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Analysis of the reaction products of N-(O,O-diisopropyl)phosphorylthreonine with uridine by capillary zone electrophoresis with diode array detection and capillary electrophoresis–mass spectrometry

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Abstract

The reaction products of N-(O,O-diisopropyl)phosphorylthreonine with uridine were analyzed by capillary zone electrophoresis with diode array detection using 50 m*M* boric acid buffer at pH 8.5. Peaks in the electropherograms were identified by the on-line UV spectra as well as some authentic standards. The influence of buffer composition and capillary coating on separations were studied. The products were also analyzed by capillary electrophoresis–mass spectrometry with a sheath-flow electrospray ionization interface. Identification was achieved by reconstructed ion electropherograms. The existence of nucleotides, homooligonucleotides and their derivatives in the products was confirmed by both methods, which supports a hypothesis for the origin of life.

Keywords: Diisopropylphosphorylthreonine; Uridine; Nucleotides; Capillary electrophoresis–mass spectrometry

1. Introduction

Which came first, nucleic acid or protein? This has long been a major question in origin of life studies [1–3]. In recent years, Zhao and her coworkers [4] have found that N-(O,O-dialkyl)phosphorylated amino acids (DAP-aa), unlike common amino acids, undergo some interesting biomimicking reactions, such as self-catalysis to form self-assembly oligopeptides and ester-exchange reactions with the alcohol on the phosphoryl group [5]. This suggests that DAP-aa might have played an important role in the prebiotic synthesis of nucleic acids and proteins. An hypothesis has been proposed by Zhao and Cao [6] that DAP-aa might be regarded as the unique original seed for nucleic acids and proteins. According to this hypothesis, oligopeptides, nucleotides and oligonucleotides should form simultaneously in the reaction of DAP-aa with nucleosides. However, the experimental evidence was insufficient to prove this. A major difficulty in providing experimental evidence was the difficulty in analysing the complex reaction products of DAP-aa. There were always many constituents whose products had similar properties. Conventional separation methods, such as TLC and column chromatography, were unable to separate them effectively. Even with reversed-phase HPLC, both resolution and reproducibility were often unsatisfactory. Moreover, because authentic standards of the potential products of these reactions usually were unavailable, product identification was a major problem. In our previous papers, fast atom bombardment mass spectrometry (FAB-MS) was used to analyze the products without prior separation [7,8].

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For the reaction of DAP-aa with a nucleoside, the content of oligopeptides in the products was high, while that of nucleotides and oligonucleotides was relatively low. FAB-MS also did not provide enough evidence of the existence of nucleotides and oligonucleotides in the reaction, although the presence of oligopeptides was confirmed by observation of the molecular ion species in the mass spectra.

Since capillary electrophoresis (CE) technology was developed in the early 1980s [9], its ability to resolve complex samples has been well recognized. A recent review summarized its applications in many areas, such as biological science, pharmaceutical analysis and chiral separations [10]. The most commonly used mode of CE is capillary zone electrophoresis (CZE), which separates ionic species based on differences in their electrophoretic mobilities. CZE has been used for the analysis of various nucleotide species [11–16] and superior resolution with shorter analysis times is often possible compared with existing HPLC methods. Besides uncoated capillaries, coated capillaries are also used for CZE analysis of nucleotide samples [17,18]. The use of coated capillaries is beneficial for two reasons. Firstly, the interactions of analytes with the inner surface of the capillary, which usually cause peak tailing, can be avoided. Secondly, the compression of electroosmotic flow (EOF) can be achieved which leads to better reproducibility, and, for strongly negatively charged nucleotide species, to shorter separation times.

The coupling of CE to a mass spectrometer (CE–MS) is one of the latest developments in CE technology [19,20]. It combines the high resolving power of CE and the peak identification ability of MS, and has the potential to play an important role in the analysis of complex samples, such as those encountered in the origin of life studies. Since the coupled technique was first demonstrated in 1987 [21], CE–MS has developed significantly in both instrumentation and applications [22]. Ionization methods, such as electrospray ionization (ESI), ion spray (ISP) and continuous-flow fast atom bombardment (CF-FAB), have been used for CE–MS. Among them, ESI is the most popular due to its sensitivity, versatility and ease of implementation. So far, two types of ESI interface, known as sheath-flow and sheathless interfaces, have gained general acceptance for CE–ESI-MS [22]. Up to now, few routine applications of CE–MS have been reported, with most reports focusing on those complex samples with quite small sample size, such as proteins [23,24] and enzymatic digests [25,26].

In this paper, we used both CZE and CE–MS with a sheath-flow ESI interface to analyze the products of the reaction of N-(O,O-diisopropyl)phosphorylthreonine (DIPPThr) with uridine, which is proposed as a model of that of a DAP-aa with a nucleoside. Before CE analysis, the products were analyzed by FAB-MS without prior separation. The existence of oligopeptides such as $(Thr)_2$ and $(Thr)_3$ was confirmed by observation of the molecular ion species in the mass spectra. Most oligopeptides were then removed by column chromatography. The results from both CZE and CE–ESI-MS confirmed the existence of nucleotides, homooligonucleotides and their derivatives in the products, which strongly supported the hypothesis we mentioned above.

2. Experimental

2.1. Equipment

A P/ACE system 5510 (Beckman, Palo Alto, CA, USA) automated CE instrument with a diode array detector (190–600 nm, Beckman) was used for CZE analysis. CE–MS experiments were carried out using a P/ACE system 5000 automated CE instrument coupled to a SSQ-710 system single quadrupole mass spectrometer (Finnigan MAT, USA) with a sheath-flow ESI interface.

2.2. Chemicals and sample preparation

3-Methacryloxypropyltrimethoxysilane (MAPS) was purchased from Fluka (Buchs, Switzerland). All other chemicals were of analytical grade. HPLC-grade water was filtered (0.45 μm pore size) and used for all requirements.

DIPPThr was synthesized according to reference [27]. All physical constants and spectroscopic data of the product were consistent with the literature values. A 1-g amount of uridine and 1 g of DIPPThr were stirred in 6 ml of anhydrous pyridine at room

temperature (30°C) for one week. Pyridine was removed under reduced pressure and 0.2 g of the mixture, dissolved in 1 ml of water, was then separated by column chromatography (Sephadex LH-20, mobile phase was methanol–water, 9:1, v/v). There were two main bands on the column, one with strong and the other with quite weak ^{31}P NMR signals. The former was collected and the solvent was removed by lyophilization. The samples was kept at −20°C. A 0.1% (w/v) aqueous solution was prepared immediately prior to CE analysis.

2.3. Preparation of a linear polyacrylamide (LPA)-coated capillary

Uncoated fused-silica capillaries (370 μm O.D. and 75 μm I.D.) were purchased from Yongnian Optical Fiber Factory (Hebei Province, China). LPA-coated capillaries were prepared according to the literature [28], with some modification. The preparation procedure was automated using the P/ACE system 5510 CE instrument and was as follows. The capillary was rinsed with a 1 *M* KOH solution for 45 min, and subsequently rinsed with water for 30 min and methanol for 30 min. A solution containing 0.4% (v/v) MAPS and 0.4% (v/v) acetic acid in methanol was continuously passed through the capillary for 2 h. The capillary was then rinsed with methanol for 30 min and with water for 30 min. A 4% acrylamide solution was prepared freshly in a buffer solution (89 m*M* Tris, 89 m*M* boric acid and 2 m*M* EDTA) and was carefully degassed (ultrasonic bath for 10 min). Polymerization was initiated by the addition of 20 μl of 10% (w/v) ammonium peroxydisulphate solution and 80 μl of 10% (v/v) N,N,N′,N′-tetramethylethylenediamine (TEMED) solution into 5 ml of acrylamide solution. The polymerizing solution was quickly passed through the capillary for 10 min and left to polymerize for 15 min. The capillary was then rinsed with water for 30 min, to remove polyacrylamide not attached to the inner surface of the capillary.

2.4. Methods for CZE and CE–ESI-MS

The products were separated by CZE in an uncoated capillary with normal polarity (the anode at the inlet) and in a LPA-coated capillary with reverse polarity (the cathode at the inlet). Both capillaries were 27 cm long in total and 20 cm from the inlet to the detection window. Running buffer was 50 m*M* boric acid solution adjusted to pH 8.5 with 0.2 *M* NaOH. Injections were achieved by applying a pressure of 3.0 kPa at the inlet for 1 s. Separations were performed at 10 kV (370 V/cm) and at 25°C. Because the absorption of nucleotides at 260 nm was strong while that of oligopeptides was weak, detection was accomplished at 260 nm in order to reduce interference from oligopeptides that had not been removed by column chromatography.

In order to protect the heated capillary in the ESI interface, nonvolatile inorganic buffer solutions, especially those containing metal ions, such as the boric acid buffers that we used for CZE, could not be used for the CE–MS separations. Instead, a 100 m*M* Tris buffer adjusted to pH 7.5 with glacial acetic acid was used for CE–MS analysis. The capillary was uncoated and 80 cm in length. Injections were achieved by applying a pressure of 3.0 kPa at the inlet for 5 s. Separations were performed at 20 kV (250 V/cm) and at 25°C. ESI-MS was conducted in the negative ion mode, which resulted in the deprotonation of phosphoric acid groups in the nucleotides. The ESI voltage was −4.6 kV, the sheath liquid was isopropanol–water–acetic acid (60:40:1, v/v/v) delivered at 3 μl/min and the scan range was 100–750 u at 100 a.m.u./s.

3. Results and discussion

3.1. CZE analysis of the products

Fig. 1 shows the CZE separation of the products in an uncoated capillary (Fig. 1A) and in a LPA-coated capillary (Fig. 1B). Separations were completed within 8 min in both cases. The on-line UV spectra obtained from the diode array detector were used to determine peak correspondence between the two electropherograms. Corresponding peaks in both figures are marked with the same number. Peak 1 was identified as uridine, by spiking with authentic standard. All peaks, except peaks 10 and 11 and shoulders on peak 1, are baseline resolved. The relative standard deviations (R.S.D.) for the migra-

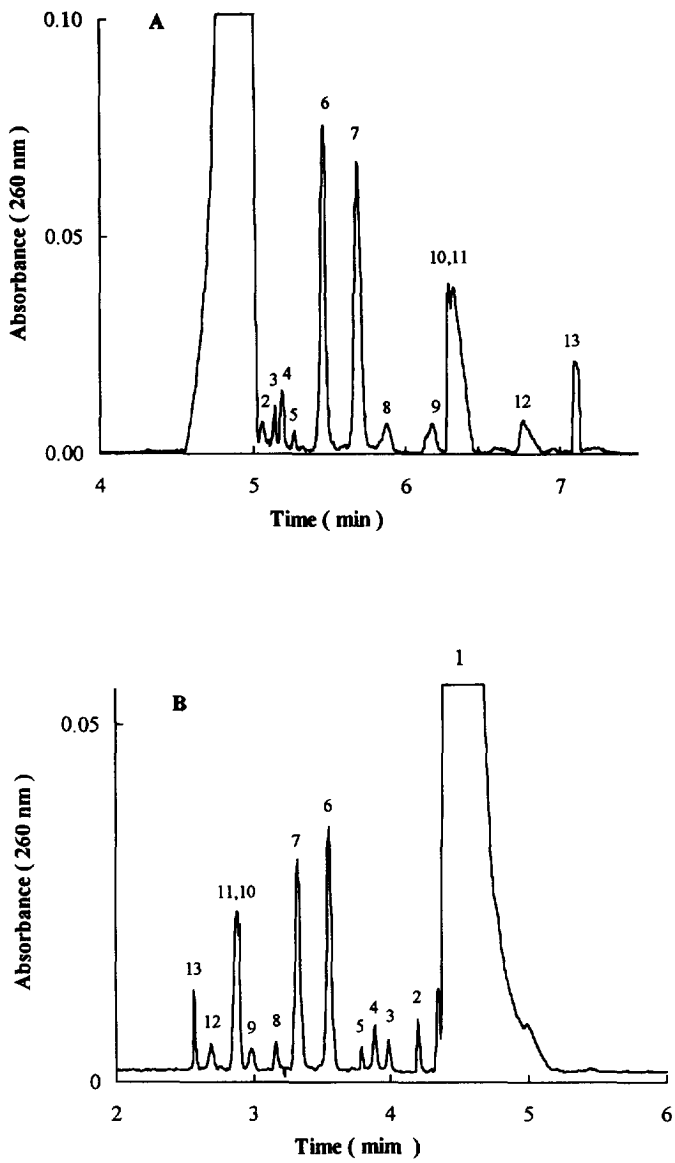

Fig. 1. CZE separations of the products, (A) in an uncoated capillary, (B) in a LPA-coated capillary. The conditions are listed in Section 2.

tion times of each peak are all less than 1.0% ($n=3$) in Fig. 1A and 0.5% ($n=3$) in Fig. 1B.

The sequence of elution in the LPA-coated capillary is reversed compared with that in the uncoated capillary. The apparent electrophoretic velocity V_{app} of a negative charged analyte in an uncoated capillary can be expressed as:

$$V_{app} = V_{eo} - V_{ep} \quad (1)$$

where V_{eo} is the electroosmotic velocity in the direction of the cathode (detection end) and V_{ep} is the electrophoretic velocity which is opposite in direction to that of V_{eo}. Generally, V_{eo} is much greater than V_{ep}, and the analytes are propelled towards the cathode to be detected. For the negatively charged analyte, the greater its V_{ep}, the smaller is its V_{app}, and in turn, the slower it emerges. In contrast, in a LPA-coated capillary, the electroosomtic flow is

compressed by the LPA coating on the inner surface of the capillary, so V_{app} of a negatively charged analyte can be expressed as:

$$V_{app} \approx V_{ep} \quad (2)$$

so, the greater its V_{ep} is, the faster it emerges.

Although CZE in either the uncoated capillary or the LPA-coated capillary can separate the products effectively, some improvements can still be seen in the latter. Firstly, the reproducibility of migration time is improved. Secondly, the separation time is reduced from 7.3 min. to 5.2 min. Thirdly, the peak shapes are improved because the interaction of analytes with the capillary's inner surface is avoided with the LPA coating. Lastly, resolution is also enhanced to some extent, as can be seen by looking at the two small peaks which are covered by peak 1 in Fig. 1A, but are partly separated in Fig. 1B.

3.2. Assignment of the electropherogram

On the basis of our previous hypothesis [6], the proposed reaction mechanism for the formation of nucleotides is given in Fig. 2. According to this mechanism, there should be nucleotides, homooligonucleotides and their derivatives, such as 5′-UMP, ^{i}PrO-pU and UpU, in the products. Fig. 3 shows the on-line UV spectra of the two reactants and the products in Fig. 1A. The absorption characteristics of all the products are quite similar to that of uridine. Because the absorption characteristics of the nucleoside, nucleotides and homooligonucleotides with the same base are usually alike [29], these products can be reasonably regarded as nucleotides, homooligonucleotides or their derivatives containing uridine. Peak 13 was further identified to be 5′-UMP by spiking with the authentic standard, however, no such standards were available for the other peaks.

Assignment of peaks 6 and 7 is approached by combining the proposed mechanism and the elution order. The V_{ep} of a charged molecular species can be approximated from the Debye-Huckle-Henry theory [30],

$$V_{ep} = \mu_{ep}E = \frac{qE}{6\pi\eta r} \quad (3)$$

where μ_{ep} is the electrophoretic mobility, q is the charge on the particle, η is the viscosity of the buffer, and r is the Stokes' radius of the particle. The mass of the particle may be related to the Stokes' radius by $M \propto r^3$, therefore V_{ep} can be expressed as:

$$V_{ep} = K\frac{qE}{M^{1/3}} \quad (4)$$

where K is a constant. According to the proposed mechanism, ^{i}PrO-pU and UpU should be two main products which should correspond to the two main peaks in Fig. 1 (peaks 6 and 7). Their pK_a values for the phosphoric acid and uracil groups are estimated to be about 6.6 and 9.5, respectively, on the basis of reference [29], so they exist mainly as monovalent anions at pH 8.5, and the molecular mass of UpU (550) is greater than that of ^{i}PrO-pU (366). Therefore, the V_{ep} of UpU is smaller than that of ^{i}PrO-pU. In turn, UpU will emerge faster than ^{i}PrO-pU (Fig. 1A), and so peaks 6 and 7 are assigned to UpU and ^{i}PrO-pU, respectively.

CZE can separate the products effectively, but due to the lack of authentic standards, peaks except uridine and 5′-UMP can not be identified exactly. Other detection methods such as MS have to be used for further identification.

3.3. CE–ESI-MS of the products

Fig. 4 shows the reconstructed ion electropherograms for the CE–ESI-MS separation of the products. There are seven peaks in the total ion electropherogram. Identification is achieved by the molecular mass of each peak which is obtained from the corresponding reconstructed ion electropherogram. Wide peaks appear due to the adsorption of the analytes on the inner surface of the capillary and to the long separation time. Because the detection limit of MS is high compared with UV, many small peaks appear in Fig. 1 that do not appear in the total ion electropherogram.

According to the proposed mechanism in Fig. 2, assignments of the seven peaks in the total ion electropherogram are listed in Table 1. The results agree with what we get from CZE. Besides uridine and the main products such as 5′-UMP, ^{i}PrO-pU and UpU, there are also some by-products in the mixture. Phosphate ester and pyrophosphate ester are the hydrolysates of DIPPThr. They do not appear in Fig.

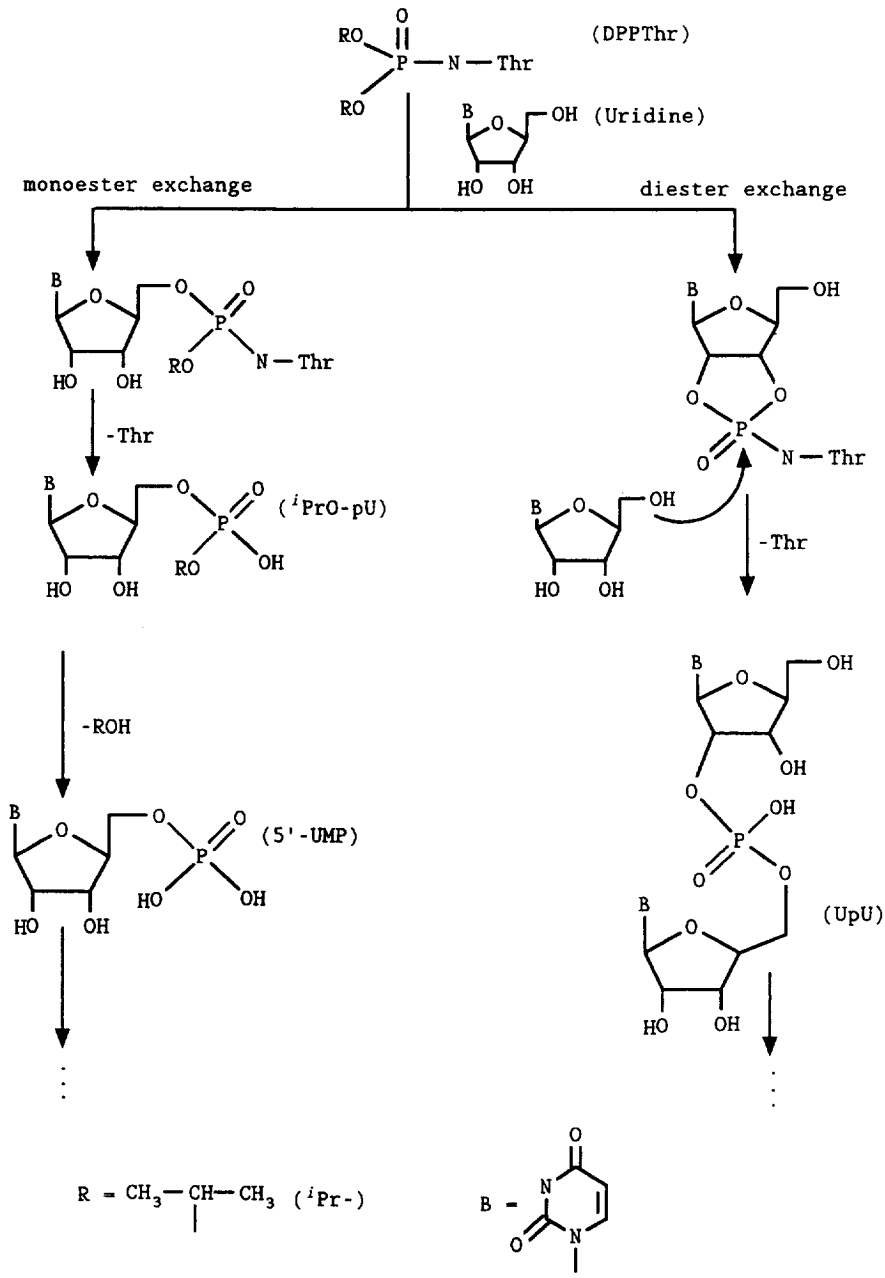

Fig. 2. The proposed main approach to form nucleotides by the reaction of DIPPThr with uridine.

1A due to their lack of adsorption at 260 nm. ⁱPrO-pUp Results from the reaction of ⁱPrO-pU and phosphate ester. Because peaks 10 and 11 in Fig. 1A correspond to only one peak in the reconstructed ion electropherogram, they have the same molecular mass ($M-1=445$), which indicates that they are two isomers of ⁱPrO-pUp.

The elution order is slightly different between Fig. 1A and Fig. 4, ⁱPrO-pUp migrates faster than 5′-UMP in boric acid buffer, while it migrates slower in

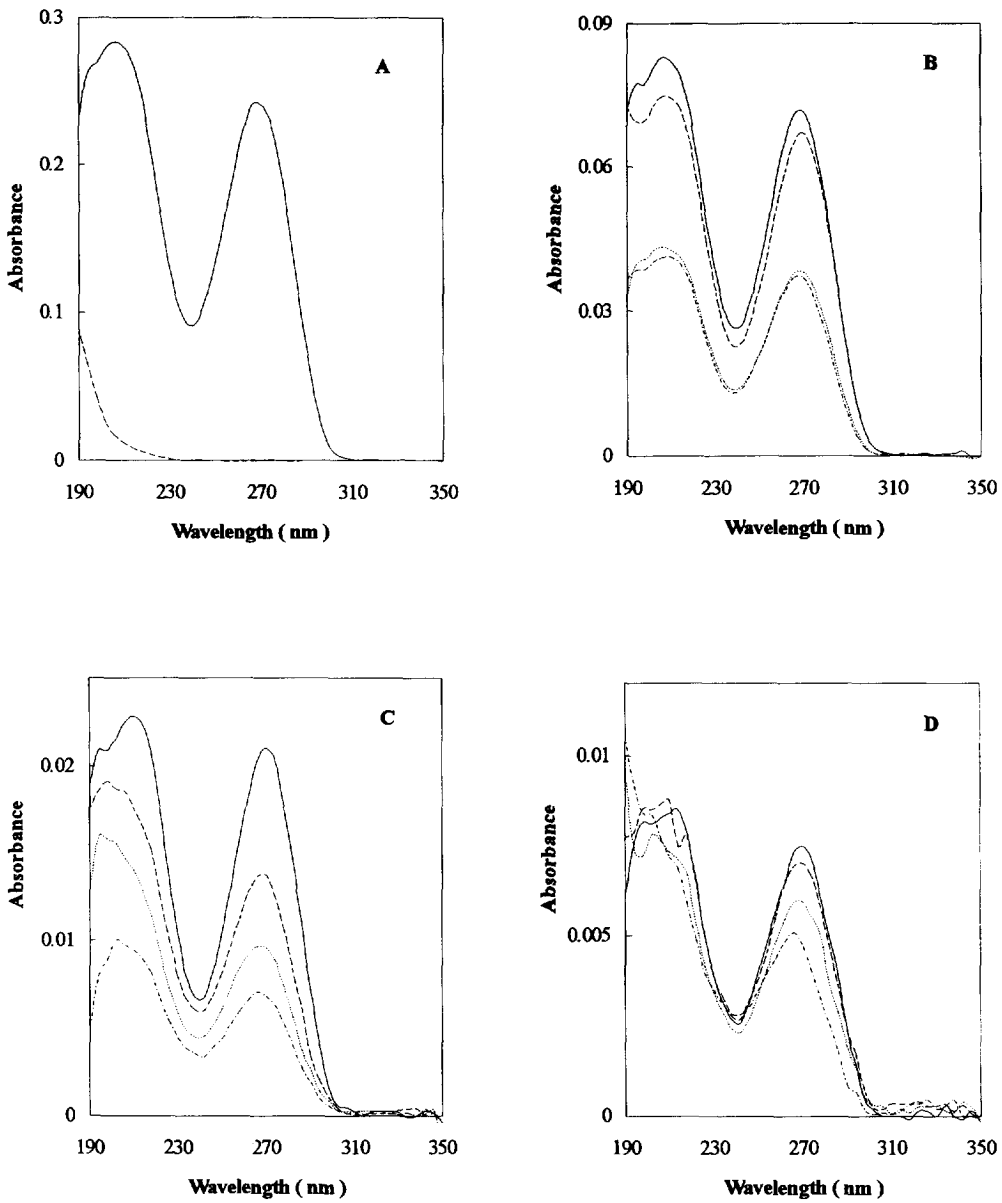

Fig. 3. The on-line UV spectra of the two reactants and the products in Fig. 1A. (A), (———) spectrum of uridine; (– – –) DIPPThr; (B), (———) peak 6; (– – –) peak 7; (· · ·) peak 11; (– · –) peak 10; (C), (———) peak 13; (– – –) peak 8; (· · ·) peak 9; (– · –) peak 12; (D), (———) peak 4; (– – –) peak 3; (· · ·) peak 2; (– · –) peak 5.

Tris buffer. Boric acid has the ability to form stable complexes with *cis*-diols such as the 2′-OH and 3′-OH of ribose in 5′-UMP, which imparts an additional negative charge to 5′-UMP. Therefore 5′-UMP migration to the cathode is slower than that of ^{i}PrO-pUp in boric acid buffer. In order to prove this, the complexation can be prevented by adding glycerin to boric acid buffer. The relative migration times (dimethyl sulfoxide is taken as a neutral marker) of 5′-UMP and ^{i}PrO-pUp are listed in Table 2. It can be seen that 5′-UMP migrates relatively faster than ^{i}PrO-pUp as the concentration of glycerin

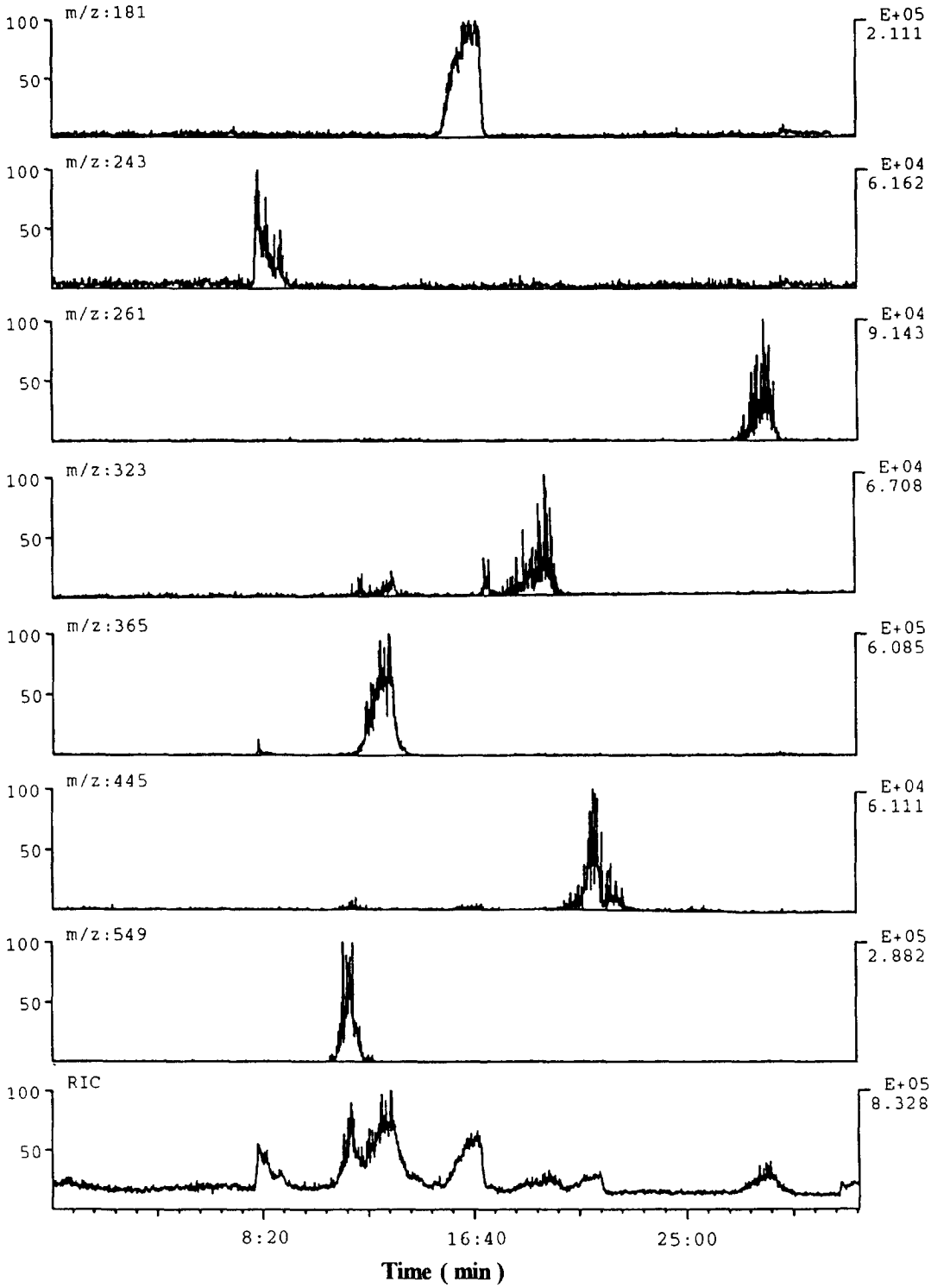

Fig. 4. Reconstructed ion electropherograms for the separation of the products (All the plots were not smoothed), conditions are listed in Section 2.

Table 1
Identification of reaction products by CE–MS

Peak number in Fig. 1	m/z	Relative intensity (%)	Possible structure
	181	34.7	phosphate ester
1	243	10.1	uridine
	261	15.0	pyrophosphate ester
13	323	11.0	5′-UMP
7	365	100	ⁱPrO-pU
10, 11	445	10.0	ⁱPrO-pUp
6	549	47.4	UpU

increases. With 400 m*M* glycerin in boric acid buffer, the same migration order as in Fig. 4 is observed. For the same reason, uridine can migrate towards the anode in the LPA-coated capillary, although it is almost a neutral molecule at pH 8.5. Due to its complexation effect, boric acid buffer is more effective for separating the products. This emphasizes the importance of buffer selection for enhancing selectivity in CE separations.

4. Conclusion

The reaction products of DIPPThr and uridine were separated effectively by CZE and the existence of at least twelve nucleotide species was confirmed by the on-line UV spectra and spiking with authentic standards. The LPA-coated capillary can compress electroosmotic flow and interactions of the analytes with the capillary inner surface, which can improve separations to some extent. Buffer composition can influence selectivity as well as resolution, with boric acid buffer having a particular advantage for the products due to its complexation with *cis*-diol-containing compounds.

CE–ESI–MS was also used for the analysis of the products. The existence of 5′-UMP, ⁱPrO-pU, UpU, ⁱPrO-pUp, as well as uridine, phosphate ester and pyrophosphate ester, was confirmed. This provides some experimental evidence for the hypothesis that DAP-aa might be the common origin for nucleic acids and proteins.

Table 2
Relative migration times of 5′-UMP and ⁱPrO-pUp

Concentration of glycerin (m*M*)	Relative migration time	
	5′-UMP	ⁱPrO-pUp
0	1.48	1.30
100	1.54	1.42
200	1.80	1.76
400	1.82	1.98

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