Highly Sensitive Fluorescence Detection of Daptomycin in Murine Samples through Derivatization with 2,3-Naphthalenedialdehyde

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Abstract

A highly sensitive high-performance liquid chromatography method has been developed using pre-column fluorescent derivatization of daptomycin (DAP) through cyclization of the amino group of ornithine with 2,3-naphthalenedialdehyde. With the proposed method, the limits of detection and quantification of DAP in murine serum were 8 and 3 nmol/L, respectively, and the calibration curve was linear across the examined dynamic range from 8 nmol/L to 1 μmol/L ($n = 8$, $r = 0.9986$). This method is suitable for animal experiments examining the side effects of DAP therapy using mice as a simple method with quantification to the order of 10 nmol/L.

**Keywords:** Daptomycin, fluorescent derivatization, 2,3-naphthalenedialdehyde, high-performance liquid chromatography, mice samples.
Introduction

Daptomycin (DAP) is a lipopeptide antibiotic derived from *Streptomyces roseosporus* that exhibits rapid and concentration-dependent bactericidal activity against gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus*.\(^1\) It is well recognized that the characteristic toxicity of DAP therapy is caused by myopathies,\(^2\)\(^-\)\(^4\) appears specifically on skeletal muscle,\(^2\) and is induced by an overdose\(^2\) or short-interval administration of DAP.\(^3\) As can be seen in a few case reports, there is concern about rhabdomyolysis as a serious side effect of DAP therapy.\(^5\)\(^-\)\(^7\) However, few factors that cause the side effects have been identified.\(^8\) Thus, to increase the effectiveness of DAP treatment, a method is required to quantify DAP with high sensitivity and accuracy in biological samples such as serum and skeletal muscle.

Analytical studies on the quantification of DAP have been conducted to develop a method that uses high-performance liquid chromatography (HPLC) and ultra-HPLC with ultra-violet (UV),\(^9\)\(^,\)\(^10\) mass spectrometry (LC-MS),\(^11\) and tandem MS (MS/MS) detection\(^12\)\(^,\)\(^13\) for the determination of DAP in plasma and serum. The UV method is not considered reliably sensitive, neither is it effective in experiments where trace analysis is required. We confirmed this in our attempt to determine the amount of DAP in murine serum and muscle samples collected 24 hours after administration in an experiment conducted on the retention of DAP in skeletal muscle.\(^14\)\(^,\)\(^15\) No peaks corresponding to DAP were observed on chromatograms of the serum and muscle samples using conventional UV detection due to lack of sensitivity of the detection method. In addition, although detection using MS is much more sensitive in general, there is a problem with the ionization suppression of biological samples influenced by a matrix effect, and with formation of polyvalent ions due to the ionization of polypeptides.\(^11\) A simple and
A fluorescence derivatization method is generally popular as well as UV detection, and the sensitivity is comparable to MS detection. In this study, we developed a highly sensitive quantification method using fluorescent derivatization of DAP using 2,3-naphthalenedialdehyde (NDA).

**Experimental**

**Reagents and chemicals**

DAP was purchased from Funakoshi Co., Ltd. (Japan) and was used without further purification. Preparation and storage of aqueous stock solutions of DAP have been reported previously. Analytical grade o-phthalaldehyde (OPA) and NDA were commercially available from Tokyo Chemical Industry Co. Ltd. (Japan) and were used without further purification. An OPA derivatizing reagent solution was prepared as a mixture of 0.4 mol/L borate buffer (pH 9), 1% aqueous solution of OPA, and 2-mercaptoethanol (100:50:1). Also, a 0.1-mmol/L methanol solution of NDA was used, mixed with 45 mmol/L KCN prepared in a 20-mmol/L borate buffer (pH 9.5) solution. HPLC-grade acetonitrile and 2-propanol used for HPLC eluent were purchased from Kanto Chemical Co. Inc. (Japan) and used as received. Ultrapure water produced by using a Milli-Q Academic A10 apparatus (Merck Millipore, USA) was used in all experiments. Mouse serum was purchased from Cosmo Bio Co. Ltd. (Japan).

**Apparatus**

We used a HPLC system (JASCO Co., Japan) consisting of a PU-980 pump, FP-2025 Plus fluorescent detector, and a CO-965 column oven equipped with a Model 7125 syringe-loading sample injector (Rheodyne, USA). The HPLC separations were
performed on a TSKgel® ODS-100V (4.6 mm I.D. × 25 cm, 3 μm particle size) column (Tosoh Co., Japan) with a mobile phase consisting of 0.1 mol/L phosphate buffer (pH 2.1), acetonitrile, and 2-propanol (47:51:4) at a flow rate of 1.0 mL/min at 40°C. The column effluent was monitored at an emission of 450 nm (excitation at 350 nm) and 481 nm (excitation at 417 nm) for the OPA and NDA derivatives, respectively.

The structures of the products of DAP derivatized with OPA and NDA were analyzed using a Shimadzu Prominence HPLC system equipped with an ion trap (IT)-time-of-flight (TOF)-MS detector (Shimadzu, Japan). In the analysis, separation was performed using an Inertsil® ODS-3 column (3 μm, GL Sciences Inc., Japan) and a mobile phase consisting of 0.1% formic acid solution and acetonitrile (1:1) with a flow rate of 0.2 mL/min. The electrospray ionization (ESI) conditions of the IT-TOF-MS system were as follows: nebulizer gas, 1.50 L/min; interface probe potential, 4.50 kV; needle potential, 0 kV (0 A); curve desolvation line temperature, 200°C; and detector potential, 1.73 kV.

Sample preparation

Pretreatment procedures for DAP quantification in murine samples were the same as previously published.14,15 Briefly, DAP in murine serum was collected with an Ultrafree-MC (0.22-μm pore size) centrifuge filter available from Merck Millipore (USA) after deproteinization using acetonitrile. DAP in murine thigh muscle was collected by the conventional Bligh-Dyer extraction method17 after trypsin digestion, followed by the application to an Ultrafree-MC (0.22-μm pore size) centrifuge filter. A sample solution collected as a filtrate passed through the centrifuge filter was further purified for fluorescent derivatization in this work as follows. The aqueous sample solution (0.6 mL) was loaded into a Sep-Pak Plus (C18) short cartridge (Waters Co.) for
solid-phase extraction, conditioned by 10 mL of methanol followed by 10 mL of water. Then, the cartridge was washed with 20 mL of water, and the DAP extracted with 3 mL of acetonitrile. The acetonitrile solution was completely evaporated and 140 μL of water was added to the residue. To the aqueous solution, 10 μL of 45 mmol/L KCN and 50 μL of 5 mmol/L NDA solution were added and reacted for 20 minutes at room temperature. A 20-μL aliquot of the reaction mixture was injected into the HPLC column.

Serum and muscle samples collected from mice who were actually administered DAP are the same as those used in previous studies\textsuperscript{14,15} and were provided by Aichi Medical University. The study was reviewed and approved by the Aichi Medical University Hospital Institutional Animal Care and Use Committee.

**Results and Discussion**

The fluorescent products of DAP through derivatization reaction with OPA and NDA were identified using IT-TOF-MS. It is well recognized that both OPA and NDA produce fluorescent substances with cyclization reactions in the presence of thiol and cyanide, respectively, for aliphatic primary amines.\textsuperscript{18} Peaks of mass-to-charge ratio (m/z) 899.3791 and 898.3810 have been observed in MS spectra of the OPA and NDA derivatization products, respectively. Previous LC-MS studies of DAP suggest that these peaks correspond to divalent ions of m/z = (M + 2H\textsuperscript{+}) corresponding to the 2H-isoindole and 2H-benz[f]isoindole substances shown in Fig. 1.\textsuperscript{11} Therefore, OPA and NDA react with DAP to produce fluorescent substances cyclized using the amino group of ornithine. In fact, the derivatization products of OPA and NDA show strong fluorescence at 450 nm (ex. 350 nm) and 481 nm (ex. 417 nm), respectively, caused by the isoindole moiety.
Figure 2 shows the stabilities of the isoindole derivatives of DAP. It is clear that the 2H-isoindole-DAP has considerably faster degradation than the 2H-benz[f]isoindole-DAP. Although it takes about 20 minutes to complete the generation reaction, the 2H-benz[f]isoindole-DAP is stable, and therefore, is suitable for pre-column derivatization analyses. Because of this, subsequent research has studied fluorescent analyses of DAP using NDA derivatization. The effects of concentrations of NDA and KCN on the reaction were examined in 1-10 mmol/L and 5-50 mmol/L, respectively. In addition, the only effect of temperature on the reaction was that warmer conditions improved the cyclization yield of DAP by NDA slightly, so room temperature was chosen for convenience. These results determined the optimal derivatization conditions as described in the experiment section.

Next, the separation of the derivatized product was investigated using murine serum. Although the composition of the eluent was examined in detail, it was not possible to detect the clear peak corresponding to 2H-benz[f]isoindole derived from DAP in serum at concentrations less than 50 nmol/L due to a hindrance of the serum components. However, solid-phase (C18) extraction before reaction with NDA efficiently removed the peaks corresponding to the matrix components, as shown in Fig. 3.

A calibration curve was created by plotting the peak intensities of 2H-benz[f]isoindole-DAP against the concentrations in the spiked serum standards, which were prepared by spiking drug-free mouse serum (360 µL) with a 40-µL aliquot of the DAP standard solutions prepared at various concentrations. The limit of detection, defined as the lowest concentration that gave a signal-to-noise ratio of at least three, was 3 nmol/L for DAP. This value is significantly lower than the values obtained for UV (0.3 µmol/L)\textsuperscript{14} and MS detection (0.2 µmol/L)\textsuperscript{11} and is approximately the same or high
sensitivity as MS/MS detection (6.2 nmol/L). The limit of quantification, defined as the drug sample concentration meeting pre-specified requirements for precision within 20% was 8 nmol/L. Linear regression calculations were unweighted and non-zero-forced, and a calibration curve was linear across the examined dynamic range from 8 nmol/L to 1 μmol/L, obtained as $\text{Int} = 401C + 48.83$ ($n = 8$, $r = 0.9986$), where $\text{Int}$ and $C$ are peak intensities and concentrations of DAP in sample solutions, respectively. The calibration curve was validated by evaluating reproducibility and recovery using three concentrations of DAP: 8 nmol/L (low level), 50 nmol/L (medium level), and 0.5 μmol/L (high level). The reproducibility for six repeated experiments was 19.5%, 9.7%, and 5.4% for the low, medium, and high concentration levels, respectively. The mean recovery values for six trials were 82.2%, 96.5%, and 98.2% for the low, medium, and high concentrations, respectively.

The proposed fluorescent method for quantification of DAP was applied to the real samples of murine serum and thigh muscle collected 24 hours after a single subcutaneous administration of 100 mg/kg DAP. Although UV could not detect DAP in the serum and muscle samples in previous studies,14,15 the fluorescence derivatization method proposed here made it possible to observe a clear peak on the chromatogram; the concentration in serum collected 24 hours after administration being $2 \times 10^{-8}$ mol/L ($n = 5$, relative standard deviation (RSD) = 23.1%). Figure 4 shows the well-separated chromatogram of $2H$-benz[f]isoindole-DAP obtained for the murine thigh muscle. DAP concentrations in murine femoral muscles collected 24 h after the single subcutaneous administration of DAP (100 mg/kg) were 20 ng/g (muscle) ($n = 5$, RSD = 28.2%).

In conclusion, the fluorescent derivatization method for quantification of DAP is simple and effective for long-term monitoring of concentrations in the blood and tissue of mice in experiments relating to treatment and side effect models with a DAP
quantification level to the order of 10 nmol/L.

References

1. C. Liu, Z. Mao, M. Yang, H. Kang, H. Liu, L. Pan, J. Hu, J. Luo, and F. Zhou, Ther. Clin. Risk. Manag., 2016, 12, 1455.

2. F. P. Tally, M. Zeckel, M. M. Wasilewski, C. Carini, C. L. Berman, G. L. Drusano, and F. B. Jr. Oleson, Daptomycin: a novel agent for Gram-positive infections. Expert Opin. Investig. Drugs, 1999, 8, 1223.

3. F. B. Jr. Oleson, C. L. Berman, J. B. Kirkpatrick, K. S. Regan, J. J. Lai, and F. P. Tally, Antimicrob. Agents Chemother., 2000, 44, 2948.

4. R. Sauermann, M. Rothenburger, W. Graninger, and C. Joukhadar, Pharmacology, 2008, 81, 79.

5. F. Sbrana, A. D. Paolo, E. M. Pasanisi, E. Tagliaferri, C. Arvia, M. Puntoni, A. Leonildi, F. Bigazzi, R. Danesi, D. Rovai, C. Tascini, and F. Menichetti, J. Chemother., 2010, 22, 434.

6. A. Kazory, K. Dibadj, and I. D. Weiner, J. Antimicrob. Chemother., 2006, 57, 578.

7. S. T. King, E. D. Walker, C. G. Cannon, and R. W. Finley, Scand. J. Infect. Dis., 2014, 46, 537.

8. T. Y. Kostrominova, C. A. Hassett, E. P. Rader, C. Davis, L. M. Larkin, S. Coleman, F. B. Oleson, and J. A. Faulkner, Muscle Nerve, 2010, 42, 385.

9. L. Baietto, A. D’Avolio, S. Pace, M. Simiele, C. Marra, A. Ariaudo, G. D. Perri, and F. G. D. Rosa, J. Pharm. Biomed. Anal., 2014, 88, 66.

10. T. Naito, T. Yamada, T. Yagi, and J. Kawakami, Biomed. Chromatogr., 2014, 28, 317.
11. L. Baietto, A. D’Avolio, F. G. D. Rosa, S. Garazzino, M. Michelazzo, G. Ventimiglia, M. Siccardi, M. Simiele, M. Sciandra, and G. D. Perri, Anal. Bioanal. Chem., 2010, 396, 791.

12. F. N. Bazoti, E. Gikas, A. Skoutelis, A. Tsarbopoulos, J. Pharm. Biomed. Anal., 2011, 56, 78.

13. M. C. Verdier, D. Bentué-Ferrer, O. Tribut, N. Collet, M. Revest, and E. Bellissant, Clin. Chem. Lab. Med., 2011, 49, 69.

14. Y. Sakai, E. Murakami, H. Kato, K. Ohyama, Y. Esaka, T. Yamamoto, M. Hagihara, H. Mikamo, and B. Uno, Biol. Pharm. Bull., 2019, 42, 751.

15. T. Kondo, M. Hagihara, Y. Esaka, T. Yamamoto, B. Uno, Y. Yamagishi, and H. Mikamo, Jpn. J. Infect. Dis., 2020, 72, 203.

16. A. Kawasaki, M. Yasuda, K. Mawatari, T. Fukuuchi, N. Yamaoka, K. Kaneko, R. Iijima, S. Yui, M. Satoh, K. Nakagomi, Anal. Sci., 2018, 34, 841.

17. E. G. Bligh and W. J. Dyer, Can. J. Biochem. Physiol., 1959, 37, 911.

18. Y. Ohkura, M. Kai, and H. Nohta, Bunseki Kagaku, 1994, 43, 259.
**Figure Captions**

Fig. 1 Structures of 2H-isoindole-DAP (left) and 2H-benz[f]isoindole-DAP (right) as plausible products of DAP through derivatization reactions with OPA and NDA, respectively.

Fig. 2 Dependence of fluorescent intensities of 2H-isoindole-DAP (left) and 2H-benz[f]isoindole-DAP (right) generated by the derivatization reaction upon the reaction time.

Fig. 3 Typical HPLC elution profile of 2H-benz[f]isoindole-DAP corresponding to 50 nmol/L DAP. The analyte-free serum (200 µL) spiked with 16.5 ng DAP was pretreated with the procedure involving solid-phase extraction.

Fig. 4 HPLC elution profile of DAP in thigh muscle collected after 24 h from mice administrated with a single subcutaneous dose of 100 mg/kg DAP. The red chromatograph is for the muscle sample added with 5 ng DAP.
$2\text{H}$-isoindole-DAP (MW = 1796.930)  $2\text{H}$-benz[f]isoindole-DAP (MW = 1794.753)

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