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Article

Development and Validation of Stability-Indicating HPLC and UPLC Methods for the Determination of Bicalutamide

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Six new process related impurities (Imp-08, Imp-09, Imp-10, Imp-12, Imp-13 and Imp-14) of bicalutamide (BCT) have been reported in this paper. BCT was subjected to oxidative, acid, alkaline, hydrolytic, thermal and photolytic degradation conditions and found to degrade in alkaline condition, yielding Imp-11. Stability-indicating high-performance liquid chromatography and ultra-performance liquid chromatography methods were developed for the determination of BCT in the presence of its 14 process-related impurities and 1 degradant by using Zorbax SB phenyl column (150 × 4.6 mm × 3.5 μm) and HSS T3 column (100 × 2.1 mm × 1.8 μm), respectively. Both the methods were validated as per International Conference on Harmonization guidelines. Quantitation limits (QL) were found be in the ranges of 0.02–0.03% for both the methods. Precision was evaluated by replicate analysis in which % relative standard deviation (RSD) values for areas were found below 2.0. Linearity for the impurities was established in the range of QL to 200% of the specification level and the correlation coefficients derived from of the respective calibration curves were approximately 0.999. The recoveries obtained for purity (90–100%) and assay (98–102%) ensured the accuracy of the developed methods.

Introduction

International Conference on Harmonization (ICH) (1) guidelines demand validated stability-indicating liquid chromatography (LC) methods established through forced degradation studies for stability testing of drug substances (2). Stability testing plays an important role in the process development and provides evidence on the quality of a drug substance that varies with time under the influence of a variety of environmental factors such as temperature, humidity and light. It allows the recommendation of storage conditions and retest periods of drug substances.

Bicalutamide (BCT), chemically described as (±)-4′-Cyano-α,α,α-trifluoro-3-[(*p*-fluoro phenyl)sulfonyl]-2-methyl-*m*-lactotoluidide is an antineoplastic drug. This study identified 14 impurities in BCT during its process development (Figure 1). Forced degradation was also performed on BCT and Imp-11 was formed in alkaline degradation. Among the 14 impurities, United States Pharmacopeia (3) reported Imp-03, Imp-04, Imp-05, Imp-06 and Imp-07; Pharmeuropa (4) reported Imp-03, Imp-04, Imp-05, Imp-06, Imp-07 and Imp-11; Rao *et al.* (5) reported Imp-02, Imp-03 and Imp-11; and Rao *et al.* (6) reported Imp-01, Imp-02, Imp-03 and Imp-11. Hence, six impurities (Imp-08, Imp-09, Imp-10, Imp-12, Imp-13 and Imp-14) were found new in this synthetic process. Because the monograph (3, 4) and literature methods (5, 6) have not reported all 14 impurities and have been unable to resolve them from each other and from BCT, there is a need for the development of new stability-indicating LC methods. Hence, this studydeveloped a high-performance liquid chromatography (HPLC) method that can resolve all 14 impurities from BCT. Ultra-performance liquid chromatography (UPLC) has significant advantages in speed, resolution, sensitivity, time saving and solution consumption (7). Hence, to reduce solvent consumption and run time in conventional HPLC analysis, a UPLC method was also developed. The integral aim of the present study is to identify process-related impurities and degradants to develop and validate (8) stability-indicating HPLC and UPLC methods, to evaluate purity, assay and stability of BCT.

Experimental

Materials

Sodium dihydrogen ortho phosphate, sodium hydroxide, octane-1-sulphonic acid, tetrabutyl ammonium hydrogen sulphate, acetonitrile hydrochloric acid, sodium hydroxide and hydrogen peroxide (30 %) were procured from Merck (India). BCT (99.8 %), Imp-01 (98.6 %), Imp-02 (99.8 %), Imp-03 (91.6 %), Imp-04 (98.8 %), Imp-05 (99.9 %), Imp-06 (95.0 %), Imp-07 (98.1 %), Imp-08 (94.0 %), Imp-09 (99.3 %), Imp-10 (99.5 %), Imp-11 (98.0 %), Imp-12 (90.3 %), Imp-13 (98.5 %) and Imp-14 (91.7 %) were obtained from synthetic division of Hetero Drugs Ltd (R&D, Hyderabad, India).

Stock solutions

For purity determination, 1 mg/mL stock solution of BCT was prepared in the respective diluents for HPLC and UPLC methods. With respect to 1 mg/mL BCT, 0.15% of impurities blend was also prepared for this purpose. For assay determination, 0.1 mg/mL stock solution of BCT was prepared in the respective diluents for the HPLC and UPLC methods.

Tablet solution

Ten tablets of BCT were finely ground using agate mortar and pestle. The ground material, equivalent to 100 mg of the active pharmaceutical ingredient, was extracted into diluent in a 100 mL volumetric flask by mixing followed by ultrasonication and made up to volume by diluent. The solution was filtered through a 0.45 micron filter. A sample with an appropriate concentration in HPLC and UPLC methods was prepared in respective diluents at the time of analysis.

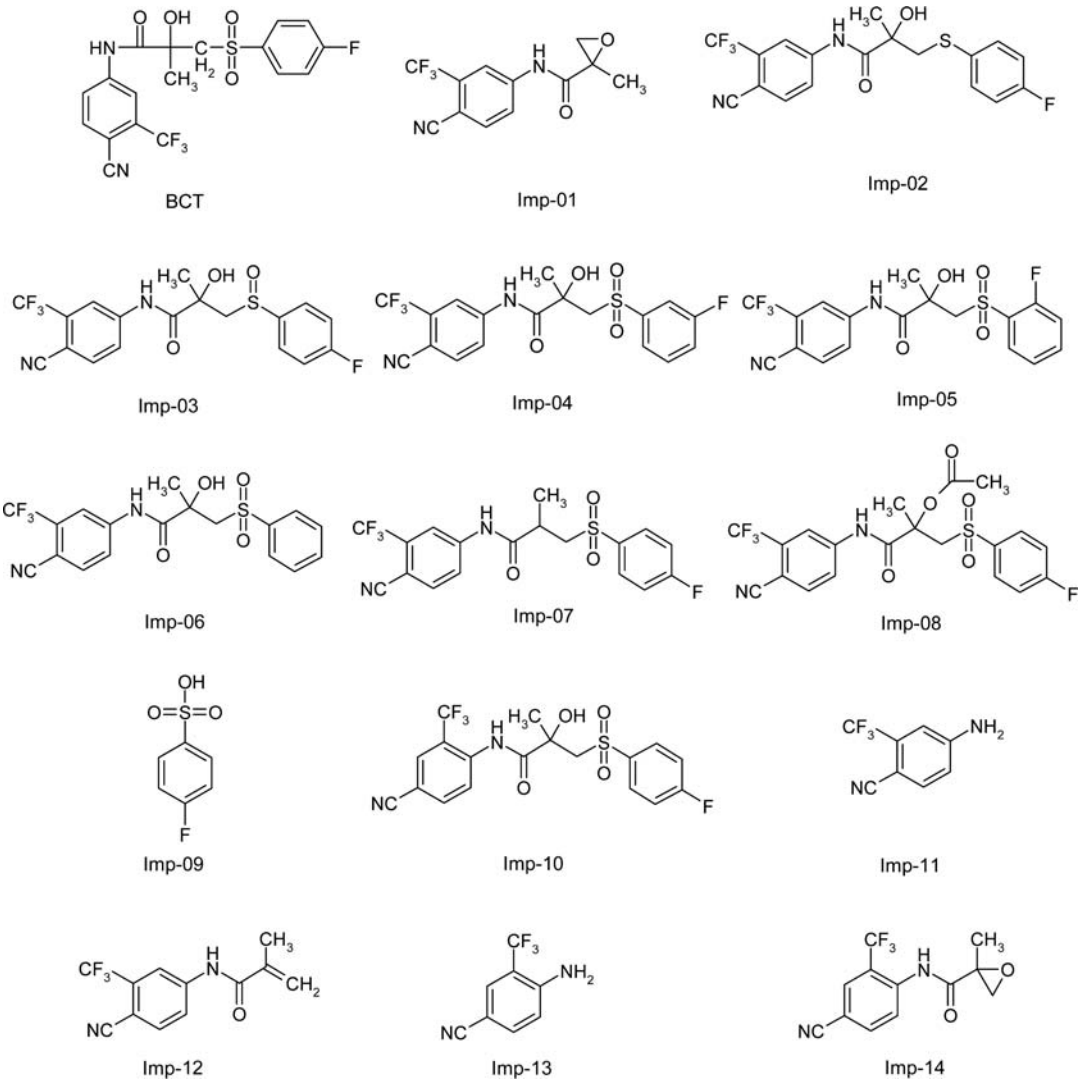

Figure 1. Structures of BCT and 14 impurities.

HPLC conditions

A Waters Alliance e2695 separation module (Waters Corporation, Milford, MA) equipped with 2489 UV/Visible detector or 2998 PDA detector (for specificity) with empower2 software was used for the analysis. Buffer consisted of 0.01M sodium dihydrogen orthophosphate, and pH was adjusted to 6.0 with sodium hydroxide and 0.001M sodium octane-1-sulphonic acid and 0.0025M tetrabutyl ammonium hydrogen sulphate. A Zorbax SB phenyl column (150 × 4.6 mm × 3.5 µm, Agilent Technologies) and a gradient mixture of solution A (buffer and acetonitrile in the ratio of 95:5 *v*/*v*) and solution B (acetonitrile and buffer in the ratio of 90:10 *v*/*v*) were used as stationary and mobile phases, respectively. The gradient program (T/%B) was fixed as 0/20, 25/20, 36/25, 49/30, 64/40, 68/45, 76/55, 85/20 and 90/20. Solution A and solution B in 1:1 *v*/*v* ratio was used as diluent. The column oven was maintained at 40°C. An injection volume of 20 µL was used. The eluted compounds were monitored at 220 nm.

UPLC conditions

A Waters Aquity UPLC (Waters Corporation, Milford, USA) equipped with TUV and PDA detector (for specificity) with empower2 software was used for the analysis. Sodium dihydrogen orthophosphate (0.001M) with pH adjusted to 6.0 with sodium hydroxide was used as buffer.An HSS T3 column (100 × 2.1 mm × 1.8 µm, Waters Corporation, Milford, MA) and a gradient mixture of solution A (buffer only) and solution B (acetonitrile and buffer in the ratio of 90:10 *v*/*v*) were used as stationary and mobile phases, respectively. The gradient program (T/%B) was set as 0.0/28, 26.0/55, 29.3/55, 31.3/28 and 34.0/28. Partial loop with needle overfill was selected as loop option. Acetonitrile and water were used as strong needle wash solution (90:10 *v*/*v*) and weak needle wash solution (10:90 *v*/*v*). Solution A and solution B in 1:1 *v*/*v* ratio was used as diluent. A flow rate of 0.5 mL/min, injection volume of 2 µL and column oven temperature of 400°C were maintained. The detection wavelength was 220 nm.

LC–tandem mass spectrometry conditions

A LC–tandem mass spectrometry (MS–MS) system (Waters 2695 Alliance) liquid chromatograph coupled with a quattromicro mass spectrometer with MassLynx software, Waters Corporation, Milford, MA) was used for the identification of impurities during process development and forced degradation studies. An Inertsil ODS-3V (250 × 4.6 mm, 5 μm, GL Sciences, Japan) column was used. Ammonium formate (0.01M) was used as buffer. A gradient mixture of solution A (buffer only) and solution B (acetonitrile and water in the ratio of 90:10 *v*/*v*) with a program of T/%B, 0.01/35, 75/35, 80/55, 90/55, 95/35 and 100/35 was used as mobile phase. Water and acetonitrile in 1:1 *v*/*v* ratio was used as diluent. The flow rate was 1.5 mL/min. Analysis was performed in electrospray negative ionization scan mode. Capillary and cone voltages were kept at 3.5 kV and 25 V, respectively. Source and dissolvation temperatures were kept at 120 and 3500°C, respectively. Dissolvation gas flow was 650 L/h.

Forced degradation conditions

Oxidative degradation

One hundred milligrams of BCT was accurately weighed and dissolved in 10 mL of 10 % H_2O_2 solution and kept at 800°C for approximately 2 h. The solution was made up to 100 mL with diluent.

Acid degradation

One hundred milligrams of BCT was accurately weighed, dissolved in 10 mL of 0.5 N HCl and kept at 800°C for approximately 2 h. The solution was neutralized with 0.5 N NaOH and the volume was made up to 100 mL with diluent.

Base degradation

One hundred milligrams of BCT was accurately weighed, dissolved in 10 mL of 0.5 N NaOH and kept at room temperature for approximately 1 h. The solution was neutralized by 0.5 N HCl and the volume was made up to 100 mL with diluent.

Hydrolytic degradation

One hundred milligrams of BCT was accurately weighed, dissolved in 10 mL of water and kept at room temperature for approximately 2 h. The solution was made up to 100 mL with diluent.

Thermal degradation

One gram of BCT sample was placed in a petri dish and kept in an oven at 105°C for 24 h. Ten milligrams of this sample was taken in to a 10 mL volumetric flask, dissolved in diluent and diluted to volume with diluent.

Photolytic degradation

One gram of BCT sample was placed in a petri dish and kept in a photo stability chamber/200Wh/m2 in ultraviolet (UV) light and 1.2 million lux h in visible light for seven days. Ten milligrams of this sample was placed a 10 mL volumetric flask, dissolved in diluent and diluted to volume with diluent.

Results and Discussion

HPLC method development

HPLC method development for the separation between BCT and its 14 impurities was initiated with the literature method (6). The blend containing 0.1 mg/mL each of BCT and 14 impurities was injected, but some of the impurities were not eluted. Hence, the reported column was replaced with a Zorbax SB phenyl (150 × 4.6 mm × 3.5 μm). All the impurities were eluted, but most of the impurities were co-eluted and some eluted at longer retention times. Then the buffer was replaced with 0.01M sodium dihydrogen orthophosphate, pH was adjusted to 6.0 with sodium hydroxide and resolutions were improved. A gradient program was also introduced to minimize retention times and optimize resolutions, and thus, buffer and acetonitrile in the ratio of 95:5 *v*/*v* was used as solution A and acetonitrile and water in the ratio of 90:10 *v*/*v* was used as solution B. Several gradient programs were tried and the current gradient program was found to be optimal. However, good resolution was not achieved between two pairs (Imp-08, Imp-02 and Imp-13, Imp-11). Then, 0.0025M tetra butyl ammonium hydrogen sulfate was introduced into the buffer, which improved the resolution between Imp-08 and Imp-02. However, the resolution between Imp-13 and Imp-11 was still low. Then, 0.001M sodium octane-1-sulphonic acid was also introduced to the buffer, which improved the resolution between Imp-13 and Imp-11 more than 2.0. A 20 μL injection volume, 1.0 mL/min flow rate, 400°C column oven temperature and 220 nm wavelength were found to be ideal for a good chromatographic performance. A typical HPLC chromatogram of BCT spiked with impurities at 0.15 % level is shown in Figure 2.

UPLC method development

The UPLC method was initiated with a gradient program and flow rate obtained by converting HPLC gradient and flow rate with a UPLC calculator supplied by Waters Corporation (Milford, USA). When optimum resolutions are not achieved on 5 μm columns, ion-pairing agents are generally added to the buffer. Because UPLC columns with sub 2 μm particle size can show enhanced resolution between critical pairs when compared to 5 μm particle size columns, ion pairing agents are not needed. Hence, ion-pairing agents were removed from buffer and HSS T3 column (100 × 2.1 mm × 1.8 μm) that resolved the two critical pairs, that is, Imp-08, Imp-02, and Imp-13, Imp-11 was introduced. Buffer concentration was also reduced. Sodium dihydrogen orthophosphate (0.001M), pH adjusted to 6.0 with sodium hydroxide, was used as buffer (solution A). Acetonitrile and buffer in the ratio of 90:10 *v*/*v* was used as solution B. A gradient program of (T/%B) of 0.0/28, 26.0/55, 29.3/55, 31.3/28 and 34.0/28, 2 μL injection volume, 0.5 mL/min flow rate and 40°C column oven temperature were found to be adequate. A typical UPLC blend chromatogram of BCT and its impurities (0.1 mg/mL each) is shown in Figure 3.

Validation of HPLC and UPLC methods

The developed HPLC (Table I) and UPLC (Table II) methods were used for both purity and assay determinations and validated as per ICH guidelines (8).

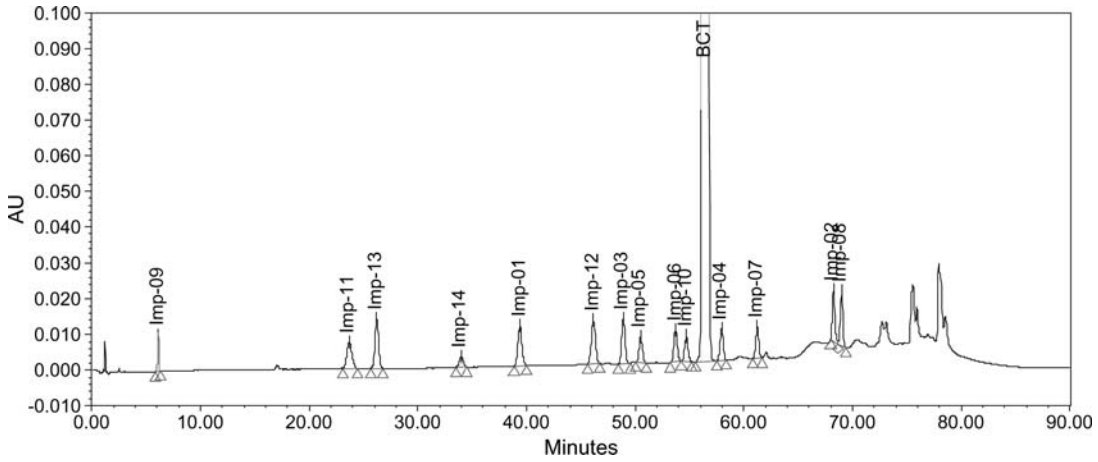

Figure 2. Typical HPLC chromatogram of BCT spiked with 14 impurities.

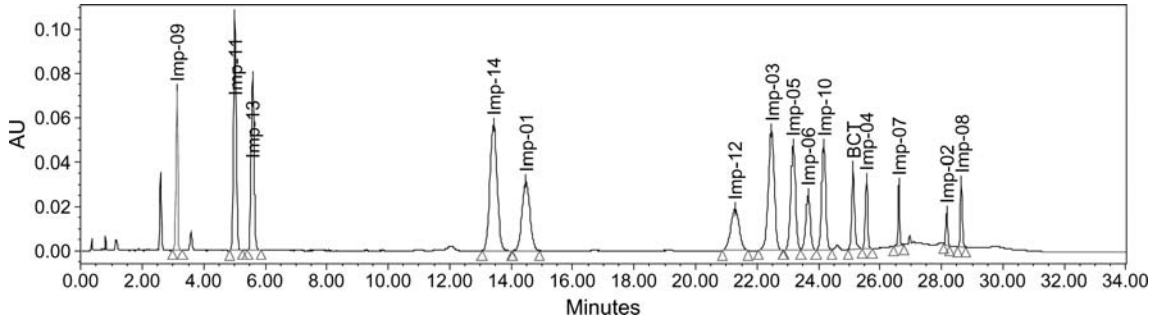

Figure 3. Typical UPLC blend chromatogram of BCT and 14 impurities.

Table I
HPLC Method Validation Data

Parameter	Purity															Assay
	BCT	Imp-01	Imp-02	Imp-03	Imp-04	Imp-05	Imp-06	Imp-07	Imp-08	Imp-09	Imp-10	Imp-11	Imp-12	Imp-13	Imp-14	
DL (%)	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	–
QL (%)	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.02	0.03	0.02	0.03	0.03	0.03	–
Slope	2579	1758	1219	1285	1928	2322	3222	1555	1542	2769	1591	1117	1341	2262	2311	1896
Intercept	203	302	212	782	225	176	125	213	302	322	181	285	142	122	162	395
Correlation coefficient	0.9998	0.9998	0.9999	0.9998	0.9997	0.9998	0.9996	0.9998	0.9997	0.9999	0.9995	0.9998	0.9996	0.9999	0.9998	0.9999

Table II
UPLC Method Validation Data

Parameter	Purity															Assay
	BCT	Imp-01	Imp-02	Imp-03	Imp-04	Imp-05	Imp-06	Imp-07	Imp-08	Imp-09	Imp-10	Imp-11	Imp-12	Imp-13	Imp-14	
DL (%)	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	–
QL (%)	0.03	0.02	0.03	0.03	0.03	0.03	0.03	0.03	0.02	0.02	0.02	0.02	0.03	0.03	0.03	–
Slope	1567	1592	2715	1791	1273	1171	3262	2951	3542	1299	3759	1283	1381	1252	1381	1925
Intercept	219	402	518	482	295	462	229	218	424	422	482	222	372	425	462	327
Correlation coefficient	0.9999	0.9997	0.9996	0.9995	0.9999	0.9998	0.9997	0.9996	0.9997	0.9999	0.9999	0.9995	0.9998	0.9999	0.9999	0.9999

Specificity

Specificity is the ability of the method to measure the analyte in the presence of its potential impurities, which might process related or degradation impurities. The specificity of the two developed methods for BCT purity and assay was checked in the presence of its 14 impurities. Photodiode array (PDA) detector was employed in both methods to check and ensure the homogeneity and purity of the BCT peak in all forced

degradation sample solutions. All the impurities were resolved from each other and from BCT peak, and the resolution between any two peaks is not less than 1.5.

Detection limits and quantitation limits To determine detection limits (DL) and quantitation limits (QL) for BCT and its 14 impurities, a series of dilute solutions of impurities and BCT with known concentrations were injected separately in both the purity methods. DL and QL values were determined at a signal-to-noise ratio of approximately 3 and 10, respectively.

Precision

A precision study of both HPLC and UPLC purity methods was carried at QL level by injecting six individual preparations of BCT and its impurities. Assay precision of both the methods was evaluated by carrying out six independent assays of test concentration of BCT against reference standard. The intermediate precision for purity and assay methods on HPLC and UPLC was evaluated on six different days as described previously. Percent relative standard deviation (%RSD) values for the areas of all the impurities and BCT were found below 2.0.

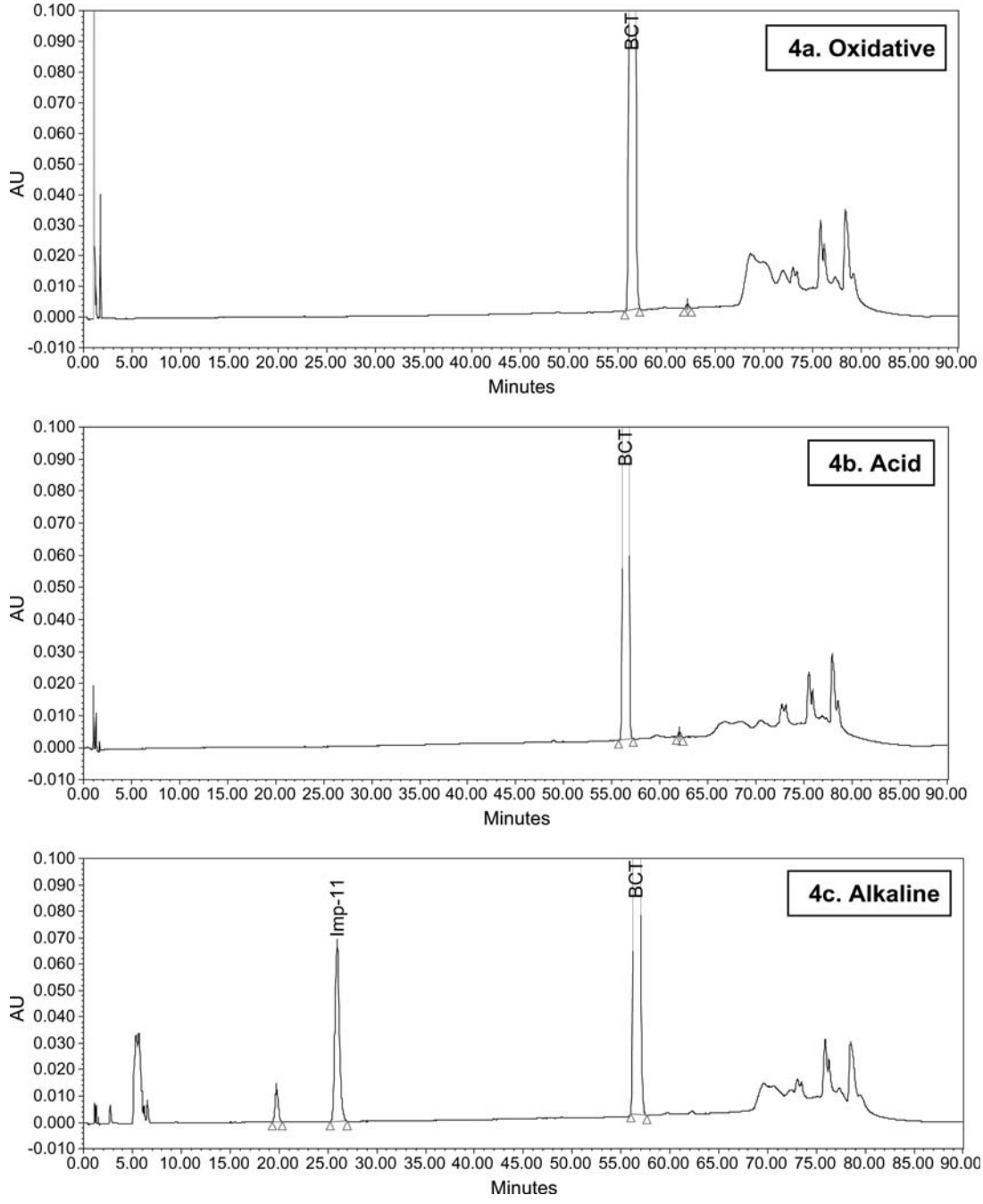

Figure 4. Typical HPLC chromatograms of BCT forced degradation samples.

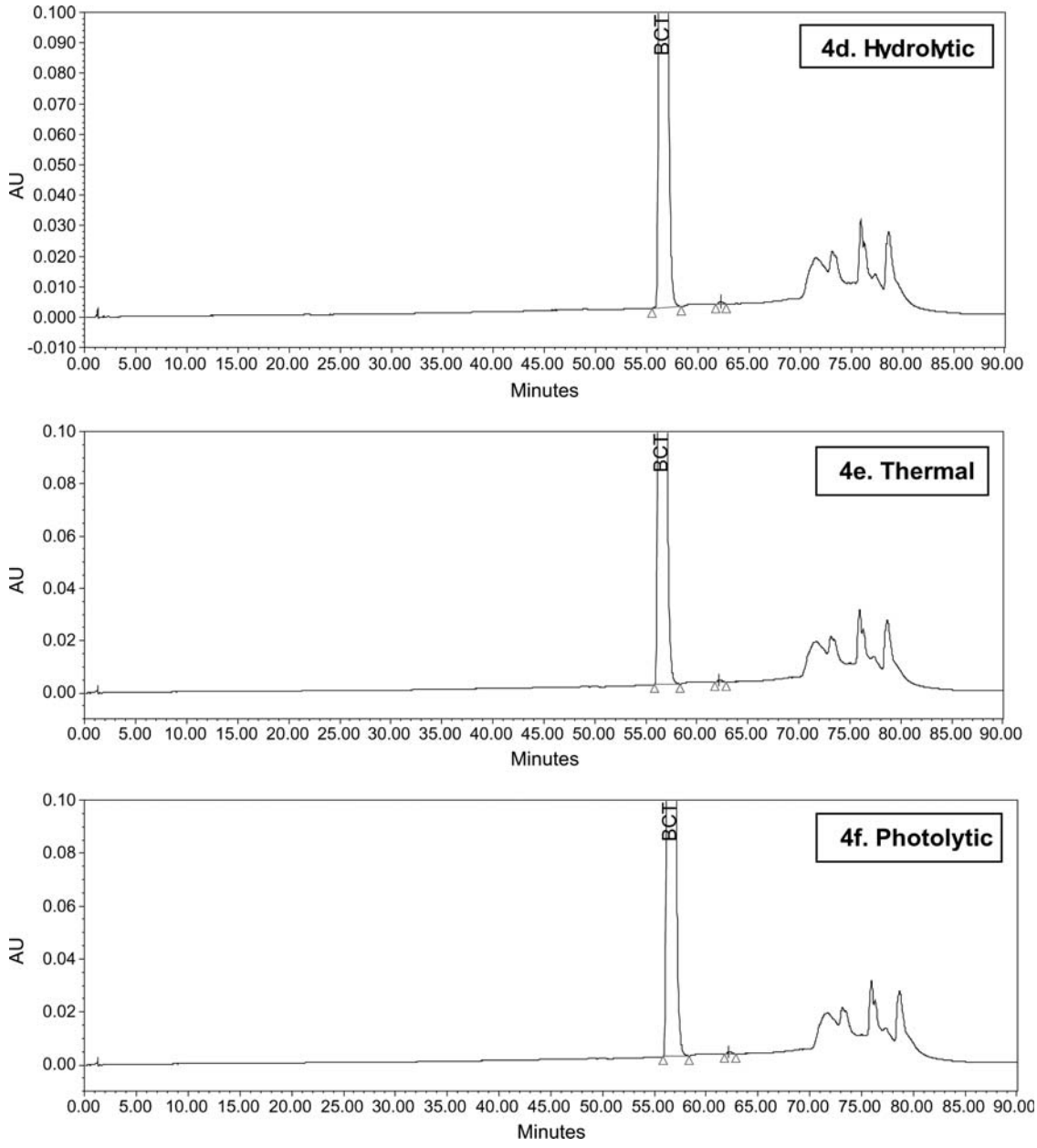

Figure 4. Continued.

Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results that are directly proportional to the concentration of the analyte in the sample. Linearity test solutions for BCT and its impurities were prepared individually at six concentration levels (each triplicate) in the range of QL to 200 % (QL, 50, 75, 100, 150 and 200%) of the specification level (0.15 % for impurities and 0.1 % for BCT). Linearity test solutions for BCT assay were prepared from stock solution at five concentration levels from 50 to 150% (50, 75, 100, 125 and 150%) of assay analyte concentration (0.1 mg/mL of BCT). Linear least-squares regression analysis was performed. Correlation coefficient of the calibration curve was calculated.

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value, which is accepted either as a conventional true value or an accepted reference value. Standard addition and recovery experiments were conducted to determine accuracy of the developed methods. The study was carried out in triplicate at QL, 100 and 150% with respect to specification level, that is, 0.15% in both the purity methods. The same procedure was adopted for tablet powder. The accuracy of the BCT assay was evaluated in triplicate at three concentration levels, 50, 100 and 150% with respect to 0.1 mg/mL of BCT test concentration. These experiments were also performed by adding 50, 100 and 150% of BCT to the

Table III
Forced Degradation Results

Forced degradation type	HPLC					UPLC				
	% Degradants formed			% Assay	Mass balance (%)	% Degradants formed			% Assay	Mass balance (%)
	Imp-11	MSUI	Total impurities			Imp-11	MSUI	Total impurities		
Unstressed	ND	0.07	0.07	–	–	ND	0.06	0.06	–	–
Oxidative degradation	ND	0.08	0.08	99.7	99.8	ND	0.06	0.06	99.5	99.6
Acid degradation	ND	0.07	0.07	99.7	99.8	ND	0.07	0.07	99.6	99.8
Base degradation	6.55	0.86	7.41	92.1	99.5	6.61	0.82	7.43	92.0	99.4
Hydrolytic degradation	ND	0.07	0.07	99.6	99.7	ND	0.06	0.06	99.4	99.5
Thermal degradation	ND	0.07	0.07	99.5	99.6	ND	0.06	0.06	99.5	99.6
Photolytic degradation	ND	0.07	0.07	99.6	99.7	ND	0.06	0.06	99.5	99.6

placebo (used for tablet preparation). The percent recovery ranges of both the methods for purity and assay are in the range of 90–100 and 98–102%, respectively.

Robustness

To determine the robustness of the developed methods, experimental conditions were purposely altered and the resolution between BCT and Imp-10 was evaluated. The resolution was found to be greater than 2.0 in both the methods, when mobile phase flow rate (± 0.2 mL/min), pH (± 0.2), organic solution ratio (± 5 %) and column temperature (± 2 °C) were deliberately varied.

Solution stability

The solution stability was determined by leaving 0.15% spiked sample solution to test BCT concentration for purity and 0.1 mg/mL BCT solution for assay in a tightly capped volumetric flask at room temperature for 24 h. The area percent of impurities was recorded for every 6 h. BCT was assayed at 6 h intervals and the results were compared with those obtained from freshly prepared solution. The mobile phase was prepared at the beginning of the study period and not changed during the experiment. The %RSD values for solution stability experiments were calculated and found to be 1.85 (HPLC purity), 1.32 (HPLC assay), 0.86 (UPLC purity) and 1.15 (UPLC assay). All samples were found to be stable for up to 24 hours.

System suitability

The system suitability for purity methods was established in terms of resolution between Imp-10 and BCT, which is more than 2.0, when a 1 mg/mL BCT solution spiked with 0.15% of Imp-10 was injected. The system suitability for assay methods was established in terms of %RSD, which should be less than 2.0 for five consecutive standard injection areas.

Forced degradation study

Forced degradation was conducted on BCT and the analysis was carried out by HPLC (Figure 4) and UPLC with a PDA detector (Table III). BCT was stable to oxidative, acid, hydrolytic, thermal and photolytic degradation conditions and degraded only in alkaline conditions resulting Imp-11. An LC–MS-MS system was used for the identification of the degradant formed. Twenty microliters each of forced degradation samples were injected at regular intervals and mass number of the degradant was identified. The identified mass number of degradants was compared time to time with available impurities. If degradant mass number was found to be equal to Imp-11, then Imp-11 was co-injected with the degraded sample and confirmed the degradant as Imp-11. Assay studies were carried out for degradation samples against reference standard and the mass balance (% assay + % sum of all process impurities + % sum of all degradation impurities) was established. The purity and assay of BCT was unaffected by the presence of its process-related and degradation impurities, and thus confirmed the stability-indicating power of the two developed methods.

Conclusions

The developed HPLC and UPLC methods for determination of BCT purity and assay were found to be specific, precise, accurate and robust. The stability-indicating nature of these methods was established by performing forced degradation, which provided degradation behavior of BCT under various conditions. Because BCT is well resolved from its process-related and degradation impurities, purity and assay of BCT was not affected during stability studies. Satisfactory results were obtained in method validation performed according to ICH guidelines. Hence, the developed methods can be conveniently used for assessing stability, impurities and assay of BCT with low cost or less time, depending on the necessity and availability of the systems.

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