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Identification, characterization and HPLC quantification of process-related impurities in Trelagliptin succinate bulk drug: Six identified as new compounds

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ABSTRACT

A sensitive, selective and stability indicating reversed-phase LC method was developed for the determination of process related impurities of Trelagliptin succinate in bulk drug. Six impurities were identified by LC–MS. Further, their structures were characterized and confirmed utilizing LC–MS/MS, IR and NMR spectral data. The most probable mechanisms for the formation of these impurities were also discussed. To the best of our knowledge, six structures among these impurities are new compounds and have not been reported previously. The superior separation was achieved on an InertSustain C18 (250 mm × 4.6 mm, 5 μm) column in a gradient mixture of acetonitrile and 20 mmol potassium dihydrogen phosphate with 0.25% triethylamine (pH adjusted to 3.5 with phosphate acid). The method was validated as per regulatory guidelines to demonstrate system suitability, specificity, sensitivity, linearity, robustness, and stability.

1. Introduction

Trelagliptin succinate (TRE), 2-[[6-[(3*R*)-3-amino-1-piperidinyl]-3,4-dihydro-3 –methyl-2,4-dioxo-1(2H)-pyrimidinyl] methyl]-4-fluoro-benzonitrile, is a highly selective and long-acting Dipeptidyl peptidase IV (DPP-4) inhibitor that is used in the treatment of Type 2 Diabetes [1]. It controls blood glucose levels by selectively and continually inhibiting DPP-4, an enzyme that causes the inactivation of glucagon-like peptide-1, glucagon dependent insulinotropic polypeptide and incretin hormones that play an important role in blood glucose regulation. The inhibition of DPP-4 increases insulin secretion depending on blood glucose concentration, accordingly controlling blood sugar levels. In addition, TRE marketed in 2015 is the first long-acting oral hypoglycemic agents with single weekly dosing, and compared to once-daily short-acting product on the market, it is expected to improve the convenience and adherence of patients significantly [2–4].

In literature, limited information is available interested in the analysis of TRE and its process-related impurities. Bao et al. described an RPLC method for quantification of eight related substances in TRE and its preparation using gradient elution however without qualitative identification [5]. Neither formation nor structural elucidation for process-related impurities could be found in current literature survey. Thus, there is a need to elaborate the impurity profiles of TRE for industry and research reference. Simultaneously, a sensitive, selective and stability indicating analytical method has been developed to monitor the levels of impurities in TRE bulk drug.

There are several synthesis routes of TRE in the previous literature [6–9]. Some of these methods [6–8] may generate positional isomers as process-impurities, because (R) –3- aminopiperidine dihydrochloride contains a primary amine and a secondary amine which are arenucleophilic attack groups. In Ref. [9], when (*R*)-3-Boc-aminopiperidine as starting material with primary amine protected to avoid generating positional isomers impurities. So the synthetic route shown in Fig. 1 was adopted in laboratory sample preparation, and after analysis of different laboratory batches of TRE, six impurities were detected in the range of 0.04–0.13%. Impurities including unreacted starting materials, intermediates, and by-product inevitably form at the end of this process, and

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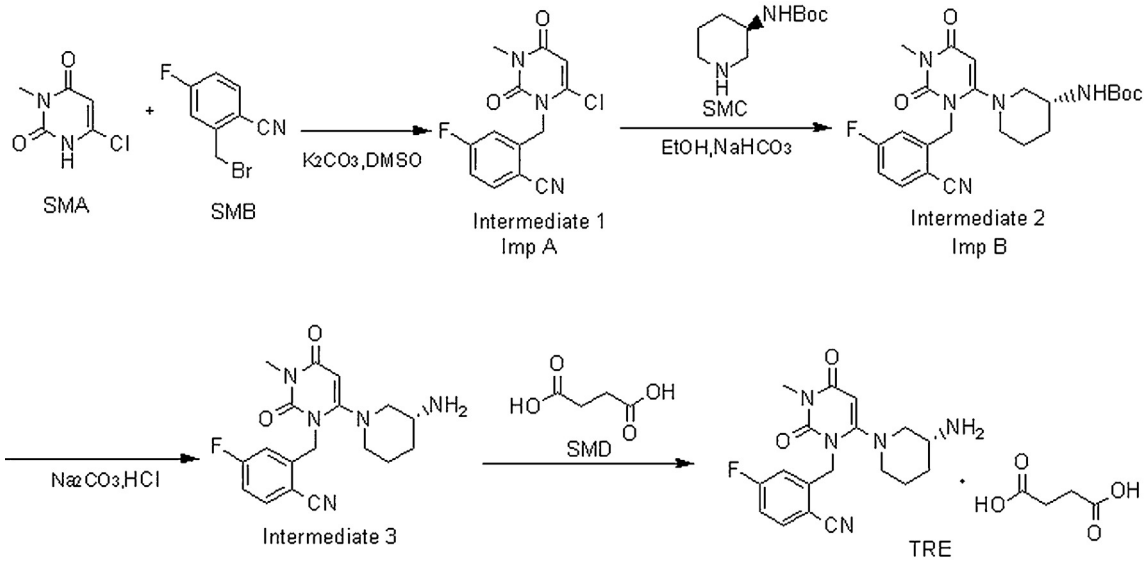


Fig. 1. The synthesis route of TRE.

the presence of impurities exceeding the accepting limit of 0.1% may have an impact on the quality and safety of the drug product [10,11]. Therefore, it is necessary for identification and characterization of the process-related impurities in TRE via NMR, MS and related techniques.

This study aims to: (1) optimize LC conditions and achieve a reliable LC method for the quantitative determination and analysis of the impurities in TRE bulk drug; (2) characterize and confirm the structures of these impurities by MS, IR and NMR; (3) obtain mechanisms for the origin and formation of these impurities with the respect to the knowledge of chemical synthesis.

2. Experimental

2.1. Chemicals and reagents

TRE and standards of 2-(6-chloro-3-methyl-2,4-dioxo-3,4-dihydro- 2H-pyrimidin-1-ylmethyl)-4-fluoro-benzonitrile (Imp-A), *N*-[(3*R*)-1-[3-[(2-cyano-5- fluorophenyl) methyl]-1,2,3,6-tetrahydro-1-methyl −2,6-oxo-4-pyrimidinyl]-3- piperidinyl] -carbamate-(1,1-dimethylethyl)ester (Imp-B), (*R*)-3-methyl-1-(3-fluorobenzyl)-6-(3-amino- piperidin-1-yl)-2, 4-dioxo-3,4-dihydro-2H-pyrimidine (Imp-C), 2-(3-methyl-2,4,6-oxo −tetrahydro-2H-pyrimidin-1-yl methyl)-4-fluoro- benzonitrile (Imp-D), (*R*)-2-[6-(3-amino-piperidin-1-yl)-3-methyl-2,4-dioxo-3,4- dihydro-2H-pyrimidin −1-yl methyl]-4-fluoro-benzoic acid (Imp-E), 2-(3-methyl-6-ethoxy-2,4-dioxo-3,4-dihydro-2H-pyrimidin-1-ylmethyl)-4-fluoro- benzonitrile (Imp-F), (*R*)-2-[6-(3-amino-piperidin-1-yl)-3-methyl-2,4-dioxo- 3,4-dihydro-2H-pyrimidin-1-yl methyl]-4-fluoro-benzamide (Imp-G), 4-((*R*)-3-amino-piperidin- 1-yl]-2-[(6-((*R*)-3- amino-piperidin-1-yl)- 3-methyl- 2,4-oxo-3,4- dihydro-pyrimidin −1(2H)-yl) methyl) benzonitrile (Imp-H) were gained from our laboratory. Starting material A (SMA; 3-methyl-6-chloro uracil), Starting material B (SMB; 2-bromomethyl-4-fluoro-benzonitrile), Starting material C (SMC; (*R*)-3-Boc-aminopiperidine), and Starting material D (SMD; Succinic acid) were purchased from Nanjing Chemlin Chemical Industry (Nanjing, China). The purity of all substances was >98% by HPLC test. Acetonitrile (HPLC grade) was purchased from Merck Ltd (Darmstadt, Germany). Water used for the preparation of mobile phase was purified using a Milli-Q pure water system (Millipore, MA, USA). Other chemicals were of analytical grade.

2.2. HPLC instrumentation and methods

All chromatographic experiments were carried out on a Waters 2695 system (Waters Technologies, MA, USA) equipped with a 2996 photo diode array detector. The system control, data acquisition and processing was accomplished by Empower data-handling system. The separation was achieved on an InertSustain C18 (250 mm × 4.6 mm, 5 μm) column (Tokyo, Japan). Mobile phase A consisting of 20 mmol potassium dihydrogen phosphate and 0.25% triethylamine (pH adjusted to 3.5 with phosphate acid) and mobile phase B of ACN were pumped at a flow rate of 1.0 mL/min. The gradient program was set as follows: Time $_{(min)}$/A: B (v/v); T_0 87/13, T_8 87/13, T_{35} 60/40, T_{60} 40/60, T_{65} 40/60, T_{66} 87/13, T_{75} 87/13. The injection volume and detection was fixed at 20 μL and 230 nm. The column temperature was maintained at 30 °C.

2.3. LC–MS instrumentation and methods

LC–MS analysis was performed on an API4000 mass spectrometer (Milwaukee, WI, USA) coupled to an Agilent 1100-LC system (Palo Alto, CA, USA) in positive or negative APCI mode. The Analysis of all compounds was carried out on an InertSustain C18 (250 mm × 4.6 mm, 5 μm) column (Tokyo, Japan) using 1 mL/min flow rate. The gradient elution employed solution A and B as mobile phase components. Mobile phase A was 20 mmol ammonium acetate buffer (pH adjusted to 3.5 with glacial acetic acid), while mobile phase B was ACN. The gradient program was the same as the HPLC chromatographic conditions described in Section 2.2. The product MS spectra were collected over the *m*/*z* range from 100 to 800 Da with the following conditions: Ion spray voltage, 4200 V; declustering potential, 70 V; entrance potential, 10 V; turbo ion spray temperature, 400 °C; collision energy, 25 V; and interface heater, on. All date were acquired and processed by Analyst Software workstation, version 1.5.2.

2.4. NMR instrumentation and methods

^{1}H, ^{13}C and DEPT NMR experiments were performed on 300 or 500 MHz NMR spectrometer using dimethyl sulfoxide (DMSO) as solvent and concentration was 50 mg/mL.

Table 1
Forced degradation results.

Stress condition	% Assay of TRE	Obeservation	Mass balance
Acid hydrolysis	97.9	Mild degradation product *i.e.* Imp-E & Imp-D formed	96.5%
Oxidation	95.2	Mild degradation product *i.e.* Imp-G formed	96.8%
thermal	96.4	3.6% unknown degradation product formed	95.8%
Base hydrolysis	89.8	Mild degradation product *i.e.* Imp-G formed, and 9.5% unknown major degradation product formed	103.1%
Photolytic	99.8	No any known and unknown degradation product formed	100.1%

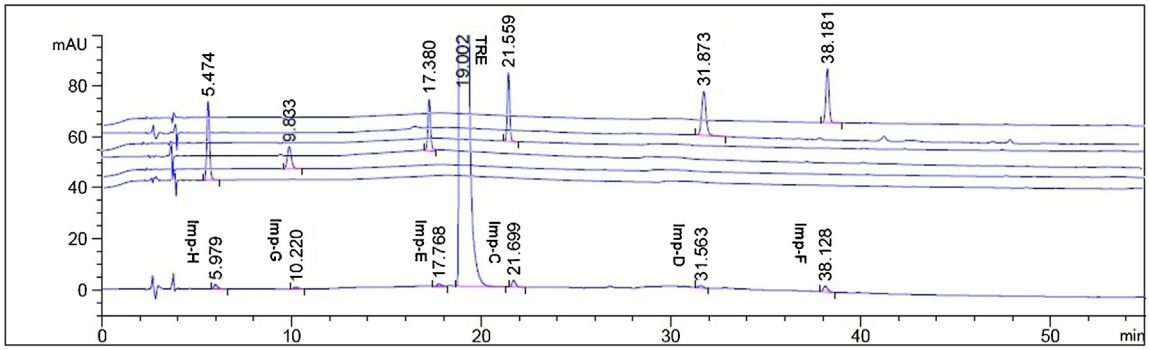


Fig. 2. The overlaid HPLC chromatogram of synthesized impurities with the impurities in TRE bulk drug.

2.5. FT-IR instrumentation and methods

The IR spectra were recorded in the solid state as a KBr powder dispersion using a Thermo Scientific Nicolet iS5 FT-IR spectrometer (Waltham, MA, USA). Data were collected between 400 and 4000 cm^{-1}, at a resolution of 4.0 cm^{-1}.

2.6. Sample preparation

Stock solution of TRE was prepared at a concentration of 0.5 mg/mL. Diluent for sample preparation is the admixture of water and acetonitrile (80:20, v/v). Spiking the 9 impurities with TRE at 0.1% of the sample concentration was used to investigate the system suitability.

The forced degradation of TRE was carried out under hydrolytic (acidic and alkaline), photo, hot and oxidative conditions. Acidic and alkaline hydrolytic degradations were carried out in 1 N HCl (5 mL) at 80 °C and 1 N NaOH (5 mL) at room temperature for 0.5 h. After the heating, the samples of acidic and alkaline hydrolysis were neutralized with NaOH and HCl, respectively. For oxidative degradation, the drug was subjected to 3% H_2O_2 at 80 °C for 0.5 h. TRE was also subjected to thermolytic (100 °C, 2 h) and photolytic (UV light, 4500 lx, 24 h) stress. Finally, all of the stressed samples were kept at a concentration of 0.5 mg/mL.

3. Results and discussion

3.1. Detection of process-related impurities and forced degradation of TRE

Analysis of TRE samples using the newly developed method described in Section 2.2 revealed the presence of six impurities (RT = 5.9, relative retention time (RRT) = 0.314; RT = 10.2, RRT = 0.537; RT = 17.7, RRT = 0.935; RT = 21.6, RRT = 1.136; RT = 31.5, RRT = 1.657; RT = 38.1, RRT = 2.005) consistently in several batches (Fig. 2). The molecular weight of these impurities were detected as 437.2, 375.4, 376.3, 332.3, 275.2 and 303.2, respectively, which correspond to Imp-H, Imp-G, Imp-E, Imp-C, Imp-D and Imp-F by analyzing by LC–MS. The typical chromatogram is shown in Fig. 3. Then on the basis of the knowledge of the route of TRE synthesis and results of MS^2 product ion analysis of impurities, we speculated that the six impurities were (*R*)-3-methyl-1-(3-fluorobenzyl)-6-(3-amino-piperidin- 1-yl)-2,4-dioxo-3,4-dihydro-2H-pyrimidine (Imp-C), 2-(3-methyl-2,4,6-oxo- tetrahydro-2H-pyrimidin-1-yl methyl)-4-fluoro-benzonitrile (Imp-D), (R)-2-[6-(3-amino-piperidin-1-yl)-3-methyl-2,4-dioxo-3,4-dihydro-2H-pyrimidin-1-yl-methyl]-4-fluoro-benzoic acid(Imp-E), 2-(3-methyl-6-ethoxy-2,4-dioxo-3,4-dihydro-2H- pyrimidin-1-ylmethyl)-4-fluoro-benzonitrile (Imp-F), (*R*)-2-[6-(3-amino-piperidin- 1-yl)-3-methyl-2,4-dioxo-3,4-dihydro-2H-pyrimidin-1-yl-methyl]-4-fluoro- benzamide (Imp-G), 4-((*R*)-3-amino-piperidin-1-yl]-2-[(6-((*R*)-3-amino-piperidin-1-yl)-3-methyl-2,4-oxo-3,4-dihydro-pyrimidin-1(2H)-yl)methyl) benzonitrile (Imp-H), respectively (Fig. 4).

TRE samples subjected to base hydrolysis and oxidation showed obvious degradation products as Imp-G. When exposed to oxidation, Imp-E and Imp-D were found. TRE was found to be stable in photolytic stress condition and labile in heat condition (Fig. 5). The mass balance results were calculated for all of the stressed samples and were found to be more than 95%. The purity and assay of TRE was unaffected by the presence of degradation products, which confirms the stability-indicating power of the developed method. The information of degradation studies is given in Table 1.

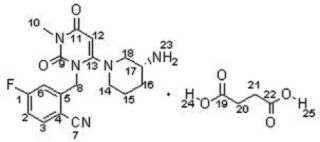
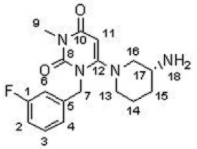
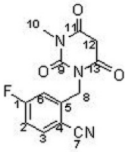
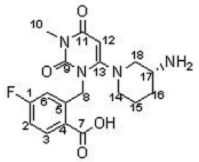
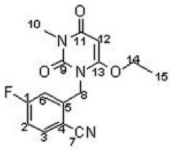
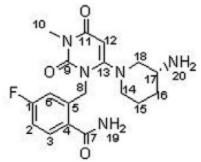
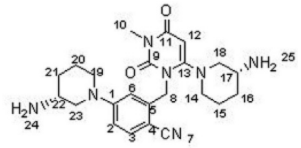
3.2. Structural elucidation of TRE and its impurities

Recently, a number of methods for structural elucidation have emerged exuberantly and been widely applied, like MS, IR, ^1H NMR, ^{13}C NMR, and DEPT. In these ways, we confirm the structures of TRE, Imp-C, Imp-D, Imp-E, Imp-F, Imp- G and Imp-H. The carbon atoms were numbered in Table 2.

3.2.1. Structural elucidation of Imp-C

The on-line LC–MS and MS^2 spectra of Imp-C in TRE suggested that it might be (*R*)-3-methyl-1-(3-fluorobenzyl)-6-(3-amino-piperidin-1-yl)-2,4-dioxo-3,4-dihydro- 2H- pyrimidine. The ^1H NMR spectrum of Imp-C showed 14 signals corresponding to 21 protons (except solvent peaks for δ: 2.50 ppm), which accords with the molecular structure of Imp-C. Imp-C misses one cyano group and succinic acid compared to that of TRE, In ^{13}C NMR, the chemical shift of cyano group and succinic acid was disappeared. The

Table 2
Determination and structure analysis of TRE and its impurities.

Compound	Structure (number assigned for NMR characterization)	% Detected values in bulk drug batches			Structure analysis				Source
		No.1	No.2	No.3	MS			NMR and IR	
					$[M+H]^+$ (m/z)	$[M-H]^-$ (m/z)	MS/MS fragmentation ions(m/z)		
TRE		99.58	99.75	99.62	358.2	–	–	^{1}H NMR, ^{13}C NMR	Target compound
Imp-C		0.08	0.04	0.09	333.9	–	333.9, 317.2, 208.1, 194.1, 109.4	^{1}H NMR, ^{13}C NMR	Process
Imp-D		0.04	0.05	0.04	276.8	274.4	276.6, 208.1, 195.7, 134.1	^{1}H NMR, ^{13}C NMR, IR	Process or degradation
Imp-E		0.07	0.06	0.04	377.9	–	378.1, 295.1, 221.3, 208.0, 153.1 100.8	^{1}H NMR	Degradation
Imp-F		0.13	0.06	0.11	304.4	–	304.5, 276.4, 219.0, 195.2, 151.5,	^{1}H NMR, ^{13}C NMR, IR	Process
Imp-G		0.04	0.04	0.05	376.8	–	376.8, 360.6, 293.3, 152.0, 101.4	^{1}H NMR	Degradation
Imp-H		0.04	–	0.05	438.6	–	438.5, 421.5, 381.8, 225.3, 214.2, 208.3, 197.3, 181.9	^{1}H NMR, ^{13}C NMR	Process

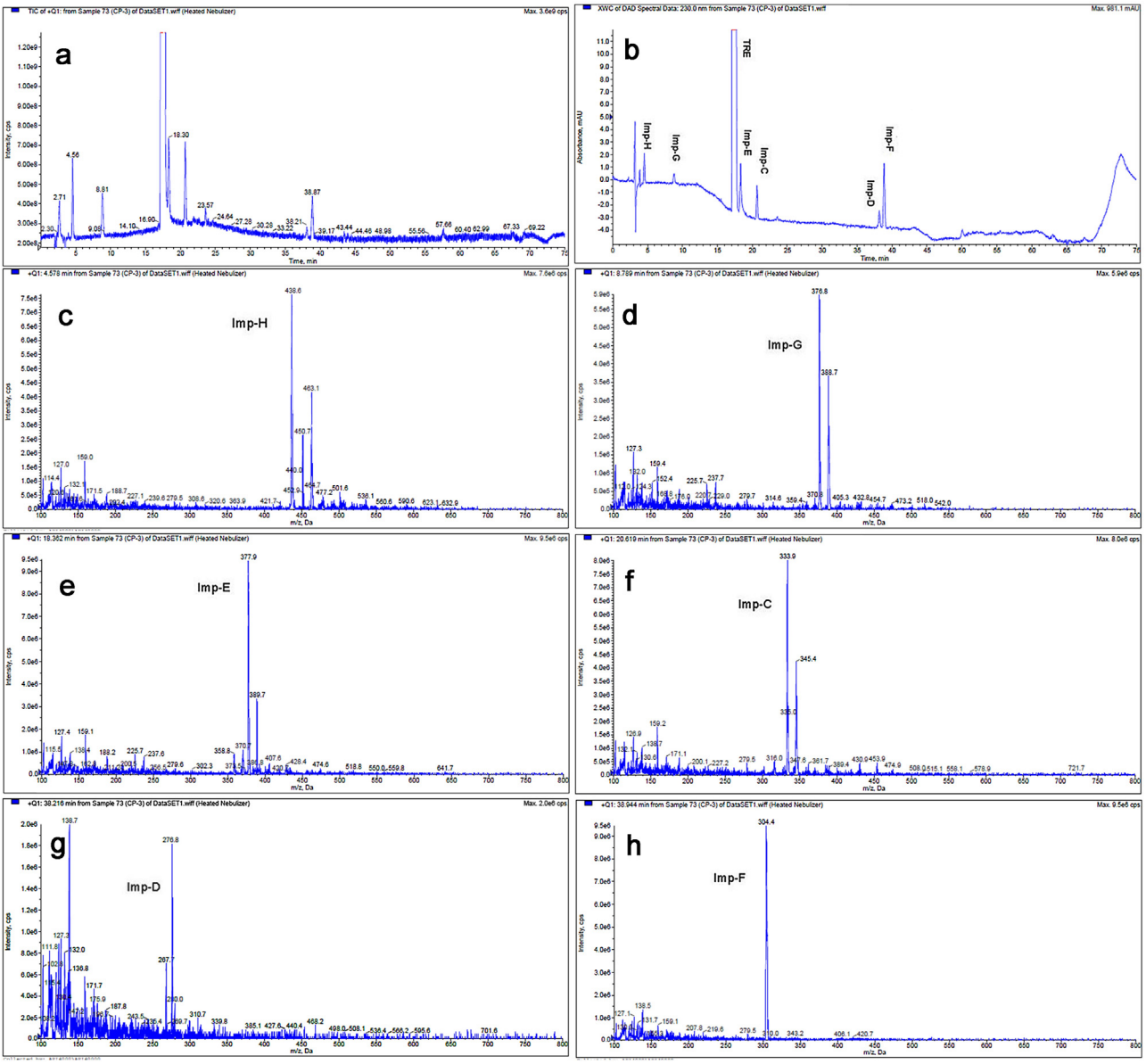


Fig. 3. The LC–MS chromatogram of TRE with bulk drug.
(a) Total ion chromatography, (b) the LC chromatogram of TRE with bulk drug, (c) the MS spectrum of Imp-H; (d) the MS spectrum of Imp-G; (e) the MS spectrum of Imp-E; (f) the MS spectrum of Imp-C; (g) the MS spectrum of Imp-D; (h) the MS spectrum of Imp-F.

detailed information of ^{1}H NMR and ^{13}C NMR spectra can be seen in Tables 3 and 4.

3.2.2. Structural elucidation of Imp-D

The on-line LC–MS and MS^{2} spectra of Imp-D suggested that it might be 2-(3- methyl-2,4,6-oxo-tetrahydro-2H-pyrimidin-1-yl-methyl)-4-fluoro-benzonitrile. The IR spectrum displays characteristic absorptions at 2983.9, 2234.0, 1703.7, 1609.8 and 1187.7 cm^{-1}, corresponding to the methyl C—H stretching mode, aromatic cyano CN stretching mode, polyimide C=O stretching mode, benzene ring, and C—F stretching mode, respectively. To further confirm the structure, ^{1}H NMR and ^{13}C NMR spectra were provided. The ^{1}H NMR spectrum of Imp-D shows 6 signals corresponding to 10 protons, which accords with the proposed molecular structure. The data ^{1}H NMR are as follows: ^{1}H NMR (500 MHz, DMSO), δ: 7.99–7.94 (m, 1H), 7.47–7.44 (m, 1H), 7.38–7.32(m, 1H), 5.08 (s, 2H), 3.84 (s, 2H), 3.14 (s, 3H). The ^{13}C NMR spectrum of Imp-D shows 13 signals corresponding to 13 protons, which further supports the proposed molecular structure. The detailed information of ^{1}H NMR and ^{13}C NMR spectra can be seen in Tables 3 and 4.

3.2.3. Structural elucidation of Imp-E

The on-line LC–MS and MS^{2} spectra of Imp-E suggested that it might be (R) –2- [6-(3-amino-piperidin-1-yl)-3-methyl-2,4-dioxo-3,4-dihydro-2H-pyrimidin-1-yl methyl]-4-fluoro-benzoic acid, which was a white powder, and the HPLC purity was found to be 99.72%. The ^{1}H NMR spectrum of Imp-E showed ten signals corresponding to nineteen protons (two active hydrogens were not detected in amino-group), which accords with the molecular structure of Imp-E. Imp-E has one additional carboxylic acid group but is missing one cyano group and succinic acid compared to that of TRE. In ^{1}H NMR, the chemical shift of the additional hydrogen was deshielded to δ8.03-7.99 ppm. The detailed information of ^{1}H NMR spectra can be seen in Table 3.

3.2.4. Structural elucidation of Imp-F

The on-line LC–MS and MS^2 spectra of Imp-F suggested that it might be 2-(3- methyl-6-ethoxy-2,4-dioxo-3,4-dihydro-2H-pyrimidin-1-yl-methyl)-4-fluoro- benzonitrile. The IR spectrum displays characteristic absorptions at 2986.7, 2227.0, 1709.0, 1609.4, 1238.5 and 1185.1 cm^{-1}, corresponding to the methyl C—H stretching mode, aromatic cyano CN stretching mode, polyimide C=O stretching mode, benzene ring, ether =C—O—C stretching mode, and C—F stretching mode, respectively. To further confirm the structure, ^1H NMR and ^{13}C NMR spectra were provided.

Imp-F has one additional ethoxy group but is missing one amidogen group and succinic acid compared to that of TRE. In ^1H NMR, the additional ethoxy group was dehielded to δ 4.12–4.05 and 1.21–1.16, and the chemical shift of amidogen group and succinic acid disappeared. The ^{13}C NMR spectrum of Imp-F shows 21 signals (six carbon splitting) corresponding to 15 protons, which further supports the proposed molecular structure. The detailed information of ^1H NMR and ^{13}C NMR spectra can be seen in Tables 3 and 4.

3.2.5. Structural elucidation of Imp-G

The on-line LC–MS and MS^2 spectra of Imp-G suggested that it might be (*R*)-2-[6-(3-amino-piperidin-1-yl)-3-methyl-2,4-dioxo-3,4-dihydro-2H-pyrimidin-1-yl methyl]-4-fluoro-benzamide, and the HPLC purity was found to be 98.89%. The ^1H NMR spectrum of Imp-G showed 13 signals corresponding to 22 protons, which accords with the molecular structure of Imp-G. Imp-G has one additional amide group but is missing one cyano group and succinic acid compared to that of TRE. In ^1H NMR, the chemical shift of the additional hydrogen was deshielded to δ7.33 and 6.12 ppm. The detailed information of ^1H NMR spectra can be seen in Table 3.

3.2.6. Structural elucidation of Imp-H

The on-line LC–MS and MS^2 spectra of Imp-H suggested that it might be 4-((R)- 3-amino-piperidin-1-yl)-2-((6-((*R*)-3-amino-piperidin-1-yl)-3-methyl-2,4-oxo-3,4- dihydro-pyrimidin-1(2H)-yl) methyl) benzonitrile.

Imp-H has one additional 3-amino-piperidine group but is missing one fluorine group and succinic acid compared to that of TRE. The ^1H NMR spectrum of Imp-H shows 13 signals (except solvent peaks for δ2.50 ppm) corresponding to 31 protons. The ^{13}C NMR spectrum of Imp-H shows 23 signals corresponding to 23 carbons. Seven carbon signals which disappeared in DEPT-135 were quaternary carbon atoms. There are nine negative carbon signals, indicating that they are secondary carbon atoms. Six carbons signals that appeared in DEPT-90 spectrum were considered as six tertiary carbon atoms. The remaining a positive carbon signal in DEPT-135 was a primary carbon. which further supports the proposed molecular structure. The detailed information of ^1H NMR and ^{13}C NMR spectra can be seen in Tables 3 and 4.

3.3. Possible mechanisms for the formation of impurities

According to the synthesis of TRE, it was surmised that five formation routes for impurities would arise (Fig. 6). In route 1, SMB may contain 3-fluorobenzyl bromide (SMB-1) as an impurity in its synthesis process, and affords Imp-C following the same reaction that yields TRE. In route 2, intermediate 1 and ethanol occurred bimolecular nucleophilic-substitution reaction and formed Imp-F in alkaline environment. In route 3, SMA may contain 1-Methyl barbituric acid (SMA-1) as an impurity, and affords Imp-D following the same reaction that gives TRE in the first step. Besides, Imp-D was speculated as an acid degradation product of TRE, which was confirmed in degradation test. In this two ways, Imp-D was formed. In route 4, intermediate 3 and SMC reacted and then remove the Boc group to form Imp-H. In route 5, the cyano group of intermediate 3 may be hydrolyzed to form Imp-E and Imp-G.

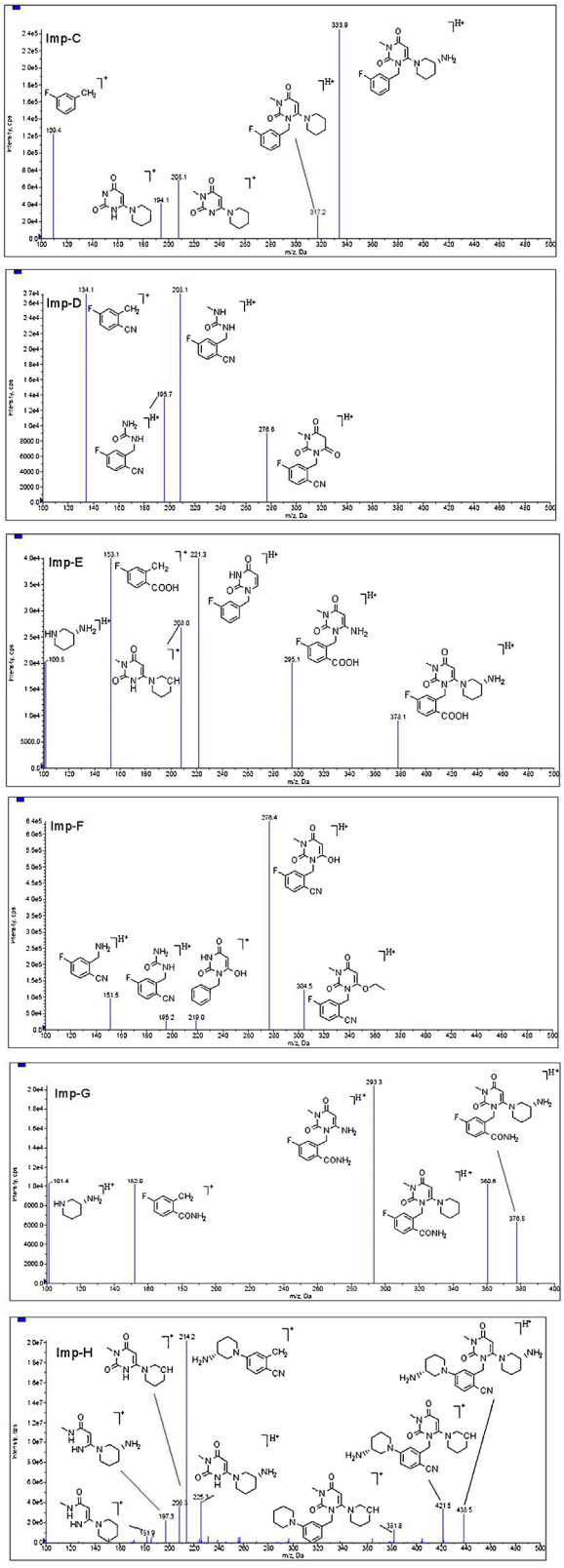


Fig. 4. MS^2 spectra and plausible fragmentation pathways for the impurities.

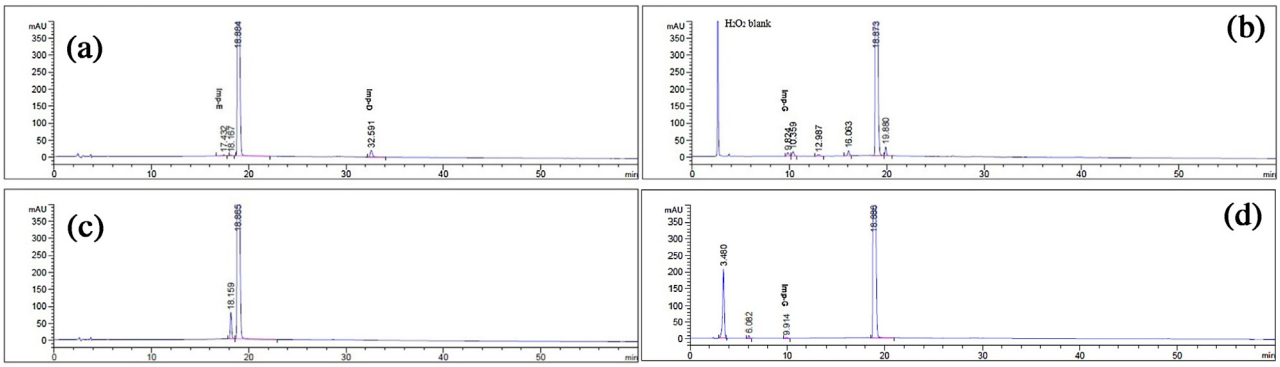


Fig. 5. The chromatogram of TRE under stress conditions (a) acid hydrolysis, (b) oxidative degradation, (c) thermal degradation and (d) base hydrolysis.

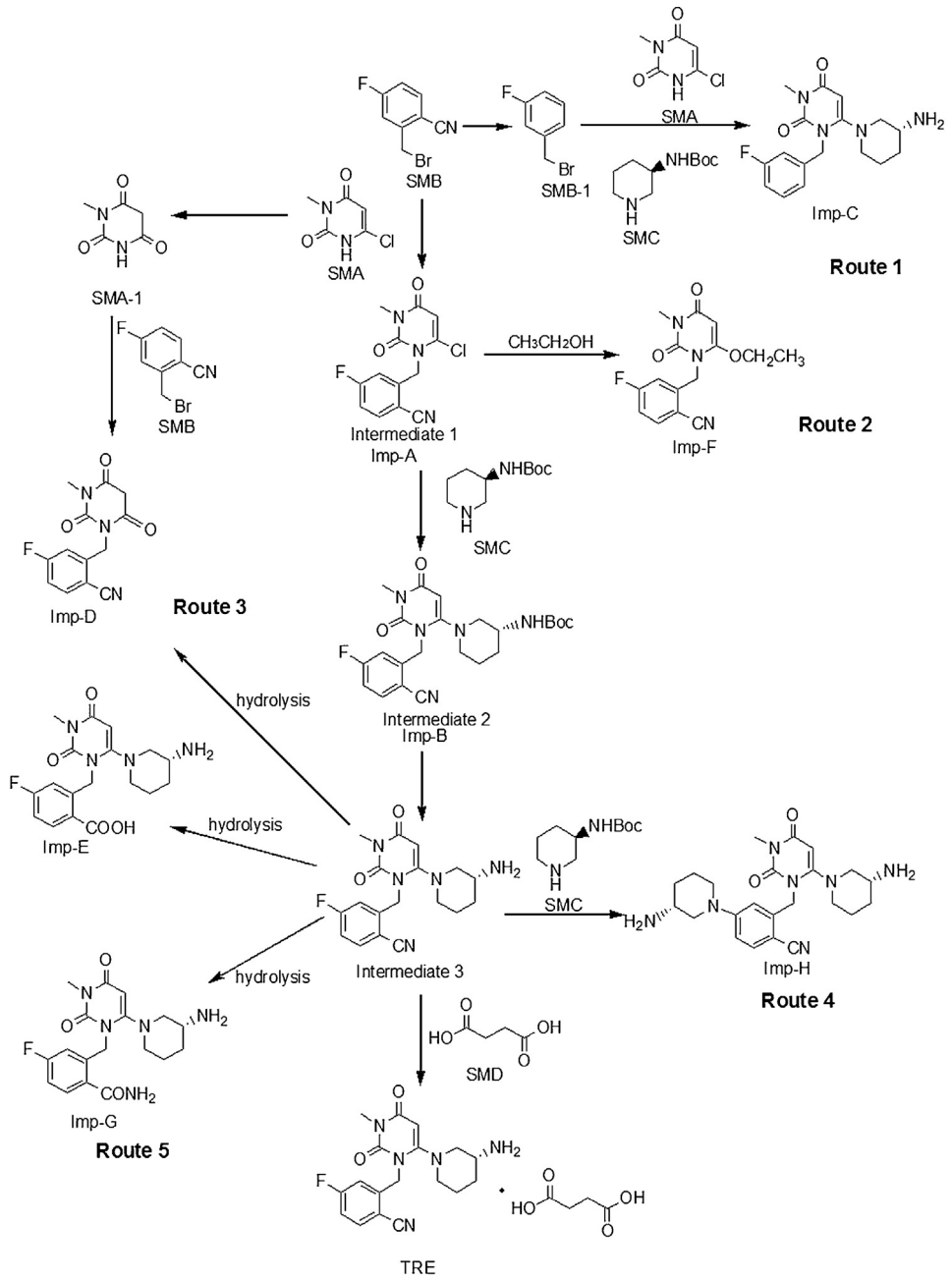


Fig. 6. Five routes of producing impurities of TRE and their relationship.

Table 3
^{1}H NMR assignment for TRE and its impurities.

position	TRE	Imp-C	Imp-D	Imp-E	Imp-F	Imp-G	Imp-H
2	7.36–7.32 (m[a],1H)	7.40–7.32 (m,1H)	7.47–7.44 (m,1H)	7.22 (m,1H)	7.39–7.36 (m,1H)	7.08–7.04 (m,1H)	6.62 (s,1H)
3	7.96–7.93 (m,1H)	7.09–7.01 (m,1H)	7.99–7.94 (m,1H)	8.03–7.99 (m,1H)	7.99–7.95 (m,1H)	7.65–7.62 (m,1H)	7.51–7.54 (d[b],1H)
4	–	7.09–7.01 (m,1H)	–	–	–	–	–
6	7.18–7.16 (m,1H)	7.09–7.01 (m,1H)	7.38–7.32 (m,1H)	6.89–6.87 (m,1H)	7.33–7.25 (m,1H)	6.67–6.64 (m,1H)	6.90–6.93 (d,1H)
7	–	5.26–4.96 (m,2H)	–	8.03–7.99 (m,1H)	–	–	–
8	5.15–5.12 (dd[c],2H)	–	5.08 (s,2H)	5.41–5.29 (m.2H)	5.18 (s,2H)	5.39–5.28 (q[d],2H)	5.07 (s,2H)
9	–	3.13 (s,3H)	–	–	–	–	–
10	3.09 (s[e],3H)	–	3.14 (s,3H)	3.22–3.14 (m,3H)	3.16 (s,3H)	3.04 (s,3H)	3.09 (s,3H)
11	–	5.74 (s,1H)	–	–	–	–	–
12	5.39 (s,1H)	–	3.84 (s,2H)	5.41–5.29 (m.1H)	5.28 (s,1H)	5.45 (s,1H)	5.32 (s,1H)
13	–	2.77–2.70 (m,1H)	–	–	–	–	–
13′	–	2.59–2.56 (m,1H)	–	–	–	–	–
14	2.90 (s,1H)	1.50–1.43 (m,1H)	–	3.05–2.90 (m,1H)	4.12–4.05 (m,1H)	1.87 (s,1H)	2.86–2.94 (m,1H)
14′	2.71 (s,1H)	1.17 (s,1H)	–	3.05–2.90 (m,1H)	4.12–4.05 (m,1H)	1.67 (s,1H)	2.59–2.62 (m,1H)
15	1.78 (s,1H)	1.80–1.76 (m,1H)	–	1.67 (m,1H)	1.21–1.16 (m,1H)	1.67 (s,1H)	1.81 (m,1H)
15′	1.52–1.50 (m,1H)	1.67–1.63 (m,1H)	–	1.37 (m,1H)	1.21–1.16 (m,2H)	1.67 (s,1H)	1.81 (m,1H)
16	1.91 (s,1H)	3.04–3.00 (m,1H)	–	1.91 (m,1H)	–	1.67 (s,1H)	1.81 (m,1H)
16′	1.52–1.50 (m,1H)	2.93–2.89 (m,1H)	–	1.37 (m,1H)	–	1.33–1.31 (m,1H)	1.81 (m,1H)
17	3.14 (s,1H)	2.36–2.30 (m,1H)	–	3.22–3.14 (m,1H)	–	3.19–3.16 (d,1H)	2.86–2.94 (m,1H)
18	3.22–3.20 (s,1H)	2.13 (s,1H)	–	3.05–2.90 (m,1H)	–	2.75 (m,1H)	3.20–3.23 (m,1H)
18′	2.71 (s,1H)	2.13 (s,1H)	–	2.68 (m,1H)	–	2.0–1.98 (d,1H)	2.59–2.62 (m,1H)
19	–	–	–	–	–	7.33 (s,1H)	3.78–3.81 (m,1H)
19′	–	–	–	–	–	6.12 (s,1H)	2.86–2.94 (m,1H)
20	2.30 (s,2H)	–	–	–	–	3.04 (s,2H)	1.33–1.44 (m,2H)
21	2.30 (s,2H)	–	–	–	–	–	1.33–1.44 (m,2H)
22	–	–	–	–	–	–	3.58–3.62 (m,1H)
23	9.47 (s.1H)	–	–	–	–	–	1.81 (m,1H)
23′	9.47 (s.1H)	–	–	–	–	–	1.81 (m,1H)
24	9.47 (s.1H)	–	–	–	–	–	1.81 (m,2H)
25	9.47 (s.1H)	–	–	–	–	–	1.81 (m,2H)

[a] Multiple.
[b] Double.
[c] Doublet of doublets.
[d] Quartet.
[e] Single.

Table 4
^{13}C NMR assignment for TRE and its impurities.

position	TRE		Imp-C		Imp-D		Imp-F		Imp-H	
	δ_C	DEPT	δ_C	DEPT	δ_C	DEPT	δ_C	DEPT	δ_C	DEPT
1	165.59, 163.57	–	162.05, 163.69	–	166.52, 163.16	–	166.3, 163.00	–	159.52	–
2	115.56, 115.38	CH	122.34, 122.38	CH	114.86–114.53	CH	115.03–114.72	CH	113.31	CH
3	135.97, 135.89	CH	130.28, 130.39	CH	135.76–135.63	CH	135.76–135.63	CH	142.51	CH
4	106.44	–	113.22, 113.93	CH	106.48–106.44	–	106.61–106.57	–	111.87	–
5	145.27, 145.20	–	140.39, 140.49	–	144.19–144.06	–	144.57–144.46	–	152.03	–
6	115.01, 114.83	CH	113.51, 113.66	CH	115.58–115.28	CH	115.95–115.65	CH	118.39	CH
7	116.62	–	46.81, 46.83	CH_2	116.47	–	116.45	–	134.34	–
8	45.73	CH_2	152.22	–	40.20	CH_2	43.29	CH_2	51.03	CH_2
9	151.77	–	27.28	CH_3	152.02	–	151.02	–	152.83	–
10	27.30	CH_3	160.46	–	27.88	CH_3	27.52	CH_3	29.87	CH_3
11	162.17	–	88.75	CH	166.04	–	162.33	–	173.51	–
12	89.87	CH	159.71	–	42.49	CH_2	78.29	CH	96.73	CH
13	159.20	–	50.91	CH_3	165.89	–	159.47	–	162.18	–
14	51.25	CH_2	22.87	CH_2	–	–	66.52	CH_2	51.91	CH_2
15	21.86	CH_2	32.66	CH_2	–	–	13.55	CH_3	22.60	CH_2
16	28.60	CH_2	58.97	CH_2	–	–	–	–	30.03	CH_2
17	46.22	CH	47.09	CH	–	–	–	–	46.89	CH
18	54.37	CH_2	–	–	–	–	–	–	63.04	CH_2
19	175.22	–	–	–	–	–	–	–	55.53	CH_2
20	31.40	CH_2	–	–	–	–	–	–	22.39	CH_2
21	31.40	CH_2	–	–	–	–	–	–	46.11	CH_2
22	175.22	–	–	–	–	–	–	–	46.38	CH
23	–	–	–	–	–	–	–	–	89.27	CH_2

3.4. HPLC method development and optimization

As starting materials and intermediates with residual potential, SMA, Imp-A and Imp-B are in need to be involved in the HPLC method for quantification. As mentioned above, Bao et al. [6] developed an RPLC method to determine TRE and its related substances however without qualitative identification of the impurities. In other words, the impurities in report are different from the impurities in our study possibly. This meant separating result was unpredictable when it's directly applied for analyzing the impurities discussed in our study. After primary trial with reported HPLC method, it showed that the impurities could not be separated

Table 5
Summary of method validation.

Compound	System suitability				Linearity				Sensitivity		Precision	
	RRT[a]	PC[b]	R[c]	SF[d]	Range(μg/mL)	r[e]	Slope	Intercept	LOD[f](μg/mL)	LOQ[g](μg/mL)	Inter-day%RSD(n = 6)	Intra-day%RSD(n = 12)
TRE	1.00	45,256	3.69	0.92	0.15–10.78	0.997	56.10	−8.87	0.07	0.15	1.0	0.8
Imp-A	2.11	223,814	4.84	0.93	0.15–1.07	0.992	52.03	−0.35	0.06	0.15	1.4	1.6
Imp-B	2.60	293,456	26.71	0.98	0.18–1.04	0.995	46.64	1.23	0.07	0.19	1.2	1.4
Imp-C	1.14	137,951	9.16	0.90	0.20–1.06	0.996	63.51	−1.77	0.07	0.20	0.8	1.2
Imp-D	1.73	119,416	36.21	0.99	0.11–1.05	0.998	54.43	−6.17	0.04	0.10	1.0	1.2
Imp-E	0.94	67,946	22.96	0.98	0.11–0.98	0.993	47.39	−0.73	0.05	0.10	0.9	1.3
Imp-F	2.02	249,503	16.27	0.92	0.17–1.14	0.996	57.90	−1.47	0.06	0.17	1.4	0.7
Imp-G	0.52	9058	11.34	0.90	0.23–1.00	0.994	33.92	0.34	0.10	0.23	1.2	1.3
Imp-H	0.29	7284	–	0.86	0.11–1.20	0.996	58.14	−1.68	0.05	0.12	1.1	1.4
SMA	0.33	14,641	3.25	0.85	0.09–0.93	0.992	52.03	−0.35	0.03	0.08	1.4	1.8

[a] Relative retention time.
[b] (USP) plate count.
[c] (USP) resolution.
[d] Symmetry factor.
[e] Correlation factor.
[f] (S/N = 3).
[g] (S/N = 10).

Table 6
The summary of accuracy and calibration factor.

Compound	Accuracy				Calibration Response Factor				
	50% MR[a]	100% MR	150% MR	RSD% (n = 9)	Slope	Intercept	MCF[b]	MRRF[c]	%RSD (n = 6)
TRE	–	–	–	–	56.62	−6.31	1.00	1.00	1.0
Imp-A	86.8	87.5	87.4	0.7	73.72	−2.48	1.31	0.76	1.4
Imp-B	84.0	84.5	92.4	5.3	45.44	1.40	0.81	1.23	0.8
Imp-C	92.1	86.3	84.3	4.6	65.77	−8.31	1.05	0.95	1.6
Imp-D	91.3	88.8	89.7	3.0	56.25	−10.13	0.92	1.08	0.7
Imp-E	88.7	86.7	85.6	2.6	47.92	−1.09	0.84	1.19	1.1
Imp-F	91.4	86.2	85.6	4.7	58.65	−1.92	1.10	0.90	3.6
Imp-G	96.7	91.9	89.2	3.8	33.93	0.92	0.63	1.58	1.5
Imp-H	93.4	89.8	90.9	2.1	59.37	−1.46	0.97	1.03	3.6
SMA	86.9	86.1	85.1	1.4	53.87	−0.56	0.93	1.07	1.3

[a] Mean recovery.
[b] Mean calibration factor.
[c] Mean relative response factor.

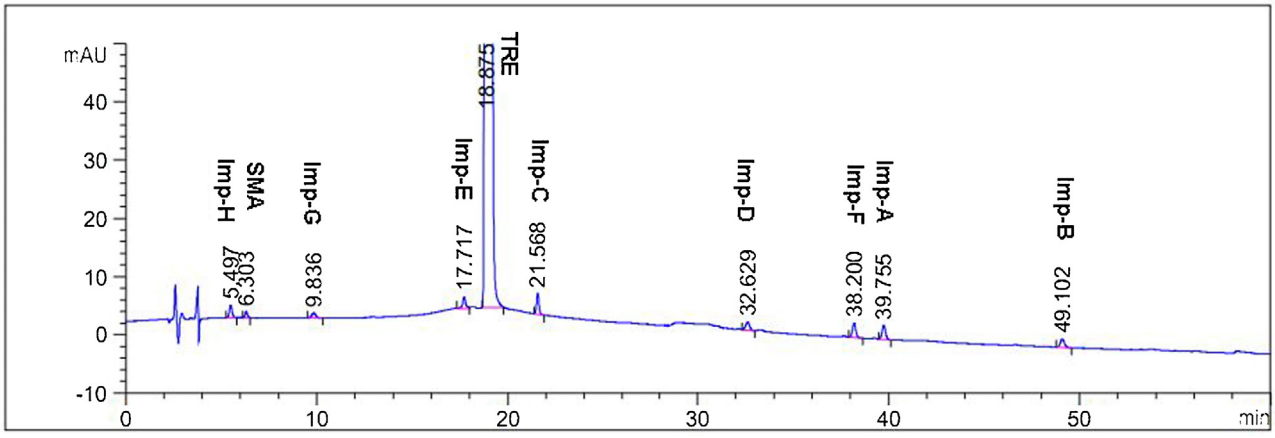


Fig. 7. The HPLC chromatogram of TRE spiked with its impurities.

well in this system, especially when focusing on the resolutions (R) between SMA and Imp-H (R = 1.41) as well as Imp-E and TRE (R = 1.05). For this reason, method optimization was performed as follows:

Efforts were taken to optimize the reported HPLC method from aspects of gradient profile, concentration and pH of potassium dihydrogen phosphate buffer in the mobile phase. Mobile phase A consisting of 20 mmol potassium dihydrogen phosphate with 0.25% triethylamine (pH adjusted with phosphate acid) and mobile phase B of ACN were chosen preliminary. First, to modify the performance, pH was adjusted to 3.0–6.0 with phosphate acid in reportorial gradient mode. The results showed that all process-related impurities and TRE had the preferable chromatographic behavior on pH 3.5. Second, different gradient elution programs were designed to optimize the HPLC condition with fixed pH 3.5. With one of the programs, sufficient resolution between SMA and Imp A (from R = 1.41 with original condition to R = 3.25 after optimization) as well as Imp-E and TRE(R = 1.05 to 3.69) was observed. Finally, the optimum separation was performed on an InertSustain C18 (250 mm × 4.6 mm, 5 μm) column in a gradient mixture of

acetonitrile and 20 mmol potassium dihydrogen phosphate with 0.25% triethylamine (pH adjusted to 3.5 with phosphate acid) as described in Section 2.2.

3.5. Method validation

The HPLC method was validated according to the ICH Q2 (R1) guidelines[12], and results of the validation study are given below.

3.5.1. System suitability

System suitability studies were performed to detect the minimum number of theoretical plates, resolution, tailing factor. The system suitability was conducted using a 0.5 mg/mL TRE solution containing 0.5 μg/mL of 9 impurities by making six replicate injections. An efficient resolution (>3.25), high number of theoretical plates (>5000/m), and good tailing factor was obtained as shown in Table 5. The HPLC chromatograms of the 9 impurities spiked with TRE is shown in Fig. 7.

3.5.2. Sensitivity and linearity

The LOD and LOQ for TRE and its 9 impurities were estimated at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of solutions diluted to known concentrations (Table 5).

A set of all 9 impurities and TRE solutions at the seven concentration levels ranging from the LOQ to 200% of the specification limit (0.1% impurity level in a 0.5 mg/mL TRE sample solution) were prepared. The calibration equations were calculated using least squares linear regression, and the correlation coefficient of regression (r) was found to be >0.99, indicating excellent linearity for the method.

3.5.3. Precision and accuracy

The precision of the method, including repeatability (interday precision) and intermediate precision (intraday precision), was tested with six different system suitability solutions with different analysts on different days using different instruments. The RSD of TRE and its impurities was within 2.0. The low RSD values of the peak areas for the principal peak and its impurities demonstrate the good precision of the method (Table 5).

The accuracy was determined by using the recovery and RSD values obtained from test TRE solutions (0.5 mg/mL) spiked with impurity levels of 0.05%, 0.10%, and 0.15% in triplicate. The percentage recovery was calculated at each level and found to be in the range of 84.0%–96.7% (Table 6).

3.5.4. Robustness and solution stability

To assess the robustness of the method, careful evaluation of deliberately changed conditions: column temperature (30 ± 5 °C), flow rate (1.0 ± 0.1 mL/min), the pH value of the buffer solution (3.5 ± 0.5), and trying different columns revealed that the resolution between any two analytes is >1.5. The stability of the TRE sample solution and the mixed impurities solution was monitored at room temperature for 12 h, and the RSD values of the peak areas of the analytes were found to be within 2.05.

3.5.5. Correction factors

The correction factor experiments were carried out on two different chromatographic instruments with three chromatographic columns injecting six concentration levels solution. The correction factor was calculated by ratio of the slope of principal components and the impurities with the linear regression equation. The results indicated that the correction factor of all impurities were within 1.31, which provided the reference to quantitative analysis of the impurities in TRE.

4. Conclusions

Six process-related impurities in TRE bulk drug obtained from our laboratory were identified and separated by an optimized HPLC method. Their structures were proposed by the synthesis route of TRE and MS^2 product ions analyses. The structures of all impurities of TRE were demonstratively confirmed and characterized using ^1H NMR, ^{13}C NMR, IR, and DEPT, and probable mechanisms for their formation were also discussed. To the best of our knowledge, six of the impurities are new compounds and the structures have not been reported previously. The LC method was validated as per ICH guidelines, and was found to be simple, sensitive, selective, cost effective and stability indicating. Thus, the method can be used in the separation and quantification of impurities in TRE bulk drugs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jpba.2016.04.041.

References

[1] K. McKeage, Trelagliptin: first global approval, Drugs 75 (2015) 1161–1164.
[2] K. Kaku, First novel once-weekly DPP-4 inhibitor, trelagliptin, for the treatment of type 2 diabetes mellitus, Expert Opin. Pharmacol. 16 (2015) 1–9.
[3] N. Inagaki, H. Onouchi, H. Maezawa, S. Kuroda, K. Kaku, Once-weekly trelagliptin versus daily alogliptin in Japanese patients with type 2 diabetes: a randomised, double-blind, phase 3 non-inferiority study, Lancet Diabetes Endocrinol. 3 (2015) 191–197.
[4] T. Klein, M. Mark, H. Niessen, L. Thomas, DPP-IV inbibitors foruse in the treatment of nafld, WO Patent 2009147125 A1 (2009).
[5] M.L. Bao, M. You, X.F. Zhao, A method for the quantification of related substances in Trelagliptin succinate and its preparation, CN Patent 104237421 A (2014).
[6] J. Feng, S.L. Gwaltney, J.A. Stafford, Z.Y. Zhang, Dipeptidyl peptidase inhibitors, WO Patent 2005095381 A1 (2005).
[7] R.C. Kelly, L.H. Koztecki, Polymorphs of succinate salt of 2-[6-(3-amino-piperldin-1-yl)3-methyl-2,4-dioxo-3,4-dihydro-2H-pyrimidin-1-ylmethy-4-fluor-benzonitril and methods of use therefor, US Patent 20080280931 A1 (2008).
[8] H. Fukuyama, K. Harada, M. Koike, Laminated tablet and manufacturing method therefor, US Patent 20140023708 A1 (2014).
[9] J.P. Yu, W. Chen, X.Y. Zhu, X.Q. Zhang, X.H. Chang, X.K. Liu, Optimized synthetic process of trelagliptin succinate, Chin. New Drug J. 24 (2015) 1061–1064.
[10] International Conference on Harmonization ICH Guidelines Q3A (R2), Impurities in New Drug Substances: Step 4 Version (2006).
[11] S. Husain, R.N. Rao, Monitoring of process impurities in drugs, J. Chromatogr. Libr. 60 (1998) 833–888.
[12] International Conference on Harmonization ICH Guidelines Q2A (R1), Validation of Analytical Procedures: Step 4 Version (2005).