Photodegradation of sparfloxacin and isolation of its degradation products by preparative HPLC

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INTRODUCTION

Sparfloxacin (Figure 1), a fluoroquinolone derivative, is a powerful antibacterial agent against a wide range of Gram-positive and Gram-negative organisms, including Streptococcus pneumoniae, Staphylococcus aureus, methicillin resistant S. aureus, Legionella spp., Mycoplasma spp., Chlamydia spp. and Mycobacterium spp. A drawback of fluoroquinolones is their photoreactivity. Sparfloxacin has been studied in terms of therapeutic activities. However, there are few published of analytical methods being applied to sparfloxacin. The aim in this study was to determine the photodegradation products of sparfloxacin, when submitted to UV light, and to characterize two of these products, designated SPAX-PDP1 and SPAX-PDP2. An accelerated study of stability in methanol solution was carried out by exposing a solution of sparfloxacin to UV light (peak wavelength 290 nm) for 36 hours at room temperature. The products were analyzed by NMR spectrophotometry, IR spectrometry and mass spectrophotometry. The results suggest that the products isolated here could be used to estimate the degradation of sparfloxacin in a stability study. However, the low activity exhibited by UV-irradiated sparfloxacin is a source of concern that demands further investigation of the mechanism of its photodegradation mechanism.

Keywords: Degradation products, fluoroquinolone, photodegradation, quality control, sparfloxacin, stability.

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conditions include the temperature, photolysis, oxidation, hydrolysis and humidity when appropriate (Singh e Bakshi, 2000).

The stability data requirements for human medicines in the European Community are based on a series of regulatory guidelines adopted by the ICH. According to Matthews (1999), stability for pharmaceutical products should be taken in the sense of ‘controlled, documented and acceptable change’. The most important factors are chemical, physical, pharmacotechnical, microbiological, toxicological and clinical, including bioavailability. Stability studies should include shipment and sufficient duration to cover storage.

Accelerated-stress stability tests are carried out, in which samples are stored under light conditions designed to stress the raw material or pharmaceutical preparations.

Stability studies of sparfloxacin have been described by Marona and coworkers (1999), who presented a rapid and sensitive method to determine the presence of any photodegradation products in the powder. An in vitro study was carried out to determine cytotoxic effects of sparfloxacin on mononuclear human culture cells. The results showed that sparfloxacin raw material and tablets, as well as sparfloxacin tablets under light, could reduce the number of cells significantly (according to Tukey Test). On the other hand, the two isolated products did not show this response under the same conditions (Marona et al., 2002).

The aim of this study was to determine photodegradation products of sparfloxacin submitted to UV light (254 nm) and to characterize these products by 1H-NMR and 13C-NMR spectrophotometry, IR spectrometry and mass spectrophotometry.

**MATERIAL AND METHOD**

All the chemicals used were of analytical reagent grade, and the solvents were of spectroscopic grade.

**Material:** Sparfloxacin reference substance (SPAX-RS), 99.5% pure, was kindly donated for the Dainippon Pharmaceutical Co. Ltd. (Suita, Osaka, Japan) and Rhone-Poulenc Rorer (U.S.A.). Sparfloxacin tablets Zagam™ (SPAX-TAB) were purchased in France. The tablets contained 200 mg of drug according to the manufacturer. SPAX-RS and SPAX-TAB were stored protected from light.

Water and methanol were HPLC grade.

Twenty tablets of sparfloxacin were carefully crushed using a glass mortar and pestle. The crushed tablets were then quantitatively transferred to a volumetric flask and dissolved in methanol, to obtain a nominal 1 mg/mL solution of sparfloxacin.

**Equipment:** NMR Spectrophotometry: The NMR data were acquired with a DTX 200 MHz spectrophotometer (Bruker, Germany). Both 1H NMR and 13C NMR spectra were obtained at 200 MHz. All spectra were recorded in D,O and DMSO. For the structural elucidation, the following spectra were obtained: 1H NMR, 13C NMR, 1H-1H COSY and 1H-13C-COSY.

Mass Spectrometry: The mass spectrometric measurements were performed in a Shimadzu 8001 (Japan) at 500 MHz.

UV-Vis Spectrophotometry: The UV-Vis data were obtained in an UV-160A at spectrophotometer (Shimadzu, Japan).

IR spectrometry: IR spectra were acquired in a Shimadzu 8001 FTIR spectrophotometer (Japan).

Semipreparative HPLC: The semiquantitative HPLC analysis was performed on a Waters Alliance 2690. A Shim-pack CLC-ODS (250 x 4.6 mm i.d., 5 µm particle size, 40 Å pore diameter) was used with 5% acetic acid : methanol : acetonitrile (75:12.5:12.5, v/v/v) as the isocratic mobile phase at a flow rate of 1.0 mL/min. The HPLC system was operated at room temperature (20 ± 1°C).

Photodegradation chamber: To degrade sparfloxacin, a fresh solution (1 mg/mL) and tablets were submitted to UV light (254 nm) for 36 hours in a chamber (16 x 16 x 100 cm).

Melting point: Koefler equipment. Phenolphthalein (WHO Melting Point reference Substance – MP 263°C was used to check the temperature).

**Method:**

**Sample irradiation**

(a) SPAX-RS (99.5%) was exposed to UV-A light, in an open Petri dish, for 90 days.

(b) SPAX-RS (99.5%) was exposed to UV-C light, in an open Petri dish, for 24 hours.

(c) SPAX-RS (99.5%) was dissolved in methanol (1 mg/mL) and then exposed to UV-C light, in an open Petri dish, for 5 hours.

(d) SPAX tablets were exposed to UV-C light in an open Petri dish, for 30 days.

(e) 20 tablets of sparfloxacin were crushed to a fine powder and dissolved in methanol, to obtain a final drug concentration of 1 mg/mL. This solution was mixed thoroughly by shaking and sonication and a 25-mL aliquot was then transferred to a Petri dish without lid and exposed to UV-C light for 24 hours.

Two different irradiation sources were used:

UV-A: Samples were placed in Petri dishes at a distance of

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**FIGURE 1 – Chemical structure of sparfloxacin (M.W. 392.4)**
8 cm from the light emission (320 - 400 nm) (HgV, 125 W, Tungsram, Hungary). The control sample was submitted to the same conditions, but covered with aluminum foil. The chamber internal temperature was 40°C ± 1°C. UV-C: Samples were placed in Petri dishes at 10 cm from a Philips TUV germicidal lamp (254 nm) of 30 W, 96 V, (0.36) with 400 mW/cm², determined with a near-UV meter, model J225 (Blak-Ray). The reaction was carried out in a chamber of 100 x 16 x 16 cm, internally mirrored, maintained at 20°C ± 1°C.

Characterization of degradation products: The degradation of SPAX samples was characterized by TLC, using two solvent systems:
- chloroform: methanol: formic acid (18: 07: 01, v/v/v)

RESULTS AND DISCUSSION

The development of stability tests for the determination of degradation products in drugs and medicines has received considerable attention in the last decade because of their importance in quality control in pharmaceutical analysis.

The World Health Organisation and Unicef carried out a study on the stability of essential drugs during international transport, to assure quality control and stability of these drugs in tropical countries. In this study, for the antibiotics chloramphenicol, ampicillin, benzylpenicillin, and tetracycline, the unprotected substance showed signs of degradation (Hogerzeil et al., 1992).

A recent survey of the literature revealed an excellent book on the advances made in drug degradation kinetics (Carstensen, 1995).

Degradation products of drugs are considered to be transformation products of the pharmaceutical compound, formed by the effect of heat, humidity, light, oxidizing agents, solvents, chemical reagents, etc (Görög, 2000). Tests should be performed on representative samples, for example, 20 capsules or tablets, in order to assure a homogenous distribution (Matthews, 1999).

After the extractive procedure using semipreparative-HPLC, the collected fractions from the SPAX-RS and SPAX-TAB samples were taken to dryness in a rotatory evaporator (<60°C) under reduced pressure and visualized by TLC using chloroform: methanol: formic acid (18: 07: 01, v/v/v) and dichloromethane: methanol: ammonium hydroxide: acetonitrile (4: 4: 2: 1, v/v/v/v) as mobile phase. Because these fractions did not exhibit high purity, they were analyzed by column chromatography with silica gel as stationary phase and 5% acetic acid : methanol : acetonitrile (75:12.5:12.5, v/v/v) as eluent. Thirty fractions (5 mL) were collected, characterized by TLC, and similar fractions were aggregated. Thus, two substances were isolated, designated as SPAX-PDP1 and SPAX-PDP2.

Identification of the two partially-purified degradation products by NMR analysis was hindered by the low solubility of the products, as well as the of the reference standard, SPAX-RS. Consequently, the solubility of these photodegradation products was tested in different solvents, such as chloroform, benzene, trifluoroacetic acid, water, methanol, pyridine and DMSO, besides evaluating effects of temperature and ultrasonic equipment.

The 1H (Fig. 2) and 13C NMR (Fig. 3) spectra were recorded in water; however, with previous solubilization in a small amount of NaOH solution, allowing the characterization of protons and carbons. The related lines in 1H-NMR patterns of SPAX-RS in D2O and other fluoroquinolone derivatives are presented in Table 1, while UV absorption peaks of SPAX are compared with literature data in Table 2. The totally-detached 13C-NMR of SPAX-RS allowed attributions presented in Table 3 to be established. For the structural elucidation 1H-13C COSY is presented in Fig. 4.

For SPAX-PDP1 infra-red spectra, under the same conditions, was not observed the absorption around 1700 cm⁻¹, related to C = O stretching was not observed, possible indicating the loss of carbonyl and carboxyl (Fig. 5). In the SPAX-PDP2 IR spectrum it is possible to see peaks of the ketone and carbonyl groups at 1638 and 1713 cm⁻¹, respectively, which are present in next to those of the undergraded substance. Substances with groups C=O and H-O possess absorption peaks corresponding to intramolecular hydrogen bonds at ~ 3070 cm⁻¹ (Fig. 6). However, SPAX does not show this peak. This suggests the C₅ amino group may react intramolecularly with the ketone oxygen. A second point also relates to this aspect. Compounds such as phenols, amides, nitriles and amines can react with iron (III) nitrate or copper. This reaction is used for the identification of various drugs and for qualitative and quantitative analyses. However, sparfloxacin cannot be analyzed using iron (III) nitrate in 1% nitric acid, as the ferric ion does not form complex with this fluoroquinolone. Moreover, the amine group at C₅ could.

![FIGURE 2 – 1H NMR spectrum of SPAX in D2O](image-url)
Table 1 - Relation among $^1$H - NMR spectra of sparfloxacin RS (D$_2$O) and other fluoroquinolones.

<table>
<thead>
<tr>
<th></th>
<th>SPAX D$_2$O</th>
<th>SPAX b</th>
<th>COMP. 10c</th>
<th>CIPX d</th>
<th>M5 e</th>
</tr>
</thead>
<tbody>
<tr>
<td>H (C2)</td>
<td>8.27</td>
<td>8.48</td>
<td>8.70</td>
<td>8.40</td>
<td>9.26</td>
</tr>
<tr>
<td>CH (cyclopropane)</td>
<td>3.77</td>
<td>3.97</td>
<td>3.77</td>
<td>3.61</td>
<td>3.5 – 4.2</td>
</tr>
<tr>
<td>CH, CH$_2$ (piperazinyl)</td>
<td>2.74-3.70</td>
<td>2.60-3.77</td>
<td>2.66-3.05</td>
<td>~ 3.0</td>
<td>-</td>
</tr>
<tr>
<td>CH$_3$ (piperazinyl)</td>
<td>1.0</td>
<td>0.75-1.20</td>
<td>0.97-1.25</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CH$_2$ (cyclopropane)</td>
<td>0.92-1.34</td>
<td>0.75-1.20</td>
<td>0.97-1.25</td>
<td>1.05 – 1.31</td>
<td>1.38 – 1.64</td>
</tr>
<tr>
<td>NH$_2$</td>
<td>-</td>
<td>7.17</td>
<td>6.03</td>
<td>-</td>
<td>-</td>
</tr>
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</table>

Table 2 - Comparison of the peak wavelengths of absorption of sparfloxacin reference substance and literature data in the UV region.

<table>
<thead>
<tr>
<th>compound</th>
<th>$\lambda$ (nm)</th>
<th>$\lambda$ (nm)</th>
<th>$\lambda$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPAX-reference substance</td>
<td>368</td>
<td>292</td>
<td>226</td>
</tr>
<tr>
<td>Compound 10 a</td>
<td>350</td>
<td>288</td>
<td>242</td>
</tr>
</tbody>
</table>

Table 3 – $^{13}$C NMR lines of sparfloxacin–RS in D$_2$O and DMSO with ciprofloxacin data.

<table>
<thead>
<tr>
<th></th>
<th>SPAX (DMSO)</th>
<th>SPAX (D$_2$O)</th>
<th>CIPX d</th>
</tr>
</thead>
<tbody>
<tr>
<td>COOH</td>
<td>180</td>
<td>178</td>
<td>172</td>
</tr>
<tr>
<td>CO</td>
<td>165</td>
<td>172</td>
<td>171</td>
</tr>
<tr>
<td>C2</td>
<td>150</td>
<td>147</td>
<td>166</td>
</tr>
<tr>
<td>C6</td>
<td>130-140</td>
<td>-</td>
<td>160</td>
</tr>
<tr>
<td>C8</td>
<td>130-140</td>
<td>-</td>
<td>160</td>
</tr>
<tr>
<td>C7</td>
<td>130-140</td>
<td>137</td>
<td>106</td>
</tr>
<tr>
<td>C8a</td>
<td>130-140</td>
<td>135</td>
<td>144</td>
</tr>
<tr>
<td>C4a</td>
<td>106</td>
<td>109</td>
<td>117</td>
</tr>
<tr>
<td>C3</td>
<td>105</td>
<td>115</td>
<td>114</td>
</tr>
<tr>
<td>C5</td>
<td>62</td>
<td>68</td>
<td>109</td>
</tr>
<tr>
<td>CH$_2$ –piperazine</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CH$_2$ – piperazine</td>
<td>-</td>
<td>-</td>
<td>46-49</td>
</tr>
<tr>
<td>CH – cyclopropane</td>
<td>40 (1C)</td>
<td>39 (1C)</td>
<td>41 (1C)</td>
</tr>
<tr>
<td>CH$_3$ – piperazine</td>
<td>20 (2C)</td>
<td>17.4 (2C)</td>
<td>-</td>
</tr>
<tr>
<td>CH$_2$ – cyclopropane</td>
<td>7 (2C)</td>
<td>7.5 (2C)</td>
<td>10 (2C)</td>
</tr>
</tbody>
</table>

The carboxylate ion gives two IR bands, an intense one at 1650 to 1550 cm$^{-1}$, associated with anti-symmetrical axial deformation, and a weaker band around 1400 cm$^{-1}$ due to symmetrical axial deformation. The SPAX-PDP2 shows a broad band in the region of 1700 cm$^{-1}$, whereas it is not possible to verify this characteristic band in the SPAX-PDP1 spectrum. The analysis by NMR of partially-purified SPAX-PDP2 discloses the presence of H on C-2 and the
loss of the cyclopropane and the piperazine rings (Fig. 7). The lines in $^1$H-NMR spectrum of SPAX-RS were attributed to groups as presented in Table 2.

The interaction of sparfloxacin with β-cyclodextrin has been shown to increase the stability of this fluoroquinolone, by several analytical procedures, including $^1$H-NMR, $^{13}$C-NMR, fluorescence spectroscopy, infrared spectroscopy, thermal analysis, and scanning electron microscopy (Chao et al., 2004). NMR techniques can provide not only quantitative information but also detailed information about the geometry of the complex. 2D nuclear effect spectroscopy, one of the many NMR tools, has proven to be a powerful technique for investigating intermolecular interaction (Chao et al., 2004).

The products of degradation of a drug can be responsible for its toxicity. On the other hand, very stable drugs can become environmental pollutants. Consequently, the study of the stability of drugs has stimulated important discussions in recent decades (Yoshida et al., 1993). Yoshida and coworkers (1993) carried out the degradation of levofloxacin, obtaining 10 products with modifications in the piperazine group. From these results, those authors concluded that the fluoroquinolone in question was sensitive to photodegradation, with oxidation in the substituent on the C-7, and that levofloxacin, being degraded by sunlight, does not come to be recognized as a pollutant. SPAX-PDP1 and SPAX-PDP2 exhibited melting points > 300°C. Such temperatures are also reached by quinolonic derivatives, as reported by Koga and coworkers (1980).

Ciprofloxacin metabolites have been isolated and their structure elucidated by Gau et al. (1986). Phillips and coworkers (1990) confirmed the loss of antibiotic activity of ciprofloxacin. However, some studies have reported antimicrobial activity in fluoroquinolone degradation products such as moxifloxacin and levofloxacin (Thoma e
Macías-Sanchez (1994) described a physico-chemical study of interaction between fluoroquinolones such as ciprofloxacin and ofloxacin and polyvalent cations. Bioassay and liquid chromatographic methods with UV detection have been used to study the stability of sparflloxacin in the raw material and tablets (Marona et al., 1999; Marona e Schapoval, 2001a). The bioassays were carried out to test for antibacterial activity in sparflloxacin and its degradation products (Marona e Schapoval, 2001a). A significant loss of antibacterial activity of sparflloxacin against \textit{E. coli} NCTC 10418 was observed after exposure to UV-C light, confirming that sparflloxacin was sensitive to photodegradation.

According to Engler and coworkers (1998), low stability is related to the high degree of fluorination, and this favored the choice of SPAX for the photodegradation study. The results demonstrate the photochemical instability of SPAX, when exposed to UV-C; however, the product proved be stable to UV-A light UV over 90 days. The existence of photodegradation products could lead to side effects as well as toxicity during antibiotic therapy. This finding is a source of great concern and suggests the need for research on the mechanism of photodegradation.

An important factor in the improvement of pharmaceutical stability testing has been the development of analytical methods that are suitable for routine use in a stability-indicating assay (Kommanaboyina e Rhodes, 1999).

The light used in this stability study was chosen in relation to a basic law of photochemistry, that only absorbed light could start a photochemical process. The sparflloxacin spectra show two characteristic bands between 300 and 200 nm. This range is typical of germicidal light emitted by a low-pressure mercury TUV lamp. This fact was used to define the irradiation source. UV-A did not induce
degradation of any sample after exposure for 90 days. For this reason, the study of photodegradation was carried out with UV-C light.

Finally, sparflouxacin was sensitive to photodegradation under the experimental conditions described. Our results showed the presence of photodegradation products when sparflouxacin was exposed to UV-C light. The applicability of the proposed method for the determination of sparflouxacin and its degradation products was demonstrated by analyzing six aliquots of each sample. HPLC, LC and TLC were carried out, resulting in the isolation of two photodegradation products, whose chemical structures were partially characterized analyzing by 'H-NMR, 13C-NMR, MS, UV and IR spectra. It is of vital importance to develop techniques that facilitate quality control in the manufacture of drugs and medicines, and allow the ideal storage conditions of pharmaceutical products to be determined.

In view of the very complex nature of photochemical decomposition of these drugs, further studies will be performed on the identification and structure elucidation of the products.

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