Vol. 6, No. 2, 2015
ISSN 2233-4203/ e-ISSN 2093-8950
LETTER www.msletters.org | Mass Spectrometry Letters

Identification of Degradation Products in the Phosphodiesterase (PDE-4) Inhibitor Roflumilast Using High Resolution Mass Spectrometry and Density Functional Theory Calculations

Saroj Kumar Paul* and Upendra N. Dash

Department of Chemistry, Institute of Technical Education and Research (ITER), Siksha 'O' Anusandhan University, Bhubaneswar, Odisha, India

Received March 18, 2015; Revised April 12, 2015; Accepted April 20, 2015
First published on the web June 30, 2015;o DOI: 10.5478/MSL.2015.6.2.38

Abstract: Roflumilast analogs are a group of drugs which act as selective photodiesterase (PDE-4) inhibitor for the treatment severe chronic pulmonary disease associated with chronic brochnonities. Structural identification of degradation products using high resolution mass spectrometry and theoretical investigation by density functional theory have been successfully carried out on roflumilast to identify four degradation products namely, 3,5-dichloropyridin-4-amine, *N*-(3,5-dichloropyridin-4-yl)-4-(difluoromethoxy)-3-hydroxy benzamide, *N*-(3,5-dichloropyridin-4-yl)-3-(cyclopropylmethoxy)-4-(difluoromethoxy) benzamide and 3-(cyclopropylmethoxy)-*N*-(3,5-dichloro-1-oxidopyridin-4-yl)-4-(difluoro methoxy) benzamide, generated in alkali, acidic and oxidative conditions.

Keywords: Roflumilast, Mass Spectrometry, Degradation products, DFT

Introduction

Phosphodiesterases (PDEs) are a group of enzymes that catalyze the breakdown of cyclic adenosine monophosphate and cyclic guanosine monophosphate to their inactive form. PDE4 is the principal selective cyclic adenosine monophosphate metabolizing enzyme in inflammatory and immune cells which is highly expressed in leukocytes and other inflammatory cells involved in the pathogenesis of inflammatory lung diseases, such as asthma and chronic obstructive pulmonary disease (COPD).[1] Roflumilast (RFL) is a second generation selective phosphodiesterase (PDE-4) inhibitor approved for the treatment of severe chronic obstructive pulmonary disease associated with chronic bronchitis. The IUPAC name for roflumilast is 3-(cyclopropylmethoxy)-*N*-(3, 5-dichloropyridin-4-yl)-4-(difluoromethoxy) benzamide; CAS 162401-32-3).[2] Roflumilast has been approved in the EU (as Daxas) and in the US (as Daliresp) for treatment of severe COPD associated with chronic bronchitis and a history of exacerbations.[3]

The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) defines a degradation product as an impurity resulting from a chemical change in the drug substance brought about during manufacture and/or storage of the new drug product by the effect of, for example, light, temperature, pH, water, or by reaction with an excipient and/or the immediate container closure system.[4] ICH guidelines also necessitate the drugs to be subjected to stress decomposition studies followed by identification and characterization of the degradation products (DP) which are formed 0.1%.[5,6]

A thorough literature survey has revealed that only a few studies have been reported for the degradation behaviour of roflumilast. Tarek S. Belal *et al.* reported significant degradation of roflumilast under acidic, alkali and oxidative conditions[7] but none have been identified or characterized. The drug has been investigated by Barhate *et al.* to be stable under neutral, thermal and photolytic conditions but unstable to acidic, alkaline and oxidative conditions at 80°C. But no attempt has been directed for the identification of DPs.[8]

However, there have been reports of process related impurities namely *N*-(3,5-dichloropyridin-4-yl)-4-difluoromethoxy-3-methoxybenzamide, 3,4-bis(cyclopropylmethoxy)-*N*-(3,5-dichloropyridin-4-yl) benzamide, *N*-(3,5-dichloropyrid-4-yl)-4-difluoromethoxy-3- hydroxybenzamide.[9,10,11]

Density Functional Theory (DFT) has been known to predict the fragmentation profile of protonated molecular

*Reprint requests to Saroj Kumar Paul
E-mail: paul.saroj@gmail.com

ions with reasonable accuracy.[12] P. Wright *et al.* have reported a rationalized approach for fragmentation profile of maraviroc using DFT.[13] Use of DFT for modelling of 15 molecules with respect to protonation-induced bond length changes and subsequent prediction of their fragmentation profiles during collision induced dissociation (CID) has also been reported.[14]

In this current study the chemical structures of the different DPs produced under acidic, alkali and oxidative stress conditions are investigated by liquid chromatography-high resolution mass spectrometry. Efforts have also been made to rationalize the fragmentation profile of the parent drug molecule by identifying the most favourable position of protonation and the changes in bond length consequent to it, DFT. The DPs were characterized by comparing their collision induced dissociation (CID) mass spectral data with that of the parent drug molecule of RFL. This rationalized fragmentation profile can also be extended to identify any other unknown transfor-mation products of RFL by comparing their daughter ions obtained under similar experimental condition.

Experimental

Materials and reagents

HPLC grade acetonitrile and methanol were purchased from Merck India limited (Mumbai, India). Ultrapure water (18.2 MΩ) was prepared using a Milli-Q plus water purification system from Millipore (Bedford, MA, USA). Formic acid and standard of RFL were obtained from Sigma-Aldrich Corporation (Bangalore, India). Analytical reagent grade ammonium acetate, sodium hydroxide, hydrochloric acid and hydrogen peroxide were obtained from Qualigens India Limited (Mumbai, India)

Liquid chromatography

All compound solutions were introduced into the ESI MS (electrospray ionisation mass spectrometry) source by high performance liquid chromatography (HPLC), Dionex ultimate 3000 (Thermo scientific, USA), using a hypersil BDS C18 column (150×4.6 mm, 5 μm, Thermo scientific, USA). A mobile phase consisting of A, 10 mM ammonium acetate adjusted to pH 3.2±0.05 with acetic acid and B, acetonitrile in gradient mode; T(min)/%B: 0/25, 10/60, 15/85, 20/85, 25/25, 30/25. Column temperature was maintained at 30 C and the flow rate was 1.0 mL/min. The samples were injected (10 μL) into the HPLC system in acetonitrile.

High-resolution mass spectrometry

The MS and MS/MS studies were performed on Thermofisher Q-exactive mass spectrometer (Thermo Electron, Bremen, Germany) using electrospray ionization source and orbitrap mass analyzer. Heated electrospray ionization source was used for ionization. The temperature of the heater was kept at 450°C and capillary of the ESI interface at 250°C. Nitrogen was used both as sheath gas and auxiliary gas. The electro spray and tube lens were set at 4.5 kv and 90 V respectively. The mass spectrometer was operated in full scan MS with data dependent MS^2 mode in positive polarity.

The selected range was from 100 to 1000 m/z and the resolution was 70,000 full width half maximum (FWHM) with an isolation window applied, followed by a data dependent scan at a resolution of 17,500 FWHM with the fragmentation energy applied. The target capacity of the C-trap was defined at 1×10^6 charges and the maximum injection time was limited to 50 ms.

Density functional theory

Gas-phase basicity, 3D structure and bond length calculations were performed using DFT, calculations at the B3LYP level using the 6-31G* basis set, with Gaussian09. The optimised geometry for the neutral molecule was calculated, basic sites were then protonated and the relating minimum energy geometry calculated for each possible structure. The energy differences between the most favourable cation (highest negative energy value in Hartrees) and all others were converted from Hartrees into kcal/mol using the conversion factor of 627.503.

Sample preparation and degradation studies

Standard solution of roflumilast (100 μg/mL) was prepared by dissolving it in acetonitrile. Stress degradation sample, acidic (1 N HCl, 80°C, 6 h), alkaline (1 N NaOH, 80°C, 6 h) and oxidative (30% H_2O_2, 80°C, 6 h) were prepared by dissolving 10 mg of sample in 1 mL of acetonitrile, followed by the addition of 2 mL 1 N HCl, 3 mL 1 N NaOH and 3 mL 30% H_2O_2 respectively. All the sample solutions were neutralized and volume made up to 10 mL with acetonitrile prior to their injection into the mass spectrometer.

Results and discussion

The drug exhibited degradation under acidic, alkali and oxidative stress conditions. The results of the degradation study have been summarized in Table 1.

In an attempt to identify the DPs by mass spectral analysis it is highly desirable to have a clear understanding of the fragmentation pathway of parent drug RFL. Under ESI conditions RFL underwent protonation at the nitrogen atom of the pyridine ring and the position of protonation has also been supported by DFT calculations. The results of DFT calculation for the energies of different protonation sites has been depicted in Figure 1. The protonated molecular ion of RFL measured accurately to be m/z 403.0449 Da and produced key fragment ions at m/z 367.0686 Da, 348.9975 Da, 241.0687 Da, 187.0213 Da and 163.9676 Da. The most abundant product ion formed at m/z 241.0687 Da due to loss of 3, 5-dichloropyridine ($C_5H_3C_{l2}N$, 146.9643) by the cleavage of C-N, which further

Table 1. The retention times (RT), measured masses, predicated elemental compositions, theoretical exact masses, mass errors and major fragment ions of degradation products (DP)

DP	RT(Min)	Measured Mass (Da)	Elemental Composition [M+H]	Theoretical Mass (Da)	Error (ppm)	Key Fragments
*RFL	14.22	403.0449	$C_{17}H_{15}C_{l2}F_2N_2O_3$	403.0422	6.69	367.0686,313.0200,348.9975 241.0687,187.0213,163.9676
DP-1	4.4	162.9821	$C_5H_5Cl_2N_2$	162.9824	1.84	90.9777
DP-2	9.2	348.9946	$C1_3H_9Cl_2F_2N_2O_3$	348.9952	1.71	313.0208,219.0471,187.0215, 163.9676,137.0240
DP-3	10.0	353.0453	$C_{16}H_{15}Cl_2N_2O_3$	353.0454	0.28	337.2584,281.0923,191.0718, 162.9838 137.0244,123.0451,103.0764
DP-4	11.1	419.0361	$C_{17}H_{15}Cl_2F_2N_2O_4$	419.0371	2.38	383.0633,344.9869,324.9794, 241.0688,187.0215,137.0244

*RFL is not a DP and stands for the parent molecule of Roflumilast.

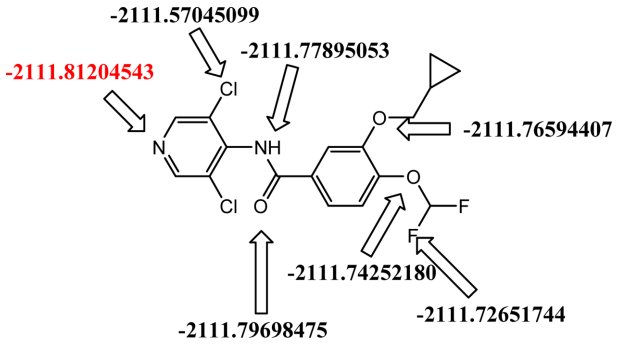

Figure 1. The energies of roflumilast molecule at different potential protonation sites in hartee units. (Hartree is the atomic unit of energy; Hartrees are converted into kcal mol^{-1} by multiplying by 627.503).

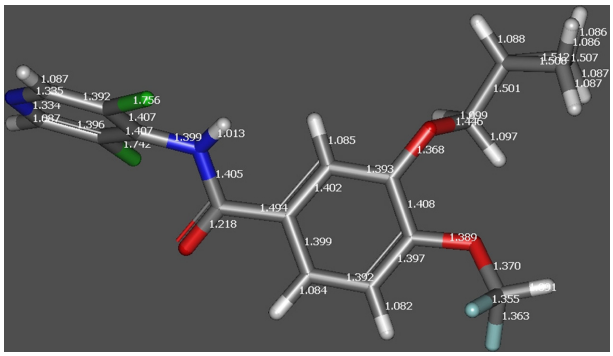

Figure 2a. DFT optimized structure of RFL indicating bond length before protonation.

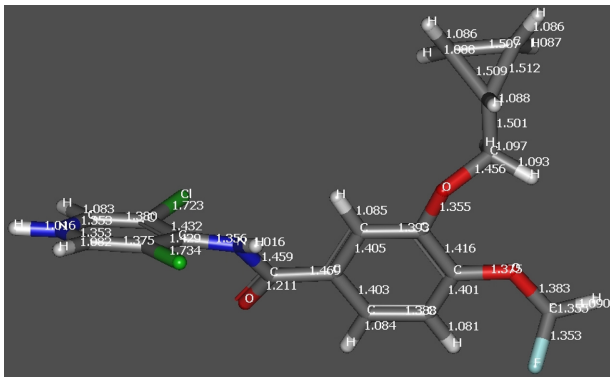

Figure 2b. DFT optimized structure of RFL indicating change in bond length after protonation.

underwent loss of methylene cyclopropane (C_4H_6, 54.047 Da) to generate fragment ion at m/z 187.0213 Da. DFT optimized structure of the protonated molecular ion of roflumilast also indicated elongation of C-N bond as depicted in Figure 2. The formation product ion at m/z 348.9975 Da is due to loss of methylene cyclopropane (C_4H_6, 54.047 Da), which in turn under goes rearrangement with a neutral loss of 185 Da to produce the cation of 3,5-dichloropyridin-4-ol.The schematic presentation of the fragmentation profile of roflumilast is shown in Figure 3a.

DP-1 formed during alkaline degradation showed its protonated molecular ion at m/z 162.9821 Da and underwent fragmentation by losing a molecule of hydrochloric acid to produce a product ion at m/z 90.9777 Da, as shown in Figure 3b. Based on the mass spectra data DP-1 identified as 3, 5-dichloropyridin-4-amine.

DP-2 with a measured accurate mass of m/z 348.9946 Da formed during acidic as well as oxidative stress conditions. The characteristic isotopic pattern of two chlorine atoms in the mass spectra and product ion spectra containing diagnostic fragment ions of m/z 163.9676 Da, 313.0208 Da (Figure 3c) evidently supported it to be *N*-(3,5-dichloropyridin-4-yl)-4-(difluoro methoxy)-3-hydroxybenzamide, formed by the loss of methyl cyclopropane moiety from roflumilast.

DP-3 was formed under alkaline as well as oxidative stress conditions and showed its protonated molecular ion at m/z 353.0453 Da with characteristic isotopic pattern of two

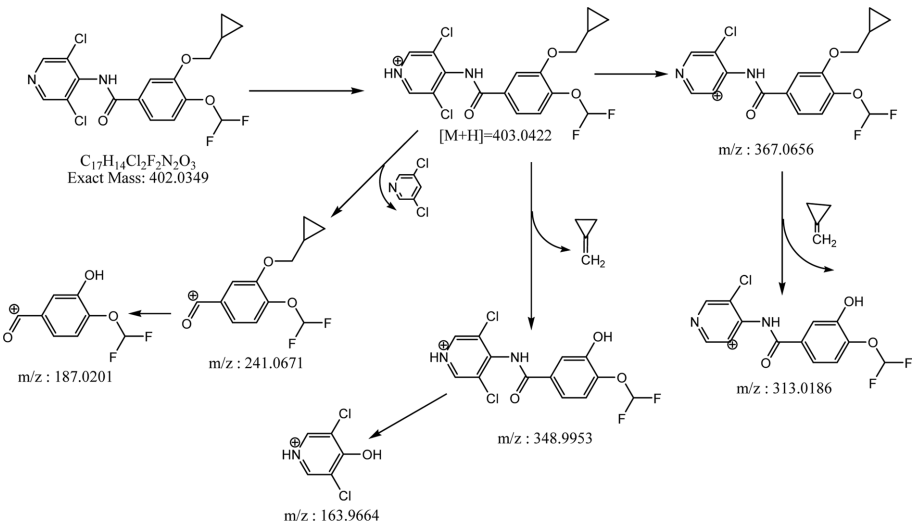

Figure 3a. Plausible fragmentation pathway of roflumilast.

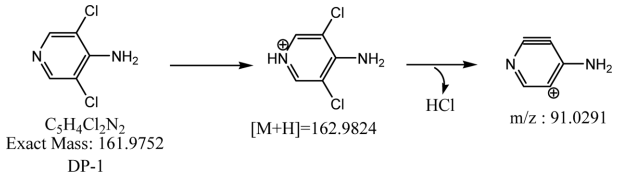

Figure 3b. Plausible fragmentation pathway of DP-1.

chlorine atoms. The diagnostic product ions formed at m/z 162.9838 Da and 191.0718 Da by the neutral loss of 3-(cyclopropylmethoxy)-4-(difluoromethoxy) benzaldehyde and 3,5-dichloropyridin-4-amine evidently supported it to be formed by the loss of CHF_2 side chain from the molecule of roflumilast. Based on this mass spectral analysis as discussed above and depicted in Figure 3d, the most plausible structure of DP-3 has been proposed as *N*-(3,5-dichloropyridin-4-yl)-3-(cyclopropylmethoxy)-4-(difluoromethoxy)benzamide.

During oxidative stress study degradant formed with an accurate mass of m/z 419.0361 Da which is 16 Da higher than that of RFL and showed isotopic pattern confirming the presence of two chlorine atoms, is assigned the code name DP-4. The product ions formed in DP-4 are identical with that of RFL and exhibited similar neutral losses. Based on this the most possible structure of DP-4 is proposed to be the *N*-oxide of roflumilast. The pathway depicting the formation of product ions is shown in Figure 3e.

Conclusion

Roflumilast was subjected to stress study and found to be sensitive in alkaline, acidic and oxidative environment. A rationalized approach based on DFT and comparative high resolution mass spectral analysis with product ion profiling was used for rapid identification of the degradation products. The four degradation products have been identified as 3,5-dichloropyridin-4-amine, *N*-(3,5-dichloropyridin-4-yl)-4-(difluoro methoxy)-3-hydroxybenzamide, (*N*-(3,5-dichloropyridin-4-yl)-3-(cyclopropylmethoxy)-4-(difluoromethoxy) benzamide and 3-(cyclopropylmethoxy)-*N*-(3,5-dichloro-1-oxidopyridin-4-yl)-4-(difluoro methoxy) benzamide.

Acknowledgements

The authors acknowledge the support of Dr. Naresh K.

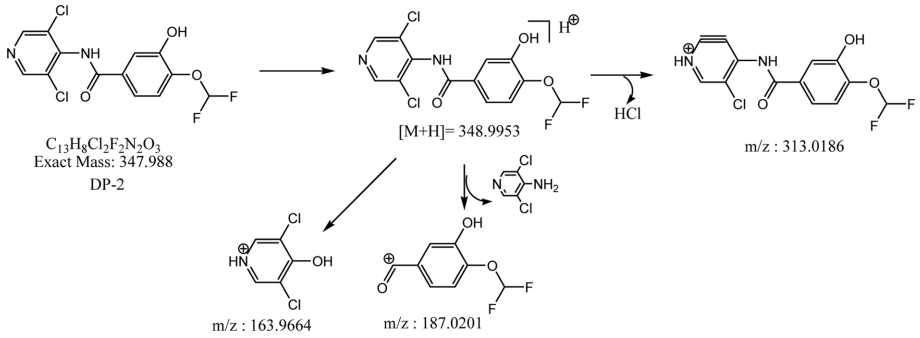

Figure 3c. Plausible fragmentation pathway of DP-2.

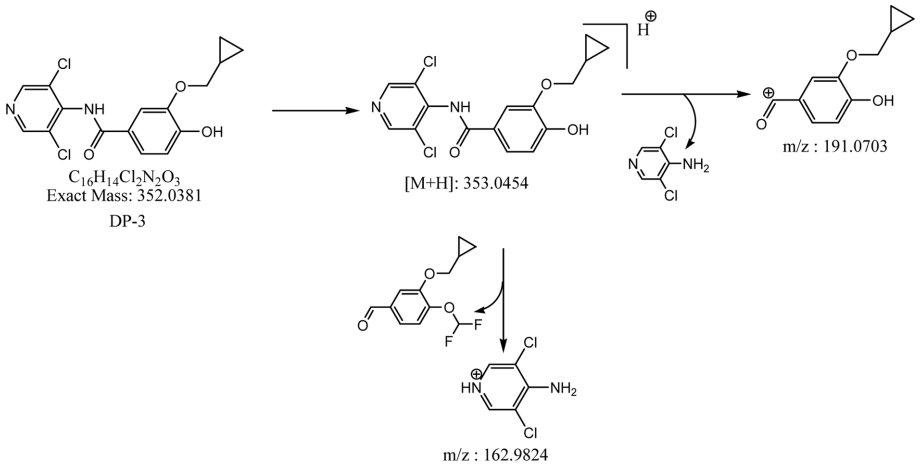

Figure 3d. Plausible fragmentation pathway of DP-3.

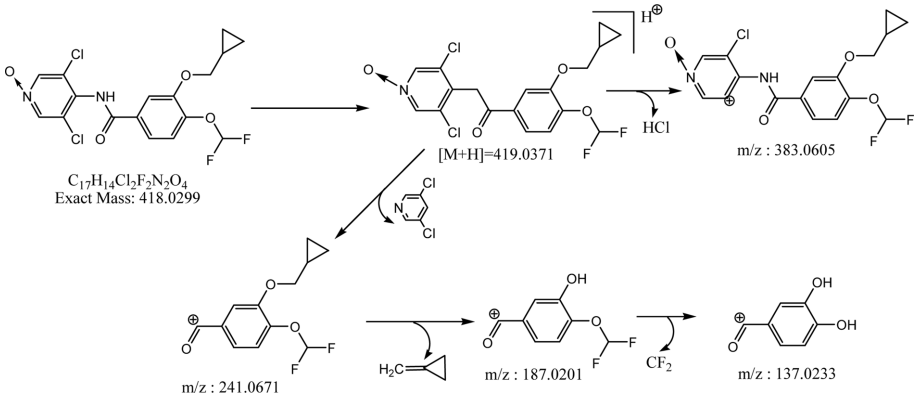

Figure 3e. Plausible fragmentation pathway of DP-4.

Jena of Stockholm University for DFT calculations, Mr. Subhrajit Rout of Indian Institute of Technology, Kanpur and, Thermo fisher application canter, Mumbai for carrying out the mass spectral analysis.

References

1. Beghe, B.; Rabe, K. F.; Fabbri, L. M. *American Journal of Respiratory and Critical Care Medicine* **2013**, 188, 271.
2. Giembycz, Mark, A.; Stephen K. F. *Drug Design, Development and Therapy* **2010**, 4,147.
3. Boland, S.; Alen, J.; Bourin, A.; Castermans, K.; Boumans, N.; Panitti, L.; Vanormelingen, J.; Leysen, D.; Defert, O. *Bioorg. Med. Chem. Lett.* **2014**, http://dx.doi.org/10.1016/j.bmcl.2014.07.016
4. ICH, International Conference on Harmonization Guidance for Industry, Impurities in New Drug Products Q3B (R2). **2006**.
5. ICH, Stability Testing of New Drug Substances and Products Q1A (R2), International Conference on Harmonization, IFPMA, Geneva. **2003**.
6. ICH, Impurities in New Drug Substances Q3A (R2), International Conference on Harmonization, IFPMA, Geneva. **2006**.
7. Tarek, S. B.; Hytham, M. A.; Mohamed, S. M.; Hoda, G. D.; Mostafa, M. B. *Bulletin of Faculty of Pharmacy Cairo University* **2014**, 5279.
8. Barhate, V. D.; Deosthalee, P. Indian *J. Pharm. Sci.* **2010**, 72, 401.
9. Sun, C.; Lin, Y.; Sun, M.; Wang, D.; Chen, L. *Research on Chemical Intermediates* **2013**, 1.
10. Zhang, H.; Lin, Y.; Li, Y.; Dong, K.; Chen, L.; Wang, D. *Journal of Chemical Research* **2014**, 38, 507.
11. Lin, Y.; Huang, P.; Qi, H.; Chen, L.; Wang, D. *Research on Chemical Intermediates* **2013**,39, 3111.
12. Alex, A.; Harvey, S.; Parsons, T.; Pullen, F. S.; Wright, P.; Riley, J. A. *Rapid Commun. Mass Spectrom.* **2009**, 23, 2619.
13. Wright, P.; Alex, A.; Nyaruwata1, T.; Parsons, T.; Pullen, F. *Rapid Commun. Mass Spectrom.* **2010**, 24, 1025.
14. Wright, P.; Alex, A.; Pullen, F. S. *Rapid Commun. Mass Spectrom.* **2014**, 28, 1127.