-pack WAX-1 (weak anion exchanger, 50 mm × 4.0 mm I.D.); flow-rate, 1.0 ml/min; eluent A, 0.01 M phosphate (pH 6.8) containing 20% acetonitrile; eluent B, 0.3 M phosphate (pH 6.8) containing 20% acetonitrile; column temperature, 40°C.

The reversed-phase chromatographic behaviour of oligonucleotides was also predicted by the computer-assisted retention prediction system under a binary-linear gradient of the volume fraction of the organic solvent, as shown in Fig. 5. The retention times and bandwidths in reversed-phase chromatography were calculated from eqns. 6 and 8 within an average error of 8%. Therefore, the simulated and the observed chromatograms in Fig. 5 were very similar in their characteristics.

The HPLC separation of oligonucleotides using a mixed-mode column was simulated and the results are illustrated in Fig. 6. The retention times and bandwidths were estimated from eqns. 2 and 4, because the linear salt gradient elution

TABLE 1

OBSERVED AND CALCULATED RETENTION TIMES IN ANION-EXCHANGE HPLC OF OLIGOADENYLATES, A_n, UNDER LINEAR GRADIENT ELUTION [25]

Column and eluents as in Fig. 3. Gradient profile is expressed as eqn. 1 using the following parameters: $x = 1$, $C_i = 0.01$ M, $C_f = 0.3$ M, $t_f = 128$ min; column temperature, 40°C.

Solute	Observed (min)	Calculated (min)	Error (%)
A_1	4.72	4.05	−14
A_2	11.6	11.6	0
A_3	19.8	20.3	2.5
A_4	28.2	30.9	9.6
A_5	35.5	40.2	13
A_6	42.1	45.9	9.0
A_7	47.9	52.3	9.2
A_8	53.3	57.9	8.6
A_9	57.2	62.8	9.8
A_{10}	61.7	67.1	8.8
A_{11}	65.8	70.9	7.8
A_{12}	69.5	74.3	6.9
A_{13}	73.0	77.2	5.8
A_{14}	76.3	79.9	4.7
A_{15}	79.4	82.3	3.7
A_{16}	82.2	84.4	2.7
A_{17}	84.9	86.4	1.8
A_{18}	87.5	88.1	0.69
A_{19}	89.8	89.7	−0.11
A_{20}	92.0	91.2	−0.87
A_{21}	94.1	92.6	−1.6
A_{22}	96.1	93.8	−2.3
A_{23}	97.4	94.9	−2.6
A_{24}	99.2	96.0	−3.2
A_{25}	101	97.0	−4.0

was used for the separation. In this instance, the chromatographic behaviour was predicted successfully with an average error of 5%. Fig. 6 clearly demonstrates that the computer-assisted retention prediction system easily reproduces the observed chromatogram, even in the mixed-mode chromatography.

These results show that the computer-assisted retention prediction system is applicable to the prediction of chromatographic behaviour of oligonucleotides in ion-exchange, reversed-phase and mixed-mode chromatography under various gradient elution conditions and to the optimization of elution conditions, as will be described in Section 5.

TABLE 2

OBSERVED AND CALCULATED RETENTION TIMES IN ANION-EXCHANGE HPLC OF OLIGOADENYLATES, A_n, UNDER CONVEX GRADIENT ELUTION [25]

Gradient elution conditions, eluents and column as in Fig. 4.

Solute	Observed (min)	Calculated (min)	Error (%)
A_1	5.04	3.74	−26
A_2	11.7	8.70	−26
A_3	17.8	14.8	−17
A_4	23.7	23.7	0
A_5	30.7	33.3	8.5
A_6	38.0	39.8	4.7
A_7	45.0	48.1	6.9
A_8	52.0	56.0	7.7
A_9	58.8	63.8	8.0
A_{10}	65.1	70.6	8.4
A_{11}	72.0	77.2	7.2
A_{12}	78.9	83.4	5.7
A_{13}	86.0	89.2	3.7
A_{14}	92.9	94.6	1.8
A_{15}	99.6	99.6	0
A_{16}	106	104	−1.9
A_{17}	112	109	−2.7
A_{18}	117	113	−3.4
A_{19}	122	116	−4.9
A_{20}	127	120	−5.5
A_{21}	132	123	−6.8
A_{22}	136	126	−7.4
A_{23}	140	130	−7.1
A_{24}	145	132	−9.0
A_{25}	149	135	−9.4
A_{26}	153	137	−10
A_{27}	158	140	−11
A_{28}	162	142	−12
A_{29}	165	144	−13
A_{30}	169	146	−14
A_{31}	172	147	−15
A_{32}	175	149	−15
A_{33}	179	151	−16
A_{34}	182	152	−16
A_{35}	185	154	−17

4. PREDICTION OF OLIGONUCLEOTIDE BEHAVIOUR IN CE SYSTEMS

CGE was principally used for the separation of oligonucleotides and polynucleotides. Emphasis will therefore be placed on the theory for CGE. In the CGE separations, several parameters af-

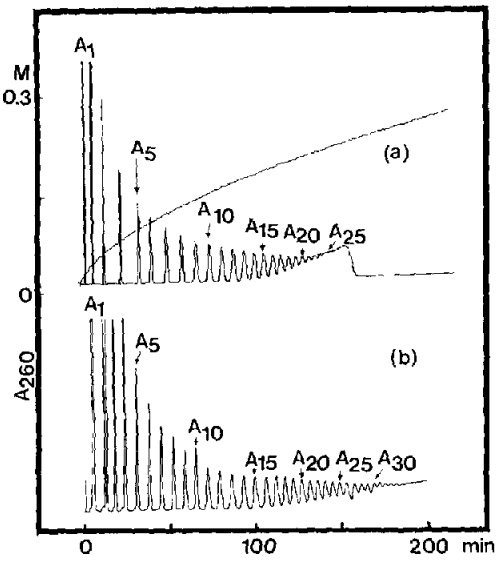

Fig. 4. (a) Simulated and (b) observed chromatograms for oligoadenylate, A_n, in ion-exchange chromatography. Gradient profile expressed as eqn. 1 using the following parameters: x = 0.58, t_r = 240 min, C_i = 0.01 *M* and C_f = 0.3 *M*. Other conditions as in Fig. 3. (From ref. 25 with permission.)

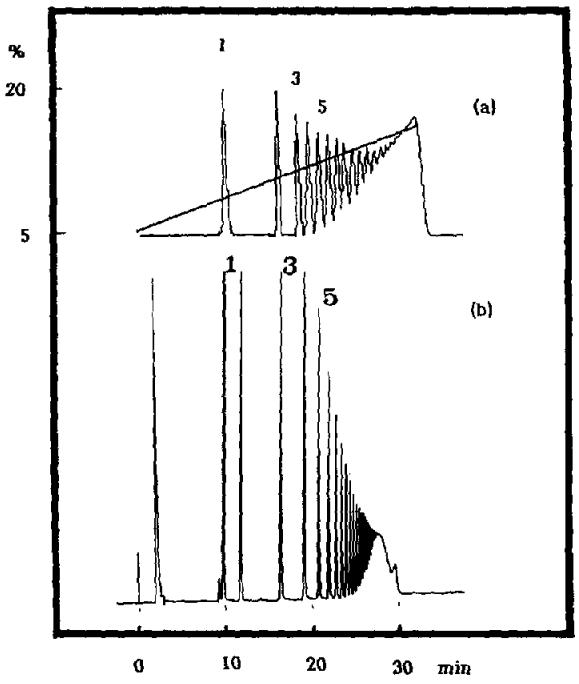

Fig. 5. (a) Simulated and (b) observed chromatograms for oligoadenylate, A_n, in reversed-phase chromatography. Numbers on the peaks represent the number of bases in the oligomer. Gradient profile expressed as eqn. 5 using the following parameters: x = 1.0, t_r = 45 min, S_i = 5% and S_f = 25%. Column, TSKgel OligoDNA RP (reversed-phase column, 150 mm × 4.6 mm I.D.); flow-rate, 1.0 ml/min; eluent A, 0.1 *M* ammonium acetate (pH 7.0) containing 5% acetonitrile; eluent B, 0.1 *M* ammonium acetate (pH 7.0) containing 25% acetonitrile; column temperature, 40°C.

fect the electrophoretic behaviour, *e.g.* the capillary length (total and effective), the electric field, the solute charge, the solute size, the base composition of oligonucleotides, the gel composition, the buffer composition, and the capillary temperature. The relationships between the electrophoretic behaviour and these parameters are formulated as eqns. 9–15, and are applicable to the prediction of the electrophoretic behaviour of oligonucleotides.

4.1. Theoretical aspects

The migration time (t') of spherical charged particles in electrophoresis, including CZE and CGE, can be expressed as eqn. 9 [5,33]:

$$t' = l \cdot 6\pi r\eta / EQ \qquad (9)$$

where l is the effective length of the capillary up to the detection point, η is the viscosity of the surrounding buffer medium, E is the applied field, Q is the net charge of spherical charged particle and r is the root-mean-square radius of the spherical particle.

The separation of oligonucleotides in CGE is based on the molecular sieving effect. The theoretical model that describes the electrophoretic mobility of oligonucleotides in a gel sieve is the Ogston model [53,55,61–63]. This model assumes that the gel pores do not greatly affect the shape of the migration oligonucleotide, but mainly serve as a molecular sieve: the pore size is considered to be much larger than the size of the DNA molecule. Separation is accomplished on the basis of the probability that the migrating solute will find a pore large enough to accommodate its passage. The equation derived by Ogston for mobility is

$$\ln \mu = \ln \mu_0 - dpT' \qquad (10)$$

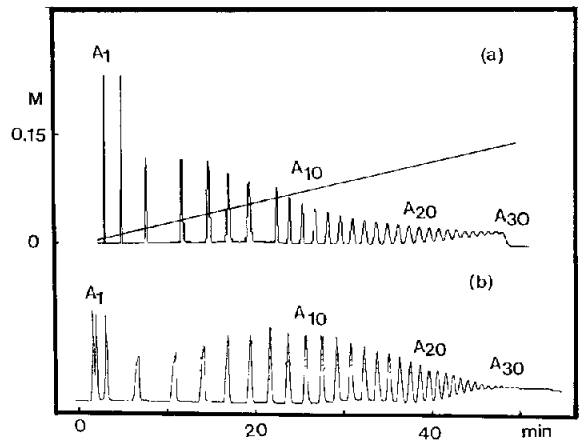

Fig. 6. (a) Simulated and (b) observed chromatograms for oligoadenylate, A_n, in mixed-mode chromatography using a salt gradient. Gradient profile expressed as eqn. 1 using the following parameters: $x = 1.0$, $t_r = 150$ min, $C_i = 0.01$ *M* and $C_f = 0.15$ *M*. Column, Neosorb-LC-N-7R (mixed-mode column, 250 mm × 4.0 mm I.D.); flow-rate, 1.0 ml/min; eluent A, 0.01 *M* sodium perchlorate with Tris acetate (pH 7.5) and 1 m*M* EDTA; eluent B, 0.15 *M* sodium perchlorate with Tris acetate (pH 7.0) and 1 m*M* EDTA; column temperature, 40°C.

where μ and μ_0 are the mobilities of the solute in the presence and the absence of gel, d is a constant of proportionality, T' is the concentration of the gel-forming polymer and p is the total chain length of the oligonucleotide expressed in nucleotide units. Substituting the term dp into the retardation coefficient, K_R, we obtain the Ferguson relationship [64] as follows:

$$\ln \mu = \ln \mu_0 - K_R T' \quad (11)$$

$$\ln t' = \ln t'_0 + K_R T' \quad (12)$$

where t' and t'_0 are the migration times of the solute in the presence and the absence of gel.

The effect of temperature on the migration time of oligonucleotides in CGE separation can be expressed by a modification of eqn. 9 [33]:

$$t' = l \cdot 6\pi r\eta/EQ \quad (9)$$

$$\eta = C_1 \cdot \exp(E_a/RT)$$

$$\ln t' = \ln(l \cdot \text{constant}/EQ) + E_a/RT \quad (13)$$

where C_1 is a constant, E_a is the activation energy for the viscous flow, R is the universal gas constant and T is the absolute temperature.

The effect of the base composition of heterooligonucleotides on the migration time can be expressed as follows [34,65]:

$$t' = (p_t/p)t'(T_p) + (p_a/p)t'(A_p) + (p_c/p)t'(C_p) + (p_g/p)t'(G_p) \quad (14)$$

where p_t, p_a, p_c and p_g are the numbers of each base (T, A, C and G) in the polynucleotide with total chain length of p ($= p_t + p_a + p_c + p_g$), and $t'(T_p)$, $t'(A_p)$, $t'(C_p)$ and $t'(G_p)$ are the migration time of each homooligonucleotides with the chain length of p, *i.e.* T_p, A_p, C_p and G_p.

The function describing the bandwidth in the CGE separation includes several sources of band broadening [53]. The net effect of the band broadening mechanisms can be expressed as the summation of the respective peak variances. All sources are assumed to be independent of one another. These variances arise from several sources: σ^2_{Inj}, the electrokinetic injection; σ^2_{Det}, the finite detection volume; $\sigma^2_{\Delta T}$, thermal gradients across the capillary; and σ^2_{Diff}, diffusion of bands in the gel. The total variance (σ^2_T) is thus:

$$\sigma^2_T = \sigma^2_{Inj} + \sigma^2_{Det} + \sigma^2_{\Delta T} + \sigma^2_{Diff} \quad (15)$$

4.2. Migration prediction in CE separation

Several factors affect the separation in CGE, including the gel composition, the capillary temperature, the base composition, the capillary length and the applied field. The effects of the capillary length and the applied field on the migration time are easily predictable from the linear relationship as expressed in eqn. 9 [32,36,44,52]. Fig. 7 illustrates the effect of the gel concentra-

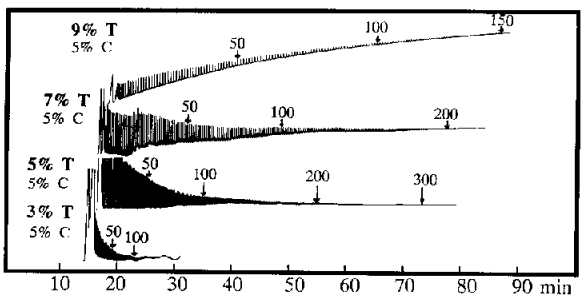

Fig. 7. Effect of the gel concentration, T', on the separation of polyadenylates. Capillary, 30 cm effective length; field, 200 V/cm. (From ref. 48 with permission.)

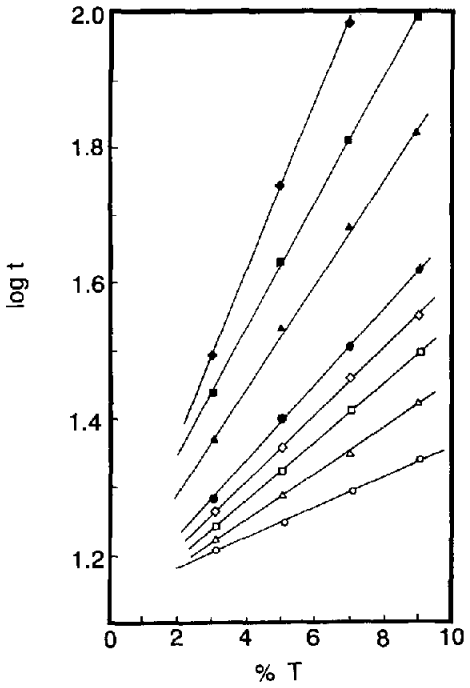

Fig. 8. Ferguson plots of polyadenylates: (○) 10mer; (△) 20mer; (□) 30mer; (◇) 40mer; (●) 50mer; (▲) 100mer; (■) 150mer; (◆) 200mer. (From ref. 48 with permission.)

tion, T' (%), on the separation of oligonucleotides [48]. Eqns. 10 and 11 predict that the mobility of oligonucleotides is higher in low $\%T'$ gels [32,37,44,48,52,53]. Therefore, a decrease in $\%T'$ led to an increase in the separation speed. Ferguson plots, as shown in Fig. 8, gave straight lines for all oligonucleotides as predicted by eqn. 12. The retardation coefficients, K_R, can be estimated from the slopes of these linear relationships and applicable to the prediction of the migration time of oligonucleotide at different gel concentrations.

The migration times of oligonucleotide and DNA fragment decrease with an increase in temperature in accordance with the relationship between the migration time and temperature as expressed in eqn. 13 [33,46]. The plots of the logarithm of the migration time *versus* reciprocal temperature showed a linear relationship, as shown in Fig. 9 [33]. These data agree well with eqn. 13, and the activation energy, E_a, can be calculated from the slope of these linear relationships. Therefore, the migration time of an oligonucleotide at a different capillary temperature is predictable from eqn. 13, using each E_a value.

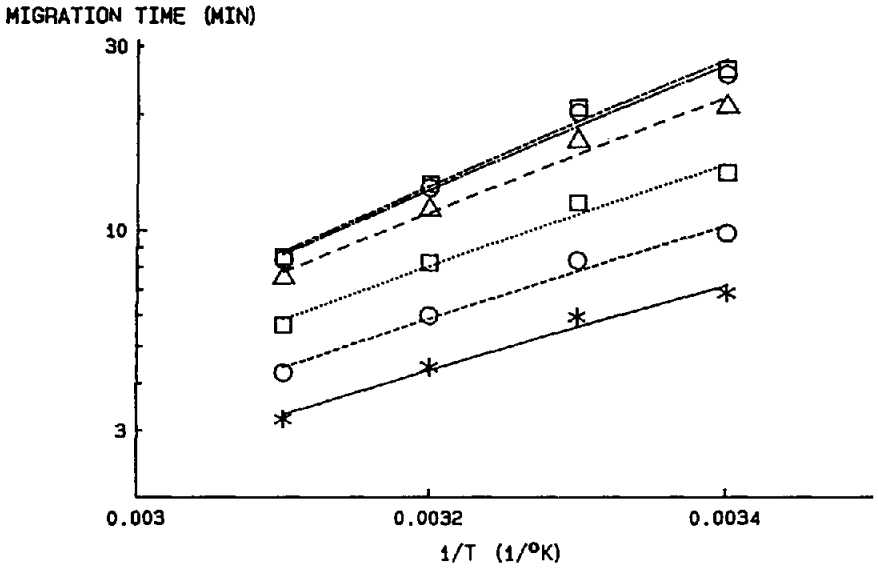

Fig. 9. Semi-logarithmic plot of the migration times of DNA restriction fragments as a function of the separation temperature. The size of DNA fragments: (*) 72 base pairs (bp); (○) 194 bp; (□) 310 bp; (△) 603 bp; (○) 1078 bp; (□) 1353 bp. (From ref. 33 with permission.)

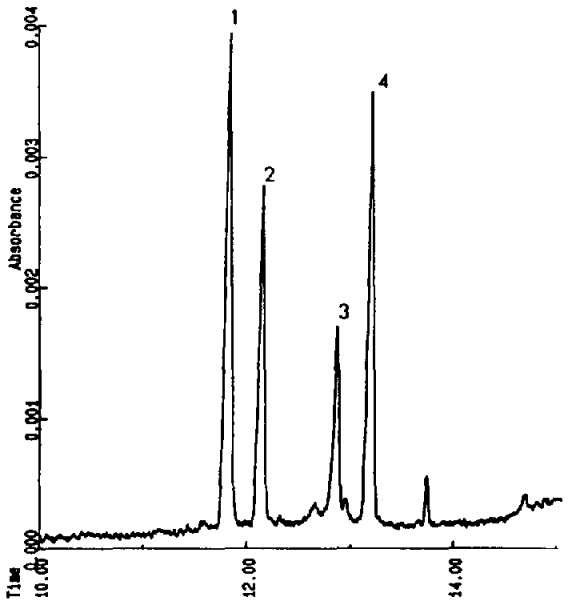

Fig. 10. Capillary gel electrophoretic separation of a homodecamer mixture on a non-denaturing gel. Peaks: 1 = dA_{10}; 2 = dC_{10}; 3 = dG_{10}; 4 = dT_{10}. Capillary, 40 cm effective length; field, 400 V/cm. (From ref. 34 with permission.)

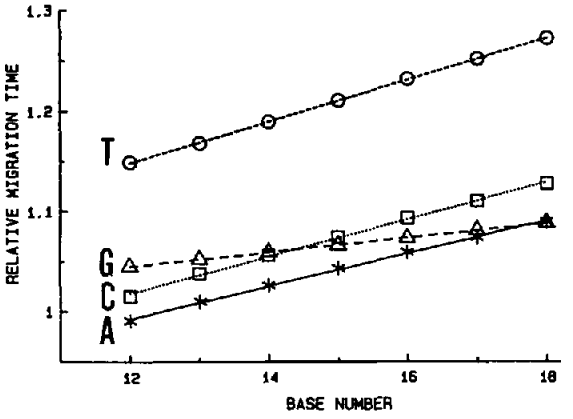

Fig. 11. Relationship between the chain length and the relative migration time of homodeoxyoligonucleotide mixtures on a non-denaturing gel-filled capillary. Conditions as in Fig. 10. (From ref. 34 with permission.)

Fig. 10 demonstrates the effect of the base composition of oligonucleotides on the migration behaviour. The migration time of each homooligonucleotide [$t'(T_p)$, $t'(A_p)$, $t'(C_p)$ and $t'(G_p)$] in eqn. 14 is estimated using the linear extrapolation from the relative migration times of the homooligomers, as shown in Fig. 11. As the base-specific retardation of oligonucleotides is an additive effect [34,65], the migration time can be easily predicted from eqn. 14. Table 3 lists the predicted and observed relative migration times of the four sample components shown in Fig. 10. There is good agreement between the predicted and observed relative migration times, within an error of ± 0.1% [34]. The migration times of heterooligomers with the same chain lengths can be predicted from eqn. 14 and show good agreement with the observed ones as listed in Table 3.

The bandwidth in CGE separation is predict-

TABLE 3

OBSERVED AND PREDICTED RELATIVE MIGRATION TIMES OF VARIOUS HOMO- AND HETEROOLIGODEOXYRIBONUCLEOTIDES IN NON-DENATURING POLYACRYLAMIDE CAPILLARY GEL ELECTROPHORESIS [34]

Relative migration times were calculated using Orange G as internal standard.

Nucleotide sequence	Relative migration time		Migration order
	Observed	Calculated	
p(dA)10	0.957	0.960	1
p(dC)10	0.983	0.981	2
p(dG)10	1.039	1.032	3
p(dT)10	1.068	1.107	4
dGTTGGAGCT–G–GTGGCGTAG	1.149	1.150	1
dGTTGGAGCT–C–GTGGCGTAG	1.156	1.155	2
dGTTGGAGCT–T GTGGCGTAG	1.160	1.161	3

TABLE 4

RELATIVE CONTRIBUTIONS TO PEAK VARIANCE [53]

Base	Relative variance (σ_x^2/σ_T^2)[a]							
	25 cm length to detector				75 cm length to detector			
	Inj.	Det.	ΔT	Diff.	Inj.	Det.	ΔT	Diff.
81	0.7790	0.0031	0.0000	0.2179	0.5418	0.0021	0.0000	0.4561
180	0.7777	0.0068	0.0000	0.2155	0.5434	0.0047	0.0000	0.4518
265	0.7144	0.0105	0.0000	0.2750	0.4609	0.0068	0.0001	0.5323
374	0.6413	0.0155	0.0001	0.3430	0.3803	0.0092	0.0002	0.6103
469	0.5886	0.0199	0.0002	0.3913	0.3295	0.0111	0.0003	0.6591
559	0.5536	0.0234	0.0003	0.4227	0.2999	0.0127	0.0004	0.6807

[a] Relative variances are expressed as the ratio of the specific variance, σ_x^2, to the total variance, σ_T^2, where x is injection (Inj.), detection (Det.), thermal gradient (ΔT) or diffusion (Diff.).

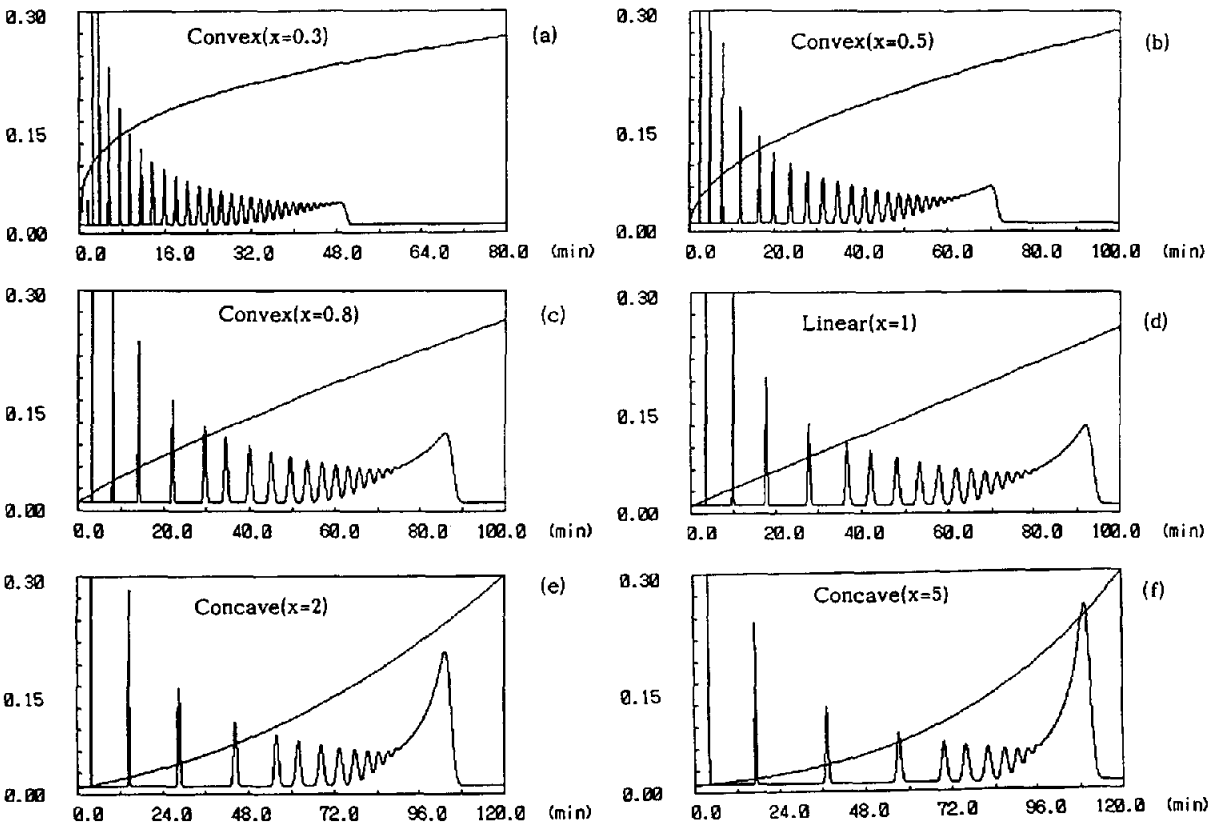

Fig. 12. Computer simulations of the gradient HPLC separation of oligonucleotides with varying gradient shape. (From ref. 26 with permission.)

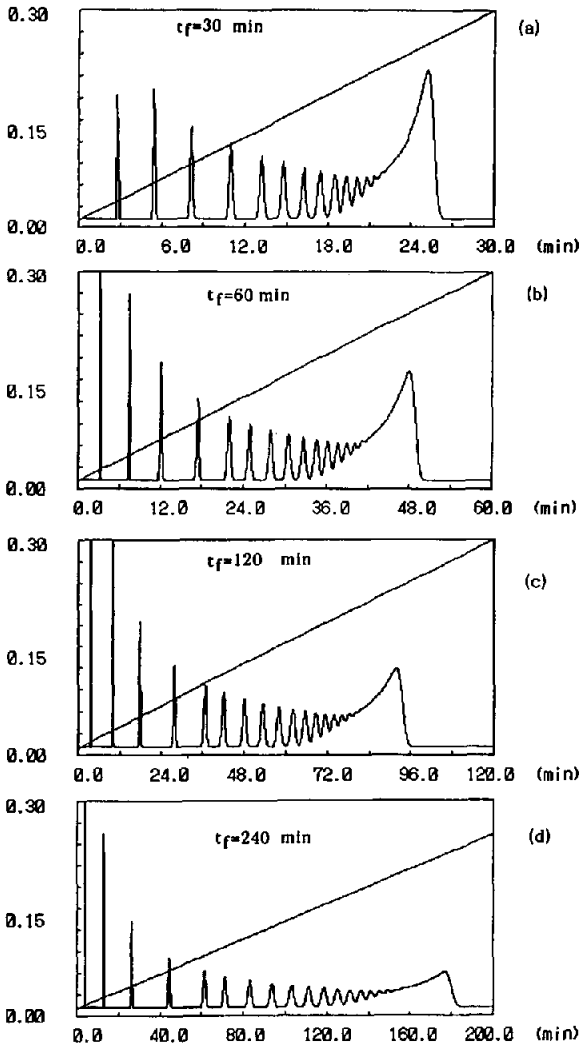

Fig. 13. Computer simulations of the gradient HPLC separation of oligonucleotides with varying gradient time. (From ref. 26 with permission.)

able from eqn. 15 [53]. The relative contribution of each variance is summarized in Table 4. The dominant sources of band broadening are the injection and longitudinal diffusion. These results, combined with the prediction of the migration time discussed above, can be applied to the prediction and the optimization of the resolution of oligonucleotides in CGE separation.

5. OPTIMIZATION OF SEPARATION CONDITIONS

To complete this review, the important area of the application of computer-assisted retention prediction systems, optimization and method development in HPLC and CGE separations will be briefly discussed.

Computer-assisted retention prediction systems and HPLC computer simulations have been successfully applied to the optimization of gradient profiles [20–27,58–60]. The computer simulation system with its capability of graphical presentation of simulated chromatograms has been demonstrated to be an efficient approach to HPLC method development, because visual simulation assists chromatographers in the selection of optimum elution conditions.

In gradient elution chromatography of oligonucleotides, computer simulation has been successfully applied to the optimization of eluent conditions to maximize sample resolution and to minimize analysis time. For example, computer simulations of changing gradient shapes give guidelines for finding the optimum gradient conditions, as shown in Fig. 12 [26]. A convex gradient

TABLE 5

EFFECTIVE RANGE OF SEPARATION OF SINGLE-STRANDED AND DOUBLE-STRANDED DNA IN CGE

Gel concentration, T' (%)	Effective range of separation	
	Single-stranded DNA (bases)	Double-stranded DNA (bp)
3.0	50–1000	100–10 000
5.0	20–500	50–5000
8.0	10–300	10–1000

shape gave a better resolution than linear and concave shapes in oligonucleotide analysis, especially for the later eluted samples. A systematic approach to obtaining an adequate separation by computer simulation has provided substantial savings in the time required for optimization over the non-systematic method (trial-and-error method).

The effect of gradient steepness on the resolution was demonstrated by computer simulations, the parameter B being varied by changing t_f. An increase in t_f resulted in a continuous improvement in resolution, as shown in Fig. 13 [26]. For example, the number of samples to be resolved completely (baseline resolution) increased from seven to ten with an increase in t_f from 30 to 240 min in linear gradient elution. The analysis time, however, increased with increasing t_f.

Computer-assisted prediction systems for electrophoretic behaviour or CE computer simulation have not yet been developed. However, optimum gel concentrations have been proposed for the effective separation of single-stranded and double-stranded DNA from experimental results [31–57] and theoretical considerations [40–42,53], as listed in Table 5. Computer-assisted prediction systems are likely to be developed to find the optimum conditions for electrophoresis, such as electric field, capillary size, capillary temperature and buffer composition.

6. CONCLUSION

Significant recent advances have been made in computer-assisted prediction and simulation systems for the HPLC and the CE separation of oligonucleotides. Fundamental theories for retention prediction have been established for ion-exchange, reversed-phase and mixed-mode chromatography. The theory is almost adequate to predict bandwidths in chromatographic separations. Fundamental theories have been formulated for the prediction of the migration times and bandwidths in the CGE separations of oligonucleotides. A computer-assisted prediction system has accurately predicted the retentions in HPLC separations of oligonucleotides. An HPLC computer simulation system has greatly simplified the task of optimizing chromatographic conditions for the HPLC separations of oligonucleotides. Although a computer-assisted prediction system for CE separations has not been developed, some guidelines are presented for the selection of the gel concentration in CGE separations.

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