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SEPARATION OF SMALL DNA AND RNA OLIGONUCLEOTIDES BY HIGH-PERFORMANCE ANION-EXCHANGE LIQUID CHROMATOGRAPHY

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SUMMARY

Small oligonucleotides from DNA and RNA have been separated according to their base composition by high-performance anion-exchange liquid chromatography on Partisil-10 SAX using triethylammonium acetate buffer as the eluent. Fifteen of the 16 possible deoxydinucleoside monophosphates and all 16 dinucleoside monophosphates have been separated. All pairs of sequence isomers were well resolved. The 15 commercially available deoxydinucleotides were resolved into 13 fractions.

A good resolution of deoxytrinucleoside diphosphates isolated from an alkaline phosphatase–Mg^{2+}-activated DNase I digest of calf thymus DNA was achieved by this technique. A large number of sequence isomers could be fully separated. The base sequence of the eluted individual constituents has been determined by their hydrolysis with snake venom and spleen phosphodiesterase followed by high-performance liquid chromatographic analysis of the nucleotides released.

The eight trinucleoside diphosphates isolated from an alkaline phosphatase–pancreatic RNase digest of yeast RNA have also been separated according to base composition. Their sequence was determined as above.

The described technique is fast and gave very good separations. Most of the sequence isomers could be separated. Moreover, the eluent triethylammonium acetate can easily be removed from column effluents by freeze-drying in order to facilitate subsequent sequence analysis of the eluted compounds. The observed elution orders of the sequence isomers obey certain rules which are discussed in detail.

INTRODUCTION**

Since Cohn[1] first separated the ribonucleotides in 1950, ion-exchange liquid

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** Abbreviations for nucleosides and nucleotides follow CBN Recommendations [see *Eur. J. Biochem.*, 15 (1970) 203].

chromatography has been used extensively in the separation of nucleic acid constituents. The fractionation of dimers and trimers of deoxyribo- and ribonucleotides according to their base composition is of great interest because of their sequential information and has been carried out by several authors using conventional ion-exchange chromatographic methods[2–15].

Column efficiency, speed and sensitivity of analysis in ion-exchange liquid chromatography have been significantly increased by using very fine ion-exchange resins and increasing the column inlet pressure[16–20]. Nucleic acid research gave a great impetus to the development of high-performance liquid chromatography (HPLC) and this method has been successfully employed to separate various nucleic acid constituents[21–26].

In this paper we report on the separation of deoxydinucleotides, deoxydinucleoside monophosphates and dinucleoside monophosphates by HPLC using triethylammonium acetate buffer as the eluent. The separation of deoxytrinucleoside diphosphates and trinucleoside diphosphates isolated from an alkaline phosphatase–DNase I digest of calf thymus DNA and an alkaline phosphatase–pancreatic RNase digest of yeast RNA, respectively, has also been carried out by this technique.

Triethylammonium acetate buffer has been chosen as the eluent because it exhibits negligible absorption at the monitoring wavelength, 260 nm, buffers well at the pH employed and is easily prepared from triethylamine and acetic acid[27,28]. Another advantage of this eluent is that it is readily removed from the eluted samples by freeze-drying[27]. This property has been found to be very important for the sequence determination of very small amounts (corresponding to about 0.1 A_{260} unit) of the eluted individual compounds, as shown in this paper in the case of deoxytrinucleoside diphosphates and trinucleoside diphosphates.

MATERIALS AND METHODS

Apparatus

A high-performance liquid chromatograph (Hewlett-Packard Model 1010B) was used together with a UV detector (Schoeffel Model SF 770) equipped with a micro flow cell (cell volume, 8 μl). The stainless-steel column had an I.D. of 3 mm and a length of 50 cm. During chromatography the eluent reservoirs were kept at 40°. The eluents were degassed at 40° under vacuum prior to chromatography. In all the separations the column temperature was 60°. The samples were introduced into the column with the help of a high-pressure sampling valve (Valco). The column effluents were monitored at 260 nm.

Preparation of the column

The column, closed by a metal frit at the bottom, was connected to a filling tube (15 × 2 cm). A suspension of the anion exchanger Partisil-10 SAX (particle size 10 μm; 3.2 g) in 20 ml of dioxane–tetrachloromethane (1:1) was transferred into the filling tube and then pumped with 150 ml of *n*-heptane into the column at a pressure of 400 bar. After passage of 100 ml of isopropanol the column was placed in the chromatographic system and washed with 200 ml of 0.01 *M* triethylammonium acetate buffer (pH 3.1).

Materials

Partisil-10 SAX [Chrompack (Middelburg, The Netherlands); particle size 10 μm] was used as the anion exchanger without any pretreatment. Dinucleoside monophosphates and 3'-deoxynucleotides were obtained from Sigma (St. Louis, Mo., U.S.A.), deoxydinucleotides from P-L Biochemicals (Milwaukee, Wisc., U.S.A.). Deoxydinucleoside monophosphates were prepared by digestion of the corresponding deoxydinucleotides with alkaline phosphatase. 5'-Deoxynucleotides, 3'- and 5'-nucleotides and yeast RNA were obtained from Boehringer (Mannheim, G.F.R.). (dC_2,dT) and (dC,dT_2) were a gift from Dr. H. Schott, Tübingen. Calf thymus DNA, urea (p.a.), triethylamine (reagent grade) and tris(hydroxymethyl)aminomethane (p.a.) were from Merck (Darmstadt, G.F.R.), and DEAE-Sephadex A-25 (3.5 mequiv./g) from Pharmacia (Uppsala, Sweden). Triethylamine was purified by refluxing it for 3 h with 2,4-diaminophenol dihydrochloride followed by distillation[28]. A stock solution of 2 *M* triethylammonium acetate (pH 4.5) was prepared by addition of purified triethylamine to an acetic acid solution. Working solutions were prepared by dilution of the stock solution and titration with glacial acetic acid to the desired pH value.

Enzymes

Pancreatic deoxyribonuclease (EC 3.1.4.5), pancreatic ribonuclease (EC 2.7.7.16), alkaline phosphatase (EC 3.1.3.1), snake venom phosphodiestrase (EC 3.1.4.1) and spleen phosphodiesterase (EC 3.1.4.18) were obtained from Boehringer.

Isolation of deoxytrinucleoside diphosphates

Deoxytrinucleoside diphosphates were isolated from an alkaline phosphatase–Mg^{2+}-activated DNase I digest of calf thymus DNA by anion-exchange column chromatography[15,29]. Deoxytrinucleoside diphosphates isostich fractions were desalted using a DEAE-Sephadex A-25 (acetate) column and triethylammonium acetate as eluent. After desalting, triethylammonium acetate was removed by freeze-drying.

Characterization of deoxytrinucleoside diphosphate

Deoxytrinucleoside diphosphates separated according to the base composition by HPLC (see Results) were characterized, as shown below, by hydrolysis with snake venom phosphodiesterase and spleen phosphodiesterase[13,30] followed by HPLC analysis of the nucleotides released (*cf.* Table I):

$$d(XpYpZ) \xrightarrow[\text{phosphodiesterase}]{\text{snake venom}} dX + pdY + pdZ$$

$$d(XpYpZ) \xrightarrow[\text{phosphodiesterase}]{\text{spleen}} dXp + dYp + dZ$$

The eluted fractions containing individual deoxytrinucleoside diphosphates were divided into two parts and freeze-dried to remove triethylammonium acetate. One part was dissolved in 0.05 ml of 0.01 *M* Tris–HCl buffer (pH 9.0) containing 0.01 *M* $MgCl_2$ and incubated with 0.005 units of snake venom phosphodiesterase at 37° for 2 h. The other part was dissolved in 0.05 ml of 0.01 *M* Tris–HCl buffer (pH 7.0) and

TABLE I

ELUTION TIMES OF NUCLEOTIDES

Eluent, 0.15 *M* triethylammonium acetate (pH 3.1). Column, Partisil-10 SAX (10 μm), 0.3 × 50 cm; temperature, 60°; pressure, *ca.* 60 bar; flow-rate, 1.0 ml/min.

5'-Nucleotide	*Elution time (min)*	*3'-Nucleotide*	*Elution time (min)*
pdC	5.6	dCp	8.1
pdA	9.2	dAp	9.6
pdT	11.0	dTp	13.0
pdG	22.0	dGp	14.8
pC	6.1	Cp	11.2
pA	9.1	Ap	12.0
pU	11.5	Up	17.6
pG	22.9	Gp	21.1

incubated with 0.005 units of spleen phosphodiesterase at 37° for 2 h. The samples were then analyzed by HPLC in order to determine the nucleotides released. The elution times of the nucleotides and the chromatographic conditions are given in Table I.

Isolation of trinucleoside diphosphates

A 100-mg amount of yeast RNA was dissolved in 100 ml of 0.01 *M* Tris–HCl buffer (pH 7.0) and incubated with 0.5 mg (25 units) of pancreatic RNase at 37° for 48 h. The digest was then treated with 40 units of alkaline phosphatase at 37° for 12 h. The isolation of trinucleoside diphosphates from this digest and their characterization were carried out as in the case of deoxytrinucleoside diphosphates.

RESULTS

Separation of deoxydinucleotides and deoxydinucleoside monophosphates

The separation of the 15 commercially available deoxydinucleotides according to the base composition on Partisil-10 SAX at 60° is shown in Fig. 1. The eluent was a linear gradient of 0.1 to 0.5 *M* triethylammonium acetate buffer. The optimum pH value for the separation of these compounds by conventional anion-exchange chromatography on DEAE-cellulose was found[15] to be 3.4 (see also ref. 1). Under our experimental conditions we obtained an optimal separation at pH 3.1 (0.1 *M*) to 3.4 (0.5 *M*).

The absorbance profile in Fig. 1 shows a resolution of 13 peaks. The sequence isomers d(pCpG) and d(pGpC), as well as d(pApG) and d(pGpA), were not resolved while a complete separation of the pairs of other sequence isomers was achieved. d(pCpA) was not available to us.

An improved resolution of this class of compounds, as shown in Fig. 2, was achieved when the 5'-terminal phosphate groups were removed by digestion with alkaline phosphatase. The elution pattern shows a separation of 15 peaks corresponding to all 15 available deoxydinucleoside monophosphates. d(CpA) was not available to us. All pairs of sequence isomers were resolved. In this case an optimal resolution was obtained with 0.01 *M* triethylammonium acetate at pH 3.1 as eluent. No gradient elution was necessary.

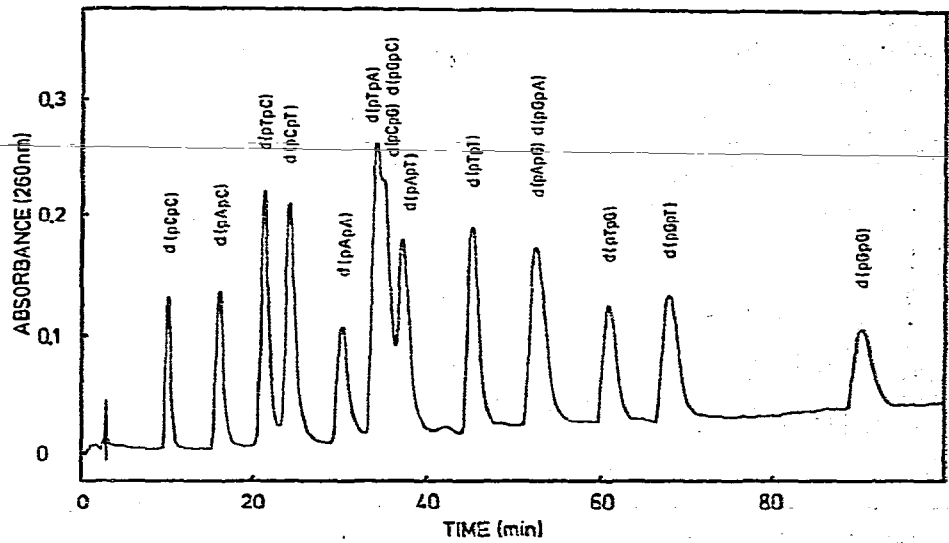

Fig. 1. Separation of deoxydinucleotides. Column, Partisil-10 SAX (particle size 10 μm), 0.3 × 50 cm; temperature, 60°; pressure, *ca.* 60 bar. Eluent: linear gradient of 0.1 *M* (pH 3.1) to 0.5 *M* (pH 3.4) of triethylammonium acetate; duration of gradient elution, 100 min; flow-rate, 1.0 ml/min.

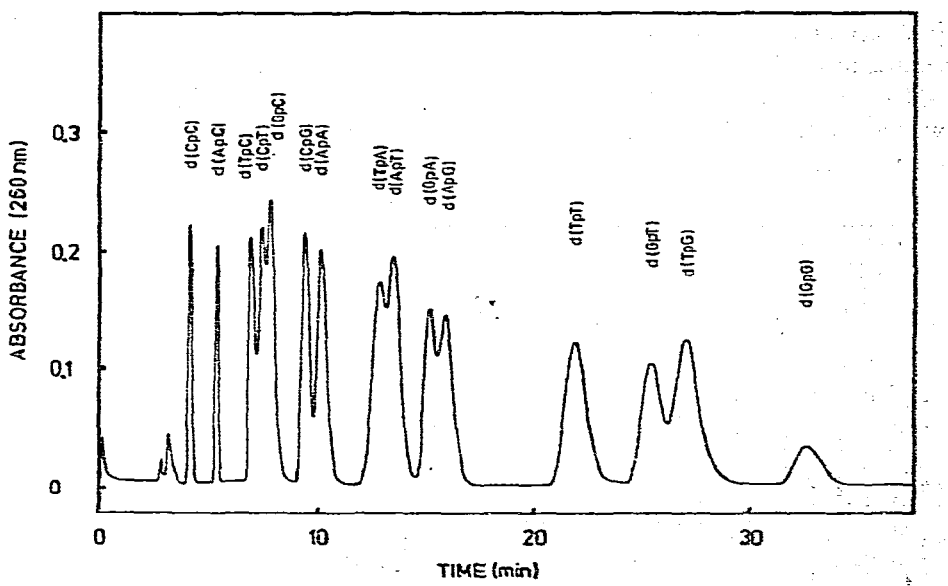

Fig. 2. Separation of deoxydinucleoside monophosphates. Eluent, 0.01 *M* triethylammonium acetate at pH 3.1; flow-rate, 1.0 ml/min. Other column details as in Fig. 1.

Separation of dinucleoside monophosphates

Fig. 3 shows the separation of the 16 possible dinucleoside monophosphates from RNA on Partisil-10 SAX at 60° with 0.01 *M* triethylammonium acetate at pH 3.1 as eluent. The absorbance profile represents a resolution of 16 peaks. All pairs of sequence isomers were completely resolved.

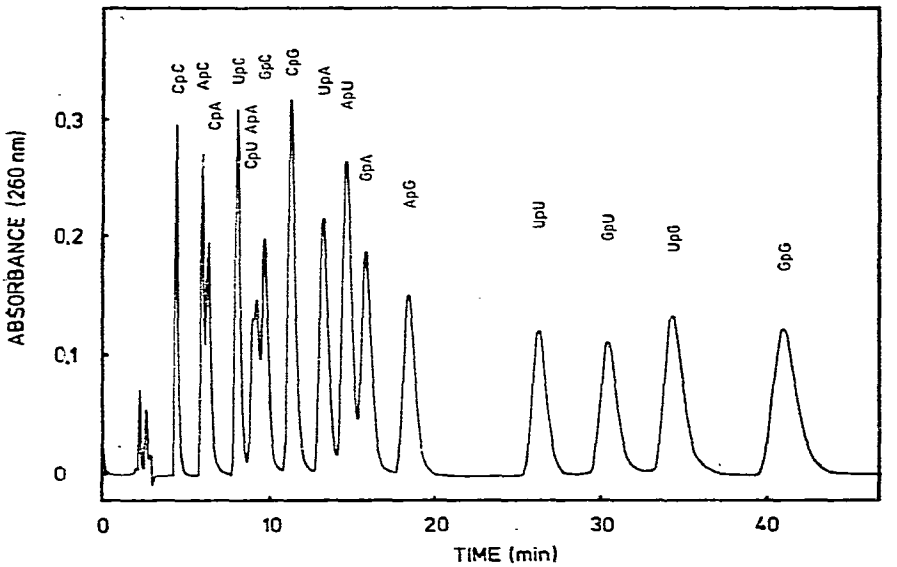

Fig. 3. Separation of dinucleoside monophosphates. Eluent, 0.01 *M* triethylammonium acetate at pH 3.1; flow-rate, 1.0 ml/min. Other column details as in Fig. 1.

Separation of deoxytrinucleoside diphosphates

The separation of deoxytrinucleoside diphosphates isolated from an alkaline phosphatase–DNase I digest of DNA is given in Fig. 4. A good resolution of these compounds was achieved with a linear gradient of 0.03 *M* (pH 3.1) to 0.4 *M* (pH 3.4) triethylammonium acetate at 60°. The base composition and sequence of well sepa-

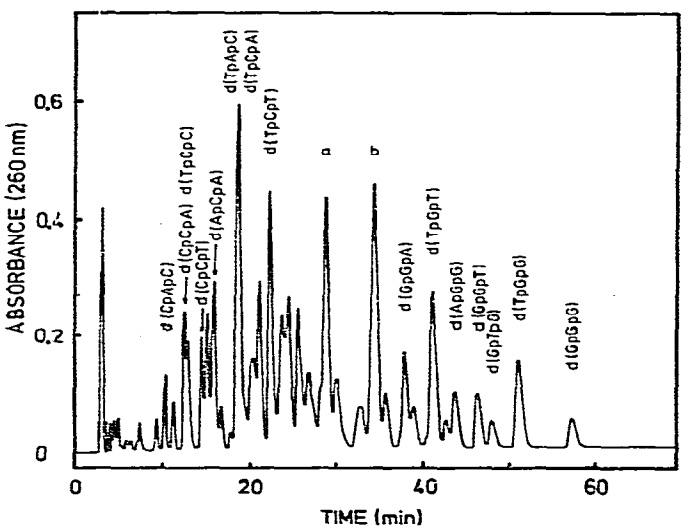

Fig. 4. Separation of deoxytrinucleoside diphosphates isolated from an alkaline phosphatase–Mg^{2+}-activated DNase I digest of calf thymus DNA. Eluent, linear gradient of 0.03 *M* (pH 3.1) to 0.4 *M* (pH 3.4) triethylammonium acetate; duration of gradient elution, 80 min; flow-rate, 1.0 ml/min. Other column details as in Fig. 1.

rated components was determined by their hydrolysis with snake venom and spleen phosphodiesterase, followed by HPLC analysis of the nucleotides released, as described in Materials and methods. Peaks a and b were found to be mixtures of compounds which contain adenine, guanine and thymine nucleosides. The other components were not characterized. The sequence isomers d(CpApC) and d(CpCpA), d(TpCpC) and d(CpCpT), d(GpGpA) and d(ApGpG) were completely separated. The three sequence isomers d(GpGpT), d(GpTpG) and d(TpGpG) were also fully resolved by this procedure.

Separation of pyrimidine deoxytrinucleoside diphosphates

The separation of the pyrimidine deoxytrinucleoside diphosphates with the general formula (dC,dT_2) and (dC_2,dT) was also attempted by this technique. The sequence isomers d(TpTpC), d(TpCpT) and d(CpTpT) were separated using 0.1 *M* triethylammonium acetate at pH 3.1 as shown in Fig. 5.

Fig. 6 shows the separation of the isomers d(TpCpC), d(CpTpC) and d(CpCpT) with a linear gradient of 0.03 *M* (pH 3.1) to 0.2 *M* (pH 3.4) triethylammonium acetate. d(TpCpC) and d(CpTpC) could not be resolved, while a complete separation of d(CpCpT) from its isomers was achieved. The sequence determination of these compounds was also carried out as described above.

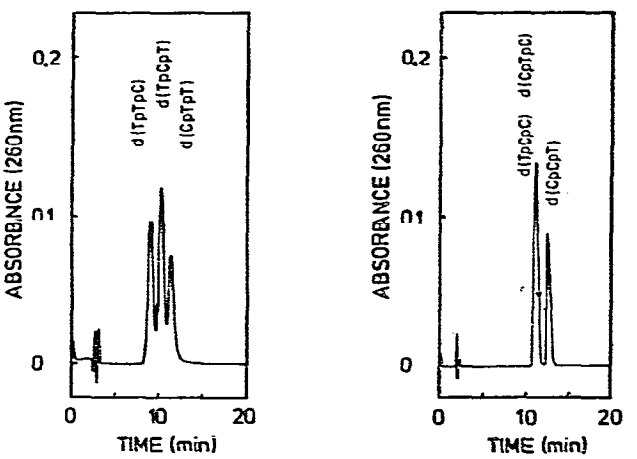

Fig. 5. Separation of the sequence isomers (dC,dT_2). Eluent, 0.1 *M* triethylammonium acetate at pH 3.1; flow-rate, 1.0 ml/min. Other column details as in Fig. 1.

Fig. 6. Separation of the sequence isomers (dC_2,dT). Eluent, linear gradient of 0.03 *M* (pH 3.1) to 0.2 *M* (pH 3.4) triethylammonium acetate; duration of gradient elution, 40 min; flow-rate, 1.0 ml/min. Other column details as in Fig. 1.

Separation of trinucleoside diphosphates

The digestion of yeast RNA with pancreatic RNase produces eight trinucleotides since this enzyme, as a highly specific endonuclease, breaks the linkage between 3'-phosphoryl pyrimidine nucleotides and adjacent nucleotides[31]. The separation of all these eight compounds after removal of their 3'-phosphate groups by digestion with alkaline phosphatase is given in Fig. 7. The characterization of these compounds was carried out as for the deoxytrinucleoside diphosphates.

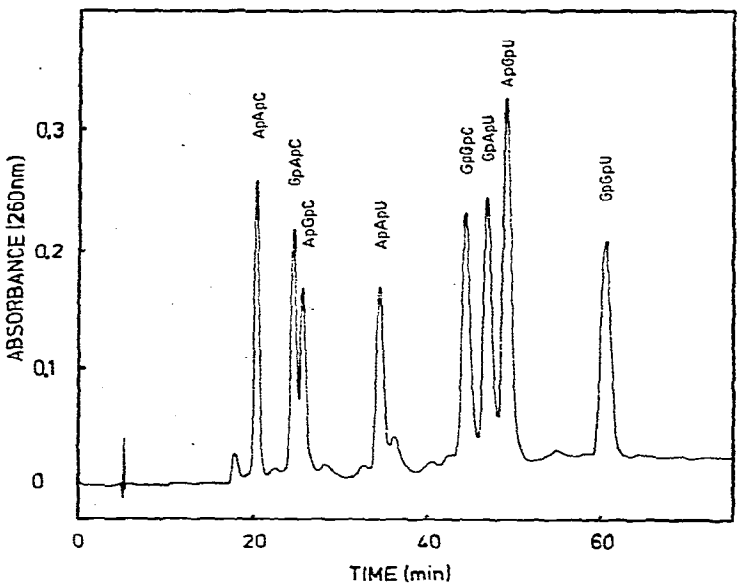

Fig. 7. Separation of trinucleoside diphosphates isolated from an alkaline phosphatase–pancreatic RNase digest of yeast RNA. Eluent, linear gradient of 0.03 *M* (pH 3.1) to 0.4 *M* (pH 3.4) triethylammonium acetate; duration of gradient elution, 80 min; flow-rate; 1.0 ml/min. Other column details as in Fig. 1.

DISCUSSION

The observed elution order of the small oligonucleotides from DNA and RNA separated by the technique described in this paper obey certain rules: the elution order of deoxydinucleoside monophosphates having one base (X) in common is the same as that of the deoxynucleotides (see also Table I):

d(XpC), d(XpA), d(XpT), d(XpG) and d(CpX), d(ApX), d(TpX), d(GpX)
pdC, pdA, pdT, pdG and dCp, dAp, dTp, dGp

A similar order was observed in the series of dinucleoside monophosphates:

XpC, XpA, XpU, XpG, and CpX, ApX, UpX, GpX
pC, pA, pU, pG and Cp, Ap, Up, Gp

With respect to a pair of sequence isomers the following elution orders were found:

d(TpC), d(CpT); d(TpA), d(ApT); d(GpA), d(ApG); d(GpC), d(CpG); d(GpT), d(TpG)

and

ApC, CpA; UpC, CpU; UpA, ApU; GpA, ApG; GpC, CpG; GpU, UpG

As a rule of thumb, the elution order of a pair of sequence isomers, d(XpY) and d(YpX), is determined by the nucleotide with the 3′-OH group within a deoxy-

nucleoside monophosphate, *i.e.*, by the elution order of pdY and pdX. Thus, d(XpY) is eluted faster than d(YpX) if pdY is eluted faster than pdX. The same rule of thumb can be applied to dinucleoside monophosphates.

This rule can be extended to sequence isomers of deoxytrinucleoside diphosphates. If these sequence isomers have a subunit (X) in common this subunit will contribute to the retention of both sequence isomers d(XpYpZ) and d(XpZpY) by a similar amount, *i.e.*, the elution order of these sequence isomers is largely governed by the elution order of the remaining dinucleoside monophosphates, d(YpZ) and d(ZpY). The same approach also holds for sequence isomers such as d(XpYpZ) and d(YpXpZ). Their elution order is determined by the elution order of d(XpY) and d(YpX). It appears that the pair d(XpYpZ) and (dZpYpX) can also be reduced to d(XpZ) and d(ZpX). This rule of thumb is obeyed in all cases studied in this paper:

d(CpApC), d(CpCpA); d(GpGpA), d(ApGpG); d(TpCpC), d(CpCpT);
d(GpGpT), d(GpTpG), d(TpGpG) (Fig. 4)
d(TpTpC), d(TpCpT), d(CpTpT) (Fig. 5)
GpApC, ApGpC; GpApU, ApGpU (Fig. 7)

The same rule has recently been found to be also followed by all sequence isomers of pyrimidine deoxytetranucleoside triphosphates separated by the same technique[32].

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