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High-performance liquid chromatographic determination of deoxycytidine monophosphate and methyldeoxycytidine monophosphate for DNA demethylation monitoring: experimental design and artificial neural networks optimisation

Jan Havliš*, John E. Madden, Alma L. Revilla[1], Josef Havel

Department of Analytical Chemistry, Faculty of Science, Masaryk University, Kotlářská 2, 611 37 Brno, Czech Republic

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Abstract

The optimal conditions for the separation of 2′-deoxycytidine-5′-monophosphate and 5-methyl-2′-deoxycytidine-5′-monophosphate in the matrix of other natural occurring nucleotides after digest of DNA were investigated. Using experimental design combined with artificial neural networks, efficient optimisation of the HPLC separation conditions was performed. The mobile phase composition was optimised on the basis of its three components (concentration of phosphate, content of methanol and pH). The best separation was obtained with a mobile phase containing 50 m*M* phosphate, pH 5.5 and 6% methanol. The final resolution achieved between 2′-deoxycytidine-5′-monophosphate and 5-methyl-2′-deoxycytidine-5′-monophosphate was equal to 2.78. Finally, the optimised system was successfully tested on the nucleotide mixture solution to determine the methylation state of 2′-deoxycytidine-5′-monophosphate in DNA in the search for FMR1 gene changes.

Keywords: Artificial neural networks; Deoxycytidine monophosphate; Methyldeoxycytidine monophosphate; DNA

1. Introduction

The content of 2′-deoxycytidine 5′-monophosphate (dC) and 2′-deoxycytidine 5′-monophosphate (mdC), originating in DNA is an important indicator of the state of genetic information in the organism. High-performance liquid chromatography (HPLC) has proved to be a powerful tool for quantitative determination of their content in DNA digests [1–3].

Attempts at optimising the separation of dC and mdC using single variable approach (SVA) have been met with disappointing results due to the complex response behaviour of the retention times of these compounds when the separation parameters are changed. Reported eluent conditions [1–3] allow for the separation of dC and mdC, but the capacity factors are unsatisfactory (up to $\alpha=7$ for mdC and up to $\alpha=3$ for dC). The complex co-dependence of the eluent components makes single variable optimi-

*Corresponding author. Fax: +420-5-4121-1214.

E-mail address: jdqh@chemi.muni.cz (J. Havliš).

[1]On leave from: UNAM, FES-C C-1, Department of Analytical Chemistry, Av. Primero de Mayo s/n, Cuautitlan Izcalli, Edo. de Mexico, Mexico.

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sation difficult and time consuming. In order to undertake an interpretive optimisation it is necessary to find a suitable method of predicting the retention times of these compounds [4]. An interpretive optimisation strategy should increase the accuracy of the optimisation process, while simultaneously decreasing the analysis time.

Artificial neural networks (ANNs) have been used for the prediction of retention times in a great number of applications [5] including HPLC [6,7], ion chromatography [8,9] and ion-interaction chromatography [10,11]. To choose relevant input data for ANN training, it was decided to apply experimental design (ED). Such a combination of ED with ANNs was used in an interpretive optimisation approach to predict dC and mdC retention times for the determination of the optimum eluent conditions for the separation of these compounds.

Resulting conditions of optimisation were tested in a model analysis of the nucleotide mixture originated from DNA as a part of an investigation of the influence of the cytosine methylation to the FMR1 gene properties.

Fragile X syndrome (fra-X) is the most frequent cause of heritable mental retardation [12]. The disease is characterised by expansion of $(CGG)_n$ repeat in the 5′ untranslated region of the FMR1 gene and subsequent methylation of the repeat and adjacent promoter [13]. It results in suppression of the gene expression and subsequent absence of specific mRNA and protein [14].

Mitotic stability of fra-X mutation in differentiated cells indicates that expansion occurs post-conceptionally in early embryogenesis [15]. This developmental stage is characterised by high extent of DNA hypomethylation [16].

The aim of our study was to develop a suitable fast HPLC method to study whether hypomethylation of DNA in lymphoblastoid cells (cultivated as tissue culture) may result in $(CGG)_n$ repeat expansion. For this purpose it was necessary to evaluate exactly the extent of DNA hypomethylation in 5-azacytidine treated cells. It is already well documented that 5-azacytidine is a potent demethylating agent [17]. After the DNA digest and sample procedure, the nucleotide mixtures were analysed in the terms of determination of the dC/mdC ratio changes by means of developed HPLC method.

2. Theory

An in-depth discussion of the use of ANNs or ED will not be presented here as details can be found in the literature [8,18–20,28]. In this work a multi-layer perceptron feed-forward neural network was used, utilising the delta-bar-delta variation of the back propagation of errors for the adjustment of the connection weights, as the training scheme.

The benefit of an ANN over a theoretical model lies in its ability to accurately describe a response surface with a minimal set of data required for training and no necessity to study and describe the physical and chemical background of the system being modelled. However, the more complex the response surface the more data points are typically required for the training phase. Simple response surfaces can be modelled accurately with only a small amount of input data in a central composite design for instance.

It must be noted that ANN modelling in this particular mode does not provide any numerical values for physical parameters (such as ion-exchange selectivity coefficients, adsorption coefficients, etc.) which can be used to assist in the interpretation of the observed trends in retention and then design improved separation systems. When the goal is to simply identify the optimal separation conditions based on an ability to accurately predict retention times (retention optimisation), these parameters are usually not necessary.

Once the ANN has been trained it can be used for the prediction of any retention times within the search area. The data predicted by the ANN can then be ranked according to any number of suitable criteria. For this work, two criteria were used, the maximum peak retention time and the resolution, as defined by:

$$R = \frac{1.177(t_{R,mdC} - t_{R,dC})}{Y_{0.5,mdC} + Y_{0.5,dC}} \quad (1)$$

where, $t_{R,mdC}$ and $t_{R,dC}$ are the retention times, in min, for mdC and dC, respectively, and $Y_{0.5,mdC}$ and $Y_{0.5,dC}$ are the peak widths at half peak height for mdC and dC, respectively. At a constant phosphate concentration of 50.0 m*M* it was found that the peak widths did not alter greatly, so constant values for

$Y_{0.5,mdC}$ and $Y_{0.5,dC}$ were assigned as 0.4 min each. Thus Eq. (1) becomes:

$$R = \frac{1.177(t_{R,mdC} - t_{R,dC})}{0.8} \quad (2)$$

The optimum condition was defined as the greatest value for resolution while having a maximum peak retention time of less than 6 min.

3. Experimental

3.1. Instrumentation

A Shimadzu HPLC 10AVP system (Kyoto, Japan) consisting of a GT-154 degasser, an SCL-10AVP system controller, an LC-10AVP pump, a CTO-10ASVP column oven, an SPD-M10AVP photodiode array detection (PDA) system, controlling software Class-VP 5.02, and a narrow-bore stainless steel column, 250×4 mm, packed with Silasorb C_{18}, particle diameter 5 μm (Lachema, Brno, Czech Republic), flow-rate 0.5 ml min^{-1} was used.

A microprocessor controlled pH meter GRYF 259 (GRYF, Havlíčkův Brod, Czech Republic) with universal combined pH electrode PCL 321/t was used for the pH measurements.

3.2. Reagents

All nucleotides (2′-deoxyadenosine-5′-monophosphate, dA; 2′-deoxyguanosine-5′-monophosphate, dG; 2′-deoxythymidine-5′-monophosphate, dT; 2′-deoxycytidine-5′-monophosphate, dC; 5-methyl-2′-deoxycytidine-5′-monophosphate, mdC) were of analytical grade, Sigma–Aldrich (Prague, Czech Republic) and used as received. The other chemicals and solvents were analytical grade supplied by Lachema. Deionised water used was produced in a commercial apparatus from Premier MFG'D Systems (Phoenix, AZ, USA). Standard pH buffers were from Merck (Darmstadt, Germany).

3.3. Procedures

3.3.1. Preparation of the DNA digest

DNA digest was comprised of several steps. In the first, immortalised cell lines were established from blood of an fra-X patient by EBV transformation of peripheral B-lymphocytes using standard protocol [21]. After reaching appropriate density the aliquot of the cells was treated by 5-azacytidine (Sigma–Aldrich) for 8 days. Fresh 5-azacytidine was added every 24 h.

At day 8, genomic DNA was isolated according to salt–chloroform protocol [22]. A 5-μg amount of DNA was digested by 20 U of *Msp*I endonuclease (New England Biolab) overnight at 37°C in appropriate buffer. Digested DNA was purified as follows:

A 250-μl volume of TE buffer pH 8.0 (10 m*M* Tris·Cl, pH 8.0; 1 m*M* EDTA, pH 8.0) was added to 50 μl of restriction mixture. A 300-μl volume of 5 *M* ammonium acetate was added and the mixture was left on ice for 1 h. Samples were centrifuged for 10 min at 14 000 rpm. Cleared supernatant was transferred to a new tube and DNA was precipitated by 0.7 volume of isopropanol and subsequent centrifugation at 14 000 rpm. The DNA pellet was washed in 70% ethanol and re-suspended in 50 μl of TE buffer.

A 2-μg amount of digested purified DNA was incubated with 100 U of Exonuclease III [23] overnight at 37°C. This exonuclease catalyses removal of mononucleotides from 3′ recessed ends (which were created by digestion of genomic DNA by *Msp*I restriction endonuclease). Digested DNA (mononucleotides) was purified by filtration through a 0.22-μm filter (Millipore, catalog No. SLGV025BS).

3.3.2. Analytical procedures

Samples of individual nucleotides for the model nucleotide mixture were dissolved to obtain the desired concentration 1 m*M* in deionised water and further diluted for HPLC analyses.

The samples were analysed in duplicate at a range of phosphate concentrations from 9.1 to 50.0 m*M*, methanol compositions from 0 to 30% and pH values from 4.0 to 8.0 (the ED-ANN search area). If duplicate measurements were found to be inconsistent (to two decimal places), then further measurements were taken to ensure repeatability. The dead time of the column was determined by injecting a sample of 0.004% aqueous solution of thiourea into the column and measuring the retention time of the related peak.

3.3.3. Calculation procedures

Statistical analysis of the performance of the ANN was carried out using retention data acquired directly from the system described above. All calculations were performed using Microsoft Excel 97 on a Pentium II 400 MHz computer with 64 MB of SDRAM, running Windows NT workstation v4.0 sp6. The ANN was simulated using the Trajan Neural Network Simulator release 3.0.D software package (Trajan Software, Durham, UK) on the same computer.

3.4. Method for training of the ANN

One of the aims of our work is to show that the combination of ED with ANN can be used to lower the number of necessary input data for ANN training. There is a heuristic rule often given in the literature [25], that there should be at least twice as many observations (data sets) as adjustable parameters in the ANN. That means, for the case solved in this paper, 64 and more data sets for (3,5,2) ANN and/or 22 and more data sets for (2,4,2) ANN. Based on previous results [29,30], there is a need for a maximum of 15 data sets in the frame of a central composite experimental design for ED-ANN training. Moreover, close and watchful input data selection should lead to relevant results, although the above described rule will be not consistently fulfilled. This was solved using two independent input data selections for general mapping of the response surface. Later on, the third data selection was made for detailed results, based on previous, primary observations.

For the simultaneous optimisation of the three eluent components, at least a two-level three-factor factorial design [24] should be used to define the end points of the data set. These points were set as: 9.1 and 50.0 m*M* phosphate, 0 and 30% methanol and pH 4 and 8, i.e., the corners of a cube. The more statistically robust central two-level three-factor factorial design adds an extra point (22.5 m*M* phosphate, 15% methanol and pH 6) to the two-level three-factor factorial design (see Table 1), i.e., the centre of the cube.

To exclude the possibility of accidentally ''good'' results of the ANN training, further a central composite design [24] was added, such that the outlying points of the star design (the faces of the cube) reached the end points of the original data set. This added a further 14 points to the existing data set (see Table 2). It must be pointed out that a central composite design in three factors results in 15 data points being defined, but due to difficulties in

Table 1
Retention times observed for dC and mdC for the three eluent parameters in a central two-level three-factor factorial experimental design

[P] (m*M*)	pH	MeOH (%, v/v)	$t_{R,dC}$ (min)	$t_{R,mdC}$ (min)
50.0	8	30	3.55	3.56
50.0	8	0	4.35	11.81
50.0	4	30	3.80	3.83
50.0	4	0	7.38	11.96
25.5	6	15	3.90	4.17
20.0	4	24	3.88	3.94
9.1	8	0	3.55	11.66
9.1	8	30	3.22	3.24
9.1	4	0	7.38	11.61
9.1	4	30	3.85	3.87

[P], Phosphate concentration; MeOH, methanol.

Table 2
Retention times observed for dC and mdC for the three eluent parameters in a central composite design such that the outlying points of the star design reaches the end points of the original data set (Table 1)

[P] (m*M*)	pH	MeOH (%, v/v)	$t_{R,dC}$ (min)	$t_{R,mdC}$ (min)
45.1	7	6	3.79	4.29
45.1	5	6	4.52	5.47
42.5	6	15	3.80	4.05
36.5	7	24	3.45	3.49
36.5	5	24	3.76	3.86
30.0	6	0	5.67	8.61
26.1	6	15	3.83	4.14
25.5	4	15	4.12	4.37
21.0	6	30	3.49	3.53
11.3	7	6	3.31	3.50
11.3	5	6	4.26	5.02
9.1	5	24	3.55	3.60
9.1	7	24	3.07	3.09
0.0	6	15	3.41	3.71

creating some of the eluents only 14 could be defined.

For the simultaneous optimisation of two components, i.e., methanol concentration and pH, a central two-level two-factor factorial design was used (see Table 3). Additional data points were collected and used as needed.

For the simultaneous optimisation of three components, a network with a (3,5,2) architecture was used: three input nodes (eluent composition given by phosphate concentration, % methanol and pH), five hidden nodes and two output nodes (retention times for the two analytes). For the simultaneous optimisation of two components, a network with a (2,4,2) architecture was used: two input nodes (eluent composition given by % methanol and pH), four hidden nodes and two output nodes. The ANN was trained using the delta-bar-delta algorithm [25–27]. The following values for the training parameters were found to be optimal; initial learning rate, 0.1; learning rate increment, 0.01; learning rate decay, 0.8; smoothing, 0.5 and noise, 0. Training was conducted until the RMS (root mean square) of the training error fell below 0.0001 or the number of epochs exceeded 500 000. The verification set was used to search for any sign of over-training by means of applying of the already trained ANN on the verification set. Any time the over-training was observed the training was stopped and the best network prior to over-training was used. To prove that the results are correct, the random scrambling experiments were done with independent variable values left untouched and the dependent variable values randomly scrambled. Pre/post-processing of the data was set up to normalise the data between a maximum value of 0.6 and a minimum value of 0.4.

4. Results and discussion

Initially, seven sets of randomly assigned experimental results were obtained during the single variable approach (see Table 4). This data set was kept as a verification set for the purpose of monitoring for evidence of over-training. Also all the random scramble experiments done resulted in non-identical results.

4.1. Central two-level three-factor factorial design

For the first ANN training a central two-level three-factor factorial design was used to define experimental parameters. The retention data obtained for the range of eluent concentrations studied are shown in Table 1. This data set was used for the training of the ANN. Comparison of the predicted retention times versus the retention times of the verification set indicate that the response surface is more complex than a simple central two-level three-factor factorial design is capable of describing. Some points, such as the retention times defined by phosphate concentration 20 m*M*, pH 4 and 24% methanol, are very close to the experimental data. Other data points are as much as 7.3 min away from the experimentally determined retention times.

It was decided to add a further 14 data points in a central composite design to the existing nine data

Table 3
Retention times observed for dC and mdC for two eluent parameters, pH and percentage of methanol, in a central two-level two-factor factorial design

pH	MeOH (%, v/v)	$t_{R,dC}$ (min)	$t_{R,mdC}$ (min)
8.0	6	3.78	7.03
8.0	0	4.35	11.81
6.5	3	4.50	8.37
5.0	6	4.52	5.47
5.0	0	6.97	10.23

Table 4
Retention times observed for dC and mdC for randomly chosen eluent parameters

System No.	[P] (m*M*)	pH	MeOH (%, v/v)	$t_{R,dC}$ (min)	$t_{R,mdC}$ (min)
1	40.0	8	0	3.98	4.51
2	25.0	7	0	4.47	6.03
3	35.0	8	24	3.52	3.55
4	20.0	7	10	3.48	3.68
5	50.0	6	15	3.80	4.05
6	20.0	4	24	3.88	3.94
7	45.0	5	30	3.77	4.24

points. The central composite design was constructed in such a way that the points of the star design would meet the end points defined by the original experiments.

4.2. Central composite design

The retention data obtained for the range of eluent conditions described by the central composite design is given in Table 2. The central composite design is slightly skewed due to restrictions during the preparation of the eluents. This data combined with the central two-level three-factor factorial design data was used for the training of the ANN. The trained ANN was then used to predict retention times across a range of eluent conditions within the search area. It was noted that phosphate concentration had very little impact on the retention of the two analytes, but had a large impact on the peak shape and width. It was thus decided to reduce the search area to a two-dimensional search area by selecting a phosphate concentration of 50.0 m*M*, where the peak shapes were considered optimal.

Retention times for eluent compositions of 50.0 m*M* phosphate, pH values of 4 to 8 in increments of 0.5 and methanol concentrations of 0 to 30% in increments of 2% were calculated and plotted as response surfaces, see Figs. 1 and 2. The response surface for the resolution is given in Fig. 3.

From Fig. 3 it is obvious that dC and mdC are poorly resolved for eluent compositions with methanol concentrations above about 6%. At pH levels below 5 separation was also found to be unacceptable because of the same reason. Therefore it was decided to redefine the search area to a two-dimensional search with pH values between 5 and 8 and methanol concentration between 0 and 6%. Phosphate concentration was kept constant at 50.0 m*M*.

4.3. Two-dimensional central two-level two-factor factorial design

The retention data obtained for the range of eluent conditions described by the two-dimensional central two-level two-factor factorial design is give in Table 3. This data was used for the training of the ANN. The trained ANN was then used to predict retention times across a range of eluent conditions with pH values varying from 5 to 8 in increments of 0.5 and methanol concentrations ranging from 0 to 6% in 0.5% increments. Resolutions were calculated and the response surface for resolution is given in Fig. 4.

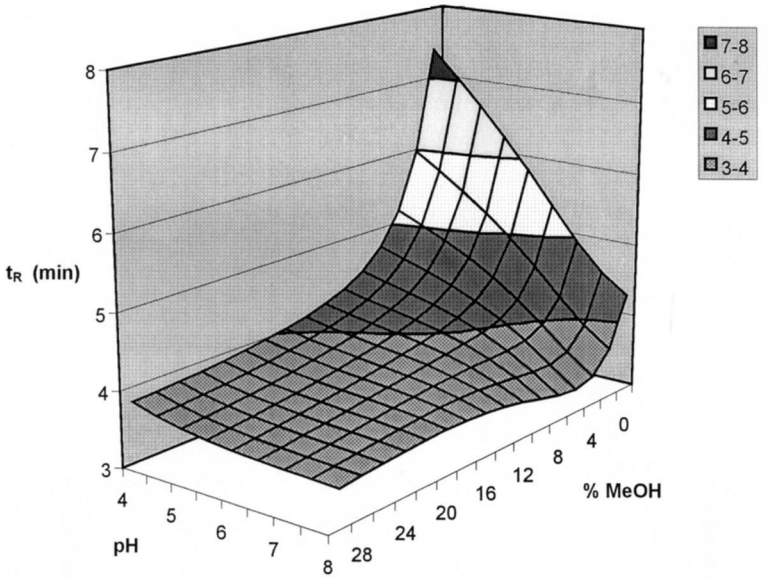

Fig. 1. Response surface for the retention of dC as predicted by an ANN trained as described in Section 4.2 for eluents composed of 50 m*M* phosphate, methanol concentrations of 0 to 30% and pH values 4 to 8.

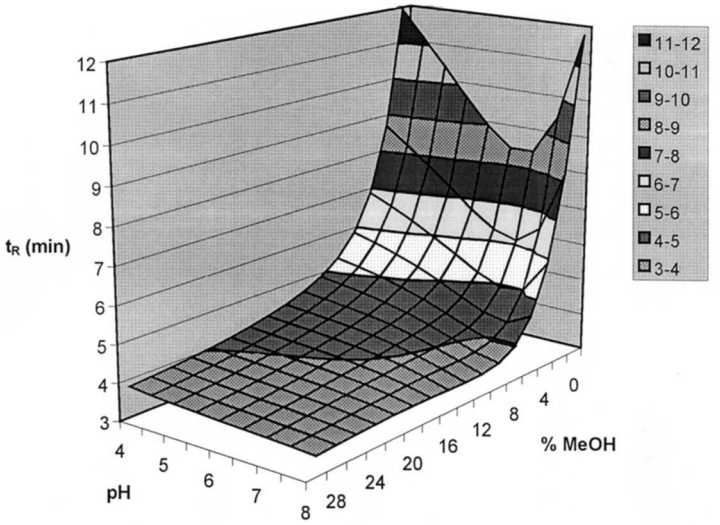

Fig. 2. Response surface for the retention of mdC as predicted by an ANN trained as described in Section 4.2 for eluents composed of 50 m*M* phosphate, methanol concentrations of 0 to 30% and pH values 4 to 8.

The greatest value for resolution where the retention times of the analytes in question remained below 6 min was found at 6.0% methanol and pH 6, giving a maximum peak retention time of 5.94 min and a resolution of 2.64. Other acceptable optima were found at 5.5% methanol and pH 6.0 (5.70 min, 2.04) and 5.0% methanol and pH 5.5 (5.78 min, 1.67).

An iterative approach was chosen to get as close to the optimum conditions as possible while preserv-

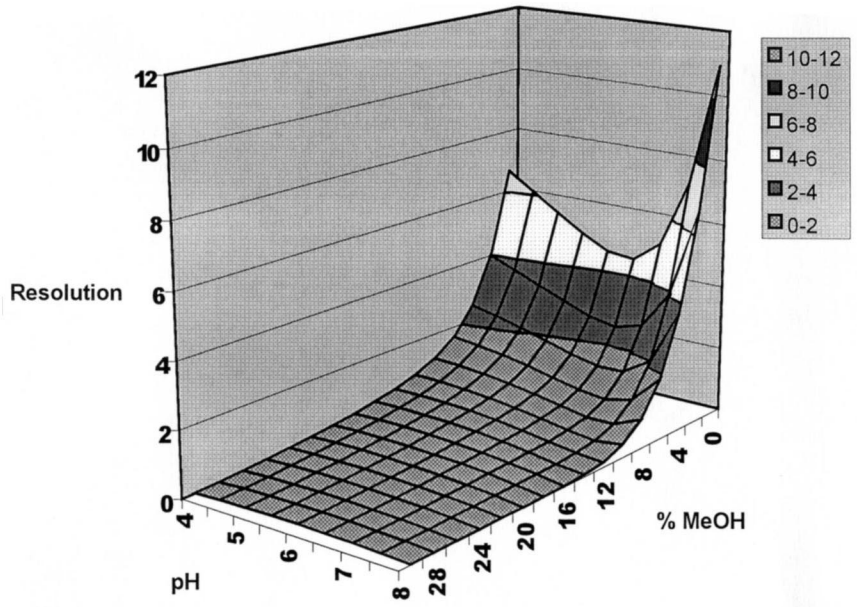

Fig. 3. Response surface for the resolution of mdC and dC as predicted by an ANN trained as described in Section 4.2 for eluents composed of 50 m*M* phosphate, methanol concentrations of 0 to 30% and pH values of 4 to 8.

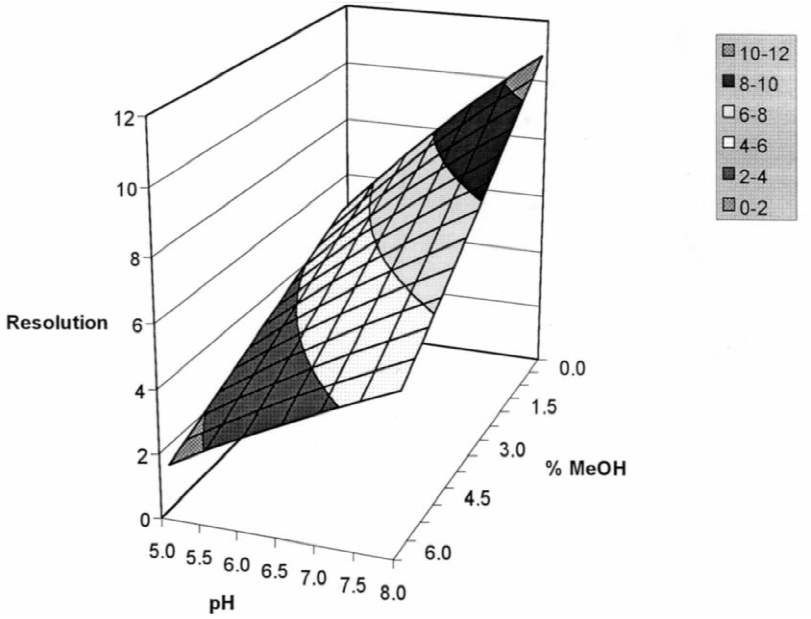

Fig. 4. Response surface for the resolution of mdC and dC as predicted by an ANN trained as described in Section 4.3 for eluents composed of 50 m*M* phosphate, methanol concentrations of 0 to 6% and pH values of 5 to 8.

ing the low number number of experiments needed. So another experiment was conducted at the eluent condition calculated as optimum, 6.0% methanol and pH 6.0. The retention times were determined to be 4.51 min for dC (4.15 min predicted) and 6.75 min for mdC (5.94 predicted). This difference in retention times was considered large enough that another iteration of the optimisation process was considered necessary. The response surface profile is very steep in this relatively narrow region of variable parameters chosen. Thus the extra data point was added to the training set and the ANN was retrained.

After retraining, the optimum eluent condition was calculated to be 4.5% methanol at pH 5.0, giving a maximum peak retention time of 5.81 min and a resolution of 1.79. Using these eluent conditions the retention times were determined to be 5.06 for dC (4.59 predicted) and 6.89 min for mdC (5.81 predicted). This extra data point was added to the training set and the ANN trained a third time.

The newly trained ANN predicted no optimum conditions that met the original criteria, so the maximum peak retention time criterion was changed from 6.0 min to 6.5 min. Given these new criteria the optimum was calculated to have eluent conditions of 6.0% methanol and pH 5.5. The retention times were measured for this eluent condition and the retention times found to be 4.56 min (4.67 predicted) and 6.55 min (6.54 predicted). These separation conditions obtained from final ANN training were found to be optimal under conditions given.

4.4. Model applications of the optimised method

The optimised separation conditions were tested on the model mixture containing dC, mdC, dG, dT and dA. All the compounds are well separated, see Fig. 5. The separation is conditioned by presence of phosphate buffer in the certain range of pH and methanol concentration. Using no other buffer type were we able to sufficiently separate these compounds. A possible explanations lies in the interaction of these substances with phosphate. Another possibility is the different acido–basic properties caused by different tendencies of these nucleotides to tautomerise.

Fig. 6 shows the successful application of the optimised method for the separation of the sample of the nucleotide mixture originated from 5-azacytidine treated lymphocyte cell DNA.

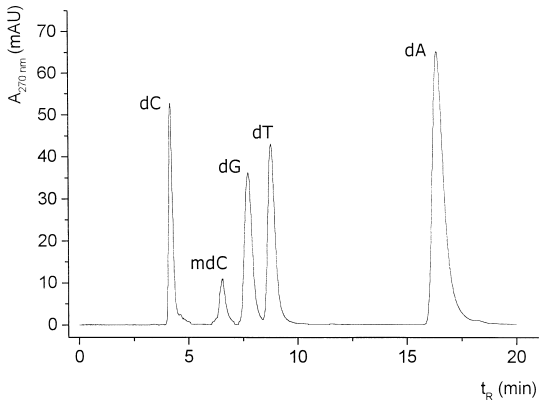

Fig. 5. Chromatogram for the separation of the model nucleotide mixture (dC, mdC, dA, dG, dT) conducted at the calculated optimum eluent conditions 50 m*M* phosphate, 6.0% methanol and pH 5.5.

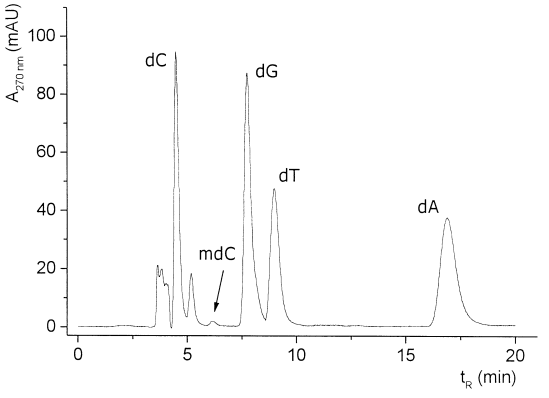

Fig. 6. Chromatogram for the separation of the nucleotide mixture from the lymphocyte cell culture treated with 5-azacytidine conducted at the calculated optimum eluent conditions 50 m*M* phosphate, 6.0% methanol and pH 5.5.

4.5. Comparison of SVA and ED-ANN approaches

Table 5 compares our obtained results of the nucleotide mixture separation with results from various sources, where the separation method was optimised using SVA [2,3]. It can be seen that the capacity factors (α) are significantly lower in our case, where separation of dC and mdC has been complete within $\alpha = 2.18$ for mdC, compared with $\alpha = 5.35$ and $\alpha = 6.89$, respectively, for the two SVA methods. The total time for complete separation of the entire matrix has been reduced from $\alpha = 27.78$ and 22.91, respectively, in the SVA optimised systems to $\alpha = 5.63$ in our system. The iterative optimisation by means of application of ED-ANN allows the application of separation conditions with much shorter retention times than obtained by conventional methods like SVA or classical ANN optimisation.

5. Conclusions

The response surfaces for the retention times of dC and mdC on a Silasorb C_{18} HPLC column were found to be very complex. Because of this, a large number of experiments were required to be able to predict the retention times with any level of accuracy. On the other hand, the use of a simplex optimisation procedure would have almost certainly taken a much larger number of experiments with a less likely chance of finding a global maximum.

Due to the complex nature of the search area it is impossible to be certain that the global maximum has indeed been found, but the calculated optimum gave significantly better separation while requiring less time than previous conditions. As it was demon-

Table 5
Comparison of used separation systems for separation and determination of dC and mdC

	Capacity factor		
	10 m*M* [P], pH 5.6, 2.5% MeOH [2]	10 m*M* [P], pH 2.0, 0% MeOH [3]	50 m*M* [P], pH 5.5, 6.0% MeOH
dC	2.78	2.93	1.52
mdC	6.89	5.35	2.18
dA	27.78	7.40	5.63
dG	9.72	13.27	2.99
dT	11.67	22.91	2.58

strated, an excellent baseline separation of the dC and mdC within 6.5 min with excellent peak shape was achieved with the optimised method.

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