International Journal of Mass Spectrometry 304 (2011) 83–90

Contents lists available at ScienceDirect

International Journal of Mass Spectrometry

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

Study on the loss of nucleoside mono-, di- and triphosphates and phosphorylated peptides to a metal-free LC–MS hardware

Thomas De Vijlder[a,*], Jasper Boschmans[a,b], Erwin Witters[a,c], Filip Lemière[a,b]

[a] CEPROMA (Center for Proteome Analysis and Mass Spectrometry), University of Antwerp, Groenenborgerlaan 171, B-2020 Antwerp, Belgium
[b] Nucleoside Research and Mass Spectrometry Unit, Department of Chemistry, University of Antwerp, Groenenborgerlaan 171, B-2020 Antwerp, Belgium
[c] Flemish Institute for Technological Research (VITO), Boeretang 200, B-2400 Mol, Belgium

ARTICLE INFO

Article history:
Received 31 March 2010
Received in revised form 16 May 2010
Accepted 19 May 2010
Available online 27 May 2010

Keywords:
Nucleotide
Phosphopeptide
Fused silica
Sample loss
Metal-free

ABSTRACT

In our earlier LC–MS experiments on the analysis of phosphocompounds like nucleotides (mono-, di- and triphosphate) and phosphopeptides and in literature, low sensitivity and severe losses of analyte to the instrumental setup were observed. Since we noticed that the stainless steel parts of the setup (e.g., the electrospray needle) adsorbed important quantities of the phosphorylated analytes, we made a LC–ESI setup without metallic surfaces. The first results were disappointing since also the fused silica surface of the LC-ESI coupler adsorbed part of the nucleotides and phosphopeptides injected in flow analysis experiments. We present experiments documenting the contribution of the different components of the setup. A number of potential solutions to the adsorption problem are proposed and tested. Only dimethyldichlorosilane deactivation of fused silica capillaries gave satisfactory results as adsorption of nucleotides and phosphopeptide was minimised.

1. Introduction

1.1. Importance of phosphorylated compounds

Phosphorylated organic compounds such as (oligo-) nucleotides and phosphoproteins represent an important class of biologically active compounds. Nucleotides are the building blocks of our genome and nucleoside triphosphates (e.g., ATP) are the main universal energy carriers of cells. Several nucleoside and nucleotide analogues are often used as pro-drugs in the treatment of various viral infections and cancer [1–3]. In most cases, their mode of action includes metabolic phosphorylation [4]. (Cyclic-)nucleotides are chemical carriers of signals within the cell, signals that are transduced by (de-)phosphorylation of target proteins. Phosphorylation of proteins is a ubiquitous posttranslational modification that dramatically influences structure, activity, subcellular location and degradation of the protein [5]. The research area connected to this – phosphoproteomics – is an increasingly important research domain [6].

These phosphorylated biomolecules (nucleotides, nucleotide analogues and phosphorylated peptides) are intensively studied by means of different chromatographic methods such as reversed phase [7], ion-pairing [8–10], ion exchange [11,12] and affinity chromatography [13–16] for both concentration and separation. These analytical chromatographic techniques are often coupled to tandem mass spectrometry for identification and/or structural elucidation of the compounds.

1.2. Analytical challenge

Although comprehensive studies have been published recently [17,18] the chromatographic and mass spectrometric analysis of phosphorylated organic compounds has never been straightforward (e.g., Piggee [19] and references therein). Due to their high polarity, multiply phosphorylated peptides and nucleotides show only limited retention in reversed phase LC–MS analysis [20,21]. Moreover, because of their lower isoelectric point resulting from the low pK_a's of the phosphate moiety they are inherently more difficult to detect in positive ion mode mass spectrometry. This is especially unfavourable for phosphopeptide analysis since positive ion mode tandem MS is the method of choice for peptide sequencing and subsequent protein identification.

A major technical challenge that needs to be tackled when dealing with (multiply) phosphorylated compounds, is the fact that they interact strongly with specific parts of an LC–MS setup [22]. This phenomenon is of critical impact when dealing with low con-

Abbreviations: AMP, 5′-adenosine monophosphate; ADP, 5′-adenosine diphosphate; ATP, 5′-adenosine triphosphate; LC, liquid chromatography; MS, mass spectrometry; ICP, inductively coupled plasma; PEEK, polyether ether ketone; PAEK, olyaryl ether ketone; EDTA, 2,2′,2″,2‴-(ethane-1,2-diyldinitrilo)tetraacetic acid; PA, phosphoric acid; ID, internal diameter; LOD, limit of detection; DMDCS, dimethyldichlorosilane.

* Corresponding author. Tel.: +32 3 265 3406; fax: +32 3 265 3233.
E-mail address: filip.lemiere@ua.ac.be (T. De Vijlder).

1387-3806/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijms.2010.05.022

centrations of phosphorylated analyte. Especially stainless steel parts show high affinity for phosphorylated molecules. This problem becomes even more apparent when these metal surfaces get corroded over time, a process that is known to happen in stainless steel emitters [23].

1.3. New approach and new problem

To avoid undesirable adsorption of phosphorylated compounds and consequent sample loss, a new capillary LC–MS setup was designed, free of metal surfaces that are in contact with the analyzed samples. The standard electrospray probe was replaced by electrospray chip technology [24].

Yet the problem of adsorption of multiply phosphorylated organic compounds at low pH could not be overcome by this approach and the main site of adsorption in this case could be traced back to the fused silica tubing used in the setup. Silanol moieties on silica surfaces are notorious in liquid chromatography for their capacity to adsorb and cause tailing of organic bases [25]. In order to minimise this problem, different strategies have been developed which included modifying the free silanol groups of the silica by endcapping. Though even then some silanol moieties remain available [26]. Castillo and colleagues also reported unexpected secondary interactions between hydroxyl containing analytes and fused silica capillaries in an LC-ICP–MS approach [27]. Little information is found in the literature about the specificity and pH dependent interaction of silanols with phosphorylated organic compounds [28,29].

With the current tendency towards analysis of minute amounts of highly complex samples, these adsorption phenomena become a tangible concern. In high throughput experiments that produce vast amounts of data, processes like the adsorption of phosphorylated compounds could easily be overlooked, resulting in the underrepresentation of entire classes of these important biomolecules. Moreover, when accurate quantification of compounds is desired, loss and carryover of any compounds on parts of the analytical setup becomes an issue.

2. Experimental

2.1. Chemicals

Raffinose was obtained from Sigma-Aldrich (Bornem, Belgium). Nucleotides (AMP, ADP and ATP) were purchased as sodium salts from Sigma-Aldrich as well and were of the highest grade available. The phosphopeptide control set consisted of a monophosphopeptide and a tetraphosphopeptide from β-casein. This phosphopeptide control set and the non-phosphorylated (Glu^1)fibrinopeptide B were also obtained from Sigma-Aldrich, as was the EDTA (Na^+-salt). Water (H_2O) and acetonitrile (ACN) were from HPLC grade quality and obtained from Acros (Geel, Belgium). n-Hexane 95% was obtained from Acros as well. Ammonium acetate (NH_4OAc, p.a.) was from Janssen Chimica (Geel, Belgium). Acetic acid (HOAc, p.a.) and ammonium hydroxide (NH_4OH, 25%, p.a.) were purchased from Merck (Overijse, Belgium). Methanol (MeOH; LC–MS grade) came from Biosolve (Valkenswaard, The Netherlands). Phosphoric acid (H_3PO_4; 85%) was obtained from Chem-lab (Zedelgem, Belgium). The dimethyldichlorosilane (DMDCS) was purchased from Fluka (Bornem, Belgium).

Stock solutions of all samples were prepared in HPLC grade water at stock concentrations of 10^{-3} or 10^{-4} M and stored at −20 °C until use. Just before analysis, samples were diluted to working concentrations of 10^{-5} M for each compound. EDTA was dissolved in 50% MeOH at a concentration of 50 mM and stored at −20 °C until use.

Two sets of test mixtures were prepared. The nucleotide test mixture consisted of equal concentrations of raffinose, 5′-AMP, 5′-ADP and 5′-ATP. The peptide test mixture consisted of fibrinopeptide B (EGVNDNEEGFFSAR) as an unphosphorylated reference peptide, a monophosphorylated (FQpSEEQQQTEDELQDK) and tetraphosphorylated (RELEELNVPGEIVEpSLpSpSpSEESITR) peptide obtained from bovine β-casein.

The mobile phases used throughout this study were A: 0.1% (v/v) HOAc (50:50 H_2O/MeOH), B: 100% ACN and C: 25:25:50 NH_4OH/H_2O/MeOH.

2.2. Instruments

For all experiments, a capillary ternary gradient pump with integrated injector was used (CapLC, Waters, Manchester, UK). The mobile phases were delivered at a flow rate of 8 μL/min unless stated otherwise. Samples were injected trough a 10 μL PEEK loop (Upchurch scientific, Oak Harbor, WA, USA) mounted on the 6 port valve type injector (Valco Instruments, Houston, TX, USA), modified with a PAEK rotor (C2-13R6) and PEEK stator (C-1C46) to omit contact of the sample with stainless steel surfaces (standard stator).

The injector was connected to the modified PEEK splitting tee (Upchurch scientific) of the Nanomate via PEEK tubing (360 μm OD, 50 μm ID, Upchurch scientific). The mobile phase was split in order to obtain an analytical flow of approximately 500 nL/min through the 30 cm long, 15 μm ID fused silica LC-coupler of the Nanomate ESI-source. As needed, an auxiliary injector with a 200 μL PEEK loop was used. A scheme of the LC setup is outlined in Fig. 1a.

Mass spectra were recorded on a Q-TOF 2 mass spectrometer (Waters) used in negative (−) or positive (+) ion mode. The Waters ESI-source was replaced by a Nanomate ESI-chip system (Advion biosystems, Ithaca, NY, USA). The ESI-chip consists of an integrated 20 × 20 array of nanoelectrospray nozzles that are etched from a planar silicon wafer. The electrospray was initiated by applying −1.75 to −1.9 kV (negative ion mode) or 1.7 kV (positive ion mode) to the polymeric coupler on the backside of the chip. Both ion modes gave the same adsorption results. Operation of the nanospray proved to be more stable in positive ion mode whereas negative ion mode yields better sensitivity for the nucleotides. Voltage and chip position were optimised using the Chipsoft 7.1.1 software (Advion Biosystems). For all nucleotide experiments, a mass range of m/z 50–750 was chosen and a cone voltage of 30 V was used. In case of the (phosphorylated) peptides, the mass range was m/z 300–2000 with a 40 V cone voltage. A scan time of 1 s was always used.

2.3. LC conditions

The adsorption of nucleotides and phosphopeptides to the LC-ESI setup was examined using the same sequence as used earlier [20]. The 10 μL sample was injected in solvent A at a flow rate of 8 μL/min, the setup washed with solvent B and any adsorbed compound eluted with solvent C (see Fig. 1b). Finally the system was reset to initial conditions (solvent A) for the next sample injection.

To assess which components of the LC hardware adsorbed the injected phosphorylated compounds, the LC setup was divided into two parts: the *INJECTOR (INJ)-part* on one hand, which included the injector, sample loop, and PEEK tubing. On the other hand the *ESI-part*, which comprised the PEEK splitting tee, the LC-coupler and the ESI-chip (Fig. 1a). To test the different parts separately, they were disconnected and a PEEK loop of 200 μL (750 μm ID) was placed upstream of the part to be tested (connection point is marked with * in Fig. 1a). 200 μL 10^{-5} M of the nucleotide mixture was injected at a flow rate of 8 μL/min and the part was rinsed in solvent A afterwards. Then both parts were reconnected and rinsed for 10 more minutes before switching to alkaline solvent C for elution. During separate experiments to test the capacity of the complete setup,