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Evaluation of column hardware on liquid chromatography–mass spectrometry of phosphorylated compounds

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ABSTRACT

The influences of column hardware, such as chromatographic tubes and frits, on liquid chromatography–mass spectrometry (LC–MS) analysis of phosphorylated compounds were evaluated. The signal to noise ratio (S/N) and the intensity of flavin adenine dinucleotide (FAD) using a glass lined tube and polyethylene frit (GL–PE) column was approximately 170 and 90 times higher, respectively, than those using conventional stainless steel tube and stainless steel frit (S–S) column. In addition, the retention time of FAD using GL–PE column was the shortest compared to other columns. Interaction between phosphorylated compounds and metal ions in the flow path in the S–S column was stronger than that between them and the GL–PE column. Thus, the metal ions in the flow path in GL–PE column were low. Since the specific surface area of a pair of frits was 70 times larger than that of a chromatographic tube (150 mm × 2.1 mm), the frits were found to have more effective improvement of the S/N as well as the intensity than the chromatographic tubes, when phosphorylated compounds were analyzed by LC–MS. When the evaluated phosphorylated compounds were analyzed by LC–MS(/MS) using a GL–PE column, the intensity and S/N were increased.

1. Introduction

In HPLC, columns are packed with minute packing materials under high pressure, and in order to avoid the outflow of the packing materials, filters which are called "frits" are used at both ends of the column. As for the column hardware materials, stainless steel, PEEK (polyetheretherketone), glass, fused-silica capillary and *etc.* are normally used. In reversed-phase HPLC, stainless steel columns are widely used while fused-silica capillaries are used for capillary columns with inner diameter smaller than 0.5 mm [1]. On the other hand, PEEK columns are normally used in ion chromatography; however, PEEK or glass columns have low pressure resistance, they are difficult to be used as separation columns without further modification. Aluminum sleeve PEEK column packed with 3-μm ODS particle under a pressure of 60 MPa obtained 130,000 plates per meter [2]. A glass lined stainless steel capillary column (300 mm × 0.3 mm) packed with 3-μm ODS particle obtained 40,000 theoretical plates [3]. For the frit materials, sintered stainless, mesh stainless, titanium, glass, Teflon, PEEK and *etc.* are used. However, no frit is needed for monolithic columns [4]. The frits in fused silica capillary for capillary LC and electrochromatography were formed in silica monolith and sintered stainless steel [5–9]. In addition, for the case of "integrated spray columns", which are normally used in nano LC, self-assembled particle frits are normally utilized [10].

Due to samples loss, the intensity of phosphorylated compounds on LC–MS is normally low; this is caused by the adsorption of the silanol groups and metal ions. [11]. In order to overcome this drawback, three methods of improvement could be used; (1) deactivation of chromatographic system such as valves, needles and tubes [9,12–15]; (2) addition of chelate reagents in pretreatment and sample solvent [11,16–18]; (3) using alkaline mobile phase as in immobilized metal affinity chromatography [16,19]. The improvements with methods (1) and (2) were due to prevention from interaction between phosphate compounds and metal ions on the surface of flow path.

Deactivation of the inner wall of fused silica capillary could be carried out by treatment with dimethyldichlorosilane to prevent hydrogen bonding between the silanol groups on the inner wall surface of the fused silica capillary and phosphorylated compounds, thus the adsorption could be minimized [15].

Kim et al. [11] improved peak intensity of phosphopeptides by adding phosphoric acid to sample solutions. On the other hand,

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Wakamatsu et al. [13] improved peak intensity of nucleic acids by flowing 400 mM phosphoric acid to the chromatographic system involving stainless steel components as a pretreatment. Asakawa et al. [16] used carbonate mobile phase for nucleic acids, which are phosphorylated compounds, in order to reduce the adsorption of the target compounds. It was concluded that this effect would be due to the desorption of nucleic acid in alkaline mobile phase and the strong buffer capacity of carbonate mobile phase. However, this was the results of improvement without the separation column [16].

Our approach was to develop a new column by optimizing the column hardwares for LC/MS analysis of phosphorylated compounds. To the best of our knowledge, the column hardwares were not evaluated on semi-micro flow LC–MS. In this paper, we evaluated the influence of chromatographic tubes and frits on LC–MS analysis of some phosphorylated compounds.

2. Materials and methods

2.1. Chemicals

Adenosine monophosphate (AMP), 5-deoxyadenosine monophosphate (dAMP) and cyclic adenosine monophosphate (cAMP) were purchased from Sigma–Aldrich (Tokyo, Japan). Flavin adenosine dinucleotide (FAD), lysophosphatidic acid (LPA), formic acid and ammonium acetate were purchased from Wako Pure Chemical (Osaka, Japan). Phosphopeptide standard kit was purchased from Funakoshi (Tokyo, Japan). Three phosphopeptides (SFVLNPTNIGM-pS-KSSQGHVTK, TRDIYETD-pY-YRK, DLDVPIPGRFDRRV-pS-VAAE) contained in the kit were used in this paper. Iron standard solution of 1000 mg/L, phosphoric acid, acetonitrile and methanol were purchased from Kanto Chemicals (Tokyo, Japan). All other reagents were of analytical grade and were used without further purification. Water was purified by a Milli-Q purification system obtained from Millipore (Bedford, MA, USA).

2.2. Sample treatment

AMP, dAMP, cAMP and FAD were dissolved separately in water at a concentration of 100 mg/L as stock solutions. The mixtures composed of AMP, dAMP, and cAMP were dissolved in water at a concentration of 500 μg/L each as sample solution. FAD was dissolved in water at a concentration of 10 mg/L as sample solution while LPA was dissolved in methanol at a concentration of 100 mg/L as a stock solution. LPA was then diluted to a concentration of 10 mg/L using methanol and used as sample solution.

3 phosphopeptides were dissolved separately in a mixture of 20 mM phosphoric acid and acetonitrile (1/1) at a concentration of 20 mg/L as stock solutions. The mixtures composed of phosphopeptides at a concentration of 2 mg/L were dissolved in 0.1% formic acid as sample solution. All stock solutions were stored at −20 °C, just before analysis, the sample solutions were prepared.

2.3. Chromatographic and mass spectrometric conditions

The chromatographic system used was a Waters 2695 Alliance System (Milford, MA). The stainless steel tubing from autosampler to column and from column to MS were replaced with PEEK tubing of 400 mm × 0.13 mm and 600 mm × 0.13 mm, respectively. Methanol was used as the needle washing solvent. The sample vials used were Waters Polypropylene vials for preventing any possible adsorption of phosphorylated compounds.

The MS used was a Waters ZQ single quadrupole mass spectrometer with electrospray ionization (ESI) performed in positive and negative mode. Instrument control and data acquisition were achieved using Waters Masslynx Software. The processing was achieved using Waters Empower Software. MS conditions were optimized by post-column infusion of the standard solutions.

2.3.1. FAD

The chromatographic mobile phase was a mixture of 0.1% formic acid and acetonitrile (10:90), at a flow rate of 0.3 mL/min. The column temperature was kept at 40 °C throughout the experiment. The injection volume was 5 μL. The MS detection was carried out by selected-ion monitoring (SIM) mode at m/z 348 in positive mode. The capillary sprayer voltage was 4.0 kV and the sample cone voltage was 30 V. The source temperature was 100 °C and the desolvation temperature was 400 °C. The desolvation and cone gas flow-rates were set to 400 and 50 L/h, respectively.

2.3.2. LPA

The chromatographic mobile phase was a mixture of 5 mM ammonium acetate and methanol (5:95), at a flow rate of 0.3 mL/min. The injection volume was 1 μL. The MS detection was carried out by SIM mode at m/z 435.5 in negative mode. The capillary sprayer voltage was −3.5 kV and the sample cone voltage was −35 V. The source temperature was 100 °C and the desolvation temperature was 400 °C. The desolvation and cone gas flow-rates were set to 500 and 50 L/h, respectively.

2.3.3. AMPs

The chromatographic mobile phase was a mixture of 0.1% formic acid and acetonitrile (99:1), at a flow rate of 0.3 mL/min. The injection volume was 5 μL. The MS detections were carried out by SIM mode at m/z 348 (AMP), 332 (dAMP) and 330 (cAMP) in positive mode. The capillary sprayer voltages were 3.5 kV and the sample cone voltages were 20 V. The source temperature was 100 °C and the desolvation temperature was 400 °C. The desolvation and cone gas flow-rates were set to 400 and 50 L/h, respectively.

2.3.4. Phosphopeptides

The chromatographic mobile phases A and B were 0.1% formic acid and 0.1% formic acid in acetonitrile, respectively. The gradient flow started with 95% A and 5% B, and linearly reached 50% A and B in 10 min, at a flow rate of 0.3 mL/min. Next, the washing of chromatographic system including a column was carried out by passing through 5% A and 95% B for 2 min, at a flow rate of 0.4 mL/min. The injection volume was 1 μL. In order to minimize carryover of the phosphopeptides, a mixture of 50% 20 mM phosphoric acid and 50% acetonitrile was used as the needle washing solvent.

The MS detections were carried out by SIM mode at m/z 688 (SFVLNPTNIGM-pS-KSSQGHVTK), 568 (TRDIYETD-pY-YRK) and 731 (DLDVPIPGRFDRRV-pS-VAAE) in positive mode. The capillary sprayer voltages were 3.5 kV. The sample cone voltage for DLDVPIPGRFDRRV-pS-VAAE and TRDIYETD-pY-YRK were 30 V, and that for the SFVLNPTNIGM-pS-KSSQGHVTK was 20 V. The source temperature was 100 °C and the desolvation temperature was 400 °C. The desolvation and cone gas flow-rates were set to 400 and 50 l/h, respectively.

2.4. Combinations of chromatographic tubes and frits for column

Chromatographic tubes used were stainless steel tube (150 mm × 2.1 mm I.D.) and glass lined stainless tube (150 mm × 2.0 mm I.D.) without deactivated treatment. The sintered frits were made of stainless steel, titanium, PEEK and PE (polyethylene). Table 1 shows combination of the tubes and the frits for the column. All columns were packed with L-column2 ODS 5 μm particle (Chemicals Evaluation and Research Institute, Tokyo, Japan) under a pressure of 50 MPa. The numbers of theoretical plates of naphthalene as a sample were measured for these