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A novel LC-MS/MS technique for identification and characterization of degradation products of Bilastine and Montelukast sodium and its greenness assessment using AGREE tool

Sapna M. Rathod[1]*, Nisarg C. Patel[2], Riyaz Dantroliya[1], Bhupendra G. Prajapati[3]

[1]Department of Pharmaceutical Chemistry and Quality Assurance, APMC College of Pharmaceutical Education and Research, Himatnagar, India.
[2]Department of Pharmacognosy, APMC College of Pharmaceutical Education and Research, Himatnagar, India.
[3]Department of Pharmaceutics, Shree S. K. Patel College of Pharmaceutical Education and Research, Ganpat University, Mehsana, India.

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ABSTRACT

The degradation products (DPs) of Bilastine and Montelukast sodium have been identified and characterized utilizing a unique liquid chromatography-tandem mass spectrometry (LC–MS/MS) technique. Both medications are anti-allergic, and they were subjected to hydrolysis, oxidation, photolysis, and heat stimuli in accordance with International Council for Harmonisation guidelines. Under circumstances of oxidative, acidic, and base hydrolysis stress, the cited drugs exhibited widespread degradation. The drug products were stable under thermal and photolytic stress. Six DPs were found and resolved. The drug and its breakdown products were separated chromatographically on an Agilent Zorbax C18 (150 × 4.6 mm, 5µm) column with an eluent of 10 mM ammonium acetate (pH 4): acetonitrile (25:75, *v/v*). The hypothesized structure of DPs and their corresponding routes are based on precise mass and MS/MS fragmentation patterns; obtained using LC-ESI-TQ-MS/MS. The suggested degradant structures and pathways will be crucial for optimizing the production and quality control parameters of the pharmaceuticals under consideration.

INTRODUCTION

Antihistamines are a family of medications used to treat allergies brought on by histamines physiological effects [1]. The current guidelines state that non-sedating antihistamines are mostly used nowadays to treat allergic rhino conjunctivitis, which includes urticaria. Bilastine (BLS) is a new second-generation H1-antihistaminic medication that is used to treat chronic urticaria and allergic rhinitis (AR) in patients over the age of 12 [2]. Its structure is represented in Figure 1A.

**Corresponding Author*
Sapna M. Rathod, Department of Pharmaceutical Chemistry and Quality Assurance, APMC College of Pharmaceutical Education and Research, Himatnagar, India.
E-mail: srathod456 @ gmail.com

Cysteinyl leukotriene receptor antagonists like Montelukast (MTK) and Zafirlukast are prescribed to prevent and treat persistent asthma [3]. A cysteinyl leukotriene receptor blocker, MTK sodium is employed to manage symptoms of seasonal allergies and asthma. MTK functions by attaching to a cysteinyl leukotriene receptor in the lungs and bronchial tubes and obstructing leukotriene D4's function on it [4]. Its structure is shown in Figure 1B.

A new combination of BLS and MTK was approved by DCGI on 09 March 2020 [5] to treat adult cases of AR. This combination is beneficial in the management of seasonal allergic rhino conjunctivitis and mild to moderate asthma. As per International Council for Harmonisation (ICH) [6] and other regulatory guidelines [7], forced degradation studies are crucial for examining how drugs and drug products change during storage and for figuring out how environmental conditions such as light exposure, oxidation, and hydrolysis (acid, base,

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Figure 1. Chemical structure of BLS (A) and MTK (B).

and neutral) affect things. In contrast to general stability investigations, forced degradation must produce the greatest number of degradation products (DPs) in the shortest amount of time [8,9]. Since the degradants could have harmful qualities, it is important to understand DPs, their structures, and the processes by which they degrade. For structural analysis of DPs without isolating the molecule, hyphenated techniques namely liquid chromatography-tandem mass spectrometry (LC-MS/MS), LC-NMR, and LC-MS have all been widely used; LC-MS/MS have been used mostly because of their sensitivity [10]. In the field of drug development, the use of forced degradation in conjunction with the identification and characterization of DPs is essential to understanding drug stability [11].

The majority of public and business sectors now place a high priority on and employ green chemistry concepts. Understanding the influence of analytical procedures on the ecosystem and economy requires evaluating their sustainability. But sustainability is a complex concept that takes into account several aspects, including waste reduction, effectiveness, safety, greenness, and cost. Sustainability cannot be fully assessed by a single method in all of these dimensions. AGREE is therefore used in this work as a methodology to facilitate a more comprehensive examination. For a personalized greenness assessment, 12 key green chemistry ideas can be customized for weighting using the AGREE tool. The measure presents the outcome as a color image and is meant to be readily comprehended [12]. Moreover, AGREE is easy to utilize because readily accessible software can be used to apply it [13]. Anywhere from dark red (0) to dark green (1) could be the color. When evaluating environmental standards-based greenness, AGREE is a priceless tool [14].

A thorough literature search reveals that some spectrophotometric [15–22], few chromatographic methods [18, 23–42], and spectrofluorometric methods [43] for the concurrent determination of MTK and BLS in API and dosage form were reported. The comprehensive characterization and molecular mechanism of DPs of BLS and MTK under stress conditions have not yet been documented in any study. The primary goals of the current investigation were to identify the primary DPs and examine the drug's overall degrading behavior. In order to determine the fragmentation pattern of the drug and its DPs, the drug was exposed to the ICH-recommended stimuli of hydrolysis, heat, oxidation, and photolysis. The resultant solutions were then analyzed using optimized LC–MS/MS and precise mass measurements.

MATERIALS AND METHODS

Chemical and reagents

BLS (99.94% pure) and MTK (99.86% pure) were gifted from Shagun Pharmaceuticals (Indore, Madhya Pradesh, India) and Sehat Pharma Pvt Ltd (Himmatnagar, Gujarat, India) respectively. Acetonitrile, methanol and ammonium acetate (HPLC grade), sodium hydroxide, formic acid, hydrochloric acid, and hydrogen peroxide (analytical grade) were acquired from Rakesh Chemicals, Ahmedabad, Gujarat, India.

Instrumentation and mass spectrometric conditions

The instrument utilized was an API-2000 Liquid chromatography-mass spectrometer, which was outfitted with a column oven, auto injector, auto sample, ion source electron spray ionizer (ESI) with Q1, and collision energy. To separate each component, an Agilent Zorbax C18 column (150 × 4.6 mm, 5 µm) was used. The ABScix API 2000 series online ion trap MSD mass spectrometer, which was outfitted with an auto sampler and an ESI source in positive mode, was used to perform LC–MS/MS. Software called Analyst QS managed the data acquisition process. An ion trap (quadrupole) mass spectrometer was used for the MS^n investigations, fitted with an ionization source (electrospray). The mass spectra were captured in the positive detection mode. With helium serving as the collision gas, collision-induced dissociation (CID) was accomplished. The conditions (ion source) were fixed as dry gas (5 l/minute), nebulizer gas (30 psi), dry temperature (400°C), electro multiplier voltage (2100 V), vaporizer temperature (400°C), capillary current (81.787 nA), corona current (4000 nA), dwell time (200 ms), and capillary exit (113.5 V). Capillary voltage of 5 kV (positive mode 4 kV), entry potential (10 V), resolution (10,000), focusing potential (400 V), and declustering potential (20 V) were the typical source settings. As a collision gas and curtain, ultra-high pure nitrogen was utilized, while zero air served as the nebulizer. The quadruple analyzer 1 was used to pick the precursor ion for the CID studies, while the quadrupole analyzer 3 examined the product ions.

LC-MS/MS analysis

Method development and optimization

The sample's solubility, pKa, molecular weight, and type (ionic or neutral molecules) all have a role in the appropriate choice of LC-MS/MS technique. The impact of chromatographic factors such as flow rate, pH, mobile phase, and solvent ratio was investigated in order to optimize the chromatographic settings. For LC-MS/MS method development, C8 and C18 columns and different mobile phases like formic acid, methanol, water, and acetonitrile with or without buffer (ammonium acetate) in different proportions were tried. For every drug and chromatogram, the retention period and tailing factor were computed. After infusing the drug solution into the mass spectrometer, the tuning parameters (ion spray voltage, temperature, ion source gas, curtain gas, declustering potential, etc) were also adjusted to get the higher intensity of selected fragments.

Diluent preparation

The diluent is made up of water and acetonitrile in a 50:50 v/v ratio.

Standard solution preparation

Precisely weighed masses of 10 mg MTK and 20 mg BLS were added into a separate volumetric flask of 100 ml quantity and sonicated to dissolve in diluent and made to mark. This solution represents a standard stock solution. One ml of each stock solution was aliquot into a 10 ml volumetric flask. Diluent was utilized to make up the volume. Furthermore, dilution with diluent was done to prepare a combined working standard solution comprising of MTK 1 µg/ml and BLS 2 µg/ml.

Preparation of eluent

In 25:75 *v*/*v* acetonitrile and water (pH 4; adjusted with formic acid), prepare a 10 mM ammonium acetate buffer. Stir thoroughly and use sonication to degas.

Preparation of sample solution

Weigh and powder 10 tablets. Weigh the quantity of powder equivalent to 10 mg MTK (20 mg BLS) in a 100 ml volumetric flask. This solution represents a sample stock solution. After adding the required amount of diluent, place the flask in a sonicator for 15 minutes. Dilute it upto the mark and filtration was carried out. Further dilutions were made to get the concentration within the linearity range. 20 µl volume was injected into the system and outcomes were reported.

Forced degradation studies

In accordance with ICH recommendations, stress degradation experiments of BLS and MTK were conducted. Every sample that was under stress was removed at appropriate intervals and diluted with mobile phase within the linearity range. Prior to LC-MS/MS analysis, all of the samples were passed through a 0.22 µm membrane filter.

Acid degradation

In two distinct 100 ml volumetric flasks, each ml of stock solution (standard) and sample stock solution were taken separately. Add 1 N of HCl in both flasks and were refluxed. For 4 hours, keep the mixture in a water bath at 60°C. The content was neutralized by adding an equal amount of 1 N NaOH, followed by the addition of a diluent to achieve MTK 1 µg/ml and BLS 2 µg/ml.

Base degradation

In two distinct 100 ml volumetric flasks, each ml of stock solution (standard) and sample stock solution were taken separately. Add 1 N of NaOH in both flasks and were refluxed. For 4 hours, keep the mixture in a water bath at 60°C. The content was neutralized by adding an equal amount of 1 N HCl, followed by the addition of a diluent to achieve MTK 1 µg/ml and BLS 2 µg/ml.

Oxidative degradation

In two distinct 100 ml volumetric flasks, each ml of stock solution (standard) and sample stock solution were taken separately. Add 1 ml of the 30% H_2O_2 solution in both flasks and were refluxed to conduct oxidative breakdown investigations. For 5 hours, keep the contents at 60°C in a water bath. Next, diluent was used to achieve MTK 1 µg/ml and BLS 2 µg/ml.

Thermal degradation and photolytic degradation

Place Petri dishes containing approximately 100 mg of the drug standard in a hot air oven; set to 105°C (Thermal) and in a photostability chamber (Photolytic) for 5 days individually for respective degradation. Five days later, weigh and transfer roughly 10 mg of MTK and 20 mg of BLS into separate 100 ml volumetric flasks, adding diluent to make up the volume. Dilution was made further to get 1 µg/ml MTK and 2 µg/ml BLS. The same procedure was followed for the formulation.

After making final solutions for all the degradation studies, it is injected with a syringe into the mass spectrometer to identify and characterize the DPs of the Drug, and its fragmentation pathway is identified by MRM scan (Q1-Q3).

Method validation

Specificity

The blank solution, working standard solution, and working sample solution of MTK (1 µg/ml) and BLS (2 µg/ml) are injected into the LC-MS/MS system. The chromatogram of blank, standard, and sample was checked for interference.

Calibration curve

Different aliquots like 0.5, 0.75, 1.0, 1.25, and 1.5 ml from the standard stock solution of BLS and MTK were transferred to a series of 100 ml volumetric flasks to obtain the concentration in the range of 0.5–1.5 µg/ml for MTK and 1–3 µg/ml BLS correspondingly. Diluent was utilized for the dilution.

Precision

Repeatability

A standard solution comprising MTK (1 µg/ml) and BLS (2 µg/ml) was injected six times and areas of peaks were measured and % RSD was computed.

Intermediate precision

A standard solution containing (0.5, 1, 1.5 µg/ml) MTK and (1, 2, and 3 µg/ml) BLS were analyzed three times on the same day and on three consecutive days for the determination of intraday and inter day variations correspondingly. The outcomes were noted.

Accuracy

The recovery study was accomplished at three levels (80%, 100%, and 120%) by adopting the standard addition method. The standard solutions of BLS (1.6, 2, and 2.4 µg/ml) and MTK (0.8, 1, and 1.2 µg/ml) were spiked to the predetermined tablet solution (1 µg/ml MTK and 2 µg/ml BLS). The determinations were made thrice and outcomes were reported.

LOD and LOQ

It was determined by substituting the respective values in the equations described in ICH guideline [44].

Robustness

Variations in method parameters like flow rate (± 0.2 ml/minute), ratio of mobile phase (± 2%), and pH (± 0.2) were done to assess the robustness of the proposed approach.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

Preliminary chromatographic experiments were tried on Agilent ZORBEX C8 (150 × 4.6 mm, 5 µm) and Agilent ZORBEX C18 column (150 × 4.6 mm, 5 µm) and different solvents (water, methanol, formic acid, acetonitrile, acetate buffer) were tried as eluent for the resolution of BLS, MTK and its DPs. While using water: methanol (20:80 *v*/*v*), broad peak of BLS is observed. In the case of water: acetonitrile (20:80 *v*/*v*), no peaks were observed for both drugs. Upon the addition of formic acid, in both mobile phases, drug peaks were observed but retention time increases as well as tailing were observed for BLS. Hence, ammonium acetate buffer (pH 4 adjusted with formic acid) was employed in place of water to achieve a satisfactory resolution between the drug and its DPs. Aqueous ammonium acetate buffer (pH 4): acetonitrile (25:75, *v*/*v*) in isocratic mode and Agilent ZORBEX C18 (150 × 4.6 mm, 5 µm) column was finalized as sharp peak and good resolution was obtained for cited drugs and their DPs. There was a 10-minute run time, 35°C column temperature, and a 1 ml/minute flow rate. These ideal chromatographic conditions were applied to the separation of MTK, BLS, and their respective DPs. The procedure was verified in accordance with the specifications given in ICH standards Q2 (R1). To provide a better signal and high sensitivity for LC-MS experiments, the Triple Quadrupole with ESI source conditions was optimized. In order to identify and characterize the DPs, a number of parameters, including drying gas temperature, capillary voltage, skimmer voltage, spray voltage, nebulizing gas flow, and drying gas flow were optimized to maximize ionization in the source and sensitivity even at extremely low quantities.

Degradation behavior of MTK and BLS

The DPs can be identified using the optimized LC-MS approach. Under varied stress circumstances, the LC–ESI–MS total ion chromatograms (TICs) were acquired. Tandem mass spectrometric analysis was used to identify and characterize a total of six DPs. The DPs generated under hydrolysis and oxidative degradation were given different notations, viz. DP1, DP2, and DP3 for MTK and DP1, DP2, and DP3 for BLS, individually. The drug (MTK and BLS) is hydrolyzed in an acidic and basic manner to produce two degradants, DP1 and DP2. Under the oxidative condition (30% H_2O_2; 60°C, 5 hours), one DP(DP3) was formed for both the drugs. It was discovered that both drugs remained stable when exposed to thermal stress and UV light in both their solid and solution forms. Figures 2 and 3 represent the typical chromatograms of BLS and MTK (standard) and their DPs formed under hydrolysis and oxidative stimuli correspondingly. Figures 4 and 5 represent the typical

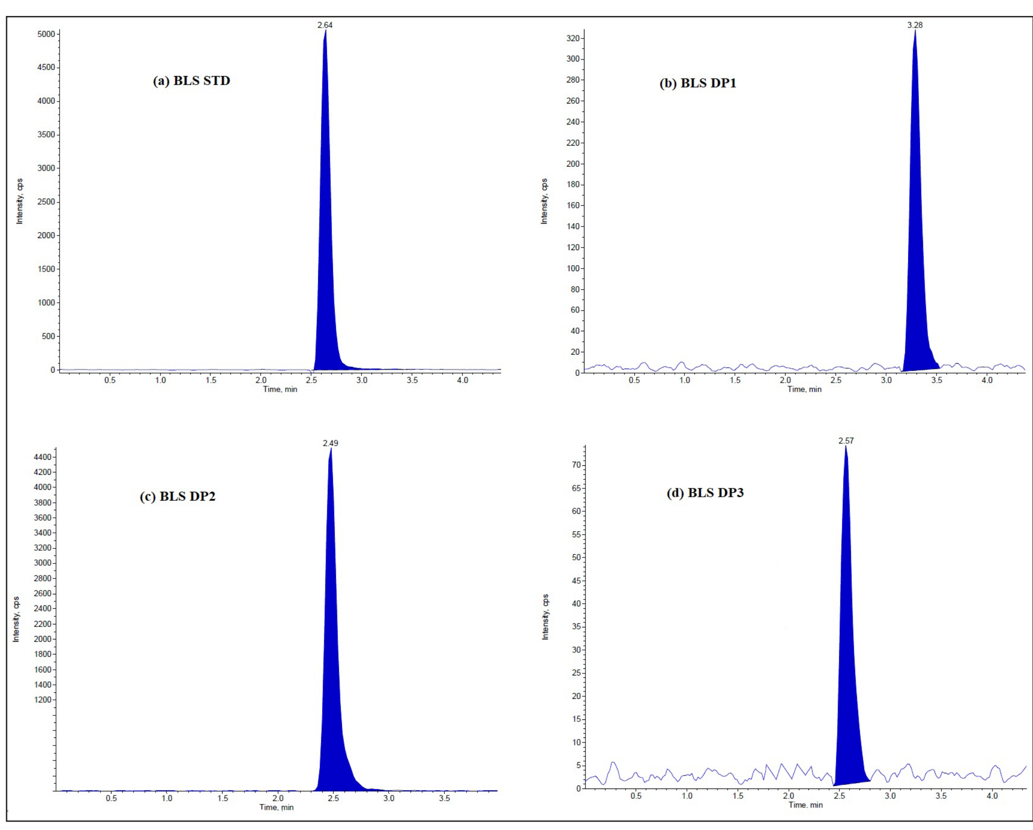

Figure 2. LC/ESI-MS TIC of (a) BLS standard drug (b) Acid degradant (DP1) (c) Base degradant (DP2) (d) Oxidative degradant (DP3).

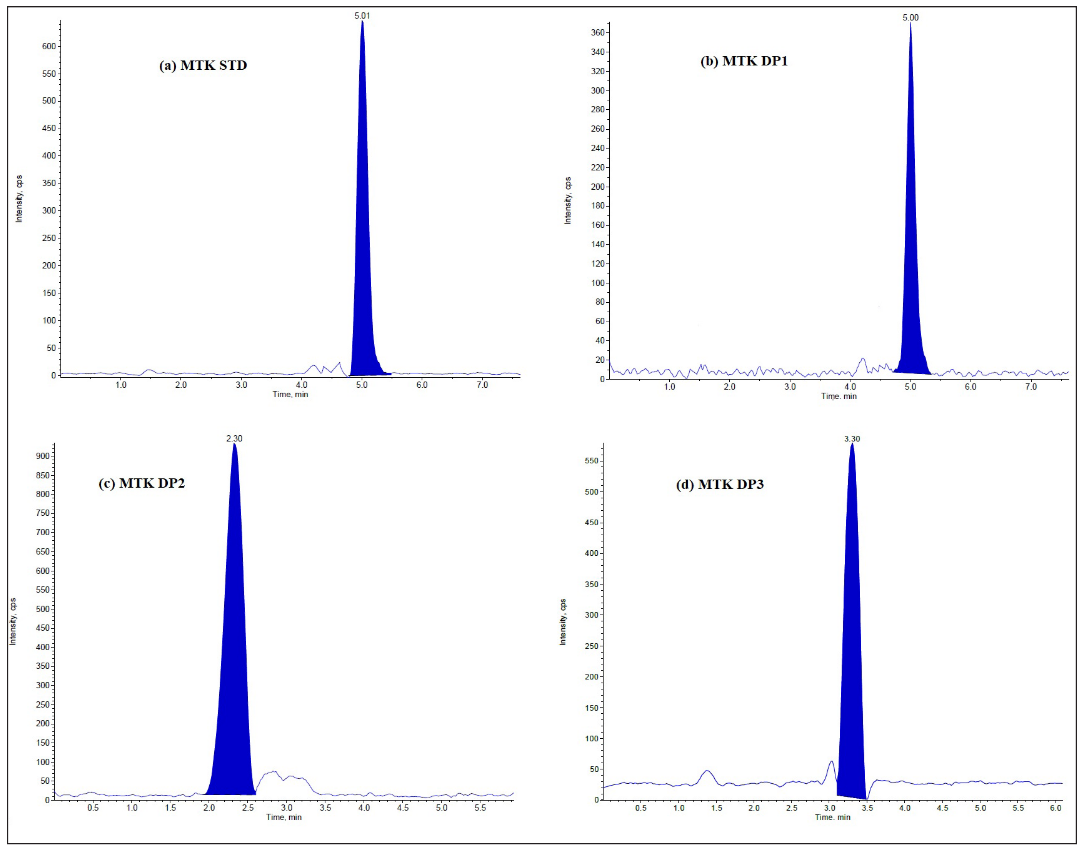

Figure 3. LC/ESI-MS TIC of (a) MTK standard drug (b) Acid degradant (DP1) (c) Base degradant (DP2) (d) Oxidative degradant (DP3).

chromatograms of BLS and MTK (sample) and their DPs formed under hydrolysis and oxidative stimuli correspondingly.

Identification and characterization of DPs by LCMS/MS

With the aid of their fragments from LC-MS/MS investigations and a comparison with the drug's fragmentation pattern from MS/MS and MS^n analysis, all of the DPs were identified. The stressed solution exposed to different conditions was exposed to LC-MS/MS analysis for characterization of the DPs.

MS^n study of MTK and BLS

The MS^n spectra of MTK and BLS were performed at reduced collision energy (0.2 mA), drugs underwent protonation and the molecular ion peak at *m/z* 586.3 Da was noted. For MTK, the ESI/MS of $[M+H]^+$ ion at *m/z* 586.3 Da demonstrates abundant fragment ion at *m/z* 568.4 Da (Removal of - OH from *m/z* 586.3), Mass Difference (MD) of 17, *m/z* 528.1 Da (Removal of – C_3H_5 from *m/z* 568.4), MD of 41, *m/z* 423.6 Da (Removal of – C_8H_9 from *m/z* 528.1), MD of 105, *m/z* 265.3 Da (Removal of – $C_7H_{10}O_2S$ from *m/z* 423.6), MD of 158. For BLS, the ESI/MS of $[M+H]^+$ ion at *m/z* 464.3 Da illustrates abundant fragment ion at *m/z* 378.2 Da (Removal of - $C_4H_7O_2$ from *m/z* 464.3), MD of 87, *m/z* 272.3 Da (Removal of – C_8H_9 from *m/z* 378.2), MD of 105, *m/z* 190.9 Da (Removal of – C_5H_9N from *m/z* 272.3), MD of 83. From the findings of the MS/MS analysis utilizing optimal mass parameters and the LC/ESI/MS in positive mode, the mass fragmentation pathway of the BLS and MTK (Fig. 6) was identified.

Characterization of DPs of MTK

Characterization of acid DP; DP1

The ESI-MS/MS of MTK exhibited an abundant protonated molecular ion at *m/z* 586.300 Da. DP1 appeared at *m/z* 602.100 Da ($[M+H]^{+;}$ $C_{35}H_{36}ClNO_4S$) and was eluted at 5 minutes (Fig. 7). DP1 formed as a result of hydrolysis of the sodium acetate group in the presence of acid. The LC-ESI-MS/MS spectrum of DP1 represents abundant fragment ion at *m/z* 544.300 Da (removal of C_3H_8O from *m/z* 602.1), MD of 58, fragment 2 at *m/z* 439.500 Da (loss of - C_8H_{10} from *m/z* 544.300), MD of 105, and *m/z 281.6* (loss of – $C_7H_9SO_2$ from *m/z* 439.500).

Characterization of base DP; DP2

The ESI-MS/MS of MTK exhibited an abundant protonated molecular ion at *m/z* 586.300 Da. DP2 appeared at *m/z* 568.100 Da ($[M+H]^+$; $C_{35}H_{36}ClNO_4S$) and was eluted at 2.30 minutes (Fig. 7). DP2 formed as a result of hydrolysis of chloroquinoline moiety in the presence of a base. The LC-ESI-MS/MS spectrum of DP2 shows abundant fragment ion at *m/z* 510.300 Da (removal of C_3H_8O from *m/z* 568.1), MD of

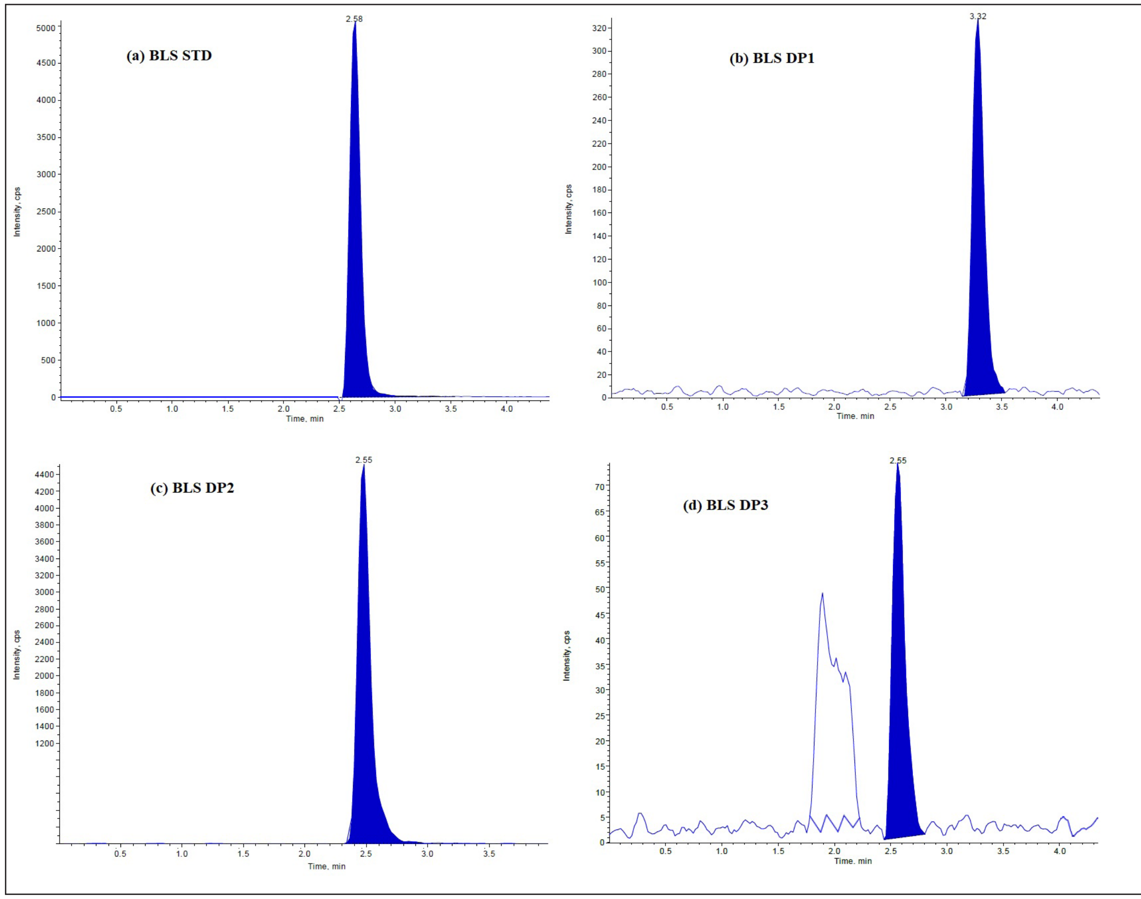

Figure 4. LC/ESI-MS TIC of (a) BLS standard drug (b) Acid degradant (DP1) (c) Base degradant (DP2) (d) Oxidative degradant (DP3) of Tablet dosage form.

58, fragment 2 at *m/z* 406.400 Da (loss of - C_8H_{10} from *m/z* 510.300), MD of 104, and *m/z 237.2* (loss of - $C_{11}H_8NO$ from *m/z* 406.400), MD of 170.

Characterization of oxidative DP; DP3

The ESI-MS/MS of MTK shows an abundant protonated molecular ion at *m/z* 586.300 Da. DP3 appeared at *m/z* 602.300 Da ($[M+H]^+$; $C_{35}H_{35}ClNO_4S$) was eluted at 3.30 minutes (Fig. 7). DP3 formed due to oxidation of sulphur (sulfoxide formed) in the presence of H_2O_2. The LC-ESI-MS/MS spectrum of DP3 demonstrates abundant fragment ion at *m/z* 585.300 Da (removal of -OH from *m/z* 602.3), MD of 17, and fragment 2 at *m/z* 543.600 Da (removal of - C_3H_7 from *m/z* 585.300), MD of 42.

The proposed structures of MTK and its DPs along with the degradation pathway are represented in Figure 8.

Characterization of DPs of BLS

Characterization of acid DP; DP1

The ESI-MS/MS of BLS represents an abundant protonated molecular ion at *m/z* 464.300 Da. DP1 appeared at *m/z* 274.300 Da ($[M+H]^+$; $C_6H_{19}N_3O$) and was eluted at 2.64 minutes (Fig. 7). DP1 formed due to hydrolysis of piperidine and cleavage of –ethyl-phenyl-2-methyl propanoic acid. The LC-ESI-MS/MS spectrum of DP1 represents abundant fragment ions at *m/z* 245.200 Da (removal of C_2H_5 from *m/z* 274.3), MD of 29, and fragment 2 at 201.300 Da. (loss of - C_4H_9O from *m/z* 274.3), MD of 73.

Characterization of base DP; DP2

The ESI-MS/MS of BLS shows an abundant protonated molecular ion at *m/z* 464.300 Da. DP2 appeared at *m/z* 486.400 Da ($[M+H]^+$; $C_{28}H_{36}N_3ONa$) was eluted at 2.49 minutes (Fig. 7). DP2 formed due to hydrolysis of carboxylic acid to form sodium salt. The LC-ESI-MS/MS spectrum of DP2 represents abundant fragment ion at *m/z* 413.200 Da (removal of - C_4H_9O from *m/z* 486.400), MD of 73, fragment 2 at 298.400 Da (loss of $C_7H_6N_2$ from *m/z* 413.2 Da), MD of 115 and 187.100 Da (loss of $C_7H_{14}N$ from *m/z* 298.4), MD of 112.

Characterization of oxidative DP; DP3

The ESI-MS/MS of BLS shows an abundant protonated molecular ion at *m/z* 464.300 Da. DP3 appeared at *m/z* 480.400 Da ($[M+H]^+$; $C_{28}H_{37}N_3O_4$) was eluted at 2.57 minutes (Fig. 7). DP3 formed as a result of the oxidation of nitrogen of the piperidine ring. The LC-ESI-MS/MS spectrum of DP3 shows abundant fragment ion at *m/z* 394.500 Da (loss of $C_4H_8O_2$ from *m/z* 480.4), MD of 86 and fragment 2 at 289.400

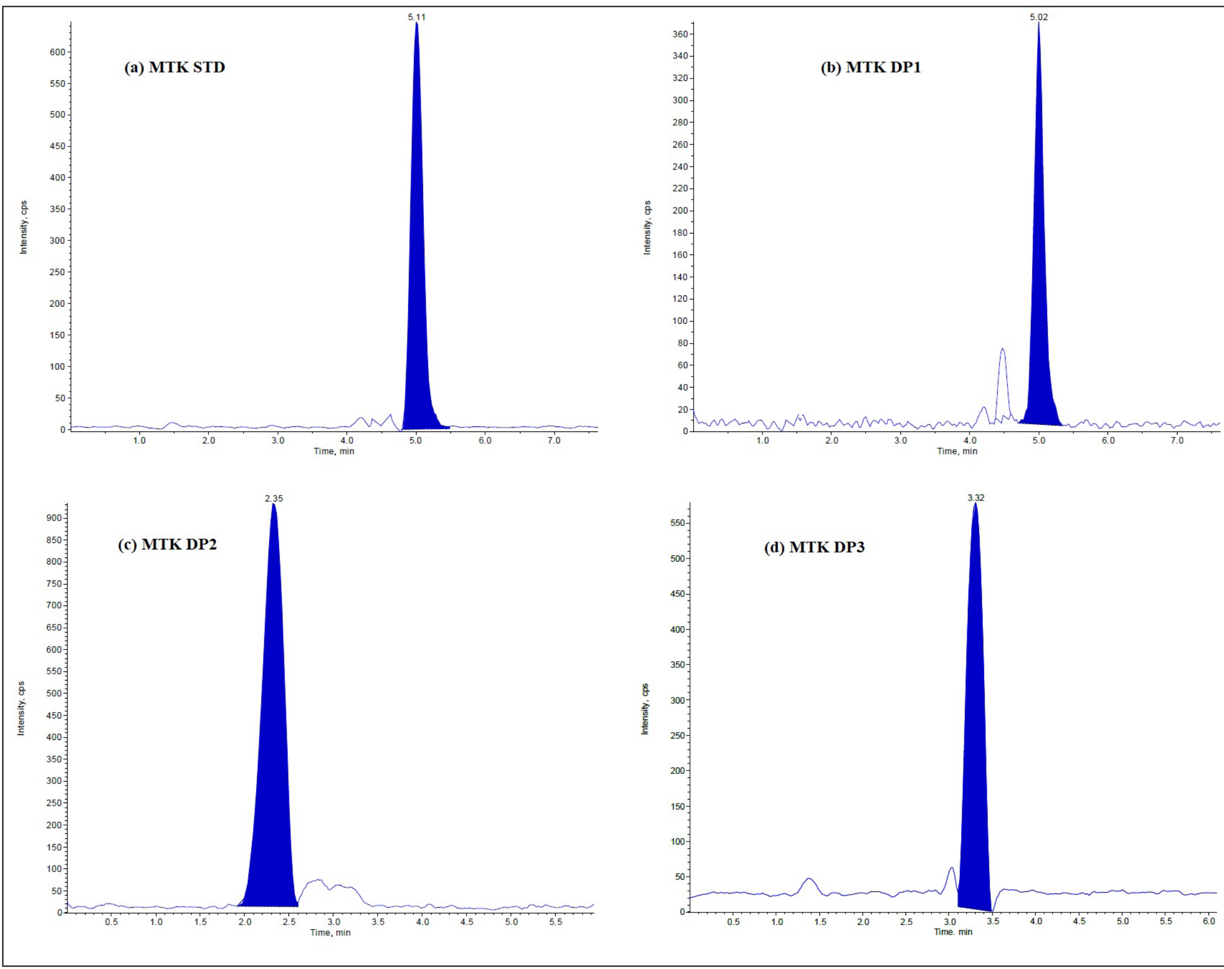

Figure 5. LC/ESI-MS TIC of (a) MTK standard drug (b) Acid degradant (DP1) (c) Base degradant (DP2) (d) Oxidative degradant (DP3) of Tablet dosage form.

(A)

$[M+H]^+$ *m/z* 464.3

$- C_4H_7O_2$

m/z 378.2
Mass Difference: 87

m/z 273.2
Mass Difference: 105

m/z 190.9
Mass Difference: 83

(B)

$[M+H]^+$ *m/z* 586.3

- OH

m/z 568.4
Mass Difference: 17

$- C_3H_5$

m/z 528.1
Mass Difference: 41

$- C_8H_9$

m/z 423.6
Mass Difference: 105

m/z 265.3
Mass Difference: 158

Figure 6. Proposed MS/MS fragmentation pathway of (A) BLS and (B) MTK.

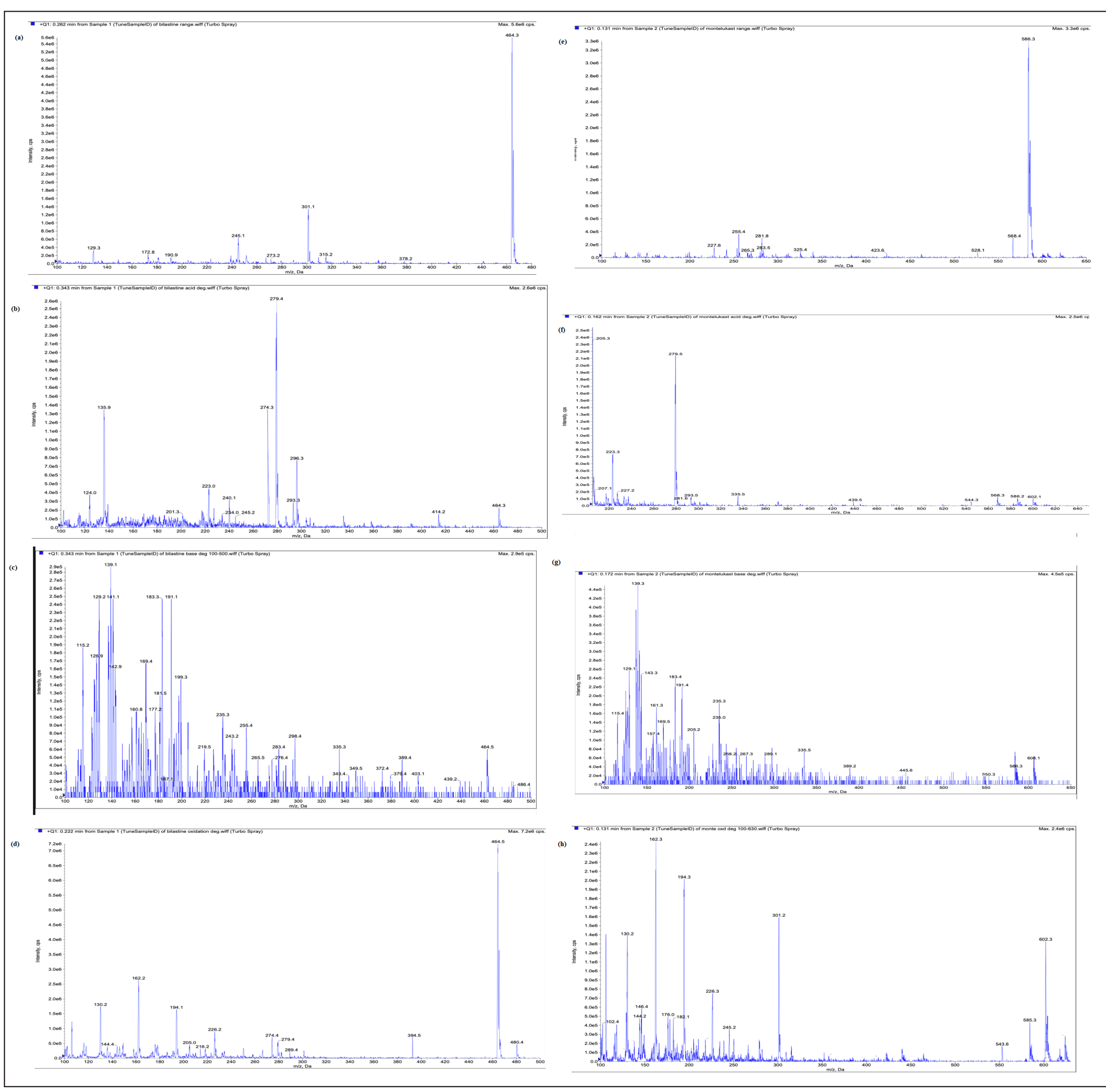

Figure 7. LC/ESI-MS/MS (product ion tandem mass) spectra of $[M+H]^+$ ions of (a) BLS standard drug, (b) DP1 of BLS, (c) DP2 of BLS, (d) DP3 of BLS, (e) MTK standard drug, (f) DP1 of MTK, (g) DP2 of MTK, (h) DP3 of MTK.

Da (loss of - C_8H_{10} from *m/z* 394.500), MD of 105 and 216.200 Da (loss of - C_4H_9O from *m/z* 289.400), MD of 73.

The proposed structures of BLS and its DPs along with the degradation pathway are represented in Figure 9.

Method validation

The optimized LC–MS/MS method was validated with respect to various parameters summarized in the ICH guidelines [44]. The specificity of the method was established by comparing the chromatograms of blank, placebo, sample, and standard, and no interference was observed (Fig. 10). The mass detector showed an excellent purity for BLS and MTK and every DP, which unambiguously proves the specificity of the method. The linearity was established in the range of 0.5–1.5 µg/ml MTK and 1–3 µg/ml BLS. The data were subjected to statistical analysis using a linear regression model; the linear regression equation and correlation coefficient (R^2) were y =7027.3x + 78.271, 0.9993 for MTK and y = 19434x + 9379.8, 0.9991 for BLS correspondingly. The determinations indicate a good correlation exists between the concentration of the drug and response (peak area). The LOD values were found to be 0.098 and 0.236 µg/ml for MTK and BLS correspondingly. The LOQ values were found to be 0.297 and 0.716 µg/ml for MTK and BLS correspondingly. Table 1 shows that the % RSD

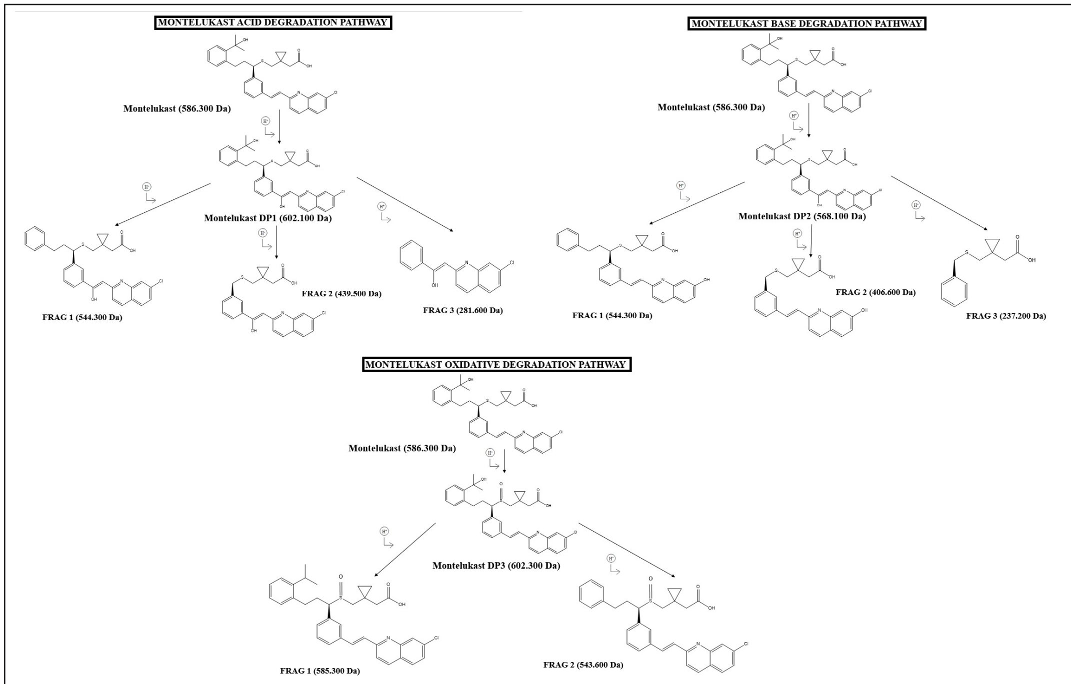

Figure 8. Proposed degradation pathway of MTK.

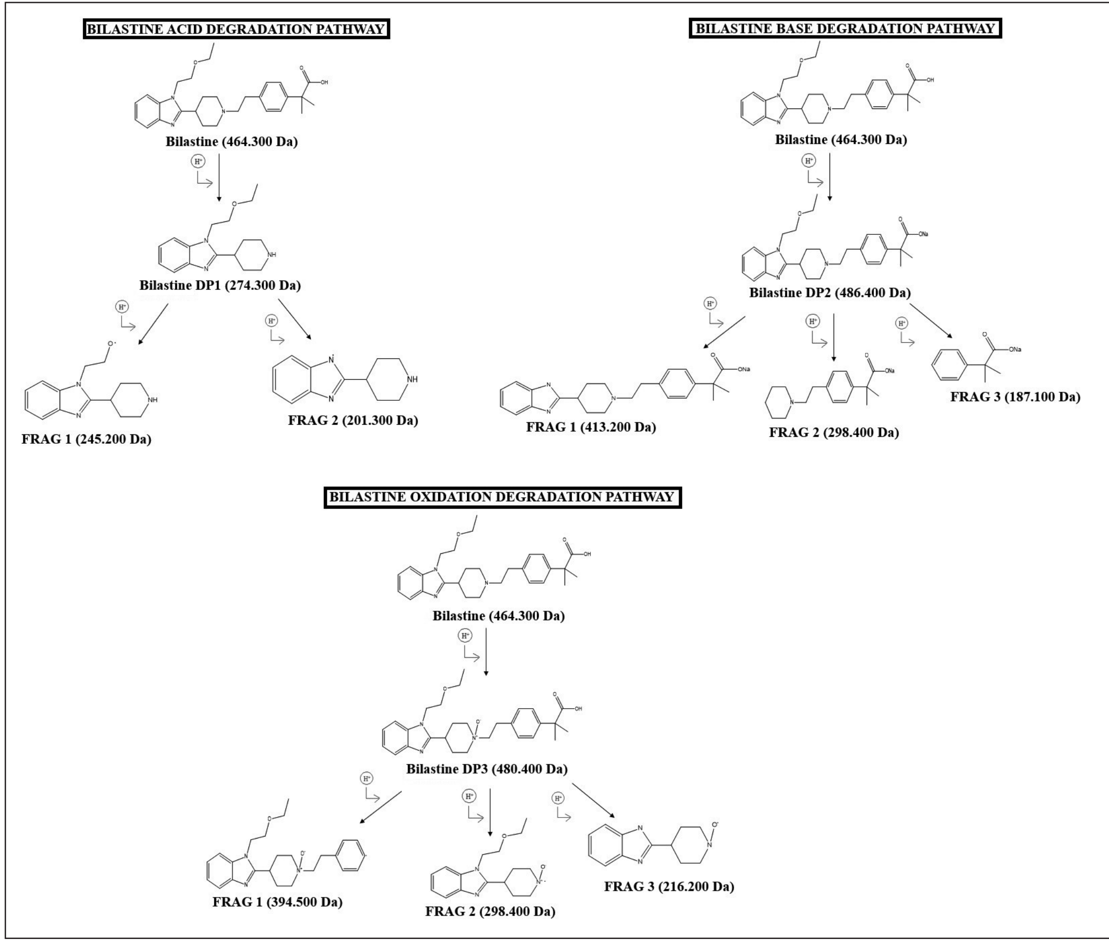

Figure 9. Proposed degradation pathway of BLS.

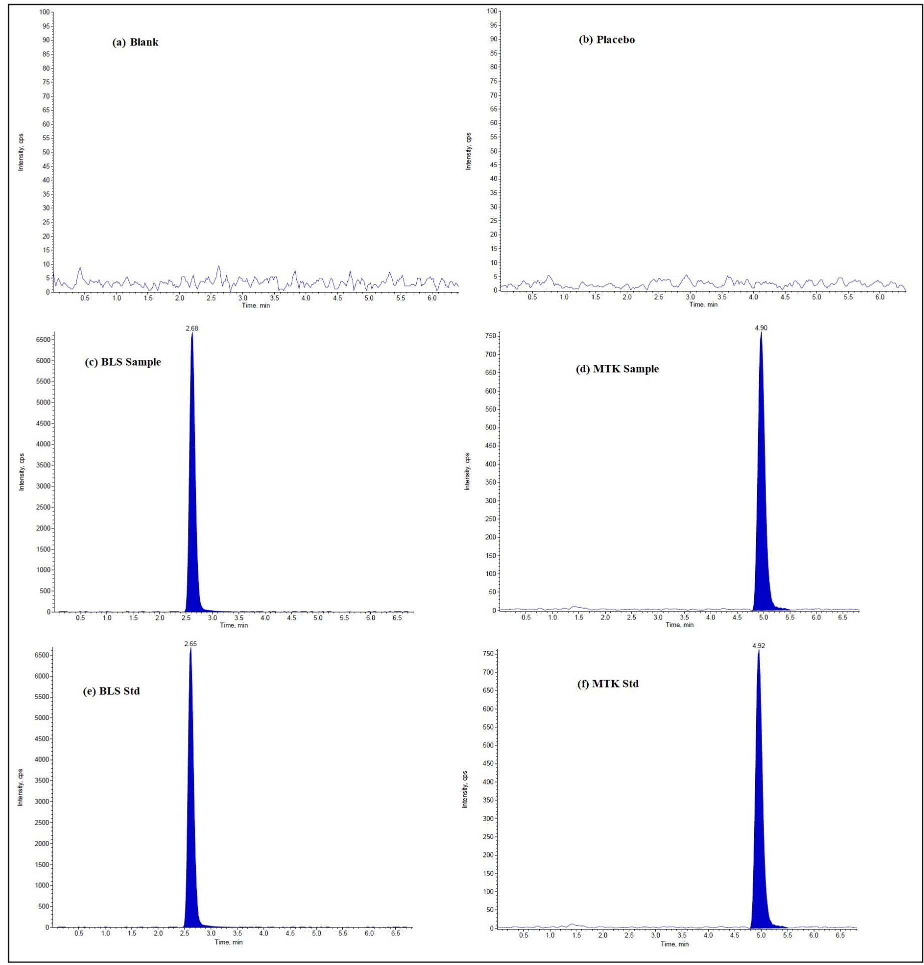

Figure 10. LC/ESI-MS TIC of (a) Blank (b) Placebo (DP1) (c) BLS sample (d) MTK sample (e) BLS standard (f) MTK standard.

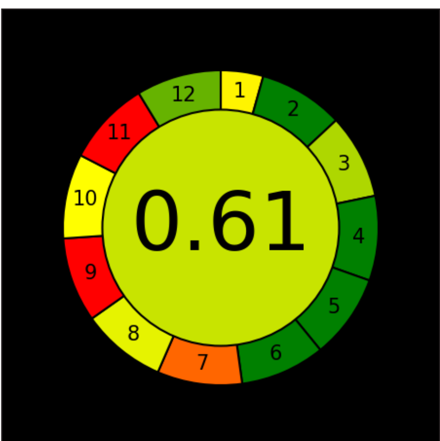

Figure 11. AGREE assessment pictogram.

for intra and inter-day precision was <2%, indicating that the method was precise. Recovery results were within an acceptable limit (98%–102%) which indicates that none of the excipients components have any interference peak under the peak of cited drugs. The outcomes of robustness of the proposed approach showed % RSD <2%, indicates that the method is robust with respect to variations in selected parameters within range. The outcomes of all the validation parameters are summarized in Table 1.

Assessment of method greenness

The most recent tool for evaluating greenness, "AGREE", was released in 2020. The AGREE tool was utilized to evaluate the created method's greenness. It is an advanced and updated method for figuring out how eco-friendly analytical procedures are. It is a qualitative analysis of the method's interactions with the surroundings. The outcome of AGREE will be a circular pictogram with 12 pieces, each of which will stand for 1 of the 12 green analytical chemistry principles. A number of 0–1 is assigned to each principle or component; a score of 1 indicates the most environmentally friendly. The outcome was a score of 0.61 based on the present AGREE tool approach (Fig. 11). Because of their problems and potential health risks, the most popular organic solvents, such as methanol and acetonitrile, ought to be used less frequently. Acetonitrile, for example, is poisonous, volatile, and combustible [ICH Q3C (R8)]. Therefore, in the AGREE pictogram, section 11 (Toxicity of the Reagents) is shown in red. The suggested approach's Sections 7 (amount of garbage generated) and 9 (energy usage)

Table 1. Outcomes of validation parameters.

Sr. No.	Parameter		MTK	BLS
1	Specificity		Specific	Specific
2	Linearity range (μg/ml)*		0.5–1.5	1–3
3	Regression equation		y = 7027.3x + 78.271	y = 19434x + 9379.8
4	Correlation coefficient (R2)		0.9993	0.9991
5	Precision	Repeatability#	1.52	1.84
	(% RSD)	Intraday*	0.95–1.44	0.89–1.84
		Interday*	0.52–1.59	1.12–1.33
6	Accuracy (% recovery)*		99.14–101.97	98.29–101.44
7	Robustness (% RSD)	Flow rate (± 0.2 ml/minute)*	(+) 1.75, (-) 1.29	(+) 0.72, (-) 1.89
		Ratio of mobile phase (± 2%) *	(+) 0.66, (-) 1.57	(+) 1.47, (-) 1.84
		pH (± 0.2)*	(+) 0.87, (-) 1.45	(+) 1.18, (-) 1.38
8	% Assay*		100.05 ±1.215	100.29± 1.896

*Average of three determinations, #Average of six determinations.

are red, which impacts the greenness of the method. According to the assessment, the suggested strategy is overall green.

CONCLUSION

In accordance with ICH recommendations, the degradation behavior of MTK and BLS under photolysis, thermal, oxidation, and hydrolysis (acid and base) stress was investigated. Under different stress environments, the current method can resolve all DPs from MTK and BLS as well as from each other. While both drugs were stable under thermal and photolytic stress conditions, they both showed significant changes under acid, base hydrolysis, and oxidative stress. Based on LC-MS/MS data, a total of six DPs were identified, and fragmentation mechanisms were suggested.

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All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

This study paper includes all generated and analyzed data.

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USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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