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Short communication

Fluorous-assisted metal chelate affinity extraction for nucleotides followed by HILIC-MS/MS analysis

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ABSTRACT

We herein developed a selective method for the determination of nucleotides by fluorous-assisted metal chelate affinity extraction followed by hydrophilic interaction liquid chromatography (HILIC) combined with tandem mass spectrometric (MS/MS) analysis. In this study, the nucleotides were selectively chelated by Fe(III)-immobilized perfluoroalkyliminodiacetic acid, and the resulting chelates were subsequently extracted into a fluorous solvent. The nucleotides present in the fluorous solvent were then back-extracted into a non-fluorous solution, such as a solution of ammonia in aqueous acetonitrile. The resulting non-fluorous solution containing the nucleotides was then directly injected into an amide-type HILIC column using a mixture of acetonitrile and aqueous ammonium bicarbonate as the mobile phase for gradient elution, and the nucleotides were detected using the negative electrospray ionization MS/MS mode. In this method, the extraction recoveries of the nucleotides ranged from 43.2 to 94.7% within a relative standard deviation of 17%. This method enabled the determination of intracellular concentrations of nucleotides.

1. Introduction

Nucleotides and related compounds are present in all cells as the basic components of nucleic acids and they play essential roles in various biological functions, such as glycosylation, cell growth, and energy metabolism [1,2]. The determination of intracellular nucleotide concentrations therefore provides a route to understanding the energy status of cellular processes that are associated with the mechanism and regulation of certain disease states. To successfully analyze the nucleotides present in biological samples, an initial extraction step is generally required to prevent enzymatic degradation. Thus, various extraction methods, such as solvent-based extractions [3,4] and solid-phase extractions using ion exchanger [5] and/or graphitized carbon [6] have been developed to date. In this case, to improve the extraction selectivity towards nucleotides, we employ an extraction method that utilizes the specific affinity of perfluoroalkyl compounds, otherwise known as the fluorous affinity or fluorophilicity. This affinity enables the highly selective enrichment and purification of perfluoroalkyl (fluorous) derivatives of target compounds using either a liquid-liquid extraction system with fluorous solvents and/or a solid-phase extraction system and liquid chromatographic (LC) separation using a fluorous-modified stationary phase column [7]. Although this specific affinity has attracted significant interest in various fields of organic chemistry, it has recently also been applied in the selective analysis of biomolecules and related compounds [8,9]. As biogenic molecules are generally non-fluorous, chemical derivatization is usually required to introduce perfluoroalkyl groups into the compounds of interest. In contrast, we previously performed the selective extraction of phosphate-containing peptides through coordination interactions based on the metal chelate affinity technique without any requirement for derivatization [10]. In this method, perfluoroalkyliminodiacetic acid (PFIDA) was used as chelating reagent with fluorous affinity for the immobilization of metal ions, such as Fe(III). The resulting chelate was then employed as an extraction reagent through interactions between the coordination site of the immobilized Fe(III) and negatively charged phosphate groups, and the obtained complex was selectively extracted into a fluorous solvent. In the present study, this fluorous-assisted metal chelate affinity method is applied to the selective extraction of nucleotides and deoxynucleotides, as indicated in Fig. 1. We expect that in this case, the coordinated nucleotides in the fluorous solvent can be easily dechelated and back-extracted into a non-fluorous aqueous solvent containing a dechelating agent, then subjected to LC analysis.

In addition, a combination of hydrophilic interaction liquid chromatography (HILIC) with tandem mass spectrometry (MS/MS) is employed for analysis of the selectively extracted nucleotides. Previously, nucleotides have been analyzed using reversed-phase ion-pair [1,4,6] and porous graphitized carbon LC methods [11]. However, more recently, HILIC has been employed in the analysis of various highly polar

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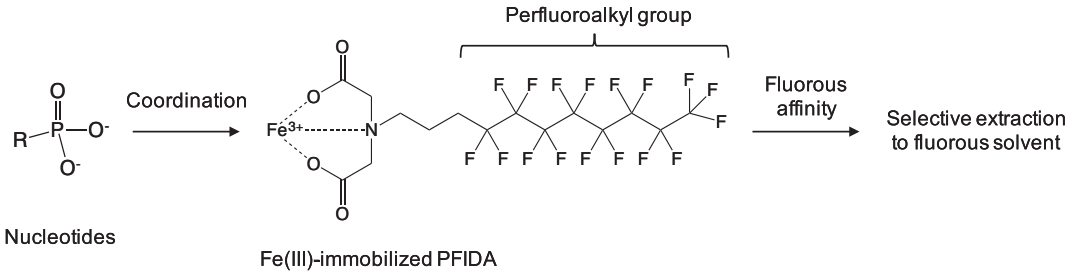

Fig. 1. The principal of the nucleotide extraction method developed herein based on the fluorous-assisted metal chelate affinity technique.

compounds, including nucleotides [12,13]. We therefore selected an amide-type HILIC column to obtain moderate retentions and separations for examined nucleotides.

The applicability of this method is also demonstrated through its application in the analysis of nucleotides in cell extract samples.

2. Experimental

2.1. Reagents and materials

Nucleotides (AMP, ADP, ATP, CMP, CDP, CTP, GMP, GDP, GTP, IMP, IDP, ITP, UMP, UDP, and UTP) and their deoxy forms (dAMP, dATP, dCMP, dCDP, dCTP, dGMP, dGDP, dGTP, dIMP, dITP, dTMP, dTDP, dTTP, dUMP, and dUTP) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Tetradecafluoro-*n*-hexane (TDFH) and tridecafluoro-1-*n*-octanol (TFO) were purchased from Wako Pure Chemicals (Osaka, Japan) and Tokyo Chemical Industry (Tokyo, Japan), respectively. 4,4,5,5,6,6,7,7,8,8,9,9,10,10,11,11,11-Heptadecafluoroundecylamine (HFUA) was obtained from Fluorous Technologies (Pittsburgh, PA, U.S.A.). PFIDA was synthesized from HFUA and Fe(III)-immobilized PFIDA was prepared according to a previously reported procedure [10]. Deionized water was purified using a Merck-Millipore EQG system (Billerica, MA, USA) and was used to prepare all aqueous solutions employed herein. All other reagents and solvents were obtained from Wako Pure Chemicals as LC-grade or the highest purity available, and were used as received.

2.2. Extraction of nucleotides using the Fe(III)-immobilized PFIDA

To an aliquot of the sample solution containing 0.2% trifluoroacetic acid (TFA) (400 μL), was added a 50 mM solution of the Fe(III)-immobilized PFIDA (20 μL), and the resulting mixture was vortex mixed for 10 min at room temperature. After centrifugation at 17,500 × *g* for 10 min, the supernatant (*i.e.*, the non-fluorous phase) was removed, and the sedimented fluorous phase was washed with a 50% aqueous methanol solution containing 0.1% TFA (100 μL). The nucleotides present in the fluorous phase were then back-extracted using a solution of 1% ammonia in 70% aqueous acetonitrile (50 μL) with mixing for 10 min at room temperature. Following subsequent centrifugation at 17,500 × *g* for 10 min, the obtained supernatant (*i.e.*, the non-fluorous phase containing the nucleotides) was directly injected into HILIC-MS/MS system.

2.3. HILIC-MS/MS conditions

The LC separations were performed using a Shimadzu (Kyoto, Japan) Nexera X2 LC system consisting of two LC-30AD pumps, a high-pressure gradient unit, a DGU-20A5R online degasser, an SIL-30AC autosampler, a CTO-20A column oven, and a CBM-20A system controller. An Acquity UPLC® BEH Amide column (100 × 3.0 mm ID, particle size 1.7 μm; Waters, Milford, MA, USA) was also employed. Mobile phase A consisted of an 80:20 (v/v) mixture of acetonitrile and a 250 mM aqueous ammonium bicarbonate solution, while mobile phase B consisted of a 60:40 (v/v) mixture of the same components. The flow rate was maintained at 0.6 mL/min with the following gradient elution conditions: 0–10 min, linear increase from 20 to 30% B; 10–12 min, linear increase from 30 to 60% B, 12–25 min, linear increase from 60 to 100% B, 25–25.01 min, linear decrease from 100 to 20% B; and 25.01–40 min, 20% B. An injection volume of 5 μL and a column oven temperature of 40 °C were also employed. Furthermore, MS/MS analysis was performed in the negative ESI mode using a Shimadzu LCMS-8050 triple quadrupole mass spectrometer, and the operating conditions were as follows: interface voltage, −4.0 kV; interface temperature, 300 °C; nitrogen gas nebulizer flow rate, 3.0 L/min; and nitrogen drying gas flow rate, 10 L/min. Argon gas (purity, > 99.9%) was used for collision-induced dissociation (CID), and the temperature was set at 400 °C. The selected reaction monitoring (SRM) transitions including the collision-induced dissociation energies (CEs) of the target nucleotides are shown in Table 1.

2.4. Method validation

Calibration curves for determination of the nucleotide quantities were obtained from the SRM measured at concentrations of 0.1–10 μM (0.1, 0.5, 1.0, 5.0, and 10 μM). The precision of this method was estimated by analyzing standard solutions (0.5, 1.0, and 10 μM) at three different times on the same day. The limits of detection (LODs) and limits of quantification (LOQ) were defined as the sample concentrations that gave signal-to-noise (S/N) ratios of 3 and 10, respectively. The extraction recoveries were determined by comparing the peak areas of the nucleotides obtained from the analysis of standard solutions (0.5, 1.0, and 10 μM) before and after the extraction process (n = 3).

2.5. Cell cultures and sample preparation

Leukemia Jurkat cells (an HTLV-1-infected T-cell line) were cultured in an RPMI-1640 medium supplemented with a mixed solution of 100 U/mL penicillin-streptomycin-glutamine (Nacalai Tesque, Kyoto, Japan) and 10% heat-inactivated fetal calf serum at 37 °C (5% CO_2). The resulting cells (1.0×10^6 cells) were then harvested and washed twice with ice-cold phosphate buffered saline (PBS). A 0.2% solution of TFA in a mixture of ice-cold methanol (320 μL) and ice-cold water (220 μL) was then added to the residue and mixed for 1 min. Following centrifugation at 17,500 × *g* for 5 min, an aliquot of the supernatant (400 μL) was taken and employed as the sample solution for analysis.

3. Results and discussion

3.1. Optimization of the HILIC-MS/MS conditions

We selected an amide-type HILIC column for analysis of the nucleotides, as this column is stable over a wide pH range, and the negatively charged nucleotides can be retained on the column under the alkaline mobile phase conditions [12]. A number of organic solvents were examined as potential mobile phases, including acetonitrile,

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