Asian Journal of Chemistry; Vol. 34, No. 9 (2022), 2386-2392

ASIAN JOURNAL OF CHEMISTRY

https://doi.org/10.14233/ajchem.2022.23878

Isolation and Characterization of Novel Degradation Product of Pidotimod

MADHURI BAGHEL[1,*], MEENAKSHI BHARKATIYA[2], FALGUNI TANDEL[3] and SADHANA J. RAJPUT[4]

[1]Shri Shankaracharya Institute of Pharmaceutical Sciences and Research, Junwani, Bhilai-491001, India
[2]Bhupal Nobles Institute of Pharmaceutical Sciences, Bhupal Nobles University, Udaipur-313001, India
[3]Parul Institute of Pharmacy, Parul University, Vadodara-391760, India
[4]Department of Chemistry, The Maharaja Sayajirao University of Baroda, Vadodara-390002, India

*Corresponding author: E-mail: banchhormadhuri@gmail.com

Received: 22 April 2022; *Accepted*: 6 July 2022; *Published online*: 19 August 2022; AJC-20938

A synthetic dipeptide, pidotimod (3-L-pyroglutamyl-L-thiaziolidine-4-carboxylic acid, PTD) is an immunomodulator and it possesses anti-infective activity against variety of infections. The objective of this study was to explore into and identify pidotimod's major degradation product. Pidotimod was degraded in ICH prescribed stress conditions. pidotimod was degraded 90% in 1.0 N NaOH at 80 ºC for 6 h and the degradation product (DP2) was determined using HPLC. The HPLC method was used to separate the primary and subsidiary degradation products and carried out on a C_{18} column with a mobile phase of ammonium acetate buffer (pH 4.5; 10 mM) and MeOH/ACN (90:10 v/v) at a ratio of 97:03 v/v at 40 ºC, flow rate of 1.0 mL/min, and detection at 215 nm. IR, NMR and LC-MS-MS were used to identify degradation product (DP2) as 3,8-dihydroxy-tetrahydro-bisthiazolo[3,4,4a,3′,4′-*d*]pyrazine-5,10-dione.

Keywords: Pidotimod, Stress degradation, Stability indicating assay method, LC-MS-MS, Acute respiratory tract infections.

INTRODUCTION

A synthetic dipeptide, pidotimod (PTD) has biological and immunological modifying properties. It is been used for a long time in the treatment and prevention of recurrent respiratory infections. It is the first peptide like biological response modulator to trigger innate and adaptive immune responses to bacteria and viruses [1,2]. Pidotimod has been studied *in vitro* and *in vivo* for the treatment and cure of acute respiratory tract infections (ARTI's) in children [3]. Regardless of the fact that much research on pidotimod was published over 25 years ago, it is still utilized to prevent ARTI's today. There is a research void in terms of extensive pharmacological and analytical studies [4-6] despite the prevalence of articles at the time of pidotimod approval. In pidotimod research, novel approaches for creating both innate and adaptive immunity have also been examined [7,8].

Only limited analytical approaches for evaluating pidotimod have been established using HILIC-MS-MS [9], HPLC-MS-MS [10], HPLC-MS [11] and HPLC-UV [12]. A bioequivalence evaluation of pidotimod formulations was also found [13] as well as a method for GC measurement of residual organic solvents in pidotimod [14]. In 1994, Magni *et al*. [15] reported the synthesis and preliminary pharmacological assessment of pidotimod, its carboxamido derivatives and enantiomer, diastereomers in addition to, the analytical and chemical profile of pidotimod was reported by Crimella *et al*. [16]. In an industrial batch of pidotimod, they discovered two process related impurities along with two more impurities unrelated to the process. The chiral separation of pidotimod and its enantiomers has been reported on the CHIRALPAK [17] and CHIRADEX [18] as stationary phases. Thermodynamic properties, crystal structure and molecular docking of pidotimod enantiomers have recently been investigated [19].

Only one study on pidotimod impurities detection has been reported [16], despite its extensive use and usefulness in preventing ARTIs. Stress degradation and structural elucidation of pidotimod degradation products (DPs) have still not been mentioned in the literature, to our best of knowledge. As a result, the present study investigated pidotimod degradation behaviour using the ICH recommended stress degradation criteria. A liquid chromatographic technique was developed to separate

various degradation products. The structure of one prominent degradation product formed under acid degradation conditions was anticipated using IR, mass and NMR spectra.

EXPERIMENTAL

The pidotimod bulk drug was supplied by Swapnaroop Pharmaceuticals, India. Spectrochem Ltd., India, ammonium acetate, methanol and acetonitrile which were HPLC graded. Unless otherwise mentioned, all samples were filtered before being used using nylon 6,6 membrane filters (0.2 µm; Ultipor N66®) from Pall Life Sciences USA. S.D. Fine Chemicals, India provided the analytical quality H_2O_2, HCl and NaOH.

Chromatographic conditions: Precision waterbaths having temperature regulators were furnished by Thermal lining company, India, for degradation trials. Photolytic degradation was explored in a photostability chamber (Thermolab Scientific Equipment & Materials Ltd. India) equipped with a fluorescence (OSRAM- L20) and four UV (OSRAM- L73) lights. The temperature and humidity conditions were measured using a thermal humidity chamber (S.R. Labs Instrumentation, India).

For HPLC analysis, a Waters Acquity from Waters Corporation (Milford, USA) was used, along with Empower-2 program software and a PDA detection. A Phenomenex RP C_{18} column (5 µm; 250 mm; 4.6 mm) was used to separate the samples at a wavelength of 215 nm. The mobile phase consisted of a 97:03 v/v buffer (ammonium acetate; 10 mM, pH 4.5 corrected with glacial acetic acid) and 90:10 v/v MeOH-CAN, with an isocratic elution at flow rate of 1.0 mL/min. The studies were carried out in a column oven at 40 ºC with a 20 µL injection volume. The LC-MS-MS experiments were performed in both positive and negative ESI modes using the software Xcalibur, on the LCQ fleet, a Thermo Fisher scientific equipment paired to an Agilent HPLC 1100 series; quaternary system module. The spectra were taken at 20 psi nebulizer pressure, 5500 V capillary voltage, 30 psi nitrogen drying gas pressure and 2500C gas temperature. A Bruker IR Affinity 1 FTIR system and IR solutions program were used to capture IR spectra. The ^{1}H and ^{13}C NMR spectra were recorded using the Bruker Advance II 400 NMR spectrometer with TMS (tetramethylsilane) and DMSO as an internal standard and solvent, respectively.

Preparation of degradation products (DPs): In the stress degradation tests, various stress treatments were applied to the bulk drug. To compare to the stress degraded samples, placebo samples were generated. In brief, 10 mL of 1 mg/mL of pidotimod was prepared in recently prepared 0.8 N HCl or 0.1 N NaOH and refluxed at 80 ºC for 3 h in the dark, for acid and base degradation. Before analysis, 2 mL aliquot of these samples was withdrawn, neutralized using the same strength of base or acid and stored in the freezer at -10 ºC. Sample of 10 mL of 1 mg/mL was produced in double distilled water and refluxed at 80 ºC for 6 h, in the dark for neutral degradation. Pidotimod (10 mL of 1 mg/mL) was generated in 0.01% H_2O_2 and refluxed at 80 ºC in dark for 1.5 h for oxidative degradation. For photolytic degradation, API was spread 1 mm thick on a petri-plate and exposed to 144 UW/cm^2 and 5382 LUX irradiation for 21 days. To produce the thermal degradation product, API was distributed 1mm thick on a petri-dish and heated at 80 ºC for 21 days. For thermal humidity induced degradation, API was placed in the stability chamber for 21 days at 40 ± 2 ºC and 75 ± 5% RH.

Isolation of major degradation product (DP): One major degradation product (DP2) formed during acid degradation was isolated by liquid extraction (LLE) and preparative thin layer chromatography (Prep-TLC). Finally, the degradation product was recrystallized using suitable solvent.

RESULTS AND DISCUSSION

Method development: The degradation products (DPs) produced in all stress conditions were mingled and diluted to 150 ppm with regard to pidotimod to identify the peaks of pidotimod and DPs. Because of its dipeptide backbone, pidotimod is polar. Since DPs (except DP4) were rather polar than pidotimod, a higher buffer ratio has been used to prolong R_t and resolving the DPs. The retention time (R_t) of pidotimod was significantly affected by the buffer pH. The R_t was delayed and DP1, DP2 and DP3 were resolved by decreasing the buffer pH to 3.8. When the pH was elevated to 5.8, the R_t of pidotimod decreased, and DP1, DP2 and DP3 were co-eluted. The DP4 was well separated at all pH values.

Despite having a higher sensitivity, acetonitrile provides a shorter R_t of pidotimod. Co-elution of DP1, DP2 and DP3 made resolution difficult at higher buffer ratios. Methanol raises the R_t of pidotimod and aids in the resolution of DPs at higher buffer ratios, but it lowers sensitivity. A mixture of MeOH and acetonitrile (90:10 v/v) was employed to optimize sensitivity, R_t and resolution. Column temperature affects sensitivity, Rt, and peak symmetry of pidotimod and DPs. As a result, optimization necessitated mixing CH_3COONH_4 buffer with MeOH-ACN. A mobile phase of ammonium acetate buffer (10 mM; pH 4.5) and MeOH-ACN (90:10 v/v) at a ratio of 97:03 v/v at 40 ºC produced the best separation.

Pidotimod and DPs had good sensitivity at 215 nm; hence detection was done at this wavelength. Stressed samples were scanned in a PDA detector at wavelengths spanning from 200 to 400 nm. The final chromatogram obtained with the selected mobile phase is shown in Fig. 1a, which reveals highly resolved pidotimod and DPs peaks.

Stress degradation studies: Peaks of 5 degradation products were observed under stress conditions when a blend of degradants was examined in LC-PDA, as shown in Fig 1a. Table-1 illustrates the forced degradation parameters with percent degradation [20,21] in a variety of situations. For computing percent degradation, the following formula was used:

$$\text{Degradation } (\%) = \frac{A - B}{A} \times 100$$

where A = area of unstressed stock solution, B = reduced area of stressed stock solution.

Pidotimod is the most susceptible to oxidative deterioration. It deteriorates significantly when subjected to acid and basic degradation. Only negligible deterioration was seen under thermal humidity, neutral and photolytic degradation conditions.

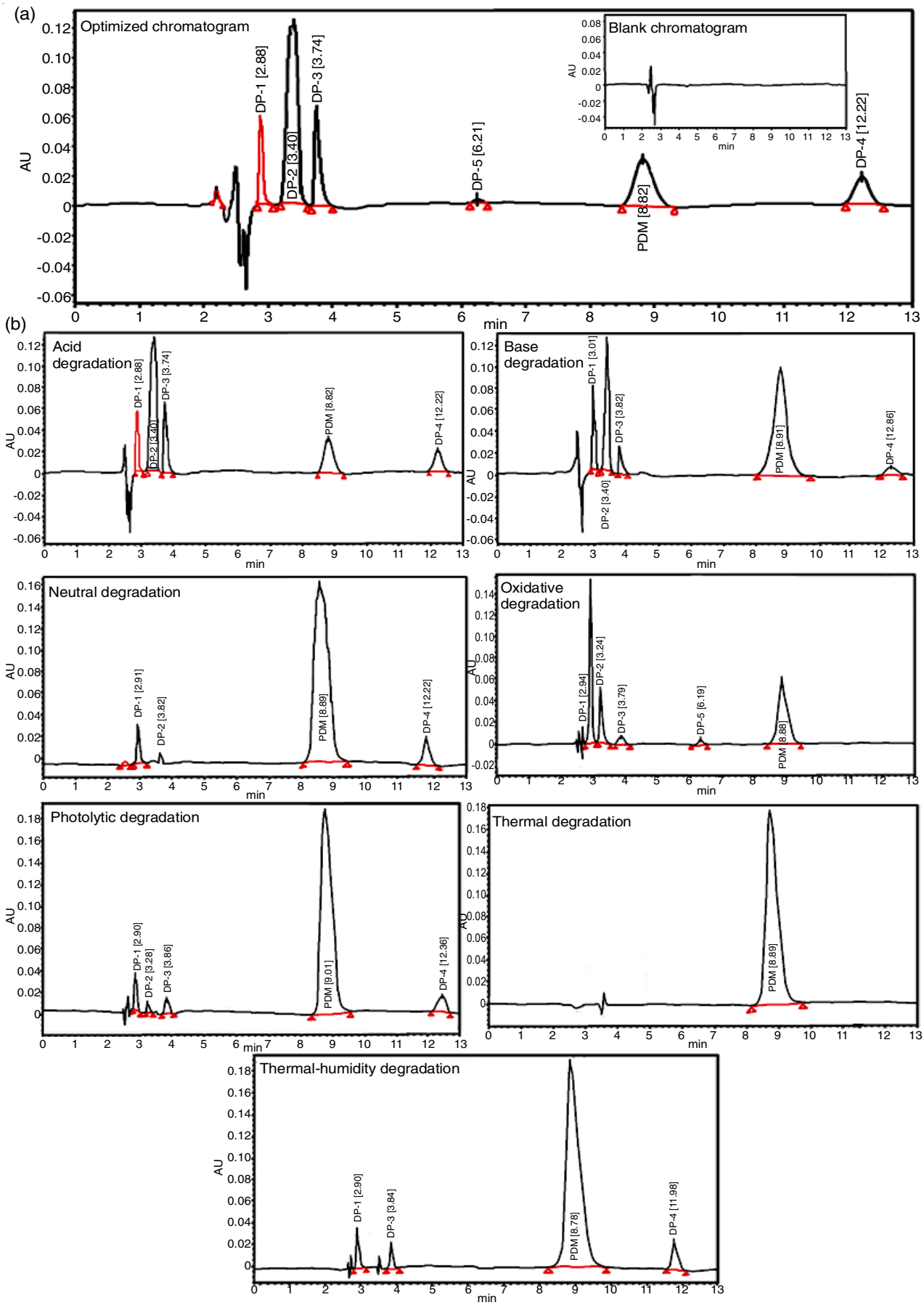


Fig. 1. (a) Optimized chromatogram, (b) Individual chromatograms

TABLE-1
SUMMARY OF PIDOTIMOD DRUG AND SYNTHETIC MIXTURE STRESS DEGRADATION

Stressor type	Time	Stressor conc.	% Degradation (API)	% Degradation (synthetic mixture)	DPs formed with R_t
Acid degradation	3 h	0.8 N, 80 °C	46.44	46.21	DP 1 (2.88), DP2 (3.40), DP 3 (3.74), DP 4 (12.22)
Base degradation	3 h	0.1 N, 80 °C	61.25	60.99	DP 1 (3.01), DP2 (3.43), DP 3 (3.82), DP 4 (12.86)
Neutral degradation	6 h	80 °C	10.4	10.1	DP 1 (2.91), DP 3 (2.84), DP 4 (12.22)
Oxidative degradation	1.5 h	0.01 %, 80 °C	75.38	74.14	DP 1 (2.94), DP2 (3.24), DP 3 (3.79), DP 5 (6.19)
Photolytic degradation	21 days	–	8.25	8.0	DP 1 (2.90), DP2 (3.28), DP 3 (3.86), DP 4 (12.36)
Thermal degradation	21 days	80 °C			No degradation
Thermal humidity induced degradation	21 days	40 ± 2 °C and 75 ± 5 °C	5.2	5.0	DP 1 (2.91), DP 3 (3.84), DP 4 (11.98)

The drug is most stable when subjected to heat deterioration. The chromatograms of distinct stressed samples are shown in Fig. 1b.

Isolation of major degradation product (DP): The DP2 was isolated from acid degradation since the HPLC peak areas of this DP was highest in acid degradation. For enrichment of DP2, pidotimod bulk drug, 500 mg, was dissolved in 50 mL of 1.5 N HCl and refluxed in the thermostatically controlled water bath at 80 ºC for 6 h. The solution was neutralized with NaOH and the percent degradation was determined using HPLC peak area, revealing that about 90% of DP2 was present. The sample was subjected to liquid-liquid extraction (LLE) with *n*-butanol (4 times) to extract the crude residue and the organic layer was evaporated on a rotary vacuum evaporator under reduced pressure. The DP2 was purified by preparative TLC with a mobile phase of *n*-butanol, ethyl acetate and glacial acetic acid (08:02:0.05 v/v).

The bands were visualized with ninhydrine solution, then scraped and extracted (3 times) with an ethyl acetate:methanol mixture (08:02). To get pure DP2, the organic layer was extracted and then evaporated at reduced pressure in a rotary evaporator before being recrystallized with hot ethyl acetate.

Structural characterization of DP2: The molecular masses of pidotimod and DPs were determined using LC-MS-MS data in positive and negative ion modes as shown in Fig 2.

MS-MS spectra: The molecular weight of DP2 is 262, which is 18 amu higher as compared to pidotimod; which has molecular weight 244. Fig. 3 depicts the ESI-MS-MS spectra and predicted fragmentation patterns for pidotimod and DP2, respectively.

Pidotimod (*m/z* 245): Pidotimod's $[M+H]^+$ ion's ESI-MS-MS spectrum revealed that the loss of water resulted in the most prevalent fragment ion at *m/z* 227. Fragmentations of INH-2 (Inherent impurity-2) *i.e*. thiazolidine carboxylic acid [16], gives rise to fragment ion at *m/z* 88 as a result of the loss of -COO+H_2. The spectra also revealed a low abundance fragment ion with *m/z* 187.

DP-2 (*m/z* 263): The ESI-MS-MS spectrum of the ion at *m/z* 263 showed two different fragmentation patterns, which was detected as a shouldered peak in LC-MS-MS, while it was recorded as a single peak in LC-PDA. A possible explanation is the existence of an isomeric peak. Due to the loss of H_2O, both had an abundance of fragment ions at *m/z* 245. There was also an abundance of the fragmentation product (DP2e) at *m/z* 227) as well as DP2 (*m/z* 148).

IR studies: Fig. 4 illustrate the IR spectra of pidotimod standard and the DP2. At 3431 cm^{-1} and 1659 cm^{-1} in pidotimod standard, the stretching vibrations of -OH and C(6)O could be seen at 3447 cm^{-1} and 1654 cm^{-1} in DP2, respectively. In DP2, the vibrations of C(12)O at 1705 cm^{-1} and of -NH at 3275 cm^{-1} disappeared.

NMR studies: Four different carbon atoms have been found in the ^{13}C NMR spectra of DP2 (with δ values of 67.41, 36.43, 52.60 and 178.11 ppm), whereas in pidotimod nine different carbon atoms are found (with δ values of 177.10, 170.85, 170.17, 61.06, 53.76, 47.20, 31.69, 28.75 and 23.80 ppm). This observation suggests the presence of identical carbon atoms since molecular weight increases by 18 amu in mass spectrum.

The ^1H NMR spectral data of pidotimod and DP2 indicate the change in carbon skeleton. Protons at positions 1 and 5 in DP2 are identical, the coupling of hydrogen with -OH group was observed that showed doublets at 4.297, 4.274 and 3.935, 3.911 ppm for -OH and -H, respectively. Even though positions

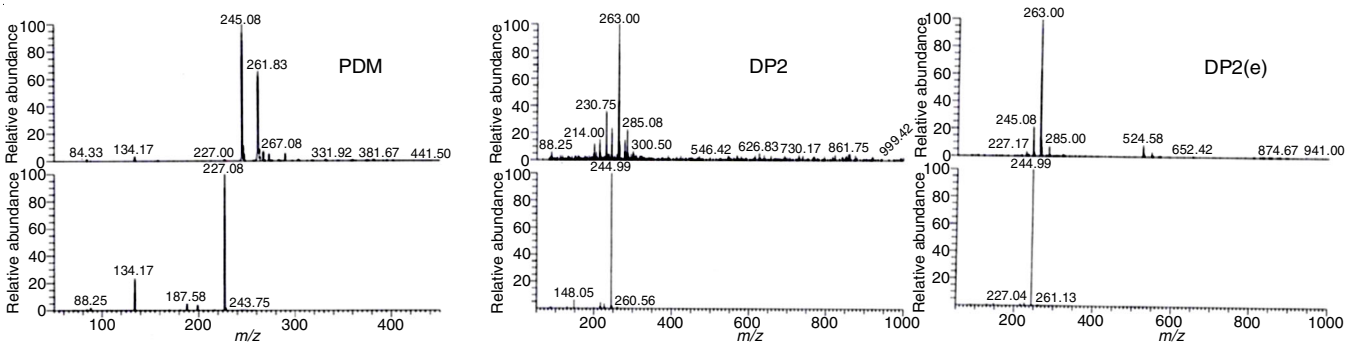


Fig. 2. ESI-MS-MS spectra of pidotimod and DPs

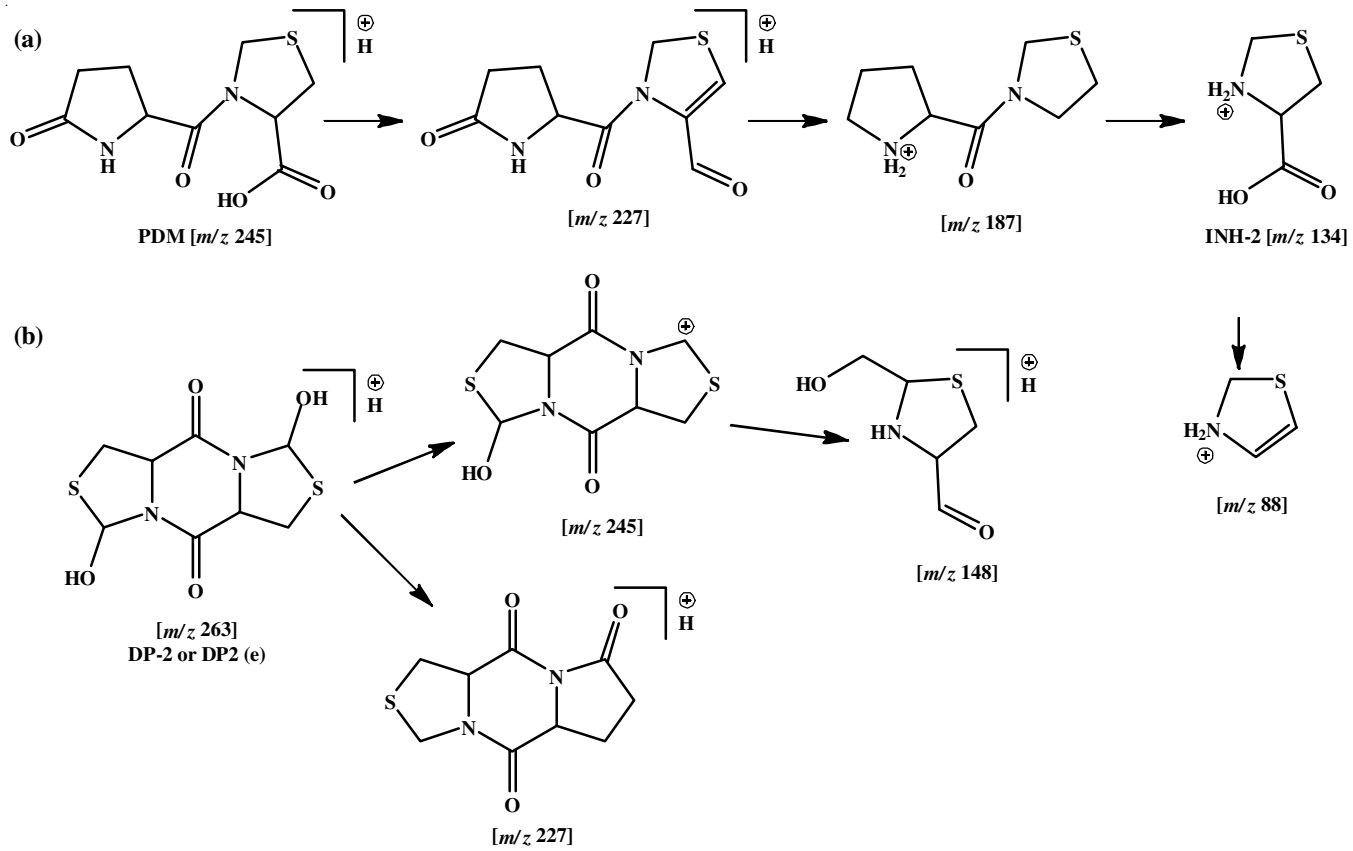


Fig. 3. Proposed fragmentation pathway of pidotimod and degradation products (DPs)

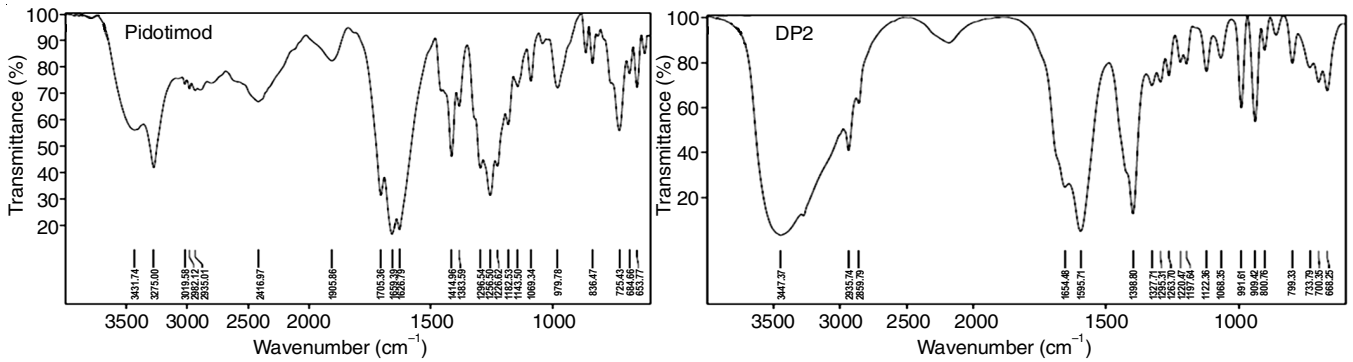


Fig. 4. IR spectra of pidotimod and DP2

2 and 6 seem to be identical, it showed double doublets at 3.228, 3.226, 3.204, 3.186 and 2.748, 2.723, 2.701, 2.655 ppm due to geminal coupling. Diastereotopic protons can couple with each other at stereogenic centers, each geminal proton will show a different chemical shift and proton will show a different *J* value with other neighboring proton. The positions 3 and 7 showed triplet at 3.572, 3.554, 3.536 ppm due to presence of two equivalent protons at adjacent carbon.

Further the integration values support that the protons are in five different environments (due to diestereotopic protons at position 2 and 6. The ^{1}H & ^{13}C spectra of pidotimod and DP2 are illustrated in Fig. 5. The NMR spectral assignments for pidotimod and DP 2 are given in Table-2.

The DP2 was assigned the chemical name 3,8-dihydroxy-tetrahydro-bisthiazolo[3,4,4a,3′,4′-*d*]pyrazine-5,10-dione on the basis of observations gathered from different spectral data for DP2. The thio group may be incorporated in DP2 from INH-2. The compound is chiral in nature with presence of two chiral centers at position 1 and 5.

Finally, based on the aforementioned findings and interpretations, the structures and degradation details for the studied compound and its degradation products (DPs) are presented in Table-3.

Conclusion

The LC-PDA detection and LC-MS-MS investigation were used to track the impurities and determine the degradability of pidotimod. LC-PDA detected four impurities in total. One major degradation products DP2 (based on HPLC peak area) formed from acid degradation condition was isolated and succe-

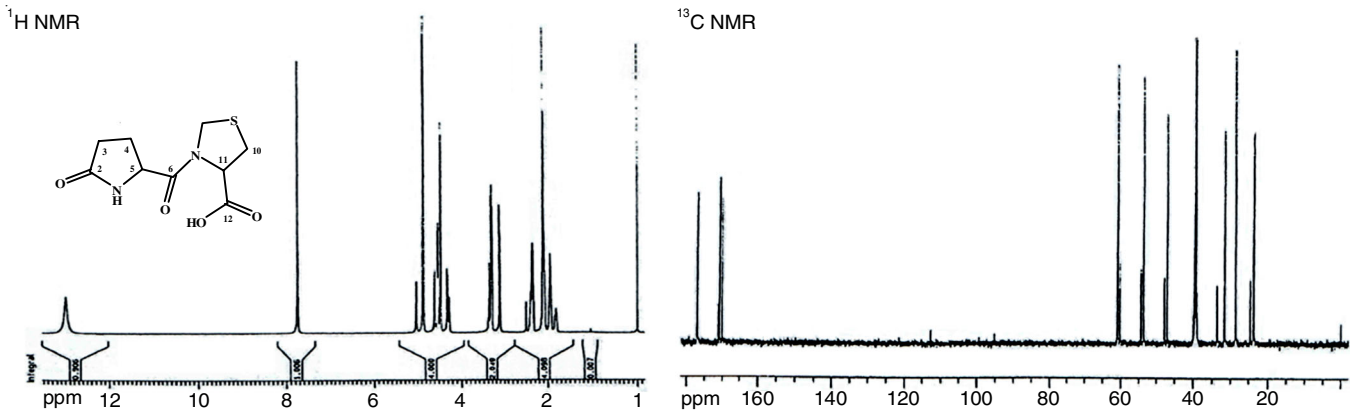


Fig. 5a. ^{1}H and ^{13}C NMR spectra of pidotimod

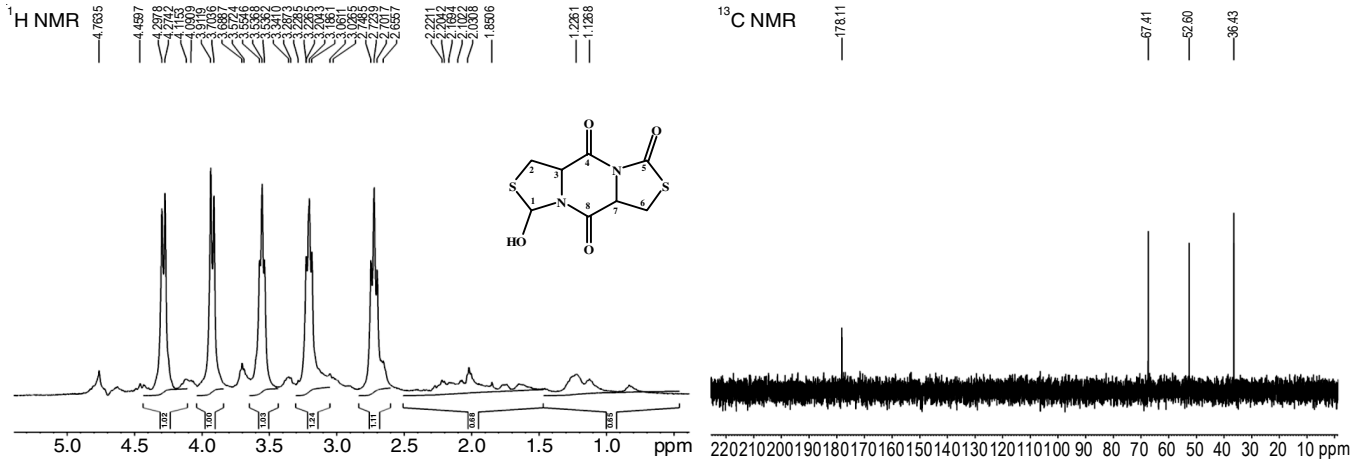


Fig. 5b. ^{1}H and ^{13}C NMR spectra of DP2

TABLE-2
^{1}H NMR AND ^{13}C NMR SPECTRAL ASSIGNMENT FOR PIDOTIMOD AND DP2

^{1}H NMR spectral assignment						^{1}H NMR spectral assignment			
Pidotimod (standard)			DP-2			Pidotimod (standard)		DP-2	
Position	Chemical shift (δ, ppm)	Multi-plicity	Position	Chemical shift (δ, ppm)	Multi-plicity	Position	Chemical shift (δ, ppm)	Position	Chemical shift (δ, ppm)
1	7.76	Broad	2	3.228, 3.226, 3.204, 3.186/8.88	dd	2	177.10, 170.85, 170.17	1, 5	67.41
3	2.12	m	6	2.748, 2.723, 2.701, 2.655/9.84	dd	3	28.75	2, 6	36.43
4	2.36/1.95	m	3, 7	3.572, 3.554, 3.536/7.12	t	4	23.80	3, 7	52.60
5	4.53	dd	4, 8	–	–	5	53.76	4, 8	178.11
8	4.87	m	1, 5 (-OH)	4.297, 4.274/9.44	d	6	177.10, 170.85, 170.17		
10	3.32/3.12	dd/m	1, 5 (-H)	3.935, 3.911/9.44	d	8	47.20		
11	4.87	m				10	31.69		
12	13.00	broad				11	61.06		

The ^{1}H chemical shift values were reported on the δ scale in ppm, relative to TMS (δ = 0.00) as internal standard.

ssfully characterized as 3,8-dihydroxytetrahydro-bisthiazolo-[3,4,4a,3′,4′-*d*]pyrazine-5,10-dione.

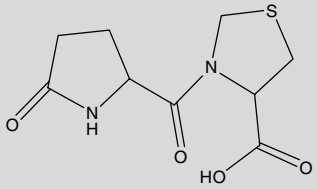
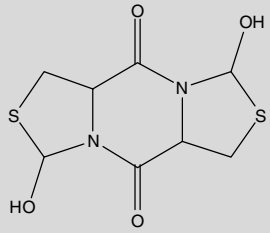
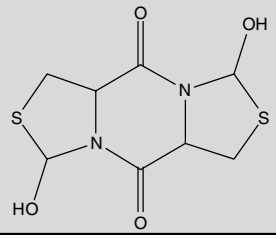
CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

REFERENCES

1. M. D'Amato, D. Paris, A. Molino, P. Cuomo, A. Fulgione, N. Sorrentino, L. Palomba, M. Maniscalco and A. Motta, *Front. Pharmacol.*, **10**, 1115 (2019); https://doi.org/10.3389/fphar.2019.01115
2. G.V. Zuccotti and C. Mameli, *Int. J. Pediatr.*, **39**, 75 (2013); https://doi.org/10.1186/1824-7288-39-75

TABLE-3
CHEMICAL STRUCTURES OF PIDOTIMOD AND DPs, THEIR ORIGIN, DEGRADATION ROUTE, R_t AND OBSERVED *m/z* VALUES FOR MAJOR FRAGMENTS

Analyte	Structure	m.f., m.w. (*m/z* of fragments)	Origin	Degradation condition	R_t
Pidotimod		$C_9H_{12}N_2O_4S$ 244.27 (227.08, 187.58, 134.17, 88.25)	API	–	8.821
DP2		$C_8H_{10}N_2O_4S_2$ 262.31 (244.99, 227.04)	Degradation product (unk)	Acid, base, photolytic, degradation	3.4
DP2 (e)		$C_8H_{10}N_2O_4S_2$ 262.31 (244.99, 148.05)	Degradation product (unk)	Acid, base, degradation	3.4

3. F. Mailland, G. Signorelli and G. Coppi, *Drugs Future*, **16**, 1096 (1991); https://doi.org/10.1358/dof.1991.016.12.158317
4. C. Mameli, A. Pasinato, M. Picca, G. Bedogni, S. Pisanelli and G.V. Zuccotti, *Pharmacol. Res.*, **97**, 79 (2015); https://doi.org/10.1016/j.phrs.2015.04.007
5. C. Giagulli, M. Noerder, M. Avolio, P.D. Becker, S. Fiorentini, C.A. Guzman and A. Caruso, *Int. Immunopharmacol.*, **9**, 1366 (2009); https://doi.org/10.1016/j.intimp.2009.08.010
6. K. Masihi, *Int. J. Antimicrob. Agents*, **14**, 181 (2000); https://doi.org/10.1016/S0924-8579(99)00161-2
7. P. Riboldi, M. Gerosa and P. Meroni, *Int. J. Immunopathol. Pharmacol.*, **22**, 255 (2009); https://doi.org/10.1177/039463200902200201
8. L. Ye, Y. Luo and X. Ou, *China Pharmacy,* **1,** 17 (2012).
9. H. Chen, M. Shen and L. Chen, *Chromatographia*, **73**, 767 (2011); https://doi.org/10.1007/s10337-011-1930-9
10. Y. Zhang, Z. Xiong, F. Qin, S. Lu, W. Liu and F. Li, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **877**, 2566 (2009); https://doi.org/10.1016/j.jchromb.2009.06.038
11. H. Lou, Z. Ruan and B. Jiang, *Arzneimittelforschung*, **62**, 99 (2012); https://doi.org/10.1055/s-0031-1297983
12. G. Coppi and M. Barchielli, *J. Chromatogr. B Biomed. Appl.*, **563**, 385 (1991); https://doi.org/10.1016/0378-4347(91)80046-F
13. J.H. Huang, X.H. Huang, K. Wang, J.C. Li, X.F. Xie, C.L. Shen, L.J. Li and Q.S. Zheng, *Biomed. Pharmacother.*, **67**, 475 (2013); https://doi.org/10.1016/j.biopha.2013.03.009
14. L. Junfang, *China Pharmacy,* **31**, 28 (2009).
15. A. Magni, G. Signorelli and G. Bocchiola, *Arzneimittelforschung*, **44**, 1402 (1994); https://doi.org/10.1002/chin.199524213
16. T. Crimella, R. Orlandi, G. Bocchiola, U. Anders and R. Stradi, *Arzneimittelforschung*, **44**, 1405 (1994).
17. L.X. Zhang, T.J. Yin, W.Y. Shen, Y. Cheng, C.P. Jiang and X.Y. Zhang, *Zhongguo Yaoke Daxue Xuebao*, **42**, 238 (2011).
18. M. Baghel and S.J. Rajput, *Indo Am. J. Pharm. Res.*, **06**, 6182 (2016).
19. X. Dou, X. Su, Y. Wang, Y. Chen and W. Shen, *Chirality*, **27**, 802 (2015); https://doi.org/10.1002/chir.22493
20. M. Baghel and S. Rajput, *J. Chromatogr. Sci.*, **55**, 899 (2017); https://doi.org/10.1093/chromsci/bmx047
21. M. Baghel and S. Rajput, *Biomed. Chromatogr.*, **32**, e4146 (2018); https://doi.org/10.1002/bmc.4146