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Purification of chemically synthesised dinucleoside(5′,5′) polyphosphates by displacement chromatography

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Abstract

Dinucleoside(5′,5′) polyphosphates (Ap_nA, Ap_nG, Gp_nG, $n=3–6$) are new group of hormones controlling important biological processes. Because some of the dinucleoside(5′,5′) polyphosphates are commercially not available purification of chemical synthesised dinucleoside(5′,5′) polyphosphates became necessary in order to test their physiological and pharmacological properties. It was the aim of this study to find a method which allows purification of 0.1–0.2 g quantities of dinucleoside polyphosphates by analytical HPLC columns yielding products with impurities lower than 1.0%. Adenosine(5′)-polyphospho-(5′)guanosines were synthesised by mixing the corresponding mononucleotides. The reaction results in a complex mixture of Ap_nA, Ap_nG and Gp_nG (with $n=3–6$ in all cases). The reaction mixture was concentrated on a preparative C_{18} reversed-phase column. The concentrate was displaced on a reversed-phase stationary. As a result of displacement chromatography, anion-exchange chromatography in gradient modus yielded baseline separated dinucleoside polyphosphates (homogeneity of the fractions>99%). The identity of the substances were determined by matrix assisted laser desorption ionisation mass spectrometry.

Keywords: Displacement chromatography; Dinucleoside polyphosphates; Adenosine–oligophosphoguanosine

1. Introduction

Diadenosine(5′,5′) polyphosphates are modulators of blood pressure regulating vascular tone and cardiac functions [1]. Recently, adenosine(5′)-oligophospho-(5′)guanosines have been described as a further group of dinucleoside polyphosphates regulating the resistance of the renal microvasculature and growth of vascular smooth muscle cells [2]. Chemical structures of diadenosine(5′,5′) polyphosphates, adenosine(5′)-oligophospho-(5′)guanosines and diguanosine(5′,5′) polyphosphates are given in Fig. 1. For characterisation of biological, pharmacological as well as physiological functions of these adenosine(5′)-oligophospho(5′)-guanosines the substances are required in a very pure form. Because adenosine (5′)-oligophospho(5′)-guanosines are not commercial available these substances have to be synthesised. Ng and Orgel published a simple synthesis of the diadenosine polyphosphates [3]. The method is based on activation of mononucleotides (e.g. ATP and GTP) with a water-soluble carbodiimide. Unfortunately the reaction yields a lot of byproducts (Ap_5A, Ap_5G, Gp_5G, Ap_6A, Gp_6G etc.) which are chemically very similar to the desired product (Ap_6G). Moreover the dinucleoside polyphosphates are contaminated with nonreacted mono-

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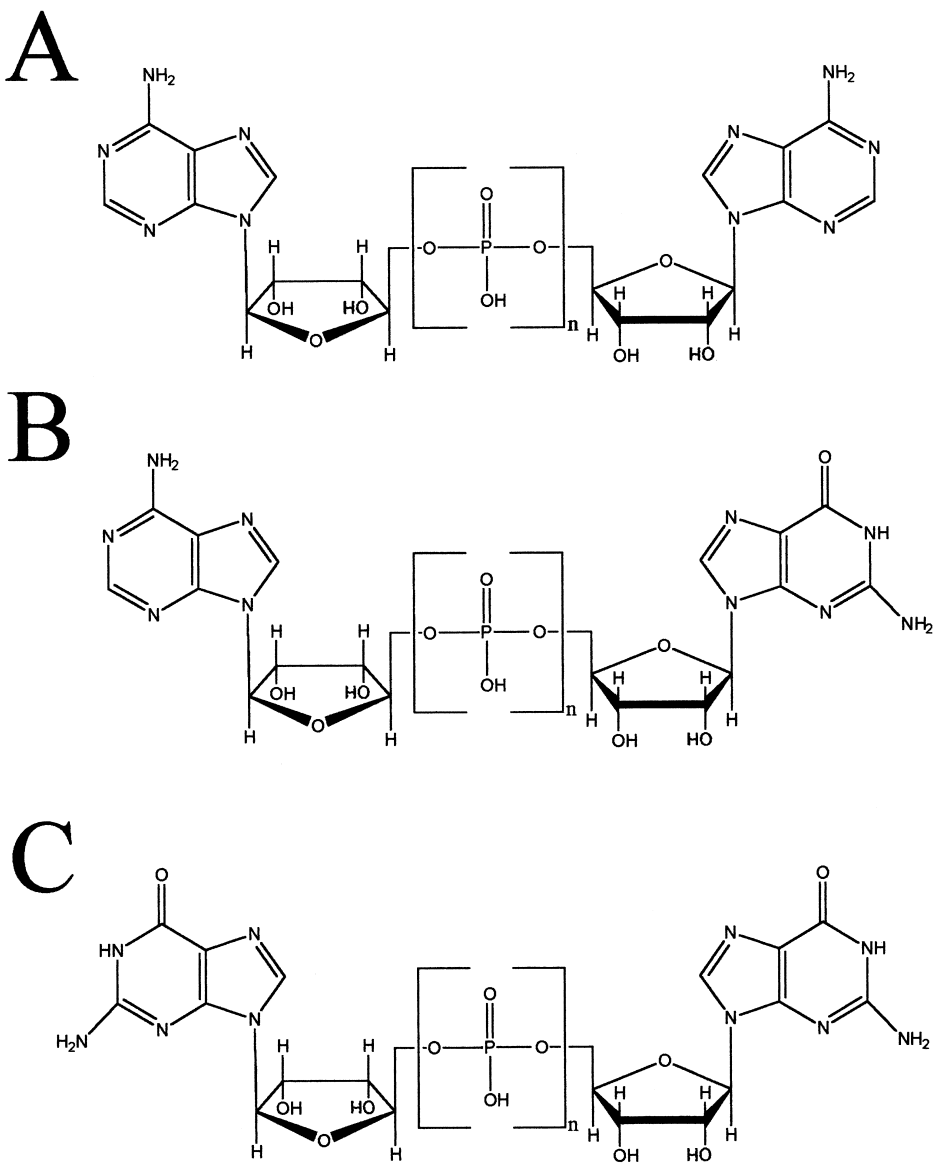

Fig. 1. Chemical structure of (A) diadenosine(5′,5′) polyphosphates, (B) adenosine(5′)-oligophospho-(5′)guanosines and (C) diguanosine(5′,5′) polyphosphates (*n* indicates number of phosphate groups).

nucleotides as well as carbodiimide adducts and hydrolysis products. Pharmacological characterization of an individual dinucleoside polyphosphate requires a homogeneous substance because its relatives as well as the mononucleotides bind to the same family of receptors, the purinoceptors [4]. Therefore a separation of the reaction products is necessary. In a set of pharmacological experiments quantities of 10–50 mg of an individual dinucleoside polyphosphate are usually needed. For purification of these quantities preparative HPLC equipment is obligatory but often not available in a biochemists

laboratory at the university. Therefore a method was developed in this study to purify 0.2 g quantities of the reaction mixture to a purity grade of >99% by semipreparative HPLC columns by employing displacement chromatography – first described by Tiselius [5] – instead of gradient elution chromatography.

2. Experimental

HPLC water (gradient grade), adenosine-5′-triphosphate (ATP), guanosine-5′-triphosphate (GTP) as sodium salts, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (CDI) and (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]) were obtained from Sigma (Deisenhofen, Germany). All other substances were purchased from Merck (Darmstadt, Germany).

2.1. Synthesis of adenosine(5′)-oligophospho(5′)-guanosines

Adenosine-oligophospho-guanosine were synthesised according to Ng and Orgel [3]. ATP (0.2 mmol≙0.11 g), GTP (0.2 mmol≙0.11 g), HEPES (16 mM≙3.81 g), CDI (0.02 *M*≙3.83 g) and magnesium chloride ($MgCl_2$) (1 m*M*≙95.21 mg) were dissolved in water, thoroughly mixed with a vortex mixer and incubated at 37°C at pH 6.5 for 48 h.

2.2. Chromatography

2.2.1. Preparative reversed-phase chromatography

Dinucleoside polyphosphates were concentrated on a C_{18} reversed-phase column (LiChroprep, 310×65 mm, 65–40 μm, Merck) using 40 mmol l^{-1} aqueous triethylammonium acetate (TEAA) in water (eluent A; flow-rate: 2 ml min^{-1}). After removing nonbinding substances with aqueous 40 mmol l^{-1} TEAA (flow-rate: 2 ml min^{-1}) nucleotides were eluted with 30% acetonitrile in water (eluent B; flow-rate: 2 ml min^{-1}). The elution was detected by UV absorption at 254 nm. The eluate was lyophilised and stored frozen at −80°C.

2.3. High-performance liquid chromatography

2.3.1. Displacement chromatography

The concentrate of the synthesis mixture (about 0.2 g) was dissolved in aqueous 40 mmol l^{-1} TEAA and injected on two C_{18} reversed-phase columns connected in series (Supersphere, 300×8 mm, 4 μm, Merck) which were equilibrated with aqueous 40 mmol l^{-1} TEAA (eluent A, carrier) earlier The carrier was pumped through the system with a flow-rate of 100 μl min^{-1} during injection of the sample. After the injection was finished *n*-butanol (100 mmol l^{-1}) in 40 m*M* TEAA was used as displacer (eluent C; flow-rate: 100 μl min^{-1}). The displacement chromatography was monitored by UV absorption at 254 nm. The fraction size was 1 ml. Every fraction of the displacement chromatography was analysed by matrix assisted laser desorption ionisation mass spectrometry (MALDI-MS). The fractions containing dinucleoside polyphosphates were collected and lyophilised.

2.4. Semipreparative anion-exchange chromatography

The individual fractions of the displacement-chromatography containing dinucleotides were lyophilised, dissolved in 1 ml of 20 mmol l^{-1} K_2HPO_4 in water, pH 8, (eluent D) and each chromatographed by using an anion-exchanger (column: Mono Q, 100×10 mm, 10 μm [Pharmacia, Sweden]; eluent E: 20 mmol l^{-1} K_2HPO_4 and 1 mol l^{-1} NaCl, (pH 8), in water; gradient: 0–10 min: 0–5% E; 10–115 min: 5–40% E; 115–120 min: 40–100% E; flow-rate: 1.0 ml min^{-1}; UV absorption wavelength: 254 nm).

2.5. Reversed phase chromatography

The fractions of the anion-exchange chromatography were desalted by HPLC reversed-phase C_{18} chromatography. The reversed-phase column (Supersphere, 300×8 mm, 4 μm, Merck) was equilibrated with eluent A. The sample dissolved in eluent A was pumped with a flow-rate of 1.0 ml min^{-1} onto the column. After washing the column with 15 ml eluent A, the substances were eluted with 35%

acetonitrile in water (eluent F). The resulting fractions were lyophilised and stored at −80°C.

2.6. Analytical procedure

2.6.1. Purity control by reversed-phase chromatography

To test the fractions for homogeneity 1/1000 of the desalted fractions of anion-exchange chromatography were chromatographed by reversed-phase gradient chromatography (conditions: Poros, R 2/H, 100×2.1 mm [Perseptive Biosystems, USA]; eluent G: 10 mmol l^{-1} K_2HPO_4 and 2 mmol l^{-1} tetrabutylammonium hydrogen sulfate in water; eluent H: 80% acetonitrile in water; gradient: 0–30.5 min: 0–30% H; 30.5–31 min: 30–50% H; 31–34.5 min: 50% H; 34.5–35 min: 0% H; flow-rate: 300 μl min^{-1}; UV absorption: 254 nm).

2.7. Identification by MALDI-MS

The lyophilised fractions from the displacement chromatography as well as the desalted and lyophilised fractions from the anion-exchange chromatography were examined by MALDI-MS [6]. A reflectron-type time-of-flight mass spectrometer (Lamma 1000, Leybold Heraeus) was used according to Hillenkamp and Karas [6]. The sample was mounted on an *x, y, z* movable stage allowing irradiation of selected sample areas. In this study, a nitrogen laser (Laser Science) with an emission wavelength of 337 nm and 3 ns pulse duration was used. The laser beam was focused to a diameter of typical 50 μm at an angle of 45° to the surface of the target. Microscopic sample observation was possible. Ten to 20 single spectra were accumulated for a better signal-to-noise ratio. The concentrations of the analysed substances were 1–10μmol l^{-1} in double distilled water. One μl of the analyte solution was mixed with 1 μl of matrix solution (50 mg ml^{-1} 3-hydroxypicolinic acid in water). To this mixture cation-exchange beads (AG 50 W-X12, 200–400 mesh, Bio-Rad) equilibrated with NH_4^+ as counter ion were added to remove Na^+ and K^+ ions. The mixture was gently dried on an inert metal surface before introduction into the mass spectrometer. The mass accuracy was in the range of approximately 0.2%.

2.8. Statistics

In this study results are presented as means±S.D..

3. Results

Fig. 2A shows the reversed-phase displacement chromatography of the desalted synthesis mixture of dinucleoside polyphosphates. The resulting fractions from the displacement chromatography were analysed by reversed-phase chromatography as well as MALDI-MS. Mass spectrometry showed that a separation was achieved between mononucleotides and dinucleotides (Fig. 2B). First mononucleotides eluted from the reversed-phase column. In the second part of the displacement train dinucleotides eluted from the column. The dotted line in Fig. 2A indicates the border of the fractions containing mononucleotides

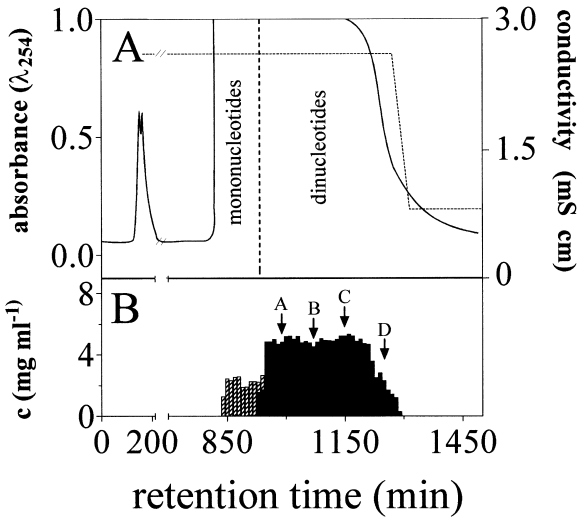

Fig. 2. (A) Displacement chromatography of the reaction mixture (two columns connected in series: C_{18} Supersphere, 300×8 mm, 4 μm, [Merck]; eluent A (carrier): 40 mmol l^{-1} TEAA in water; eluent C (displacer): 100 mmol l^{-1} *n*-butanol in eluent A; flow-rate: 100 μl min^{-1}; UV detection: 254 nm; fraction size: 1 ml). The line indicates the UV absorbance, the dotted line indicates the conductivity. The dotted vertical line indicates the border of fractions containing mononucleotides and dinucleotides (abscissa: retention time [min]; right ordinate: UV absorption [arbitrary units]; left ordinate: conductivity [mS cm^{-1}]). (B) Concentrations of nucleotides in the fractions of displacement chromatography (mononucleotides [▤]; dinucleotides [■]). The letters indicate fractions chromatographed by anion-exchange chromatography.

and fractions containing dinucleotides (Fig. 2A). *n*-Butanol is detectable by a decreasing conductivity after all nucleotides elute from the column (dotted line in Fig. 2).

The individual fractions of the displacement chromatography containing dinucleotides were chromatographed by anion-exchange chromatography. Fig. 3 shows typical anion-exchange chromatograms of some of the fractions of the displacement chromatography (labelled in Fig. 2B as A, B, C and D). After desalting the fractions of the anion-exchange chromatography were tested for homogeneity by reversed-phase chromatography. In Fig. 4 typical reversed-phase chromatograms of some fractions of the anion-exchange chromatography (labelled in Fig. 3D as I–IV) are shown. As shown in the figure the fractions of the anion-exchange chromatography were free of secondary components (Fig. 4). The

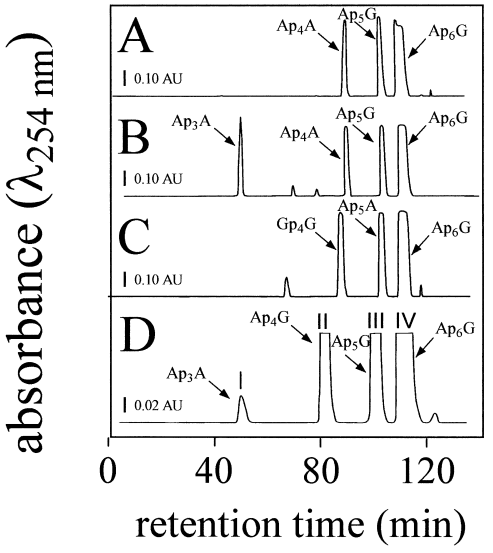

Fig. 3. Typical anion-exchange chromatograms of some of the fractions from the displacement chromatography (Fig. 2A): (A) fraction 990–1000 min, indicated with A in Fig 2B; (B) fraction 1070–1080 min, indicated with B in Fig 2B; (C) fraction 1150–1160 min, indicated with C in Fig 2B; (D) fraction 1250–1260 min, indicated with D in Fig 2B (column: Mono Q, 100×10 mm, 10 μm [Pharmacia]; eluent D: 20 mmol l^{-1} K_2HPO_4 in water; eluent E: 20 mmol l^{-1} K_2HPO_4 and 1 mol l^{-1} NaCl (pH 8) in water; gradient: 0–10 min: 0–5% E; 10–115 min: 5–40% E; 115–120 min: 40–100% E; flow-rate: 1.0 ml min^{-1}; fraction size: 2 ml; abscissa: retention time [min]; ordinate: UV absorption at 254 nm [arbitrary units]). Peak I, II, III and IV (labelled in D) were analysed by reversed-phase chromatography (Fig. 4).

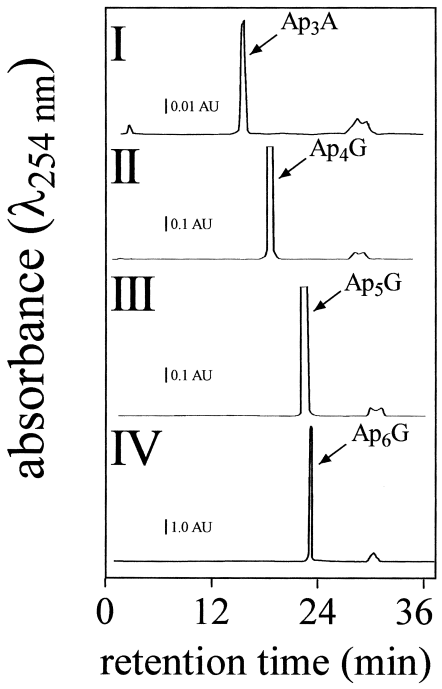

Fig. 4. Chromatography of 1/1000 of the individual fractions I–IV (labelled in Fig. 3D) on a reversed-phase C_{18} column (column: Poros, R 2/H, 100×2.1 mm [Perseptive Biosystems] flow-rate: 300 μl min^{-1}; eluent G: 10 mmol l^{-1} K_2HPO_4 and 2 mmol l^{-1} tetrabutylammonium hydrogen sulfate in water; eluent H: 80% acetonitrile in water; gradient: 0–30.5 min: 0–30% H; 30.5–31 min: 30–50% H; 31–34.5 min: 50% H; 34.5–35 min: 0% H; abscissa: retention time [min]; ordinate: UV absorption at 254 nm [arbitrary units]).

small peaks at a retention time of 2.5 min and 30.0 min are caused by the system used. The reversed-phase chromatography indicates that the purity of the substances were above 99% (Fig. 4). The yield of dinucleotides in each peak labelled in Fig. 3 amounted to about 2–4 mg by calibration curves. The identity of the purified individual dinucleoside polyphosphates was determined by MALDI-MS. Table 1 shows the monoisotopic experimental and calculated molecular weights of isolated dinucleoside polyphosphates.

4. Discussion

In this study it is shown that displacement chromatography is suitable for the problem of separation of

Table 1
Monoisotopic experimental and calculated monoisotopic molecular weight of dinucleoside polyphosphates isolated in this study

Monoisotopic experimental molecular weight $m/z \pm$S.D.	Calculated monoisotopic polyphosphate [MW+H$^+$]	Dinucleoside
757.6±0.2	757.4	Ap_3A
837.9±0.1	837.4	Ap_4A
853.6±0.3	853.4	Ap_4G
869.6±0.1	869.4	Gp_4G
917.6±0.4	917.4	Ap_5A
933.4±0.4	933.4	Ap_5G
1014.0±0.2	1013.4	Ap_6G

larger quantities of dinucleotides by the use of semipreparative columns instead of preparative columns. Here the separation of a mixture of about 0.2 g reaction mixture by displacement chromatography is demonstrated. Due to the separation mechanism, displacement chromatography enables much higher sample loads compared to conventional elution chromatography [7]. In displacement chromatography, interactions of the solute with the stationary phase are predominant. In contrast in gradient elution, chromatography partition equilibria of the solute between the two systems, the stationary phase and the mobile phase drive the separation. In displacement chromatography the sample molecules compete with each other for the binding sites of the stationary phase. This competition starts with the injection of the sample to the column. The displacement train arranges itself into its final form after moving a certain distance down the column, driven by the displacer. The length of this distance mainly depends on relative affinities of the species for the adsorbent. The substance with the highest affinity can be found at the head of the column (nearest to the injector) followed by the substance with the next lower affinity. The substance with the lowest affinity is the nearest to the detector. The zones of the individual substances border on each other. The zones are characterised by high concentrations of the individual substances. The concentration depends on the affinity of the substance to the stationary phase, the higher the affinity the higher the concentration. The zones will not move down the column until the displacer binds to the stationary phase. The displacer binding to the head of the column starts the displacement-train (sum of all zones including the displacer zone). Because *n*-butanol was detectable at the end of the column after all nucleotides have eluted from the column, a conventional isocratic elution chromatography with *n*-butanol at a very slow flow-rate may be excluded. The separation was consequently determined by *n*-butanol as displacer.

During movement of the displacement train the substances continuously compete for the binding sites of the stationary phase until they leave the column. This competition results in a self sharpening of the square wave shaped zones, in reducing overlapping of the zones and in concentrating the individual substances in the zones. In contrast in isocratic elution and in some cases in gradient elution chromatography also, the individual sample components get diluted during chromatography. Samples elute within Gaussian curves which beside dilution of the sample causes much more overlapping and as a result impurified fractions. The optimal sample size depends on the affinity of the sample to stationary phase. If the sample load is too low, the zones of the sample components may be too narrow and thus in one fraction several zones of different substances may be present [8]. On the other hand on an overloaded column the ''displacement train'' can not completely develop. Concentration zones of overloaded columns do not develop rectangular shapes and overlapping will be noticed [9]. In the application described here separation is optimal if the sample size covers 50–80% of the total column capacity. In gradient elution chromatography high resolution separations are guaranteed only if the sample size is smaller than 5–10% of the total binding capacity of the column. For this reason displacement chromatography is much more econ-

omical because high sample loads are needed for optimal separations.

The choice of triethylammonium acetate in water as mobile phase ensures binding of the nucleotides to the stationary phase. The displacer *n*-butanol was chosen because it adsorbed more strongly to the stationary phase than the dinucleoside polyphosphates [10], did not react with any of the sample components and was completely removable by lyophilisation from the sample. The low flow-rate (here 100 $\mu l\,min^{-1}$) compared to gradient elution chromatography (usually 1 $ml\,min^{-1}$) is necessary to develop the competition equilibria.

The separation of mono- and dinucleotides by displacement chromatography (dotted line in Fig. 2) was nearly quantitative. The separation of Ap_4A, Gp_4G and Ap_4G is in unison with the theory of displacement chromatography also. Individual fractions of the displacement chromatography (Fig. 2) contained more than one dinucleoside polyphosphate as can be recognised in the chromatogram of the anion-exchange chromatography (Fig. 3). This observation cannot be due to wrong fraction collection, because Ap_5G and Ap_6G are present in most displacement fractions (A to D) shown in Fig. 3 whereas Ap_4A appears in fraction A and B, Gp_4G in C and Ap_4G in D. The presence of Ap_5G and Ap_6G in displacement fractions containing dinucleoside polyphosphates does not fit with the theory of displacement chromatography at first sight. This phenomenon was observed by the authors in many different displacement chromatographies of dinucleoside polyphosphates using different column dimensions and/or sample loads. A declaration of this elution behaviour may be an incomplete developed displacement-train. However, this hypothesis seems unlikely because the mononucleotides and the dinucleotides are completely separated by the displacement chromatography (Fig. 2A). The phenomenon may be explained by the formation of complexes, in this case of Ap_3A, Ap_5G and Ap_6G with other molecules present in the sample. These complexes will behave differently compared to the uncomplexed nucleotides. Noncovalent complexes of dinucleoside polyphosphates with other molecules are already described in the literature [11]. The formation of cationic complexes have been measured for a series of dinucleoside polyphosphates that contain adenosine and guanosine [12]. Different complexes of Ap_5A and Ap_6A with Na^+ were showed by MALDI-MS also [13]. Traces of metal ions may be responsible for multiple elution of individual dinucleoside polyphosphates forming different species of complexes. Impurities of the ion-pair reagent triethylammonium with cationic amines may be another source for complexing substances. Most likely the triethylammonium cation itself may form complexes with the dinucleoside polyphosphates with different stoichiometries (e.g. $[Ap_5G+TEA]^{4-}$, $[Ap_5G+2TEA]^{3-}$ etc.). These complexes will differ significantly in their hydrophobicities resulting in multiple elution of dinucleoside polyphosphates. Nevertheless displacement fractionation reduces the complexity of the components in the individual fractions. Ap_4A, present in fraction B and Gp_4G, present in fraction C (Fig. 3) differ only slightly in their retention times. Without prior displacement chromatography separation of these two substances by anion-exchange chromatography would have been difficult. Analysis of the desalted individual fractions from the anion-exchange chromatographies (Fig. 4) showed that these fractions are purified to homogeneity (purity >99%) therefore sufficient for pharmacological experiments. Each fraction contains about 2–4 mg quantities of dinucleotides, which can be needed for a whole set of pharmacological experiments.

In conclusion, the separation procedure describes an effective, simple and economic method to yield highly pure dinucleotides in mg quantities. Furthermore this study is an additional example demonstrating the benefits of displacement-chromatography.

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