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Research Article

Optimization of mobile phase for the determination of Esomeprazole and related compounds and investigation of stress degradation by LC–MS

In this study, the objective was to investigate the degradation behavior of Esomeprazole under different recommended stress conditions according to International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use [1] by HPLC. Our research showed that the effect of mobile phase species on separation was significant for the determination of Esomeprazole and its related compounds. Successful separation of the drug from its related impurities and degradation products formed under different stress conditions was achieved using ammonium acetate buffer/ACN by a gradient elution. Compared with phosphate buffer/ACN, ammonium acetate buffer/ACN under same pH and gradient showed a great improvement in resolution due to the change of elution order. The drug was subjected to stress conditions including acidic, alkaline, oxidative, photolytic, and thermal conditions. Extensive degradation occurred in acidic and oxidative conditions, while mild degradation was observed in alkaline and photolytic conditions. Besides, it turned out the drug was extremely stable under thermal condition. The stability-indicating LC–UV method was validated with respect to linearity, precision, accuracy, specificity, and robustness. The LC–MS method was also adopted for the characterization of degradation products. Based on the m/z values and fragmentation patterns, the degradation pathway of the drug has been proposed.

Keywords: Esomeprazole / Improved separation / LC-MS Characterization / Mechanism / Related compounds
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1 Introduction

Esomeprazole, 5-methoxy-2-((*S*)-((4-methoxy-3,5-dimethyl-2-pyridinyl)methyl)sulfinyl)-1H-benzimidazole, is the optically stable *S*-enantiomer of omeprazole [2]. It is the first proton pump inhibitor (PPI) developed as an optical isomer. Just as other PPIs, Esomeprazole plays an important role in acid suppression by inhibiting the gastric H^+/K^+-ATPase [3]. Because of its advantageous metabolism, the enhanced clinical efficacy and predictability of Esomeprazole can lead to higher AUC values, higher bioavailability, and more consistent pharmacokinetics between individuals, increasing delivery of the drug to the proton pump and providing better acid control [4]. For patients with gastro-oesophageal reflux disease (GERD), Esomeprazole is effective and well tolerated in the maintenance of healing of erosive oesophagitis [5]. Meanwhile, it can reduce nighttime heartburn and sleep disturbances, thus improving sleep quality and work productivity [6]. It is reported that Esomeprazole demonstrates significantly greater efficacy than omeprazole in the treatment of GERD patients with erosive esophagitis [7]. On the other side, unlike omeprazole, Esomeprazole-based triple therapy can effectively eradicate *Helicobacter pylori* infection and heal patients with duodenal ulcer without the need of follow up monotherapy [8].

Impurity profile of a drug substance is critical for its safety assessment and manufacturing process. Therefore, it is important to study the impurity profile for the manufacturing of a drug product. Currently the vast majority of drug-related impurity determinations are performed by HPLC, which can offer the desired sensitivity for trace level determinations and offer a high degree of automation.

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Abbreviations: GERD, gastro-oesophageal reflux disease; **PPI**, proton pump inhibitor

Up to now, a few works about Esomeprazole analysis based on chromatography have been reported. Önal et al. have published a stability-indicating HPLC method for the determination of Esomeprazole in tablets [9]. Reddy et al. have published an RP-HPLC method for simultaneous determination of Esomeprazole and Naproxen in pharmaceutical formulations [10]. Zanitti et al. have published a method, using HPLC, for enantioseparation and determination of Esomeprazole and its chiral impurities [11]. Nalwade et al. published an ultra performance liquid chromatography (UPLC) method for the determination of impurities in Esomeprazole magnesium [12]. So far to our knowledge, no analytical determination of Esomeprazole and its related compounds was studied by HPLC. So it is essential to develop an HPLC procedure that will serve as a reliable and convenient method for the determination of Esomeprazole and its related compounds. In our work, chromatographic conditions of HPLC, including column and mobile phase, were investigated and optimized, and a validated LC–UV method for simultaneous determination of Esomeprazole and its related compounds was established. According to the reference, such PPIs were always analyzed in phosphate buffer system [13]; however, the separation was poor when phosphate buffer/ACN was used in our study, especially for Esomeprazole and its oxide. Baseline separation could not be achieved despite the adjustment of the mobile phase proportion. In order to improve this problem, ammonia buffer system was introduced into our method to replace phosphate buffer system. Finally, it turned out that the baseline separation of Esomeprazole and all of its related compounds (a total of ten compounds) were realized. This result coincided with Rosés's research work that cationic ammonia buffer showed its advantage over anionic phosphate buffer of the same pH in terms of the resolution due to the change of elution order [14]. Furthermore, in order to explore the degradation pathway of Esomeprazole, an LC–MS method was employed. The stress degradation behaviors of Esomeprazole under different ICH suggested conditions were investigated, including the degradation induced by acidic, alkaline, oxidative, photolytic, and thermal stress [1]. The impurity peaks were characterized, respectively. Furthermore, based on the m/z values and fragmentation patterns, the degradation pathway of the drug has been proposed.

2 Experimental

2.1 Materials and equipment

Esomeprazole and all its related compounds were supplied by Shanghai Institute of Pharmaceutical Industry and were used without further purification (Fig. 1). The HPLC grade ACN was purchased from Merck (Darmstadt, Germany). All other chemicals and reagents were of analytical grade and were purchased from Sinopharm (Shanghai, China). Pure water was obtained by using Milli-Q Advantage A10 system (Millipore, USA).

Precision water bath DC-12H (Anpel, Shanghai, China) was used for the degradation experiment in alkaline condition. Photo stability chamber LHH-150GP (Bluepard, Shanghai, China) was adopted for the degradation experiment in photolytic condition. And thermal degradation study was performed in dry-air oven DHG-9070A (Yiheng Instruments, Shanghai, China).

The HPLC system L-2000 (Hitachi, Tokyo, Japan) consisted of an L-2130 pump, an L-2400 UV detector, an L-2200 autosampler, an L-2300 column oven, and an organizer module. Data were acquired and processed using Hitachi Model D-2000 Elite Chromatography Data Station Software HPLC System Manager Version 2.0. The column in the method was XTerra® MS C8 (4.6 × 150 mm, 3.5 μm) (Waters, Milford, America).

LC–MS studies were carried out on a system in which LC part consisted of 1100 series HPLC (Agilent Technologies, Waldbronn, Germany), including a G1322A on-line degasser, a G1312A binary pump, a G1313A auto injector, a G1316A column oven, and a G1315B PDA detector. And the MS system consisted of a G1946D quadrupole MS detector (Agilent Technologies, Waldbronn, Germany). Data were acquired and processed using Agilent Chemstation Version A10.02. Besides, LC-TOF-MS studies were carried out on AcQuity UPLC and Xevo QTOf MS (Waters, Milford, America).

2.2 Stress degradation of Esomeprazole

Stress degradations were carried out, and then the samples were injected into HPLC system. The conditions about stress degradations were as follows, respectively, (i) 0.3% hydrogen peroxide at room temperature for 30 min, (ii) 1 M sodium hydroxide for 8 h at 80°C water bath, (iii) strong light (4500LX) for 24 h, (iv) 0.01 M hydrochloric acid at room temperature for 30 min, (v) heated in oven at 140°C. For acid and base induced degradation, the samples were neutralized before injected.

2.3 Optimization of the stability-indicating LC–UV method

The LC–UV method was optimized to develop the stability-indicating method. Pure drug mixed with its related compounds (degradation and process related impurities) was injected into the system. The mobile phase was optimized in several aspects, including different aqueous buffers, pH value, and the proportions of ACN and aqueous buffer. It turned out that the alkaline aqueous could help to improve the peak shape, and aqueous buffer was adjusted to pH 7.6 owing to the better resolution after several attempts. According to the resolution, peak shape and analytical period from

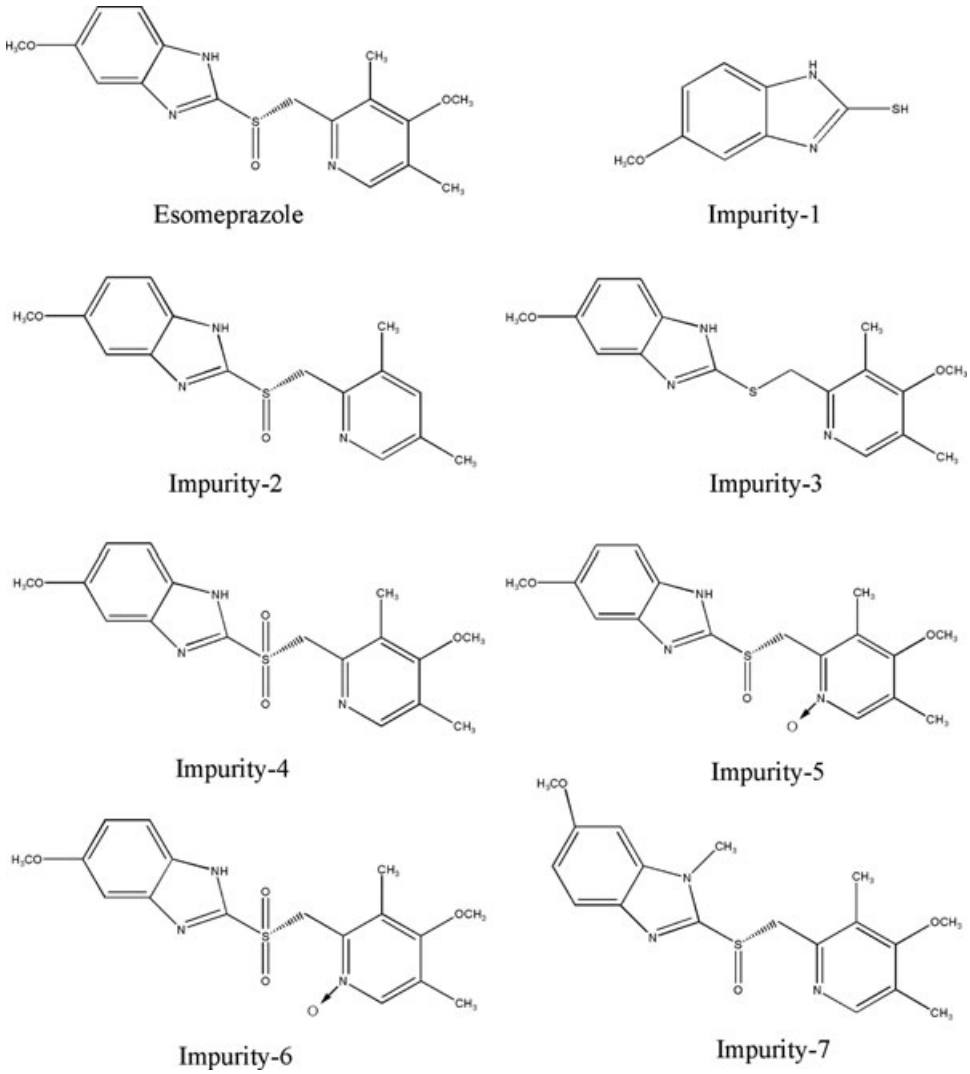

Figure 1. Chemical structures of Esomeprazole and its related impurities.

experimental results, ammonium acetate buffer-ACN system was chosen as the final mobile phase, and the gradient elution procedure was adopted. Several columns were also investigated, such as Welch Xtimate™ C8 (4.6 × 150 mm, 5 μm), Welch Xtimate™ C8 (4.6 × 100 mm, 3 μm) and Waters XTerra® MS C8 (4.6 × 150 mm, 3.5 μm). As a result, Waters XTerra® MS C8 (4.6 × 150 mm, 3.5 μm) was chosen as the chromatography column because of its column efficiency and durability under the alkaline condition.

Finally, after optimization, the stability-indicating method has been determined to be carried out on Waters XTerra® MS C8 (4.6 × 150 mm, 3.5 μm) employing ACN and ammonium acetate buffer (pH 7.6; 20 mM) in the gradient program as: time (min)/% ACN: 0/15, 40/25, and 60/35. The injection volume was 40 μL and the flow rate was 1 mL/min. All samples were detected under the wavelength of 280 nm in 25°C. The optimized stability-indicating LC–UV method showed suitable retention time and good resolution.

2.4 Validation of the stability-indicating LC–UV method

Linearity of the method was studied by injecting five concentrations of the drug in the range of 0.01–0.5 mg/mL in mobile phase in triplicate into the HPLC system. Repeatability study was carried out by the detection of drug in three different concentrations (0.05, 0.1, and 0.5 mg/mL), while intermediate precision (interday precision) study was carried out by repeating repeatability study on three different days. LOD and LOQ were determined by injecting a series of diluted known concentrations of standard solution. LOD was considered to be the concentration of the drug when the S/N was 3:1, while 10:1 for LOQ. Robustness was determined by changing the flow rate, the ratio of ACN and the pH value of mobile phase. Specificity of the method was determined by studying the resolution factor of the main peak from the nearest resolving peak. Meanwhile, purity of each degradation product peak was also determined using its retention

time by DAD detector. At last, the accuracy of the method was determined through the recovery of the drug at various concentrations according to the calibration curve.

2.5 Development of LC–MS method and characterization of degradation products

Since ammonium acetate buffer-ACN system was suitable for LC–MS study, the chromatographic condition described in LC–UV could be conveniently used without any modification to characterize degradation products. The mass spectra of Esomeprazole and its degradation products formed under different conditions were taken in ESI positive mode in mass range of 50–1000 and the source voltage was kept at 70 mV. Since flow rate was kept at 1 mL/min, the flow splitter was introduced into MS, which split volume of mobile phase and delivered minimum amount of mobile phase into MS. The split ratio was 3:1 (1:4 into the mass spectrometer). The obtained m/z values and retention time were compared to those of the known related compounds. The fragmentation pattern was also investigated. Based on the molecular weight, retention time and the fragmentation pattern, the presence of known degradation products was confirmed and also structures could be speculated for those unknowns. In the end, the degradation pathway could be outlined based on the results.

3 Results and discussion

3.1 Establishment and optimization of stability-indicating LC–UV method

3.1.1 The influence of buffer in mobile phase

Different aqueous buffers (phosphate buffer and ammonium acetate buffer), pH value, and the proportions of ACN and aqueous buffer were studied in our LC–UV method. Pure drug mixed with its related compounds (degradation and process related impurities) was injected into the system, the resolution and peak shape were observed in the experiments. Phosphate buffer was first studied as aqueous buffer. The results showed that the alkaline aqueous could help to improve the peak shape, and for the better separation between Esomeprazole and its related compounds, phosphate buffer was finally adjusted to pH 7.6. The resolution became poor with the rise of ACN ratio; especially the peaks of impurity-5 and impurity-6 had almost overlapped. However, when ACN ratio was decreased, the improvement of the resolution had led to very long retention time and broadening of peak shape. Considering the resolution, peak shape, and analytical period, the gradient elution procedure was finally determined through several attempts.

A total of 20 mM ammonium acetate was then used to replace phosphate buffer as aqueous buffer in the same gradient program, pH was also adjusted to 7.6 by ammonia solution. The typical chromatogram is shown in Fig. 2A and B. The result indicated that the retention behavior of impurity-4 was significantly affected by the buffer components in mobile phase, which was eluted before main peak in phosphate buffer/ACN system, eluted after main peak in ammonium acetate buffer/ACN system. Meanwhile, the chromatogram showed that the resolution was greatly improved in ammonium acetate buffer/ACN system. This behavior can probably be explained because of the different ionization trends of the compounds with the addition of ACN to both aqueous buffers, which has already been studied in previous research. The pH value of anionic phosphate buffer increases when ACN is added, while cationic ammonia buffer shows the reverse trend. The pK_a variation of analytes may follow the similar tendency: with the addition of ACN, the same analyte in two different aqueous buffers of the same pH may show a different ionization trend, thus leading to the different chromatographic retention [14]. In our study, in anionic phosphate buffer, impurity-4 was the more ionized analyte compared to Esomeprazole, whereas in the cationic ammonia buffer, Esomeprazole became more ionized, which finally contributed to the change of elution order in two buffer systems. Considering its better resolution, ammonium acetate buffer and ACN were chosen as the mobile phase in our stability-indicating LC–UV method.

3.1.2 The investigation of LC columns

Several columns were investigated in our study, such as Welch Xtimate™ C8 (4.6 × 150 mm, 5 μm), Welch Xtimate™ C8 (4.6 × 100 mm, 3 μm), and Waters XTerra® MS C8 (4.6 × 150 mm, 3.5 μm). It turned out that Welch Xtimate™ C8 (4.6 × 100 mm, 3 μm) and Waters XTerra® MS C8 (4.6 × 150 mm, 3.5 μm) offered much better peak shape and resolution than Welch Xtimate™ C8 (4.6 × 150 mm, 5 μm). However, when it comes to durability, Waters XTerra® MS C8 (4.6 × 150 mm, 3.5 μm) showed its obvious advantage over other two. As a result, Waters XTerra® MS C8 (4.6 × 150 mm, 3.5 μm) was finally chosen as the chromatography column because of its column efficiency and durability under the alkaline condition.

3.2 The investigation of degradation products of Esomeprazole

3.2.1 Oxidative condition-induced degradation

Esomeprazole was found to be extremely unstable under oxidative condition. After exposed to 0.3% H_2O_2 for 30 min, about 5.3% degradation was formed at 33.2 min (Fig. 2C). LC–MS analysis of the degradation drug indicated that the degradation product at 33.2 min had molecular weight of m/z 362.1 (Supporting Information Fig. S1a). Besides, the retention time of the oxidative degradation product was also

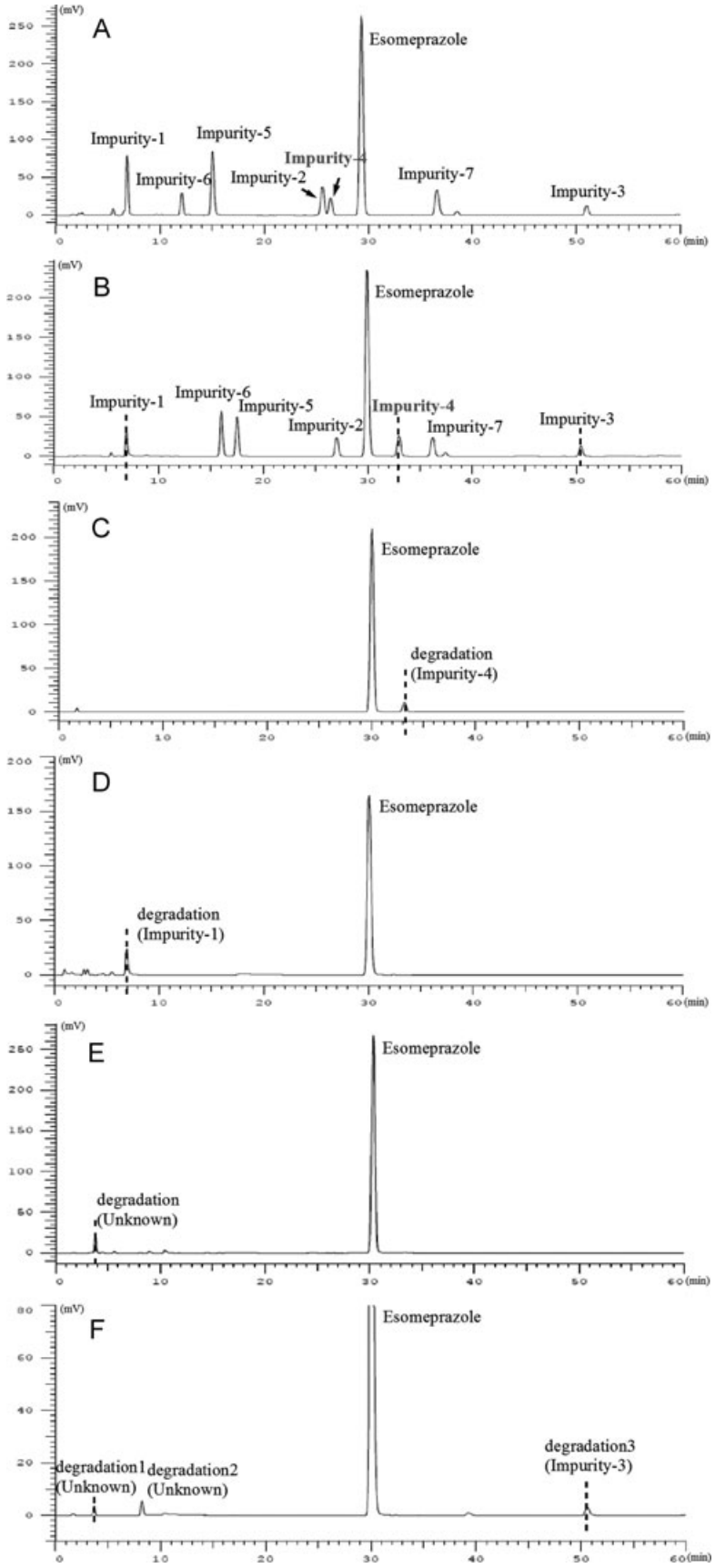

Figure 2. Typical chromatograms of Esomeprazole with its related impurities and stress degradation samples under different stress conditions: Esomeprazole with its related impurities in phosphate buffer/ACN system (A), Esomeprazole with its related impurities in ammonium acetate/ACN system (B), sample degraded in 0.3% hydrogen peroxide at room temperature for 30 min (C), sample degraded in 1 M sodium hydroxide for 8 h at 80°C water bath (D), sample degraded in strong light (4500LX) for 24 h (E), sample degraded in 0.01 M hydrochloric acid at room temperature for 30 min (F).

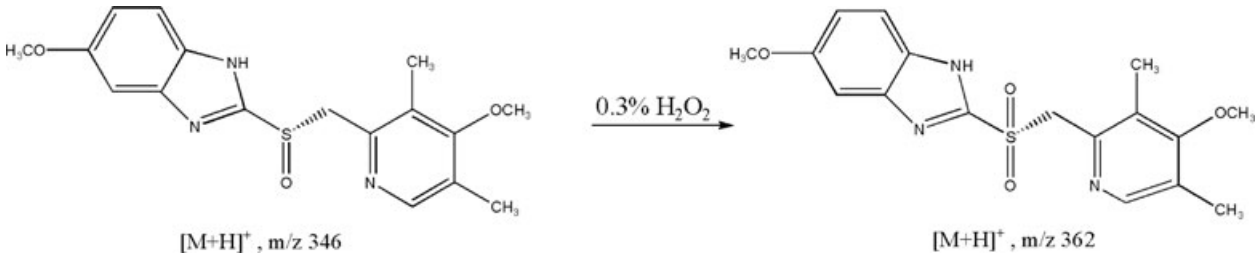

Figure 3. Pathway for oxidative degradation of Esomeprazole.

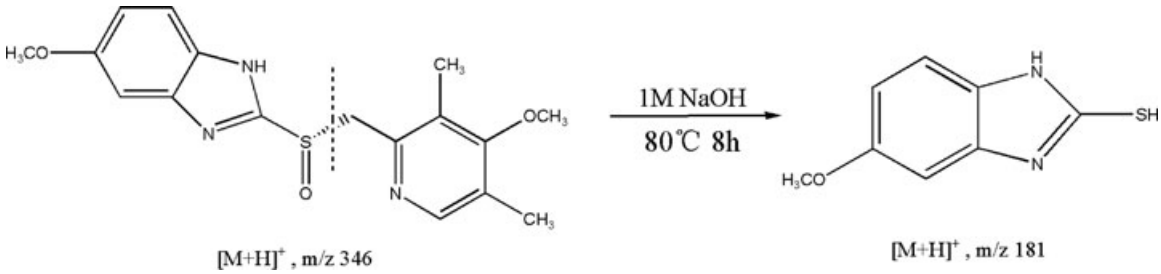

Figure 4. Pathway for alkaline degradation of Esomeprazole.

in accordance with impurity-4 in the mixed standard chromatogram. So it could be presumed that under H_2O_2 oxidative condition, the sulphoxide was converted into sulfonyl group through the increase in molecular weight by 16 (Fig. 3).

3.2.2 Alkaline condition induced degradation

Esomeprazole was found to be very stable under alkaline condition. When mixed with 0.1 M sodium hydroxide for 8 h at 80°C water bath, no obvious degradation was found. As a result, 1 M sodium hydroxide was taken as the alkaline condition, and about 10% degradation was observed at 6.98 min (Fig. 2D). LC–MS analysis indicated that the major degradation at 6.98 min had molecular weight of *m/z* 181.1 (Supporting Information Fig. S1b), and the retention time was also consistent to impurity-1 in the mixed standard chromatogram. So, we could speculate that under alkaline conditions, Esomeprazole was broken at sulpho bond, leading to the formation of impurity-1 with *m/z* 180 and other minor fragments (Fig. 4).

Under oxidative and alkaline conditions, all the degradation products obtained were in accordance with the related compounds in both retention time and molecular weight, which enabled us to perform the characterization easily. However, for the other conditions, unknown degradation products emerged. Therefore the LC-TOF-MS procedure was introduced to help to do the characterization.

3.2.3 Photolytic condition induced degradation

When exposed to strong light (4500LX), Esomeprazole was also found to be very stable. After 24 h exposure in the light chamber, only 3% degradation was observed at 3.68 min (Fig. 2E), which had molecular weight of *m/z* 328.1 (Supporting Information Fig. S1c). Compared with above mixed standard chromatogram, no retention time was matched with it. So the degradation product was unknown. For the characterization, the LC-TOF-MS procedure was performed. According to the exact mass, the molecular weight of *m/z* 328.1292 from the LC-TOF-MS (Supporting Information Fig. S2a), and database search, the unknown was characterized. Furthermore, we have presumed the degradation product and its possible mechanism. The drug first followed the Smiles Rearrangement to form the sulfenic acid. Then the sulfenic acid was removed, which formed hydroxymethyl. And the hydroxymethyl was finally turned into carboxymethyl during the long exposure under strong light (Fig. 5).

3.2.4 Acidic condition induced degradation

According to our study, Esomeprazole was found to be very labile under acidic condition. When mixed with 1 M hydrochloric acid, the drug was degraded completely in no time. After several attempts, 0.01 M hydrochloric acid was finally chosen as the acidic condition. Reacted with 0.01 M hydrochloric acid for 30 min at room temperature, the drug showed about 10% degradation. Three main degradation products were observed at 3.73 min (degradation 1), 8.30 min (degradation 2), and 50.61 min (degradation 3) (Fig. 2F) and showed molecular weight of *m/z* 328.1, 330.1, and 330.1 separately by LC–MS analysis (Supporting Information Fig. S1d). Actually, degradation 1 showed the same retention time and molecular weight as that of the degradation induced by photolytic condition, which turned out that they were the same substance and followed the same mechanism. Degradation 3 had molecular

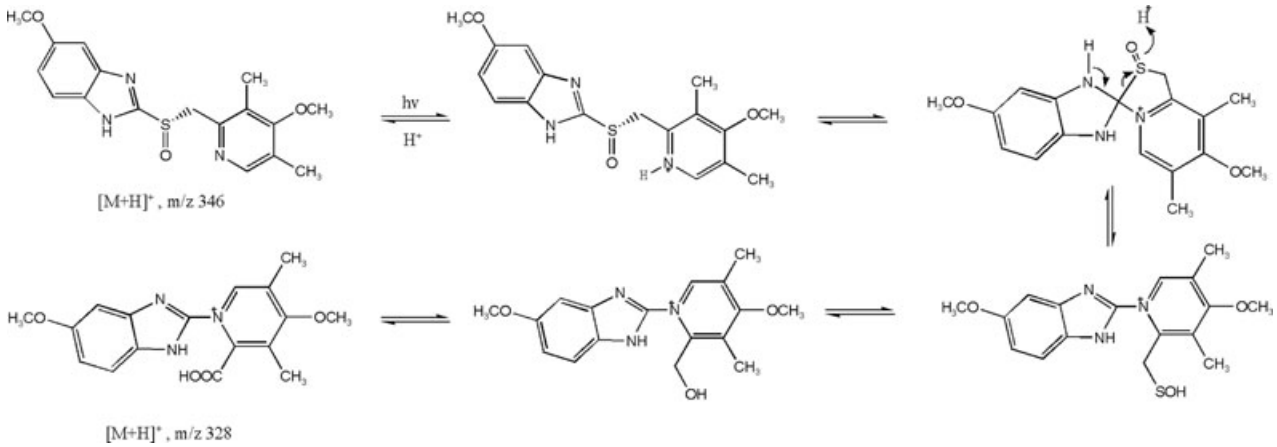

Figure 5. Mechanism for photolytic degradation of Esomeprazole.

weight of m/z 330.1, and the retention time was also consistent to impurity-3 in the mixed standard chromatogram. So it could be deduced that under acidic condition, just as omeprazole, Esomeprazole followed a similar pathway. The protonation led to radical cation and OH radical formation, which might cause the oxidation reaction on the aromatic group of the drug, leading to the formation of degradation impurity-3 [15]. For degradation 2, no literature has been mentioned till now. So according to the molecular weight of m/z 330.1273 from the LC-TOF-MS (Supporting Information Fig. S2b) and database search, considering its polarity, we presumed that after following the Smiles Rearrangement to form the sulfenic acid, the compound proceeded to dehydrate, then finally led to the formation of thiol through open loop (Fig. 6).

3.2.5 Thermal condition induced degradation

Solid Esomeprazole was placed into a dry-air oven to study the thermal stability of the drug. It turned out that even under extreme high temperature 140°C for 4 h, there was no obvious degradation for the drug, which indicated that Esomeprazole was very stable under thermal condition.

3.2.6 The comparison of degradation findings between Esomeprazole and omeprazole

Based on our research, we compared the degradation findings for Esomeprazole with those found for omeprazole in previous literature. Both of them reacted in the same way under oxidative condition, converting the sulfoxide group to the corresponding sulfone [16]. For alkaline condition, they were both very stable. Only in very strong base environment could Esomeprazole degrade through the cleavage of sulpho bond, while omeprazole was just deprotonated in the benzimidazole [16]. When under strong light, Esomeprazole and omeprazole made a great difference. Esomeprazole was very stable and it was slightly turned into carboxymethyl after long exposure, while omeprazole was unstable and completely degraded into sulfide and benzimidazolone compounds [15]. Under acidic condition, Esomeprazole and omeprazole were both very labile and followed a similar pathway. They were predominantly protonated on the pyridine nitrogen, and finally Esomeprazole was degraded into three different compounds mentioned in Section 3.2.4, while omeprazole was mainly degraded into sulfenic acid compound [16]. Besides, just like Esomeprazole, omeprazole in powder was also very stable under thermal condition, since no decomposition was observed when it was placed subjected to dry heat at 105°C for 5 h [17].

3.3 Validation of the stability-indicating LC–UV method

3.3.1 Linearity

The response for the drug was found to be linear in the concentration range between 0.01 and 0.5 mg/mL. The value of slope was 50 786 310 and the value of intercept was 48 956, with $r^2 = 0.9999$.

3.3.2 Precision

The results of repeatability and intermediate precision obtained from precision studies were shown in supporting Information Table S1. The RSD values were <2% for both repeatability and intermediate studies, which confirmed that the LC–UV method was sufficiently precise.

3.3.3 LOD and LOQ

LOD and LOQ were considered to be the concentration of the drug when the S/N was 3:1 and 10:1, respectively. According to the study, the LOD turned out to be 0.04 μg/mL, and the LOQ turned out to be 0.12 μg/mL.

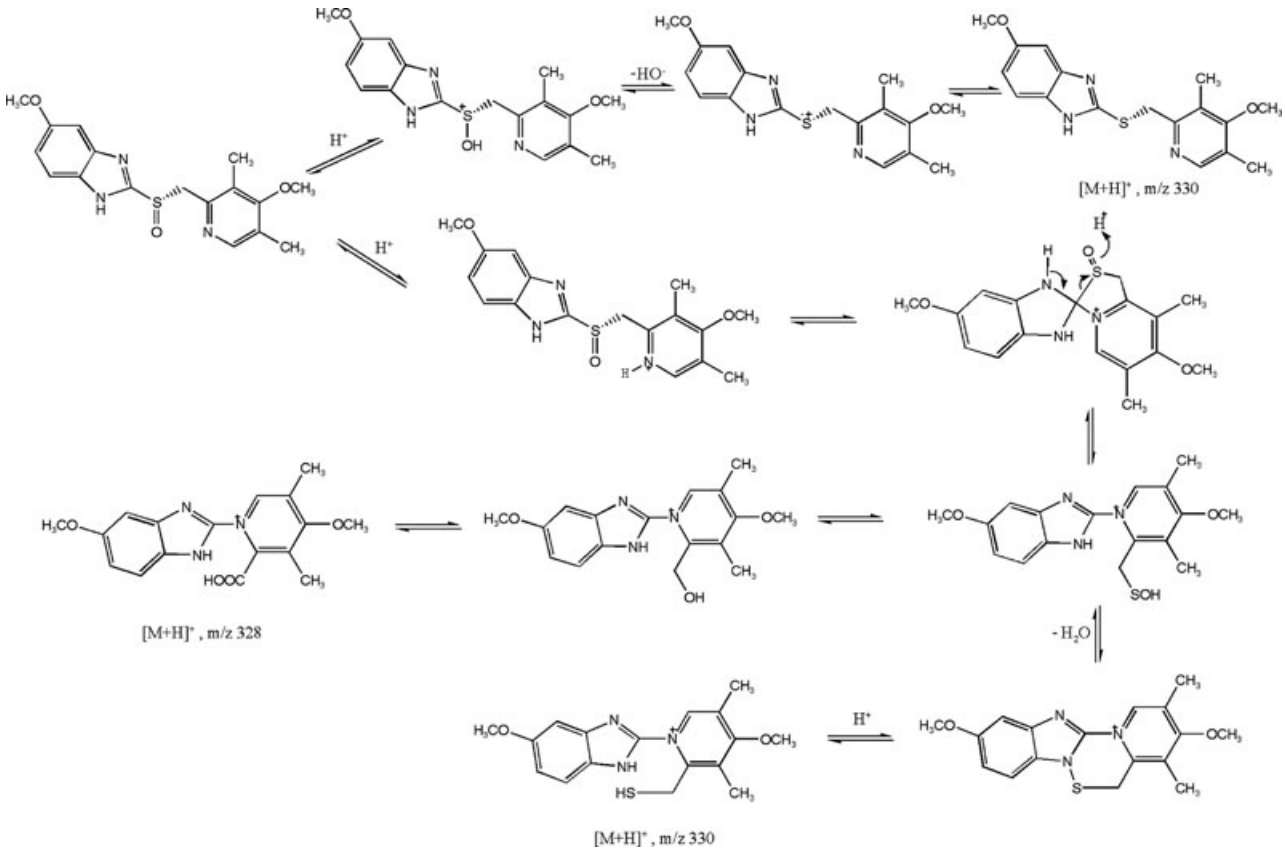

Figure 6. Mechanism for acidic degradation of Esomeprazole.

3.3.4 Robustness

The flow rate, the ratio of ACN, and the pH value of mobile phase were chosen as the three parameters in our robustness study. They were changed at three levels. One parameter was changed to see the result while other two remained unchanged. The mixed standard solution at 0.1 mg/mL was injected under small changes of these three parameters. As a result, retention time, capacity factor, and asymmetry of the peak changed insignificantly and remained relatively stable (Table 1). Besides, the resolution of the drug with its degradation products turned out to be similar on different chromatographic system on different days. So it can be concluded that the LC–UV method had sufficient robustness.

Table 1. Robustness testing

	Chromatographic changes			
	Factor	Retention time (min)	Capacity factor	Asymmetry
Flow rate (mL/min)	0.9	31.47	3145.6668	1.04
	1.0	29.75	2973.6668	1.03
	1.1	28.05	2804.3335	1.04
Ratio of ACN in the mobile phase v/v	14→24→34	32.83	3281.6668	1.00
	15→25→35	29.75	2973.6668	1.03
	16→26→36	26.43	2641.6668	1.04
pH of mobile phase	7.5	29.51	2949.6668	1.14
	7.6	29.75	2973.6668	1.03
	7.7	29.04	2903.0001	1.21

14→24→34: time (min)/% ACN: 0/14, 40/24 and 60/34.
15→25→35: time (min)/% ACN: 0/15, 40/25 and 60/35.
16→26→36: time (min)/% ACN: 0/16, 40/26 and 60/36.

3.3.5 Specificity

In our LC–UV method, Esomeprazole and its degradation products achieved a clear baseline separation and all the peaks were sharp and symmetrical. The resolution factor for the drug peak was >2 from the nearest resolving peak. The DAD detector scanned all the degradation samples from 200 to 400 nm, which turned out that no degradation peak was hiding under the drug peak. This LC–UV method has sufficient specificity.

3.3.6 Accuracy

Three different concentrations of the drug were injected into the system, and good recoveries in the range from 98.40 to 99.05% were made at last (Supporting Information Table S2).

4 Concluding remarks

In this study, a validated stability-indicating LC–UV method was developed for Esomeprazole according to ICH guidelines, providing information about degradation behaviors of the drug under different stress conditions, including acidic, alkaline, oxidative, photolytic, and thermal condition. The drug was found to undergo extensive degradation under acidic and oxidative condition, degrade to a mild extent under alkaline and photolytic condition, and be extremely stable under thermal condition. LC–MS method was carried out for the characterization of degradation products. The m/z values, retention time and fragmentation patterns obtained enabled us to confirm the presence of known products under alkaline and oxidative conditions, and to propose the structures of unknown products under photolytic and acidic conditions. Thus, a more extensive degradation pathway of the drug was proposed, which might be useful for the study on characterization of process related impurities, drug-excipient interaction products, and metabolic pathway of the drug under different conditions.

The authors have declared no conflict of interest.

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