Identification, Characterization and HPLC Quantification of Process-Related Impurities in Bepotastine Besilate Bulk Drug

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ABSTRACT

Bepotastine besilate (here after referred to as BTST), chemically known as ({d(S)4[4[(4chlorophenyl) (2pyridyl) methoxy] piperidino} butyric acid monobenzene sulphonate), is a second-generation antihistamine drug. To the best of our knowledge, no studies concerning the isolation or identification of process-related impurities have been reported so far. The current study reports the development and validation of a stability-indicating RP-HPLC method for the separation and identification of 5 potential impurities in bepotastine besilate. In this experiment, the structures of 3 process-related impurities were found to be new compounds. They were characterized and confirmed by NMR and MS spectroscopy analyses. These 3 new compounds were proposed to be (S)-4-[(phenyl)-2-pyridinylmethoxy]-1-piperidinebutanoic acid,(Imp-A); 4-[(S)-(4-chlorophenyl)-2-pyridinylmethoxy]-1- piperidinebutyric acid, N-oxide (Imp-B) and (S)-4-[(4- chlorophenyl)-2-pyridinylmethoxy]-1-piperidylethane (Imp-C). In addition, an efficient optimized chromatographic method was performed on a Shimadzu Inertsil C8–3 column (150 mm × 4.6 mm, 3 µm) to separate and quantify these 5 impurities. It was using 15 mmol ammonium formate buffer in water (pH adjusted to 3.8 with formic acid) and acetonitrile as the mobile phase in gradient mode. The method was developed to separate and quantify these 5 impurities obtained in the range of 0.05–0.75 µg/mL. It was validated and proven to be selective, accurate and precise and suitable. It is the first publication of identification and characterization data of the 3 new compounds. It is also the first effective HPLC method for separation and quantification of all of process-related impurities in bepotastine besilate.

Introduction

Bepotastine besilate, chemically known as ({d(S)4[4[(4chlorophenyl) (2pyridyl) methoxy] piperidino} butyric acid monobenzene sulphonate), is a second-generation antihistamine drug. As a potent and long-acting histamine H(1) receptor antagonist and with platelet activating factor antagonist effects, BTST has a high-level selectivity and has no affinity for 5-HT2, α1, α2 receptor.[1] Experiments on human peripheral blood mononuclear cells showed that the anti-allergic effect of this compound may be related to interleukin-5 mediator. BTST can quickly and effectively inhibit 3 major symptoms of allergic rhinitis: sneezing, runny nose and stuffy nose. BTST can inhibit eosinophil infiltration, while eosinophils releasing cytotoxin protein. BTST can also cause infiltration of inflammatory parts and inhibit the production of activated eosinophils IL-5. [2–3]

Bepotastine besilate can inhibit the infiltration of eosinophils into peripheral tissues, so that tulipamustine is effective against nasal mucosal inflammation than existing drugs. In addition, bepotastine besilate rarely passes through the blood-brain barrier, which means it has almost no sedative effect. It acts on anticholinergic effect and antihistamines separately. With excellent pharmacodynamic effects and clinical effects, it has extremely small adverse reactions, which makes it better than terfenadine, cetirizine, epipitabine. So the clinical application has broad prospects.[4–7]

Bepotastine besilate tablet was developed by Tanabe Seiyaku and Ube Industries in Japan, was approved in July 2000 and January 2002 for the treatment of allergic rhinitis and urticaria, eczema/dermatitis, prurigo and pruritus cutaneous in human patients.

A thorough literature survey revealed that most reports of bepotastine besilate are focused on synthesis and pharmacokinetics. Narasimha RK et al. described a HPLC method for the quantification of bepotastine besilate and its related substances.[8] They also studied on the forced degradation of bepotastine besilate. However, the study was not comprehensive enough. The impurities they studied were not characterized, and 3 new impurities were found during our synthesis. All the 3 impurities have never been identificated or characterizated before thus, a simple, sensitive, and effective analytical method needs to be developed to monitor the levels of all possible impurities in BTST, from the initial production process, to ensure its safety in the formulations. However, to the best of our knowledge, no studies concerning the formation or identification of these 3 new-found process-related impurities have been reported so far. Several syntheses of BTST have been published [7–10]; the route shown in ▶ **Fig. 1** for manufacturing BTST has several advantages such as the use of low-cost materials, a simple preparation process, and good yields [10]. Impurities inevitably form at the end of this process, and include unreacted starting materials, by-products, and intermediates. It is essential to carryout identification and structural elucidation of potential impurities in drug development. In this study, the potential process-related impurities of BTST were isolated and then analyzed by HPLC-UV–ESI–MS.

The aims of the current research are as follows: (i) to propose potential process-related impurities in bulk BTST by reference to the synthetic methodology; (ii) to identify and elucidate the structures of these impurities by NMR and MS; (iii) to optimize LC conditions and develop an effective HPLC method for the quantitative determination of the potential impurities in BTST.

Materials and Methods

Chemical and reagents

Bepotastine besilate and standards of 4-[(S)-(4-chlorophenyl)-2-pyridinylmethoxy]-1- Piperidinebutyric acid, ethyl ester (BT-01); (S)-4-[(phenyl)-2-pyridinylmethoxy]-1-Piperidinebutanoic acid,(Imp-A) ;4-[(S)-(4- chlorophenyl)-2-pyridinylmethoxy]-1- Piperidinebutyric acid,N-oxide (Imp-B) and (S)-4-[(4- chlorophenyl)-2-pyridinylmethoxy]-1-piperidylethane (Imp-C) were obtained from School of Pharmaceutical Sciences in Nanjing Tech University (Nanjing, China). Starting material A (SMA; (S)-4-[(4-chlorophenyl)-2 pyridinylmethoxy]-Piperidine and starting material B (SMB; 1-Bromo-3-(carboethoxy)-propane were purchased from Lianben Pharm-chemicals Tech. Co., Ltd. (Beijing, China). The purity of all substances was > 99 %. HPLC grade acetonitrile was purchased from Merck Ltd. (Darmstadt, Germany). Ammonium formate, formic acid, sodium hydroxide, hydrochloric acid, hydrogen peroxide (30 %) and acetone were purchased from Aladdin Reagent Co., Ltd. (Shanghai, China). Double-distilled water was produced through a Milli-Q pure water system (Milford, MA, USA). The other chemicals were all of analytical grade.

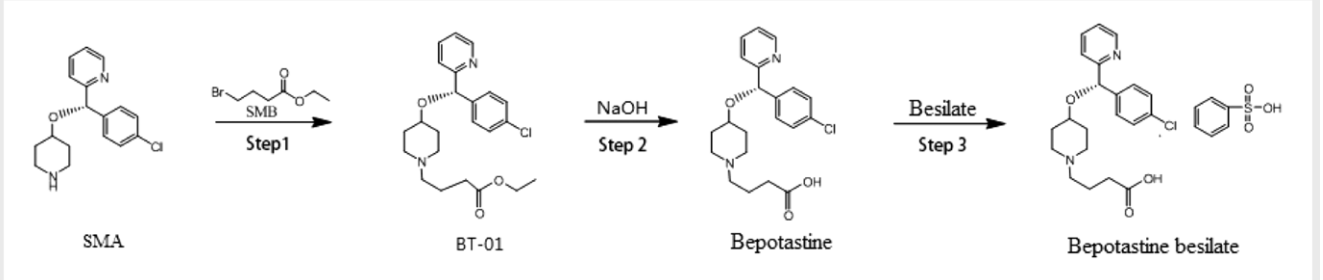
HPLC instrumentation and methods

Chromatographic studies were performed on an Agilent 1220 series HPLC (Palo Alto, CA), equipped with a binary gradient pump, degasser, variable wavelength detector, autosampler, and ChemStation software. The peak homogeneity was studied by using Agilent 1220 series DAD detector. The separation was performed on a Shimadzu Inertsil C8–3 column(150 mm × 4.6 mm, 3 µm) (Tokyo, Japan). Mobile phase A was ammonium formate buffer (15 mmol with the pH adjusted to 3.8 using formic acid) mixed with 10 % ACN, and phase B was ACN.

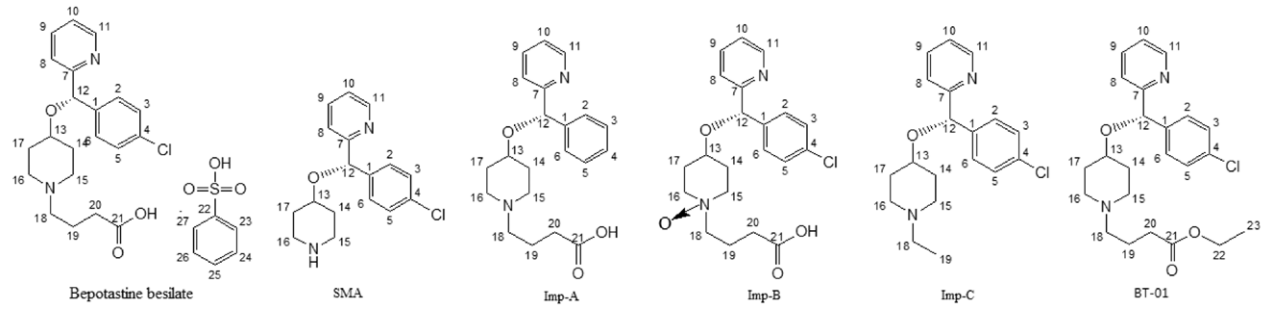
The HPLC gradient program was as follows: $Time_{(min)}$/A:B (v/v); $T_0$100/0, T_{15}77.8/22.2, T_{18}77.8/22.2, T_{23}66.7/33.3, T_{33}100/0, T_{40}100/0. The UV detector wavelength was chosen at 220 nm. The flow rate was 1.0 mL/min, and the column temperature was maintained at 25◦C. A water and acetonitrile mixture (70:30,v/v) was used in the BTST samples preparing, which was at 1 mg/mL concentration. In this experiment, 10 µL of sample solution was injected into the HPLC system for analysis.

LC–MS instrumentation and methods

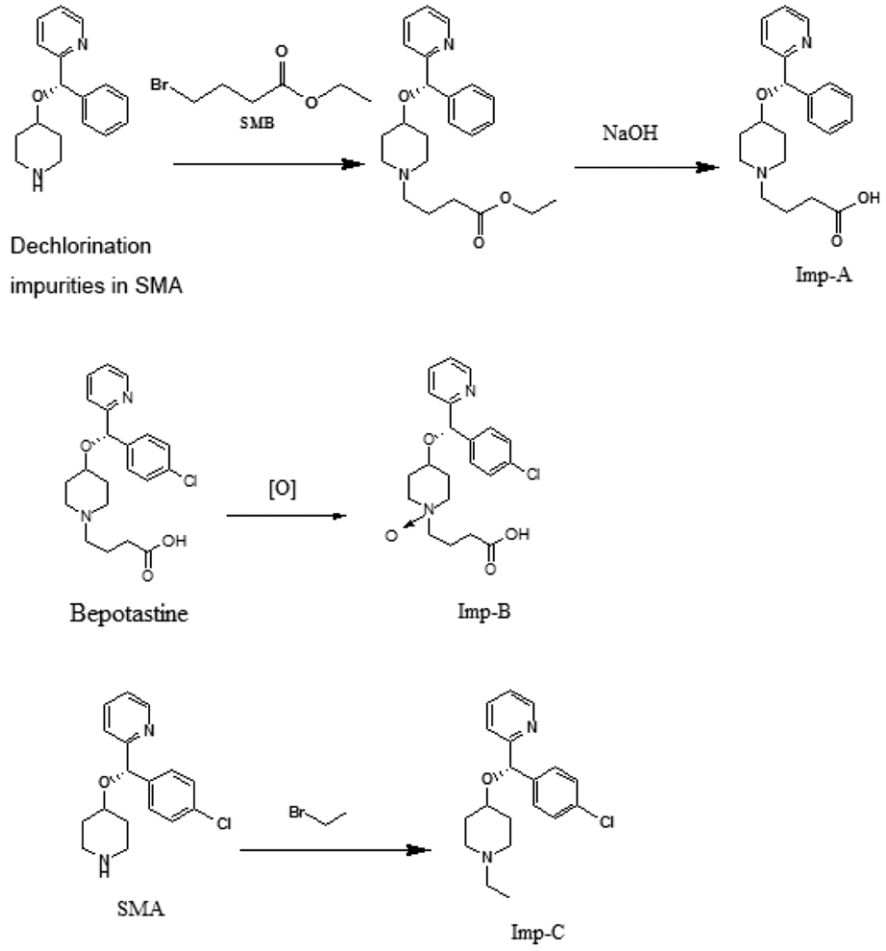
LC-MS analysis in this experiment was performed using an API4000 mass spectrometer (Milwaukee, WI, USA) equipped with an electrospray ionization (ESI) source interface coupled to an Agilent 1200-LC system (Palo Alto, CA, USA). The HPLC gradient program was as follows: $Time_{(min)}$/A:B (v/v); $T_0$100/0, T_{15}77.8/22.2, T_{25}77.8/22.2, T_{35}100/0, T_{40}100/0. And 10 µL of sample solution was injected. The UV detector wavelength was chosen at 220 nm. The column temperature was maintained at 25◦C.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 electrospray mode, and the operating param-



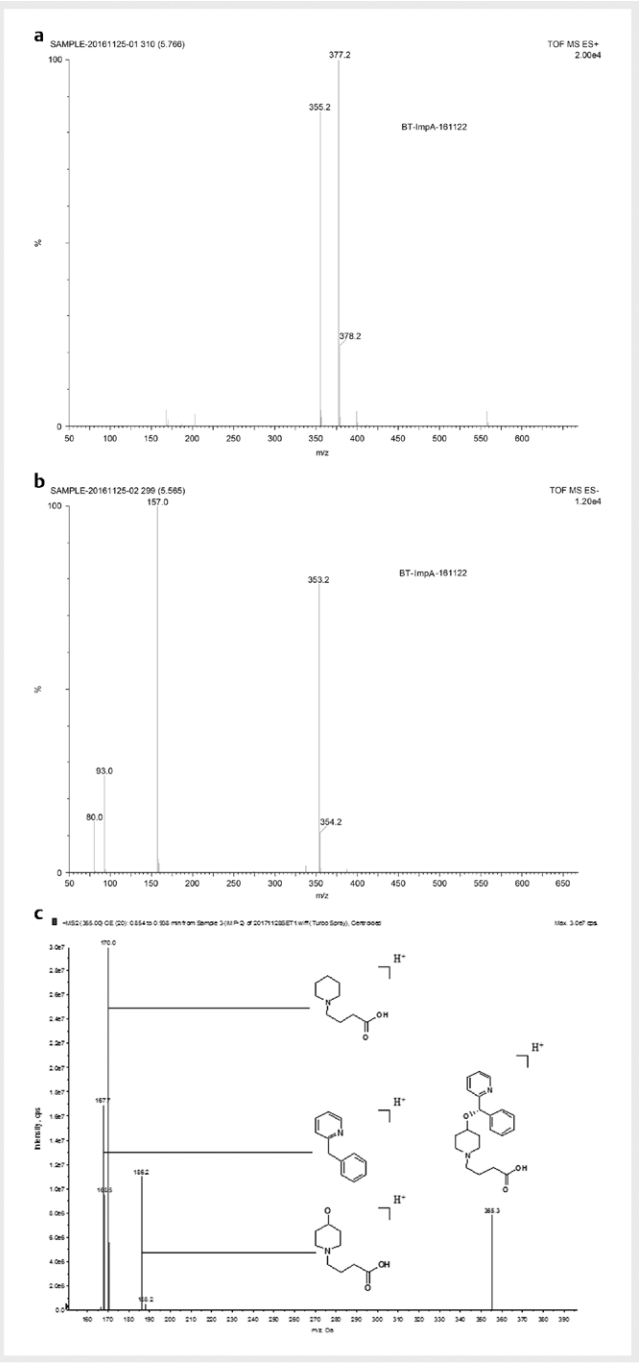
▶ **Fig. 1** The synthesis route of BTST. Link text: Several syntheses of BTST have been published [7–10]; the route shown in ▶ **Fig. 1**.



▶ **Fig. 2** Structures of BTST and its process-related impurities with numbering assigned for NMR characterization. Link text: we speculated that the exist of Bepotastine besilate and 5 impurities , which were 1-piperidinebutanoic acid, 4-[(*S*)-(4-chlorophenyl)-2-pyridinylmethoxy]-1- piperidinebutyric acid, ethyl ester (BT-01); (S)-4-[(phenyl)-2-pyridinylmethoxy]-1-piperidinebutanoic acid,(Imp-A) ;4-[(*S*)-(4-chlorophenyl)-2-pyridinylmethoxy]-1- piperidinebutyric acid,N-oxide (Imp-B); (S)-4-[(4- chlorophenyl)-2-pyridinylmethoxy]-1-piperidylethane (Imp-C) and starting material A (SMA; (S)-4-[(4-chlorophenyl)-2pyridinylmethoxy]-piperidine respectively (▶ **Fig. 2**).



▶ **Fig. 3** The possible routes of formation of Imp-A, Imp-B, and Imp-C. Link text: The possible routes of formation of Imp-A, Imp-B, and Imp-C are shown in ▶ **Fig. 3**.



▶ **Fig. 4** **a** The TOF MS spectra of Imp-A (+), **b** The TOF MS spectra of Imp-A (-) , **c** MS^2 spectra of Imp-A. Link text: The TOF MS spectra and MS^2 spectra of Imp-A is shown in ▶ **Fig. 4**.

eters were as follows: ion spray voltage, 5000 V; declustering potential, 70 V; entrance potential, 10 V; turbo ion spray temperature, 500 ◦C; collision energy,40 V; collision cell exit potential, 15 V; interface heater, on; and mass range, 100–800 Da. Nitrogen was used as both curtain and auxiliary gas. The system was operated using an Analyst Software workstation, version 1.5.2.

NMR instrumentation and methods

The ^{1}H NMR, ^{13}C NMR, and distortionless enhancement by polarization transfer (DEPT) spectra were recorded at 300 or 500 MHz on a Bruker AVANCE NMR spectrometer (Fallanden, Switzerland). The samples were dissolved in chloroform-d or dimethyl sulfoxide-d6 at a concentration of 50 mg/mL.

Preparation of sample solution

The stock solutions of BTST were prepared with acetonitrile at a concentration of 2 mg/mL, and its related impurities were prepared with acetonitrile at a concentration of 1 mg/mL. The solutions were adequately diluted by a water and acetonitrile mixture (70:30, v/v) ,which was used as the diluent in the sample preparation. The concentration of the BTST sample was 0.5 mg/mL, and the solutions used to investigate the system suitability were prepared by spiking the BTST sample with 5 impurities at 0.2 % of the sample concentration.

Results and Discussion

HPLC method development and optimization

The first step of establishing the HPLC method in this experiment was to determine the appropriate wavelength, which was usually a compromise for different compounds with different absorption maximum. The suitable wavelength was found after measuring all spectra and testing the detector response of analytes at 220 nm, because of the sufficient selectivity and sensitivity for all the products in the study.

Then the selection of organic phase was considered. The object of the investigation was methanol and acetonitrile. The results of using methanol showed that the retention time (RT) of each component is too long, the peak width is very large and the number of theoretical plates (NTP) is very low. The results of using acetonitrile showed that each component has shorter RT, sharp peak, and higher NTP.

Then, Shimadzu Inertsil C8–3 column(150 mm × 4.6 mm, 5 µm) (Tokyo, Japan) was found to have the most suitable RT and good separation effect, after testing various types of chromatographic columns. In order to get better resolution (R) and higher NTP, Shimadzu Inertsil C8–3 column (150 mm × 4.6 mm, 3 µm) (Tokyo, Japan) was finally chosen. However, BTST, Imp-B and Imp-C have an extremely close RT. And the test showed that RT and peak shape of BTST and 5 impurities are greatly affected by the pH. Considering the pKa of BTST is 4.44. Several different pH conditions are tested, then the results showed 3.8 is the most suitable pH. BTST and 5 impurities have sharp peaks, and R was greater than 2. When pH is lower than 3.8, R of BTST and Imp-C is very low. However, adjusting pH greater leads to R of Imp-B and Imp-C to be lowered.

Several buffer salts were tested during the pH adjustment. Using phosphate or trifluoroacetic acid leads to the peak of each group splitting. The ammonium formate was finally chosen for it affords sharp shape of peaks and good separation result.

In order to reduce the noise, 10 % of the acetonitrile is mixed into the mobile phase A. After calculation and adjustment, the gradient method as described in Section 2.2 is determined.

▶ **Table 1** The detailed information of ^{1}H NMR spectra.

position	BTST	Imp-A	Imp-B	Imp-C	BT-01
1					
2	7.45(d,2 H)	7.45–7.26(m,8 H)	7.34–7.28(m,4 H)	7.39–7.37(d,2 H)	7.37–7.35(d,2 H)
3	7.39(d,2 H)	7.45–7.26(m,8 H)	7.34–7.28(m,4 H)	7.44–7.43(d,2 H)	7.43–7.41(d,2 H)
4	–	–	–	–	–
5	7.39(d,2 H)	7.45–7.26(m,8 H)	7.34–7.28(m,4 H)	7.44–7.43(d,2 H)	7.43–7.41(d,2 H)
6	7.45(d,2 H)	7.45–7.26(m,8 H)	7.34–7.28(m,4 H)	7.39–7.37(d,2 H)	7.37–7.35(d,2 H)
7	–	–	–	–	–
8	7.60(d,1 H)	7.65–7.57(m,3 H)	7.41–7.40(d,1 H)	7.58–7.7.56(d,1 H)	7.56–7.55(d,1 H)
9	7.81(t,1 H)	7.82(m,1 H)	7.71–7.67(m,1 H)	7.81(m,1 H)	7.80(s,1 H)
10	7.28(t,1 H)	7.45–7.26(m,8 H)	7.20–7.17(m,1 H)	7.28–7.26(m,1 H)	7.25(m,1 H)
11	8.49(d,1 H)	8.48(s,1 H)	8.51–8.58(d,1 H)	8.50–8.49(d,1 H)	8.48(s,1 H)
12	5.71(s,1 H)	5.70(s,1 H)	5.53(s,1 H)	5.68(s,1 H)	5.66(s,1 H)
13	3.68(m,1 H)	3.67(s,1 H)	3.80(s,1 H)	3.42–3.38(m,1 H)	3.39(m,1 H)
14	2.02(m,4 H)	1.85(m,6 H)	2.36–2.35(m,4 H)	1.87–1.85(m,2 H)	1.67(m,2 H)
15	3.26(m,4 H)	3.06(m,4 H)	3.64–3.59(m,4 H)	2.69–2.68(m,2 H)	2.26(m,4 H)
16	3.26(m,4 H)	3.06(m,5 H)	3.64–3.59(m,4 H)	2.02(m,2 H)	2.26(m,5 H)
17	2.02(m,4 H)	1.85(m,6 H)	2.36–2.35(m,4 H)	1.59–1.55(m,2 H)	1.55–1.54(d,2 H)
18	3.08(m,2 H)	3.28(s,2 H)	3.49–3.44(t,2 H)	2.32–2.28(m,2 H)	2.02(s,2 H)
19	1.87(m,2 H)	1.85(m,6 H)	1.91–1.89(m,2 H)	0.98(m,3 H)	1.84–1.82(d,2 H)
20	2.31(t,2 H)	2.50(s,2 H)	2.46–2.40(t,2 H)	–	2.64(s,2 H)
21	11.53(br,1 H)	10.64(s,2 H)	9.65(br,1 H)	–	–
22	–	–	–	–	4.07(m,2 H)
23	7.68(d,2 H)	7.65–7.57(m,3 H)	–	–	1.17(m,3 H)
24	7.33(m,3 H)	7.45–7.26(m,8 H)	–	–	–
25	7.33(m,3 H)	7.45–7.26(m,8 H)	–	–	–
26	7.33(m,3 H)	7.45–7.26(m,8 H)	–	–	–
27	7.68(d,2 H)	7.65–7.57(m,3 H)	–	–	–

Detection of process-related impurities of BTST

Analysis of BTST samples used the method which has been described in Section 2.2 revealed the presence of 5 impurities consistently in several batches.

The molecular weight of the 5 related impurities were detected by analyzing by LC–MS. Each was 303, 354, 405, 330 and 416, respectively corresponds to SMA, Imp-A, Imp-B, Imp-C, and BT-01. Then on the basis of the knowledge of the route of BTST synthesis and results of MS^2 product ion analysis of impurities, we speculated that the exist of Bepotastine besilate and 5 impurities, which were 1-piperidinebutanoic acid, 4-[(S)-(4-chlorophenyl)-2-pyridinylmethoxy]-1- piperidinebutyric acid, ethyl ester (BT-01); (S)-4-[(phenyl)-2-pyridinylmethoxy]-1-piperidinebutanoic acid,(Imp-A) ;4-[(S)-(4-chlorophenyl)-2-pyridinylmethoxy]-1- piperidinebutyric acid, N-oxide (mp-B); (S)-4-[(4-chlorophenyl)-2-pyridinylmethoxy]-1-piperidylethane (Imp-C) and starting material A (SMA; (S)-4-[(4-chlorophenyl)-2pyridinylmethoxy]-piperidine respectively (▶ **Fig. 2**).

Structural elucidation of BTST and its impurities

Recently, a number of methods for structural elucidation have emerged exuberantly and been widely applied, like MS, ^{1}H NMR, ^{13}C NMR, and DEPT. In these ways, we confirm the structures of BT-01, Imp-A, Imp-B, and Imp-C. And the structural elucidation of 3 new compounds (Imp-A, Imp-B and Imp-C) is shown follow.

Imp-A was presumed to be involved in the subsequent response by generated by the dechlorination impurities in starting materials A (SMA). Imp-B was presumed to be produced by the oxidation of bepotastine. Imp-C was presumed to be generated by the reaction of bromoethane and SMA. Since there could be bromoethane as impurity in starting materials B (SMB). The possible routes of formation of Imp-A, Imp-B, and Imp-C are shown in ▶ **Fig. 3**. The TOF MS spectra of Imp-A, Imp-B and Imp-C, and MS^2 spectra of Imp-A, Imp-B and Imp-C is shown follow.

Structural elucidation of Imp-A

The online LC–MS and MS^2 spectra of Imp-A in BTST suggested that it might be(S)-4-[(phenyl)-2-pyridinylmethoxy]-1-Piperidinebutanoic acid. Using benzene sulfonic acid in the process of preparing Imp-A caused that the ion peak of benzene sulfonic acid will appear in the mass spectrogram. The mass spectrogram shows that the base peak 377.2 is $[M+Na]^+$ peak, the relative strength is 100. $[M+H]^+$ peak was detected as 355.2, and the relative intensity is 85. 378.2 as the isotope peak. Under the negative ion mode, the base peak 157 is $[M-H]^-$ peak of benzene sulfonic acid, the relative strength is 100. $[M-H]^-$ peak was detected as 353.2, and the relative strength is 80. It can be deduced that the molecular weight of

▶ **Table 2** The detailed information of ^{13}C NMR spectra.

position	BTST		Imp-A		Imp-B		Imp-C		BT-01	
	δc	DEPT	δc	DEPT	δc	DEPT	δc	DEPT	δc	DEPT
1	140.91	C	137.57	C	139.2	C	140.89	C	140.89	C
2	129.28	CH	125.95	CH	128.82	CH	128.54	CH	128.52	CH
3	129.2	CH	128.19	CH	128.05	CH	128.05	CH	128.04	CH
4	132.61	C	127.39	CH	133.74	C	131.81	C	136.83	C
5	129.2	CH	128.19	CH	128.05	CH	128.05	CH	128.04	CH
6	129.28	CH	125.95	CH	128.82	CH	128.54	CH	128.52	CH
7	161.42	C	161.88	C	160.8	C	161.47	C	161.49	C
8	120.98	CH	123.1	CH	120.59	CH	120.24	CH	120.22	CH
9	137.65	CH	141.85	CH	137.25	CH	136.89	CH	136.87	CH
10	123.21	CH	120.96	CH	122.93	CH	122.34	CH	122.42	CH
11	149.36	CH	148.44	CH	149.2	CH	148.66	CH	148.66	CH
12	80.69	CH	81.53	CH	81.54	CH	79.82	CH	79.83	CH
13	70.68	CH	67.15	CH	69.21	CH	73.01	CH	72.92	CH
14	28.25	CH2	28.78	CH2	25.07	CH2	31.03	CH2	31.09	CH2
15	49.01	CH2	50.45	CH2	29.68	CH2	50.08	CH2	50.44	CH2
16	49.01	CH2	50.45	CH2	32.92	CH2	50.08	CH2	50.44	CH2
17	28.25	CH2	28.78	CH2	24.85	CH2	31.03	CH2	31.09	CH2
18	55.36	CH2	55.35	CH2	66.97	CH2	12.05	CH3	56.67	CH
19	19.61	CH2	19.61	CH2	18.65	CH2	–		21.89	CH2
20	31.04	CH2	31	CH2	58.41	CH2	–		31.45	CH2
21	173.29	C	173.88	C	176.67	C	–		172.68	C
22	148.22	C	149.23	C	–		–		59.5	CH2
23	125.95	CH	127.97	CH	–		–		13.98	CH3
24	128.74	CH	128.76	CH	–		–		–	
25	128.74	CH	129.07	CH	–		–		–	
26	128.74	CH	128.76	CH	–		–		–	
27	125.95	CH	127.97	CH	–		–		–	

the sample is 354, which is in accordance with the molecular weight of Imp-A.

The TOF MS spectra and MS^2 spectra of Imp-A is shown in ▶ **Fig. 4**.

The ^{1}HNMR spectrum of Imp-A showed 12 signals corresponding to 31 protons, which accords with the molecular structure of Imp-A. Imp-A misses one chlorine compared to that of BTST, In ^{13}C NMR, the chemical shift of C4 (which is a tertiary carbon) is less than that of BTST (which is a quaternary carbon). The detailed information of ^{1}H NMR and ^{13}C NMR spectra can be seen in ▶ **Tables 1** and ▶ **2**.

Structural elucidation of Imp-B

The online LC–MS and MS^2spectra of Imp-B in BTST suggested that it might be 4-[(S)-(4-chlorophenyl)-2-pyridinylmethoxy]-1- piperidinebutyric acid,N-oxide. The mass spectrogram shows that the base peak 405.9 is $[M+H]^+$peak, the relative strength is 100. It can be deduced that the molecular weight of the sample is 405, which is in accordance with the molecular weight of Imp-B.

The TOF MS spectra and MS^2 spectra of Imp-B is shown in ▶ **Fig. 5**.

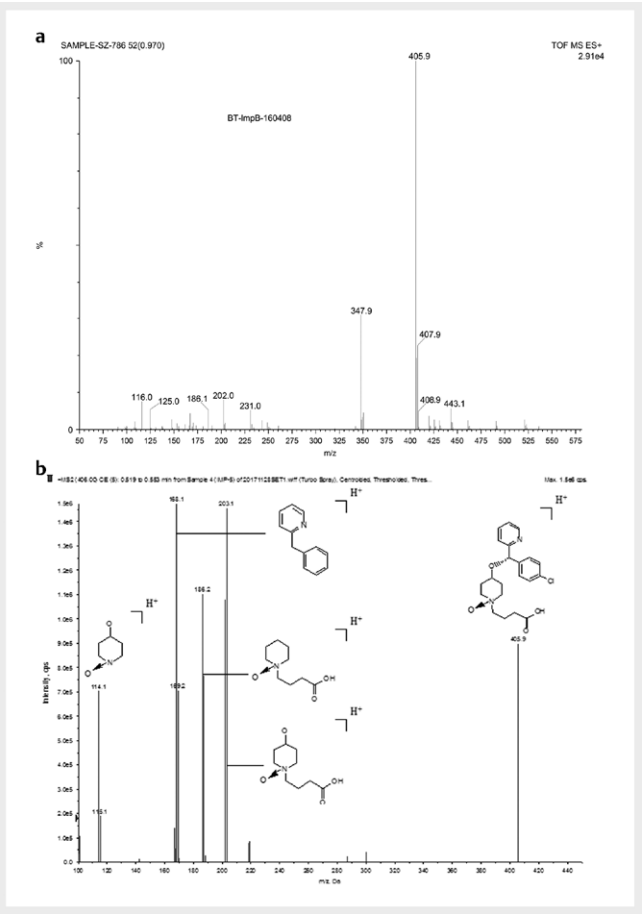
The ^{1}HNMR spectrum of Imp-B showed 15 signals corresponding to 25 protons. In ^{13}C NMR, 19 peaks were detected. Because the tested products contain 21 carbons, 2 groups (C3 and C5, C2 and C6) have the same chemical environment. Caused by N-oxide group, C14,C15, C16 and C17 have different chemical environments. The chemical shift of C15 and C16 are obviously less than those of BTST. So it is known from the ^{13}C-NMR spectrum that there are 19 peaks in addition to the DMSO solvent peak, which are in accordance with the number of carbon atoms of Imp-B. This result accords with the molecular structure of Imp-B. Imp-B has an N-oxide compared to that of BTST. The detailed information of ^{1}H NMR and ^{13}C NMR spectra can be seen in ▶ **Tables 1** and ▶ **2**.

Structural elucidation of Imp-C

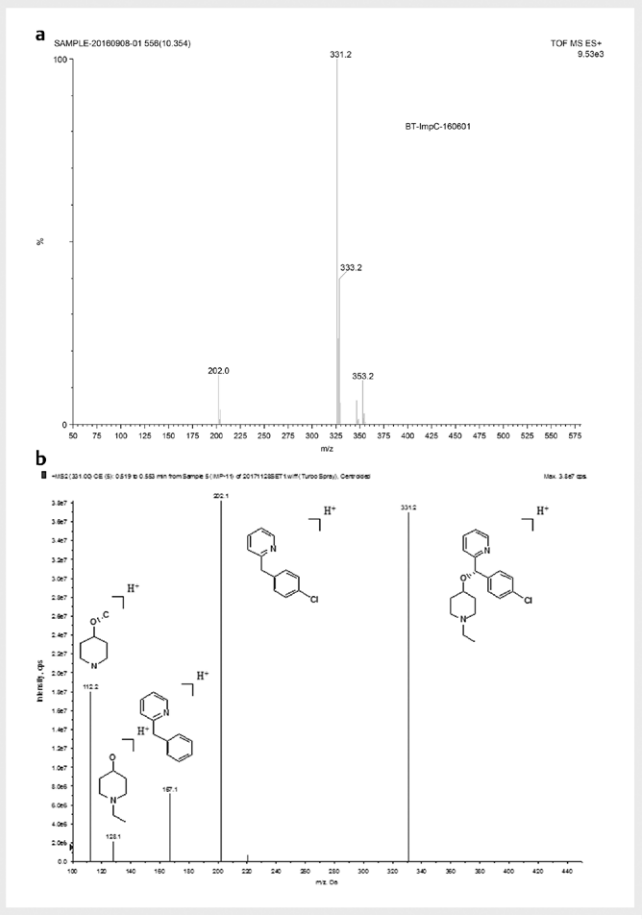
The online LC–MS and MS^2 spectra of Imp-C in BTST suggested that it might be (S)-4-[(4- chlorophenyl)-2-pyridinylmethoxy]-1-piperidylethane. The mass spectrogram shows that the base peak 331.2 is $[M+H]^+$peak, the relative strength is 100. It can be deduced that the molecular weight of the sample is 330, which is in accordance with the molecular weight of Imp-C.

The TOF MS spectra and MS^2 spectra Imp-C is shown in ▶ **Fig. 6**.

The 1HNMR spectrum of Imp-C showed 14 signals corresponding to 23 protons. In ^{13}C NMR, 15 peaks were detected. Because the tested products contain 19 carbons, but 4 groups (C3 and C5, C2 and C6, C14 and C17, C15 and C16) have the same chemical environment. So it is known from the ^{13}C-NMR spectrum that there are 15 peaks in addition to the DMSO solvent peak, which are in accordance with the number of carbon atoms of Imp-C. This result



▶ **Fig. 5** **a** The TOF MS spectra of Imp-B, **b** MS^2 spectra of Imp-B. Link text: The TOF MS spectra and MS^2 spectra of Imp-B is shown in ▶ **Fig. 5**.



▶ **Fig. 6** **a** The TOF MS spectra of Imp-C, **b** MS^2 spectra of Imp-C. Link text: The TOF MS spectra and MS^2 spectra of Imp-C is shown in ▶ **Fig. 6**.

accords with the molecular structure of Imp-C. Imp-C misses -CH2COOH compared to that of BTST. The detailed information of ^1H NMR and ^{13}C NMR spectra can be seen in ▶ **Tables 1** and ▶ **2**.

Method Validation

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 BTST solution containing 1 µg/mL of 5 impurities by making six replicate injections. An efficient resolution (> 2.15), high number of theoretical plates (> 113303 /m), and good tailing factor was obtained as shown in ▶ **Table 3**. The HPLC chromatograms of BTST spiked with 5 impurities and bulk drugs is shown in ▶ **Fig. 7**.

LOD, LOQ, and linearity

LOD and LOQ were calculated by injecting a series of BTST solution and each impuritiy solution respectively, which were diluted to known concentrations for at S/N = 3:1 and 10:1, (▶ **Table 3**).

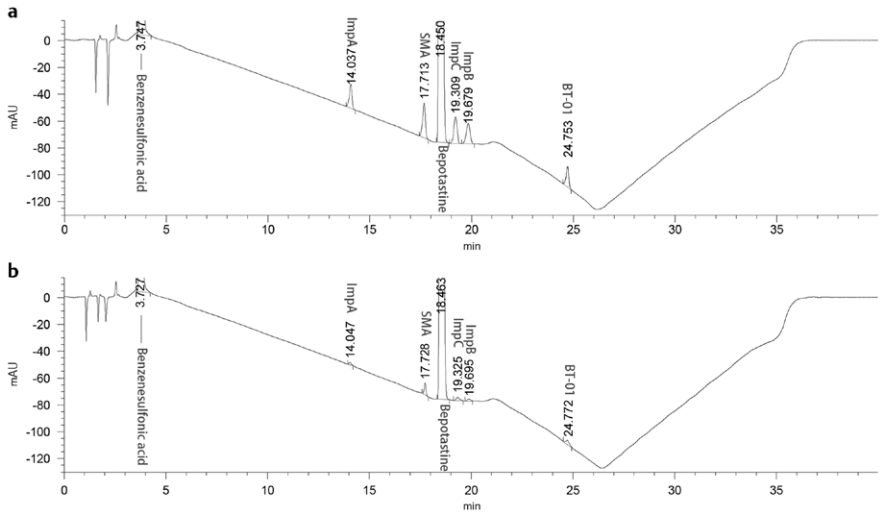
Linearity solutions of 5 impurities were prepared at 6 concentration levels ranging from the LOQ to 150 % of the specification level. The peak area of analyte versus concentration was analyzed by least squares linear regression (▶ **Table 1**). The calculated parameters of calibration curves indicated satisfactory linearity. The correction response factor (CRF) was calculated from the ratio of the slope of principal components and impurities by linear regression. The results indicate that the response factors of all impurities are within 0.63–1.41 (▶ **Table 3**).

Precision and accuracy

The precision of the method was assessed by injecting 6 individual solutions (BTST sample 500 µg/mL mixed with all 5 impurities (1 µg/mL), then estimating all the percent RSD for each peak. The precision of the method was good, for the percent RSD for BTST and 5 impurities was within 2.0 (▶ **Table 3**). The accuracy of the method was evaluated by using the recovery and percent RSD values obtained from test BTST solutions (0.5 mg/mL) spiked with impurity levels of 0.16, 0.20, and 0.24 %. All measurements were carried out in triplicate. The mean percentage of recoveries of the impurities was obtained in the range 98.97–99.56 % (▶ **Table 4**).

Robustness and solution stability

The conditions listed below were deliberately changed for assessing the robustness of the method. The column temperature



▶ **Fig. 7** The HPLC chromatograms of BTST spiked with 5 impurities **a** and bulk drugs **b**. Link text: The HPLC chromatograms of BTST spiked with 5 impurities and bulk drugs is shown in ▶ **Fig. 7**.

▶ **Table 3** The summary of method validation.

Compound	System Suitability				Linearity				Sensitivity		Calibration response factor			
	RRTa)	PCb)	SFc)	Rd)	Range (ug/mL)	Re)	Slope	Intercept	LODf) (ug/mL)	LOQg) (ug/mL)	Slope	Intercept	MCFh)	RSD (n=6)
BTST	1	175508	1.06	5.29	0.104–0.730	0.9990	17.778	−0.074	0.031	0.104	–	–	–	–
Imp-A	0.76	125504	0.97	–	0.103–0.722	0.9996	15.616	−0.075	0.031	0.103	15.691	0.862	0.53	1.20
Imp-B	1.06	156859	1.03	2.15	0.102–0.711	0.9990	17.725	−0.031	0.031	0.102	17.450	0.881	0.53	0.68
Imp-C	1.04	135164	1.02	2.49	0.053–0.744	0.9998	28.249	−0.024	0.016	0.053	28.378	1.610	1.12	0.53
SMA	0.95	135829	1.25	17.87	0.168–0.750	0.9995	26.144	−0.088	0.054	0.168	42.757	−1.942	1.41	0.60
BT-01	1.34	113303	1.27	21.73	0.106–0.740	0.9996	28.638	−0.472	0.032	0.106	23.964	1.068	0.77	0.57

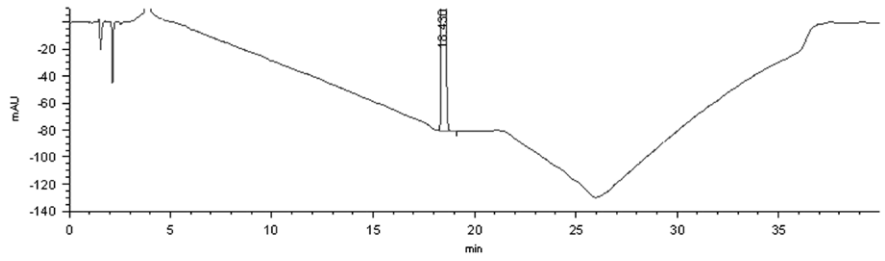
a) Relative retention time; b) (USP)plate count; c) Symmetry factor; d) (USP)resolution; e) Correlation factor; f) (S/N = 3); g) (S/N = 10); h) Mean calibration response factor.

▶ **Table 4** The summary of precision and accuracy.

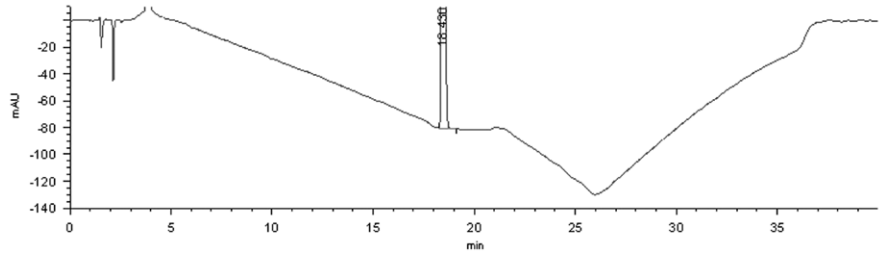
Compound	Precision	Accuracy			
	RSD (n=6)	80 %MR	100 %MR	120 %MR	RSD (n=9)
BTST	-	–	–	–	–
Imp-A	1.33	99.86	99.66	98.55	99.36
Imp-B	1.20	98.18	99.62	99.77	99.19
Imp-C	1.31	98.85	98.84	99.22	98.97
BT-01	0.74	99.69	99.28	99.72	99.56
SMA	0.75	99.35	99.06	99.13	99.18

(25 ± 5◦C), and the flow rate (1.0 ± 0.1 mL/min) showed that the resolution between any 2 analytes is > 1.5. The pH value of the buffer solution (3.8 ± 0.5), and trying different columns revealed that the resolution between BTST and Imp-C, Imp-B and Imp-C is < 1.5. When pH is less than 3.8, R of BTST and Imp-C is very low. And adjusting pH greater leads to R of Imp-B and Imp-C becomes lower.

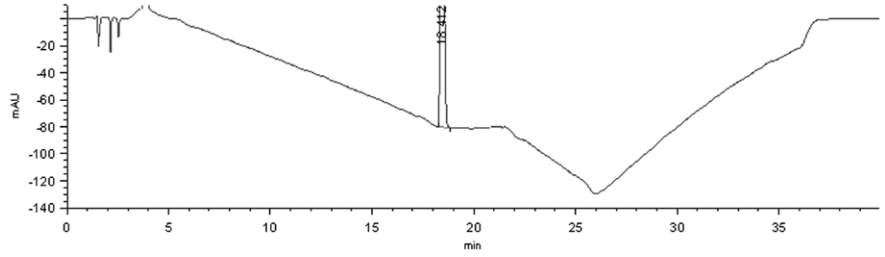
The stability of the BTST sample solution and the mixed impurities solution was monitored at room temperature for 24 h. The BTST mixed with 5 impurities solution should prepared daily, cause the solution remained stable for 10 h though, BT-01 was found decreased by 20 % after 12 h and the baseline noise increased obviously after 12 h.



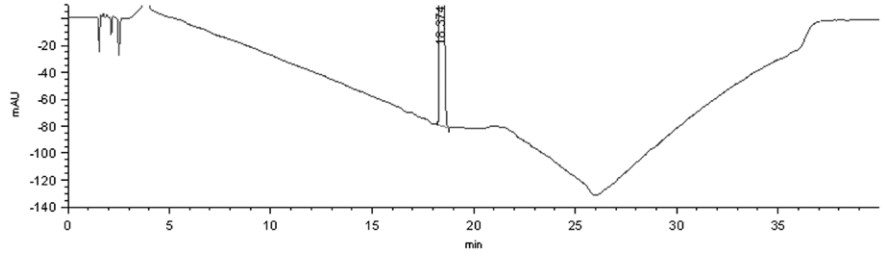
▶ **Fig. 8** The HPLC chromatograms of the elaborated product of BTST. Link text: And the HPLC chromatograms of the elaborated product is shown in ▶ **Fig. 8**.



▶ **Fig. 9** The HPLC chromatograms of BTST under the acid stress. Link text: The detailed results are shown in ▶ **Figs. 9–13**.



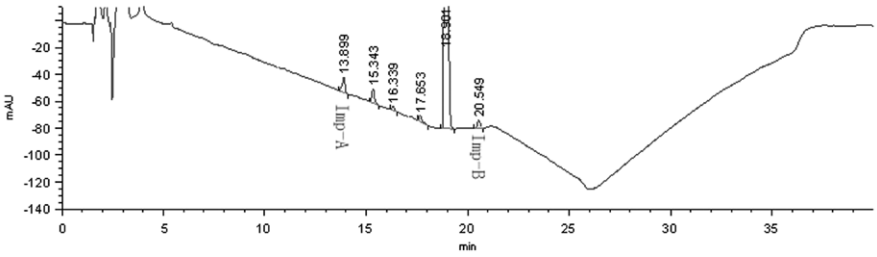
▶ **Fig. 10** The HPLC chromatograms of BTST under the base stress. Link text: The detailed results are shown in ▶ **Figs. 9–13.**



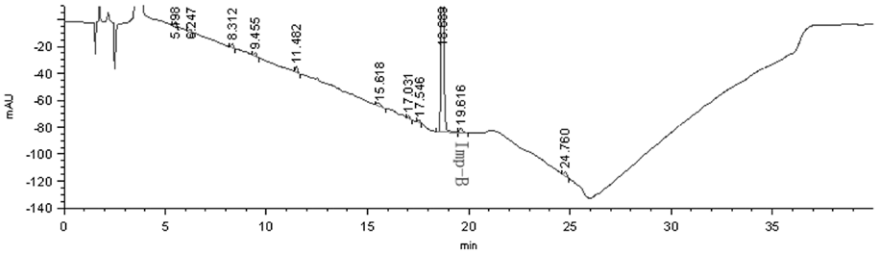
▶ **Fig. 11** The HPLC chromatograms of BTST under the water hydrolysis. Link text: The detailed results are shown in ▶ **Figs. 9–13.**

Conclusions

According to the International Conference on Harmonisation (ICH) guidelines for specificity, sensitivity, linearity, accuracy and precision, and system suitability, a stability-indicating HPLC method has been developed and validated for the separation and quantification of bepotastine besilate and its 5 related impurities. Both 5 impurities in BTST sample prepared by our synthetic route were iden-



▶ **Fig. 12** The HPLC chromatograms of BTST under the oxidation stress. Link text: The detailed results are shown in ▶ **Figs. 9–13.**



▶ **Fig. 13** The HPLC chromatograms of BTST under the photolytic stress. Link text: The detailed results are shown in ▶ **Figs.9,10–13.**

tified and separated by this optimized HPLC method. Besides, 3 of the process-related impurities (Imp-A, Imp-B and Imp-C) were found to be new compounds and their structures have not been reported previously, to the best of our knowledge. Their structures were identified and confirmed by MS and NMR. This is the first publication of Identification, characterization of the 3 new compounds, and also the first effective HPLC method for the separation and quantification of all of these 5 impurities in bepotastine besilate.

Forced degradation studies

Considering the existence of the impurities in BTST bulk drug, the elaborated product was used in the forced degradation studies. The elaborated product was prepared by recrystallized BTST bulk drug with acetone aqueous solution. And the HPLC chromatograms of the elaborated product is shown in ▶ **Fig. 8**.

Five degradation conditions were performed, including acid stress, base stress, water hydrolysis, photolytic stress and the oxidation stress.

The acid stress was performed at 0.1 N HCl for 24 h at 90℃, and the base stress was performed at 0.1 N NaOH for 24 h at 90℃. The water hydrolysis was performed at 90℃ for 24 h. The photolytic stressed study was performed for UV Indirect (200 watt hours/square meter). And the oxidation stress was done using 30 % hydrogen peroxide at 90℃ for 30 min. The stressed samples of BTST generated were checked for peak purity of by using Agilent diod array detector (DAD). The peak purity is within the limit obtained in all stressed samples, demonstrates the analyte peak homogeneity.

The result showed that BTST is stable under the acid stress, base stress and water hydrolysis. But significant degradation was found in the study of photolytic stress and the oxidation stress. Imp-A and Imp-B were found from the peroxide hydrolysis degradation. Imp-B was found from the photo degradation. The detailed results are shown in ▶ **Figs. 9–13**.

Acknowledgements

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Conflicts of interest

There are no financial/commercial conflicts of interest.

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