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Prediction of retention times of peptide nucleic acids during reversed-phase high-performance liquid chromatography

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Abstract

Peptide nucleic acids (PNAs) are synthetic biopolymers consisting of nucleobase side chains attached to an amino ethyl glycine backbone. At present this family of compounds enjoys a well deserved popularity in biomedical research, due to a number of favorable biological and chemical properties of PNAs compared to conventional synthetic oligonucleotides. PNAs are basically peptides, and are synthesized, purified and analyzed by traditional peptide chemistry, chromatography and mass spectrometry techniques. In the current report, we analyzed factors that influence the elution behavior of 29 PNAs on reversed-phase high-performance liquid chromatography using a water–acetonitrile–trifluoroacetic acid gradient elution system on C_{18} columns. We found that increasing the temperature from 25°C to 55°C resulted in improved peak shape and resolution. The retention times of the PNA analogs were dependent upon the length of the polymers with longer PNAs eluting later. Mixtures of PNAs with varying length, originating from inefficient monomer couplings during the polymer assembly, could be separated by single chromatographic runs. The retention time also depended upon the cytosine, thymine, adenine and guanine content of the polymers. These differences in the contribution to the retention times could be explained by simple hydrophobicity features of the monomer side chains at pH 1.8. Based on all data, a linear equation was generated which predicted the retention time of any synthetic PNA based on composition and length. Comparison of the predicted and observed retention times showed a remarkable reliability of the algorithm.

Keywords: Retention prediction; Peptide nucleic acids; Nucleic acids

1. Introduction

Peptide nucleic acids (PNAs) are one of the most fashionable family of biopolymers of the nineties. Their popularity is due to increased melting temperatures when hybridized to complimentary DNA sequences, their ability to perform strand invasion, their remarkable biostability, and an easy synthetic access to PNAs in large quantities with simple chemical modification possibilities [1,2]. In the PNAs, the nucleobases of the deoxyribonucleic acids are attached to an amino ethyl glycine backbone that separates the nucleobases in a distance identical to that found in DNA, making these molecules ideal DNA mimics [3]. The PNA polymers are assembled from appropriately protected and activated monomers, just like peptides, on conventional peptide synthesizers [4,5]. Purification of the crude products is achieved by traditional gradient reversed-phase high-performance liquid chromatography (RP-

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HPLC) on C_{18} columns, with water and acetonitrile as mobile phases and trifluoroacetic acid (TFA) as counterion [6], conditions typically used for purification of synthetic peptides [7]. Just like peptides, purified PNAs are analyzed by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) [8]. In spite of the widespread use of PNAs in biomedical research and the analogy to peptides, the chromatographic behavior of PNAs has not been scrutinized to the extent it has been for peptides [9]. Moreover, the prediction of the RP-HPLC retention times of peptides [10–12] yielded much success both academically and commercially.

A thorough analysis of the chromatographic behavior of PNAs is further supported by the nature of the chemistry of their preparation. The coupling efficiencies during the syntheses are substantially lower than those of peptides, resulting in a series of truncated sequences [13]. Because the PNAs are end capped with acetylation after each synthetic cycle, the hydrophobic acetyl end groups facilitate the retention of the PNAs on the RP-HPLC column, and the procedure eliminates the occurrence of deleted sequences containing polymers with the same lengths. Chromatographic separations for PNAs of various lengths would not only simplify studies aimed at structure–activity relationships, but considering the high cost of the monomers, would make these studies considerably more economical. The knowledge of the dependence of the retention times on the PNA polymer composition (in addition to instant identification of the peaks representing the target PNA or deleted homologs) would indicate the major forces of the separation process and would allow further chromatographic method development.

2. Materials and methods

2.1. PNA synthesis

PNA oligomers were assembled from Fmoc-A(Bhoc)-OH, Fmoc-C(Bhoc)-OH, Fmoc-G(Bhoc)-OH and Fmoc-T-OH monomers (purchased from PerSeptive Biosystems, Framingham, MA, USA) either on an Expedite 8900 Nucleic Acid Synthesis System starting from 2 μmol derivatized resin by using standard cycles, or on cellulose membranes derivatized with a TFA cleavable linker [14]. After the syntheses were completed, the products were cleaved at room temperature with TFA–*m*-cresol (4:1, v/v), and precipitated with cold ether. The pellets were dissolved in 0.1% aqueous TFA.

2.2. Chromatography

The HPLC system consisted of two Beckman 110B solvent delivery modules and a Beckman system organizer (containing an injection loop of 250 μl), driven by a NEC PC 8300 controller. A Rainin Dynamax absorbance detector model UV-C was set to 260 nm, and the chromatograms were recorded on a Shimadzu CR501 Chromatopac integrator. A Phenomenex Jupiter C_{18} silica column was used with 5 mm particle size, and 300 Å pore size. Column temperature was modulated by an external Systec CH 1530 single column heater. Solvent A was 0.1% TFA in water (pH 1.8), solvent B was 40% aqueous acetonitrile containing 0.1% TFA. A gradient of 0.5% solvent B/min was applied at a flow-rate of 1 ml/min starting from 5% B solvent composition.

2.3. Mass spectroscopy

The integrity and the purity all PNA oligomers used in this study were verified by MALDI-MS. Mass spectra were taken at the Wistar Institute Protein Microchemistry Facility on a Voyager Biospectrometry Workstation in conditions identical to those used by Butler and coworkers [8]. Five pmole amounts of samples were loaded. Table 1 lists all PNA sequences that were used in this study.

2.4. Calculations

The contribution of the four monomers to the retention times of any PNA was calculated by using the SYSTAT software package and linear regression of the experimentally observed retention times of the 29 oligomers listed in Table 1. The coefficients were identified by the least squares method.

Table 1
Peptide nucleic acid oligomers used in this study[a]

PNA name	Sequence	Origin
PH107a	AGCGTGTCAT	Translocation between chromosomes *bcr* and *avl* in leukemia cell lines
ALL1	CACCACCTCG	Translocation between chromosomes *bcr* and *avl* in leukemia cell lines
E6-10	AGTGTGTTGC	Human papillomavirus type 16 E6 protein gene
E6-8	TGTGTTGC	Human papillomavirus type 16 E6 protein gene
E7-10	CGCATCTCAG	Human papillomavirus type 16 E7 protein gene
E7-9	GCATCTCAG	Human papillomavirus type 16 E7 protein gene
p53	CCGTCCGTGT	Tumor suppressor protein p53 gene
PH107b-12	ATAGCAGCAGCT	Translocation between chromosomes *bcr* and *avl* in leukemia cell lines
PH107b-8	CAGCAGCT	Translocation between chromosomes *bcr* and *avl* in leukemia cell lines
RM1-7	TAATCGT	Rabies virus glycoprotein gene
RM1-5	ATCGT	Rabies virus glycoprotein gene
RM1-4	TCGT	Rabies virus glycoprotein gene
RM3	TCCCTAGTCT	Rabies virus glycoprotein gene
RM4	TACTATT	Rabies virus glycoprotein gene
RM6-10	TTCATCTACA	Rabies virus glycoprotein gene
RM6-7	ATCTACA	Rabies virus glycoprotein gene
RM6-6	TCTACA	Rabies virus glycoprotein gene
RM6-4	TACA	Rabies virus glycoprotein gene
RM9-15	ACCCGTTCATTTTTA	Rabies virus glycoprotein gene
RM9-10	TTCATTTTTA	Rabies virus glycoprotein gene
RM9-6	TTTTTA	Rabies virus glycoprotein gene
RM11	TCACAACGCC	Rabies virus glycoprotein gene
RM12	TGTAGGTTTC	Rabies virus glycoprotein gene
RM19-15	CTCTCTTCCCTCTAC	Rabies virus glycoprotein gene
RM19-12	TCTTCCCTCTAC	Rabies virus glycoprotein gene
RM19-10	TTCCCTCTAC	Rabies virus glycoprotein gene
RM19-8	CCCTCTAC	Rabies virus glycoprotein gene
RM20	TGTTCCACCA	Rabies virus glycoprotein gene
Rabies A5T	TTCCCACTAC	Rabies virus glycoprotein gene containing a T→A mutation

[a] The integrity of all PNA molecules was verified by MALDI-MS. The C-terminal residues (3′-equivalents) are aligned to demonstrate similarities among the structurally related sequences.

3. Results and discussion

3.1. Effect of column temperature

By using RP-HPLC conditions customary for peptides the PNA oligomers exhibited relatively sharp peaks at 25°C column temperature (Fig. 1). Overlapping peaks, however, could not be separated (Fig. 1). By increasing the column temperature to 40°C, the retention time of our model RM19-10 PNA decreased from 24.1 min to 21.5 min, and the PNA peak became sharper accompanied by good separation of the contaminants. When the column was operated at 55°C, the elution time further decreased to 18.9 min without additional improvement in peak shape and separation (Fig. 1). Based on this, all consecutive HPLC runs were made at 55°C external column temperature. Heating of the column was reported earlier to improve the resolution of PNAs during RP-HPLC [8] without discussion of the benefits. Indeed, increased operating temperatures are reported to improve peak shape for proteins [15] and resolution for peptides [16]. The reduced retention times and slightly improved separation of the PNAs upon higher column temperatures are in line with earlier findings on peptide standards during similar RP-HPLC conditions [17]. Nevertheless, these authors noted that the advantages of chromatography at elevated temperatures have to be weighted against risks associated with peptide degradation and acceleration of column aging. The Phenomenex Jupiter column we used is new on the market, and a large number of additional independent runs are needed to estimate its lifetime at elevated tempera-

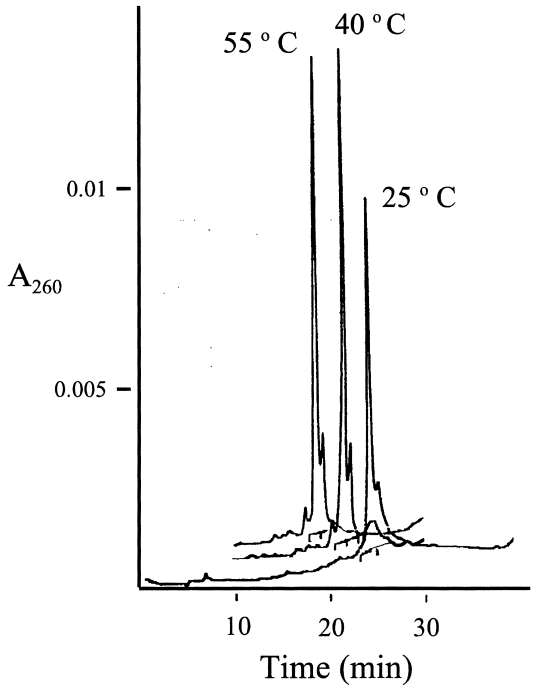

Fig. 1. Overlayed RP-HPLC chromatograms of peptide nucleic acid RM19-10 at different temperatures. Approximately 1 nmol (2.6 μg) of PNA was loaded in each run. The retention times are as follows: 25°C: 24.0 min; 40°C: 21.5 min; 55°C: 18.9 min. When the column was new, the same PNA at 55°C eluted at 19.8 min (compare with Fig. 2), demonstrating the aging of the column when used at elevated temperatures for extended periods of time. The A_{260} scale refers to the chromatogram that comes to zero.

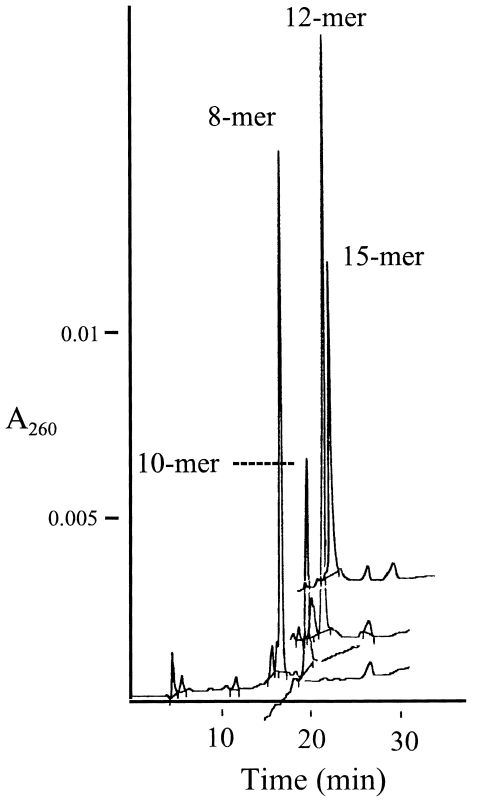

Fig. 2. A composite chromatogram of a homolog series of peptide nucleic acids consisting of PNA RM19-8, RM19-10, RM19-12 and RM19-15. Approximately 1 nmol PNA was loaded. The retention time values are: 8-mer: 16.8 min; 10-mer: 19.8 min; 12-mer: 21.4 min and 15-mer: 22.0 min. The A_{260} scale refers to the chromatogram that comes to zero.

tures. According to the manufacturer, the column is stable at pH 1.8 (0.1% TFA in water) for more than 3000 h of usage. Since the rates of the chemical reactions are approximately doubled by a temperature increase of 10°C, it is expected that an increase of 10°C would decrease the lifetime of the column by 50%. Based on these calculations, after raising the operating temperature of 25°C to 55°C the column should last for at least 300 h. The aging of the Jupiter column when used at the elevated temperatures for extended periods of time is well reflected in the decrease of the retention time of PNA RM19-10 from 19.8 min to 18.9 min (compare Figs. 1 and 2).

Peptide degradation, and especially PNA degradation, is another issue of concern. For regular DNA, under acidic chromatographic conditions (below pH 4.0), an acid catalyzed deamination is observed [18]. The rate of this C → U transition is further increased if the column temperature is raised above 37°C [18]. To our knowledge, the extent of a possible similar modification during the purification of PNAs has not been studied yet. Such a $NH_2 \rightarrow OH$ change on the PNA cytosine side-chain would be difficult to identify solely by MALDI-MS (mass difference 1 u) or RP-HPLC. Nevertheless, the chromatographic conditions (solvents, pH, temperature, etc.) we used are identical to those that have generally been used for the purification of PNA since their introduction in 1991. By using these chromatographic conditions, more sensitive mass spectrometry methods, such as

electrospray MS [5,19], or MALDI-MS aimed at directly analyzing PNA products [8] failed to identify deaminated pyrimidine nucleobases, even if the model sequences contained multiple C analog monomers.

Another aspect of the chromatography of PNAs during standard peptide elution conditions is the derivatization of the 5′-equivalent amino terminus. To increase on-column time, the hydrophobic dimethoxy-trityl protecting group attached to the 5′-end of regular oligonucleotides is kept on the polymers during the cleavage from the synthetic solid support, and removed only after RP-HPLC purification [20]. Accordingly, it was proposed that the amino-terminal Fmoc protecting group retains the PNAs on the HPLC column to a greater extent than the end capping acetyl groups, and should be removed only after HPLC [5]. Indeed, PNA oligomers without N-terminally attached Fmoc-group eluted earlier than peptides of similar length. While synthetic peptides generally elute above 15% acetonitrile content, our PNAs eluted in the 6–10% acetonitrile range. Nevertheless, as our retention times in Fig. 1 and Table 2 indicate, by choosing a slower gradient (and diluted acetonitrile as solvent B) than those usually used for peptides (0.5%/min compared with 1–1.3%/min), the acetyl-end capped PNAs were retained on the column long enough to achieve good separation. This synthetic-chromatographic protocol eliminates the extra cleavage step that can be associated with the production of newly introduced contaminants after purification.

3.2. Separation of PNA analogs with different length

The coupling efficiencies during the automated assembly of PNAs are much lower than those during peptide synthesis resulting in the presence of a large number of truncated sequences. To prevent the extension of truncated sequences that can hybridize to unrelated DNA, each cycle is end capped with acetylation. Because it was anticipated that the side chains containing the nucleobases are the major factors of binding to the RP-HPLC column, we hypothesized that in a series of PNA homologs, longer PNA oligomers would elute later. To test this possibility we synthesized and purified four PNAs corresponding to the gene of the rabies virus glycoprotein. First an 8-mer was made, and two, four and seven more monomers were added to result in homologs consisting of 8, 10, 12, and 15 monomer units, respectively. Fig. 2 shows a composite HPLC of the four molecules. The 8-mer eluted at 16.8 min, the 10-mer eluted at 19.8 min, the 12-mer eluted at 21.4 min and the 15-mer eluted at 22.0 min. This demonstrated that the length of the PNAs indeed regulated the retention times. The per monomer difference of the retention times decreased as the PNA became longer: 1.5 min/monomer in the 8–10-

Table 2
Predicted and observed retention times of 10-mer model PNA sequences

PNA	Composition				Retention time (min)		
	A	C	G	T	Predicted	Observed	Difference
RM9-10	2	1	0	7	23.4	23.4	0
RM12	1	1	3	5	22.7	22.1	−0.6
RM3	1	1	4	4	21.1	21.4	+0.3
RM6-10	3	3	0	4	21.0	20.6	−0.4
PH107a	2	2	3	3	20.4	20.3	−0.1
RM19-10	1	5	0	4	20.1	19.8	−0.3
RM20	2	4	1	3	19.9	18.7	−1.2
Rabies A5T	2	5	0	3	19.6	19.7	+0.1
p53	0	4	3	3	19.4	19.8	+0.4
E7-10	2	4	2	2	19.2	19.9	+0.7
RM11	3	5	1	1	18.5	18.3	−0.2
ALL1	2	6	1	1	18.0	18.1	+0.1

mer range, 0.8 min/monomer in the 10–12-mer range and 0.2 min/monomer in the 12–15-mer range. This reduction in the contribution of the monomers as the PNA grew could not be explained solely on the basis of side-chain hydrophobicity of the additional monomers (compare Section 3.3). Instead, it may indicate that the somewhat hydrophobic ethyl-glycine backbone contributes to the weak column binding at low monomer numbers but becomes less and less significant as the side chains promote stronger binding. For longer peptides, the formation of various secondary structures modulates the retention times, usually by retaining the peptides on the reversed-phase columns [21]. In contrast, the PNA oligomers are achiral, and are expected to form well-defined secondary structures only in the presence of chiral molecules, such as DNA, a condition not present during our chromatographic protocol.

To demonstrate the possibility of isolating the deleted PNA sequences in single HPLC runs, we analyzed the crude products after the synthesis of PNAs E7-10 and RM6-10. Mass spectroscopy indicated that the major products for E7-10 were the C-terminal (3'-equivalent) 9-mer and the full 10-mer, and for RM6-10 the C-terminal (3'-equivalent) 4-mer (RM6-4), 6-mer (RM6-6), 7-mer (RM6-7) and the full 10-mer homologs. Fig. 3 shows the mass spectrum of the crude product of the synthesis of RM6-10. While the presence of the 3-mer and the 5-mer was also observed, the 8-mer and the 9-mer were missing indicating complete coupling in these cycles. All major PNA oligomers that were detected by mass spectroscopy could be identified by RP-HPLC (Fig. 3). Moreover, the 4-mer, 6-mer, 7-mer, and 10-mer of the RM6-10 PNA and the 9-mer and the 10-mer of the E7-10 PNA could be preparatively separated and the purified fractions were reanalyzed by mass spectroscopy. Mass analysis verified the purity of these products. Fig. 4 shows the mass spectrum of the purified 6-mer RM6-6 (HPLC retention time: 16.5 min). When the last coupling steps of the PNA assembly were incomplete, PNA homologs with as few as one monomer deletions could often be isolated with remarkable purity. Fig. 5 shows the chromatogram of the purified 9-mer E7-9 (HPLC retention time: 18.5 min; mass spectrum: 2436; 2460 and 2477, corresponding to the molecular ion, the sodium adduct and the potassium adduct,

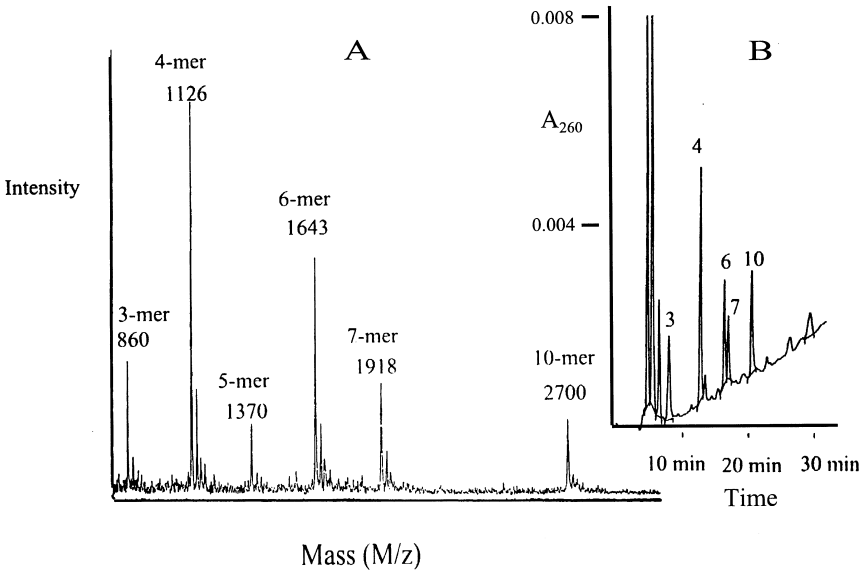

Fig. 3. Matrix-assisted laser desorption/ionization mass spectrum (panel A) and reversed-phase chromatogram (panel B) of the crude product of the synthesis of peptide nucleic acid RM6-10. Two pmol amount of PNA was used for MALDI-MS and 1 nmol for HPLC. The retention times are: 4-mer (molecular mass 1126 u): 12.9 min; 6-mer (1643 u): 16.5 min; 7-mer (1918 u): 17.1 min, and 10-mer (2700 u): 20.6 min.

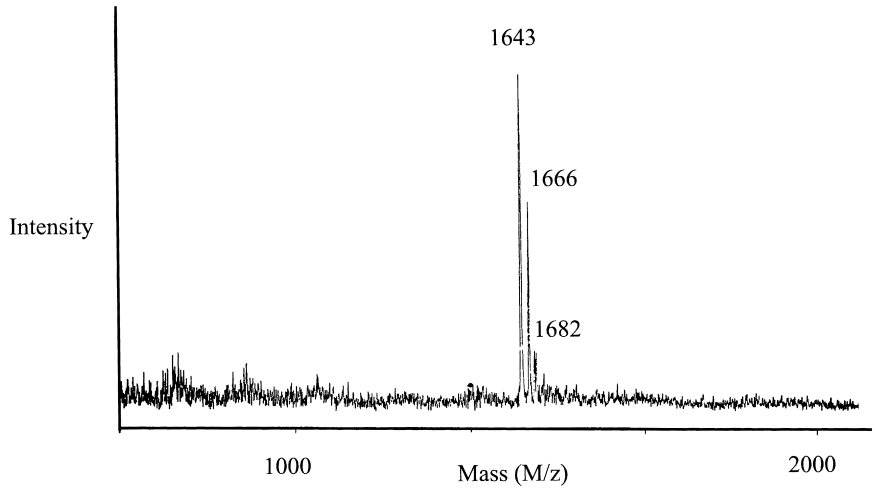

Fig. 4. Matrix-assisted laser desorption/ionization mass spectrum of purified PNA RM6-6. The peak at 1643 m/z represents the molecular ion; peaks at 1666 and 1682 represent the sodium and potassium adducts, respectively.

respectively), which was a contaminant of the synthesis of E7-10 (HPLC retention time: 19.9 min).

3.3. Contribution of individual monomer side-chains

When we compared the retention time values of all studied 29 PNA oligomers it became clear that the polymer length is not the only factor that determines the retention times of the PNA molecules. For example, the 6-mer RM9-6 sequence eluted at 20.7 min, and the 7-mer RM1-7 sequence eluted at 19.0 min, later than many 10-mers from Table 2. These sequences contain an above-average T content indicating that the side-chain of the thymidine analog PNA monomer binds more strongly to the column than those of the other three analogs. By using the retention time values of all 29 PNA oligomers, we calculated the contribution of all four individual monomers to the elution times. To precisely predict the expected retention times of any PNA, we also considered the N-terminal acetyl group, and the PNA length. First a linear equation was generated. According to this, during the chromatographic conditions we used, the following equation predicts the retention times of any PNA:

$$\text{Retention time} = 7.373 + 1.883A + 1.398C + 1.655G + 2.338T - 0.055L^2,$$

where the value A is the number of the adenine-containing monomers, C is the number of the cytosine-containing monomers, G is the number of the guanine-containing monomers and T is the number of thymine-containing monomers. L represents the length of the PNA. The constant reflects the contributions of the N-terminal (5′-equivalent) acetyl group and the C-terminal (3′-equivalent) amide group as well as the dead volume of our HPLC

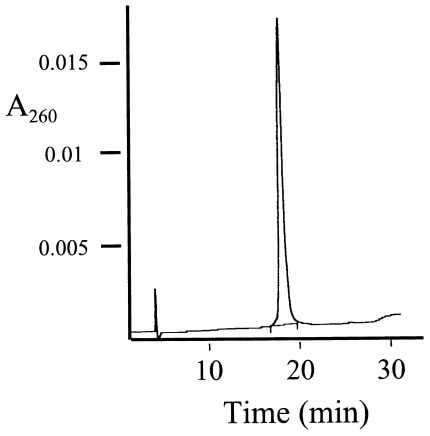

Fig. 5. Reversed-phase chromatogram of 2 nmol (4.8 μg) purified peptide nucleic acid E7-9 which was a side-product of the synthesis of E7-10. The purity of the isolated PNA was verified by mass spectrometry.

system. The above equation was derived taking into account of correlation between retention time and the number of A, C, G and T analog monomers. The length L was added to the equation because it significantly improved the ability of the equation to predict retention times. Length was added as a quadratic term because its relationship with retention time is curvilinear. The coefficients in the equation were estimated by the standard statistical method of least squares. The coefficients are optimal in the sense that they minimize the sum of the squared distances between the observed and predicted retention times. The equation is most reliable over the range of A, C, G and T levels summarized in Table 2.

The $T>A>G>C$ contribution to the retention times can be explained on the basis of the side-chain hydrophobicity of the monomers. Only thymine contains a hydrophobic methyl group. Of the three nucleobases without hydrophobic groups, those with a purine ring are more hydrophobic than the cytosine with a pyrimidine ring. The oxo-group increases the hydrophilicity of the guanine compared to the otherwise identical adenine. Elution of C prior to T could also be rationalized by the fully ionized amino group of C at pH 1.8 (T does not have an amino group). This explanation is unlikely, however, as G is eluted before A, in spite of the only partial ionization of the amino group of G and fully ionized amino group of A at pH 1.8 (pK_a 1.6 compared with pK_a 3.5). Hydrophobic surfaces or the lack thereof appear to be more important to column binding than the ionization status of the exocyclic amino groups. The negative coefficient for the length reflected the decreased contribution of the additional monomer units as the PNA grew and corrected the implicit length contribution already present in the model with the $A+C+G+T$ sum.

While the linear equation was calculated by considering the retention times of all 29 PNA oligomers, its accuracy was tested on the twelve 10-mer sequences (Table 2). Nine of the twelve PNAs eluted within 0.4 min of the predicted values, and only one oligomer eluted with a difference of more than a minute. The observed retention times were statistically spread around the predicted values: correct prediction was provided for one sequence, the prediction underestimated the real retention times five times and overestimated them six times. This prediction provided a good estimate of the PNA studied. Moreover, it contains only constant and coefficient terms with clear physical meaning. In our attempts to further improve the equation, we introduced a non-linear GT term:

$$\text{Retention time} = 6.885 + 1.842A + 1.383C + 2.544G + 2.553T - 0.246GT - 0.058L^2$$

The above equation was derived by taking account of the noisy relationship between retention time and the number of A, C, G and T analog monomers. The nature of the relationship was not perfectly linear, so nonlinear terms of the form GT, AC, G^2, T^2, etc. were added to the model with the hope of improving prediction. Only the addition of GT term significantly improved prediction. Again, the equation is most reliable over the range of A, C, G and T levels summarized in Table 2. This formula provided a better estimate for the 29 PNAs sequence set than the original linear equation, but the non-linear term cannot be justified from a chemical point of view. Clearly, the linear formula is the first step to predict the unknown retention times of PNA oligomers, yet the database needs to be significantly increased to obtain even more precise estimates with well conceivable physical meaning of the terms. The equations above are useful for the standard peptide and PNA chromatographic conditions we used. Although these are generally accepted and employed for the separation of these biooligomers, other mobile phase compositions were also reported, especially for DNA–PNA chimeras [22]. The utility of our equation for the modified mobile phase systems is yet to be evaluated.

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