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Isolation and quantification of dinucleoside polyphosphates by using monolithic reversed phase chromatography columns

V. Jankowski [a], R. Vanholder [b], L. Henning [a], S. Karadogan [a], W. Zidek [a], H. Schlüter [a], J. Jankowski [a,*]

[a] *Charité—CBF, Medizinische Klinik IV, Hindenburgdamm 30, D-12200 Berlin, Germany*
[b] *Nephrology Section, Department of Internal Medicine, University Hospital, Gent, Belgium*

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Abstract

In former studies, dinucleoside polyphosphates were quantified using ion-pair reversed-phase perfusion chromatography columns, which allows a detection limit in the μmolar range. The aim of this study was both to describe a chromatographic assay with an increased efficiency of the dinucleoside separation, which enables the reduction of analytical run times, and to establish a chromatographic assay using conditions, which allow MALDI-mass spectrometric analysis of the resulting fractions. We compared the performance of conventional silica reversed phase chromatography columns, a perfusion chromatography column and a monolithic reversed-phase C18 chromatography column. The effects of different ion-pair reagents, flow-rates and gradients on the separation of synthetic diadenosine polyphosphates as well as of diadenosine polyphosphates isolated from human platelets were analysed. Sensitivity and resolution of the monolithic reversed-phase chromatography column were both higher than that of the perfusion chromatography and the conventional reversed phase chromatography columns. Using a monolithic reversed-phase C18 chromatography column, diadenosine polyphosphates were separable baseline not only in the presence of tetrabutylammonium hydrogensulfate (TBA) but also in the presence of triethylammonium acetate (TEAA) as ion-pair reagent. The later reagent is useful because, in contrast to TBA, it is compatible with MALDI mass-spectrometric methods. This makes TEAA particularly suitable for identification of unknown nucleoside polyphosphates. Furthermore, because of the lower backpressure of monolithic reversed-phase chromatography columns, we were able to significantly increase the flow rate, decreasing the amount of time for the analysis close to 50%, especially using TBA as ion-pair reagent. In summary, monolithic reversed phase C18 columns markedly increase the sensitivity and resolution of dinucleoside polyphosphate analysis in a time-efficient manner compared to reversed-phase perfusion chromatography columns or conventional reversed-phase columns. Therefore, further dinucleoside polyphosphate analytic assays should be based on monolithic silica C18 columns instead of perfusion chromatography or conventional silica reversed phase chromatography columns. In conclusion, the use of monolithic silica C18 columns will lead to isolation and quantification of up to now unknown dinucleoside polyphosphates. These chromatography columns may facilitate further research on the biological roles of dinucleoside polyphosphates.

Keywords: Dinucleoside monophosphates; Isolation; Quantification; Monolithic columns; Vasoregulatory hormones

1. Introduction

Diadenosine polyphosphates have previously been isolated from human tissues and cells such as platelets [1–6], erythrocytes [7], heart [8–10], placenta [11], and human plasma [12]. Diadenosine polyphosphates are involved as intra- and extracellular mediators in the regulation of numerous physiological functions, e.g. growth of vascular smooth muscle cells and control of vascular tone [3,5,12–14]. The book entitled "Ap_4A and other dinucleoside polyphosphates" edited by McLennan gives an excellent overview on the biology and physiology of dinucleoside polyphosphates [15]. Reviews about the role of diadenosine polyphosphates

* Corresponding author. Tel.: +49 30 8445 1773; fax: +49 30 8445 1762.
E-mail address: joachim.jankowski@charite.de (J. Jankowski).

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in the cardiovascular system have been published recently [16,17]. Vascular effects of Ap_nA vary with the number of phosphate groups linking the adenosine molecules [3,18,19].

Perfusion chromatography is generally used for purification of biomacromolecules [20,21]. Nevertheless, diadenosine polyphosphates are also quantified in speed-vac-dried eluates of reversed-phase chromatographies by ion-pair reversed-phase perfusion chromatography [22]. Reversed-phase gradient systems are used in order to ensure the simultaneous separation of molecules with a broad range of hydrophobicity. The chromatographic quantitation of the diadenosine polyphosphates is in general based on the method described by Brüggemann et al. [23] with tetrabutylammonium sulfate as ion-pair reagent and perfusion reversed phase chromatography columns [21].

In contrast to the method of Brüggemann et al. [23], the flow-rate used for quantification was reduced from 1 ml/min to 300 μl/min in recent studies [12,24]. Although high flow rates are typical of perfusion chromatography, the reduction of the flow-rate increases the intensity of the peaks and thus decreases the detection limit. This approach is appropriate for the quantification of diadenosine polyphosphates if the concentration is sufficiently high as described in several publications [12,22,24,25], but fails, if the concentrations are low.

Therefore, we developed based on a monolithic silica HPLC reversed phase column two strategies: one with an increased efficiency of the dinucleoside separation for dinucleoside polyphosphates quantification, and one for dinucleoside polyphosphates identification using conditions which allows MALDI-mass spectrometric analysis of the resulting fractions. For a broad variation of the stationary phase characteristics, we used two conventional silica reversed phase columns, a perfusion chromatography column, and a monolithic reversed phase chromatography column.

We investigated the effects of different ion-pair reagents, gradients and flow rates on the chromatographic resolution. In particular, our attention was turned to the compatibility of the chromatographic strategy with mass spectrometry methods like MALDI-MS.

2. Materials and methods

HPLC water (gradient grade) and acetonitrile were purchased from Merck (Darmstadt, Germany), and all other substances from Sigma–Aldrich (Taufkirchen, Germany). A porous reversed phase column ("Poros R2/H" (50 × 4.6 mm I.D., Perseptive Biosystems, Freiburg, Germany)), a monolithic reversed phase column ("Chromolith™ SpeedROD" (50 × 4.6 mm I.D., Merck, Darmstadt, Germany)), and two conventional reversed-phase columns ((a) LiChrospher 100 RP-18e (55 × 4 mm I.D., Merck, Darmstadt, Germany); (b) Superspher 100 RP-18e (55 × 4 mm I.D., Merck, Darmstadt, Germany)) were compared.

2.1. Reversed-phase chromatography with triethylammonium acetate (TEAA) as ion-pair reagent

Diadenosine polyphosphates (Ap_nA with $n = 2$–6; each 3 μg) were separated by gradient elution on each of the four above mentioned reversed-phase columns in the presence of the ion-pair reagent triethylammonium acetate (TEAA; 40 mmol/1 (final concentration)) as eluent A and water–acetonitrile (80:20%, v/v) as eluent B. The column temperature was ambient (22 ± 1 °C). The mobile phase was pumped at a flow-rate of 1 ml/min by a high-pressure gradient pump system (Merck, Darmstadt, Germany). The column eluate was monitored with a variable wavelength UV detector (759 A, Absorbance Detector, Applied Biosystems, Darmstadt, Germany). The diadenosine polyphosphate mixture was dissolved in eluent A. The diadenosine polyphosphates were eluted with the following gradient: 0–2 min: 0% eluent B; 2–62 min: 0–60% B; 62–63 min: 60–100% eluent B. The concentration of eluent B of 60% corresponds to an acetonitrile concentration of 12% in the total eluate volume. UV absorption was measured at 254 nm. Data were recorded and processed with the Chromeleon Lab System 6.0 (Dionex, Idstein, Germany).

Low backpressure is one of the important characteristic features of perfusion and monolithic reversed-phase chromatography columns in comparison to conventional silica reversed phase chromatography columns, allowing high flow rates. To investigate the effect of increasing flow rate on the resolution, the flow rate was increased in the range between 1 ml/min and 6 ml/min, in a separate set of experiments.

2.2. Reversed-phase chromatography with tetrabutylammonium hydrogensulfate (TBA) as ion-pair reagent

To evaluate whether the ion-pair reagent would have an impact on the performance of the reversed-phase columns, the ion-pair reagent tetrabutylammonium hydrogensulfate (TBA) was used instead of TEAA.

Diadenosine polyphosphates Ap_nA (with $n = 2$–6; each 3 μg) were separated by gradient elution on each of the four above mentioned reversed-phase columns in the presence of ion-pair reagent 2 mmol/1 tetrabutylammonium hydrogensulfate in a phosphate buffer (10 mmol/1 K_2HPO_4, final concentration; pH 6.8) as eluent A and water–acetonitrile (20:80%, v/v) as eluent B. The phosphate buffer was necessary to adjust the pH of the ion-pair reagent tetrabutylammonium hydrogensulfate solution to a value of 6.8. The column temperature was ambient (22 ± 1 °C). The diadenosine polyphosphates were eluted with the following gradient: 0 min: 100% eluent A; 0–30 min: 0–45% B; 30–33 min: 45–100% eluent B; 33–36 min: 100% B. The concentration of eluent B of 45% corresponds to an acetonitrile concentration of 36%. All other experimental conditions were identical as described above. To investigate

the effect of the flow rate on the resolution, the flow rate was increased in the range between 1 ml/min and 6 ml/min.

2.3. Isolation of dinucleoside polyphosphates from human platelets

Dinucleoside polyphosphates were isolated from human platelets as described elsewhere [2,5]. Briefly, human platelets were washed with an isotonic solution of NaCl and centrifuged (4000 rpm, 4 °C, 10 min) twice. The supernatant was aspirated and the pellets frozen to −30 °C. The platelet pellets were rethawed in double distilled water (10 ml). The resulting suspension was deproteinized with 0.6 mol/1 (final concentration) perchloric acid and centrifuged (4000 rpm, 4 °C, 5 min). After adjusting pH to 7.0 with 5 mol/1 KOH the precipitated proteins and $KC1O_4$ were removed by centrifugation (4000 rpm, 4 °C, 5 min). 1 mol/1 triethylammonium acetate (TEAA) was added to the supernatant up to a final concentration of 40 mmol/1. Supernatant was concentrated on a preparative reversed phase column (LiChroprep RP-18 B, Merck, Darmstadt, Germany) in the presence of the ion-pair reagent TEAA (40 mmol/1 (final concentration)) as eluent A and water–acetonitrile (80:20%, v/v) as eluent B. The column temperature was ambient (22 ± 1 °C). The mobile phase was pumped at a flow-rate of 5 ml/min by a high-pressure gradient pump system (Merck, Darmstadt, Germany). The diadenosine polyphosphates were eluted with a stepwise gradient.

The lyophilized eluate of the reversed phase chromatography dissolved in 1 mol/1 ammonium acetate (pH 9.5) was loaded to a phenyl boronic acid resin. The resin was prepared according to Barnes et al. [26]. The adsorbed substances were eluted with 1 mmol/1 HC1 (flow rate: 1 ml/min). The eluate from the phenyl boronic acid resin to which 1 mol/1 TEAA was added to a final concentration of 40 mmol/1 was desalted by a reversed phase chromatography (LiChroprep RP-18 B, Merck, Darmstadt, Germany; equilibration and sample buffer: 40 mmol/1 TEAA in water; flow rate: 5 ml/min). The lyophilized eluate was used for reversed-phase chromatography using the four different reversed phase columns.

2.4. Matrix assisted laser desorption/ionisation mass spectrometry (MALDI-MS) and post-source decay (PSD)-MALDI-MS

The identity of the diadenosine polyphosphates was confirmed by matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS) [27] and post-source decay (PSD)-MALDI-MS [28]. A reflectron-type time-of-flight mass spectrometer (Reflex III, Bruker-Daltronic, Bremen Germany) was used according to Hillenkamp et al. [27]. The sample was mounted on *x*, *y*, *z* movable stage allowing for irradiation of selected sample areas. In this study, a nitrogen laser (Laser Science Inc., Franklin, MA, USA) with an emission wavelength of 337 ran and 3 ns pulse duration was used. Typically, the laser beam was focused to a diameter of 50 μm at an angle of 45° to the surface of the target. Microscopic sample observation was possible via a diachronic mirror in the beam path. 10–20 single spectra were accumulated for a better signal-to-noise ratio. In MALDI-MS large fractions of the desorbed analyte ions undergo post-source decay during flight in the field free drift path [28]. Using a RETOF set-up, sequence information from PSD fragment ions of precursors produced by MALDI were obtained. Sample preparations for MALDI- and PSD-MALDI experiments were identical. The concentrations of the analysed dinucleoside polyphosphates were 1–10 μmol/1 in bidistilled water. One microliter of the analyte solution was mixed with 1 μl of the matrix solution (50 mg/ml 3-hydroxy-picolinic acid in water). The mixture was gently dried on an inert metal surface before introduction into the mass spectrometer. For calibration of the mass spectra, synthetic diadenosine hexaphosphate (Ap_6A) was used as external standard. The mass accuracy was in the range of 0.05%.

3. Results and discussion

Besides the broad variation of the experimental chromatographic conditions like flow rate, ion-pair reagent, this study focused on the broad variation of the stationary phase characteristics. The chromatographic conditions used for testing the different chromatography columns were identical—within the limits of the column backpressure.

We compared the performance of conventional silica reversed phase columns with the performance of a perfusion chromatography column and a monolithic reversed phase chromatography column. As examples for conventional reversed phase chromatography columns, we used a Lichrospher and Superspher of Merck (Darmstadt, Germany). These reversed phase chromatography columns are characterized by the composition of spherical particles of silica with endcapped octadecyl derivative. The particle size of Lichrospher versus Superspher amounts to 5 μm versus 4 μm, resulting in a different number of theoretical plates (55,000 N/m versus <100,000 N/m).

In recent studies, the low molecular weight and highly charged dinucleoside polyphosphates, which also contain hydrophobic fragments, were fractionated by perfusion chromatography (e.g. [24,29,30]), although the latter technique is generally only used for purification of high molecular weight biomacromolecules [20,21].

The main advantage of perfusion chromatography is that the resolution does virtually not depend on flow rate, whereas conventional materials exhibit a marked reduction in resolution with increased flow rates. In general, synthetic polymers such as polystyrenedivenylbenzene are used as matrix building blocks due to their excellent physical stability (allowing pressures up to 200 bar) and chemical stability compared to most other substances used [21]. This makes column cleaning easy because even aggressive chemicals such as acids and bases can be used. This in turn enables longer lifetimes

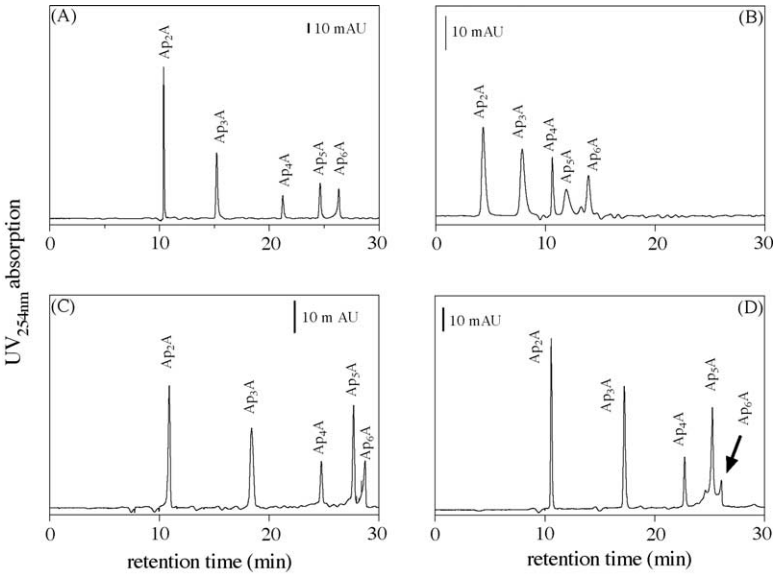

Fig. 1. Reversed phase chromatography of synthetic diadenosine polyphosphates (Ap_nA; with $n = 2$–6) on: (A) an analytical, monolithic reversed-phase high performance liquid chromatographic column (Chromolith^{TM} SpeedROD (50 × 4.6 mm I.D., Merck, Darmstadt, Germany)); eluent A: 2 mmol/1 tetrabutylammonium hydrogensulfate and 10 mmol/1 K_2HPO_4 in water (pH 6.8); eluent B: water–acetonitrile (20:80%, v/v); gradient: 0 min: 0% eluent B; 0–30 min: 0–45% B; 30–33 min: 45–100% eluent B; 33–36 min: 100% B; flow rate: l.0 ml/min). (B) An analytical reversed-phase high performance liquid chromatographic column (Poros R2/H (50 × 4.6 mm I.D., Perseptive Biosystems, Freiburg, Germany)). The conditions were identical to the conditions identical to gradient in (A). (C) An analytical reversed-phase high performance liquid chromatographic column (LiChrospher 100 RP-18e (55 × 4 mm; Merck, Darmstadt, Germany)). The conditions were identical to the conditions in (A). (D) An analytical reversed-phase high performance liquid chromatographic column (Superspher 100 RP-18e (55 × 4 mm; Merck, Darmstadt, Germany)). The conditions were identical to the conditions in (A).

of the chromatography columns. In addition, the amount of time needed for column cleaning is minimal. A high resolution and unchanged binding capacity are characteristic for perfusion reversed-phase columns. In addition, costs are generally lower for perfusion chromatography, compared to conventional reversed phase chromatography, either due to intrinsic financial advantages, or to a gain in use of time.

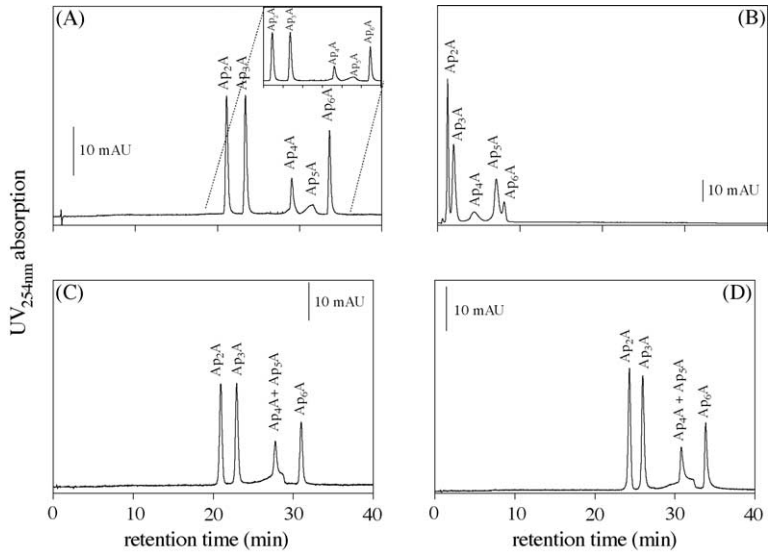

Fig. 2. Reversed phase chromatography of synthetic diadenosine polyphosphates (Ap_nA; with $n = 2$–6) with triethylammonium acetate (TEAA) as ion-pair reagent on: (A) an analytical, monolithic reversed-phase high performance liquid chromatographic column (Chromolith^{TM} SpeedROD (50 × 4.6 mm I.D., Merck, Darmstadt, Germany)); eluent A: 40 mmol/1 TEAA in water; eluent B: water–acetonitrile (80:20%, v/v); gradient: 0–2 min: 0% eluent B; 2–62 min: 0–60% B, respectively; 62–63 min: 60–100% eluent B. The concentration of eluent B of 60% corresponds to an acetonitrile concentration of 12%. (B) An analytical reversed-phase high performance liquid chromatographic column (Poros R2/H (50 × 4.6 mm I.D., Perseptive Biosystems, Freiburg, Germany)). The conditions were identical to the conditions in (A). (C) An analytical reversed-phase high performance liquid chromatographic column (LiChrospher 100 RP-18e (55 × 4 mm; Merck, Darmstadt, Germany)). The conditions were identical to the conditions in (A). (D) An analytical reversed-phase high performance liquid chromatographic column (Superspher 100 RP-18e (55 × 4 mm; Merck, Darmstadt, Germany)). The conditions were identical to the conditions in (A).

The previous reports mentioned above indicate that diadenosine polyphosphates are quantifiable by perfusion chromatography with tetrabutylammonium hydrogensulfate as ion-pair reagent (e.g. [1,24]).

An exemplary reversed phase chromatographic separation of synthetic diadenosine polyphosphates Ap_nA (with $n = 2–6$) is shown in Fig. 1 using TBA as ion-pair reagent and a monolithic silica reversed phase column (Fig. 1A), a perfusion reversed-phase column (Fig. 1B), and two conventional silica reversed-phase columns, a LiChrospher (Fig. 1C) and a Superspher (Fig. 1D). The peaks labelled in the figure represent the UV absorption of synthetic diadenosine polyphosphates Ap_nA (with $n = 2–6$). Using TBA and the monolithic silica reversed phase column, the differences in retention time of the dinucleoside polyphosphates can markedly be increased in comparison to perfusion reversed phase as well as conventional silica reversed phase columns. Moreover, the peak widths of the dinucleoside polyphosphates decrease using TBA as an ion-pair reagent. As a consequence, less concentrated dinucleoside polyphosphates are quantifiable by using monolithic silica reversed phase columns with TBA as ion-pair reagent. The monolithic silica reversed-phase columns are based on the "sol–gel" technology, which employs highly porous monolithic rods of silica with a bimodal pore structure. The column consists of both a macroporous and mesoporous structure. The macropores are on average 2 μm in diameter and together form a dense network of pores through which the eluent can rapidly flow to reduce the separation time.

This approach, however, fails in combination with mass-spectrometric analysis and in the case of low concentrations of dinucleoside polyphosphates. For identification, dinucleoside polyphosphates are generally analysed by MALDI-mass spectrometry (e.g. [30–33]). Due to the strong ionic bonding of TBA with the phosphates of the dinucleoside polyphosphates and because of the low steam pressure of TBA, this ion-pair reagent is not removable by lyophilisation in the presence of dinucleoside polyphosphates. For that reason, the use of TBA as ion-pair reagent precludes the identification of the diadenosine polyphosphates by MALDI-mass spectrometry. But for the separation of ionic solutes such as dinucleoside polyphosphates the addition of an ion-pair reagent as a counterion is essential to ensure the retention of the ionic species. This indicates that an alternative to the ion-pair reagent TBA is necessary.

Therefore, the identities of the diadenosine polyphosphates as showed in Fig. 1 were confirmed by comparing the respective retention times with those of single synthetic dinucleoside polyphosphates. Because of the use of TBA as ion-pair reagent and perfusion reversed-phase columns, in former studies, two strategies were necessary, one for the identification and one for the quantification of dinucleoside polyphosphates. TBA was used as ion-pair reagent for the chromatographic quantification of the dinucleoside polyphosphates; for the chromatographic isolation and identification, triethylammonium acetate was used as an alternative cationic ion-pair reagent. In contrast to TBA, TEAA is completely removable from dinucleoside polyphosphates by lyophilisation and therefore subsequent MALDI mass analysis of dinucleoside polyphosphates is possible.

However, in contrast to monolithic reversed phase chromatography, reversed-phase perfusion chromatography and chromatography with conventional silica reversed-phase columns with TEAA as the ion-pair reagent do not lead to sufficient separation of dinucleoside polyphosphates. Fig. 2 presents characteristic reversed-phase chromatograms showing the separation of synthetic diadenosine polyphosphates Ap_nA (with $n = 2–6$) in the presence of triethylammonium acetate as ion-pair reagent using the four columns under study (Fig. 2A–D). The resolution of perfusion chromatography as well as of conventional reversed phase chromatography is lower than that of the monolith reversed-phase chromatography. Dinucleoside polyphosphates are obviously only separable in the presence of the cationic ion-pair reagent TEAA if monolithic silica reversed phase columns are used for the chromatography.

In contrast to reversed-phase chromatography with the ion-pair reagent TEAA, in the case of reversed-phase

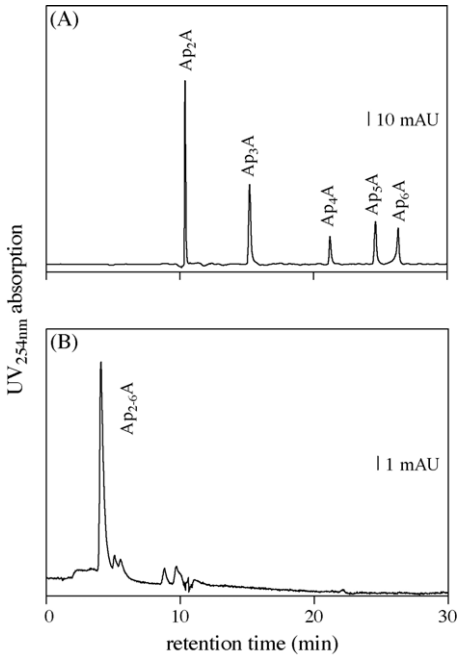

Fig. 3. Reversed phase chromatography of synthetic diadenosine polyphosphates (Ap_nA; with $n = 2–6$) on an analytical, monolithic reversed-phase high performance liquid chromatographic column (Chromolith™ SpeedROD (50 × 4.6 mm I.D., Merck, Darmstadt, Germany) using the ion-pair reagent tetrabutylammonium hydrogensulfate in the presence (A) and in the absence the buffer system K_2HPO_4 (B). (A) Conditions: eluent A: 2 mmol/1 tetrabutylammonium hydrogensulfate and 10 mmol/1 K_2HPO_4 in water (pH 6.8); eluent B: water–acetonitrile (20:80%, v/v); gradient: 0 min: 0% eluent B; 0–30 min: 0–45% B; 30–33 min: 45–100% eluent B; 33–36 min: 100% B; flow rate: 1.0 ml/min). (B) Eluent A: 2 mmol/1 tetrabutylammonium hydrogensulfate without K_2HPO_4 in water (pH 6.8); eluent B: water–acetonitrile (20:80%, v/v). The gradient and the flow rate were identical to the conditions identical to gradient in (A).

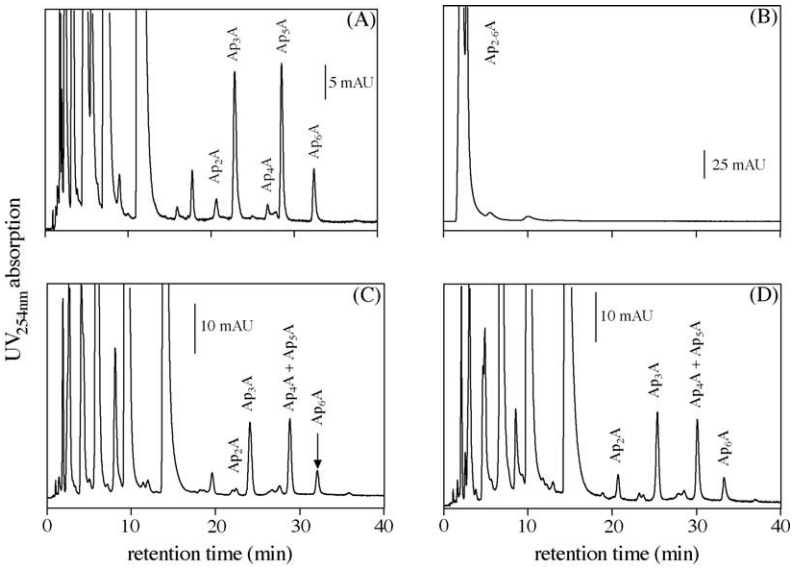

Fig. 4. Reversed phase chromatography of a homogenate of human platelets after reversed phase and affinity-chromatography on: (A) an analytical, monolithic reversed-phase high performance liquid chromatographic column (Chromolith^{TM} SpeedROD (50 × 4.6 mm I.D., Merck, Darmstadt, Germany)); eluent A: 40 mmol/1 triethylammonium acetate (TEAA) in water; eluent B: water–acetonitrile (80:20%, v/v); gradient: 0–2 min: 0% eluent B; 2–62 min: 0–25% B; 62–63 min: 25–100% eluent B; flow rate: 1.0 ml/min). (B) An analytical reversed-phase high performance liquid chromatographic column (Poros R2/H (50 × 4.61 mm I.D., Perseptive Biosystems, Freiburg, Germany)). The conditions were identical to the conditions in (A). (C) An analytical reversed-phase high performance liquid chromatographic column (LiChrospher 100 RP-18e (55 × 4 mm; Merck, Darmstadt, Germany)). The conditions were identical to the conditions in (A). (D) An analytical reversed-phase high performance liquid chromatographic column (Superspher 100 RP-18e (55 × 4 mm; Merck, Darmstadt, Germany)). The conditions were identical to the conditions in (A).

chromatography with TBA as ion-pair reagent a buffer system is essential to adjust the pH at 6.8 (Fig. 3A). Diadenosine polyphosphates are insufficiently retained and fractionated by the ion-pair reversed phase chromatography with TBA in the absence of a buffer system (Fig. 3B). In addition, chromatography of diadenosine polyphosphates at acid pH values bears the risk of hydrolysis of the diadenosine polyphosphates.

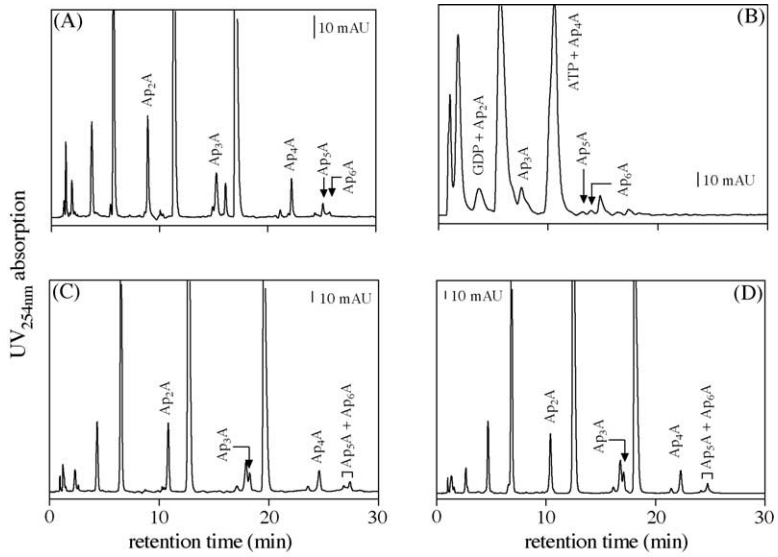

Fig. 5. Reversed phase chromatography of a homogenate of human platelets after reversed phase and affinity-chromatography on: (A) an analytical, monolithic reversed-phase high performance liquid chromatographic column (Chromolith^{TM} SpeedROD (50 × 4.6 mm I.D., Merck, Darmstadt, Germany)); eluent A: 2 mmol/1 tetrabutylammonium hydrogensulfate and 10 mmol/1 K_2HPO_4 in water (pH 6.8); eluent B: water–acetonitrile (20:80%, v/v); gradient: 0 min: 0% eluent B; 0–30 min: 0–45% B; 30–33 min: 45–100% eluent B; 33–36 min: 100% B; flow rate: l.0 ml/min). (B) An analytical reversed-phase high performance liquid chromatographic column (Poros R2/H (50 × 4.6 mm I.D., Perseptive Biosystems, Freiburg, Germany)). The conditions were identical to the conditions in (A). (C) An analytical reversed-phase high performance liquid chromatographic column (LiChrospher 100 RP-18e (55 × 4 mm; Merck, Darmstadt, Germany)). The conditions were identical to the conditions in (A). (D) An analytical reversed-phase high performance liquid chromatographic column (Superspher 100 RP-18e (55 × 4 mm; Merck, Darmstadt, Germany)). The conditions were identical to the conditions in (A).

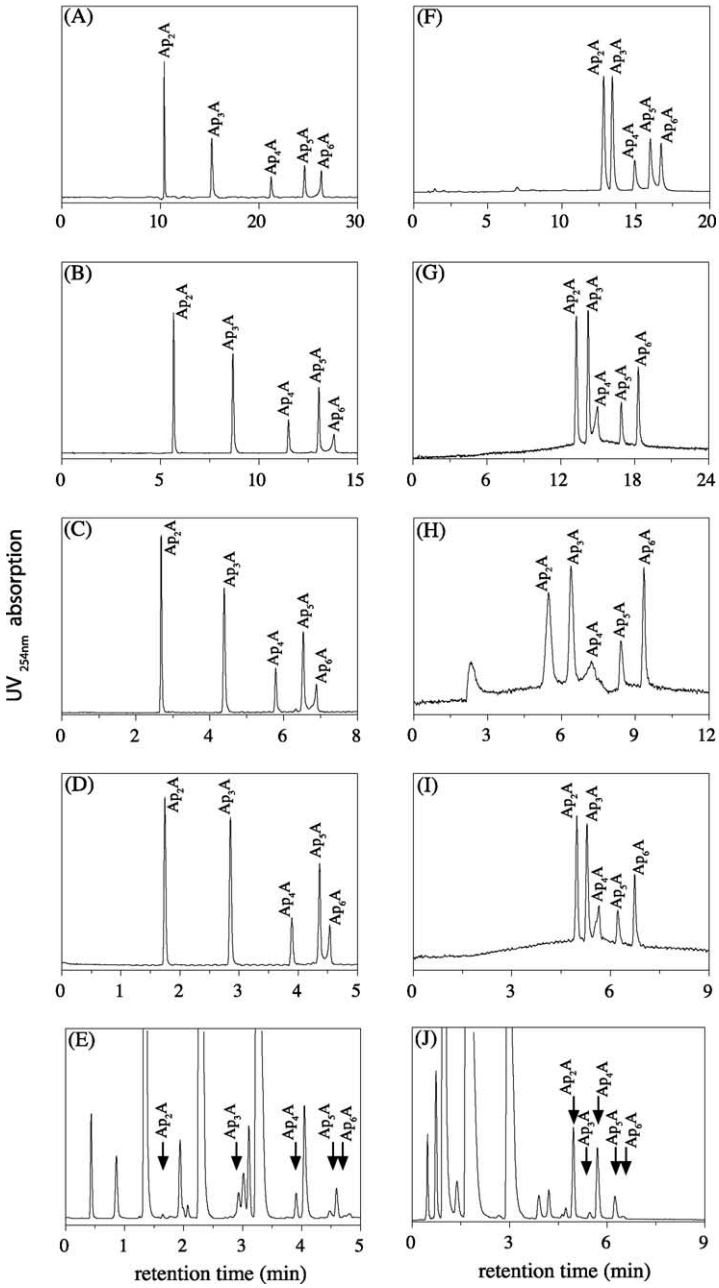

Fig. 6. Reversed phase chromatography of synthetic diadenosine polyphosphates (Ap_nA; with $n = 2$–6) on an analytical, monolithic reversed-phase high performance liquid chromatographic column (Chromolith SpeedROD^{TM} (50 × 4.6 mm I.D., Merck, Darmstadt, Germany) using TBA (A–D) respectively TEAA (E–I) as ion-pair reagent and flow rate in the range of 1 ml/min up to 6 ml/min. E and J showed the chromatography of a platelet extract using TBA and TEAA as ion-pair reagent. (A) Eluent A: 2 mmol/1 tetrabutylammonium hydrogensulfate and 10 mmol/1 K_2HPO_4 in water (pH 6.8); eluent B: water–acetonitrile (20:80%, v/v); gradient: 0 min: 0% eluent B; 0–30 min: 0–45% B; 30–33 min: 45–100% eluent B; 33–36 min: 100% B; flow rate: 1 ml/min). (B) Eluent A: 2 mmol/1 tetrabutylammonium hydrogensulfate and 10 mmol/1 K_2HPO_4 in water (pH 6.8); eluent B: water–acetonitrile (20:80%, v/v); gradient: 0 min: 0% B; 0–15 min: 0–40% B; 15–16 min: 40–100% eluent B; 16–17 min: 100% B; flow rate: 2 ml/min). (C) Eluent A: 2 mmol/1 tetrabutylammonium hydrogensulfate and 10 mmol/1 K_2HPO_4 in water (pH 6.8); eluent B: water–acetonitrile (20:80%, v/v); gradient: 0 min: % B; 0–7.5 min: 0–40% B; 7.5–8.2 min: 40–100% eluent B; 8.2–9.2 min: 100% B; flow rate: 4 ml/min). (D) Eluent A: 2 mmol/1 tetrabutylammonium hydrogensulfate and 10 mmol/1 K_2HPO_4 in water (pH 6.8); eluent B: water–acetonitrile (20:80%, v/v); gradient: 0 min: 0% B; 0–5 min: 0–40% B; 5–5.5 min: 40–100% eluent B; 5.5–6.5 min: 100% B; flow rate: 6 ml/min). (E) Reversed phase chromatography of a platelet extract using the conditions as described in (D). (F) Eluent A: 40 mmol/1 TEAA in water; eluent B: water–acetonitrile (80:20%, v/v); gradient: 0–2 min: 0% eluent B; 2–62 min: 0–60% B, respectively; 62–63 min: 60–100% eluent B. The concentration of eluent B of 60% corresponds to an acetonitrile concentration of 12%. Flow rate: 1 ml/min. (G) Eluent A: 40 mmol/1 TEAA in water; eluent B: water–acetonitrile (80:20%, v/v); gradient: 0 min: 0% B; 0–32 min: 0–35% B; 32–33 min: 35–100% eluent B; 33–34 min: 100% B; flow rate: 2 ml/min. (H) Eluent A: 40 mmol/1 TEAA in water; eluent B: water–acetonitrile (80:20%, v/v); gradient: 0–1 min: 0% eluent B; 1–16 min: 0–35%; 16–17 min: 35–100% eluent B; flow rate: 4 ml/min. (I) Eluent A: 40 mmol/1 TEAA in water; eluent B: water–acetonitrile (80:20%, v/v); gradient: 0–1 min: 0% eluent B; 1–11 min: 0–35%; 11–12 min: 35–100% eluent B. Flow rate: 6 ml/min. (J) Reversed phase chromatography of a platelet extract using the conditions as described in (I).

The chemical stability of conventional silica reversed phase columns is generally limited. Silica-based reversed-phase sorbents operate within the pH limits of $2 < pH < 8$, because at $pH > 8$ silica slowly dissolves [34] and at $pH < 2$ the covalently bound silane ligands are hydrolyzed [35]. Due to this limited chemical stability only restricted purification procedures can be performed with limited operating life times as a result. This is another reason to prefer monolith reversed-phase chromatography columns for separation of dinucleoside polyphosphates.

Fig. 4 shows characteristic reversed-phase chromatograms of a homogenate of human platelets using TEAA as ion-pair reagent and with the four columns under study (Fig. 4A–D). In contrast to the other tested reversed-phase chromatography columns, the resulting chromatogram of the monolithic silica reversed phase column shows no interference with individual dinucleoside polyphosphates.

While TEAA is superior as an ion-pair reagent if subsequent mass spectrometry is needed for identification of individual compounds, for all other chromatographic conditions, TBA seems to be superior regarding the analysis time. Fig. 5 depicts analogous separations with TBA as the ion-pair reagent. The resolution of the separation is further increased compared to the corresponding resolution of the chromatography with TEAA as the cationic ion-pair reagent. The distinct elution of the dinucleoside polyphosphates using the latter chromatographic conditions, allows quantification of very low concentrated derivatives. Using TBA instead of TEAA as ion-pair reagent, the analysis time can obviously be decreased without a decreasing of the resolution of the chromatography.

The combination of TBA as the cationic ion-pair reagent with monolithic silica reversed phase columns yields a chromatogram with baseline separated and sharp UV-absorption peaks. Using this combination of chromatographic conditions, not only the abundant and known dinucleoside polyphosphates like Ap_2A, Ap_3A, Ap_4A, Ap_5A and Ap_6A, are baseline separated, but also less concentrated, yet unknown nucleoside polyphosphates may be in all probability separated with high resolution and small peak width.

Next, the effect of increasing the flow rate on the resolution of the monolithic reversed-phase column was analyzed. Due to the greater back-pressure of the reversed-phase columns compared to the monolith reversed-phase and the perfusion reversed-phase chromatography columns, the flow rates used in chromatography with conventional silica reversed phase chromatography columns are in general lower than those used in chromatography with monolithic or perfusion chromatography columns. Higher flow rates result in a decrease in the analysis time. Fig. 6 shows characteristic reversed-phase chromatograms of synthetic diadenosine polyphosphates using the monolithic reversed phase column in the presence of the ion-pair-reagent TBA (Fig. 6A–D) and in the presence of the ion-pair-reagent TEAA (Fig. 6F–I) using a flow rate of 1–6 ml/min. Analysis time can be reduced by close to one magnitude without a significant decrease in the resolution. The identity of the substances underlying the UV absorption was determined by retention time comparison with the single diadenosine polyphosphates. Moreover, the identity of these substances fractionated by using TEAA as ion-pair reagent was determined by MALDI mass-spectrometry analysis. Characteristic MALDI mass-spectra of diadenosine polyphosphates fractionated using the conditions as described in Fig. 6H are shown in Fig. 7. As mentioned above, the use of TBA as ion reagent prevents the analysis by MALDI mass-spectrometry (data not shown).

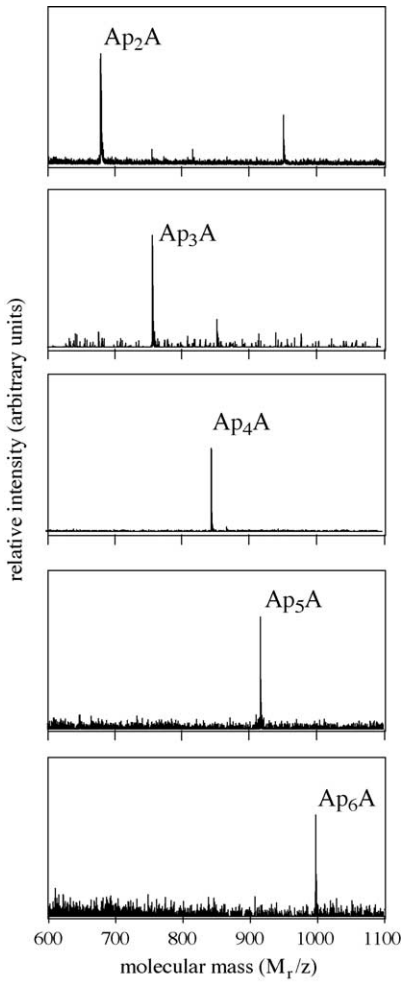

Fig. 7. Exemplary MALDI mass spectra of the substances underlying the UV absorption-peaks (labelled as Ap_2A, Ap_3A, Ap_4A, Ap_5A and Ap_6A in Fig. 6H).

The separation of a platelet extract in the presence of TBA and TEAA using a flow rate of 6 ml/min is shown Fig. 6E and J, respectively. In the presence of TBA, the differences of retention times of diadenosine polyphosphates increase.

Therefore we recommend the use of a monolithic reversed phase chromatography column, TBA as ion-pair reagent and a flow rate in the range of 6 ml/min for fast dinucleoside polyphosphate separation for quantification (as shown in Fig. 6E). For isolation of unknown dinucleoside polyphosphate, we recommend the use of a monolithic reversed phase

chromatography column, flow rates in the range of 1 ml/min and – because of its compatibility to mass-spectrometric methods – TEAA as ion-pair reagent (as shown in Fig. 4A).

In summary, isolation and quantification of dinucleoside polyphosphates by using monolithic silica C18 columns has obviously essential advantages compared to chromatography with perfusion le-versed phase media or conventional silica reversed-phase media. In the future, the usage of monolithic silica reversed phase columns will lead to isolation and quantification of yet unknown dinucleoside polyphosphates.

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