Identification of Furosemide Photodegradation Products in Water–Acetonitrile Mixture

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The aim of this study was to identify the chemical structure of the photodegradation products of furosemide in a water–acetonitrile mixture (1 : 1). Furosemide solution was irradiated with a D65 fluorescent lamp and the products were isolated by preparative HPLC. The fractions were evaporated to dryness in vacuo.

The purity of the photodegradation products was measured by HPLC. The purity of products 1, 3, and 4 was greater than 90%, whereas that of product 2 was 13%, therefore, photodegradation product 2 was unstable. We identified photodegradation products 1 and 3 as 4-chloro-5-sulfamoylanthranilic acid and 4-hydroxy-N-furfuryl-5-sulfamoylanthranilic acid, respectively, by LC/MS and NMR. Additionally, we assumed that photodegradation product 4 was methyl 2-((furan-2-ylmethyl)amino)-4-hydroxy-3-(methyleneamino)-5-sulfamoylbenzoate by LC/MS and NMR. This showed that furosemide underwent hydrolysis and substitution, and reacted with the acetonitrile under the light of a D65 fluorescent lamp. We were furthermore able to determine the elution times of the photodegradation products of furosemide by applying the Japanese Pharmacopoeia chromatographic method for related substances to the isolated products.

Key words furosemide; photodegradation; water–acetonitrile mixture; identification; Japanese Pharmacopoeia

Furosemide (4-chloro-N-furfuryl-5-sulfamoylanthranilic acid) is widely used as a diuretic in the treatment of edema. It is available in solid and liquid dosage forms, but it is well known that furosemide is unstable in aqueous and organic solution. In aqueous solutions, furosemide is hydrolyzed to saluamine (4-chloro-5-sulfamoylanthranilic acid (CSA)) and furfuryl alcohol.1,2) Regarding organic solutions, Moore and Sithipitaks reported a photodegradation pathway in oxygen-free methanol.3) Furosemide degrades rapidly at acidic pH, although it is stable at alkaline pH under laboratory conditions and diffuse daylight.4–6) In addition, furosemide is a photosensitive drug and its degradation products and impurities have been reported.7–10) Asker and Ferdous also reported that furosemide is most stable at pH 7 and that the photodegradation of furosemide follows first-order kinetics.10) Because of such backgrounds, numerous analytical methods have been developed for assaying furosemide in pharmaceutical samples.11–13)

Furosemide related substances are determined by HPLC in the purity test in the Japanese Pharmacopoeia. In this method, water–tetrahydrofuran–acetic acid mixture is used as mobile phase and acetic acid/water–acetonitrile mixture is used to dissolve the sample. On the contrary, recent progress in LC/MS method has spurred a great deal of research into the determination of related substances in purity test for active pharmaceutical ingredients and drug formulations. We have developed a new analytical method for determining the structure of furosemide impurities using by LC/MS method. In this novel method, the mixture of formic acid/acetonitrile as mobile phase and the mixture of water–acetonitrile as solvent to dissolve the sample are selected, respectively. Although the photodegradation product of furosemide in methanol has been reported,13) to the best knowledge of the authors, no research has yet been carried out with respect to determination of photodegradation products of furosemide derived from water–acetonitrile mixture.

Therefore, we have isolated and identified the photodegradation products of furosemide in water–acetonitrile mixture by preparative HPLC, LC/MS and NMR. Furthermore, we were able to determine the elution times of the photodegradation products of furosemide by applying the Japanese Pharmacopoeia chromatographic method for related substances to the isolated products.

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Experimental

Materials  Furosemide was purchased from a commercial source. Acetonitrile, methanol, tetrahydrofuran, acetic acid and formic acid were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Kanto Chemical Co., Inc. (Tokyo, Japan). The polytyrosine-1,3,6 LC/MS calibration standard was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, U.S.A.). Dimethyl sulfoxide-d$_6$ (DMSO-d$_6$) containing 0.05% (v/v) tetramethylsilane (TMS) was purchased from Wako Pure Chemical Industries, Ltd. All reagents were commercially available and analytical grade.

Preparation of Furosemide Photodegradation Products  Furosemide (1 g) was dissolved in water–acetonitrile mixture (1:1) (100 mL) and an aliquot (10 mL) was transferred into a glass vial. The solution was exposed to D65 fluorescent lamp light in a light stability cabinet (LTL200 D3 CJH, Nagano Science, Japan) for 1 week at an illumination intensity of 4000 lx. The temperature was 25°C and the relative humidity was 60%.

Isolation of Furosemide Photodegradation Products  Water–acetonitrile mixture (1:1, 5 mL) was added to a portion of the photolyzed furosemide solution (5 mL). The solution (2 mL) was injected into a preparative HPLC system. The preparative HPLC system consisted of a UV-Vis detector (UV702, GL Sciences, Tokyo, Japan), a column oven (CO-705C, GL Sciences), an auto-sampler (G-Prep AS, GL Sciences), a fraction collector (FC204, GL Sciences) and a degasser (GASTORR PG-12, GL Sciences). An Inertsil ODS-3 column (20 mm i.d.×250 mm, 5 µm particle size, GL Sciences) and an Inertsil ODS-3 guard column (20 mm i.d.×50 mm, 5 µm particle size, GL Sciences) were used. The detection wavelength was 272 nm. The temperature of column oven was 30°C. The flow rate was 16 mL/min. The mobile phases were 0.1% formic acid in water (solvent A) and acetonitrile (solvent B). The gradient program was as follows: 0 to 30 min, 50% A and 50% B; 30 to 35 min, linear gradient from 50 to 10% A; 35 to 65 min, 10% A and 90% B; 65 to 70 min, linear gradient from 10% to 50% A; 70 to 90 min, 50% A and 50% B. The fractions containing the photodegradation products were evaporated to dryness in vacuo. The vacuum evaporation system consisted of a vacuum evaporator (SD905, GL Sciences), an organic solvent recovery system (SC907, GL Sciences), a water chiller (CCM-1100, GL Sciences), and a nitrogen generator (NM910, GL Sciences). The temperature of the heat block and chiller were 30 and 5°C, respectively, and the nitrogen flow rate was 2.0–5.0 L/min.

Purity of Furosemide Photodegradation Products  The photodegradation products (2 mg) were dissolved in 2.2% acetic acid in water–acetonitrile mixture (1:1, 2 mL). The sample solutions were analyzed by HPLC. The HPLC system consisted of a separation module (Alliance W2695, Waters, Milford, MA, U.S.A.) and a photodiode array detector (W2998, Waters). The purity of the sample solutions was calculated by the area percentage method. The HPLC method conformed to the Japanese Pharmacopoeia for substances related to furosemide. A Mightysil RP-18 GP column (4.6 mm i.d.×250 mm, 5 µm particle size, Kanto Chemical) was used. The photodiode array detector was set at 210–400 nm. The temperature of the column oven was 25°C. The mobile phase was water–tetrahydrofuran–acetic acid (70:30:1) and the flow rate was 1.25 mL/min. The sample solution (20 µL) was injected into the HPLC system and the run time was 60 min.

Mass Spectra of Furosemide Photodegradation Products  The photodegradation products were dissolved in water–acetonitrile mixture (1:1) and the concentration was adjusted to 0.005 mg/mL. The sample solutions were analyzed by LC/MS. The LC/MS system consisted of an HPLC system (UltiMate3000, Dionex, Sunnyvale, CA, U.S.A.) and a mass spectrometer (LTQ Orbitrap Discovery, Thermo Fisher Scientific, Waltham, MA, U.S.A.). The HPLC system consisted of a pump (HPG-3200RS, Dionex), an auto-sampler (WPS-3000RS, Dionex), a column compartment (TCC-3000RS, Dionex), and a diode array detector (DAD-3000RS, Dionex). The mass spectrometer consisted of a linear ion trap (LTQ XL, Thermo Scientific) and a spectrometer (LTQ Orbitrap, Thermo Scientific). An Inertsil ODS-3 column (2.1 mm i.d.×150 mm, 5 µm particle size, GL Sciences) was used. The wavelength of the diode array detector was 210–400 nm. The temperature of the column oven was 30°C. The mobile phases were 0.1%
formic acid in water (solvent A) and acetonitrile (solvent B). The flow rate was 0.2 mL/min. The gradient program was as follows: 0 to 10 min, 80% A and 20% B; 10 to 18 min, linear gradient from 80 to 65% A; 18 to 50 min, 65% A and 35% B; 50 to 51 min, linear gradient from 65 to 80% A; 51 to 75 min, 80% A and 20% B. The sample solution (2 μL) was injected into the LC/MS system. The spectrometer was operated in electrospray ionization (ESI) and negative ion mode, with a resolution of 30000. The sheath gas flow rate and auxiliary gas flow rate were 50 and 10 arb. units, respectively. The ion spray voltage was 3 kV. The capillary temperature was 330°C. The MS2 and MS3 measurements were performed by collision-induced dissociation (CID) with collision energy of 35 eV. The spectrometer was calibrated with a polytyrosine-1,3,6 standard.

Table 1. Purity Data of Furosemide Photodegradation Products

<table>
<thead>
<tr>
<th>Sample</th>
<th>Retention time (min)</th>
<th>Relative retention time$^a$</th>
<th>Purity (%)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Photodegradation product 1</td>
<td>5.94</td>
<td>0.32</td>
<td>94.9</td>
</tr>
<tr>
<td>Photodegradation product 2</td>
<td>12.54</td>
<td>0.68</td>
<td>13.0</td>
</tr>
<tr>
<td>Degradation product of photodegradation product 2</td>
<td>3.44</td>
<td>0.19</td>
<td>86.4</td>
</tr>
<tr>
<td>Photodegradation product 3</td>
<td>9.13</td>
<td>0.49</td>
<td>99.5</td>
</tr>
<tr>
<td>Photodegradation product 4</td>
<td>13.79</td>
<td>0.74</td>
<td>98.5</td>
</tr>
</tbody>
</table>

$^a$ Relative retention to furosemide (retention time = 18.53 min). $^b$ Calculated by area percentage method (except blank peaks).

Fig. 3. HPLC Chromatograms at 272 nm and UV Spectra of Furosemide Photodegradation Products and Furosemide
(a) Furosemide. (b) Photodegradation product 1. (c) Photodegradation product 2. (d) Photodegradation product 3. (e) Photodegradation product 4. (f) Blank.
Identification of Furosemide Photodegradation Products

The photodegradation products (5 mg) were dissolved in DMSO-\textit{d}_\textsubscript{6} containing 0.05\% (v/v) TMS (0.6 mL). The samples were analyzed by NMR (JNM-ECA-500, JEOL, Tokyo, Japan). The NMR spectrometer consisted of a 4 mm CP-MAS probe (JEOL), a 500 MHz spectrometer (ECA-500, JEOL), and a compressor (SLP-07E-S53BP, Anest Iwata, Yokohama, Japan). One- and two-dimensional NMR spectra (\textsuperscript{1}H-, \textsuperscript{13}C-, \textsuperscript{1}H–\textsuperscript{1}H correlation spectroscopy (COSY), \textsuperscript{1}H–\textsuperscript{13}C heteronuclear multiple quantum correlation (HMQC), \textsuperscript{1}H–\textsuperscript{13}C heteronuclear multiple bond correlation (HMBC)) were recorded at 25°C. Tetramethylsilane (TMS) was used as an internal standard, and all chemical shifts were assigned relative to its peak at \(\delta\ 0.00\) ppm. The relaxation delays for the \textsuperscript{1}H- and \textsuperscript{13}C-NMR were 5 and 2 s, respectively, and the relaxation delay for the \textsuperscript{1}H–\textsuperscript{1}H COSY, \textsuperscript{1}H–\textsuperscript{13}C HMQC, and \textsuperscript{1}H–\textsuperscript{13}C HMBC NMR was 1.5 s. The number of scans for the \textsuperscript{1}H- and \textsuperscript{13}C-NMR was 32 and 1000, respectively. The number of scans for the \textsuperscript{1}H–\textsuperscript{1}H COSY, \textsuperscript{1}H–\textsuperscript{13}C HMQC, and \textsuperscript{1}H–\textsuperscript{13}C HMBC NMR was 32.

Results and Discussion

Preparation of Furosemide Photodegradation Products

Bundgaard et al. and Yagi et al. reported that neutral furosemide (furosemide in its unionized form) is extremely susceptible to photodegradation in aqueous solution.\textsuperscript{5,6}) Rapid degradation was also observed in mixtures of water and organic solvents, such as methanol and acetonitrile. This suggests that furosemide is not ionized in water–acetonitrile mixture (1:1) and that the photodegradation of furosemide is accelerated by...
Fig. 5. Negative Ion Mode ESI-MS\textsuperscript{1} Spectra of Photodegradation Products of Furosemide
(a) Photodegradation product 1. (b) Photodegradation product 2. (c) Photodegradation product 3. (d) Photodegradation product 4.

Table 2. MS\textsuperscript{1} Data of Furosemide and Furosemide Photodegradation Products

<table>
<thead>
<tr>
<th>Sample</th>
<th>Molecular ion</th>
<th>Accurate mass (m/z)</th>
<th>Elemental composition$^{a}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Furosemide</td>
<td>[M−H]$^{-}$</td>
<td>329.0001</td>
<td>C\textsubscript{12}H\textsubscript{10}ClN\textsubscript{2}O\textsubscript{5}S</td>
</tr>
<tr>
<td>Photodegradation product 1</td>
<td>[M−H]$^{-}$</td>
<td>248.9741</td>
<td>C\textsubscript{9}H\textsubscript{5}ClN\textsubscript{2}O\textsubscript{4}S</td>
</tr>
<tr>
<td>Photodegradation product 2</td>
<td>[M−H]$^{-}$</td>
<td>555.0495</td>
<td>C\textsubscript{20}H\textsubscript{19}N\textsubscript{4}O\textsubscript{11}S\textsubscript{2}</td>
</tr>
<tr>
<td>Degradation product of photodegradation product 2</td>
<td>Ni</td>
<td>Ni</td>
<td>Ni</td>
</tr>
<tr>
<td>Photodegradation product 3</td>
<td>[M−H]$^{-}$</td>
<td>311.0337</td>
<td>C\textsubscript{12}H\textsubscript{11}N\textsubscript{2}O\textsubscript{4}S</td>
</tr>
<tr>
<td>Photodegradation product 4</td>
<td>[M−H]$^{-}$</td>
<td>352.0598</td>
<td>C\textsubscript{14}H\textsubscript{14}N\textsubscript{3}O\textsubscript{6}S</td>
</tr>
</tbody>
</table>

$^{a}$ NI, not ionized. $^{a}$ Calculated from the accurate mass.

Table 3. MS\textsuperscript{2}–MS\textsuperscript{3} Data of Furosemide and Furosemide Photodegradation Products

<table>
<thead>
<tr>
<th>Sample</th>
<th>MS\textsuperscript{a}</th>
<th>Precursor ion (m/z)</th>
<th>Product ion accurate mass (m/z)$^{b}$</th>
<th>Product ion elemental composition$^{b}$</th>
<th>Mass change (Da)</th>
<th>Neutral loss composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Furosemide</td>
<td>2</td>
<td>329.00</td>
<td>285.0101</td>
<td>C\textsubscript{10}H\textsubscript{8}ClN\textsubscript{2}O\textsubscript{3}S</td>
<td>−44</td>
<td>CO\textsubscript{2}</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>285.01</td>
<td>204.9842</td>
<td>C\textsubscript{9}H\textsubscript{5}ClN\textsubscript{2}O\textsubscript{3}S</td>
<td>−84</td>
<td>C\textsubscript{9}H\textsubscript{5}O</td>
</tr>
<tr>
<td>Photodegradation product 1</td>
<td>2</td>
<td>248.97</td>
<td>204.9841</td>
<td>C\textsubscript{9}H\textsubscript{5}ClN\textsubscript{2}O\textsubscript{3}S</td>
<td>−44</td>
<td>CO\textsubscript{2}</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>204.98</td>
<td>126.0114</td>
<td>C\textsubscript{6}H\textsubscript{5}ClN\textsubscript{2}O\textsubscript{2}S</td>
<td>−79</td>
<td>H\textsubscript{2}NO\textsubscript{2}</td>
</tr>
<tr>
<td>Photodegradation product 2</td>
<td>2</td>
<td>555.05</td>
<td>511.0589</td>
<td>C\textsubscript{19}H\textsubscript{18}N\textsubscript{4}O\textsubscript{9}S\textsubscript{2}</td>
<td>−44</td>
<td>CO\textsubscript{2}</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>511.06</td>
<td>467.0696</td>
<td>C\textsubscript{19}H\textsubscript{18}N\textsubscript{4}O\textsubscript{9}S\textsubscript{2}</td>
<td>−44</td>
<td>CO\textsubscript{2}</td>
</tr>
<tr>
<td>Photodegradation product 3</td>
<td>2</td>
<td>311.03</td>
<td>267.0441</td>
<td>C\textsubscript{11}H\textsubscript{9}N\textsubscript{2}O\textsubscript{5}S</td>
<td>−44</td>
<td>CO\textsubscript{2}</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>311.03</td>
<td>246.0403</td>
<td>C\textsubscript{10}H\textsubscript{9}N\textsubscript{2}O\textsubscript{5}S</td>
<td>−65</td>
<td>H\textsubscript{2}NO\textsubscript{2}</td>
</tr>
<tr>
<td>Photodegradation product 4</td>
<td>2</td>
<td>352.06</td>
<td>310.0496</td>
<td>C\textsubscript{12}H\textsubscript{12}N\textsubscript{3}O\textsubscript{6}S</td>
<td>−42</td>
<td>C\textsubscript{9}H\textsubscript{3}O</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>310.05</td>
<td>266.0599</td>
<td>C\textsubscript{11}H\textsubscript{12}N\textsubscript{3}O\textsubscript{6}S</td>
<td>−44</td>
<td>CO\textsubscript{2}</td>
</tr>
</tbody>
</table>

$^{a}$ Base peak ion monitored. $^{b}$ Calculated from the accurate mass.
Fig. 6. Fragmentation Patterns of Photodegradation Products 1 and 3 by CID with Collision Energy of 35 eV
(a) Photodegradation product 1. (b) Photodegradation product 3.

Fig. 7. 500 MHz $^1$H–$^{13}$C HMQC NMR Spectrum of Photodegradation Product 1 in DMSO-$d_6$ at 25°C
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acetonitrile. Figure 1 shows the color change of furosemide in water–acetonitrile mixture (1:1) from colorless to yellow under D65 fluorescent lamp light (600000 lx·h), suggesting that it underwent photodegradation.

Isolation of Furosemide Photodegradation Products in Water–Acetonitrile Mixture (1:1) Figure 2 shows the preparative HPLC chromatogram of the photolyzed furosemide solution. The furosemide photodegradation products were isolated by preparative HPLC. Products 1–4 had retention times of 8–10, 11–12, 13–15, and 25–26 min, respectively. The HPLC fractions were dried in vacuo to obtain 1 as yellow crystals, 2 as brown crystals, and 3 and 4 as white crystals.

Purity of Photodegradation Products Figure 3 shows the HPLC chromatograms and UV spectra of furosemide and its photodegradation products. Product 2 contained two large peaks. We assumed that the elution order of the photodegradation products was the same as for preparative HPLC. Thus, the two peaks at 3.44 and 12.54 min were assigned as the product 2 degradation product and product 2, respectively.

Changes in the UV spectra of furosemide in various sol-
vents after exposure to fluorescent light and UV light were reported by Rowbotham et al., Moore and Sithipitaks, and Asker and Ferdous. The UV spectra of photodegradation products 2 and 3 were similar to the spectrum of furosemide. However, the UV spectra of products 1 and 4 were different from that of furosemide. The purity of photodegradation products 1, 3 and 4 were greater than 90%, whereas that of photodegradation product 2 was about 13% (Table 1). Therefore, photodegradation product 2 was unstable.

Mass Spectra of Furosemide Photodegradation Products

Figure 4 shows the UV chromatograms of furosemide and its photodegradation products at 272 nm. The peaks at 14.32 and 5.08 min were identified as photodegradation product 2 and its degradation product, respectively, because the UV spectrum of the peak at 14.32 min was similar to that of Fig. 2(c) (peak at 12.54 min). In addition, product 1 and the degradation product of product 2 could not be separated under this LC condition. To obtain a mass spectrum of product 1, a pure sample was required.

Figure 5 shows the negative ion mode ESI-MS spectra of the furosemide photodegradation products. The negative ion mode ESI-MS spectra show a deprotonated molecular ion ([M-H]−) and the MS and MS2,3 results are shown in Tables 2 and 3, respectively. Figure 6 shows the fragmentation patterns of photodegradation products 1 and 3 by CID with collision energy of 35 eV. These results suggest that products 1 and

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Fig. 9. 500 MHz 1H–13C HMQC Spectrum of Degradation Product of Photodegradation Product 2 in DMSO- d6 at 25°C
3 were CSA and 4-hydroxy-N-furfuryl-5-sulfamoylanthranilic acid, respectively. In addition, photodegradation product 4 was identified by the MS² spectrum as a product of the reaction between furosemide and acetonitrile.

**Identification of Furosemide Photodegradation Products**

¹H-NMR has been used to identify and assay furosemide and its degradation product (CSA) in tablet and injection formulations.¹¹,¹⁴ Figures 7 and 8 show the 500 MHz ¹H–¹³C HMQC NMR spectra of photodegradation products 1 and 3 in DMSO-d₆ at 25°C, respectively. The NMR spectra were consistent with products 1 and 3 being CSA and 4-hydroxy-N-furfuryl-5-sulfamoylanthranilic acid. Furthermore, the carboxyl group of the furosemide degradation products underwent H-D exchange in DMSO-d₆.

Figure 9 shows the 500 MHz ¹H–¹³C HMQC NMR spectrum of the degradation product of photodegradation product 2 in DMSO-d₆ at 25°C. The degradation product of photodegradation product 2 was identified as 4-hydroxy-2-(prop-2-ynylamino)-5-sulfamoylbenzoic acid. However, photodegradation product 2 could not be identified by NMR and LC/MS.

![Fig. 10. 500 MHz ¹H–¹³C HMQC Spectrum of Degradation of Photodegradation Product 4 in DMSO-d₆ at 25°C](image-url)
Figures 10 and 11 show the 500 MHz $^1$H–$^{13}$C HMQC and $^1$H–$^{13}$C HMBC NMR spectra of photodegradation product 4 in DMSO-$d_6$ at 25°C. Although the signal at $\delta$ 6.38–6.43 ppm was observed as a singlet in the $^1$H-NMR spectrum of product 4, $^1$H–$^{13}$C HMQC NMR in acetone-$d_6$ confirmed that the $^1$H-NMR signal of H-3 overlapped with that of H-2 in DMSO-$d_6$ (data not shown). We assumed that the product 4 might be methyl 2-((furan-2-ylmethyl)amino)-4-hydroxy-3-(methyleneamino)-5-sulfamoylbenzoate from the LC/MS and NMR results.

We irradiated the related compounds methyl 4-hydroxybenzoate, ethyl 4-hydroxybenzoate, propyl 4-hydroxybenzoate,
and butyl 4-hydroxybenzoate with D65 lamp light to determine whether they react with acetonitrile. The compounds did not react with acetonitrile, suggesting that this reaction is specific to furosemide.

**Conclusion**

To identify the furosemide photodegradation products in a water–acetonitrile mixture (1:1), we isolated the products by preparative HPLC and identified their structures by LC/MS and NMR. The results suggested that furosemide in water–acetonitrile mixture (1:1) underwent hydrolysis and substitution of the Cl moiety with OH, and reacted with acetonitrile. Furthermore, we were able to determine the elution times of the photodegradation products of furosemide by applying the Japanese Pharmacopoeia chromatographic method for related substances to the isolated products.

**Conflict of Interest**

Shinji Katsura, Nobuo Yamada, Atsushi Nakashima and Sumihiro Shiraishi are employees of Teva Pharma Japan Inc.

**References**