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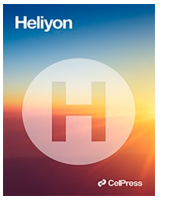

Research article

Development and validation of stability-indicating method of etrasimod by HPLC/DAD/MS/MS technique with greenness profiling

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ABSTRACT

Etrasimod, a novel selective sphingosine-1-phosphate receptor modulator, was recently approved by the U.S. Food and Drug Administration and the European Medicinal Agency for the treatment of moderately to severely active ulcerative colitis in adults. In this research, the forced degradation study as an integral part of drug product and packaging development, which generates data on degradation mechanisms, is published. The development and validation of the stability-indicating method using a superior high-performance liquid chromatography technique coupled with a diode array detector and tandem mass spectrometer was performed to support the forced degradation study and monitor the formation of degradation products. Etrasimod demonstrated good stability under elevated temperature and basic stress conditions, while acidic hydrolysis, oxidative, and photolytic degradation produced eight degradation products. The knowledge of degradation products will be useful in the long-term stability study for establishing the acceptance criteria of etrasimod as a drug substance and dosage form during quality control and stability assessment. The eco-friendliness of the developed forced degradation procedure was evaluated using various greenness appraisal tools. The green metric tools showed that the forced degradation procedure obeys eco-friendly conditions.

1. Introduction

Inflammatory bowel disease (IBD) is known to be a polygenic disorder in which gene-environment interactions play a role, although the aetiology remains poorly understood. IBD includes two major forms: ulcerative colitis (UC) and Crohn's disease (CD), although there is a small group of patients with an intermediate form. The pathological process of both UC and CD leads to bowel damage driven by an extreme inflammatory reaction against luminal antigens. UC is characterized by diffuse and superficial inflammation of the colonic mucosa and rectum, while CD causes inflammation of the full thickness of the bowel wall in any part of the digestive tract. Common symptoms of this chronic and relapse-remitting inflammatory disease of gastro-intestinal tract during the

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active phase of the disease include diarrhea, rectal bleeding, abdominal pain, weight loss, fecal urgency, fatigue, nausea, and vomiting [1–3]. Current treatment recommendations for IBD emphasize the importance of early therapeutic intervention, treat-to-target strategies, and therapeutic drug monitoring. The major goals of IBD treatment are the induction and maintenance of clinical remission, the prevention of complications, and mucosal healing. Current pharmacological options include several drug classes such as aminosalicylates, glucocorticoids, and immunomodulators in case of steroid dependence, followed by biologic therapy. In the last two decades, IBD therapeutic strategies have been completely reshaped after the introduction of biomolecular drugs. They demonstrated unique and differentiative therapeutic mechanisms that can alleviate the inflammation more effectively. But despite these advances, up to one-third of patients fail to respond adequately, while another third of initial responders experience secondary loss of response due to the generation of antibodies to the drug [4]. In the context of searching for novel therapies, the development of oral small drug molecules comprising alternative mechanisms of action has driven a significant investment in drug development, with numerous unique compounds currently being tested for the treatment of IBD [5,6].

There is a growing interest in the clinical development of sphingosine-1-phosphate (S1P) receptor modulators in IBD but also in other domains of medicine, such as rheumatoid arthritis, psoriasis, and atopic dermatitis. S1P is a multifunctional, membrane-derived lysophospholipid signaling molecule implicated in a vast array of physiological and pathophysiological processes. Intracellular S1P synthesis consists of sphingosine phosphorylation. Once generated, S1P is translocated by ABC transporters outside the cells, where it acts as an extracellular lipid mediator via activation of five different subtypes of receptors termed $S1PR_{1–5}$. Activation of $S1PR_2$ and $S1PR_3$ may be linked to cardiovascular, pulmonary, and cancer-related adverse events, while subtypes $S1PR_1$, $S1PR_4$, and $S1PR_5$ are involved in the mitigation of intestinal inflammation. This finding represents a milestone in the development of selective modulators with the preserved efficacy of S1P receptor modulation and minimal adverse events related to $S1PR_2$ and $S1PR_3$ receptor subtypes [7–10].

In 2021, the U.S. Food and Drug Administration (FDA) approved ozanimod, an agonist of $S1PR_1$ and $S1PR_5$ receptors, for the treatment of moderate-to-severe UC [11]. Etrasimod (2-[(3R)-7-[[4-cyclopentyl-3-(trifluoromethyl)phenyl]methoxy]-1,2,3,4-tetrahydrocyclopenta[*b*]indol-3-yl]acetic acid) represents a next-generation S1P receptor modulator that selectively activates S1P receptor subtypes 1, 4, and 5, with no evident activity on $S1PR_2$ and $S1PR_3$ (Fig. 1). The results of phase 3 clinical trials have shown that all key secondary endoscopic, symptomatic, and endohistological endpoints were met. Moreover, the treatment with etrasimod has shown durable efficiency as well as corticosteroid-free clinical remission at week 52. During clinical studies, an increased incidence of adverse events was not observed, showing a favorable safety profile [2]. These findings have shown that etrasimod is a rising star of the IBD therapeutic armamentarium [2,9,10,12,13]. Etrasimod, under the trade name “Velsipity” by Pfizer, was approved by the FDA on October 18, 2023, for the treatment of moderately to severely active UC in adults with the recommended dosage of 2 mg orally once daily. It received marketing authorization from the European Medicinal Agency (EMA) on February 16, 2024 [14,15].

Preformulation and formulation studies are the key steps of the drug development. For marketing approval, stability testing needs to be conducted according to the appropriate regulatory requirements to predict drug performance in the final dosage form. Drug degradation may result in a low amount of drug given to the patient. On the other hand, it may generate hazard degradation products. Forced degradation study can be considered an integral part of drug product and packaging development as it enriches understanding of the decomposition behavior of drugs in various environmental conditions. It generates data on degradation mechanisms and possible degradants that might be produced during product storage [16]. The evaluation of these degradants is essential, as their presence can affect the quality, efficacy, and safety of new drug products [17].

To our knowledge, this is the first report of a forced degradation study of etrasimod, the new, innovative medication for the treatment of IBD. For this study, it was of interest to develop and validate the stability-indicating method to determine the percentage of degradation of etrasimod during forced degradation study. The degradation products formed under stress conditions using a superior high performance liquid chromatography technique coupled with a diode array detector and tandem mass spectrometer (HPLC-DAD-QqQ-MS/MS) were separate. The degradation kinetics of etrasimod is discussed. During method development, the principles of green chemistry were implemented to develop eco-friendly and sustainable analytical methods.

2. Materials and methods

2.1. Chemical and reagents

Etrasimod standard was purchased from MedChemExpress (Monmouth Junction, NJ, USA) with purity of 99.82 % (according to supplier certificate analysis). HPLC gradient grade methanol and acetonitrile were supplied from J. T. Baker (Griesheim, Germany). Formic acid (LC-MS grade) was acquired from Merck (Darmstadt, Germany). Sodium hydroxide pellets (ACS reagent, 97.0 %) were

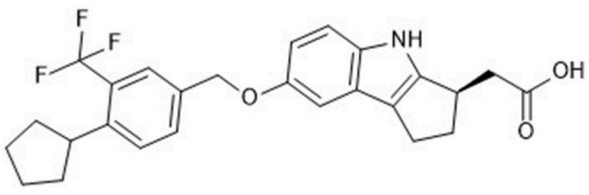

Fig. 1. Chemical structure of sphingosine-1-phosphate (S1P) receptor modulator etrasimod.

supplied by Sigma-Aldrich (St. Louis, MO, USA), while hydrochloric acid (37 %, for analysis) by Carlo Erba (Val-de-Reuil, France). All experiments used ultra-pure water obtained from the WaterPro water system Labconco (Kansas City, MI, USA) with a resistivity of 18.2 mΩ cm (25 °C).

2.2. Preparation of stock and standard solution

The stock solution of etrasimod (100 μg/mL) was prepared by dissolving an appropriate amount of analytical standard in methanol. Further dilutions of the stock solution with a methanol/water (50:50, *v/v*) mixture produced a series of standard working solutions in the concentration range of 2–10 μg/mL. Standard solution samples for method validation were independently prepared in the same matrix at low (2 μg/mL), middle (6 μg/mL), and high (10 μg/mL) concentrations representative of the range of calibration curves. The solutions were kept in the dark at 4 °C until they were used. As the etrasimod was soluble in a mixture of water and methanol (50:50, *v/v*), all the stress samples were prepared using the same diluent. Final concentrations of 10 μg/mL, including the added stressor, were injected (5 μL) into the HPLC/DAD/QqQ-MS/MS system.

2.3. Chromatographic analysis

In the analysis of the samples, the Agilent 1260 series HPLC system was used with a DAD detector and Ultivo Triple Quadrupole with electrospray ionization (ESI) source all by Agilent Technologies (Santa Clara, CA, USA). Standard solutions and stress degradation samples were analyzed under gradient elution conditions using chromatographic column Poroshell 120 SB-C18 (3.0 × 100 mm, 2.7 μm particle size; Agilent Technologies, Santa Clara, CA, USA) with an operating temperature of 40.0 ± 0.1 °C. The mobile phase consisted of two eluents, ultra-pure water (eluent A) and acetonitrile (eluent B), both acidified with formic acid (0.1 %) and delivered at a flow rate of 0.8 mL/min with a gradient system (0–6 min 15–50 % B, 6–13 min 50–95 % B, 13–14.5 min 95 % B, and 14.5–18 min 95–15 % B). The injection volume was 5 μL. The DAD quantitation was performed at 227 nm with a slit of 4 nm. The absorbance of the analytes during a chromatographic run was collected in the spectral range of 200–600 nm. Structural characterization of etrasimod and degradation products was carried out using a triple quadrupole. The MS settings were optimized to obtain the maximum signal. Data was generated in the positive mode obtained from an ESI source, operating with the capillary voltages of 3500 V and nebulizer pressure of 30 psi. The gas temperature was set at 350 °C with a gas flow of 11 L/min. A tuning mix (Agilent Technologies, Santa Clara, CA, USA) was used daily as a calibrant. The full scan mass spectra were acquired over a range of *m/z* 100–600. Highly pure N_2 (>99.9995) was used as the collision gas with the collision energy of 40 V for the etrasimod fragmentation study. Fragmentor voltage was set at 135 V. Data acquisition and processing were performed and carried out using OpenLab ChemStation and MassHunter Workstation software.

2.4. Analytical method validation

The newly developed method was validated for the determination of etrasimod according to the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use guidelines (Validation of Analytical Procedures ICH Q2 (R2)) [18].

2.5. Forced degradation study

Stress study was carried out as per ICH guidelines (Stability Testing of New Drug Substances and Drug Products Q1A(R2) and Photostability Testing of New Active Substances and Medicinal Products Q1B) under different acid, base, oxidative, photolytic, and thermal conditions [19,20]. Degradation study was carried out to demonstrate the specificity of the developed stability-indicating analytical method to investigate the degradation pathways of etrasimod and its stability under stressful conditions. Acid and basis hydrolysis were investigated by dissolving etrasimod to a final concentration of 10 μg/mL using different aqueous hydrochloric acid or sodium hydroxide solutions (0.01–1 M), separately, into amber screw cap vials and samples were left at room temperature. For oxidative studies, etrasimod solutions (final concentration 10 μg/mL) were separately incubated into amber screw cap vials with hydrogen peroxide aqueous solutions (3–33 %) at room temperature. Photostability was investigated by exposing etrasimod solution (10 μg/mL) stored in transparent screw cap vials to direct sunlight. Lastly, a thermal stress testing study was carried out by keeping etrasimod solution in amber screw-cap vials in the thermostatically controlled incubator (ES-20/60 by Biosan, Riga, Latvia) maintained at 60 °C. Samples were withdrawn at regular intervals to monitor the degradation process using the proposed HPLC/DAD/QqQ-MS/MS method. The forced degradation studies were carried out until the limit of degradation of 10–20 % was reached, or up to 7 days. For every stress condition, the blank solution is subjected to stress in the same manner as drug solution.

2.6. Method greenness profiling

Several theoretical approaches based on principles of green analytical chemistry were used for the evaluation of the eco-friendliness of forced degradation procedure [21–24].

The AMGS approach summarizes data from various sources that contribute to the overall AMGS score, such as solvent preparation of standards and samples, system suitability, sensitivity, mobile phase selection, instrument conditions, and instrument energy utilization. The outcome is given as the overall calculated greenness score, which is obtained by adding the following three scores: solvent energy, instrument energy, solvent environment, health, and safety [21].

The Analytical Eco-scale is based on the penalty points assigned due to the reagents and instrumentation selection. The method's point total is determined by counting from 100. The method can be categorized as "excellent green analysis" (more than 75 points), "acceptable" (between 75 and 50 points), or "inadequate" (less than 50 points) based on the final score [22].

The greenness evaluation by ComplexGAPI is presented in the form of a pictogram that includes a pentagon in the center that represents the general method type, a circle in the center denoting quantitative analysis, and four additional pentagons mirroring across each of the shape's edges, representing four main categories of assessment criteria: sample handling (fields from 1 to 4), sample preparation (fields from 6 to 8), required solvents and reagents (fields from 9 to 11), and instrumentation (fields from 12 to 15). An additional hexagonal field at the bottom corresponds to the category of pre-analysis processes (fields from I to VI). These sectors are highlighted by means of three colors (red, yellow, and green) based on their contribution to the eco-friendliness of the method [23].

To evaluate the greenness of analytical procedure, the AGREE approach converts each of the twelve principles of green analytical chemistry into scores. AGREEprep is the first tool designed for the assessment of analytical sample preparation greenness based on ten consecutive steps of assessment that correspond to the ten principles of green sample preparation. Assessment is also based on the possibility of distinguishing between criteria of importance by assigning them weights. Both approaches present results as a round pictogram with a circle in the center that shows the overall score using a reference scale ranging from 0 to 1 (where 1 represents the highest level of eco-friendliness) [24].

3. Results and discussion

3.1. Method development and optimization

Chromatographic conditions were optimized during the method development, considering the chemical structure and physico-chemical properties of etrasimod. Based on data calculated by the ACD/Labs program, high lipophilicity (ACD/LogP value was 6.43) and polar surface area (62 $Å^2$) of etrasimod were predicted [25].

Various reversed-phase chromatographic columns (solid phase type: C8 and C18; dimensions: 100 and 150 mm; particle size: 2.7, 3.5, and 5.0 μm), organic phase type (methanol, acetonitrile) and percentage (10–90 %) in the mobile phase, elution type (isocratic, gradient), and flow rate (0.5–1.0 mL/min) were investigated to optimize chromatographic parameters. Because of the structural similarity between etrasimod and its stress-induced degradation products, simultaneous separation was challenging. Different reversed-phase columns were evaluated, and Poroshell 120 SB-C18 gave high efficiency at lower pressures and the best resolution with outstanding sharp peaks due to its superficially porous particles that have a solid silica core and a porous outer layer. The next step in the method development process was the mobile phase selection. Methanol and acetonitrile were utilized for method development since they are by far the preferred organic modifiers used in reversed-phase chromatography due to their remarkable properties. Among them are complete miscibility with water, relatively low viscosity of their aqueous solutions (particularly in the case of acetonitrile), low UV cut-off wavelength (190 nm and 205 nm for acetonitrile and methanol, respectively), availability in the high purity required for liquid chromatography and mass spectrometry, and low chemical reactivity with most analytes, as well as with analytical equipment and chromatographic column surfaces. During the mobile phase optimization process, several isocratic chromatographic conditions were used in initial scouting, like methanol/water and acetonitrile/water in different proportions. It was observed that acetonitrile was found to be better in terms of resolution and peak shapes as compared with methanol. Even if methanol is less toxic and more easily biodegradable than acetonitrile, it was used as an organic solvent for further investigations. Formic acid as a mobile phase modifier was used mostly due to its compatibility with ESI and the superior ionization efficiency of the analytes. The UV spectrum of etrasimod in the mobile phase showed a maximum at 227 nm, and there were not any interferences that might come from the matrix components. Thus, all the stress samples were analyzed using an easy-to-operate DAD detector in a scan mode covering a range of 200–600 nm, and the final chromatogram was recorded at 227 nm to detect all degradation products.

System suitability parameters were calculated to verify the performance characteristics of the chromatographic analysis for the intended separation. Results are presented in Table 1 and a set of parameters is given: retention time, peak area, peak purity, retention factor, peak symmetry, and theoretical plate count.

3.2. Method validation

The stability-indicating method was successfully validated for determination of etrasimod as per ICH guidelines for the parameters:

Table 1
System suitability parameters of stability-indicating method (concentration level 6 μg/mL, n = 6).[a]

Parameter	Symbol	Value	RSD (%)
Retention time (min)	t_R	12.01	0.10
Peak area (mAU·s)	A	164.41	0.20
Peak purity	P	999.99	0.01
Retention factor	K	14.49	0.68
Symmetry	A_s	0.96	0.29
Theoretical plate count	N	311311	0.20

[a] Acceptance criteria: System Repeatability – Relative Standard Deviation (RSD) value less than 2 %, Peak purity – more than 999.0, Retention factor – more than 2.0, Symmetry – between 0.8 and 1.8, Theoretical plate count – more than 2000.

linearity, limits of detection (LOD) and quantitation (LOQ), precision, accuracy, and standard solution stability [18].

The linearity of method is presented in Table 2. Each calibration standard was prepared in triplicate, and the validity of the method was proved by evaluating the regression line statistically. The high value of the correlation coefficient (0.9998) indicates the good linearity of the method over the specified concentration range. Furthermore, the accuracy of the back-calculated concentrations of each calibration standard was evaluated, and it was found to be within ±2.1 % of the nominal concentration at all investigated levels. Finally, the residual analysis was performed to check the assumptions of linearity and homoscedasticity (Fig. S1). The residuals appeared to be randomly scattered about zero in residual plots versus fits and versus order, revealing that the errors had constant variance and each error was independent of all other errors.

LOD and LOQ values represent the concentration of the analyte that would yield a signal-to-noise ratio of 3 for LOD and 10 for LOQ. The values were found to be 0.10 and 0.34 μg/mL, respectively (Table 2).

The repeatability, or intra-day precision, expresses the precision under the same operating conditions over a short interval of time. It was evaluated with six standard solutions (6 μg/mL), and the assay value was expressed in terms of relative standard deviation (RSD) and the box-and-whisker plot (Fig. S2). The intermediate precision, or inter-day precision, expresses within-laboratories variations. It was performed at the same concentration level on three consecutive days in three replicates. The inter-day variation is also expressed in terms of the RSD value. Both values were found to be below 0.39 %, indicating good precision, as depicted in Table 2.

The accuracy was determined at three concentration levels: low (2 μg/mL), medium (6 μg/mL), and high (10 μg/mL) in three replicates. Recoveries ranging from 100.3 to 102.0 % with RSD values lower than 0.28 % and accuracy errors lower than 0.04 demonstrate satisfactory accuracy of the method (Table 2).

Stability experiments were conducted using etrasimod standard solution (6 μg/mL). Benchtop, autosampler, and long-term stability were checked at different time intervals and storage conditions. The sample was stored at room temperature for a period of 4 h and analyzed thereafter to determine the benchtop stability of etrasimod. The autosampler stability was evaluated using a sample stored inside the autosampler for 8 h at 4 °C. The long-term stability was carried out by storing the standard solution of etrasimod at −20 °C for seven days. The stability testing solutions were assayed against fresh samples. All stability tests showed no significant difference in etrasimod concentrations, with recoveries between 100.1 and 101.2 %.

3.3. Forced degradation study

A forced degradation study was performed to define the chemical stability of etrasimod under different stress conditions. We conducted the study based on the ICH guidelines (Q1A(R2) and Q1B) [19,20]. The primary stress conditions we chose were 0.01 M HCl, 0.1 M HCl, 1 M HCl, 0.01 M NaOH, 0.1 M NaOH, 1 M NaOH, 3 % H_2O_2, 33 % H_2O_2, light, and a temperature of 60 °C. A degradation of 10–20 % can be found as a recommendation for stress testing to produce relevant degradation products. The above-described validated chromatographic method was used for the determination of etrasimod concentration in stress samples. The etrasimod concentration of 10 μg/mL was used due to its low solubility. Stressed sample chromatograms are illustrated in Fig. 2.

A total of eight novel degradation products were formed under different stress conditions that were never reported earlier. Relative

Table 2
Stability-indicating method validation data for determination of etrasimod.

Validation parameter	Value
Linearity	
Range (μg/mL)	2–10
Equation	y = 28.031x–4.8094
Standard error of slope	0.26
Standard error of the intercept	1.74
Correlation coefficient (*r*)	0.9998
Regression sum of squares	31445
Residual sum of squares	8.25
Total sum of squares	31453
Sensitivity [a]	
Limit of detection (μg/mL)	0.10
Limit of quantitation (μg/mL)	0.34
Precision [b]	
Repeatability (*n* = 6; RSD, %)	0.26
Intermediate precision (*n* = 9; RSD, %)	0.39
Accuracy [c]	
Low (*n* = 3; mean recovery, %/RSD, %/accuracy error)	102.0/0.26/0.04
Medium (*n* = 3; mean recovery, %/RSD, %/accuracy error)	100.5/0.28/0.03
High (*n* = 3; mean recovery, %/RSD, %/accuracy error)	100.3/0.09/0.03

[a] The limit of detection (LOD) and quantitation (LOQ) were obtained by diluting the standard solution based on a signal-to-noise ratio 3:1 and 10:1, respectively.

[b] The repeatability was performed by analyzing the standard solution (6 μg/mL) within the same day in six replicates, while the intermediate precision was performed by analyzing same solution on three consecutive days in three replicates.

[c] Accuracy was performed by analyzing standard solutions (2, 6, and 10 μg/mL) in three replicates.

retention times (RRTs) of all those products are lower than 1.0, indicating lower hydrophobicity of degradation products compared to etrasimod. As per the elution pattern, these degradation products were denoted from DP1 to DP8 (Tables 3 and 4).

Seven of eight degradation products at relative retention times of 0.73, 0.83, 0.87, 0.90, 0.94, 0.96, and 0.99 concerning peaks DP1-DP6 and DP8 were formed under the photolytic stress condition within 5 h. Moreover, the most hydrophilic degradation product, DP1, as well as degradation products DP3 and DP5, were produced only by exposing etrasimod to daylight.

Degradation in an acidic environment using 0.01 HCl occurred rapidly, achieving more than 10 % degradation in 5 h. Acidic stress on etrasimod at room temperature resulted in four key degradation products marked as DP2, DP4, DP6, and DP8.

The rate of degradation under oxidative conditions was slower as compared to that of acidic hydrolysis. The chromatographic data

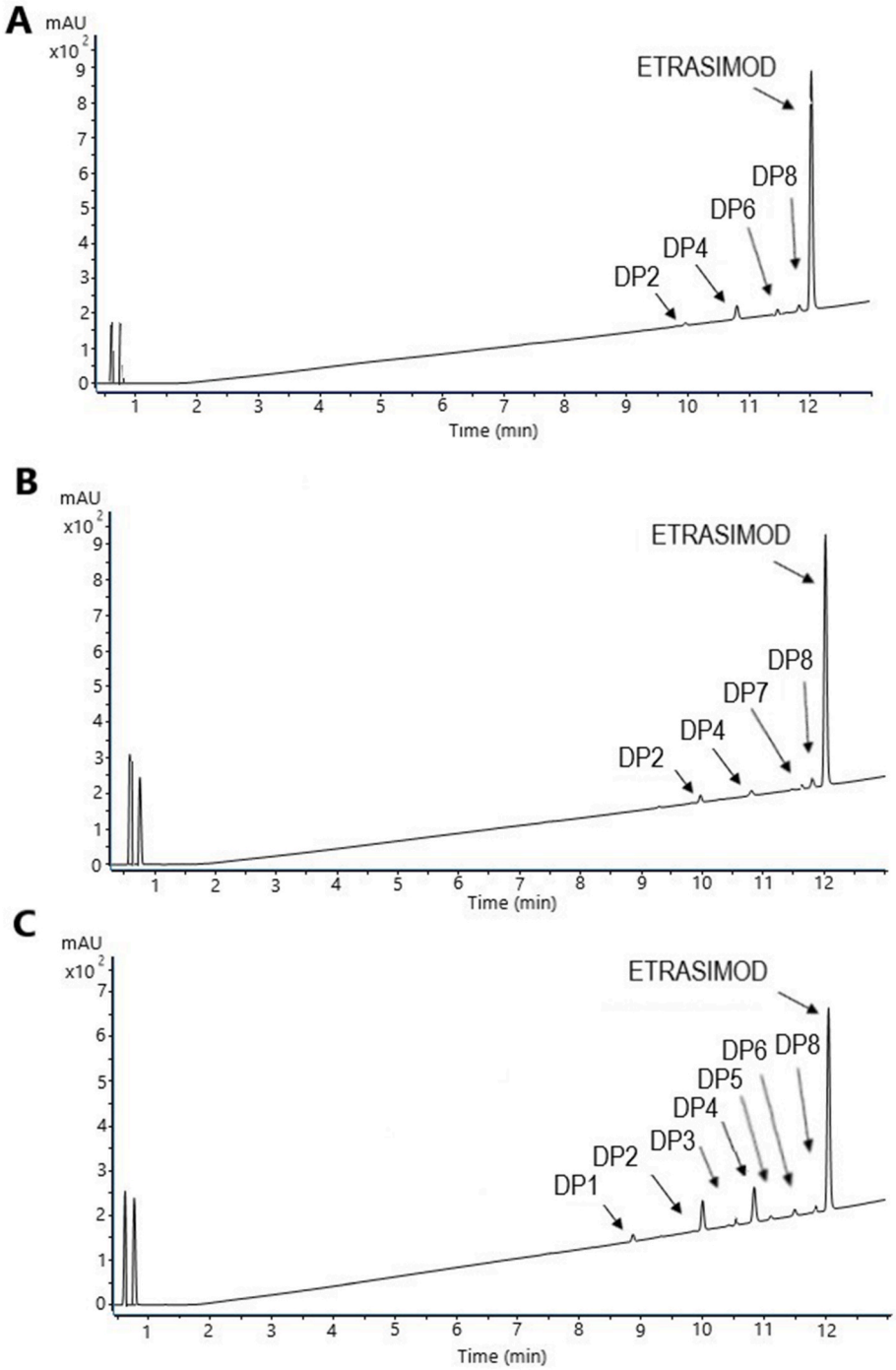

Fig. 2. Representative chromatograms of etrasimod under acidic (A), oxidative (B), and photolytic (C) stress conditions.

Table 3
Forced degradation study results.

Stress condition	Temperature/time	Degradation of etrasimod (%)	Reaction rate constant (k), 1/h	Correlation coefficient (r)	Observation
Base hydrolysis (1 M NaOH)	room temperature/ 7 days; 60 °C/7 days	–	–	–	No degradation observed
Acid hydrolysis (0.01 M HCl)	room temperature/ 5 h	10.46	0.0220	0.987	Formation of degradant products: DP2, DP4, DP6, DP8
Oxidation 33 % H_2O_2	room temperature/ 5 h	10.12	0.0047	0.990	Formation of degradant products: DP2, DP4, DP7, DP8
Thermal degradation	60 °C/7 days	–	–	–	No degradation observed
Photolytic degradation	room temperature/ 5 h	10.32	0.0161	0.950	Formation of degradant products: DP1, DP2, DP3, DP4, DP5, DP6, DP8

Table 4
Retention and mass spectrometric data of etrasimod and degradation products obtained by forced degradation study.

Analyte	Retention time (min)	Relative retention time (RRT)	Retention factor (k)	Base peak (m/z)	Major fragments (m/z)
Etrasimod	12.01	0.0	14.49	458	430, 398, 389, 231, 227, 203, 186, 171, 159, 144
DP1	8.80	0.73	10.35	288	310, 272, 149
DP2	10.02	0.83	11.93	490	528, 512, 472, 458, 412, 227, 159
DP3	10.47	0.87	12.51	508	556, 522, 490, 472, 458, 428, 412, 338, 316, 280, 227, 159, 149
DP4	10.86	0.90	13.01	490	528, 512, 472, 458, 402, 338, 227, 159, 149
DP5	11.24	0.94	13.59	428	338, 284
DP6	11.50	0.96	13.83	504	556, 526, 458, 430, 338, 284, 149
DP7	11.62	0.97	14.00	561	583, 531, 458, 430, 338, 272, 149
DP8	11.89	0.99	14.34	326	364, 338, 284, 257, 149

on the oxidative stress sample showed the formation of four degradation products. DP2, DP4, and DP8 were commonly formed under acidic hydrolysis and photolytic degradation conditions, as well as oxidative degradation conditions. The formation of DP7 was only observed under oxidative degradation conditions.

Degradation in basic conditions using 1 M NaOH did not provide noteworthy etrasimod degradation in seven days. However, a rise of a potentially relevant degradation product was noticed at a level of 0.92 % of the total peak area. On the other hand, basic conditions did not provide further degradation of etrasimod even when an elevated temperature of 60 °C was applied. Likewise, there was no major degradation observed in thermal degradation conditions even after exposure of the drug to stress conditions for 7 days, indicating that the drug is highly stable at elevated temperatures.

In order to determine the selectivity of the method, peak purity analysis was done by using diode array detection. The peak purity of etrasimod in all stress samples was higher than 999. The forced degradation study proved that the developed method is stability-indicating and capable of separating the closely eluting degradation products (resolution between adjacent peaks was higher than 1.5 in all stressed samples).

Furthermore, the order of etrasimod degradation was evaluated to support the establishment of its shelf life and optimum storage conditions for drug substance and dosage forms. To perform a degradation kinetics study of etrasimod the samples were collected at predefined time intervals (data collected at six time points: 0, 1, 2, 3, 4, and 5 h). The concentrations of the remaining etrasimod were calculated, and the reaction order was established by plotting the drug concentration (zero-order), the logarithm (first-order), and the reciprocal (second-order) versus time. The regression equations were computed, the correlation coefficients (r) were obtained, and the best-observed fit indicated the reaction order. The results of the forced degradation study found clear support for the first-order degradation reaction of etrasimod, as the degradation reaction rate under all stress conditions was proportional to a decrease in the initial concentration of the drug as a function of time (Table 3). The highest degradation rate coefficient (k) was achieved for acid degradation ($k = 0.022\ h^{-1}$), indicating the fastest degradation of etrasimod in an acidic environment.

3.4. Mass spectrometry of etrasimod and degradation products

Monitoring of the degradation products relied on the in-depth study of the mass spectrum of etrasimod and its degradation products to pinpoint the characteristic fragmentation processes. A highly sensitive Triple Quadrupole mass spectrometer coupled with an electrospray ionization source was used to characterize etrasimod and its degradation products [26].

Initially, etrasimod was subjected to an MS scan to determine the m/z value and its prominent signals. The response of etrasimod to ESI was evaluated by recording the full-scan mass spectra in both positive and negative ionization modes. Although the negative ionization mode showed lower background noise, a higher signal intensity was obtained in the case of the positive ionization mode (Fig. 3). Etrasimod was observed with a major ion corresponding to the protonated molecule $[M+H]^+$ at m/z of 458, with the presence

of the adduct ions $[M+Na]^+$ and $[M+K]^+$ at *m/z* of 480 and 496, respectively.

Subsequently, an MS/MS experiment was performed to gain comprehensive knowledge of the fragmentation behavior of the drug, aiding in the establishment of a fragmentation pattern for etrasimod. The detailed ESI-MS/MS spectrum of etrasimod is depicted in Fig. 3. The fragmentation behavior of etrasimod shows the breaking of the precursor ion *m/z* 458 into many different product ions (*m/z* 430, 398, 389, 231, 227, 203, 186, 171, 159, and 144). One of the most prominent peaks (*m/z* 398) was obtained by McLafferty rearrangement occurring next to the carboxylic group. The *m/z* of 389 was produced from the parent ion with the loss of the

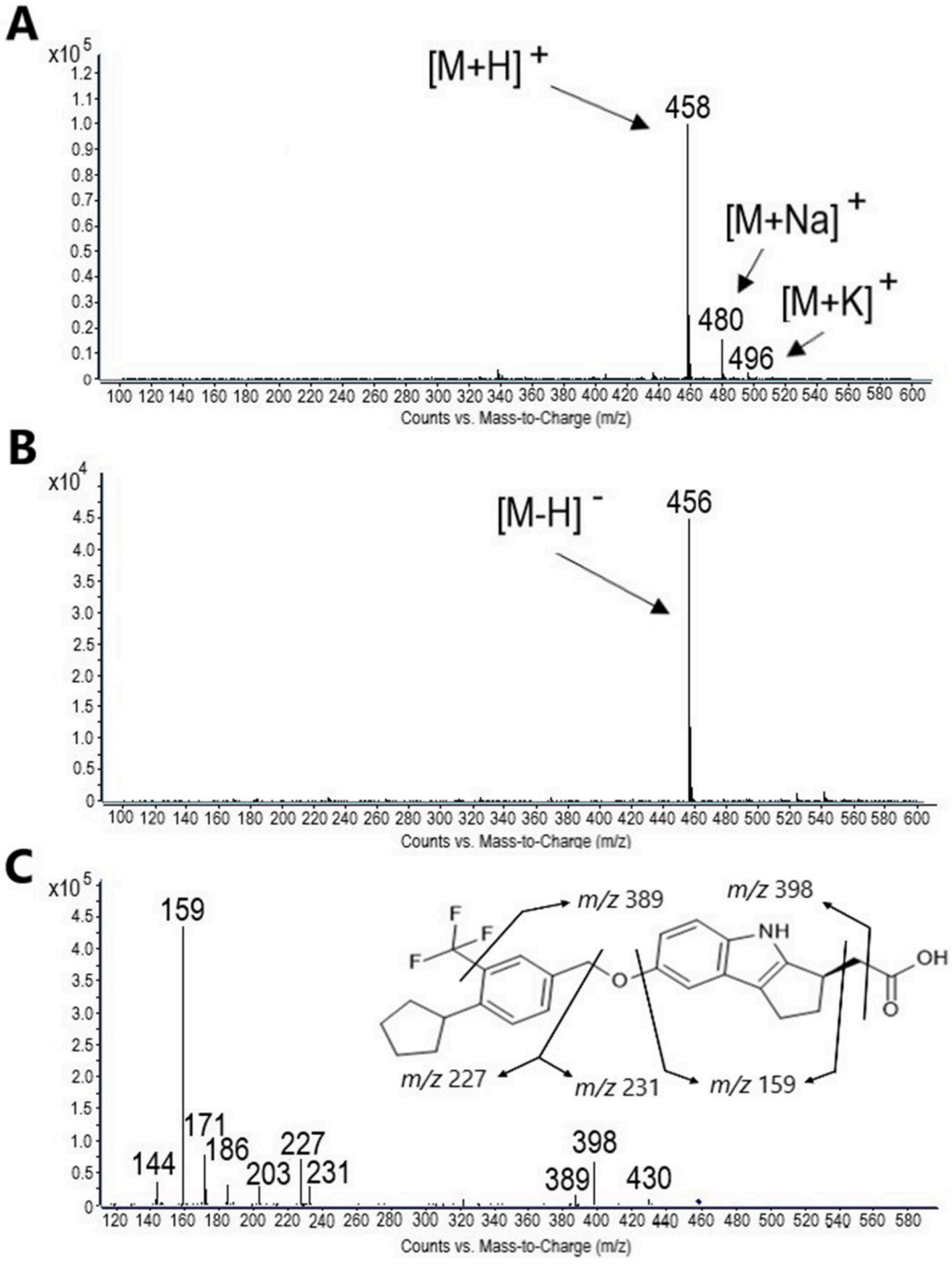

Fig. 3. MS and MS/MS spectra of etrasimod: positive ESI-MS spectrum (**A**), negative ESI-MS spectrum (**B**), and ESI-MS/MS spectrum of $[M+H]^+$ ion (*m/z* 458) with proposed fragmentation pattern (**C**).

trifluoromethyl group through cleavage of the C–C bond. The heterolytic cleavage of the C–O bond between the phenyl ring and central tricyclic cyclopenta[*b*]indol ring resulted in the generation of two ions with *m/z* of 227 and 231. HPLC/MS/MS analysis demonstrated the base peak at *m/z* of 159. This fragment ion was suspected to correspond to the central tricyclic ring produced by the removal of the 4-cyclopentyl-3-(trifluoromethyl)phenyl]methoxy fragment, followed by the neutral loss of 60 Da observed by the cleavage of the $–CH_2COOH$ fragment. To our knowledge, only one report on the disposition of etrasimod in healthy subjects giving mass spectrometric data of etrasimod without fragmentation pathways is available in the literature [27].

Following the evaluation of the etrasimod fragmentation pathway, LC-MS data were acquired for each stress sample. Fig. 4 presents the spectra of all eight degradation products (DP1-DP8) obtained when etrasimod is treated under different stress conditions.

The formation of the degradation product DP1 was caused only by photolytic degradation. The MS spectrum of the most hydrophilic degradation product (RRT = 0.73) revealed a pseudomolecular ion $[M+H]^+$ at *m/z* of 288 and an adductor ion $[M+Na]^+$ at *m/z* of 310. A mass difference of 170 Da compared to etrasimod accounts for the prominent degradation of the etrasimod molecule. The MSTools simulator was used to propose the molecular formula for DP1. The program revealed that DP1 might be formed by the loss of $–CH_2COOH$ and $–CF_3$ groups with a molecular formula of $C_{20}H_{17}NO$.

MS spectra of degradation products DP2 (RRT = 0.83) and DP4 (RRT = 0.90) show pseudomolecular ion $[M+H]^+$ at *m/z* of 490 with adductor ions $[M+Na]^+$ and $[M+K]^+$ at *m/z* of 512 and 528, respectively. Both the MS spectra with a mass difference of 32 Da compared to etrasimod are similar, except that the intensities of fragment ions were varying, suggesting an isomeric structure for DP2 and DP4. Moreover, etrasimod contains one chiral center, supporting the isomeric structure of its degradation products. The spectra of both degradation products show an ion at *m/z* 472 suggesting loss of H_2O from pseudomolecular ions $[M+H–H_2O]^+$. Furthermore, the

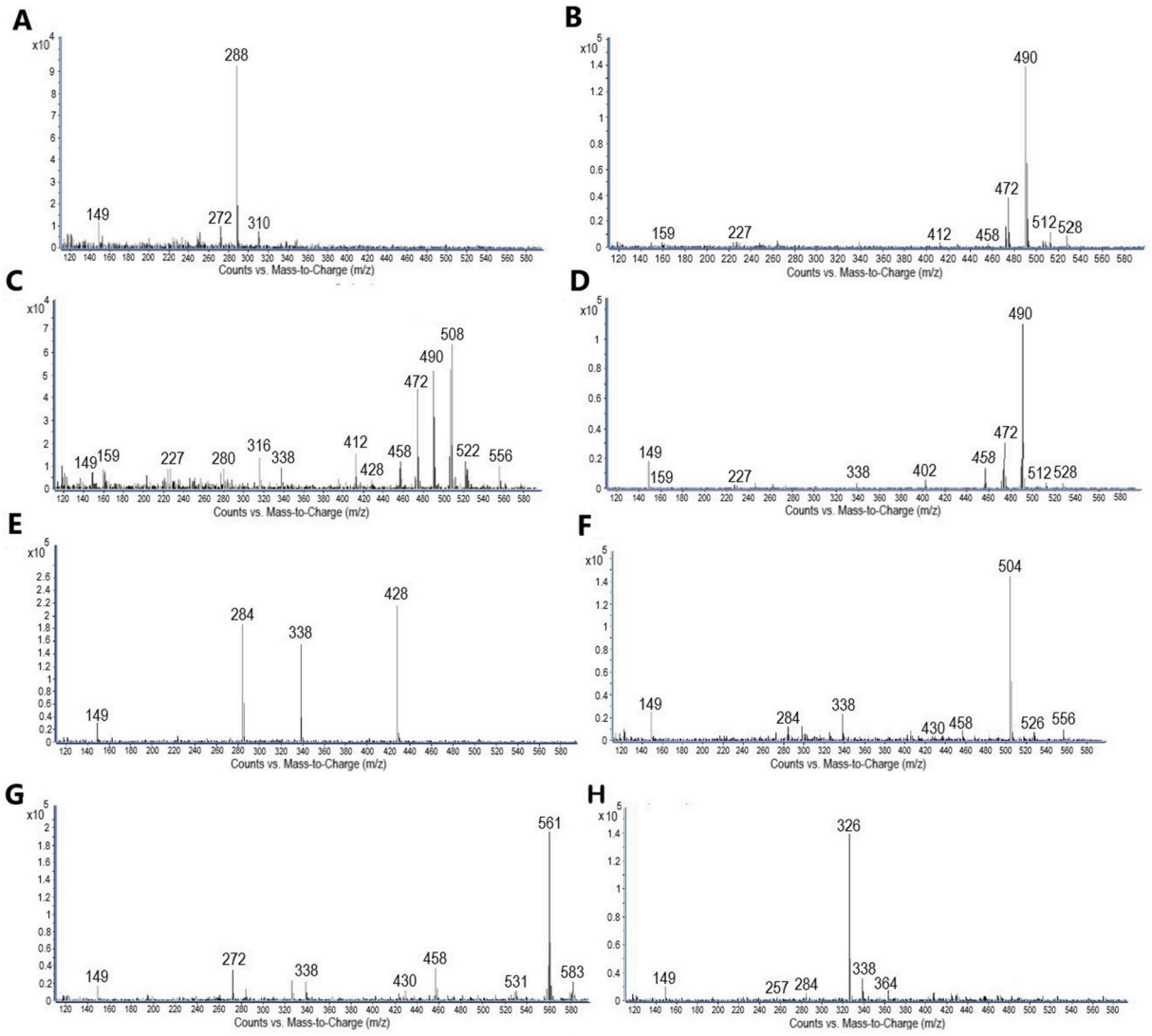

Fig. 4. ESI-MS spectra of degradation products: DP1 (A), DP2 (B), DP3 (C), DP4 (D), DP5 (E), DP6 (F), DP7 (G), and DP8 (H).

ion corresponding to the pseudomolecular ion of etrasimod at *m/z* of 458 was observed in their spectra. Although some authors explored the possibility of resolving isomers by MS/MS on the basis of "diagnostic fragments", these degradation products need to be further distinguished using a chiral chromatographic system [28].

Under photolytic degradation, the degradation product DP3 was formed. Spectrum analysis of this degradation product demonstrated the base peak at *m/z* of 508 and other prominent ions at *m/z* of 490 (formed by loss of H_2O from the base peak) and 472 (formed by further loss of H_2O). The ions at *m/z* 458, 227, and 159, which are similar to those obtained for etrasimod, are also observed.

Photolytic degradation formed the product DP5 (RRT = 0.94), whose mass spectrum demonstrated three prominent peaks (*m/z* 428, 338, and 284). The base peak of DP5 showed a mass difference of 30 Da compared to etrasimod, and thus the MSTools simulator proposed that the photolytic degradation caused the loss of two oxygens and the formation of a degradant with the molecular formula $C_{26}H_{28}ONF_3$.

The MS spectrum of degradation product DP6 (RRT = 0.96) shows pseudomolecular ion $[M+H]^+$ at *m/z* 504 with a mass difference of 46 Da compared to etrasimod. The low abundance adductor ion $[M+Na]^+$ at *m/z* 526 is observed in the spectrum as are the ions at *m/z* 458 and 430, which are similar to those obtained for etrasimod.

Oxidative degradation led to the formation of the degradation product DP7 (RRT = 0.97) with the pseudomolecular ion $[M+H]^+$ at *m/z* 561 and the low abundance adductor ion $[M+Na]^+$ at *m/z* 583, as well as ions at *m/z* 458 and 430, which are similar to those obtained for etrasimod.

The degradation product DP8 (RRT = 0.99) formed under acidic hydrolysis, oxidation, and photolytic degradation showed pseudomolecular ion $[M+H]^+$ at *m/z* 326 and the low abundance adductor ion $[M+K]^+$ at *m/z* 364. The *m/z* 257 was produced from the parent ion with the loss of the trifluoromethyl group (loss of 69 Da) through cleavage of the C–C bond.

Based on the data presented in US patent US8853419B2 (Processes for the preparation of (R)-2-(7-(4-cyclopentyl-3-(trifluoromethyl)benzyloxy)-1,2,3,4-tetrahydrocyclopenta[*b*]indol-3-yl)acetic acid and salts thereof) for enantiomeric separation of etrasimod and its impurity (3S), chiral chromatographic columns need to be employed. In our future research, we plan to evaluate the applicability of chiral separation in the forced degradation study of etrasimod. Furthermore, future research should certainly further test the applicability of the developed stability-indicating method to analyze dosage forms.

3.5. Green profiling of forced degradation procedure

The criteria of ideal green analysis include the elimination or minimal use of organic solvents and reagents, reduced energy consumption, reduced sample preparation, and no waste generation. In liquid chromatographic analysis, elimination of the use of organic solvents and mobile phase modifiers cannot be applicable, while some practices can be adopted in order to make such procedures greener, such as the replacement of hazardous reagents with their safer and easily degradable equivalents, eliminating or reducing the hazard of waste, reducing the number of stages in a given analytical procedure, and applying miniaturized sample preparation procedures [29]. Finding the most appropriate way to evaluate the green character of an analytical procedure is challenging since many different parameters must be taken into consideration. The sustainability of the developed forced degradation procedure was evaluated using four different greenness appraisal tools.

The first assessment tool was AMGS, which includes the safety, health, and environmental assessment of the solvents utilized, as well as solvent energy demand, instrument energy consumption, and waste production [21]. The calculated greenness score of the forced degradation procedure of etrasimod using the developed stability-indicating method was 629. Based on calculation data, the

Fig. 5. The ComplexGAPI pictogram of etrasimod forced degradation study. Legend: 1 – collection, 2 – preservation, 3 – transport, 4 – storage, 5 – type of method, 6 – scale of extraction, 7 – solvents/reagents used, 8 – additional treatment, 9 – amount of reagents and solvents, 10 – health hazard of reagents and solvents, 11 – safety hazard of reagents and solvents, 12 – instrumentation energy, 13 – occupational hazard, 14 – waste, 15 – waste treatment, I – yield, II – pre-analysis temperature and time, III – relation to green economy, IV_a – health hazard of reagents and solvents used in pre-analysis, IV_b – safety hazard of reagents and solvents used in pre-analysis, V_a – technical set up, V_b – energy of instruments used in pre-analysis, V_c – occupational hazard of pre-analysis, VI_a – end products workup, VI_b – purity. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

highest contribution to the greenness score was contributed to instrument energy (almost 50 %), followed by solvent influence (more than 35 %). Although the main objective of this tool is to compare methods during method development and an absolute measure of method greenness is not given, the analyst can gain insight into the weak and strong points of the developed analytical procedure. We are aware that the energy consumption of the HPLC/MS/MS technique per single run is about six times higher than that of HPLC, but still, MS detection offered us valuable data on the structural characterization of degradation products.

The Analytical Eco-Scale introduced by Gałuszka and co-workers [22] was used to evaluate the penalty points of the forced degradation procedure. The procedure collected 21 penalty points, and it was ranked on the scale with a score of 79. Although the highest number of penalty points (in total 5) was achieved due to reagents used for the promotion of degradation as well as waste production (in total 5) the procedure was ranked among excellent green analytical procedures with Analytical Eco-Scale above 75.

The greenness evaluation of the proposed analytical procedure performed by ComplexGAPI is presented in the form of a pictogram (Fig. 5). The components of the analytical procedure that correspond to the principles of green analytical chemistry are given in green (15 components), components that somewhat correspond are given in yellow (5 components), and components that do not correspond with the principles are given in red (5 components). Red (fields 1, 7, and 9) and yellow (fields 10 and 11) fields are related to the stability-indicating method due to the usage of organic solvents and formic acid as mobile phase modifiers. Furthermore, each sample analysis produces approximately 7 mL of waste. Still, numerous samples had to be analyzed during method development and validation, making the forced degradation procedure "less green" (fields 14 and 15). The hexagonal field at the bottom of the pictogram describes the green character of pre-analysis processes related to the preparation of stress samples. The ComplexGAPI pictogram indicated yellow fields due to the conduction of thermal stress (field II) and chemical stress (IVa and IVb), although low amounts of these reagents were used.

In an attempt to provide a more complete greenness profile two recently developed complementary metric tools were used, namely, the Analytical GREEnness Metric Approach (AGREE) and the Analytical GREEnness Sample Preparation Metric Approach (AGREEprep) [24]. To evaluate the greenness of the forced degradation procedure, the AGREE approach shows the overall score of 0.71, colored in green, indicating that the developed method is obeying the eco-friendly conditions with several limitations (Fig. 6). The performance of the forced degradation procedure in each of the assessment criteria is reflected by the color in the segment with the number corresponding to each criterion. The scores are in the range of 0.33–1.00, reflected in the yellow, orange, and green colors. The inspection of 12 criteria used for procedure greenness assessment highlighted that 9 out of 12 criteria are marked with various hues of green with scores between 0.67 and 1.00. The most critical points of our procedure are related to the usage of hazardous chemicals used for the induction of stress conditions, none of which originates from bio-based sources, making the score equal to 0.5 (criteria 10, highlighted in yellow). Those chemicals produced analytical waste due to stress samples and mobile phase preparation (criterion 7, score 0.33, highlighted in orange). Each sample analysis produces approximately 7 mL of waste that positions this criterion in the middle of the scale. Method development and validation, as well as degradation kinetics of etrasimod evaluation includes numerous sample analysis that need to be considered. The direct analytical methodology in a forced degradation study cannot be applied; thus, criterion 3 is scored with 0.33 and highlighted in orange.

During the forced degradation study of adalimumab, we identified sample preparation as the most critical step from an eco-friendly analytical chemistry point of view [30]. To obtain a more comprehensive approach to the greenness profiling of sample preparation, the AGREEprep approach was applied [29]. The evaluation produced a round pictogram with a circle in the center that shows the overall score of 0.64 colored in light green and ten trapezoid bars corresponding to the ten criteria, each having a length equivalent to the assigned weight and color responding to the greenness of the method parameter (Fig. 7). Compared to green profiling of the overall forced degradation procedure obtained by the AGREE approach, the AGREEprep approach demonstrated lower eco-friendliness of

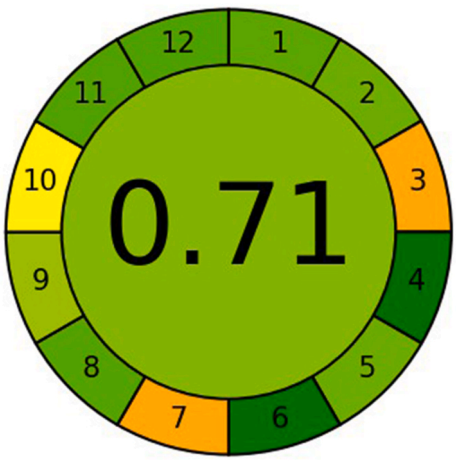

Fig. 6. The AGREE pictogram of etrasimod forced degradation study. Legend: 1 – sample procedure, 2 – sample size, 3 – sampling, 4 – sample preparation steps, 5 – automatization, 6 – derivatization, 7 – waste, 8 – analysis throughput, 9 – energy consumption, 10 – renewable reagents, 11 – toxicity of reagents, 12 – operator' safety.

sample preparation. The trapezoid bars showed scores in the range of 0.25–1.00, demonstrating the eco-friendliness of each of them with yellow, orange, and green colors. Five green scores with values above 0.66 are earned due to low sample size, energy consumption, and high sample preparation throughput. Criterion 3 promotes materials that are sustainable, renewable, and reusable. As we used recycled materials and reusable laboratory glassware whenever possible, a score of 0.5 was obtained for criterion 3 and is highlighted in yellow. The criteria focused on the application of non-toxic reagents (criterion 2, score 0.43), automatization of analytical procedure (criterion 7, score 0.38), and analyst safety (criterion 10, score 0.25) are highlighted in orange, propounding the weakest point of the forced degradation procedure. We found 5 criteria (1, 3, 4, 5, and 8) focused on sample and waste size more significant in terms of greenness evaluation, and thus modifications of weight lengths were done.

Although the green metric tools for analytical method assessment provided data of diverse complexity, all of them highlighted two major weak points of the performed forced degradation study. Firstly, the energy consumption of the HPLC/MS/MS technique is pointed out; still, we can justify the implementation of this advanced technique as it offered us data on the structural characterization of etrasimod and its degradation products published for the first time. Furthermore, special attention was given to method selectivity, as eight structurally related analytes were determined within the method runtime of 15 min. Secondly, all green metric tools highlighted the use of reagents as the weakest point of the procedure. As there is no alternative to those chemicals for forced degradation conduction in the relevant guidelines, we tried to improve the ecological sustainability of the developed procedure by reducing the volume size of the sample as much as possible without influencing method reliability.

4. Conclusions

In the present work, the forced degradation study of etrasimod was performed using a developed and validated HPLC-DAD-QqQ-MS/MS stability-indicating method. The proposed method proved to be linear, precise, accurate, and selective for the quantitative analysis of etrasimod in stress samples. Etrasimod demonstrated good stability under elevated temperatures and basic stress conditions. The stress conditions produced eight degradation products of etrasimod. The knowledge of the degradation process is useful in establishing the acceptance criteria for etrasimod as a drug substance and drug product during quality control and stability assessment.

The eco-friendliness of the developed forced degradation procedure was evaluated using four different greenness appraisal tools. This aspect of research suggests that the established forced degradation procedure is obeying eco-friendly conditions. As the sample preparation procedure was highlighted as one of the weakest points of the analytical procedure, ecological sustainability was improved by reducing the volume size.

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Data availability statement

Data will be available on request.

CRediT authorship contribution statement

Jelena Kovačić: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Daniela Amidžić Klarić:** Writing – review & editing, Visualization, Conceptualization. **Nikša Turk:** Writing – review & editing, Conceptualization. **Željko Krznarić:** Writing – review & editing, Conceptualization. **Ana Mornar:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

List of abbreviations

CD	Crohn's Disease
IBD	Inflammatory Bowel Disease
ICH	International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use
DAD	Diode Array Detector
EMA	European Medicinal Agency
ESI	Electrospray Ionization
FDA	U.S. Food and Drug Administration
LOD	Limit of Determination

Fig. 7. The AGREEprep pictogram of etrasimod forced degradation study. Legend: AGREEprep: 1 – sampling, 2 – hazardous materials, 3 – sustainability, renewability, and reusability of materials, 4 – waste, 5 – economy of a sample, 6 – sample throughput, 7 – automatization, 8 – energy consumption, 9 – analytical instrumentation, 10 – operator's safety.

LOQ	Limit of Quantitation
MS/MS	Tandem Mass Spectrometry
RRT	Relative Retention Time
RSD	Relative Standard Deviation
S1P	Sphingosine-1-Phosphate
UC	Ulcerative Colitis
HPLC	High-Performance Liquid Chromatography

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e34066.

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