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Spectrofluorimetric Determination of Bisphosphonates in Biological Sample with a Fluorescent Chemosensor, NadDPA-2Zn^{2+}

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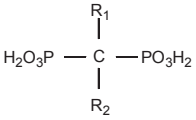
The accurate determination of bisphosphonate levels in bone and biological fluids is important in both clinical and pharmacological/toxicological studies; however, the quantitative analysis of the bisphosphonate is difficult because its concentration is quite low in most of biological sample. A novel fluorescent chemosensor (FCS)-based measurement method of bisphosphaonate levels using Naphta-diDPA-2Zn^{2+} (NadDPA-2Zn^{2+}, DPA = dipycolylamine), an excellent FCS previously used for detecting PPi, was developed. By the FCS method, the concentration of bisphosphonates having no fluorophores can be determined analyzed with sufficient sensitivity. The results of this study indicate that the FCS-based measurement can be a useful method to analyze bisphosphonates in biological samples.

Key Words: Chemosensor, Fluorescent chemosensor, Bisphosphonate, Alendronate, Risedronate

Introduction

Because of their inhibitory effect on bone resorption, various types of bisphosphonates (Fig. 1) are used in the treatment of bone metastasis and several bone disorders such as Paget's disease, and for the prevention of osteoporosis in postmenopausal women.[1] Several administration methods of bisphosphonates are available, but the absorption rate is quite low; The absorption rate in oral administration ranges from 0.5% to 1%.[2,20] Moreover, because the concentration of bisposphonates in therapeutic range is very low in case of intravenous or transdermal administration, the quantitative estimation of bisphosphonates is quite complicated. For approximately 30 years, researchers have been attempting to develop various sensitive analytical methods that can detect low concentrations of bisphosphonates in blood.



Name of Bisphosphonate	R_1	R_2
Alendronate	$-CH_2CH_2CH_2NH_2$	-OH
Clodronate	-Cl	-Cl
Etidronate	$-CH_3$	-OH
Ibandronate	$-CH_2CH_2N(CH_3)(CH_2CH_2CH_2CH_2CH_3)$	-OH
Neridronate	$-CH_2CH_2CH_2CH_2CH_2NH_2$	-OH
Olpadronate	$-CH_2CH_2N(CH_3)_2$	-OH
Pamidronate	$-CH_2CH_2NH_2$	-OH
Risedronate	$-CH_2$-(3-pyridyl)	-OH
Tiludronate	-S-(4-chlorophenyl)	-H
Zoledronate	$-CH_2$-(imidazol-1-yl)	-OH

Figure 1. Various bisphosphonates which are medically used for osteoporosis therapy.

Liquid chromatography (LC) is the most frequently used method for bisphoshponate analysis.[3,4,5,6,21] However, alendronate, pamidronate, and neridronate cannot be analyzed using ultraviolet/visible (UV-vis) and fluorescence detector because these compounds do not contain any strong chromophore; before analysis, they must be modified with derivatizing agents such as 9-fluorenylmethyl chloroformate (FMOC), naphthalene-2,3-dicarboxaldehyde (NDA), fluorescamine, or *N*-isobutoxycarbonyl methyl ester to introduce the strong chromophore or fluorophore.[7,8,9,10] Strong absorption of UV can also be obtained by the chromophoric complexation between the bisphosphonates and Cu(II)/Fe(III) cations.[11,12] Even though other analytical techniques including liquid chromatography with tandem mass spectrometry (LC/MS/MS), capillary electrophoresis (CE), and gas chromatography (GC) with an improved limit of detection (LOD) or limit of quantitation (LOQ) have also been developed,[10,13,14] they all require additional complicate procedures such as derivatization with chromophores or careful control of chromatography conditions.

Meanwhile, there have been many studies that have used fluorescent chemosensors (FCSs) for the recognition of certain molecules or ions. Among them, NadDPA-2Zn^{2+} was used for the determination of pyrophosphate (PPi) concentration, the signaling molecule which is resulted from the decomposition from adenosine triphosphate (ATP) to adenosine monophosphate (AMP). The NadDPA-2Zn^{2+} (host)-PPi (guest) complexes exhibit strong fluorescence and allow highly sensitive quantitative/qualitative analysis using a spectrofluorometer. Because of the structural similarity between the P-C-P frame of the bis-

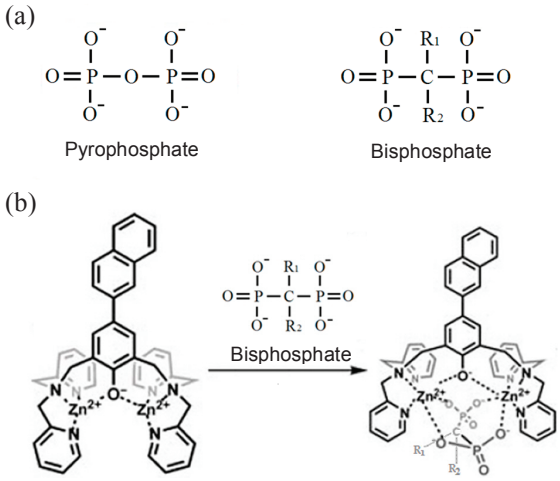


Figure 2. (a) Structural similarity between pyrophosphate (PPi) and bisphosphonate and (b) schematic illustration of the formation of FCS (NadDPA-2Zn^{2+})-bisphosphonate complex.

phosphonate and the P-O-P frame of PPi (Fig. 2a), the 4 oxygens of bisphosphonates were expected to bind to the FCS with the same coordination. Possibly, the association constant (K_a) of NadDPA-2Zn^{2+}-bisphosphonate complex is similar to that of NadDPA-2Zn^{2+}-PPi complex, which is approximately 10^8.[18]

The aim of this study was to confirm if NadDPA-2Zn^{2+} FCS can be used to detect bisphosphonates in a simple assay. Alendronate and risedronate, two important bisphosphonate medicines for the treatment of osteoporosis, were selected as the test drugs. Because alendronate has an aliphatic backbone but risedronate has an aromatic one, the potential of the FCS method for general bisphosphonate drugs could be confirmed by the analysis of both drugs.

This method has been successfully validated in the terms of stoichiometry, linearity, accuracy, precision, and sensitivity. When compared to other reported methods for analyzing bisphosphonates, our method showed sufficient sensitivity without any time-consuming procedures.

Experimental

Reagents and materials. Alendronate (sodium salt) and risedronate (risedronic acid) were purchased from Langfang Shinya Chemical Co., Ltd., China, and 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethane sulfonic acid (HEPES) buffer solution (pH 7.0 - 7.5; 1 M in water), tetrachlroacetic acid, sodium hydroxide (powder), and calcium chloride were obtained from Sigma-Aldrich (USA). NadDPA-2Zn^{2+} was used as a fluorescent chemosensor.

Instrument & data-processing tools. Fluorescence detection was performed using a spectrofluorometer (FT-650; Jasco, Japan) and all samples were dispensed in cuvettes (No. 1916, Italy). PC softwares for data processing were Sigma plot (ver. 8.0) and MS excel 2007.

Determination of stoichiometry (Job's plot). We prepared stock solutions A (50 μM bisphosphonate/HEPES buffer; pH 7.4; 0.01 M) and B (50 μM NadDPA-2Zn^{2+}/HEPES buffer; pH 7.4; 0.01 M). The two solutions were mixed in the following ratios (by volume, A:B); 0:10, 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1, and 10:0 (final volume: 2 mL). All samples were analyzed using a spectrofluorometer (FT-650; Jasco, Japan) with an excitation and emission wavelengths of 316 nm and 460 nm, respectively.

Preparation of calibration curve and determination of LOD and LOQ of organic samples. We prepared stock solutions C (1 mg/mL bisphosphonate, 10 μM NadDPA-2Zn^{2+}/HEPES buffer; pH 7.4; 0.01 M) and D (10 μM NadDPA-2Zn^{2+}/HEPES buffer; pH 7.4; 0.01 M). The standards in the concentration range of 0.01-2.5 μg/mL were prepared by mixing of two solutions. The fluorescence of each solution was analyzed using a spectrofluorometer. The results were used to construct a calibration curve and to confirm the LOD and LOQ values.

Accuracy & precision. The alendronate-plasma and risedronate-plasma samples (0.5 μg/mL and 2 μg/mL, respectively) were analyzed to confirm the accuracy and precision of the FSC method. Plasma fraction was obtained from the supernatant after centrifugation (6,300 × g, 10 min) of the blood collected from a Sprague-Dawley rat (male, 6 ~ 8 weeks; weight, 230 ~ 250 g). Bisphosphonate-plasma samples were prepared by adding 5 μL of bisphosphonate solution into 145 μL of rat plasma to achieve the alendronate or risedronate concentrations of 0.500 μg/mL and 2.000 μg/mL.

Next, proteins in each sample were removed by adding 450 μL of 10% (w/v) trichloroacetic acid (TCA) to each sample and vortexing the samples for 3 min; subsequently, the samples were centrifuged for 15 min at 6,300 × g at room temperature. The supernatant was transferred to another tube, and 80 μL of 1.25 M $CaCl_2$ and 200 μL of 30% (w/v) NaOH were added to the supernatant to induce precipitation. The sample was vortexed for 30 s and then centrifugated for 15 min at 6,300 × g. The supernatant was discarded and the pellet was dissolved in 0.8 mL of 0.5 M HCl; this solution was heated at 90 ^{o}C for 30 min to hydrolyze pyrophosphates in the plasma.[6] Further, 50 μL solution of 1.25 M $CaCl_2$ and 90 μL of 30% NaOH were added and the samples were centrifuged for 15 min at 6,300 × g. The white precipitate was washed 3 times with water and dissolved in 0.15 mL of 0.5 M EDTA-Na solution and 0.85 mL of HEPES buffer (pH 7.4; 0.01 M).

For the fluorescence detection, a 2 μL aliquot of 5 mM NadDPA-2Zn^{2+}/HEPES buffer (pH 7.4; 0.01 M) was added to the sample prepared above. The concentration of NadDPA-2Zn^{2+} in the sample was approximately 10 μM. The fluorescent emission was measured at 460 nm. A negative control in HEPES buffer (pH 7.4; 0.01M) instead of rat plasma was also analyzed with the same procedure as above.

Results and Discussion

The stoichiometric ratio of FCS (NadDPA-2Zn^{2+}) and bisphosphonate (alendronate or risedronate) in the complexes with different mole fractions of bisphosphonate was estimated by using Job's method of continuous variation. Job's plots of FCS against alendronate and risedronate (Fig. 3) showed the highest value at a mole fraction of 0.5, which confirmed that the ratio between FCS and bisphosphonate in the complex was 1:1. Therefore, we assumed that a bisphosphonate molecule can bind with a NadDPA-2Zn^{2+} molecule in the same manner with the PPi-

NadDPA-2Zn^{2+} complex. A slight asymmetry in the Job's plot in the risedronate-NadDPA-2Zn^{2+} complex formation is probably due to the steric effect of hydrophobic pyridine ring in risedronate molecule.

From the calibration curve of the fluorescence, linearity, LOD/LOQ values, accuracy (recovery), and precision of the FCS-based analysis of bisophosphonates can be checked. A linear relationship between fluorescence emission and alendronate (or risedronate) concentration was obtained over a concentration range of 0.01 ~ 2.5 μg/mL. As shown in Table 1, R^2 values are 0.9974 (alendronate) and 0.9965 (risedronate), respectively. LOD, defined as the concentration at the signal-to-noise ratio of 3:1, was 0.031 μg/mL for alendronate and 0.027 μg/mL for risedronate. LOQ, the concentration at the signal-to-noise ratio of 10:1, was 0.104 μg/mL for alendronate and 0.090 μg/mL for risedronate. In each determination, the noise value was determined from the standard deviation of blank solution (10 μM NadDPA-2Zn^{2+} only).

As commented in the introduction, significant amount of PPi is produced during the decomposition from ATP to AMP, and PPi can be a potential competitor against bisphosphonate during formation of complex with NadDPA-2Zn^{2+}. This can cause a considerable deviation on the accurate determination of bisphosphonate concentration in biological samples. We can solve this problem by a solid phase extraction (SPE) process and following thermal hydrolysis of pyrophosphate during the pretreatment of biological samples.

The accuracy and precision of this method was confirmed by the comparison of the fluorescence in HEPES buffer and rat plasma. Table 2 summarizes the results as the average of 9 independent experiments. The recovered concentration of bisphosphonates after the SPE process and thermal hydrolysis is expressed as Found value. Comparing with the original concentration (Mixed value), the recovery percentage can be calculated. All the recovery rates were in the range of 97.8% - 100.8%, and the relative standard deviations (RSDs) of the recovery rate were in the range of 1.1% - 2.6%. Therefore, our method has enough reliability and reproducibility to be utilized for a detailed analysis of biological samples.

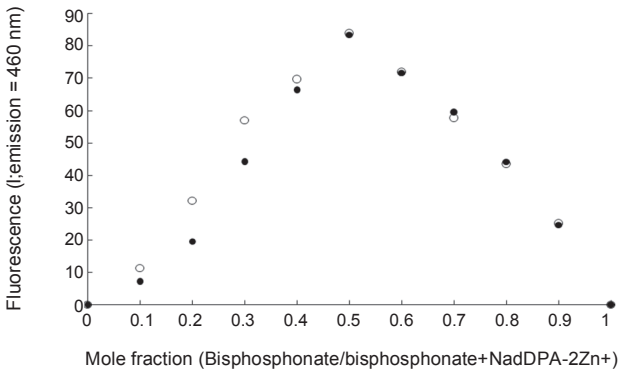


Figure 3. Job's plots for bisphosphonate-NadDPA-2Zn^{2+} complex (○ : alendronate, ● : risedronate) dissolved in HEPES (pH 7.4; 0.01 M) (Excitation/Emission = 316 nm/460 nm).

Conclusion

We developed a new analytical method for detecting bisphosphonates by using a FCS (NadDPA-2Zn^{2+}) as the molecular recognizer. The sensitivity, accuracy, and precision of this method are sufficient to analyze bisphosphonate concentration in plasma sample. Considering the rapid and easy analytical procedure, the NadDPA-2Zn^{2+} chemosensor can be a useful tool for the determination of bisphosphonate concentrations in biological sample such as blood. The chemosensor-based analysis can be applied for both clinical and pharmacological/toxicological purposes. Further studies using other bisphosphonates such as pamidronate, ibandronate, and etidronate as guests and other fluorescent chemosensors as hosts are required to ensure that the chemosensor-based method is appropriate for the analysis of general bisphosphonates.

Table 1. Linearity, range, LOD, and LOQ. The equations of the calibration curve were obtained from 9 points. LOD and LOQ were defined as the concentrations of at 3 × S/N and 10 × S/N ratios, respectively

Bisphosphonate	Line equation	R^2	Range (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)
Alendronate	y = 249.20 X + 93.256	0.9974	0.01 ~ 2.5	0.031	0.104
Risedronate	y = 295.61 X + 75.669	0.9965	0.01 ~ 2.5	0.027	0.090

Table 2. Accuracy and precision of FCS (NadDPA-2Zn^{2+}) for alendronate and risedronate (RSD = Deviation/Average × 100)

Sample (n = 9)	Mixed (μg/mL)	Found (μg/mL)	Recovery (%)	RSD (%)	Mixing solution
Alendronate	0.500	0.499	99.8	1.829	HEPES buffer
	2.000	2.001	100.0	1.101	HEPES buffer
	0.500	0.504	101	2.243	Plasma
	2.000	1.972	98.60	1.621	Plasma
Risedronate	0.500	0.498	99.6	1.899	HEPES buffer
	2.000	2.000	100.0	1.190	HEPES buffer
	0.500	0.489	97.8	2.622	Plasma
	2.000	1.978	98.90	1.839	Plasma

References

1. Shaw, N. J.; Bishop, N. J. *Arch. Dis. Child.* **2005**, *90*, 494.
2. Cremers, S. C. L. M.; van Hogezand, R.; Bänffer, D.; Hartigh, J. d.; Vermeij, P.; Papapoulos, S. E.; Hamdy, N. A. T. *Osteoporos Int.* **2005**, *16*, 1727.
3. Raoa, B. M.; Srinivasua, M. K.; Rania, Ch. P.; Kumara, S. S.; Kumarb, P. R.; Chandrasekharc, K. B.; Veerender, M. *J. Pharm. Biomed. Anal.* **2005**, *39*, 781.
4. Tsai, E. W.; Chamberlin, S. D.; Forsyth, R. J.; Bell, C.; Ip, D. P.; Brooks, M. A. *J. Pharm. Biomed. Anal*. **1994**, *12*, 983.
5. Quitasol, J.; Krastins, L. *J. Chromatogr. A* **1994**, *671*, 273.
6. Jia, H.-J.; Li, W.; Zhao, K. *Anal. Chim. Acta* **2006**, *562*, 171.
7. De Marco, J. D.; Biffar, S. E.; Reed, D. G.; Brooks, M. A. *J. Pharm. Biomed. Anal*. **1989**, *7*, 1719.
8. Kline, W. F.; Matuszewski, B. K. *J. Chromatogr*. **1992**, *583*, 183.
9. Wong, J. A.; Renton, K. W.; Crocker, J. F. S.; O'Regan, P. A.; Acott, P. D. *Biomed. Chromatogr*. **2004**, *18*, 98.
10. Saktyama, N.; Kataokab, H.; Makita, M. *J. Chromatogr. A* **1996**, *724*, 279.
11. Ostovic, D.; Stelmach, C.; Hulshizer, B. *Pharm. Res*. **1993**, *10*(3), 470.
12. Kuljanin, J.; Jankovic, I.; Nedeljkovic, J.; Prstojevic, D.; Marinkovic, V. *J. Pharm. Biomed. Anal*. **2002**, *28*, 1215.
13. Zhu, L. S.; Lapko, V. N.; Leey, J. W.; Basir, Y. J.; Kafonek, C.; Olsen, R.; Briscoe, C. *Rapid Commun. Mass Spectrom*. **2006**, *20*, 3421.
14. Bertinatto Rodriguez, J. A.; Desimone, M. F.; Iglesias, S. L.; Giogieri, S. A.; Diaz, L. E. *J. Pharm. Biomed. Anal*. **2007**, *44*, 305.
15. Kim, S. K.; Lee, D. H.; Hong, J.-I.; Yoon, J. *Acc. Chem. Res*. **2009**, *42*, 23.
16. Kim, S. K.; Seo, D.; Han, S. J.; Son, G.; Lee, I. J.; Lee, C.; Lee, K. D.; Yoon, J. *Tetrahedron* **2008**, *64*, 6402.
17. Lee, D. H.; Kim, s. Y.; Hong, J.-I. *Tetrahedron Lett*. **2007**, *48*, 4477.
18. Lee, D. H.; Kim, s. Y.; Hong, J.-I. *Angew. Chem., Int. Ed.* **2004**, *43*, 4881.
19. Connors, K. A. *Binding Constants-The Measurement of Molecular Complex Stability*; Wiley press: New York, 1987; pp175-183.
20. Ezra, A.; Hoffman, A.; Breuer, E.; Alferiev, I. S.; Mönkkönen, J.; Hanany-Rozen, N. E.; Weiss, G.; Stepensky, D.; Gati, I.; Cohen, H.; Törmälehto, S.; Amidon, G. L.; Golomb, G. *J. Med. Chem*. **2000**, *43*, 3641.
21. Al Deeb, S. K.; Hamdan, I. I.; Al Najjar, S. M. *Talanta* **2004**, *64*, 690.