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Characterization of Potential Eluxadoline Impurities: Identification and Synthetic Approach

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KEYWORDS

Eluxadoline;

Impurities synthesis;

Characterization;

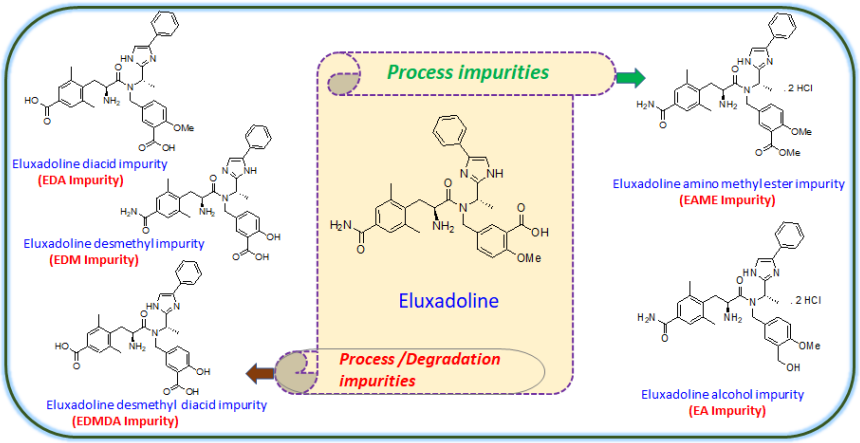
Process impurities;

Degradation impurities.

ABSTRACT:

Eluxadoline is a novel active pharmaceutical ingredient (API) used in the treatment of diarrhea and abdominal pain associated with diarrhea-predominant irritable bowel syndrome (IBS-D). During its synthesis, five unknown impurities were identified, synthesized, and comprehensively characterized using various analytical techniques such as ^{1}H NMR, ^{13}C NMR, mass spectrometry, and IR spectroscopy. These five process/degradation impurities are Eluxadoline diacid impurity (EDA impurity), Eluxadoline desmethyl impurity (EDM impurity), Eluxadoline desmethyldiacid impurity (EDMDA impurity), Eluxadoline amino methyl ester impurity (EAME impurity), and Eluxadoline Alcohol impurity (EA impurity). These impurities are crucial as reference standards for the development and quality control, regulatory compliance of the active pharmaceutical ingredient manufacturers and research

GRAPHICAL ABTRACT:



1. Introduction

Eluxadoline, or 5-({[(2S)-2-amino-3-(4-carbamoyl-2,6-dimethylphenyl)propanoyl][(1S)-1-(4-phenyl-1H-imidazol-2-yl)ethyl]amino}methyl)-2-methoxybenzoic acid is a mixed opioid receptor agonist and antagonist, acting as an agonist for the mu (μ) receptor and an

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antagonist for the delta (δ) receptor [1]. It acts locally in the gastrointestinal (GI) tract, helping to relieve abdominal pain and regulate bowel movements while minimizing constipation. Eluxadoline is marketed as Viberzi in the US and Truberzi in Europe [2]. It is an oral medication for treating diarrhea and abdominal pain in diarrhea-predominant irritable bowel syndrome (IBS-D) [3], approved in the US in May 27, 2015 [4]. Eluxadoline is developed by Actavis and originates from Janssen Pharmaceuticals. Eluxadoline's positive benefits in treating diarrhea-predominant irritable bowel syndrome (IBS-D) are due to its local action within the GI tract, where opioid receptors are widely expressed and play a crucial role in regulating GI motility, secretion, and visceral sensation [5] [6] [7]. Eluxadoline's multimodal opioid pharmacology appears to enable it to efficiently relieve abdominal pain and bowel movements in IBS-D patients while limiting the risk of constipation Figure 1 represents the chemical structure of Eluxadoline.

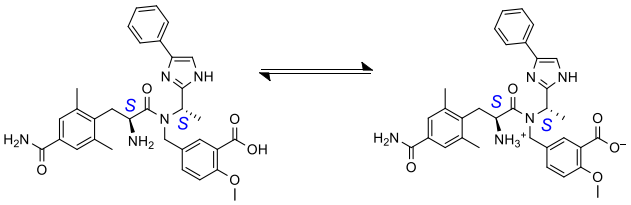

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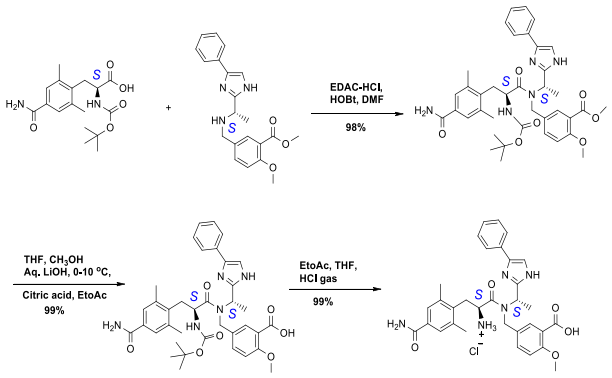
Figure 1: Chemical structure of Eluxadoline

Several clinical studies showed that Eluxadoline is effective in the treatment of severe irritable bowel syndrome with diarrhea (IBS-D) in adult men and women. Identification and control of impurities in drug compounds and drug products are crucial and critical for their safety assessment. These impurities affect the drug's efficacy, efficiency, quality, and safety measures. The maximum recommended daily dosage of eluxadoline (Viberzi) in adults is 100 mg(milligram) for some patients, 75 mg twice daily, taken with food [8]. International Council for Harmonization (ICH) tripartite guidelines suggest impurities in new drug substances identification threshold is 0.10%, and the reporting threshold is 0.05% maximum for a daily dose of ≤2 g/day [9]. The efficacy and safety of the drug compound primarily depend on the presence of impurities in the drug. Thus, the identification and isolation of these impurities formed during the synthesis of active pharmaceutical ingredient (API) products is crucial to address the key requirements for approval from regulatory agencies. Due to limited literature availability, the synthesis of these impurities becomes a challenge for manufacturers. Hence, the requirement for the identification of unknown impurities in pharmaceutical substances is inevitable [9-11]. Impurity formation and its structural identification become significant tasks in the synthesis of any drug substance, which widely depend on the synthetic route and reaction conditions.

In 2009, Henry and coworkers [12, 13] reported the synthesis of Eluxadoline, depicted in Scheme 1. Later, several researchers synthesized Eluxadoline using a similar procedure with slight modifications [14].



Scheme 1: Chemical synthesis of Eluxadoline HCl.

Various related impurities observed during the synthesis of Eluxadoline have been identified in the literature [15-17]. During pilot-scale synthesis, unknown impurities were observed in the final product at levels of 0.05 to 0.15% using HPLC. These compounds were identified using liquid chromatography-mass spectrometry (LC-MS) and named according to IUPAC nomenclature, as shown in Figure 2. This article reports the synthesis and structural illustration of four major process/degradation impurities identified during the synthesis of Eluxadoline [18-20].Figure 2 depicts the new impurities identified during the process development of Eluxadoline

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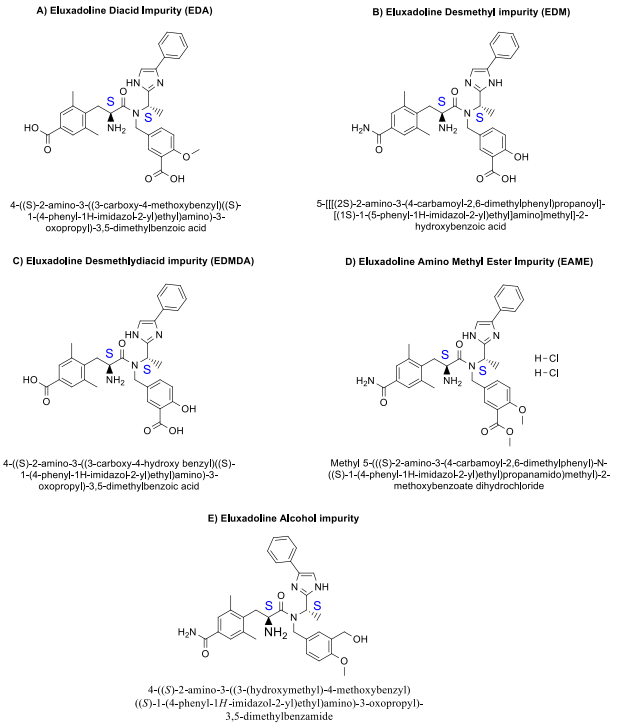

Figure 2: New impurities were identified during the process development of Eluxadoline

2.0 Materials and Methods

2.1 Chemicals

All the reagents and solvents were purchased from vendors of commercial sources and used as is without any purification.

2.2 Instrumentation and conditions

Nuclear magnetic resonance spectra 1H NMR and 13C NMR were recorded in DMSO-d6, CD3OD, or CDCl3 using a Bruker Ultrashield NMR spectrometer (Bruker CO., Switzerland) at 300 MHz and 75 MHz, respectively. The chemical shift values (δ) were reported in parts per million (ppm), splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; q, quadruplet; m, multiplet; dd, doublet of doublet, and the coupling constant (J) is expressed in Hertz (Hz). Thin-layer chromatography was carried out on silica gel F-254 plates purchased from Merck with visualization of components by UV light (254 nm). The IR spectra were recorded with an FT-IR spectrophotometer (Perkin-Elmer FTIR-4200). A Shimadzu LC-MS-2020 was used to generate mass spectra (MS). SOR used an Autopol V instrument, analyzing a 1% solution in MeOH at 589 nm and 25 °C.

2.3 HPLC method

The identification of Eluxadoline impurities was performed using HPLC chromatography. Details are Waters e2695 HPLC. Column: Cosmosil MS-II C18 (250 x 4.6) mm,5 µM. Flow rate: 0.8 mL/min l: 210 nm. Injection vol.: 15 µL. Mobile phase A: Water, methanol, and orthophosphoric acid in the ratio of 900:100:1 (v/v), Disodium phosphate buffer solution (dissolved 0.7 g disodium phosphate in 100 mL water, adjusted to pH 6.8±0.05 with perchloric acid. Mobile phase B: Methanol. Run time: 50 min. Temperature: 50°C. Gradient (A: B, 0e15 min: 95:5; 15e34 min: 75:25; 34e42 min: 50:50; 42e42.1 min: 95:5; 42.1e50 min: 95:5).

2.4. Experimental Procedures:

2.5 Synthesis of compound A (Eluxadoline diacid impurity- EDA impurity):

4-((S)-2-amino-3-((3-carboxy-4-methoxybenzyl)((S)-1-(4-phenyl-1H-imidazol-2-yl)ethyl) amino)-3-oxopropyl)-3,5-dimethylbenzoic acid.

Eluxadoline (2 g, 3.5 mmol) in concentrated HCl (conc. HCl) (4.0 g, 1.5 equiv.) was stirred for 6-8 h at 90–100 ºC. The reaction was monitored with Thin Layer Chromatography (TLC) with solvent system 9:1 methylene dichloride (MDC): methanol (MeOH). After completion, the product was cooled to RT and then distilled out of water under vacuum until the reaction mass became a residue. Then, the residue was charged with water and stirred at RT for 15-30 minutes, with pH adjusted from 5.5 to 6.5 using ammonium hydroxide (NH4OH) and conc. HCl. The solid precipitate at RT was stirred for 1-2 h. Then, the slurry was washed with 50 mL of water. The compound A was dried at 50-60°C to afford 1.7 g as an off-white solid.1H NMR (300 MHz, D2O): δ/ppm= 6.74-7.71 (m, 11H), 5.61-5.63 (m, 1H), 4.93-4.986 (m,1H), 4.39-4.56 (m, 1H), 3.71 (s, 3H), 3.01 -3.28 (s, 2H), 2.11-2.35 (s, 6H), 0.82-1.42 (2d, 6H); 13C NMR (75 Hz, DMSO-d6) δ/ppm= 172.19, 170.77, 168.42, 168.64, 156.98, 156.87, 138.18, 137.98, 137.89, 137.11, 133.92, 131.98, 131.30, 130.50, 130.90, 129.17, 129.72, 128.59, 128.87, 126.50, 126.61, 124.88, 123.87, 122.66, 116.85, 111.94, 112.65, 56.00, 56.07, 49.83, 51.02, 48.15, 49.24, 45.63, 32.85, 33.38, 20.22, 20.38, 17.28, 17.46; HRMS (ES+): calculated for

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$C_{32}H_{34}N_4O_6^+$: 571.2551[M+H]+; found: 571.2551; HPLC purity: 96.30%.

2.6 Synthesis of compound B (Eluxadoline desmethyl impurity- EDM impurity):

5-[[[(2S)-2-amino-3-(4-carbamoyl-2,6-dimethylphenyl)propanoyl]-[(1S)-1-(5-phenyl-1H-imidazol-2-yl)ethyl]amino]methyl]-2-hydroxybenzoic acid

In a 20 mL vial, Eluxadoline (2.0 g, 35.0 mmol, 1.0 equiv.) was dissolved in a 33% w/w HBr in acetic acid solution (20 mL, 10 vol) and heated to 60-70 °C under stirring for overnight. HPLC analysis of the mixture after 20 h showed 0.6% eluxadoline as the starting material and ~98% desmethyl eluxadoline. The majority of acetic acid was removed using rotavapor, and the thick, oily residual was diluted with water (50 mL) and extracted with IPAc (isopropyl acetate) twice (2 x 50 mL). The aqueous layer was slowly drop-wise added into a 500 mL beaker with a pH meter and 10-15 °C water (100 mL) with simultaneous addition of ~5% aqueous NH4OH solution to maintain the pH between 4-6.5 pH throughout the addition. After complete addition of the acidic aqueous layer resulted in a white slurry resulted, which was adjusted to ~6.5 pH and stirred for 30 min at 10-15 °C. The solid was then filtered and washed with cool water (5 mL), resulting in compound B as an off-white solid (1.8 g, 92% yield). 1H NMR (300 MHz, Acetic acid-d4): δ/ppm= 6.70-7.69 (m, 11H), 5.29 (bs, 1H), 4.85-4.98 (m,1H), 4.18-4.28 (m, 1H), 3.49-3.63 (s, 2H), 2.41-2.45 (s, 6H), 1.04-1.72 (2s, 6H); 13C NMR (75 Hz, Acetic acid-d4) δ/ppm= 172.85, 171.75, 170.28, 170.59, 161.55, 160.76, 144.34, 146.02, 138.41, 138.75, 135.28, 136.26, 134.40, 134.54, 132.10, 133.51, 130.56, 130.88, 129.11, 129.42, 128.17, 128.96, 126.49, 126.66, 125.89, 126.19, 124.23, 117.15, 117.91, 116.13, 114.95, 112.75, 113.95, 50.60, 51.76, 49.04, 49.33, 48.51, 45.72, 31.37, 32.03, 14.31, 15.23; HRMS (ES+): calculated for $C_{31}H_{33}N_5O_5^+$:556.2554 [M+H]+; found: 556.2570; HPLC purity: 96.00%.

2.4.7 Synthesis of compound C (Eluxadoline desmethyldiacid impurity- EDMDA):

4-((S)-2-amino-3-((3-carboxy-4-hydroxybenzyl)((S)-1-(4-phenyl-1H-imidazol-2-yl)ethyl) amino)-3-oxopropyl)-3,5-dimethylbenzoic acid

Compound B (2.0 g, 3.6 mmol) was added in conc. HCl (4.0 g, 10 equiv.) and stirred for 3-4 h at 90-100 °C. The complete reaction was monitored with TLC (solvent system, MDC: MeOH, 9:1). After reaction completion, the reaction mixture was cooled down to RT. Then, excess water was evaporated under vacuum until the reaction mixture turned into an oily residue. The residue was charged with water and stirred at RT for 15-30 minutes, the reaction mass pH was adjusted to 5.5-6.5 with NH4OH and conc. HCl. The precipitated solid was stirred at RT for 1-2 h. The slurry was then filtered and washed using 50 mL of water. The compound was dried at 50-60 °C to manage the acquired 1.7 g of compound C as an off-white solid. 1H NMR (300 MHz, Acetic acid-d4): δ/ppm= 6.71-7.84 (m, 11H), 5.31-5.32 (s, 1H), 4.84-5.04 (m, 1H), 4.19-4.31 (m, 1H), 3.45-3.64 (m, 1H), 2.43 & 2.44 (s, 2H), 1.05-1.75 (d, 1H). 13C NMR (75 Hz, Acetic acid-d4) δ/ppm= 170.64, 170.24, 161.55, 160.79, 145.94, 144.28, 138.41, 138.81, 136.23, 136.66, 134.27, 134.55, 133.55, 130.87, 130.24, 129.28, 129.10, 128.94, 126.68, 126.71, 126.14, 126.53, 125.87, 124.34, 117.84, 117.18, 115.05, 50.76, 51.77, 49.01, 49.32, 48.45, 45.68, 31.45, 32.13, 14.29, 15.31. HRMS (ES+): calculated for $C_{31}H_{32}N_4O_6^+$: 557.2394 [M+H]+; found: 557.2407; HPLC purity: 98.04%.

2.8 Synthesis of compound D (Eluxadoline amino methyl ester impurity (EAME impurity)

Methyl 5-(((S)-2-amino-3-(4-carbamoyl-2,6-dimethylphenyl)-N-((S)-1-(4-phenyl-1H-imidazol-2-yl)ethyl)propanamido)methyl)-2-methoxybenzoate

Dimethylformamide (DMF) (14 mL, 4.8 vol) was added slowly to a mixture of compound 2 (CAS# 623950-02-7, 3.01 g, 9.0 mmol), amine compound 3 (CAS# 1391712-57-4, 3.00 g, 8.2 mmol), HOBt (85% assay, 1.39 g, 8.2 mmol) while maintaining the temperature between 8-15 °C under N2 atmosphere. The reaction mixture was stirred for 30 min, and then a solution of EDAC. HCl ((4.25 g, 22.2 mmol) dissolved in DMF (4.8 mL) N, N'-diisopropylethylamine (5.00 mL, 28.7 mmol))) was added for 45 min while maintaining the temperature

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below 20 °C. The reaction mixture was slowly warmed to RT for 1 h and then stirred for 2-6 h at RT. After confirmation of the complete reaction using HPLC, IPAc (15 mL) and water (18 mL) were added sequentially for 30 min while keeping the temperature below 25 °C. The separation of layers was observed and was separated. The bottom aqueous layer was extracted with IPAc (3 × 15 mL) and separated. The combined rich IPAc layer was washed with 5% NaHCO3 (2 × 20 mL), water (20 mL), and 5% brine (20 mL). The organic layer was separated and concentrated to ~6.0 mL under vacuum. The rich IPAc layer was then diluted with IPAc (30 mL) and MeOH (4.5 mL) and stirred. HCl in IPA (5 N, 1.8 mL, 9.4 mmol) was added over 30 min, and the mixture was stirred for 1 h at RT. The solid precipitate was filtered, washed with IPAc (6.0 mL), and vacuum dried in an oven at 40 °C for 18 h to give compound 4 as a white solid.Weight: 5.6 g; Yield: 95%. 1H-NMR (400HZ, DMSO-d6): δ/ppm= 14.80 – 15.02 (m,1H),7.35 – 8.27 (m, 12H), 6.65 – 7.30 (m, 3H), 4.44 – 5.93 (m, 4H), 3.60 – 3.69 (m, 6H), 3.423.42 (t, 2H), 1.71 – 2.33(m, 9H), 0.741 – 1.63 (m, 9H). 13C NMR (100 Hz, DMSO-d6) δ/ppm= 171.28, 1167.83, 165.78, 157.24, 154.64, 145.39, 145.39, 137.57, 137.43, 136.92, 133.11(3C), 132.52, 131.69, 131.42, 128.87, 128.04(2C), 127.42, 127.02, 125.59(2C), 125.26(3C), 119.63, 79.91, 61.97, 59.72, 55.78, 49.88, 27.93, 26.88, 21.57, 20.73(3C), 19.96, 15.56. HRMS (ES+): calculated for $C_{32}H_{37}N_5O_4^+$:556.29 [M+H]+; found: 682.16; HPLC purity: 99.73%.

In a mixture of compound 4 (4.00 g, 5.5 mmol) and MeOH (28 mL, 7 vol), HCl gas was bubbled slowly over 20 min, maintaining the temperature below 20 °C. The reaction mixture was warmed to RT for 1.0 h and then stirred at RT (18-23 °C) for 2.0 h. After confirmation of the complete reaction using HPLC, the reaction mixture was concentrated to 25 mL under vacuum (bath temp; 20-25 °C) and cooled to 10 °C over 30 min. MTBE (30 mL) was added over 30 min, and the mixture was stirred at 10 °C for 1.0 h. The precipitated solid was filtered, washed with MTBE (10 mL), and dried in a vacuum oven at 40 °C for 18 h to give compound D as a solid white compound. Weight: 2.92 g; Yield: 80%. 1H-NMR (300HZ, DMSO-d6): δ/ppm= 6.68-8.02 (m, 11H), 5.52 (bs, 1H), 4.71-4.92 (m, 1H), 4.48-4.52 (m, 1H), 4.019 - 3.98 & 3.66 (1H), 3.73 (s, 1H), 3.28-3.58 (d, 1H), 2.32 & 2.35 (2s, 2H), 0.66-1.43 (m, 1H). 13C NMR (75 Hz, CD3OD-d4) δ/ppm= 170.09, 170.44, 166.38, 166.58, 157.90, 158.84, 143.18, 145.88, 138.18, 138.69, 134.80, 135.18, 133.56, 133.74, 133.10, 132.87, 132.45, 130.32, 130.51, 129.31, 129.39, 128.66, 128.96, 127.12, 127.87, 126.30, 125.89, 125.38, 125.29, 119.93, 118.70, 116.53, 114.35, 114.59, 112.10, 112.71, 55.24, 54.71, 51.15, 51.38, 49.56-50.56, 47.70-48-32, 44.84, 31.24, 32.13, 19.25, 19.37, 13.02, 14.72. HRMS (ES+): calculated for $C_{33}H_{37}N_5O_5^+$: found: 584.2888; HPLC purity: 98.57%.

2.9 Synthesis of compound E (Eluxadoline alcohol impurity (EA impurity) 4-((S)-2-amino-3-((3-(hydroxymethyl)-4-methoxybenzyl)((S)-1-(4-phenyl-1H-imidazol-2-yl)ethyl)amino)-3-oxopropyl)-3,5-dimethylbenzamide dihydrochloride

In a 50 mL vial, Tetrahydrofuran (THF) (15 mL, 5 vol) was added to Amine compound 3 (CAS# 1391712-57-4, 3.00 g, 8.2 mmol). Then cool the reaction mixture up to 5-10 °C. Slowly added LiAlH4 lot-wise into the reaction mixture while keeping temp 5-10 °C and stirred for 4-6 h at 5-10 °C. The reaction completion was monitored by TLC (solvent system, MDC: MeOH, 9:1). After reaction completion, the reaction mixture was quenched with saturated ammonium chloride solution 6 mL. Stirred reaction mixture for 10 minutes at 10-15 °C. Again, charged water 6ml and 15 ml of THF into the reaction mixture. The reaction mixture was stirred at RT. Filtered reaction mass through HyFlow and washed by 15 ml THF. Collected filtrate and then separated layers. The THF layer evaporated under vacuum, resulting in compound 5 as an oily residue. Weight: 2.5 g; Yield: 90%. 1H-NMR (300HZ, CDCL3): δ/ppm= 7.65-7.67 (d, 2H), 7.31-7.36 (m, 2H), 7.18-7.26 (m, 3H), 7.09-7.10 (d, 1H), 6.74-6.76 (d, 1H), 4.64(s.2H), 4.01-4.08 (q,1H),3.77(s,3H),3.55-3.64(q,2H) & 1.41-1.43 (d,3H). 13C NMR (75 Hz, DMSO-d6) δ/ppm= 156.02, 151.88, 137.35, 132.85, 131.25, 1129.45, 128.65(2C), 128.32, 128.26, 126.62, 124.64 (2C), 116.08, 109.98, 59.94, 55.20, 51.56, 51.04, 21.32. LCMS (ES+): calculated for $C_{20}H_{23}N_3O_2^+$: 338.18 $[M+H]^+$; found: 338.29; HPLC purity: 97.12%.

Dimethylformamide (DMF) (10 mL, 4.0 vol) was added slowly to a mixture of compound 2 (CAS# 623950-02-7, 2.74 g, 8.15 mmol), amine alcohol compound 5 (2.5 g, 7.4 mmol), HOBt (85% assay, 1.14 g, 8.43 mmol) while maintaining the temperature between 8-15 °C under N2 atmosphere. The reaction mixture was stirred for 30 min,

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and then a solution of EDAC. HCl (4.15 g, 21.66 mmol) dissolved in DMF (5.0 mL) N, N'-diisopropylethylamine (2.4 g, 18.52 mmol) was added for 45 min while maintaining the temperature below 20 °C. The reaction mixture was slowly warmed to RT for 1 h and then stirred for 2-4 h at RT. After confirmation of the complete reaction using HPLC, IPAc (20 mL) and water (20 mL) were added sequentially for 30 min while keeping the temperature below 25 °C. The separation of layers was observed and was separated. The bottom aqueous layer was extracted with IPAc (3 × 20 mL) and separated. The combined rich IPAc layer was washed with 5% NaHCO3 (2 × 20 mL), water (20 mL), and 5% brine (20 mL). The organic layer was separated and concentrated to ~5.0 mL under vacuum. The rich IPAc layer was then diluted with IPAc (25 mL) and MeOH (5.0 mL) and stirred. HCl in IPA (5 N, 2.25 mL, 11.14 mmol) was added over 30 min, and the mixture was stirred for 1 h at RT. The solid precipitate was filtered, washed with IPAc (5.0 mL), and vacuum dried in an oven at 40 °C for 6 h to give compound 6 as a white solid. Weight: 4.6 g; Yield: 90%. 1H-NMR (300HZ, CDCl3): δ/ppm= 7.61-7.64 (d, 4H), 6.87-7.35 (m, 6H), 6.49-6.52 (d, 1H), 6.28 (s, 1H), 6.00-6.02 (d, 1H), 4.95-5.03 (m, 1H), 4.79-4.81 (m, 1H), 3.75-4.59 (m, 7H), 3.60 (s,3H), 3.28-3.36 (t, 1H), 2.99-3.03 (t, 1H), 2.23 (s,6H), 1.49 (m,9H). 13C NMR (75 Hz, CDCl3) δ/ppm= 172.71, 170.75, 169.78, 156.90, 155.94, 146.45, 137.92 (2C), 136.42, 132.28, 129.98, 129.31, 128.74, 128.58 (2C), 128.43 (2C), 127.89, 127.29, 127.00, 124.82, 124.63 (2C), 81.15, 67.68, 61.01, 55.09, 50.30, 45.24, 32.43,28.24(3C), 19.99 (2C), 16.08. LCMS (ES+): calculated for $C_{37}H_{45}N_5O_6^+$: 656.34; found: 656.3; HPLC purity: 96.90%.

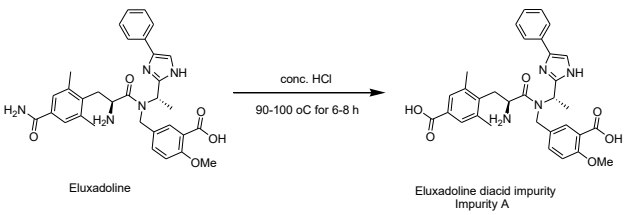
In a mixture of compound 6 (4.5 g, 6.86 mmol) and MeOH (48 mL, 7 vol), HCl gas was bubbled slowly over 20 min, maintaining the temperature below 20 °C. The reaction mixture was warmed to RT for 1.0 h and then stirred at RT (18-23 °C) for 2.0 h. After confirmation of the complete reaction using HPLC, the reaction mixture was concentrated to 18 mL under vacuum (bath temp; 20-25 °C) and cooled to 10 °C over 30 min. MTBE (24 mL) was added over 30 min, and the mixture was stirred at 10 °C for 1.0 h. The precipitated solid was filtered, washed with MTBE (7 mL), and dried in a vacuum oven at 40 °C for 6 h to give compound E as a solid white compound. Weight: 3.43 g; Yield: 90%. 1H-NMR (300HZ, MeOD-d4): δ/ppm= 7.59- 7.77 (m, 3H), 7.56 - 7.46 (m, 2H), 7.40-7.01 (m, 4H), 6.84-6.64 (d, 2H), 6.52-6.54 (m,1H), 5.23-5.58(m, 2H), 5.07-4.57 (m, 3H), 4.30-4.34 (d, 2H), 3.80 (s, 1H),3.21-3.38 (m, 2H),2.36 & 2.49 (2s, 6H), 0.70-0.73 & 1.58-1.61(2d, 3H) . 13C NMR (75 Hz, CD3OD-d4) δ/ppm= 170.28, 169.96, 157.43, 156.40, 145.95, 143.15, 138.67, 138.19, 135.17,134.81, 133.82, 133.67, 130.04, 129.66, , 129.59, 129.42 1 129.27, 129.03, 128.98, 128.62,127.88, 127.25, 126.67, 126.31, 1 126.05, 125.88, 125.51, 125.34, 125.17, 114.62, 114.53, 111.14, 110.29, 54.91, 54.34, 50.29, 48.69, 44.86, 40.50, 40.33, 32.11, 31.23, 19.22, 19.18, 14.57, 12.92; LCMS (ES+): calculated for $C_{32}H_{37}N_5O_4^+$: 556.29 [M+H]+; found: 556.4; HPLC purity: 98.57%.

RESULTS AND DISCUSSION

During pilot-scale synthesis, unknown impurities were observed in the final Eluxadoline (API) at levels of 0.05 to 0.15% using HPLC. These impurities were identified using liquid chromatography-mass spectrometry (LC-MS) and named as compounds **A-E**. Upon identifying, our objective is to synthesize and characterize each impurity in a pure form. The details of seven impurities, along with IUPAC nomenclature, are depicted in **Figure 2**.

3.1 Eluxadoline diacid impurity-EDA impurity (A)

The amide hydrolysis of eluxadoline API under acidic conditions can result in the formation of a diacid impurity. This impurity, designated as EDA (Compound A) and synthesized by treating eluxadoline with concentrated hydrochloric acid at 90–100 °C, as shown in Scheme 2. The formation of EDA was confirmed by mass spectrometry, which showed a molecular ion peak at m/z 571.2 [M+1] in positive mode, corresponding to the molecular weight of eluxadoline diacid (570.63 g/mol) having molecular formula $C_{32}H_{34}N_4O_6$. Further structural confirmation was provided by IR spectroscopy and proton exchange experiments in the 1H NMR spectrum using D2O.



Scheme 2: Synthesis of EDA impurity

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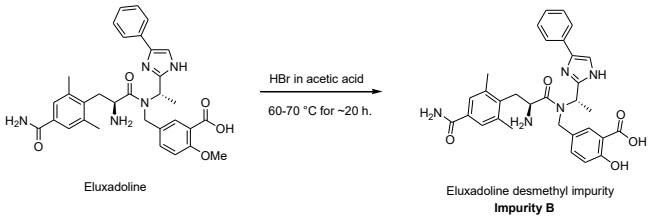
3.2 Eluxadoline desmethyl impurity-EDM impurity (B)

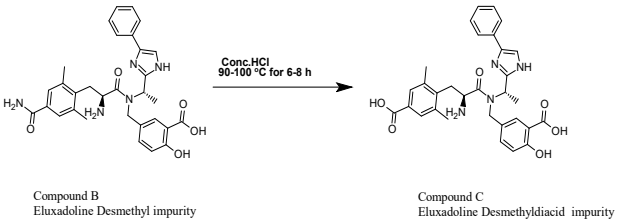
The demethylation of eluxadoline under photolytic exposure or upon acidic, oxidative, or thermal degradation leads to the formation of the desmethyl impurity. The synthesis of this impurity was achieved by treating the Eluxadoline and hydrobromic acid in acetic acid at 60-70 °C for 20 h, as depicted in Scheme 3. The formation of the desmethyl impurity (Compound B) was confirmed by mass spectrometry, which exhibited a molecular ion peak at m/z 556.5 [M+1] in positive mode, consistent with the calculated molecular weight of 555.62 g/mol. The absence of a proton at δ 3.7 ppm as compared to the starting material in 1H NMR spectra confirmed the demethylation. The IR spectrum showed key peaks at 3181.94 cm-1 (O-H stretching), 1651.47 cm-1 (C=O stretching), and 1172.5 cm-1 (C-N stretching).



Scheme 3: Synthesis of EDM impurity

3.3 Eluxadoline desmethyldiacid impurity- EDMDA impurity (C)

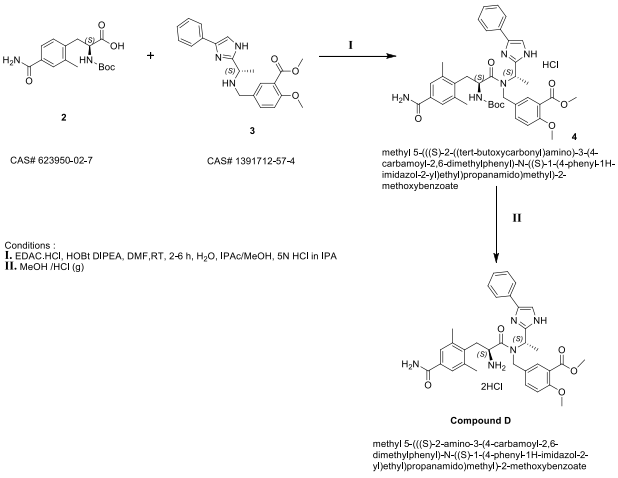
Under acidic conditions, concurrent amide hydrolysis and O-demethylation of eluxadoline result in the formation of the desmethyldiacid impurity (compound C), a degradation product incorporating both structural modifications. The treatment of compound B with conc. HCl solution at a higher temperature resulted in the synthesis of Eluxadoline desmethyldiacid impurity as shown in **Scheme 4.** The obtained product was confirmed by MS, showing a molecular ion peak at 557.43 [M+1], consistent with a molecular weight of 556.61 g/mol. The IR analysis identified key peaks as 2967.43 cm-1 (O-H stretching) and 1629.37 cm-1 (C=O stretching of amides).



Scheme 4: Synthesis of EDMDA impurity

3.4 Eluxadoline amino methyl ester impurity- EAME impurity (D)

The eluxadoline methyl ester impurity typically arises from the carryover of an unreacted Stage-1 intermediate (methyl ester compound), which subsequently undergoes the same reaction conditions as eluxadoline, leading to its persistence as an impurity in the final product (compound **D**). The synthetic protocol involves the coupling of intermediates **2** and **3**, followed by the deprotection of the Boc group of resultant **D** under acidic conditions (**Scheme 5**). The product was confirmed by MS, showing a molecular ion peak at 584.2 [M+1] in a negative mode, consistent with a molecular weight of 583.67 g/mol. The IR spectrum revealed the key peaks at 3333.08 cm^{-1} and 3145.63 cm^{-1} (N-H stretching of amide), 1713.66 cm^{-1} (C=O stretching of ester), 1656.86 cm^{-1} (C=O stretching of amide), and 1156.53 cm^{-1} (C-N stretching).



Scheme 5: Synthesis of EAME impurity

3.5 Synthesis of compound E (Eluxadoline alcohol impurity (EA impurity)

The eluxadoline alcohol impurity typically arises from the carryover of a compound 3 content alcohol impurity, which subsequently undergoes the same reaction

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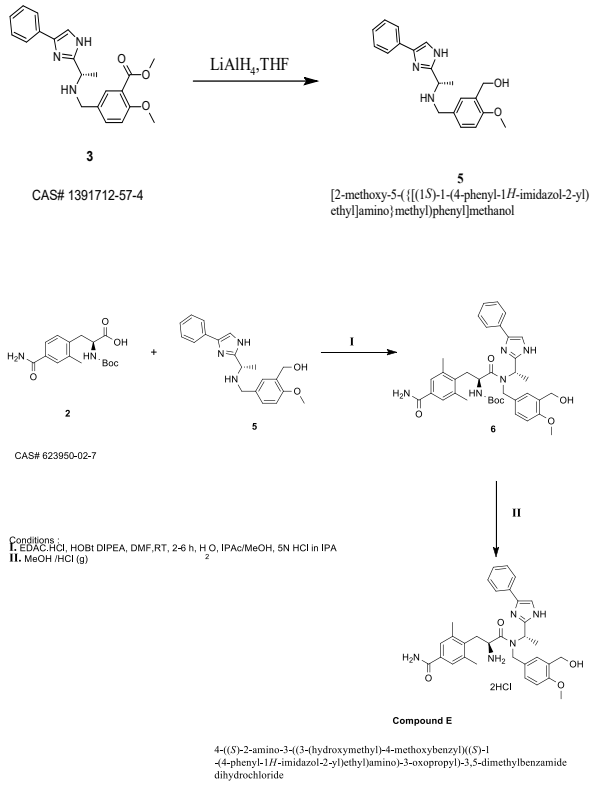
conditions as Eluxadoline, leading to its persistence as an impurity in the final product (compound **E**). The synthetic protocol involves the coupling of intermediates **2** and **5**, followed by deprotection of the Boc group of resultant **E** (**Scheme 6**). The product was confirmed by MS, showing a molecular ion peak at 556.4 [M+1] in a positive mode, consistent with a molecular weight of 555.66 g/mol.



Scheme 6: Synthesis of Eluxadoline alcohol impurity

After synthesis and characterization of all impurities, the spike chromatogram of these impurities, along with Eluxadoline. All impurities are well separated as represented in **Figure 4.**

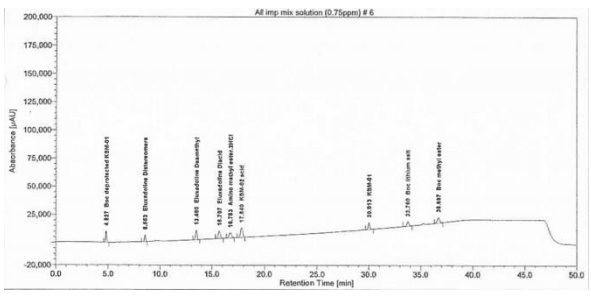


Figure 4: Spike HPLC chromatogram of identified Eluxadoline impurities.

3. Conclusion

In conclusion, five unknown impurities of Eluxadoline were identified, synthesized, and structurally confirmed through comprehensive analytical techniques using ^{1}H NMR, ^{13}C NMR, IR, and mass spectrometry. A plausible mechanism and explanation were deduced for all five impurities formed during synthesis. Furthermore, the detailed characterization of these impurities provides a foundation for establishing reference standards, thereby supporting the synthesis of highly pure Eluxadoline drug substances. This study will help researchers identify and control the synthesis of all seven impurities during the scale-up and manufacturing process. This work will elevate interest in the researchers working on the process and formulation development of the Eluxadoline API.

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Conflict of interest

The authors declare no conflict of interest, financial or otherwise.

Ethical approval

No human/animal studies were carried out in the present work.

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Supplementary material

Full experimental procedure, ^{1}H NMR, ^{13}C NMR spectra, HPLC traces, and MS spectra for impurities A to E. This material can be found via the “Supplementary Content” section of this article.

Abbreviations

UV: Ultraviolet; IR: Infrared; FTIR: Fourier Transform Infrared Spectroscopy; ATR: Attenuated Total Reflection; mmol: Millimole; MDC: Methylene dichloride; MeOH: Methanol; API: Active

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Pharmaceutical Ingredient; mg: Milligram; HPLC: High-Performance Liquid Chromatography; EtOAc: Ethyl Acetate; MHz: Megahertz; TLC: Thin Layer Chromatography; NH4OH: Ammonium Hydroxide; Conc. HCl: Concentrated hydrochloric acid; EtOAc: Ethyl Acetate; ml or mL: Milliliters; LC-MS: Liquid Chromatography-Mass Spectrometry; THF: Tetrahydrofuran; RT: Room Temperature; equiv: Equivalent; °C: Degree Centigrade; DMA: N, N'-Dimethylacetamide; ppm: Parts Per Million; DMF: Dimethylformamide; SOR: Specific Optical Rotation; DMSO: Dimethyl Sulfoxide; HRMS: High-Resolution Mass Spectrometry; ee: Enantiomeric Excess; DSC: Differential Scanning

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