Journal of Chromatography A, 1216 (2009) 7488–7492

Contents lists available at ScienceDirect

Journal of Chromatography A

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

Determination of phosphorus using capillary electrophoresis and micro-high-performance liquid chromatography hyphenated with inductively coupled plasma mass spectrometry for the quantification of nucleotides

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ARTICLE INFO

Article history:
Available online 18 May 2009

Keywords:
Nucleotides
Phosphorus
Quantification
Capillary electrophoresis
Micro-high-performance liquid chromatography
Inductively coupled plasma mass spectrometry

ABSTRACT

We performed the quantification of phosphorus in deoxynucleotides using capillary electrophoresis (CE) and micro-HPLC (μHPLC) hyphenated with inductively coupled plasma mass spectrometry (ICP-MS). DNA and its component units have conventionally been determined by photometry; however, more selective and sensitive methods are needed for small biological samples. CE and μHPLC offer the advantages of good separation and small consumption of samples, and ICP-MS is a highly sensitive technique for the determination of a chemical element. Therefore, we have developed an interface device for combining CE and μHPLC with ICP-MS for quantifying nucleotides based on phosphorus content. The interface utilizes 4.5 μL/min for nebulizing and effective introduction of the sample into ICP. The samples of nucleotides and free phosphoric acid were well separated in the CE–ICP-MS measurement, and the calibration curves (1–100 μg/mL) of the nucleotides showed a linear ($R^2 > 0.999$) increase in intensity. Similarly, the samples of nucleotides were baseline separated using μHPLC–ICP-MS, and the calibration curves of the nucleotides were linear ($R^2 > 0.998$). The detection limits of these species and phosphorus in nucleotides using CE–ICP-MS and μHPLC–ICP-MS were 0.77–6.5 ng/mL and 4.0–6.5 ng/mL, respectively. These values were about one or two orders lower than those in a previous report. The sample volumes of these experiments were calculated to be about 10 nL and 50 nL per analysis. Therefore, these analytical methods have the potential to be useful for the determination of biological samples, such as DNA and RNA molecules.

1. Introduction

In recent years, the demand for the quantification of nucleotides and oligonucleotides has increased in many areas, such as food analysis, clinical diagnostics, and molecular biology. Especially, a highly sensitive analytical method is needed to analyze the small volume of real biological samples. DNA is one of the most primitive materials in biological compounds. DNA and its component units have conventionally been determined by the photometric method. However, an accurate method for the quantification of nucleic acids in small complex samples has not been established yet. DNA is composed of deoxynucleotides (dNMPs), which consist of deoxyribose, a base, and phosphate. Therefore, the determination of phosphorus is one of the most effective methods to quantify the nucleotides. The determination of phosphorus can be used for the quantification of macromolecules, such as DNA and/or RNA, due to the fixed stoichiometry of these elements in that molecule. The quantification of a DNA sample using inductively coupled plasma optical emission spectrometry (ICP-OES) on the basis of the phosphorus content of DNA with a high precision of measurement has been reported [1–3]. There are various species of nucleotides, and they need to be discriminated for quantification. However, nucleic acid analysis using ICP-OES cannot discriminate all these species, such as dNMPs. Furthermore, this method requires a large volume of sample material. On the other hand, capillary electrophoresis (CE) and micro-HPLC (μHPLC) offer the advantages of good separation and small consumption of samples, and inductively coupled plasma mass spectrometry (ICP-MS) is a highly sensitive technique for the determination of a chemical element. Some interface devices for CE–ICP-MS [4–10] and HPLC–ICP-MS [11–14] are commercially available. However these devices are difficult to maintain due to the choking and breakage of inner capillaries, the unstable state of nebulizing, and the dilution of the sample with a large amount of sheath liquid.

Therefore, we have developed a novel interface device for combining CE and/or μHPLC hyphenated with ICP-MS, as reported previously [15,16]. This interface facilitates easy maintenance for

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doi:10.1016/j.chroma.2009.05.019

choking and capillary breakage as well as sensitive determination of many elements using an ultra-low consumption nebulizer and a drainless mini-size chamber. In the present article, we applied the interface device for the determination of phosphorus using CE and μHPLC hyphenated with ICP-MS for the quantification of nucleotides.

2. Experimental

2.1. Chemical reagents

The free acid forms of deoxyadenosine 5′-monophosphate (dAMP), deoxyguanosine 5′-monophosphate (dGMP), deoxycytidine 5′-monophosphate (dCMP), and thymidine 5′-monophosphate (dTMP) were purchased from Sigma–Aldrich Japan (Tokyo, Japan) at >98% purity. The nitric acid solution was adjusted to 0.1% by dissolving an appropriate amount in ultra-pure water. Potassium hydroxide (0.5%) was prepared by dissolving potassium hydroxide in methanol. A phosphate ion standard solution (Kanto, Tokyo, Japan), assured by the Japan Calibration Service System (JCSS), was used as the phosphorus calibration solution. Selenium, cesium and cerium were purchased from Kanto (Tokyo, Japan). Cetyltrimethylammonium bromide (CTAB) and ammonium acetate were purchased from Wako (Osaka, Japan). Citric acid and γ-aminobutyric acid (GABA) were purchased from Sigma–Aldrich Japan.

2.2. Apparatus

Images of the apparatus for the interface device are shown in Fig. 1. This nebulizer consisted of a three-layered nebulizer (I.D.: 200 μm, back pressure: 40 psi (Ar: 1 L/min), costum-designed in Glass Expansion, West Melbourne, Australia) and a low-volume (10 mL, I.D.: 16 mm, O.D.: 19 mm) vaporizing chamber (custom-designed in Glass Expansion; Fig. 1a). The nebulizer capillary (I.D.: 75 μm for CE and 50 μm for μHPLC) and nebulizer were connected with a polymer-based fitting. The nebulizer system with a nano-liter scale injector, C4-0344-.05 (Valco, TX, USA), was used for the flow injection analysis (FIA)–ICP-MS experiment. An Agilent 7500a (Agilent Technologies, CA, USA) ICP-MS system was used for the FIA–ICP-MS experiment. The operation condition of the ICP-MS system was as reported in Table 1. The interface device for CE–ICP-MS consisted of the same nebulizer and the vaporizing chamber reported in FIA–ICP-MS and was equipped with an electrode connector with a sheath solution (laboratory-made system, Fig. 1b). The electrophoresis capillary was connected with this cross connector. The sheath solution was flowed using natural drawing to obtain stable electrophoretic conditions and minimize the introduction of the

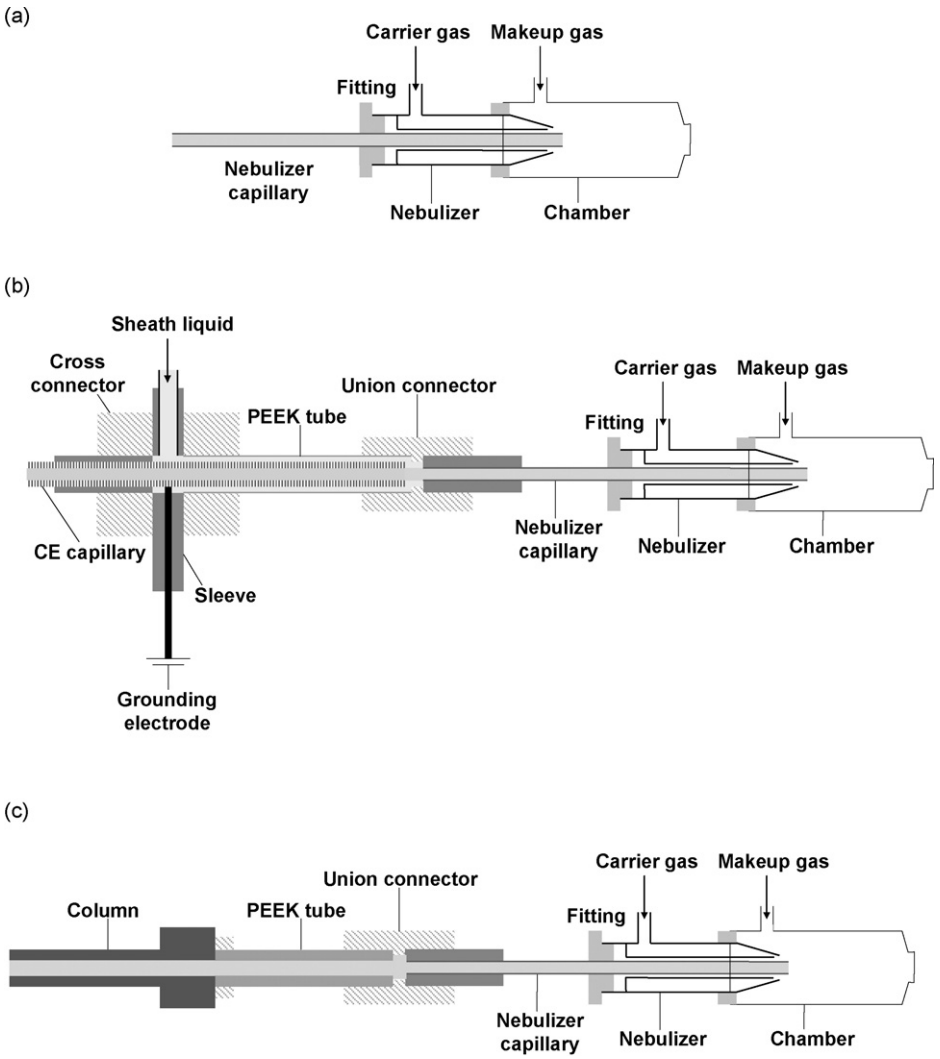

Fig. 1. Schematic images of the nebulizer and interface device. (a) Three-layered nebulizer and low-volume chamber system; (b) interface device for CE–ICP-MS; (c) interface device for μHPLC–ICP-MS.

Table 1
Instrumental and operating conditions of ICP-MS.

ICP-MS system	Agilent 7500a
Plasma conditions	
Incident Rf power	1.5 kW
Reflected power	<1 W
Outer gas flow rate	Ar 15 L/min
Intermediate gas flow rate	Ar 0.9 L/min
Carrier gas flow rate	Ar 1.00 L/min
Make-up gas flow rate	Ar 0.25 L/min
Sampling depth	7 mm from work coil
Data acquisition	
Scanning mode	Peak hopping
Data points	1 points/peak
Dwell time	10 ms/point
Measured m/z	^{31}P, ^{82}Se, ^{133}Cs, ^{140}Ce

sheath flow into the plasma. The interface device for μHPLC–ICP-MS is shown in Fig. 1c. This interface device for μHPLC–ICP-MS consisted of the same nebulizer and vaporizing chamber as for FIA–ICP-MS and was simply connected with a column without an electrode connector.

2.3. Separation condition for CE–ICP-MS

The instrumental set-up in all experiments for CE–ICP-MS consisted of a P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Tokyo, Japan) attached with a fused silica capillary (I.D.: 50 μm, O.D.: 375 μm; GL Science, Tokyo, Japan) and Agilent 7500a ICP-MS. Both instruments were connected *via* the interface device. A 0.1% HNO_3 solution was used as the sheath liquid. A spike of 5 μg/mL of Se was used as the internal standard in the sample solution, and 0.1 μg/mL of Cs in the sheath solution was used as the monitoring material to observe the stability of the nebulization during all experiments. Before use, the capillary was pre-conditioned by washing with 0.5% of KOH in methanol for 3 min and 0.1 M NaOH for 1 min followed by a flushing period of 1 min with ultra-pure water. After each run, the capillary was washed with 0.1 M NaOH (3 min, 20 psi), water (3 min, 20 psi), and an electrophoresis buffer (3 min, 20 psi). The electrophoresis eluent consisted of 5 mM of CTAB and 5 mM of a citric acid buffer (pH 3.3 adjusted by GABA). The sample was injected with 0.5 psi for 10 s with pressure. The separation mode was micellar electrokinetic chromatography (MEKC) in the reverse mode, and the applied voltage was set at 30 kV. The operation condition of the ICP-MS system was as mentioned in Table 1. The samples containing dNMPs (dAMP, dTMP, dGMP, and dCMP) were analyzed using CE–ICP-MS and calibrated using a standard phosphate solution.

2.4. Separation condition of μHPLC–ICP-MS

An LC10-AD (Shimadzu, Kyoto, Japan) system using the interface with the nL scale injector was used for μHPLC–ICP-MS experiment. For nucleotide separation, a Develosil C30AQ (150 mm × 0.3 mm, 3 μm; Nomura, Aichi, Japan) column was used. The eluent consisted of 20 mM CH_3COONH_4 and MeOH (95:5 (v/v), pH 8.6). The flow rate was set to 3 μL/min in all experiments. The injection volume was set to 50 nL. The chromatographic system was connected with the interface device without an electrode connector. The operation condition of the ICP-MS system was as mentioned in Table 1. The samples containing dNMPs (dAMP, dTMP, dGMP, and dCMP) were analyzed using μHPLC–ICP-MS and calibrated with a standard phosphate solution.

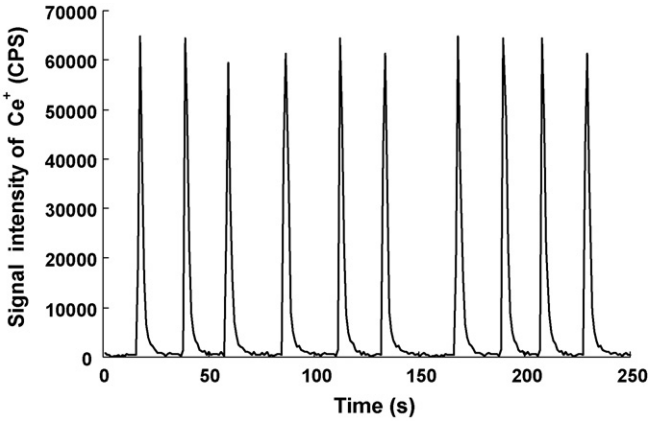

Fig. 2. Result of Ce measurement with FIA–ICP-MS. Standard solution of Ce (3 ng/mL) measured on the mass of ^{140}Ce for the comparison of the measurement time.

2.5. Sample preparation

A different dNMP stock solution (about 1 g/L) was gravimetrically prepared by dissolving an appropriate amount of deoxyribonucleotide except dAMP in ultra-pure water. Tris–HCl (10 mM, pH 8.0) was used to dissolve dAMP due to its poor solubility in pure water. Each test solution was prepared by diluting the stock solution using ultra-pure water. The determination of water content of the dNMPs was performed using a Karl Fisher titrator (Mitsubishi Chemical, Tokyo, Japan). The water content measured from Karl Fisher titration are 7.6% for dAMP, 1.6% for dTMP, 5.8% for dGMP and 2.6% for dCMP, respectively. When the stock solution was made, these values were considered.

3. Results and discussion

3.1. Instrumental conditions

To evaluate the reproducibility of the interface device coupled with ICP-MS, FIA–ICP-MS was performed. The flow rate of the sheath solution was 4.5 μL/min. A cerium solution (3 ng/mL) was prepared and injected 10 times. Each injection volume was set to 50 nL. The result of the Ce measurement with FIA–ICP-MS is shown in Fig. 2. The repeatability of the peak area was *ca.* 1% (n = 10), and the half width was 1.1 ± 0.1 s (n = 10). The flow rate in a conventional nebulizer is required to be ~0.7–1 mL/min. The flow rate became 1/200 or less using this nebulizer system, realizing highly efficient

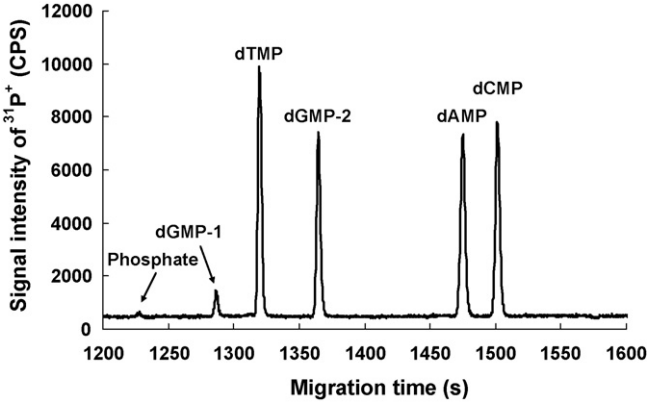

Fig. 3. Electropherogram of dNMPs analysis in CE–ICP-MS. Mixture of dAMP, dTMP, dGMP, and dCMP (25 mg/L of each species) measured on the mass of ^{31}P for the comparison of the migration time.

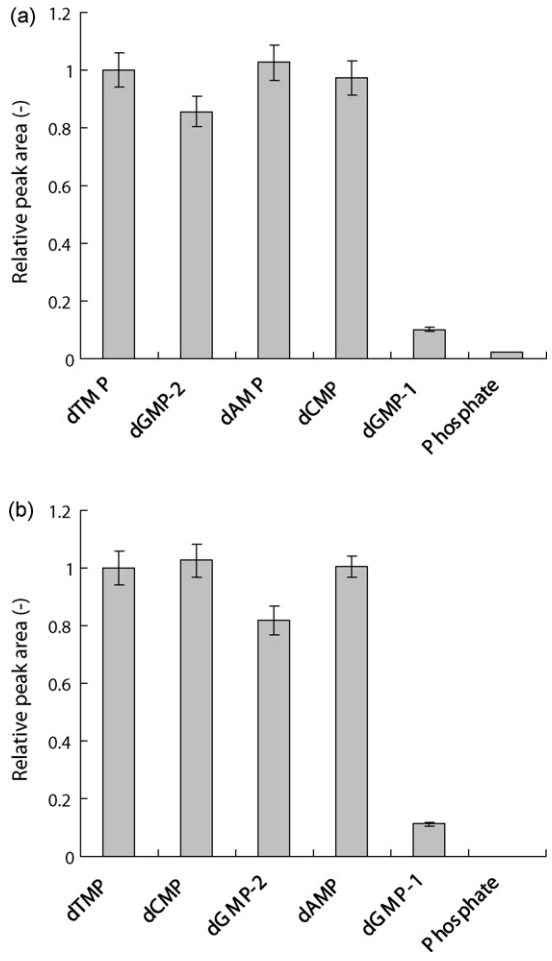

Fig. 4. Relative value of each peak area. (a) CE–ICP-MS measurement; (b) μHPLC–ICP-MS measurement.

nebulizing. Moreover, the fitting in the nebulizer allowed for easy adjustment of the top position of the nebulizer capillary. In short, the regulation of the introductory volume (1–10 μL/min) and easy replacement of the inner capillary were realized.

3.2. dNMP measurement using CE–ICP-MS

A CE–ICP-MS experiment using the interface device was performed to investigate the separation efficiency of CE and its possible application to phosphorus determination for the quantification of nucleotides. The CE capillary was connected using a cross connector, where the sheath liquid and grounding electrode were combined. The same nebulizer and low-volume chamber as for the FIA–ICP-MS were adopted in this experiment. The interface achieved nebulizing at 4.5 μL/min and effective introduction of the sample to ICP. Using ICP-MS, the following elements were measured in this experiment: ^{31}P, ^{82}Se, and ^{133}Cs.

The electropherogram of the dNMP analysis monitoring ^{31}P in CE–ICP-MS is shown in Fig. 3. The sample was separated with an eluent consisting of 5 mM of CTAB and 5 mM of citric acid buffer (pH 3.3 adjusted by GABA) using the MEKC reverse mode at 30 kV. No influence of the detergent in the eluent for the detection in ICP-MS was observed. In this experiment, a 0.1% HNO_3 solution was used as the sheath liquid. The influence of the sheath liquid for the measurement of mass ^{31}P was not observed. When more concentrate HNO_3 solution (e.g. 1%) was used, the interferences caused by polyatomic ion (NO^+, NOH^+) on mass ^{31}P were observed (data not shown). Although the peak of dGMP had two peaks (dGMP-1 and dGMP-2), the samples of dNMPs and free phosphoric acid were separated at the baseline within approximately 25 min. As can be seen in Fig. 3, these peaks in the electropherogram were assigned to dAMP, dTMP, dGMP, and dCMP by comparing their migration times. The RSD of the migration time of the phosphorus analysis was 3.98% (n = 3). Fig. 4a shows the relative value of each peak area in the CE–ICP-MS measurement of phosphorus in each species. Each relative peak area (in dGMP, the peaks of dGMP-1 and dGMP-2 were combined) was 0.96–1.03, while that of dTMP was 1. The relationships of the calculated concentration calibrated with the phosphate standard and the concentration from the mass fraction of the dNMPs were 0.81–1.06. Therefore, the concentration of nucleotides could be obtained from the measured value of phosphorus. The calibration curves (1–100 μg/mL) of dNMPs show linearity (R^2 > 0.999) increasing in intensity. The detection limit of each species, phosphorus in dNMP and absolute phosphorus, is shown in Table 2. These values were one or two orders lower than those in a previous report [12]. The detection limit and absolute detection limit of phosphorus were 0.87 μg/L and 0.009 pg, respectively. The flow rate and the volume of the chamber in the previous report using CEI-100 were 8 μL/min and 5 mL, respectively. Therefore, it is likely that the dilution of the sample and collision of the sample to the chamber wall were controlled in the measurement using the new interface. Moreover, the three-layered nebulizer performed highly efficient nebulizing. It might be expected, therefore, that these factors lead to the improvement of sensitivity. The sample volume of this experiment was calculated to be approximately 10 nL per analysis, demonstrating that this analytical method is effective for the samples of small volume.

3.3. dNMP measurement using μHPLC–ICP-MS

The interface device was used for the determination of dNMPs with μHPLC–ICP-MS. The same nebulizer and low-volume chamber as for the FIA–ICP-MS were adopted in this experiment. The nebulizer was simply connected with a column *via* a union connector without an electrode or a sheath solution. The elements of ^{31}P and ^{82}Se were measured using ICP-MS in this experiment.

The chromatogram of dNMP analysis with μHPLC–ICP-MS is shown in Fig. 5. The sample was separated with an eluent consisting of 20 mM CH_3COONH_4 and MeOH (95:5 (v/v), pH 8.6) using a Develosil C30AQ column. Although the peak of dGMP had two peaks (dGMP-1 and dGMP-2), as in the CE–ICP-MS measurement, the samples of dNMPs were separated at the baseline within about

Table 2
Detection limit of dNMP and phosphorus in CE–ICP-MS and μHPLC–ICP-MS analysis.

Species	CE–ICP-MS Detection limit			μHPLC–ICP-MS Detection limit		
	Species (ng/mL)	P in dNMP (ng/mL)	P absolute (pg)	Species (ng/mL)	P in dNMP (ng/mL)	P absolute (pg)
dAMP	47	4.4	0.044	45	4.3	0.22
dTMP	8	0.77	0.008	41	4	0.2
dGMP	73	6.5	0.065	72	6.5	0.32
dCMP	18	1.8	0.018	43	4.3	0.22

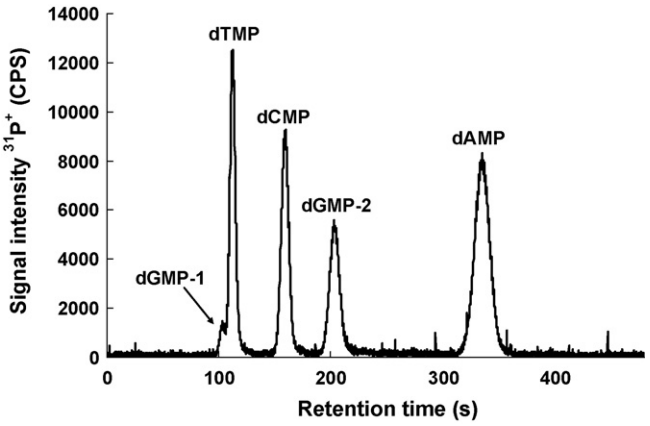

Fig. 5. Chromatogram of dNMP analysis with μHPLC–ICP-MS. Mixture of dAMP, dTMP, dGMP, and dCMP measured on the mass of ^{31}P for the comparison of the retention time.

7 min. As can be seen in Fig. 5, these peaks in the chromatogram were assigned to dAMP, dTMP, dGMP, and dCMP by comparing their retention times. The RSD of the retention time of the dNMP analysis was 1.0–1.9% ($n = 4$). Fig. 4b shows the relative value of the each peak area in μHPLC–ICP-MS measurement of phosphorus in each species. Each relative peak area (dGMP was combined to the peak of dGMP-1 and dGMP-2) was 0.93–1.03 compared with dTMP as 1. And the relationships of the calculated concentration calibrated with phosphate standard and the concentration from the mass fraction of the dNMPs were 0.90–1.21. Therefore, it was enabled to obtain the concentration of nucleotide from the measured value of phosphorus. The calibration curves (1–100 μg/mL) of dNMPs show a linearity ($R^2 > 0.998$) increasing in intensity. The detection limit of species, phosphorus in dNMP and absolute phosphorus were shown in Table 2. The detection limit and absolute detection limit of phosphorus were 0.24 μg/L and 0.012 pg, respectively. These values were one or two orders lower than those in a previous report [12]. The detection limit using μHPLC–ICP-MS was the same as that in the CE–ICP-MS experiment, which showed the possibility to apply the interface device to the HPLC system. Moreover, the detection limit of phosphorus in μHPLC–ICP-MS was better than that in a previous report using a He collision instrument. For large amounts of a sample, the μHPLC–ICP-MS equipped with this interface device is an effective method for their determination because of the interface of this extremely simple device.

4. Conclusion

We have achieved the separation and quantification of phosphorus in dNMPs using CE and μHPLC hyphenated with ICP-MS. The developed interface for the CE and/or HPLC connected with ICP-MS effectively introduced the sample to the ICP. Four kinds of dNMPs were separated at the baseline with either CE or μHPLC and detected as phosphorus in ICP-MS. These analytical results were better than those in a previous report; in particular, the detection limit of nucleotides in CE–ICP-MS was one or two orders lower that in the previous report. On the other hand, the μHPLC–ICP-MS works well for the determination of these samples, since these instruments could be connected more easily using the interface device than the CE–ICP-MS. These devices of separation can be selected by the purpose of analysis, sample volume, sensitivity, and so on. This analytical method of phosphorus in dNMPs has the potential to determine the accurate concentration of the DNA and/or RNA molecule.

Acknowledgment

Part of this study was sponsored by the New Energy and Industrial Technology Development Organization (NEDO).

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