Journal of Chromatography B, 878 (2010) 763–768

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Replacement of acetonitrile by ethanol as solvent in reversed phase chromatography of biomolecules

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ARTICLE INFO

Article history:
Received 6 October 2009
Accepted 21 January 2010
Available online 29 January 2010

Keywords:
Dinucleoside polyphosphates
Peptides and proteins
Acetonitrile
Ethanol
Methanol
n-Propanol
Reversed phase chromatography

ABSTRACT

Acetonitrile, which is a by-product of acrylonitrile synthesis, is the commonly used solvent in ion-pair reversed phase chromatography. In consequence of the decreasing demand for acrylonitrile due to the financial crisis, a worldwide shortage of acetonitrile is observed. Therefore, the aim of this study was to establish ion-pair reversed phase chromatographic assays using alternative eluents for acetonitrile and to decrease costs incurred hereby. We compared the performance of ion-pair reversed phase chromatography using acetonitrile with the alternative eluents methanol, ethanol and *n*-propanol, using monolithic reversed phase C5 as well as C18 chromatography columns. We used triethylammonium acetate (TEAA) and tetrabutylammonium sulfate (TBA) as representative cationic ion-pair reagents and trifluoroacetic acid (TFA) as representative anionic ion-pair reagent. For covering a large field of applications, we fractionated representative low, middle and high-molecular weight biomolecules, in particular dinucleoside polyphosphates, peptides, proteins and tryptic digested human serum albumin. Whereas the chromatographic characteristics of both methanol and *n*-propanol were partly insufficient, ethanol was characterised equally or partly even better in the matter of elution strength and separation quality compared to the eluent water–acetonitrile. In conclusion, ethanol is an appropriate alternative for acetonitrile in ion-pair reversed phase chromatography of biomolecules.

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1. Introduction

Isolation, purification and quantification of biomolecules are the essential procedures in current biochemical and medical research. Ion-pair reversed phase chromatography is a common method for separation and purification of biomolecules, both for low molecular weight and highly charged biomolecules like dinucleoside polyphosphates and for middle and high-molecular weight ampholytic biomolecules like peptides and proteins [1–4]. Therefore, ion-pair reversed phase chromatography is used for desalting steps to avoid an interference of salt in the following physiological/pathophysiological characterisation steps, chromatographic purification and mass-spectrometric analysis. Furthermore, in reversed phase chromatography of ionic substances the use of ion-pair reagents is applied in order to mask their charge and allow their retention on the hydrophobic resin. For instance, the ion-pair reagent tetrabutylammonium sulfate (TBA) is commonly used for chromatographic quantification of highly negatively charged low molecular weight biomolecules like dinucleoside polyphosphates [3]. The positively charged ion-pair reagent TBA enhances the hydrophobicity of the complex by its four butylene groups. Unfortunately, TBA is not removable by lyophilisation in the presence of negatively charged substances like dinucleoside polyphosphates because of its low vapour pressure. Therefore, the use of TBA does not allow subsequent mass-spectrometric analysis of the biomolecules of interest. The volatile, positively charged ion-pair reagent triethylammonium acetate (TEAA) is an appropriate alternative for TBA in reversed phase chromatography followed by mass-spectrometric analysis [5], but the resulting chromatographic resolution is reduced compared to TBA. Trifluoroacetic acid (TFA) is a well-suited ion-pair reagent in ion-pair reversed phase chromatography of amphoteric molecules like peptides and proteins (e.g., [4,6–8]). The protons of TFA are masking anionic amino acid residues and the anionic part of TFA trifluoroacetate interacts with cationic amino acid residues. Because of its volatility, TFA is removable by lyophilisation.

The aprotic, polar and water-miscible solvent acetonitrile (ACN) is a commonly used eluent in reversed phase chromatography [9], especially in ion-pair reversed phase chromatography. The decreasing demand for acrylonitrile results in a worldwide shortage of acetonitrile (ACN), since ACN is the by-product of acrylonitrile production. Furthermore, metabolic derivatives of ACN like hydrogen cyanide cause toxic effects. ACN is characterised by its sufficient elution strength for biomolecules paired with minor UV absorption (>190 nm) and low backpressure. Therefore, ACN has been the most suitable solvent for high resolution reversed

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1570-0232/$ – see front matter © 2010 Published by Elsevier B.V.
doi:10.1016/j.jchromb.2010.01.029

phase separation and fractionation of dinucleoside polyphosphates, peptides and proteins [3,5,10–12]. Primary alcohols like methanol, ethanol or *n*-propanol are water-miscible, polar and protic solvents too. The polarity of these primary alcohols decreases with increasing number of methylene groups.

Therefore, the present study compares the chromatographic characteristics of ACN with methanol, ethanol and *n*-propanol as eluents in chromatographic separation of biomolecules, using both conventional C18 reversed phase columns as well as conventional C5 reversed phase columns.

2. Materials and methods

Acetonitrile, ethanol, methanol and tetrabutylammonium sulfate (TBA) were purchased from Merck (Germany); trypsin (modified sequencing grade) was purchased from Roche (Switzerland). *n*-Propanol and all other substances were purchased from Sigma–Aldrich (Germany). Amicon Ultra centrifugal filter devices (10 kDa) were purchased from Millipore (Germany). HPLC water was generated with a Seralpur Delta ultra filtration unit from ELGA LabWater (Germany). Reversed phase chromatographic separation was performed using conventional C18 monolithic reversed phase columns (Chromolith® Performance RP-18e (100 mm × 4.6 mm I.D., pore size 0.01–2 μm, Merck, Germany)) and conventional C5 reversed phase columns (Discovery® Bio Wide Pore C5-3 (100 mm × 4.6 mm I.D., particle size 3 μm, Sigma–Aldrich, Germany)).

2.1. Comparison of the eluents of interest in reversed phase chromatography in the presence of Ion-pair reagent triethylammonium acetate (TEAA)

Diadenosine polyphosphates (Ap_nA with $n = 2–6$; each 1 μg) were separated by gradient elution using water and the ion-pair reagent triethylammonium acetate (TEAA) (40 mmol/l (final concentration; pH 6.5; adjusted with triethylamine and formic acetic acid)) as eluent A, and the Chromolith® Performance RP-18e reversed phase column. Eluent and gradient conditions are shown in Table 1. Elution gradient of water–acetonitrile (eluent B_1), water–ethanol (eluent B_2), water–*n*-propanol (eluent B_3) was performed with 10% B_1, B_2, B_3, respectively, within 30 min. Elution gradient of methanol (eluent B_4) was performed with 20% B_4 within 30 min. The column temperature was ambient (22 ± 1 °C). The mobile phase was pumped at a constant flow rate of 1 ml/min by a high-pressure gradient pump system (Merck, Germany). The diadenosine polyphosphate mixture was dissolved in water and was mixed with eluent A. Injection volume was 110 μl. UV absorption was measured at 254 nm. The column eluate was monitored with a variable wavelength UV detector (759 A, Absorbance Detector, Applied Biosystems, Germany). Data were recorded and processed with the Chromeleon Lab System 6.60 (Dionex, Germany).

2.2. Comparison of the eluents of interest in Reversed phase Chromatography in the presence of Ion-pair reagent tetrabutylammonium hydrogen sulfate (TBA)

Diadenosine polyphosphates Ap_nA (with $n = 2–6$; each 1 μg) were separated by gradient elution on the Chromolith® Performance RP-18e reversed phase column in the presence of TBA (4 mmol/l) in a phosphate buffer (20 mmol/l K_2HPO_4, final concentration; pH 6.5 (adjusted with H_3PO_4 and KOH)) as eluent A. Eluent and gradient conditions are shown in Table 2. Additional experimental conditions were identical as described above.

2.3. Comparison of the eluents of interest in Reversed phase Chromatography in the presence of Ion-pair reagent Trifluoroacetic acid (TFA)

The peptide mixture (bradykinin, angiotensin II, angiotensin I (each 2 μg)) was separated by gradient elution on the Chromolith® Performance RP-18e reversed phase column in the presence of ion-pair reagent 0.1% trifluoroacetic acid (TFA) in water as eluent A. Eluent and gradient conditions are shown in Table 3 in detail.

Table 1
Conditions of chromatographic fractionation of diadenosine polyphosphates ($Ap_{2–6}A$) using a monolithic reversed phase C18 column.

Eluent A	Eluent A	Eluent A	Eluent A
Water/TEAA (40 mmol/l) (pH 6.5)	Water/TEAA (40 mmol/l) (pH 6.5)	Water/TEAA (40 mmol/l) (pH 6.5)	Water/TEAA (40 mmol/l) (pH 6.5)
Eluent B_1	Eluent B_2	Eluent B_3	Eluent B_4
Water–acetonitrile (20:80, v/v)	Water–ethanol (50:50, v/v)	Water–*n*-propanol (50:50, v/v)	Methanol (100%)
Gradient	Gradient	Gradient	Gradient
0% B_1 at 0 min 10% B_1 at 30 min	0% B_2 at 0 min 10% B_2 at 30 min	0% B_3 at 0 min 10% B_3 at 30 min	0% B_4 at 0 min 20% B_4 at 30 min

Table 2
Conditions of chromatographic fractionation of diadenosine polyphosphates ($Ap_{2–6}A$) using a monolithic C18 reversed phase column.

Eluent A	Eluent A	Eluent A	Eluent A
Water/TBA/K_2HPO_4 (4 mmol/l/20 mmol/l) (pH 6.5)	Water/TBA/K_2HPO_4 (4 mmol/l/20 mmol/l) (pH 6.5)	Water/TBA/K_2HPO_4 (4 mmol/l/20 mmol/l) (pH 6.5)	Water/TBA/K_2HPO_4 (4 mmol/l/20 mmol/l) (pH 6.5)
Eluent B_1	Eluent B_2	Eluent B_3	Eluent B_4
Water–acetonitrile (20:80, v/v)	Ethanol (100%)	*n*-Propanol (100%)	Methanol (100%)
Gradient	Gradient	Gradient	Gradient
0% B_1 at 0 min 40% B_1 at 30 min	0% B_2 at 0 min 20% B_2 at 30 min	0% B_3 at 0 min 10% B_3 at 30 min	0% B_4 at 0 min 50% B_4 at 30 min

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