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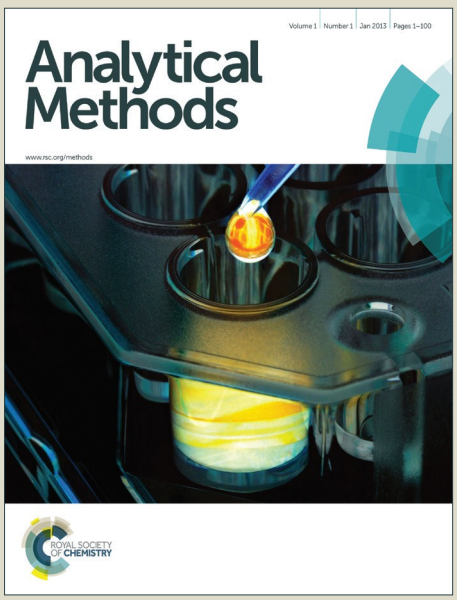

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Identification, characterization and HPLC quantification for impurities of Apremilast

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Abstract

A sensitive, selective and stability indicating LC-UV method was developed for the determination of process-related impurities of Apremilast. High performance liquid chromatography (HPLC) investigation of Apremilast bulk samples revealed the presence of six impurities (Imp-A, Imp-B, Imp-C, Imp-D, Imp-E and Imp-F). Additionally, based on the characterization data, Imp-F is a new compound proposed to beN-{2'-[1-(3-Ethoxy-4-methoxy-phenyl)-2-methanesulfonyl-ethyl]-1,3,1',3'-tetraoxo-1,3,2',3'-tetrahydro-1'H-[2,4']biisoindolyl-4-yl}-acetamide.The structures of Imp-A and Imp-B were speculated based on LC-MS, UV and the synthetic process. The structures of other four impurities were characterized and confirmed by IR, LC-MS and NMR techniques. The newly developed LC-UV method was validated by its satisfactory specificity, precision, accuracy and sensitivity.Quantitation limitsfor impurities were in the range of 0.795-1.498ng. Correlation coefficient values of linearity were higher than 0.9985 for Apremilast and four impurities. The mean recoveries of four impurities were between 92.5% and 103.2%. Thus, the developed HPLC method was suitable for the separation and quantification of all discovered impurities in Apremilast at present. The possible mechanism for the formation of these impurities is also discussed.

Keywords: Apremilast/structural elucidation/process-related impurities/quantification

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Abbreviations:TNF, tumor necrosis factor; IL, interleukin; ACN, acetonitrile; ESI, electrospray ionization source; Imp-A, N-[1-(3-Ethoxy-4-methoxy-phenyl)-2-methanesulfonyl-ethyl]-acetamide; Imp-B, N-(1-{1-[1-(3-Ethoxy-4-methoxy-phenyl)-2-methanesulfonyl-ethylamino]-ethyl}-3-methyl-butyl)-acetamide; Imp-C, 3-(Acetylamino)-1,2-benzenedicarboxylic acid; Imp-D,

2-[1-(3-Ethoxy-4-methoxyphenyl)-2-(methylsulfonyl)ethyl]-4-nitro-1H-isoindole-1,3(2H)-dione; Imp-E, 4-Amino-2-[1-(3-ethoxy-4-methoxy-phenyl)-2-methanesulfonyl-ethyl]-1H-isoindole-1,3(2H)-dione; Imp-F, N-{2'-[1-(3-Ethoxy-4-methoxy-phenyl)-2-methanesulfonyl-ethyl]-1,3,1',3'-tetraoxo-1,3,2',3'-tetrahydro-1'H-[2,4']biisoindolyl-4-yl}-acetamide

SMA, starting material A; SMB, starting material B; SMB-1, (3-nitro-1,2-benzenedicarboxylic acid);Resolving agent, N-Acetyl-L-leucine; intermidate-2', (4-amino-1,3-isobenzofurandione);R, resolution; *r*, regression; RRT, relativeretention time; RSD, relative standard deviation;

1. introduction

Apremilast (CC-10004;Celgene Corporation, Summit, NJ, USA), N-[2-[(1S)-1-(3-ethoxy-4-methoxyphenyl)-2-(methylsulfonyl)ethyl]-2,3-dihydro-1,3-dioxo-1H-isoindol-4-yl]acetamide, is an oral small molecule inhibitor of phosphodiesterase 4 (PDE4) [1]which has been shown to be effective and well tolerated in clinical trialsin psoriasis (phase III),psoriatic arthritis (phase III), and Behçet's disease (phase II).Targeted inhibition of PDE4 results in partial inhibition of proinflammatory mediator production, such as TNF- α, interferon- γ, andIL-23, and increases in anti-inflammatory mediator production, suchas IL-10 [2], which in turn results in reduced infiltration of immune cells and changes in resident cells of the skin and joints[3].

In March 2014, the US Food and Drug Administration approved apremilast for the treatment of adult patients with active psoriatic arthritis [4-5]. Several synthetic methods for apremilast have been published [6-9]. Combined with the manufacturing process of apremilast, we design a simple and feasible synthetic route to manufacture apremilast as shown in Fig.1. Based on our synthetic route, starting materials are easy to obtain and this route is suitable for industrialized production. The potential process-related impurities of apremilast were speculated based on the different aspects of studies and analyzed by HPLC-UV-ESI-MS. The structure of Apremilast and

impurities were shown in Fig.2. The analytical method for the determination of Apremilast was based on HPLC method 20mmol/L phosphate buffer in water(pH=3.5) as mobile phase A and ethyl alcohol as mobile phase B by using a Untron Chiral ES-OVS column[10]. Chen, Xiaole *et. al.*[11] have reported a method entitled "Determination of Apremilast in Rat Plasma by UPLC-MS/MS and its Application to a Pharmacokinetic Study". In this article, a ultra performance liquid chromatography-tandem mass spectrometry method for determination of Apremilast in rat plasma was developed and validated. However,extensive literature research reveals thatno reports concerning quantitative determination of impurities and characterization of potential impurities of apremilast have been revealed so far. Thus, there is a need for development of an effective analytical method to monitor the levels of impurities in apremilast during process development and it is essential and mandatory to identify and characterize any impurities in drug substances exceeding the accepting limit of 0.1% [12].

Objectives of the current study were: (1) to speculate the potential process-related impurities in the apremilast drugaccording to the synthetic routes; (2) to detect, identify and elucidate the impurities of Apremilast by spectral data(UV, NMR, MS and IR); (3) to optimize LC conditions and develop an effective HPLC method for the quantitative determination of the potential process-related impuritiesaccording to ICH guidelines[13].Through a series of research we solve the above problem. At the same time, we obtain a newly and effective HPLC method and elucidate the impurities including a new compound (Imp-F, N-{2'-[1-(3-Ethoxy-4-methoxy-phenyl)-2-methanesulfonyl-ethyl]-1,3,1',3'-tetraoxo-1, 3,2',3'-tetrahydro-1'H-[2,4']biisoindolyl-4-yl}-acetamide) .

2. Experimental

2.1.Materials and reagents

Apremilast and its standards of Imp-C, Imp-D, Imp-E, Imp-F were obtained in our laboratory. Starting material A (SMA; 1-(3-Ethoxy-4-methoxy-phenyl)-2-methanesulfonyl-ethylamine), and Starting

material B (SMB; 3-Amino-1, 2-benzenedicarboxylic acid) were purchased from Enantiotech Corporation Ltd (Guangdong, China). Imp-C, 3-(Acetylamino)-1,2-benzenedicarboxylic acid; Imp-D, 2-[1-(3-Ethoxy-4-methoxyphenyl)-2-(methylsulfonyl)ethyl]-4-nitro-1H-isoindole-1,3(2H)-dione; Imp-E, 4-Amino-2-[1-(3-ethoxy-4-methoxy-phenyl)-2-methanesulfonyl-ethyl]-1H-isoindole-1,3(2H)-dione; Imp-F, N-{2'-[1-(3-Ethoxy-4-methoxy-phenyl)-2-methanesulfonyl-ethyl]-1,3,1',3'-tetraoxo-1,3,2',3'-tetrahydro-1'H-[2,4']biisoindolyl-4-yl}-acetamide.The purity of all substances was >98%. HPLC grade acetonitrile (ACN) was purchased from Merck (Darmstadt, Germany).Anhydroustrifluoroaceticacid(TFA) was purchased from Thermo Scientific(Rockford, IL, USA). Water was produced through a Milli-Q pure water system (Millipore , USA).Other chemicals were of analytical grade.

2.2. Preparation of the impurities (Imp-C, Imp-D, Imp-E, Imp-F)

Imp-A and Imp-B were detected by LC-MS and speculate their structure combining with the synthetic route of Apremilast in Fig 4.

Imp-C and Imp-D are obtained by our designed synthetic route, the detail synthetic method as follow:

3-Acetylamino-phthalic acid (Imp-C) A mixture of SMB(5g, 0.024mol) and 25ml 1mol/L NaOH was stirred in ethanol water (25ml) at 30℃ for 0.5h. Then 20ml 1mol/L HCl was added, a large amount of solid precipitated out from the solution. The solid was dried in vacuum drying oven at 30℃ to obtain Imp-C 3.12g, yield 62.4%

2-[1-(3-Ethoxy-4-methoxy-phenyl)-2-methanesulfonyl-ethyl]-4-nitro-isoindole-1,3-dione(Imp-D) intermediate-1(4.5g, 0.01mol) and 3 –nitrophthalicanhydride(2.03g, 0.01mol) acidwere stirred in the acetic acid solution(45ml) at 120℃ for 2h. When the temperature of solution was at 50℃, the solution was concentrated by vacuum rotary evaporation at 60℃. Then, the residuum was extracted by ethyl acetate and dried by sodium sulphate anhydrous about 2h.

Filter liquor was concentrated by vacuum rotary evaporation at 40℃ to obtain crude product of Imp-D 4.56g, yield 96%

Imp-E and Imp-F are obtained by column chromatography and preparative liquid chromatography (Pre-HPLC) from the stock solution of Apremilast.

(4-Amino-2-[1-(3-ethoxy-4-methoxy-phenyl)-2-methanesulfonyl-ethyl]-isoindole-1,3-dione) Imp-E and (**N-{2'-[1-(3-Ethoxy-4-methoxy-phenyl)-2-methanesulfonyl-ethyl]-1,3,1',3'-tetraoxo-1,3,2',3'-tetrahydro-1'H-[2,4']biisoindolyl-4-yl}-acetamide)Imp-F** The stock solution of Apremilast was concentrated by vacuum rotary evaporation at 40℃. Then acetone (270mL) and ethyl alcohol(540mL) was added into the residue and was stirred for 3-5h at 30-50℃.The solution was disposed by suction filtration and eluted by ethyl alcohol (500mL). Filter liquor was concentrated by vacuum rotary evaporation at 40℃ to obtainyellow solid 17.6g. A mixture of the yellow solid, acetone(53mL) and ethyl alcohol(106mL) was stirred for 3h at 35℃. The solution was disposed by suction filtration and eluted by ethyl alcohol (100mL). Filter liquor was concentrated by vacuum rotary evaporation at 40℃ to obtainyellow solid 3.4g faint yellow solid. The solid was separated silica gel column chromatography to obtain Imp-E (500mg, purity: 97.6%) and Imp-F (200mg, purity: 88%). Then the Imp-F was further separated and purified by Pre-HPLC under 30℃ and was freeze-dried to obtain pure white solid (150mg, purity:99.8%)

2.3.High performance liquid chromatography (HPLC) and Pre-HPLC

Chromatographic studies were performed on Agilent 1100 HPLC system (Agilent Technologies, USA). The chromatograms were recorded and analyzed employing Agilent Chemstation chromatographic workstation.The HPLC was performed on a Wondersil C_{18}(250mm×4.6mm, 5μm). The column was held at 30 ℃. The mobile phase A was 0.03% TFA in the water, while the mobile phase B was ACN (0.03% TFA). The gradient program was as follow: Time $_{(min)}$/A:B (v/v); T_0 95/5, T_{40} 30/70, T_{45} 30/70, T_{50} 95/5, T_{60} 95/5. The elution flow rate was 1.0mL/min, and the detection wavelength was set at 230nm. The Apremilast samples were prepared in

diluents (water and acetonitrile, 40:60, v:v) at 300μg/mL concentration and 20μL of sample solution was injected into HPLC system.

Imp-F was obtained from the method of the preparative liquid chromatography. The purity of Imp-F was 88% after purifying by column chromatography. A preparative liquid chromatography method was used to improve the purity of Imp-F to 99%. GX - 281 high flux automatic preparative chromatography system (Gilson,Inc USA) equipped with a Gilson 332 pump(prep-scale HPLC), Gilson 156 detector(UV/Vis) with high pressure flow cell, Gilson 231XL sample injector and Gilson 402 dilutor-dispenser with 25ml syringe and 25ml transfer tubing was used in the study. Trilution LC software was used to control the Imp-F fraction collection and data collection. The Pre-HPLC was performed on a Gemini 5μ C_{18} 110A(250mm×10.00mm, 5μm). The column was held at 30 ℃. The mobile phase A was water and the mobile phase B was ACN. The gradient program was as follow: Time $_{(min)}$/A:B (v/v); T_0 40/60, T_{20} 60/40, T_{30} 60/40, T_{40} 40/60. The elution flow rate was 4.0mL/min, and the detection wavelength was set at 230nm. The Apremilast samples were prepared in diluents (water and acetonitrile, 40:60, v:v) at 200mg/mL concentration and 100μL of sample solution each time was injected into Pre-HPLC system.

2.4.Liquid chromatography-tandem mass spectrometry method (LS-MS)

LC-MS was performed on an API4000 mass spectrometer (AB Sciex, USA) equipped with an electrospray ionization (ESI) source interface coupled to an Agilent 1100-LC system (Agilent Technologies, USA). Mobile phase A (0.05% acetic acid in the water) and phase B (acetonitrile) were as mobile phases. The LC gradient program was the same as HPLC chromatographic conditions. The flow rate was maintained at 1 mL/min and was split at the column outlet to allow 0.2 mL of eluent to flow into the mass spectrometer.The MS was in positive-ion electrospraymode, and the operating parameters were as follows: Ion spray voltage, 5000 V; declustering potential, 70 V; entrance potential, 10 V; turbo ion spray temperature, 500 ℃; interface heater, on; and mass range, 100–800 Da. Nitrogen was used as both curtain and auxiliary gas. The system was operated using Analyst Software

workstation, version 1.5.2 (AB Sciex, USA).

2.5.NMR spectroscopy

One-dimensional(^{1}H NMR, ^{13}C NMR) and 2D(distortionless enhancement by polarization transfer (DEPT), ^{1}H-^{1}H correlation spectroscopy (COSY), heteronuclear multiple bond correlation (HMBC) , and heteronuclear singular quantum correlation (HSQC)) NMR spectra were recorded on a Bruker AVANCE 500MHz NMR system (Fallanden, Switzerand). The samples were dissolved in DMSO-d_6, and tetramethylsilane was added as an internal standard at 25℃.

2.6.FT-IR spectroscopy

The IR spectra were recorded from KBr pellets using Thermo Scientific Nicolet iS5 FT-IR spectroscopy. Dates were collected between 4000 cm^{-1} and 400 cm^{-1}, with a resolution of 4.0 cm^{-1}. A total of 16 scans were obtained and processed using OMNIC software version 6.0.

3. Results and discussion

3.1 HPLC method development and optimization

Characterization and controlling stereoisomer impurity is especially important for Apremilast as a chiral drug. Impurities Imp-A, Imp-B, Imp-D and Imp-E are generated in the process from the intermidate-1 to Apremilast. The intermidate-1,whose configuration is determinate, is obtained from the stereospecific synthesis with resolving agent. Based on the formation mechanism, impurities(Imp-A, Imp-B, Imp-D and Imp-E) originated from intermidate-1, which means the configurations of impurities can be probably controlled. Moreover, Romualdo Benigni *et. al.*[14] have reported the structural alerts for the in vivo micronucleus assay in rodents. There are no structural alerts in the impurities Imp-A, Imp-B, Imp-D and Imp-E. The analysis of chiral impurity has been not taken into consideration for the low probability of formation and toxicity.

The first step was to determine the appropriate wavelength, which was usually a compromise for different compounds with different absorption maximum. The proper wavelength was targeted after measuring all spectra and testing the detector response

of analytes at 230nm, because of the sufficient selectivity and sensitivity for all the related substances in the study.

A HPLC method, which described the analysis of Apremilast, using 80% mobile phase A (20mmol/L phosphate buffer in water and adjusting pH to 3.5) was shown in literature [10]. However, a chiral column not a regular reverse phase column was used and this method was only used to detected the content of Apremilast.This method can't get good separation of the impurities in Apremilast. Thus, to obtain an adequate separation of Apremilast and its impurities, the types of columns, the proportions of mobile phase, flow rate and column temperature were optimized. In our study, preliminary column screening studies were conducted using Wondersil C18 (250mm×4.6mm, 5μm) , Diamonsil C18 and Inertsil ODS-3 C18. Combined with the chromatographic behavior of the three chromatographic columns, Wondersil C18 column showed the best analysis result. However, there are some difficulties for the HPLC optimization, one is how to enhancing the retention time of Imp-C, and the other is improving the resolution of Imp-E/Apremilast.

To improve the resolution and peak symmetry, TFA was added into the mobile phase. Because Apremilast is a tertiary amine, a tailing could be produced when high injection amount is injected to HPLC system. TFA is an ion pair reagent and can avoid this kind tailing. According to the chemical structure of Imp-C, Imp-C owned two carboxyl groups with a pKa of 2.54. The high proportion of water and slight TFA were used to increase the retention of high polar substances.An impurity-spiked solution was successively injected to HPLC system to investigate the HPLC conditions for each trial. ACN and 0.01% TFA were initially chosen to optimize the HPLC conditions. The results showed that the retention time of Imp-C was Increased to 7.87min, but the resolution (R) of Apremilast and Imp-E was low (Rs<1.5).

To further modify the performance, ACN and TFA mixtures, with the acid concentration adjusted over the range 0.01%-0.05%, was taken as mobile phase in a different gradient mode. The trifluoroacetic acid value of the mobile phase has a significant effect on capacity and tailing factor of all the compounds and the optimum symmetry of all the analytes was achieved with acid concentration of 0.03%. The

resolution was improved(Rs>1.5) using the suitable TFA concentration and gradient analysis. In this trial, all process related impurities can be observed with separation.

Finally, the optimum separation was attained using Wondersil C_{18} column(250×4.6 mm, 5μm) , the detection wavelength was 230nm and the flow rate was 1.0mL/min in a gradient elution mode described in Section 2.2.

3.2 HPLC method validation

The proposed method was validated in accordance with the current ICH guidelines.Linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy, and calibration factor were investigated and summarized in Tables 1 and 2.

3.2.1 System suitability and specificity

The system suitability of the method was carried out by improving of the number of theoretical plates, symmetry factor, and resolution. The system suitability solution was prepared by the four impurities spiked with Apremilast (0.3μg/mL ;0.1% of sample concentration 0.3mg/mL). An efficient resolution (>1.7), the United States Pharmacopoeia (USP) were greater than 18894 and good symmetry factor were obtained with the chromatography method shown in Table 1. The peak purity test of these samples was carried on PDA detector revealing that there is no merging to any known peak with any unknown peak. HPLC chromatogram of Apremilast bulk samples spiked with six impurities is shown in Fig.3

3.2.2 Sensitivity and linearity

Sensitivity was investigated by the LOD and LOQ for Apremilast and its four impurities at S/N 3:1 and 10:1, respectively, by injecting a series of solution diluted with known concentration. The linearity of the impurities in Apremilast was obtained from a series concentration ranging from 0.03μg/ml -0.63μg/ml. The peak area of analyte versus concentration was analyzed by the least squares linear regression, and the correlation coefficient of regression (r) was > 0.9985, indicating the excellent linearity of the method.

3.2.3 Precision and accuracy

The precision of the method, including repeatability (inter-day precision) and intermediate precision (intra-day precision) was preformed with six individual system suitability solutions, and the evaluation was estimated by different experimenter on a different day using different instruments. The RSD of Apremilast and its impurities was within 1.95, indicating the good precision of the method. The accuracy of the method was assessed with standard addition and recovery experiments in triplicate at three concentration levels, 80%, 100%, 120% of the analyte concentration. The mean recovery of four impurities was between 97.60% and 100.24% (Table 2)

3.2.4 Robustness and solution stability

Robustness of the method was studied by changing the column temperature(30±5℃), the TFA content of mobile phase(0.01%, 0.03%, 0.05%), the ratio of organic phase(5%±2%), the flow rate(1.0±0.2mL/min) and column-to-column variation. The robustness was tested with a 0.3mg/mL Apremilast spiked with all the impurities solution(0.1% of Apremilast concentration). The results revealed that the resolution between any of two analytes was > 1.77. The stability of Apremilast and its mixed impurities solution was monitored at room temperature for 12h, and the RSD values of the peak area of all the analytes were found to be within 1.95.

3.2.5 Correction factors

For quantitative study of the impurities in bulk drug, the main methods of the determination are impurity reference substance, the addition (or not) of the correction factor of principal components. So, it is essential to calculate the correction factors for all impurities, when using area normalization to quantify the impurities. The correction factor experiments were carried out on two different chromatographic instruments with three different chromatographic columns by injecting solutions with impurities at six concentration levels (LOQ to 200% limit of the impurities). The correction factor was calculated from the ratio of the slope of principal components and impurities by linear regression. The results indicate that the correction factors of all impurities are within 1.29-2.82, and these values are used in the quantitative analysis of the impurities in Apremilast.

3.3 Detection of impurities

Apremilast was obtained by synthetic route as described in Fig.1. The water and acetonitrile mixture(40:60) was used as the diluents in the preparation of Apremilast bulk samples solution. The concentration of bulk samples solution was 0.3mg/mL. HPLC analysis of Apremilast (Fig.3) revealed the presence of six impurities in several batches. Theretention time (RT) and relative retention time (RRT) of all six impurities were RT=7.87, RRT=0.25; RT=16.94, RRT=0.54;RT=22.13, RRT=0.71;RT=23.71, RRT=0.76;RT=30.06, RRT=0.98; RT=34.12, RRT=1.10 and marked as Imp-C, Imp-A, Imp-B, Imp-D, Imp-E, Imp-F.

Moreover, A LC-ESI/MS method was described in section2.4 to identify these impurities. The molecular weights respectively were 223.05, 315.11, 428.23, 448.09, 418.12, 605.15, which were in correspondence with Imp-C, Imp-A, Imp-B, Imp-D, Imp-E, Imp-F.(Electronic Supplementary Information Table S1)

3.4 Structure elucidation of Apremilast and its related impurities

Apremilast and its impurities (Imp-C, Imp-D, Imp-E, Imp-F) were obtained inour laboratory and further confirmed by IR, LC-MS,^{1}H-NMR, ^{13}C-NMR, DEPT, H-HCOSY, etc. Imp-A and Imp-B were elucidated by LC-MS and combined withsynthetic process. The numbered carbon atoms were shown in Fig.2.

3.4.1 Structural elucidation of Apremilast

The negative ion ESI-MS spectrum of Apremilast exhibited a base peak of $[M-H]^-$ ion at m/z 459.1 and M+Na at m/z 483.1. The IR spectrum displayed characteristic absorptions at 3363.3, 2837, 1764, 3002 and 1338 cm^{-1} which was indicative of acylamino N-H stretching, methylene C-H, isoindole C=O, benzene ring and sulphone $-SO_2-$. The structure was further supported by ^{1}H-NMR, ^{13}C-NMR and DEPT spectrum.

The imino proton (H_{21}) was deshielded to δ9.69ppm. Six aromatic protons (H_3, H_4, H_6, H_{17}, H_{18}, H_{19})were deshielded to δ8.45-6.92ppm. The tertiary carbon proton (H_{10}) was deshielded to δ5.80-5.77ppm. The four methylene protons (H_8, H_{11}) were deshielded to δ4.38-4.00ppm. The twelve methyl protons (H_1, H_9, H_{12}, H_{23}) were deshielded to δ3.73-1.30ppm. ^{13}C-NMR account for twenty-two carbon atoms. Nine carbon signals disappearing in DEPT-135 spectrum were considered as nine

quaternary carbon atoms. DEPT-135 spectrum revealed eleven carton (CH_3 or CH_2) signals and displays two negative carton (CH_2) signals which were C_8 and C_{11}. Seven cartons signals appearing in DEPT-90 spectrum were considered as seven tertiary cartons and four carbons signals also appear in DEPT-135 spectrum which were considered as four primary carbons. The detailed information of ^{1}H-NMR, ^{13}C-NMR and DEPT can be seen in Electronic Supplementary Information Table S2 and Table S3.

3.4.2Structural analysis of Imp-A and Imp-B

The impurity A and impurity B have been speculated as N-[1-(3-Ethoxy-4-methoxy-phenyl)-2-methanesulfonyl-ethyl]-acetamide and N-(1-{1-[1-(3-Ethoxy-4-methoxy-phenyl)-2-methanesulfonyl-ethylamino]-ethyl}-3-methyl-butyl)-acetamide, respectively.

Based onLC-MS, Imp-A has quasi-molecular ions $[M+H]^+$ at 316.2 and $[M-H]^-$ at 314.4. The maximum UV absorption wavelength (λmax) of Imp-A is 221.4nm which is the conjugated double bondπ→π*transition of benzene ring.

The ion LC-MS spectrum of Imp-B exhibited base peaks of $[M+H]^+$ at 429.0 and $[M-H]^-$ at 427.3. The Imp-B has a strong absorption in the UV absorption wavelength of 222.0nm of the conjugated double bondof benzene ring and a weak absorption of 348.20 of the n→π*transition of amide.

3.4.3 Structural elucidation of Imp-C

The impurity has been identified as 3-(Acetylamino)-1, 2-benzenedicarboxylic acid.

The negative ion ESI-MS spectrum of Imp-C exhibited a base peak of $[M-H]^-$ ion at m/z 222 which accords with Imp-C's molecular mass. The IR spectrum displayed characteristic absorptions at 3256.4, 2895.9, 2634.1 and 1474.3 which was indicative of secondary amino group -NH-, methyl C-H, carboxylic acid C=O and benzene ring. The ^{1}H-NMR spectrum showed peak integral ratio (from low to high field) for 2:1:1:1:1:3, the total number of protons is 9, which accord with the molecular structure of Imp-C. The ^{13}C-NMR spectrum displayed ten carbon signals. The details information of ^{1}H-NMR and ^{13}C-NMR can be seen in Electronic Supplementary Information Table S2 and Table S3.

3.4.4 Structural elucidation of Imp-D

This impurity has been identified as 2-[1-(3-Ethoxy-4-methoxyphenyl)-2-(methylsulfonyl)ethyl]-4-nitro-1H-isoindole-1,3(2H)-dione, based upon the following observations:

The ESI-MS showed a quasi-molecular ion $[M+Na]^+$ at m/z 471.1(calcd. For $[C_{20}H_{20}N_2O_8S+Na]^+$:471.1), which was consistent with the molecular formula of $C_{20}H_{20}N_2O_8S$. The IR spectrum analysis of Imp-D revealed the characteristic absorptions at 2984.7, 2934.3, 1718.7, 1539.4, 1337.9, 1265.5 and 3092.7 which was indicative of methyl C-H, ethyl C-H, carboxylic acid C=O, nitro $–NO_2$, sulphone $-SO_2-$, ether –C-O-C- and benzene ring. The ^{1}H-NMR spectrum displayed peak integral ratio (from low to high field) for 1:1:1:1:1:1:1:2:2:3:3:3:3, the total number of protons is 20, which accord with the molecular structure of Imp-D. The structure of Imp-D was closely related to this of Apremilast, except for acylamino turning to the nitro. The ^{13}C-NMR spectrum showed twenty carton signals corresponding to twenty carton atoms. The detailed information of ^{1}H-NMR, ^{13}C-NMR and DEPT can be seen in Electronic Supplementary Information Table S2 and Table S3.

3.4.5 Structural elucidation of Imp-E

This impurity has been identified as4-Amino-2-[1-(3-ethoxy-4-methoxy-phenyl)-2-methanesulfonyl-ethyl]-1H-isoindole-1,3(2H)-dione.

The negative ion ESI-MS spectrum of Imp-E exhibited a base peak of $[M-H]^-$ ion at m/z 417.1 and M+Na at m/z 441.1. The IR spectrum displayed characteristic absorptions at 3473.3, 1405.9, 2958.0, 2925.4, 1753.9, 1694.8, 1592.7 and 1351.6 which was indicative of primary amine N-H, aromatic primary amine C-N, methyl C-H, ethyl C-H, isoindole ring C=O, phenyl isoindole ring Ar-CO-, benzene ring C=C and sulphone $-SO_2-$. The ^{1}H-NMR spectrum showed peak integral ratio (from low to high field) for 1:1:4:2:1:1:3:3:3:3, the total number of protons is 22, which accord with the molecular structure of Imp-E. Imp-E loses one acetyl group compared to that of Apremilast. In ^{1}H-NMR, the losed methyl group were deshielded to δ2.19. The

^{13}C-NMR spectrum displayed twenty carton signals corresponding to twenty carton atoms. The detailed information of ^{1}H-NMR, ^{13}C-NMR and DEPT can be seen in Electronic Supplementary Information Table S2 and Table S3.

3.4.6 Structural elucidation of Imp-F

This impurity has been identified asN-{2'-[1-(3-Ethoxy-4-methoxy-phenyl)-2-methanesulfonyl-ethyl]-1,3,1',3'-tetraoxo-1,3,2',3'-tetrahydro-1'H-[2,4']biisoindolyl-4-yl}-acetamide, its structure is identified based upon the following observations:

The negative ion ESI-MS spectrum of Imp-F exhibited a base peak of $[M-H]^-$ ion at m/z 604.2 and M+Na at m/z 628.0. The IR spectrum showed characteristic absorptions at 3551.2, 3413.7, 2978.0, 2935.6, 1776.8, 1706.8, 1524.1 and 1334.9 which was indicative of secondary amine N-H, acylamino N-H, methyl C-H, ethyl C-H, isoindole ring C=O, phenyl isoindole ring Ar-CO-, benzene ring and sulphone $-SO_2-$. In ^{1}H-NMR, The imino proton (H_{21}) was deshielded to δ9.77-9.75ppm. Nine aromatic protons (H_3, H_4, H_6, H_{17}, H_{18}, H_{19}, H_{24}, H_{25}, H_{26}) were deshielded to δ8.55-6.93ppm. The tertiary carbon proton (H_{10}) was deshielded to δ5.72-5.70ppm. The four methylene protons (H_8, H_{11}) were deshielded to δ4.30-3.97ppm. The twelve methyl protons (H_1, H_9, H_{12}, H_{31}) were deshielded to δ3.73-1.27ppm. The ^{13}C-NMR spectrum displayed thirty-seven carton signals including two peaks overlap and nine peak splitting corresponding to thirty carton atoms. Twelve carbon signals disappearing in DEPT-135 spectrum were considered as fourteen quaternary carbon atoms (There were two groups of symmetric cartons: C_{13}, C_{14} and C_{21}, C_{22}). DEPT-135 spectrum revealed two negative carton (CH_2) signals which were C_8 and C_{11}. Eleven cartons signals appearing in DEPT-90 spectrum were considered as eleven tertiary cartons. The remaining four carbon signals appearing in ^{13}C-NMR were considered as four carbon atoms. The detailed information of ^{1}H-NMR, ^{13}C-NMR and DEPT can be seen in Electronic Supplementary Information Table S2 and Table S3.

Based on a variety of database retrieval information having been reported so far, we haven't found Imp-F related information, so the compound is a new compound.

3.5 Possible formation mechanism of impurities

According to the manufacturing process of Apremilast and combined with some references, an impurity formation processwasshown in Fig 4. In route 1, the Imp-A might be the result of intermediate-1 reacting with acetic anhydride which was residual in the synthesis process of intermediate-2. In this reaction, the amino of intermediate-1 combining with acetic anhydride was an acylation reaction. In route 2, the amino of intermediate-1 and the carboxyl of resolving agent reacted to form Imp-B under the catalysis of acetic anhydride. In route 3, the anhydride in intermidate-2 might be hydrolyzed to form Imp-C. In route 4, SMB may contain SMB-1 as an impurity, the SMB-1 and the amino of intermediate-1 under the catalysis of acetic anhydride might react to form Imp-D. In route 5, SMB and acetic anhydride reacted to form intermidate-2', intermidate-1 and intermidate-2'might react to form Imp-E. Then the amino of Imp-Eand the anhydride in intermidate-2 might react to form Imp-F.

4. Conclusion

In this study, we have speculated six process related impurities and illustrated their possible mechanism of formation. The six impurities in the bulk sample were firstly reported as impurities in the synthetic process of Apremilast. The Imp-F was easily broken down in the solution above 30℃ which was a new compound obtained by column chromatography and preparative liquid chromatography (Pre-HPLC). The structures of Imp-A and Imp-B were speculated based on LC-MS, UV and the synthetic process. Thestructures of the other four impurities were characterized and confirmed viaUV, IR, LC-MS and NMR. At the same time, we developed a new HPLC and validated according to ICH guidelines with specificity, precision, accuracy and sensitivity. There was no report developing an effective HPLC method to quantify all impurities in Apremilast. The newly developed HPLC can be applied to the separation and quantification the process related impurities in Apremilast samples.

Acknowledgements

We would like to thank the cooperation from employees of Nanjing Healthnice Medical Technology Co.Ltd

Highlight:

1. obtain a new compound Imp-F and elucidate the structures by spectral data (NMR, MS, and IR)
2. speculate the potential process-related impurities in the apremilast drug which was the first report impurities, according to the synthetic routes and identify and elucidate the structures of these impurities by spectral data (NMR, MS, and IR)
3. to optimize LC conditions and develop an effective HPLC method for the quantitative determination of the potential process-related of Apremilast according to ICH guidelines

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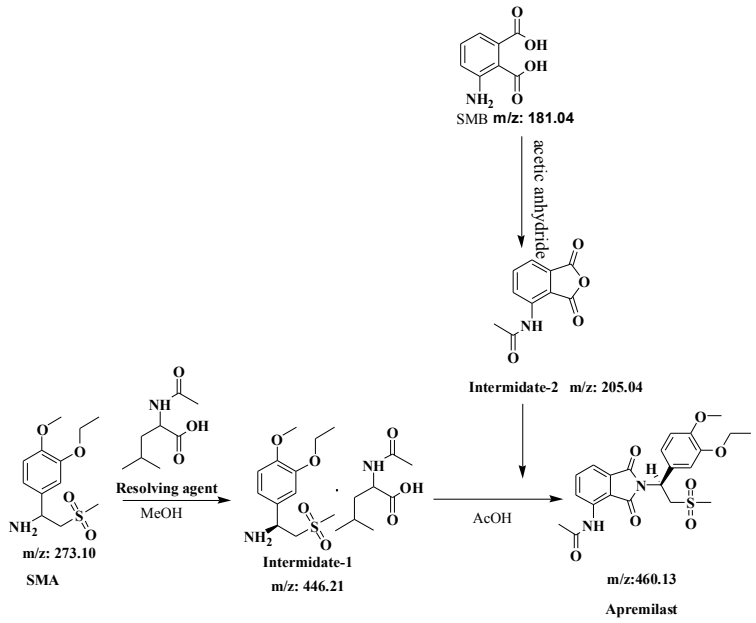
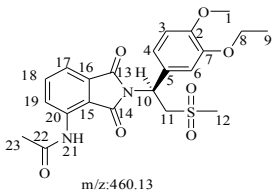
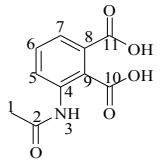


Fig 1 The synthesis route of Apremilast

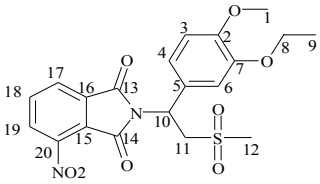


m/z:460.13

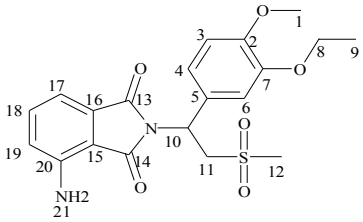
Apremilast



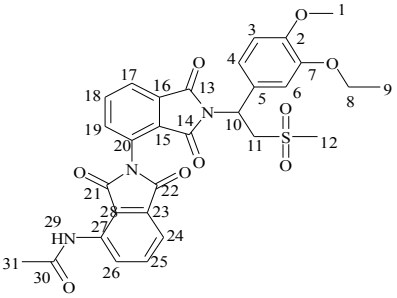
Imp-C **m/z: 223.05**



Imp-D **m/z:448.09**



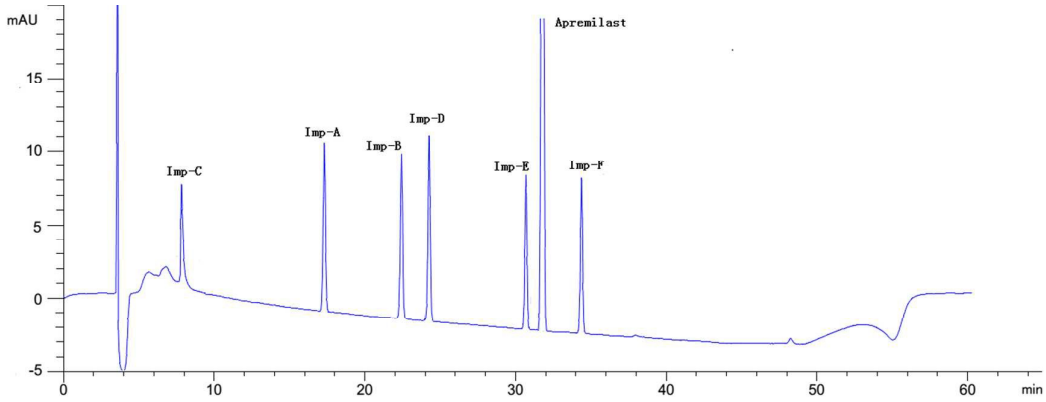
Imp-E **m/z:418.12**



Imp-F **m/z:605.15**

Fig 2 Structure of Apremilast and impurities with numbering assigned for NMR characterization

(a)



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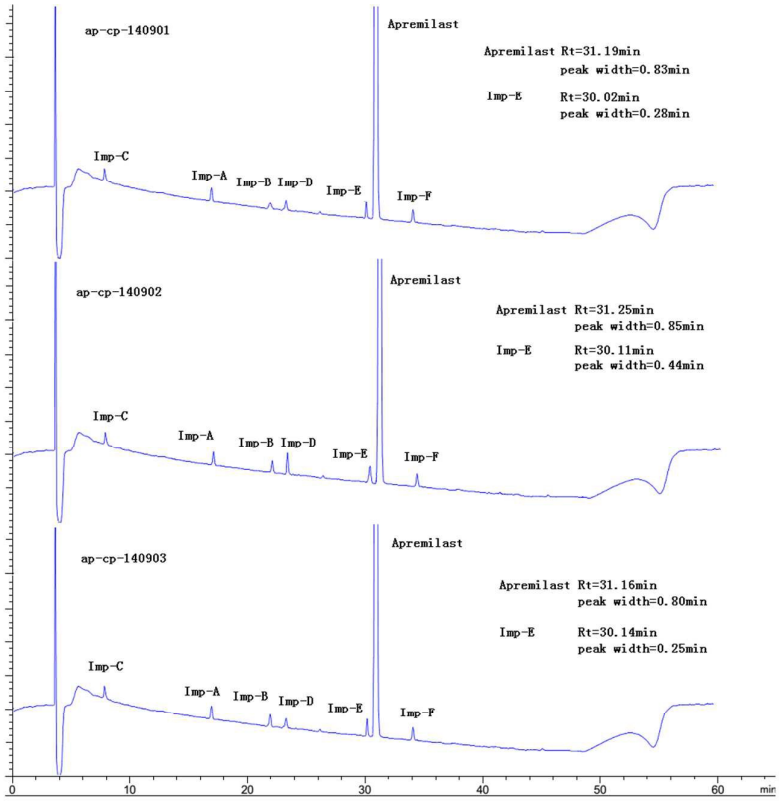

Fig 3 The HPLC chromatograms of Apremilast sipked with its impurities (a) and the HPLC analysis of three batches of bulk Apremilast (b)

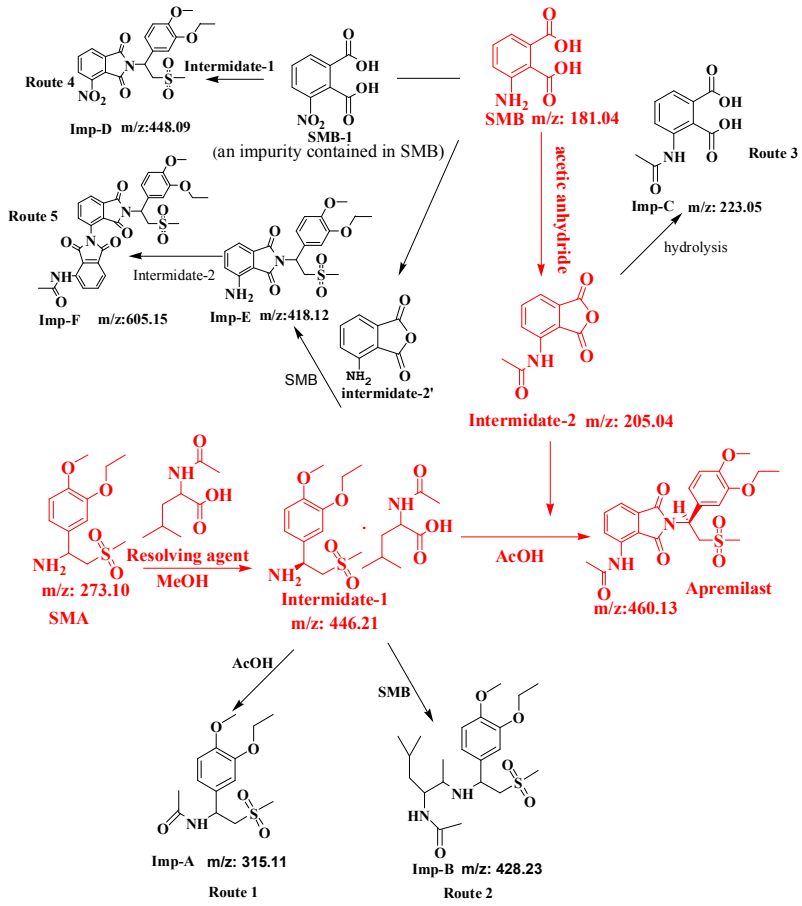

Fig 4 Five routes of producing impurities of Apremilast and their relationship

Table 1
Summary of method validation

Compound	System suitability					Linearity			Sensitivity		Precision	
	RTT	NTP	TF	Rs	Range (ug/mL)	r	Slope	Intercept	LOD(ng)	LOQ(ng)	Inter-day %RSD(n=6)	Intra-day %RSD(n=12)
Apremilast	1.00	222206	1.02	1.77	0.03-0.63	0.9993	107709	3212.1	0.426	0.798	0.90	1.52
Imp-C	0.25	18894	0.81	-	0.06-0.62	0.9985	76805	-1374.4	0.499	1.498	1.51	1.28
Imp-D	0.76	272974	1.03	7.37	0.03-0.66	0.9993	79724	601.77	0.428	0.803	0.86	1.73
Imp-E	0.98	225859	1.04	79.2	0.03-0.57	0.9991	73594	621	0.392	0.735	0.80	1.65
Imp-F	1.10	423692	1.05	4.97	0.03-0.65	0.9993	83388	299.93	0.424	0.795	1.27	1.54

Table 2
The summary of accuracy and calibration factor

Compound	Accuracy				Calibration Factor			
	80% MR	100% MR	120% MR	RSD (n=9)	Slope	Intercept	MCF	RSD (n=6)
Aremilast	-	-	-	-	-	-	-	-
Imp-C	96.11	99.02	97.7	2.06	38997	265.6	2.75	0.0125
Imp-D	97.1	97.9	99.5	1.42	78517	124.7	1.37	0.0178
Imp-E	97.6	99.6	99.5	2.44	74528	652.6	1.44	0.0149
Imp-F	98.9	100.4	101.3	1.97	81207	-311.73	1.32	0.0177