Identification, characterization and in silico ADMET prediction of Roflumilast degradation products

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ABSTRACT

The present study reports the degradation behavior of roflumilast (RFL), a new drug developed for the treatment of chronic obstructive pulmonary disease. The degradation of RFL was tested under various stress conditions as per the guidelines of the International Conference on Harmonization. The degradation products (DPs) of RFL were identified, characterized and in silico predictions were made of their pharmacokinetic properties, absorption, distribution, metabolism, excretion and toxicity (ADMET). RFL was subjected to various stress conditions including photodegradation, alkaline and acidic hydrolysis, oxidative and metallic degradation. After analysis by HPLC-DAD, the DPs were isolated by preparative TCL and characterized by high resolution mass spectrometry (HRMS), 1H NMR, 13C NMR and infrared (IR) spectroscopy. RFL tablets were prepared by the addition of solid stress inducing substances such as excipients and storage in an accelerated stability chamber (40 °C; 75% r.h.) for sixteen months. Resulting DPs from the tablets were analyzed by UFLC-QTOF. The most drastic degradation conditions for RFL were 5 M NaOH, 6 M HCl, 7.5% v/v peracetic acid, which resulted in the isolation of four DPs. However, milder degradation conditions (1 M NaOH and photoysis) generated six DPs (DP-1, 2, 3, 5, 7 and 8), and are more similar to the actual conditions the drug will be exposed. For tablets containing RFL exposed to an alkaline reagent, two DPs were formed: DP-1 and DP-11. Whereas RFL-containing tablets exposed to acid and oxidizing agents, formed one product DP-11. Forced degradation of RFL led to the formation of eleven DPs, seven of which have never been previously reported. RFL is stable under metabolic stress and it is relatively stable during photodegradation testing. The UFLC-QTOF methodology detected a greater number of DPs that formed during the stress conditions tested when compared to the HPLC-DAD methodology. In silico prediction of the ADMET properties of the RFL degradation products and metabolites produced in this study are potentially hepatotoxic.

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1. Introduction

Roflumilast (RFL), 3-(cyclopropylmethoxy)-N-[3,5-dichloropyridin-4-yl]-4-(difluoromethoxy) benzamide, is used in the treatment of chronic obstructive pulmonary disease (COPD) and was launched to the pharmaceutical market in 2010 [1]. RFL and its active metabolite (roflumilast N-oxide) are selective inhibitors of phosphodiesterase-4, which exerts anti-inflammatory effects by preventing the breakdown of cAMP, thus allowing the activation of protein kinase A. Anti-inflammatory effects attributed to RFL include a reduction in neutrophil release of inflammatory mediators, monocytes, and cytokines from CD8+ and CD4+ T cells; inhibition of fibrotic lung remodeling; and a reduction in oxidative stress [1,2].

According to the International Conference on Harmonization (ICH) [3], it is of fundamental importance that the mechanisms governing degradation of newly developed long-term use drugs, such as RFL, are well understood. It is also essential that the identity of the degradation products (DPs) and their potential toxicities are characterized. The identification of DPs allows the development of more selective and secure analytical methods, and facilitates the
study of their pharmacokinetic properties, absorption, distribution, metabolism, excretion and toxicity (ADMET).

The structure of RFL indicates its apparent lability, specifically related to the cyclopropane substituent, which is unstable at acidic pH. Subsequently, its amide group renders it more highly prone to hydrolysis, making stabilization of the drug pharmaceutically challenging. No RFL monographs has been reported by any pharmacopoeia and a comprehensive survey of existing literature reveals the absence of detailed studies reporting the forced degradation of RFL and the toxicity of its degradation products. The objectives of these studies differed: some were directed towards validating HPLC-DAD analytical methods and showed forced degradation results (acidic, alkaline and neutral hydrolysis, oxidation, heating and photostability) of RFL. However, these previous studies did not report isolation or characterization of the resulting degradation products [4–8]. Furthermore, no study was conducted evaluating the degradation products that formed in the final pharmaceutical form produced, which is essential for effectively ensuring safety and efficacy. 

Dash and Paul (2017) established a HPLC method for the quantitative determination of RFL along with its degradation products, and identified the structure of three main degradation impurities: N-(3,5-dichloropyridin-4-yl)-4-(difluoromethoxy)-3-hydroxybenzamide, N-(3,5-dichloropyridin-4-yl)-3-(cyclopentyloxymethoxy)-4-hydroxybenzamide and roflumilast N-oxide [9]. Paul and Dash (2015) reported the structural identification of four RFL degradation products using high resolution mass spectrometry (HRMS) and theoretical investigation by density functional theory (DFT) namely: 3,5-dichloropyridin-4-amine, N-(3,5-dichloropyridin-4-yl)-4-(difluoromethoxy)-3-hydroxybenzamide, N-(3,5-dichloropyridin-4-yl)-3-(cyclopentyloxymethoxy)-4-hydroxybenzamide and N-(3,5-dichloro-1-oxo-pyridin-4-yl)-3-(difuoro methoxy) benzamide [10]. Some of these studies concluded that RFL is stable during dry heat (80 °C, 24 h to seven days), neutral hydrolysis (80 °C, 8–24 h) and ultraviolet radiation conditions (254 nm for seven days) [4,7–9]. Assessment of the toxicity and safety profiles of RFL degradation products is essential and not described in these previous studies. This information is necessary for the reduction of risk-based control limits in drug substances and/or drug products as applicable.

The aim of the present study was to investigate the degradation profile of RFL under various stressors including alkaline and acidic hydrolysis, metallic, oxidation and photostability conditions. The degradation profile of RFL tablets formulated with different excipients (acidic, alkaline or oxidative) was also evaluated. The structural elucidation of the DPs allowed in silico prediction of absorption, distribution, metabolism, excretion and toxicity (ADMET) properties to be performed. The study of toxicity of RFL degradation products was not described in previous studies, despite being essential for the reduction of risk-based control limits in drug substances.

2. Experimental

2.1. Drug and reagents

Roflumilast was purchased from Tractus Company Limited (Hong Kong, China). Urea hydrogen peroxide was obtained from Sigma-Aldrich Ltda (São Paulo, Brazil). The reagents HPLC grade acetonitrile (MeCN), methanol (MeOH), ethyl acetate (EtOAc), ethanol (EtOH) and analytical reagents grade, sodium hydroxide (NaOH), chloridric acid (HCl), acetic acid glacial, triethyamine (TEA) were purchased from Tedia (Rio de Janeiro, Brazil). Deuterated dimethyl sulfoxide (DMSO-d6, 99.5% + 0.05% TMS) was obtained from Cambridge Isotope Laboratories, Inc. (Massachusetts, USA). Analytical thin layer chromatographic (TLC) (silica gel, aluminum sheets 60 F254) was purchased from Merck (Darmstadt, Germany). Additionally, ammonium acetate, sodium sulphate anhydrous, phosphomolybdic acid solution 7%, tartaric acid, sodium carbonate anhydrous, magnesium stearate, peracetic acid, microcrystalline cellulose and cupric sulphate pentahydrate were purchased from local suppliers. Purified water for preparing solutions and performing HPLC studies was obtained by distillation water treatment (Quimis, Diadema, Brazil).

2.2. Degradation conditions

Forced degradation was carried out under hydrolytic, oxidative, photolytic and metallic conditions according to ICH guidelines [3,11,12]. All of the degradation conditions were carried out using a large amount of RFL (0.5–1.0 g) to ensure that detectable amounts of DPs were produced [13,14]. The solubility of RFL was a limitation in this study since it is insoluble in water [1], therefore EtOH (± 15 ml) was used in acidic hydrolysis and oxidative degradation conditions to increase solubility of RFL. Each solution obtained from the subsequent stress conditions: alkaline/acidic hydrolysis, metallic and oxidative conditions were subjected to liquid–liquid extraction (LLE) with EtOAc, without a previous neutralization step. These extractions resulted in organic alkaline, acid, and neutral fractions (OFalk, OFac, OFmet) as well as an aqueous fraction (AF). Aqueous fractions were lyophilized in a 101L IOTOP lyophilizer (São Carlos, SP, Brazil) and the resulting solid was dissolved in MeOH and identified by TLC and HPLC-DAD. Organic solvent was removed from the samples using the rotary evaporator RV 10 (Ika, Staufen, Germany), while the obtained solid was solubilized in MeOH for analysis by TLC and HPLC-DAD.

Alkaline hydrolysis of 1 g of RFL was carried out by refluxing for 24 h into 30 ml 5 M NaOH(0.5). Whereas, the milder alkaline hydrolysis of 1 g of RFL was performed by refluxing for 48 h into 30 ml 1 M NaOH(0.5). The RFL was completely dissolved in both alkaline solutions. Acidic hydrolysis of 1 g of RFL was performed by refluxing for 24 h into 30 ml 6 M HCl hydrochloric acid. Metallic stress was performed with 500 mg of RFL refluxed for 48 h into 40 ml of 17 mM CuSO4 5H2O hydro-alcoholic solution. For oxidative stress conditions, 1 g of RFL was refluxed for 48 h into 15 ml 7.5% (v/v) peracetic acid hydroalcoholic solution.

The photodegradation study was performed using a photosatibility chamber 420-CLDTS3000 (Nova Ética, São Paulo, Brazil) equipped with both UV and fluorescent lamps. The solid drug was degraded by exposure to light inside an inert transparent glass bottle. Two drug solutions were prepared using 10 mg/ml RFL in either MeCN or MeOH, then subjected to the photodegradation test. As a control, a light protected sample was subjected to the same conditions. After 48 h of light exposure, the samples (control and test preparations) were solubilized in MeOH and then analyzed by HPLC-DAD and UPLC-QTOF.

2.3. Accelerated stability studies of RFL tablets

Accelerated stability studies were carried out with tablets composed of 20 mg of RFL, 100 mg of cellulose microcrystalline, 0.1% of magnesium stearate and 10 mg of stress agents, oxidative (urea-hydrogen-peroxide), alkaline (sodium carbonate) or acid (tartaric acid). Tablets were produced using a hydraulic press, Ola Quality (La Accessories, New Jersey, USA) and immediately placed into a stability chamber at 40 °C and 75% of r.h. (Nova Ética, São Paulo, Brazil). Tablets were organized into control (without RFL) and test groups. Both of these groups were prepared by wet (96% v/v ethanol) and dry granulation and stored in transparent glass beakers and divided into kraft paper (semi-permeable package) and
Parafilm® sealing film (impermeable package) (Heathrow Scientific, United Kingdom) groups (the beakers were covered with paper or Parafilm® to simulate semi-permeable and impermeable conditions). After sixteen months, both the control and test tablets were solubilized in MeOH for analysis by HPLC-DAD and UFLC-QTOF.

2.4. Isolation, identification and characterization of the degradation products

2.4.1. Isolation and characterization of DPs

The degradation products obtained by the LLE method were isolated with preparative TLC using silica gel, pH 7 with 400 mesh ASTM (SPLabor, São Paulo, Brazil). Preparative TLC was carried out using EtOAc:hexane (1:1, υ/υ) as the eluent. The isolated DPs obtained by preparative TLC were identified using the HPLC-DAD method.

Elucidation of structure was performed by HRMS ([M-H]− and [M+H]+) using a Bruker microTOF II mass spectrometer by electron spray ionization (ESI). NMR spectra (1H- and 13C NMR) were recorded using an Avance 200 MHz spectrometer (Bruker, Billerica, MA, USA) and solvent DMSO-d6. Chemical shifts were given in ppm (δ scale) and coupling constants (J) were given in hertz (Hz). IR spectra were obtained using a IRPrestige-21 FTIR spectrometer (Shimadzu, Tokyo, Japan) and KBr pellets.

2.4.2. Development of the HPLC-DAD method

RFL degradation products that formed during stress conditions needed to be selectively detected using specifically developed HPLC-based methods, as previously described [4-6,9]. In this study, separation of the drug and DPs was carried out using high-performance liquid chromatography (HPLC), equipped with: Ultimate 3000 pump LPG; auto sampler WPS-3000 TSL; column compartment TCC-3000 SD and diode array detector DAD (Thermo Fisher, São Paulo, Brazil). The system was controlled using the Chromeleon™ 6.8 Chromatography System Software (Thermo Fisher, São Paulo, Brazil). Column selection was based upon studies conducted by Jain & Basniwal (2013) and analysis was performed using a X Terra® C18 MS analytical column (250 × 4.6 mm; 5 μm) [15]. Conditions selected included a mobile phase (A) 5 mM ammonium acetate adjusted to pH 4.2 using acetic acid and 0.5% (υ/υ) TEA, and in (B) MeCN was used during the gradient mode: T(min)/%B, 0/30, 25/90, 26/30, 30/30. Temperature of the column was maintained at 25 °C, flow rate was 0.8 ml/min during the run and the detection was performed at 245 nm. The samples in methanol (20 μl) were injected into the HPLC system for analysis.

2.4.3. UFLC-QTOF

In the stress tests some DPs may be formed in very small amounts, below the detection limit of the HPLC-DAD. Therefore, the fractions obtained in the stress tests (alkaline hydrolysis with 1 M NaOH(aq), oxidative degradation with 7.5% υ/υ peracetic acid and photodegradation solutions) were analyzed by the analytical method UFLC-QTOF. UFLC-QTOF was used for the characterization of DPs formed without isolation by preparative TLC. HPLC methods were adapted to determine the molecular identity of the isolated DPs, using ultra-fast liquid chromatography (UFLC) (Nexera, Shimadzu, Japan). The UFLC system was equipped with a quadrupole time-of-flight detector mass spectrometer (QTOF) Compact™ and controlled using Compass Data Analysis software, version 4.2 (Bruker, Germany). The DPs were separated and characterized using an Acquity UPLC BEH Shield RP C18 column (100 × 2.1 mm, 1.7 μm) from Waters Corporation (Wexford, Ireland). A mobile phase consisting of (C) 5 mM ammonium acetate (pH 4.2) using acetic acid and (B) MeCN in gradient mode; time (minutes)% B: 0/30, 6/90, 6.5/30, 7/30. During the analysis, the column was maintained at room temperature [21 °C ± 2 °C] with a flow rate of 0.3 ml/min. The samples were solubilized in methanol since the QTOF detector is incompatible with TEA. The potential structures were defined, their exact mass values were calculated and searched using the high resolution mass spectrum. Ions within 3–5 ppm error to the exact mass normally correspond to the molecular ion, unless there is a difference in the actual structure [16].

2.5. In silico toxicity analyses

ADMET parameters were performed using ADMET Predictor™ version 6.0 (Simulations Plus, Inc., Lancaster, CA, USA) in qualitative and quantitative models. Hepatotoxicity parameters were specifically studied using relevant biomarkers: alkaline phosphatase (ALP), serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), gamma-glutamil transferase (GGT) and lactate dehydrogenase (LDH). In addition, mutagenicity was predicted in five individual strains of Salmonella typhimurium with or without metabolic activation and/or Escherichia coli. Acute toxicity measures the capability of a compound to kill 50% of the test animals/organisms within 24 h of exposure (LD50) and expresses the relative hazards associated with exposure to the DPs. Prediction of metabolic indicators was based on phase I and phase II analysis CYP P450 inhibition prediction included models for CYP 2C8, 2C9, 3A4, 1A2, 2E1, 2A6, and 2C19. All the CYP-metabolites generated for each DP, were evaluated for risk potential whenever possible. In addition, Phase II metabolism of DPs was investigated to determine the probability that human uridine 5'-diphosphate-glucuronosyltransferases (UGT) were involved. All models were accumulated and criteria measurements of ADMET Risk were assessed and scored in the 0–24 range. These values indicate the number of potential ADMET risk factors that a compound might possess.

3. Results and discussion

3.1. Obtaining DPs using stress conditions

Rolflumilast degradation was tested under forced conditions including alkaline and acidic hydrolysis, oxidation, metallic and photolysis. The OF and AF obtained were first analyzed by TLC, with the purpose of confirming degradation of the drug. Next, the fractions were analyzed by HPLC-DAD. The AF and OF obtained from RFL degradation was found to be most stable under conditions using metallic degradation, photodegradation of solids and solubilization in methanol.

The most drastic condition, alkaline hydrolysis (5 M NaOH(aq)), resulted in the production of two DPs, DP-2 and DP-3, which were separated from the acidic organic fraction (OFak) (Fig. 1A). Two DPs, DP-1 and DP-3 were separated from the alkaline organic fraction (OFal) (Fig. 1B). Under milder alkaline hydrolysis conditions (1 M NaOH(aq)), in the OFal (Fig. 1A) and the OFak (Fig. 1B) three DPs were separated, DP-2, DP-3, DP-5 and DP-1, DP-3, DP-5, respectively.

The acidic hydrolysis condition (6 M HCl(aq)) (Fig. 1C) generated only DP-1 in the OFalk fraction. Whereas, the oxidative degradation condition (peracetic acid) generated a major DP, DP-4, which was detected only in OFak (Fig. 1D).

Analysis of the RFL 1H NMR spectrum indicated that fourteen protons are present in the structure. The signal at δ 10.64 ppm corresponds to an amide proton and the signal at δ 8.77 ppm corresponds to a proton present on the pyridine ring (Table 1). The 13C NMR spectrum indicated that RFL has seventeen carbons in its structure. The signal at δ 164.38 ppm corresponds to a carbonyl group carbon and the signal at δ 121.46 ppm corresponds to a carbon in the CHF2 group.
The DP-1 structure included an amino-pyridine ring producing a proton signal at δ 8.14 ppm and a NH$_2$ group hydrogen at δ 6.64 ppm (Table 1). The $^{13}$C NMR spectrum of DP-1 has three carbon signals, which can be seen in Table 1. Data from IR spectrometry and HRMS (Table 1) was characterized as the structure of 3,5-dichloropyridin-4-amine. In the $^1$H- and $^{13}$C NMR spectra of DP-2 (Figs. 51a and S1b) the amino pyridine ring was absent. Furthermore, the proton signal of the CH$_2$ group was absent at δ 7.23 ppm and the carbon signal was absent at δ 121.46 ppm that came from the same group in DP-2. The $^1$H- and $^{13}$C NMR spectra of DP-3 have proton and carbon signals at similar ppm as RFL, indicating that the amide group had not been hydrolyzed. However, the absence of the proton signal from the CH$_2$ group at δ 7.23 ppm and the absence of the carbon signal from this same group at δ 121.46 ppm indicate that DP-3 is similar to RFL however, without the CH$_2$F group. Data from HRMS and IR spectrometry (Table 1) indicated that the structure of DP-2 was: 3-(cyclopromethyl)oxy)-4-hydroxybenzoic acid, and for DP-3 it was: N-(3,5-dichloropyridin-4-yl)-3-(cyclopromethyl)-4-hydroxybenzamide. The HRMS data allowed observation of a sodium adduct of DP-3. Infrared spectrometry was not performed with DP-2 due to the limited amount of product. The $^1$H- and $^{13}$C NMR spectra of the DP-4 are very similar to the RFL, however one difference is the displacement of the proton signal "a" at δ 8.8 ppm in RFL to δ 8.5 ppm in DP-4 (Table 1). This proton is influenced by the presence of oxygen attached to nitrogen (N-oxide group). In addition, the data obtained from infrared spectrometry corresponds to roflumilast N-oxide (Table 1). $^1$H NMR spectrum confirms that the DP-5 structure (Fig. S2a) does not show the characteristic signal of the pyridine ring protons, whereas all the other protons signals are present. In the $^{13}$C NMR spectrum of the DP-5 (Fig. S2b) three carbons referring to the pyridine ring were not present. The change in chemical shift of the carbonyl group from 164.38 ppm to δ 167.06 ppm, which changed from amide to acidic function respectively, confirms the hydrolysis of the amide group. The signal at δ 122.86 ppm corresponding to the carbon in the CH$_2$F group confirms the structure of the DP-5, 3-(cyclopromethyl)-4-(difluoromethoxy)benzoic acid. The infrared spectroscopy (Table 1 and Fig. S3) reports the intensities of the bands in the experiment.
Tables 1  
H and 13C NMR chemical shift assignments, infrared and HRMS data of roflumilast and DPs.

<table>
<thead>
<tr>
<th>Material</th>
<th>Infrared Spectrometry (cm⁻¹)</th>
<th>¹H NMR (DMSO-d₆) δ (ppm)</th>
<th>¹³C NMR (DMSO-d₆) δ (ppm)</th>
<th>HRMS-ESI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>m/z measured</td>
</tr>
<tr>
<td>RFL</td>
<td>3249, 3096, 2928, 1653, 1558, 1200, 1155, 1200, 1155, 1047, 1098</td>
<td>10.64 (s, 1H), 8.77 (s, 2H), 7.73–7.50 (m, 2H), 7.40–7.21 (m, 2H), 3.99 (d, J = 6.5 Hz, 2H), 1.30–1.25 (m, 1H), 0.65–0.56 (m, 2H), 0.47–0.37 (m, 2H)</td>
<td>164.3, 150.2, 148.8, 143.5, 141.7, 131.2, 130.9, 121.4, 121.0, 117.0, 114.3, 73.9, 10.5, 3.6</td>
<td>–</td>
</tr>
<tr>
<td>DP-1</td>
<td>3443, 1620, 1494, 1413, 1273, 1088, 892</td>
<td>8.14 (s, 2H), 6.64 (s, 2H)</td>
<td>146.9, 146.5, 115.1</td>
<td>162.9823 [M+H⁺]</td>
</tr>
<tr>
<td>DP-2 **</td>
<td>7.49–7.34 (m, 2H), 6.85 (d, J = 8.6 Hz, 1H), 3.82 (d, J = 6.9 Hz, 2H), 1.32–1.12 (m, 1H), 0.59–0.50 (m, 2H), 0.36–0.28 (m, 2H)</td>
<td>168.3, 151.6, 146.9, 123.9, 123.4, 115.6, 115.1, 73.5, 10.8, 3.7</td>
<td>207.0662 [M-H⁻]</td>
<td>C₁₁H₁₃O₄</td>
</tr>
<tr>
<td>DP-3</td>
<td>3198, 1655, 1600, 1483, 1305, 1197, 1020</td>
<td>8.71 (s, 1H), 7.60–7.47 (m, 1H), 6.33 (d, J = 8.6 Hz, 1H), 3.87 (d, J = 6.9 Hz, 2H), 1.36–1.16 (m, 1H), 0.66–0.47 (m, 2H), 0.41–0.26 (m, 2H)</td>
<td>164.9, 151.8, 148.6, 147.2, 142.5, 131.3, 123.9, 122.5, 115.7, 114.0, 73.7, 10.8, 3.7</td>
<td>375.0280 [M+Na⁺]</td>
</tr>
<tr>
<td>DP-4</td>
<td>3226, 2992, 1659, 1534, 1488, 1285, 1138, 838</td>
<td>8.55 (s, 2H), 7.74–7.52 (m, 2H), 7.31–7.15 (m, 2H), 3.94 (d, J = 6.8 Hz, 2H), 1.38–1.14 (m, 1H), 0.66–0.55 (m, 2H), 0.43–0.27 (m, 2H)</td>
<td>164.6, 149.9, 142.7, 138.2, 133.4, 130.8, 121.5, 120.7, 117.1, 114.4, 112.0, 73.7, 10.5, 3.5</td>
<td>–</td>
</tr>
<tr>
<td>DP-5</td>
<td>3093, 2927, 1683, 1212, 1158</td>
<td>7.60–7.50 (m, 2H), 7.28–7.16 (m, 2H), 3.92 (d, J = 6.9 Hz, 2H), 1.28–1.14 (m, 1H), 0.61–0.50 (m, 2H), 0.37–0.31 ppm (m, 2H)</td>
<td>168.5, 151.4, 145.3, 130.6, 124.3, 122.1, 118.4, 118.7, 75.1, 11.9, 5.0</td>
<td>257.0615 [M-H⁻]</td>
</tr>
</tbody>
</table>

*Test not performed since sufficient substrate not available. Frequency 200 MHz.
RFL: 3-(cyclopropylmethoxy)-N-(3,5-dichloropyridin-4-yl)-4-(difluoromethoxy) benzamide.
DP-1: 3,5-dichloropyridin-4-amine.
DP-2: 3-(cyclopropylmethoxy)-4-hydroxybenzoic acid.
DP-3: N-(3,5-dichloropyridin-4-yl)-3-(cyclopropylmethoxy)-4-hydroxybenzamide.
DP-4: roflumilast N-oxide.
DP-5: 3-(cyclopropylmethoxy)-4-(difluoromethoxy)benzoic acid.

tal spectra at 3093 cm⁻¹ refers to aromatic carbons (–C=H), at 2927 cm⁻¹ refers to carbons of cyclopropane (–CH), at 1683 cm⁻¹ refers to the carbonyl group (C=O), at 1212 cm⁻¹ refers to ether formation between an aromatic carbon and aliphatic carbon (C=C–O–C) and at 1158 cm⁻¹ refers to CF₃ group. HRMS data for DP-5 measured the accurate mass of m/z 257.0615 [M-H⁻] with a mass error of 0.1 ppm (Table 2).

Photostability of the solutions was analyzed by HPLC-DAD, after 48 h of UV–vis irradiation of RFL in solid form and solubilized in MeOH and MeCN. A negative result however, was obtained for HPLC-DAD analysis of solid form RFL and therefore, no degradation products were produced. On the other hand, solutions containing MeCN and MeOH, resulted in inconclusive chromatograms. The photostability solutions were also analyzed in the same way as above using the UFLC-QTOF methodology.

3.2. UFLC-QTOF for the identification and characterization of DPs

In real life systems, it is more likely that RFL will not be completely degraded. Therefore, the RFL degradation study conditions including alkaline hydrolysis 1 M NaOHaq, oxidative and photodegradation require methods with lower detection limits and sensitivity than HPLC-DAD, since DPs may be formed in very small amounts. For these reasons, the analytical method UFLC-QTOF allowed the characterization of DPs that formed without the need for isolation by preparative TLC.

The OF₃all and OF₃ac obtained by alkaline hydrolysis (1 M NaOHaq) were evaluated by the UFLC-QTOF system in both positive and negative modes. In OF₃all, the DPs identified were: DP-1, DP-3, DP-5. Whereas, DP-3 was the only DP detected in both the positive and negative modes, DP-1 was only detected in the positive mode and DP-5 was detected only in the negative mode (Table 2). Detection of DPs using either UFLC-QTOF and HPLC-DAD methodologies were found to be similar. Several DPs were identified in the OF₃ac: DP-2, DP-3, DP-5, DP-7 and DP-8 were found using the positive mode, whereas in the negative mode only the DP-7 and DP-8 were uncovered (Table 2). The use of UFLC-QTOF method allowed the detection of two new DPs (DP-7 and DP-8) in a low intensity situation, which had not been detected by the HPLC-DAD method. Table 2 presents the data obtained for OF₃ac resulting from oxidative conditions obtained by UFLC-QTOF in the positive and negative
Table 2: UPLC-QTOF analysis of RFL degradation products and their elemental compositions.

<table>
<thead>
<tr>
<th>DP</th>
<th>Positive Mode [M+H]+</th>
<th>Negative Mode [M-H]-</th>
<th>m/z theoretical</th>
<th>m/z measured</th>
<th>Ion Formula</th>
<th>RT (min)</th>
<th>m/z (ppm)</th>
<th>Intensity</th>
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<tr>
<td>DP-1</td>
<td></td>
<td></td>
<td>309.0988</td>
<td>309.0986</td>
<td>C12H13ClNO3</td>
<td>2.5</td>
<td>3.3 5</td>
<td>-</td>
</tr>
<tr>
<td>DP-2</td>
<td></td>
<td></td>
<td>331.0554</td>
<td>331.0556</td>
<td>C12H12F2NSO</td>
<td>1.7</td>
<td>3.1 3</td>
<td>-</td>
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<tr>
<td>DP-3</td>
<td></td>
<td></td>
<td>391.0073</td>
<td>391.0075</td>
<td>C12H13ClFNO3</td>
<td>1.5</td>
<td>3.1 5</td>
<td>-</td>
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<tr>
<td>DP-4</td>
<td></td>
<td></td>
<td>349.0472</td>
<td>349.0496</td>
<td>C12H13ClFNO3</td>
<td>1.5</td>
<td>3.1 5</td>
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<td>DP-5</td>
<td></td>
<td></td>
<td>373.0863</td>
<td>373.0866</td>
<td>C12H12F2NSO</td>
<td>1.7</td>
<td>3.1 3</td>
<td>-</td>
</tr>
<tr>
<td>DP-6</td>
<td></td>
<td></td>
<td>419.0398</td>
<td>419.0396</td>
<td>C12H13ClFNO3</td>
<td>1.5</td>
<td>3.1 5</td>
<td>-</td>
</tr>
<tr>
<td>DP-7</td>
<td></td>
<td></td>
<td>449.0844</td>
<td>449.0846</td>
<td>C12H13ClFNO3</td>
<td>1.5</td>
<td>3.1 5</td>
<td>-</td>
</tr>
<tr>
<td>DP-8</td>
<td></td>
<td></td>
<td>479.1193</td>
<td>479.1196</td>
<td>C12H13ClFNO3</td>
<td>1.5</td>
<td>3.1 5</td>
<td>-</td>
</tr>
<tr>
<td>DP-9</td>
<td></td>
<td></td>
<td>539.1385</td>
<td>539.1386</td>
<td>C12H13ClFNO3</td>
<td>1.5</td>
<td>3.1 5</td>
<td>-</td>
</tr>
<tr>
<td>DP-10</td>
<td></td>
<td></td>
<td>579.1638</td>
<td>579.1640</td>
<td>C12H13ClFNO3</td>
<td>1.5</td>
<td>3.1 5</td>
<td>-</td>
</tr>
</tbody>
</table>

Note: The table above shows the elemental compositions and retention times of different degradation products of roflumilast (RFL) detected by UPLC-QTOF. The products were divided into two groups: those detected positively (Pos) and negatively (Neg). The m/z theoretical and measured values are presented along with the intensity of detection.

3.3. Results of accelerated stability studies of RFL tablets

RFL in tablet form, in contact with commonly found excipients in pharmaceutical dosage forms, is stable at 45°C and 75% of r.h. for up to sixteen months. The accelerated stability provides a useful tool to evaluate liability of drugs in a pharmaceutical dosage form. Identification and characterization of DPs were only possible using the UFLC-QTOF method, due to the small amount produced. In tablets containing RFL exposed to alkaline agent, two DPs were formed: DP-1 and DP-11. In tablets containing RFL, and...
exposed to acid and oxidizing agents, one product formed, DP-11. No differences were found when RFL tablets were stored in a semi-permeable and impermeable package. Regardless of how the tablets were packaged (permeable or impermeable), RFL is more sensitive to alkaline and oxidative excipients. Thus, RFL interacted with excipients of the formulation when stored under conditions of controlled temperature and humidity leading to the formation of two degradation products. When an interaction occurs between the drug and excipients for solid dosage forms, degradation products form that may interfere with bioavailability and affect drug efficacy and safety [18].

3.4. In silico ADMET analysis

The in silico toxicity study of RFL and DPs are presented in Supplementary data Table S4. The DP-1, DP-4, DP-5, DP-7 as well as RFL were identified as potential hepatotoxic compounds, since elevation of both types of glutamate transferase in serum indicates severe hepatic injury. DP-1 and DP-7 also showed predisposition for respiratory sensitization (TOX_RESP).

Although, RFL and roflumilast O-nxide are not considered mutagenic [19], mutagenic potential was observed for the DPs DP-4 and DP-10 in TA97 and/or TA1537, and TA98 strains of S. typhimurium. DP-1 appears to be potentially mutagenic in the TA102 strain of S. typhimurium and/or WP2 uvrA strain of E. coli. The LD50 acute toxicity in rats was predicted, as well as RFL, as seen in Supplementary data Table S1. All DPs showed higher LD50 values than RFL (389 mg/kg), considered not acutely toxic in rats. This value is classified as slightly toxic according to the Hodge and Sterner toxicity classification scale [20].

The inhibitory potency of the DPs against cytochrome P450 was evaluated for different isoforms. RFL showed greater inhibitory potency against CYP 3A4, as described in the literature [21]. DP-2, DP-5, DP-6 also caused CYP3A4-mediated metabolic reactions. CYP2C9 was predicted to be inhibited for DP-7 and DP-11, and CYP2CB8 for DP-4 and DP-11. Less evidence was shown however, for CYP 2E1 and CYP 2C19 with inhibitory potency towards DP-7 and DP-10, respectively. In addition, DP-9 did not cause any significant phase I reactions. All metabolites that were generated along with their ADMET Risk are presented in Supplementary data Table S1.

The metabolites produced for each DP, excluding DP-9, exhibited an ADMET profile, an important indicator of undesirable ADMET properties. The DP metabolites from DP-1, DP-3, DP-4, DP-5, DP-7, DP-8 and DP-11 also displayed hepatotoxic potential. Mutagenicity was predicted for MI-3 (metabolite of DP-1), MI1I2 (metabolite of DP-3), MI1V3 (metabolite of DP-4), MIIV (metabolite of DP-7), MIIVIII-3, MX and MXI, metabolites of the DP-8, DP-10 and DP-11, respectively, presented potential issues for metabolism.

4. Conclusion

Forced degradation of the RFL resulted in the formation of eleven degradation products, seven never reported before as RFL degradation products (DP-2, DP-5, DP-6, DP-7, DP-8, DP-9 and DP-10). The most drastic degradation conditions (5 M NaOH, 6 M HCl, 7.5% v/v peracetic acid) allowed degradation products to be isolated in sufficient amounts for characterization by HPLC-DAD, 1H and 13C NMR, IR and HRMS. However, milder degradation conditions (1 M NaOH and photolysis) are relevant since they are most similar to conditions that the drug will be exposed. These conditions generated a larger number of degradation products, however in smaller quantities. The lower yield of DP in these milder conditions necessitated the use of UFLC-QTOF for their separation and characterization. The UFLC-QTOF methodology identified a greater number of degradation products formed during oxidative stress conditions when compared to the HPLC-DAD method. This confirmed the importance of techniques such as UFLC-QTOF, which can be used to detect and evaluate DP produced in smaller quantities, resulting from degradation protocols that may be perhaps better mimetic actual conditions to which the drug is exposed. Roflumilast is stable under metallic stress and it is relatively stable during photodegradation testing – from these condition only DP-11 was identified.

The manufacture of RFL tablets with alkaline, acid and oxidative excipients and exposure of these tablets to accelerated temperature and humidity conditions resulted in the formation of two of the eleven identified degradation products that could be potentially produced (DP-1 and DP-11).

The structural characterization of the degradation products formed during the forced degradation study, enabled the in silico prediction of ADMET properties of these products and metabolites. These studies demonstrated that most of these compounds produced are potentially hepatotoxic. All DPs exhibited LD50 values that were higher than RFL, therefore they are considered to be slightly toxic according to the Hodge and Sterner toxicity classification scale.

Conflict of interest

None of the authors of this paper have a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jpba.2017.02.012.

References


