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Isolation and characterization of process related impurities and degradation products of bicalutamide and development of RP-HPLC method for impurity profile study

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Abstract

A reversed-phase high-performance liquid chromatographic method was developed for determination of process impurities and degradation products of bicalutamide in bulk drug and pharmaceutical formulations. The separation was accomplished on a Symmetry C_{18} (4.6 mm × 250 mm; particle size 5 μm) column under isocratic mode. The mobile phase was 0.01 M KH_2PO_4 (pH 3.0):acetonitrile (50:50 v/v) and a PDA detector set at 215 nm was used for detection. Forced degradation of bicalutamide was carried out under thermal, photo, acidic, alkaline and peroxide conditions. The unknown process impurities and alkaline degradation products were isolated and characterized by ESI-MS/MS, ^{1}H NMR and FT-IR spectral data. Under alkaline conditions bicalutamide was degraded in to an acid and an amine. The kinetics of degradation was studied. The proposed method was validated and successfully applied to the analysis of commercial formulations. Thus, the developed method can be used for process development as well as quality assurance of bicalutamide in bulk drug and pharmaceutical formulations.

Keywords: Bicalutamide; Forced degradation; Process impurities; Kinetics of degradation; Isolation and characterization

1. Introduction

Bicalutamide (BCT) (Fig. 1) (*RS*)-*N*-[4-cyano-3-(trifluoromethyl)phenyl]-3-[(4-fluorophenyl)sulfonyl]-2-hydroxy-2-methyl-propanamide, under the trade name of Casodex® is one of the leading non-steroidal anti-androgens used for treatment of prostate cancer. It competes with testosterone and dihydrotestosterone for binding on to the prostate and other androgen-sensitive tissues. It binds preferentially to receptors located outside the central nervous system and causes little increase in testosterone levels with little agonist activity [1–3]. The pharmacokinetics of BCT was studied thoroughly [4,5].

A thorough literature search has revealed that, there are only a few HPLC methods reported for determination of BCT in plasma [6–8]. A few chiral HPLC methods have been reported for the determination of enantiomers of BCT [9–12]. Recently we have also reported chiral HPLC method for the determination of BCT enantiomers in bulk drug and pharmaceutical formulations [13]. Torok et al. have evaluated different chiral stationary phases for separation of enantiomers of BCT and its impurities [14]. Recently Saravanan et al. have reported a stability indicating HPLC method for separation of two of the process impurities and one of the degradation products of BCT in gradient elution mode using tetra *n*-butyl ammonium hydrogen sulphate as an ion pair reagent [15]. The method has several draw backs, viz.; (i) only one of the degradation products was separated where as more than one product were formed on degradation under alkaline conditions, (ii) use of ion pair reagent decreases not only the column life time but also increases the time required for equilibration, (iii) use of an ion pair reagent in a gradient elution mode is not preferred because of system artifacts, and (iv) the process impurities and degradation products were not characterized. Further, BCT is not yet official in any of the pharmacopoeia and no method for determination of its impurities has been reported. The present work describes the development of simple isocratic reversed-phase high-performance liquid chromatographic (RP-HPLC) method for determination of BCT, degradation products and process related impurities in bulk drug and pharmaceutical formulations. Forced degradation of BCT was carried out

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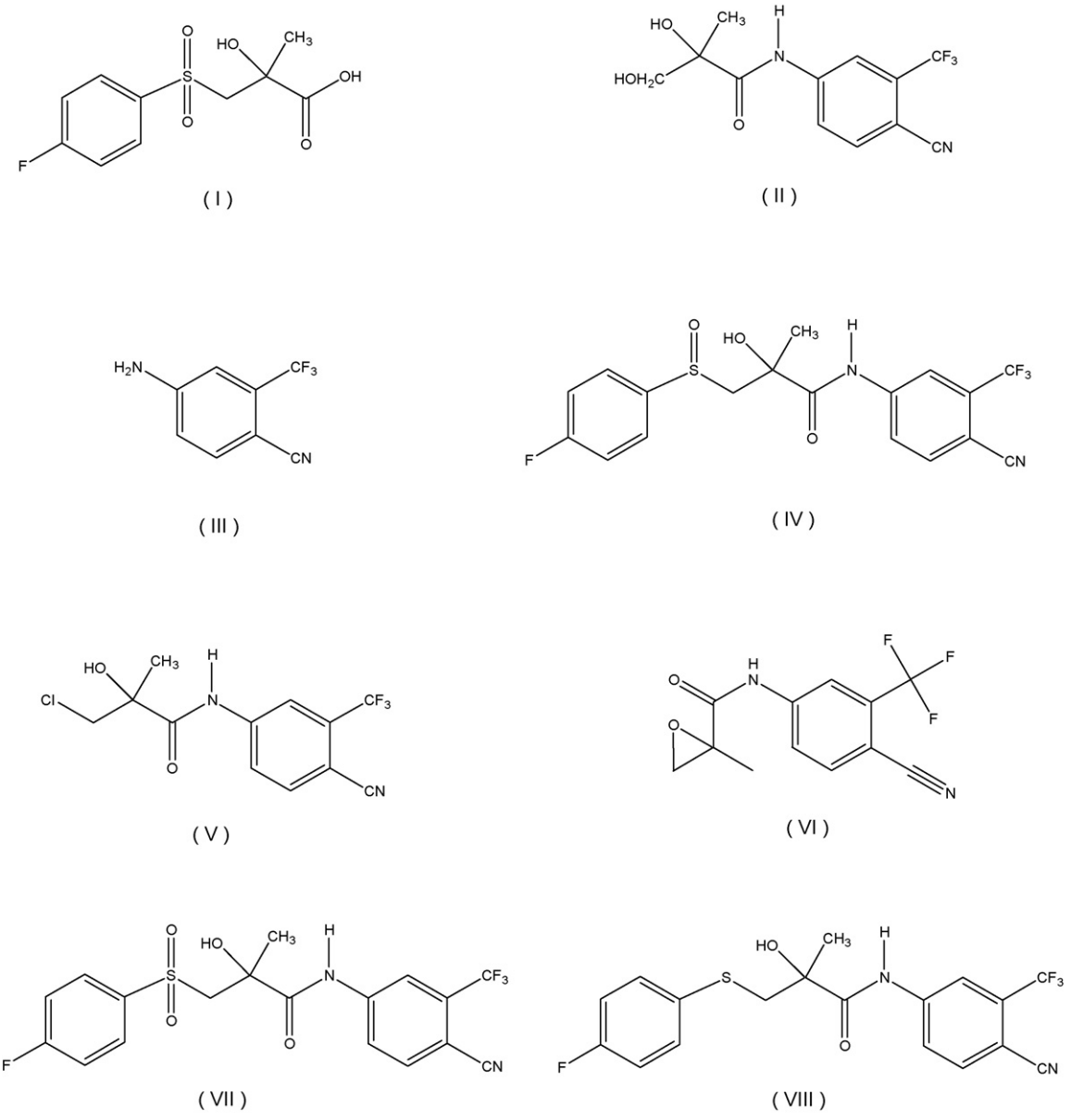

Fig. 1. Chemical structures of BCT (VII), degradation products (I and III) and its process-related impurities (II, IV, V, VI and VIII).

under thermal, photo, acidic, alkaline and peroxide conditions. Two degradation products were formed under alkaline conditions where as Saravanan et al. [15] have reported only one of them. In the present study we also discussed the kinetics of degradation. While studying the synthetic process in our laboratory, we observed (II), (IV), V, VI and (VIII) (Fig. 1) as process related impurities which are likely to be present in the finished products. Two are unknown impurities (II and IV) and detected consistently in almost all the batches. The area percentage of impurities was in the range from 0.03 to 0.1%. The impurity profile study has to be carried out for any final product to identify and characterize all the unknown impurities that are present at a level of even below 0.05% [16]. The requirement of identifying and characterizing the impurities in the final product is extremely important in the wake of stringent requirements from the regulatory authorities. Two process impurities (II and IV) and the alkaline degradation products (I and III) were isolated and characterized by ESI-MS/MS, ^{1}H NMR and FT-IR spectral data. The ESI-MS/MS profiles of BCT and all impurities were discussed during characterization.

2. Experimental

2.1. Materials and reagents

All the reagents were of analytical-reagent grade unless stated otherwise. Glass-distilled and de-ionized water (Nanopure, Barnsted, USA), HPLC-grade acetonitrile, methanol, ammonium acetate, potassium dihydrogen phosphate, phosphoric acid, hydrochloric acid, sodium hydroxide, hydrogen peroxide, chloroform, ethyl acetate and hexanes (S.D. Fine Chem., Mumbai, India) were used. $CDCl_3$ and DMSO were purchased from

Sigma–Aldrich, USA. Silica gel mesh 60–120 (S.D. Fine Chem., Mumbai, India) was used for preparing the column for isolation of impurities. The investigated samples of BCT bulk drug materials, crude samples and impurities were obtained from Organic Chemistry Department of IICT, Hyderabad, India.

2.2. Mass spectrometry

The experiments were performed using a commercial LCQ ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA), equipped with an ESI source. The data acquisition was carried out using Xcalibur software. The typical source conditions were: spray voltage, 4 kV; capillary voltage, 15–20 V; heated capillary temperature, 200 °C; tube lens offset voltage, 20 V; sheath gas (N_2) flow rate, 15 units; and helium was used as damping gas and the collision energies were 20–40 eV.

2.3. ^1H NMR spectroscopy

^1H NMR measurements were performed on a Varian Gemini 2000 model 200 MHz instrument at 25 °C. The ^1H NMR chemical shift values were reported on the δ scale in ppm, relative to TMS ($\delta = 0.00$) as internal standard.

2.4. FT-IR spectroscopy

The IR spectra for BCT, degradation products and process impurities were recorded in the solid state as KBr dispersion using PerkinElmer 1650 FT-IR spectrophotometer.

2.5. High performance liquid chromatography

The HPLC system was composed of two LC-10AT VP pumps, an SPD-M10AVP photo diode array detector, SIL-10AD VP auto injector, a DGU-12A degasser and SCL-10A VP system controller (all from Shimadzu, Kyoto, Japan). The chromatographic and the integrated data were recorded using a HP-Vectra (Hewlett Packard, Waldbron, Germany) computer system. The chromatographic columns used in the present work were

1. BDS Hypersil C_{18} (250 mm × 4.6 mm) 5 μm (Thermo Electron Corporation, Runcorn, UK).
2. Inertsil ODS 3V (250 mm × 4.6 mm) 5 μm (G.L. Sciences, Japan).
3. Kromasil KR100-5C_{18} (250 mm × 4.6 mm) 5 μm (Eka Chemicals AB, Sweden).
4. Symmetry C_{18} (250 mm × 4.6 mm) 5 μm (Waters, USA).

2.5.1. Chromatographic conditions

The analysis was carried out on Symmetry C_{18} (250 mm × 4.6 mm i.d.; particle size 5 μm) column using the mobile phase consisting of 0.01 M KH_2PO_4 (pH adjusted to 3.0 with dilute H_3PO_4) and acetonitrile (50:50 v/v). Before delivering the mobile phase in to the system, it was degassed and filtered through 0.45 μm PTFE filter using vacuum. The flow rate was kept constant at 1.0 ml/min and column was maintained at room temperature. The injection volume was 20 μl and the detection was performed at 215 nm using a photo diode array detector.

2.5.2. Semi-preparative LC conditions

Separation and isolation of the degradation products was carried out on a semi-preparative Inertsil ODS 3V (10 mm × 250 mm; particle size 5 μm) LC column using 0.05 M ammonium acetate:acetonitrile (50:50 v/v) as a mobile phase at a flow rate of 4.0 ml/min and the detector was maintained at 215 nm.

2.6. Analytical procedures

Solutions (2.0 mg/ml) of BCT, its process impurities and degradation products were prepared by dissolving known amounts of the components in the methanol first and diluted up to the mark with the mobile phase. The solutions were adequately diluted to determine the accuracy, precision, linearity and limits of detection and quantification. The specification concentration of BCT was taken as 200 μg/ml.

3. Results and discussion

The chemical structures of BCT (VIII), degradation products (I and III) and all its process-related substances (II, IV, V, VI and VIII) are shown in Fig. 1. The compounds (II and IV) are synthetic by-products and (V, VI, and VIII) are precursor impurities generated during the synthesis of BCT (Fig. 2). The present study was aimed at developing a chromatographic method for separation and quantitative determination of BCT and its impurities. Further studies were carried out to characterize the process impurities (II and IV) and degradation products (I and III) of BCT.

3.1. Forced degradation of BCT

BCT solutions (200 μg/ml) were prepared with mobile phase as described in Section 2.6 and forced degradation studies were carried out under (i) UV light at 254 nm, 60 °C temperature for 15 days and (ii) extreme conditions such as 0.2–1.0N HCl, 0.05–0.5N NaOH, and 3% H_2O_2. Under UV light, thermal and acidic conditions and in the presence of peroxide, no change in the sample purity was observed, but in alkaline conditions two degraded products (I and III) were formed and well separated from BCT under the present conditions (Fig. 11B) where as Saravanan et al. [15] had reported only one of them (III). Further studies were carried out on the effect of concentration of the NaOH and temperature on kinetics of degradation.

3.1.1. Kinetics of degradation

A series of solutions at a concentration of 200 μg/ml of BCT were prepared in 10 ml standard flasks having a concentration of 0.05, 0.1, 0.2, 0.3, 0.5N NaOH and the temperature was maintained at 30 °C. The analysis was carried out at regular intervals of time for the above solutions. With increasing the base concentration, the rate of degradation was also increased (Fig. 3A). Then effect of temperature on rate of degradation was studied in

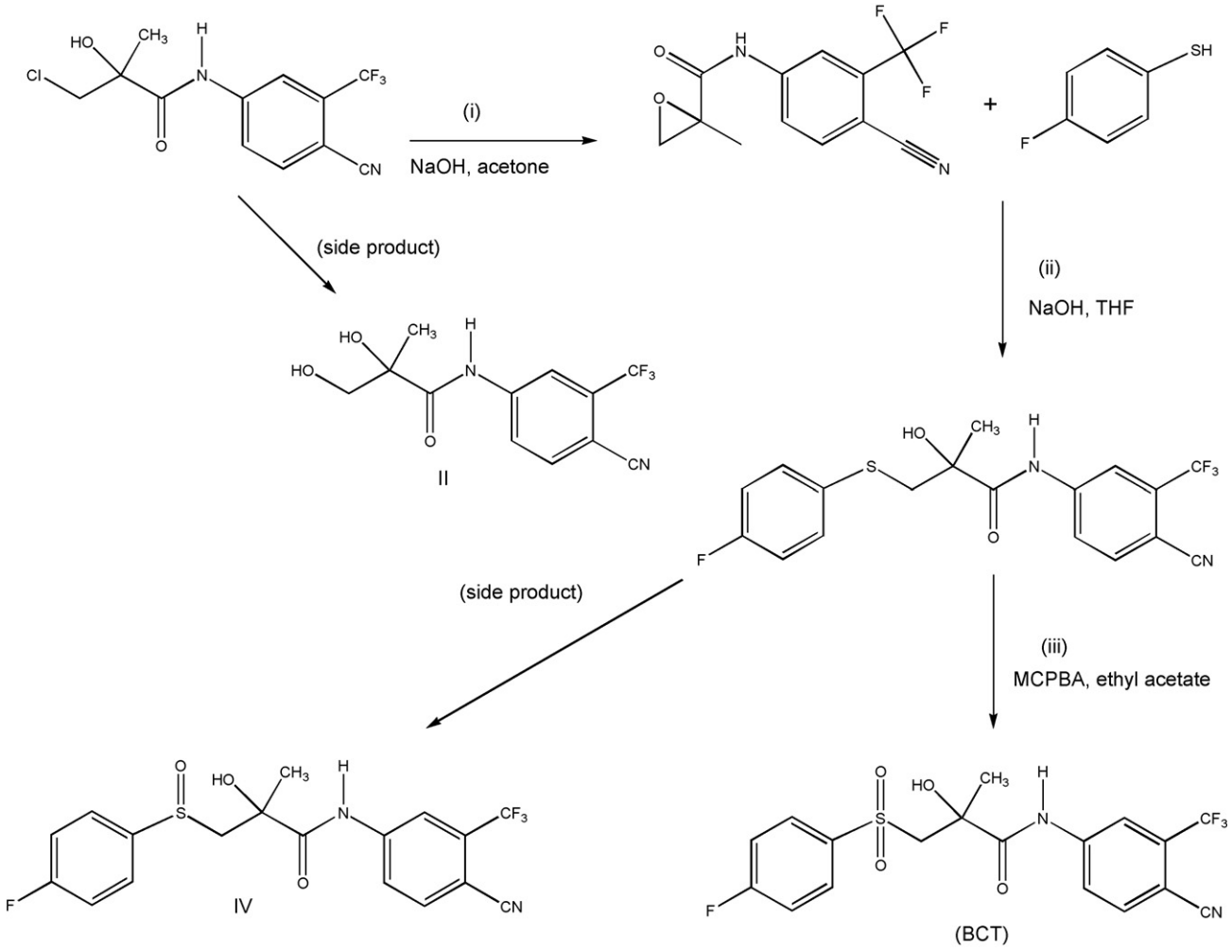

Fig. 2. The scheme of reactions involved in the synthesis of BCT.

the range 30–60 °C at 0.1N NaOH and with increasing the temperature rate of degradation increased (Fig. 3B). Using method of initial rates, order of degradation reaction was measured. Initial rates of degradation reaction was measured at different concentrations of NaOH (0.1N and 0.2N) and BCT (200 μg/ml and 400 μg/ml) as given in Table 1.

$$\text{Rate of reaction } (r) = k[\text{BCT}]^a[\text{NaOH}]^b \text{ mol L}^{-1}\text{min}^{-1}$$

where r is rate of reaction and k is reaction constant. [BCT] and [NaOH] are concentrations of BCT and NaOH, respectively. a and b are order of reaction with respect to BCT and NaOH, respectively. According to method of initial rates the reaction rates were measured by keeping the concentration of NaOH constant and varying the concentration of BCT as shown in experiments 1 and 2 (Table 1) and then the ratio of reaction rates were determined as given below

$$\frac{13.50 \times 10^{-11}}{6.50 \times 10^{-11}} = \frac{k[9.302 \times 10^{-7}]^a \times [0.1]^b}{k[4.651 \times 10^{-7}]^a \times [0.1]^b}$$
$$2.07 = [2]^a$$

From above equation a is approximately equal to 1, so order of reaction with respect to BCT is one. Similarly, reaction rates were measured by keeping the concentration of BCT constant and varying the concentration of NaOH as shown in experiments 2 and 3 (Table 1) and then the ratio of reaction rates were

Table 1
Rates of degradation reaction of BCT at different conditions

Experiment	Initial concentration of BCT (mol L^{-1})	Initial concentration of NaOH (mol L^{-1})	Rate of reaction (mol L^{-1} min^{-1})
1	4.651×10^{-7}	0.1	6.50×10^{-11}
2	9.302×10^{-7}	0.1	13.50×10^{-11}
3	4.651×10^{-7}	0.2	14.50×10^{-11}

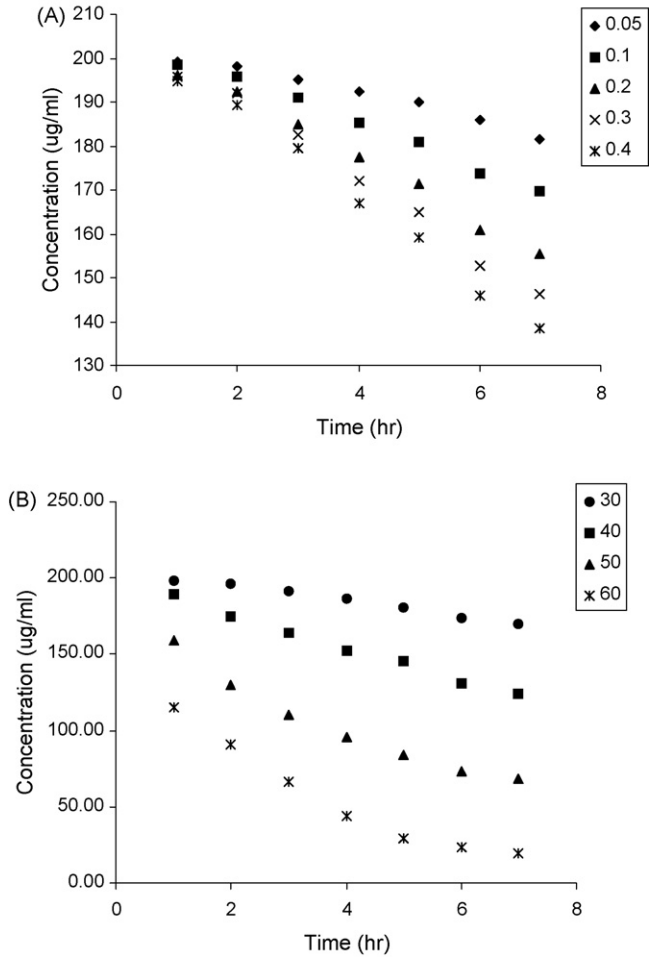

Fig. 3. The effect of (A) concentration of NaOH and (B) temperature on the degradation of BCT.

determined as given below

$$\frac{14.50 \times 10^{-11}}{6.50 \times 10^{-11}} = \frac{k[4.651 \times 10^{-7}] \times [0.2]^b}{k[4.651 \times 10^{-7}] \times [0.1]^b}$$

$$2.23 = [2]^b$$

From above equation b is approximately equal to 1, so order of reaction with respect to NaOH is one. So overall order of alkaline degradation reaction of BCT is two.

3.2. Isolation and characterization of the degradation products and synthetic by-products

BCT (250 mg) dissolved in 10 ml methanol and 5 ml 0.1N NaOH was subjected to alkaline degradation at 40 °C temperature for 24 h. About 70% of BCT was degraded and the degradation product was isolated by semi-preparative LC, using the conditions described in Section 2.5.2. The retention times of BCT and the degradation products (I and III) were 12, 3.5 and 7 min, respectively. The collected fractions of the individual degradation products I and III were concentrated separately under high vacuum on a rotavapor and the organic solvent was stripped off. The aqueous layer of degradation product III was subjected to solvent–solvent extraction with ethyl acetate and the compound was extracted into organic layer. The ethyl acetate fractions were pooled together and concentrated on rotavapor under vacuum. The aqueous layer of degradation product I was subjected to lyophilization and removed. The chromatographic purity of the degradation products were tested by analytical LC and found to be >97.0%, indicating that the fractions were quite stable during isolation. The process impurities II and IV were isolated from crude samples by column chromatography. The crude samples were loaded on to a open tubular column packed with silica gel and run with mobile phases consisting of hexane, ethyl acetate and chloroform. The fractions were collected at regular intervals of time and each fraction was analyzed by HPLC to ensure the purity. The collected fractions were pooled together separately and concentrated under high vacuum on a rotavapor and the organic solvent was stripped off. The chromatographic purity of the isolated impurities II and IV was >95%, indicating that the fractions were stable during isolation. The isolated compounds were subjected to analysis by ESI-MS/MS, ^{1}H NMR and FT-IR spectroscopy and characterized.

3.2.1. Structure elucidation of the degradation products I and III

The spectral data of degraded products was compared with that of BCT. The FT-IR spectral data of BCT and all its impurities was given in Table 2. The FT-IR spectrum of degradation product I exhibited a broad absorption band at 3350 cm^{-1}, indicating the presence of an acid functionality. Degradation product III had shown two stretching bands at 3485 and 3360 cm^{-1} adjacent to each other indicating presence of a primary amine functional group. The two degradation products I and III were subjected to ESI-MS mass spectral analysis (negative mode). The degraded product I showed a molecular ion at m/z 259 amu (atomic mass units) and III showed a molecular ion at m/z 185 amu. It suggested the formation of an acid and an amine by the hydrolysis of BCT. To get the structural information I and III were further subjected to ESI-MS/MS analysis (Fig. 4A and C). Degradation

Table 2
FT-IR spectral data of BCT (VII), degradation products (I, III) and process impurities (II, IV, V, VI, VIII)

Sample	IR (KBr) (cm^{-1})
I	3445, 1728, 1658, 1593, 1495, 1317, 1292, 1145, 1085, 840, 576, 510
II	3422, 3315, 2924, 2231, 1743, 1519, 1458, 1327, 1175, 1139, 1050, 759
III	3485, 3360, 2217, 1614, 1508, 1466, 1360, 1277, 1181, 1119, 1042
IV	3311, 2924, 2230, 1694, 1586, 1520, 1427, 1326, 1231, 1178, 1137, 1087, 1049, 1018, 835
V	3484, 3332, 2923, 2231, 1691, 1583, 1520, 1426, 1325, 1177, 1134, 1050, 840, 554
VI	3343, 2925, 2227, 1703, 1587, 1524, 1428, 1326, 1175, 1134, 1049, 847, 750
VII	3577, 3338, 3112, 2229, 1690, 1592, 1498, 1328, 1288, 1240, 1178, 1135, 1081, 1052, 577, 509
VIII	3444, 3338, 2232, 1699, 1522, 1488, 1324, 1225, 1178, 1142, 1086, 1046, 824

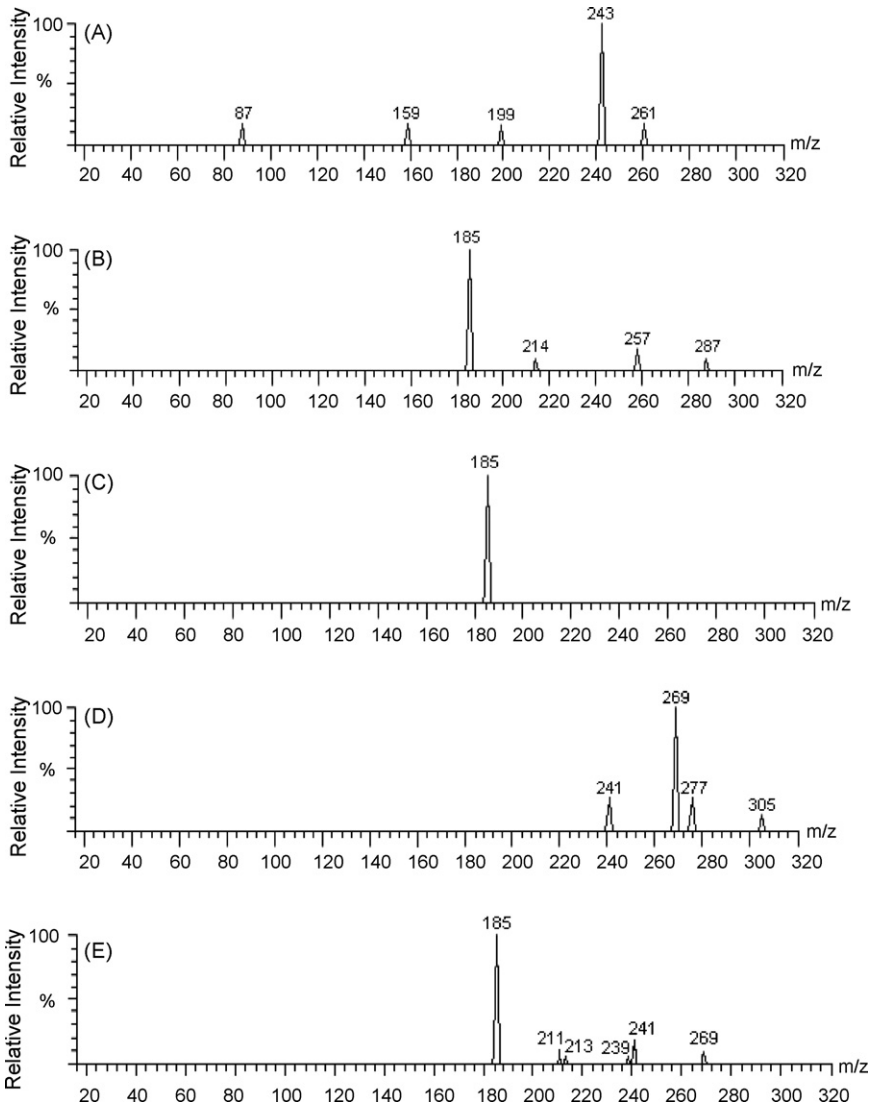

Fig. 4. ESI-MS/MS spectra of (A) I, (B) II, (C) III, (D) V and (E) VI.

Table 3
^{1}H NMR assignments for BCT (VII) and degradation products (I, III) and process impurities (II, IV, V, VI, VIII)

Sample	Solvent	^{1}H NMR assignments with respective to TMS (δ ppm)[a]
I	D_2O	6.65–6.55 (m, 2H, Ar–H), 6.05–5.95 (m, 2H, Ar–H), 2.57 (d, 1H, J = 14.92 Hz, SO–CHH$_a$–C), 2.38 (d, 1H, J = 14.92 Hz, SO–CHH$_b$–C), 1.98 (s, 3H, CH$_3$–C–CO)
II	$CDCl_3$	8.98 (brs, 1H, N–H), 8.10–8.0 (m, 2H, Ar–H), 7.82 (d, 1H, J = 9.07 Hz, Ar–H), 3.98 (d, 1H, J = 11.09 Hz, OH–CHH$_a$–C), 3.52 (d, 1H, J = 11.09 Hz, OH–CHH$_b$–C), 3.15 (brs, 1H, OH–C–CO), 2.42 (brs, 1H, OH–C–CO), 1.59 (s, 3H, CH$_3$–C–CO)
III	$CDCl_3$	7.55 (d, 1H, J = 8.31 Ar–H), 6.95 (d, 1H, J = 2.27 Hz, Ar–H), 6.79 (dd, 1H, J = 8.31, 2.27 Hz, Ar–H), 4.42 (brs, 2H, Ar–NH$_2$)
IV	$CDCl_3$	9.47 (brs, 1H, N–H), 8.26 (d, 1H, J = 1.563 Hz, Ar–H), 8.05–7.80 (m, 2H, Ar–H), 7.75–7.65 (m, 2H, Ar–H), 7.40–7.20 (m, 2H, Ar–H), 6.05 (brs, 1H, HO–C–CO), 3.50 (d, 1H, J = 13.28 Hz, SO–CHH$_a$–C), 3.03 (d, 1H, J = 13.28 Hz, SO–CHH$_b$–C), 1.29 (s, 3H, CH$_3$–C–CH$_2$)
V	$CDCl_3$	8.94 (brs, 1H, N–H), 8.05–7.95 (m, 2H, Ar–H), 7.80 (d, 1H, J = 9.07 Hz, Ar–H), 4.09 (d, 1H, J = 11.33 Hz, Cl–CHH$_a$–C), 3.69 (d, 1H, J = 11.33 Hz, Cl–CHH$_b$–C), 3.10 (brs, 1H, OH–C–CO), 1.59 (s, 3H, CH$_3$–C–CO)
VI	$CDCl_3$	8.33 (brs, 1H, N–H), 7.99–7.91 (m, 2H, Ar–H), 7.80 (d, 1H, J = 8.39 Hz, Ar–H), 2.97 (s, 2H, Cl–CH$_2$–C), 1.67 (s, 3H, CH$_3$–C–CH$_2$Cl)
VII	DMSO-d_6	10.28 (brs, 1H, N–H), 8.42 (s, 1H, J = 1.563 Hz, Ar–H), 8.20 (d, 1H, J = 8.443 Hz, Ar–H), 7.96–7.89 (m, 3H, Ar–H), 7.35–7.22 (m, 2H, Ar–H), 6.40 (brs, 1H, HO–C–CO), 3.93 (d, 1H, J = 14.784 Hz, SO–CHH$_a$–C), 3.60 (d, 1H, J = 14.784 Hz, SO–CHH$_b$–C), 1.45 (s, 3H, CH$_3$–C–CO)
VIII	$CDCl_3$	8.92 (brs, 1H, N–H), 7.95–7.7 (m, 3H, Ar–H), 7.45–7.30 (m, 2H, Ar–H), 6.95–6.80 (m, 2H, Ar–H), 3.73 (d, 1H, J = 14.57 Hz, SO–CHH$_a$–C), 3.52 (s, 1H, HO–C–CO), 3.08 (d, 1H, J = 14.57 Hz, SO–CHH$_b$–C), 1.45 (s, 3H, CH$_3$–C–CO)

[a] TMS, tetramethyl silane; ppm, parts per million; d, doublet; m, multiplet; brs, broad singlet; dd, doublet of a doublet; J, coupling constant. The ^{1}H chemical shift values were reported on the δ scale in ppm, relative to TMS (δ = 0.00) as internal standard.

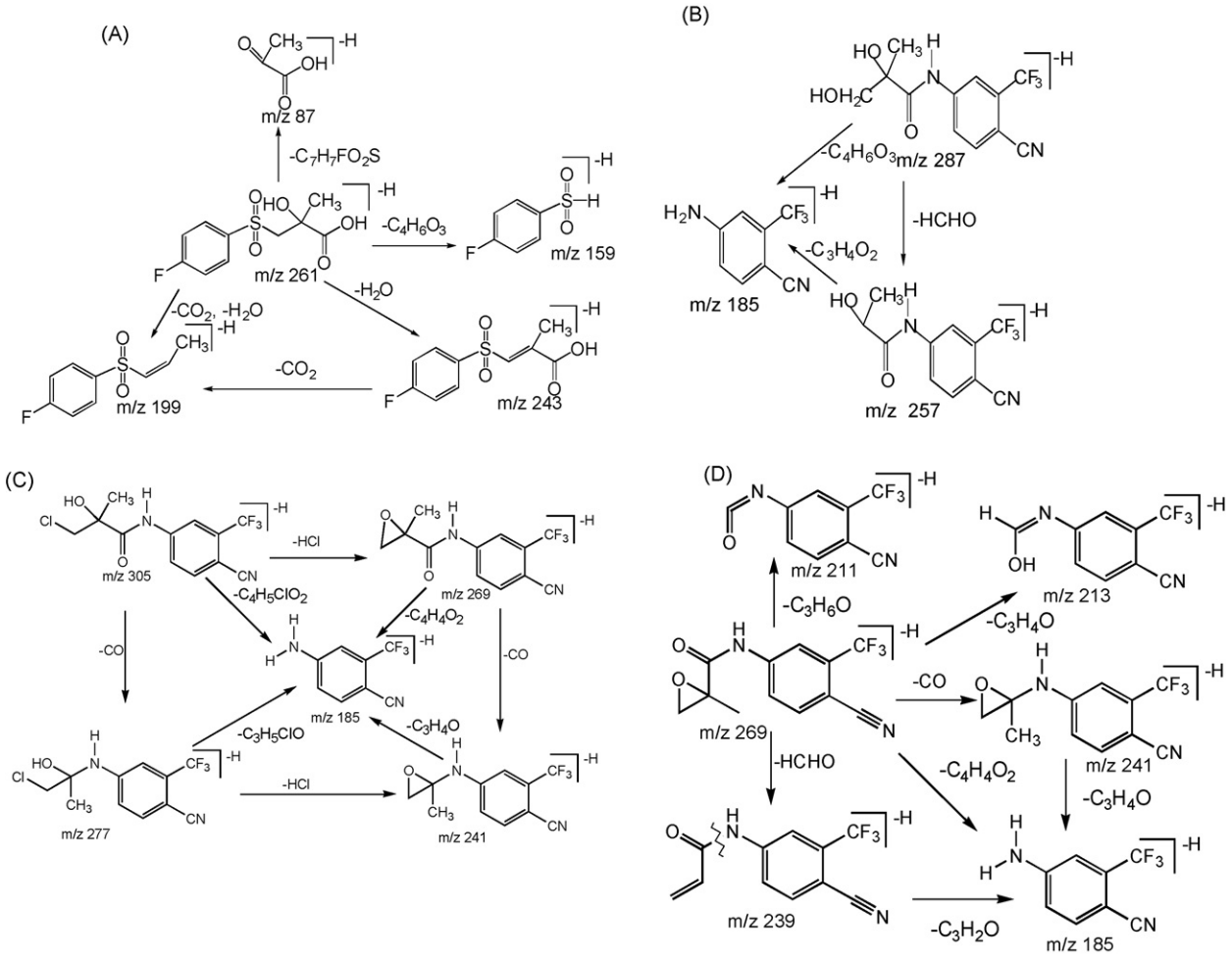

Fig. 5. ESI-MS/MS fragmentation patterns for (A) I, (B) II, (C) V and (D) VI.

product III had not shown any further fragmentation and the mass fragmentation path way for I was given in Fig. 5A. In the ESI-MS/MS spectra the peak at *m/z* 243 corresponds to loss of H_2O from $[M-H]^-$ ions, peak at *m/z* 199 corresponds to the loss of CO_2 and H_2O from $[M-H]^-$ ions or loss of CO_2 from *m/z* 243. The peak at *m/z* 159 corresponds to loss of $C_4H_6O_3$ and *m/z* 87 corresponds to loss of $C_7H_7FO_2S$ form $[M-H]^-$ ions. The ^{1}H NMR spectral data of BCT and all impurities was given in Table 3 and the ^{1}H NMR spectra of I and III are given in Fig. 6A and B, respectively. The ^{1}H NMR spectra of I has four protons in aromatic region and two aliphatic protons attached to same carbon atom beside a chiral centre with coupling constant 14.92 Hz and the three aliphatic protons corresponds to methyl group. The ^{1}H NMR spectra of III has three protons in aromatic region and a broad signal at 4.2 ppm equivalent to two protons corresponds to NH_2 group. From the above spectral information the structures of compounds I and III were conformed as 3-(4-fluoro-benzenesulfonyl)-2-hydroxy-2-methyl-propionic acid and 4-amino-2-fluoromethyl-benzenonitrile, respectively. The degradation product III matched with the impurity-A reported by Saravanan et al. [15]. The mechanism of degradation of BCT is given in Fig. 7.

3.2.2. Structure elucidation of impurity II

The spectral data of impurity II was similar to known impurities V and VI. The ESI-MS (negative mode) mass spectrum of impurity II exhibited molecular ion at *m/z* 287 amu which was less by 18 amu than that of V (*m/z* 305) and 18 amu more than that of VI (*m/z* 269). Interestingly, the characteristic $M+2$ molecular ion that was due to the presence of one chlorine atom in V was absent in the mass spectrum of impurity II, indicates absence of chlorine atom. This data indicates presence of additional OH functionality in impurity II. For structural information ESI-MS/MS (negative mode) analysis was carried out (Fig. 4B, D and E) and fragmentation path way for II, V and VI were quite different as given in Fig. 5B, C and D, respectively. In addition to this observation, the IR spectra of II showed absorption band at 3350 cm^{-1} was more intense than that of V. This indicates presence of an additional OH group in impurity II in place of chlorine atom. Compared to impurity V the ^{1}H NMR spectrum of impurity II has an additional broad signal (at 2.42 ppm) due to second OH group proton was observed (Fig. 6C). From all above spectral information the structure of II was conformed as *N*-(4-cyano-3-trifluoromethyl-phenyl)-2,3-dihydroxy-2-methyl-propionamide.

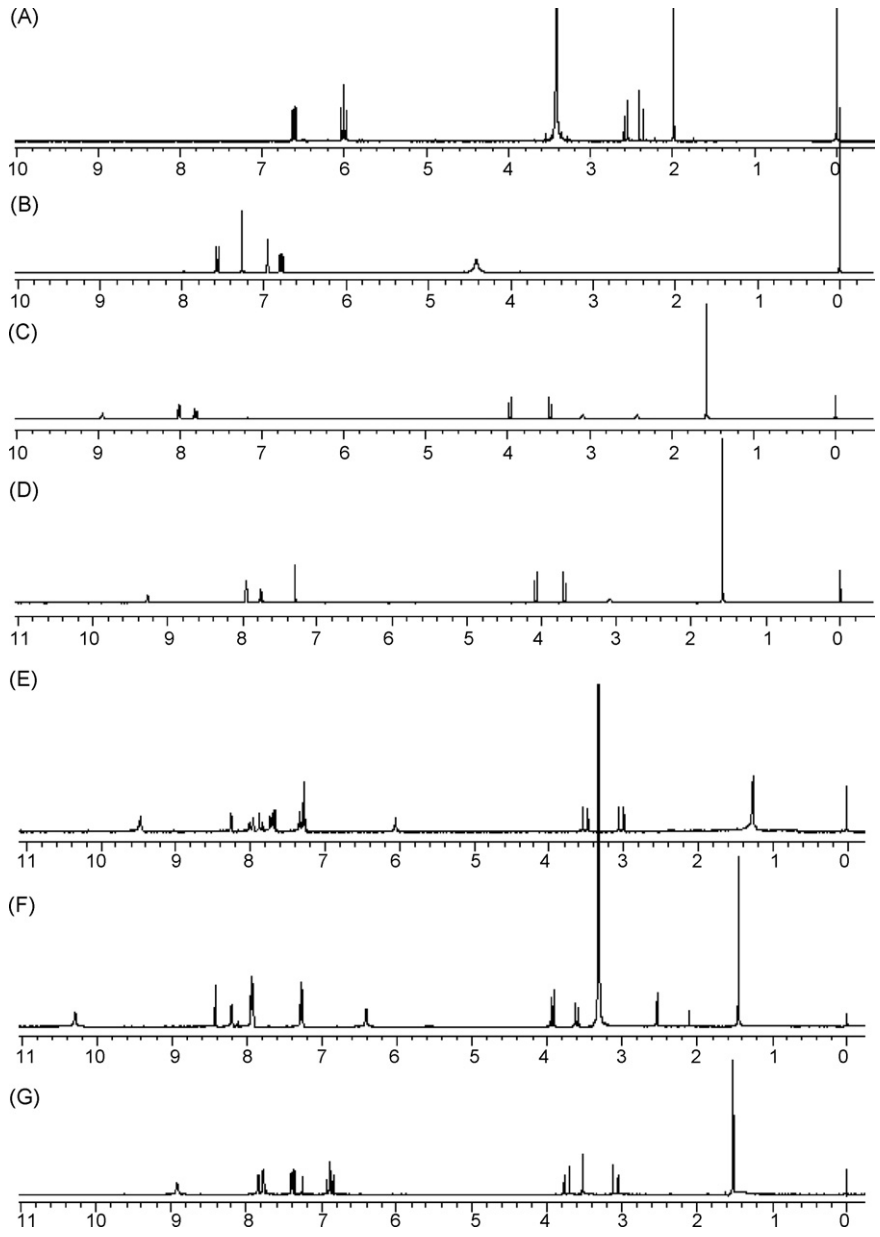

Fig. 6. The ^{1}H NMR spectra of (A) I (D_2O), (B) III ($CDCl_3$), (C) II ($CDCl_3$), (D) V ($CDCl_3$), (E) IV ($CDCl_3$), (F) VII (DMSO-d_6) and (G) VIII ($CDCl_3$).

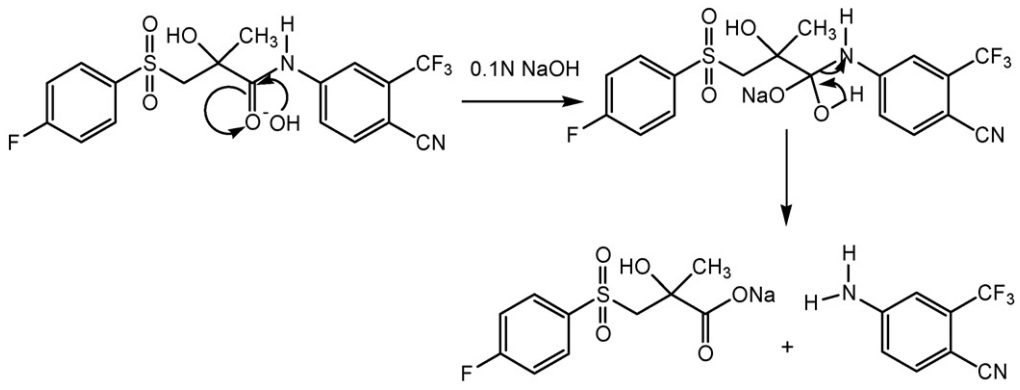

Fig. 7. The degradation of BCT by alkaline hydrolysis.

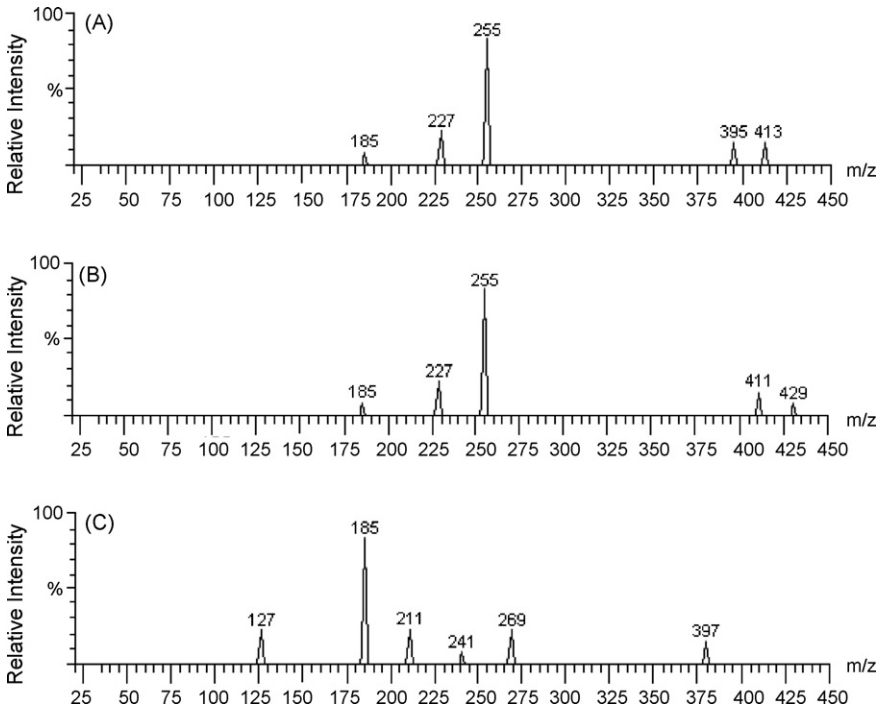

Fig. 8. ESI-MS/MS spectra of (A) IV, (B) BCT (VII) and (C) VIII.

3.2.3. Structure elucidation of process impurities IV

The spectral data of impurity IV was similar to BCT and VIII. The ESI-MS (negative mode) mass spectrum of impurity IV exhibited molecular ions at *m/z* 413 which was less by 16 amu than that of BCT (*m/z* 429) and 16 amu more than VIII (*m/z* 397). This suggests IV has one oxygen atom less than BCT and one more than VIII and it may be a sulphoxide impurity. To get structural information mass fragmental studies were taken up for IV, BCT and VIII (Fig. 8A, B and C) and the mass fragmentation path ways were given in Fig. 9. Loss of H_2O and formation of *m/z* 185 fragment ion is common for all the three compounds. Fragment ion 255 is observed for IV and VII and S-C cleavage followed by hydrogen transfer to give fragment ion 127 and 269 is occurred for VIII. The FT-IR spectral data of IV is similar to VII and VIII. The bands due to sulphone group (1288 and 1240) are absent in IV and an additional band at 1018 is observed due to presence of a sulphoxide group (Table 2). The ^{1}H NMR spectra of IV, BCT (VII) and VIII are given in Fig. 6E, F and G, respectively. The ^{1}H NMR spectra were similar but small variations in chemical shifts were observed (Table 3). From all above spectral information the structures of IV was confirmed as *N*-(4-cyano-3-trifluoromethyl-phenyl)-3-(4-fluoro-benzene sulfinyl)-2-hydroxy-2-methyl-propionamide. The process impurity IV matched with the impurity-B reported by Saravanan et al. [15].

3.3. Optimization of the chromatographic conditions

In preliminary experiments, BCT and all the impurities were subjected to separation by reverse phase HPLC, on different commercial C_{18} columns using H_2O and acetonitrile and methanol as organic modifiers. Generally the selection of the column depends on the characteristics of the compounds to be separated. Depending on the solubility of BCT and its impurities a C_{18} column was more preferred to a C_8. The peaks corresponding to BCT and all impurities were very broad and separation was poor. Peak shapes were improved when 0.01 M KH_2PO_4 buffer was used in place of H_2O, so it was chosen as a buffering system. The HPLC conditions were optimized to study the effects of different columns, concentration of organic modifiers and concentration and pH of buffer on separation of BCT and impurities.

3.3.1. Column selectivity

Four different C_{18} columns as described in Section 2.5 were evaluated. The selectivity and resolution [17] of all compounds on the columns is described in Table 4. It could be seen from Table 4 that on Symmetry C_{18}, all compounds were well separated with good peak shapes allowing accurate determination of all the compounds. So it was chosen for further development.

3.3.2. Effect of organic modifier

The separation of impurities IV, V, VI and BCT (VII) became important as they eluted very close to each other. With methanol, baseline noise and retention times were higher and separation was poor. When acetonitrile was used as organic modifier peaks became sharp and resolution was improved. Further studies were performed on effect of concentration of acetonitrile on separation and retention behavior of all compounds. As the concentration of acetonitrile was decreased, retention was increased for all compounds (Fig. 10A) and resolution increased for all compounds except for (IV, V) and (V, VI) (Fig. 10B). At 50% of acetonitrile good separation ($R_s > 2.0$) was observed for all

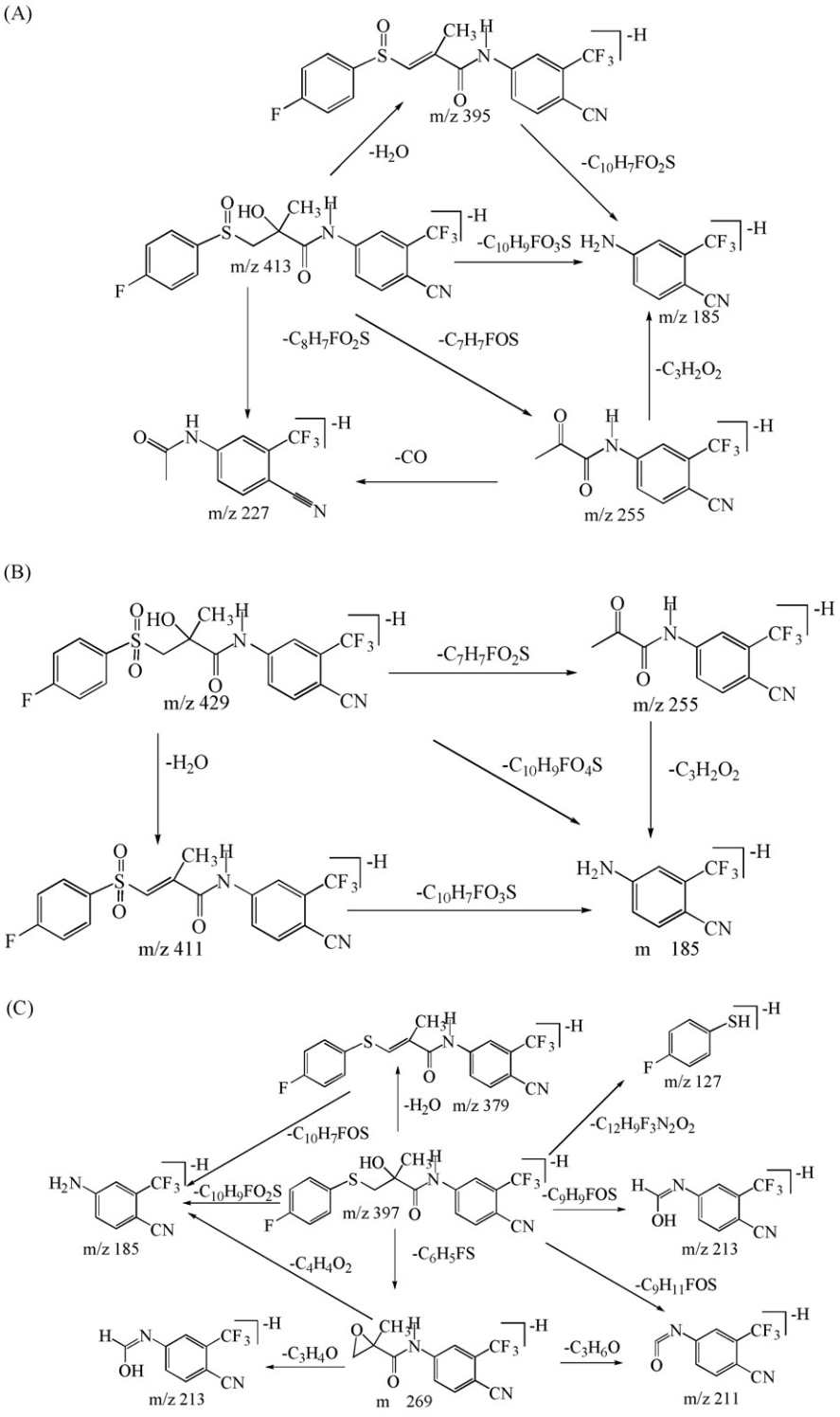

Fig. 9. ESI-MS/MS fragmentation patterns for (A) IV, (B) BCT (VII) and (C) VIII.

compounds with minimum analysis time. So, it was optimized for further development.

3.3.3. *Effect of buffer pH*

Further studies were carried out on the effect of buffer pH and concentration on resolution and retention. Buffer concentration had no effect on retention and resolution. So 0.01 M KH_2PO_4 was chosen as a buffer system. The pH of the buffer has not shown much effect on both retention and resolution (Table 5). For compounds (I, II), (II, III) and (V, VI) resolution was decreased while for remaining compounds it was slightly increased with decreasing pH of the buffer. Especially for

Table 4
Selectivity of C_{18} columns of different manufacturers in the optimized conditions

Column	Compounds	k'	α	R_s	A_s
Hypersil BDS (250 mm × 4.6 mm) 5 μm	I	0.134	4.701	7.987	1.963
	II	0.630	2.035	6.526	1.210
	III	1.282	1.263	2.955	1.261
	IV	1.619	1.275	3.544	1.224
	V	2.065	1.108	1.650	1.241
	VI	2.288	1.079	1.268	1.276
	VII	2.469	2.531	18.155	1.216
	VIII	6.249	–	–	1.191
Inertsil ODS (250 mm × 4.6 mm) 5 μm	I	0.345	3.261	10.23	1.612
	II	1.125	2.364	13.27	1.268
	III	2.648	1.221	3.951	1.118
	IV	3.232	1.293	5.456	1.099
	V	4.178	1.111	2.455	1.088
	VI	4.642	1.097	2.194	1.077
	VII	5.094	2.500	22.53	1.081
	VIII	12.736	–	–	1.059
Kromasil KR100-5C_{18} (250 mm × 4.6 mm) 5 μm	I	0.217	3.046	6.570	1.060
	II	0.661	2.756	12.97	1.220
	III	1.822	1.245	3.860	1.174
	IV	2.269	1.377	6.350	1.141
	V	3.124	1.133	2.780	1.123
	VI	3.541	1.079	1.719	1.108
	VII	3.821	2.733	24.56	1.107
	VIII	10.444	–	–	1.075
Symmetry C_{18} (250 mm × 4.6 mm) 5 μm	I	0.230	3.161	8.052	1.050
	II	0.727	2.398	12.593	1.196
	III	1.743	1.264	4.614	1.084
	IV	2.204	1.322	6.167	1.054
	V	2.913	1.109	2.509	1.040
	VI	3.230	1.096	2.266	1.029
	VII	3.539	2.592	25.534	1.030
	VIII	9.172	–	–	1.005

k': retention factor; α: selectivity; R_s: resolution; A_s: tailing factor.

impurity-I retention (0.112–0.230) and peak shape had increased as pH was decreased. At higher pH quantitation of impurity-I was problematic as it was eluting very close to dead volume. But at pH 3.0 it was eluted away from dead volume ($k' = 0.230$). Further at pH 3.0 all compounds were well separated ($R_s > 2.0$) with good peak shapes, so it was optimized.

Finally, separation was carried out on Symmetry C_{18} column maintained at room temperature with mobile phase consisting of 0.01 M KH_2PO_4 (pH 3.0):acetonitrile (50:50 v/v) with PDA detector set at 215 nm. A typical chromatogram showing the separation of 2.5% (w/w) of each of the related substances spiked to BCT (VII) at the specified relative concentration of 200 μg/ml is given in Fig. 11A. It is clear from Fig. 11A that all the compounds were eluted and separated with good peak shapes and resolution. In the present study a simple buffer 0.01 M KH_2PO_4 (pH 3.0) was used in an isocratic mode for the separation. While the method reported by Saravanan et al. was a gradient method and an ion pair reagent tetra *n*-butyl ammonium hydrogen sulphate was used as a mobile phase solvent [15]. Use of ion pair reagent decreases not only the column life time but also increases the time required for equilibration and an ion pair reagent in a gradient elution mode is not preferred because of system artifacts. The developed method was validated in terms of accuracy, precision and linearity as per ICH guidelines.

Table 5
The effect of pH on retention and resolution in the optimized conditions

pH	Retention factor (k')								Resolution (R_s)							
	I	II	III	IV	V	VI	VII	VIII	I	II	III	IV	V	VI	VII	VIII
3	0.230	0.727	1.743	2.204	2.913	3.230	3.539	9.172	8.05	12.59	4.65	6.17	2.51	2.27	25.53	–
4	0.227	0.721	1.747	2.218	2.928	3.256	3.571	9.320	7.21	12.65	4.59	6.10	2.57	2.29	25.50	–
5	0.138	0.719	1.746	2.204	2.912	3.245	3.547	9.186	11.22	12.71	4.56	6.07	2.62	2.21	25.44	–
6	0.112	0.731	1.780	2.211	2.993	3.332	3.650	9.338	10.09	12.74	4.52	6.06	2.64	2.20	25.27	–

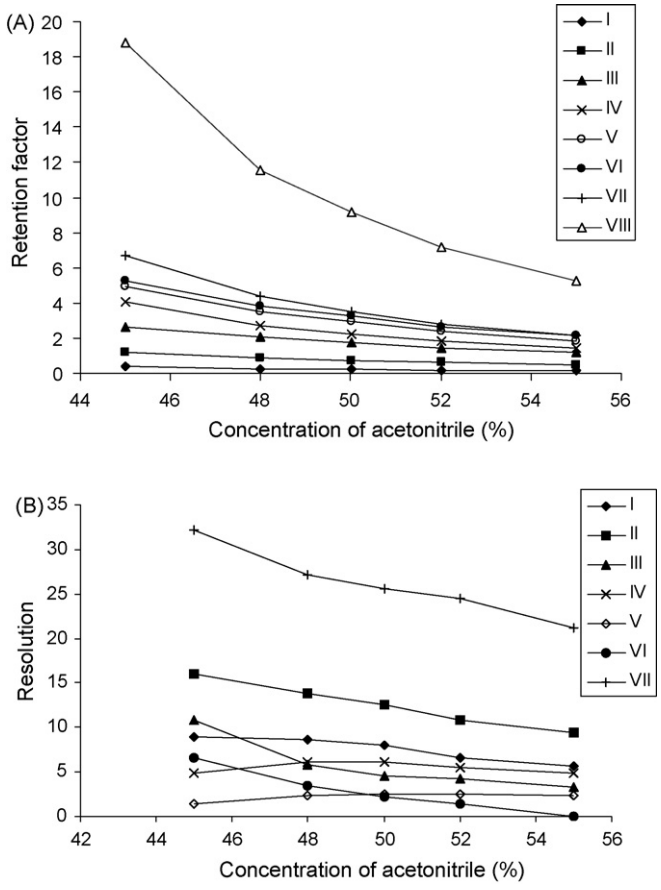

Fig. 10. The effect of organic modifier on (A) retention factors (k') and (B) resolution (R_s).

4. Validation

The developed RP-HPLC method was validated in terms of accuracy, precision and linearity.

4.1. Specificity

Specificity is the ability of the analytical method to measure the analyte concentration accurately in presence of all the potential impurities. For specificity determination, all the known impurities were added to BCT and the concentration of BCT was measured using the developed method. The assay of BCT for three determinations was found to be 99.94% with 0.041 %R.S.D., while in the presence of impurities (1.0%, w/w) it was 99.92% with %R.S.D. 0.06. It suggests that the assay results did not change in the presence of impurities. The specificity was checked by stressing the BCT under (i) UV light at 254 nm, 60 °C temperature for 15 days and (ii) extreme conditions such as 2N HCl, 0.5N NaOH, and 3% H_2O_2. Under UV light, thermal and acidic conditions and in the presence of peroxide, no change in the sample purity was observed, but in alkaline conditions two degraded products (I and III) were formed and well separated from BCT under the present conditions (Fig. 11B). In formulations, it was observed that the excipient peaks did not interfere with the peaks of interest (Fig. 11A) and were well separated from BCT and other impurities.

4.2. System suitability

The system suitability was conducted using 2.5% (w/w) of all the impurities spiked to BCT (200 μg/ml) and evaluated by making five replicate injections. The system was suitable for use if resolution for all compounds was not less than 2.27. When

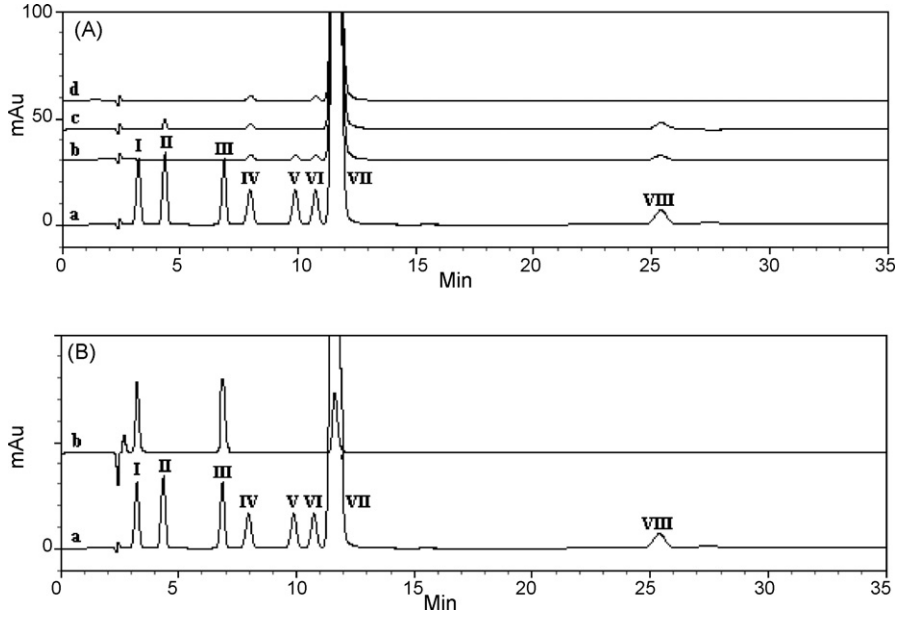

Fig. 11. Typical HPLC chromatograms of (A) (a) BCT (VII) (200 μg/ml) spiked with 2.5% (w/w) of each of the impurities (I–VI and VIII); (b) and (c) bulk drug samples from different manufacturers; (d) pharmaceutical formulation. (B) (a) BCT (VII) (200 μg/ml) spiked with 2.5% (w/w) of each of the impurities (I–VI and VIII); (b) degradation of BCT at 0.1N NaOH.

Table 6
System suitability data

Sample	t_R (min) (±S.D., $n=3$)	RRT	R_s	A_s	RRF
I	3.05 ± 0.05	0.271	8.05	1.06	0.715
II	4.28 ± 0.07	0.380	12.59	1.05	1.008
III	6.80 ± 0.11	0.604	4.65	1.08	1.346
IV	7.95 ± 0.14	0.706	6.17	1.05	1.049
V	9.70 ± 0.18	0.861	2.51	1.04	1.010
VI	10.49 ± 0.22	0.932	2.27	1.03	1.005
VII	11.26 ± 0.25	1.000	25.53	1.03	1.000
VIII	25.227 ± 0.41	2.241	–	1.01	1.062

t_R: retention time; RRT: relative retention time; R_s: resolution; A_s: tailing factor; RRF: relative response factor; S.D.: standard deviation.

synthetic mixtures and process samples were analyzed by the present method, a minimum resolution of 2.27 was observed for all impurities. This minimum resolution was taken as a system suitability parameter for the proposed method. The quantities of impurities and assay of BCT were calculated from their respective peak areas. System suitability data is given in Table 6.

4.3. Accuracy

The recoveries of impurities I, II, III, IV, V, VI and VIII were determined by spiking each impurity at five different levels ranging from 0.5–2.0 μg/ml to BCT at the specified level (200 μg/ml). The recovery range and R.S.D. for all impurities were found to be 96.31–103.54% and 0.44–2.87%, respectively (Table 7). Similarly, the accuracy in determination of the assay of BCT (VII) was checked at five concentration levels, i.e. 100, 150, 200, 250 and 300 μg/ml each in triplicate for 3 days and the percentage recoveries are recorded in Table 7. The R.S.D. values were found to be below 0.51%.

4.4. Precision

BCT was spiked with 0.5% (w/w) of each impurity and the precision of the method was tested by six ($n=6$) injections of above solution and the R.S.D. of retention time (t_R) and peak areas were determined. The R.S.D. ranged from 0.31 to 0.89%. The precision in determination of BCT and impurities was studied by repeatability, intermediate precision and reproducibility (ruggedness). Repeatability is the intra-day variation in assay obtained at different concentration levels, and is expressed in terms of R.S.D. calculated for each day. The R.S.D. values for impurities and BCT were found to be in the range 0.55–3.41% and 0.29–0.93%, respectively, indicating a good repeatability. The intermediate precision is the inter-day variation at the same concentration level determined on successive days. The inter-day variations calculated for five concentration levels from the above data of 3 days. The R.S.D. values for impurities and BCT were found to be in the range 1.32–3.85% and 0.25–0.33%, respectively, indicating a good intermediate precision. The ruggedness of the method is defined as the degree of reproducibility obtained by the analysis of the same sample under a variety of conditions at different labs, with different analysts using different instruments and different lots of reagents. The same samples of three concentrations were analyzed in triplicate on 2 days by another instrument (LC-10A Module HPLC system containing pump and UV–vis detector) by a different analyst with different lots of reagents and columns. The data obtained were within 2.0% R.S.D.

4.5. Linearity

The linearity of detector response to different concentrations of impurities was studied in the range from 0.5 to 5.0 μg/ml at 10 different levels. Similarly, the linearity of BCT was also studied by preparing standard solutions at 10 different levels ranging from 10 to 300 μg/ml. The data were subjected to statistical analysis using a linear-regression model; the regression equations and coefficients (r^2) are given in Table 8. The results have indicated good linearity.

4.6. Limits of detection and quantification

Limits of detection (LOD) and quantification (LOQ) represent the concentration of the analyte that would yield signal-to-noise ratios of 3 for LOD and 10 for LOQ, respectively. LOD and LOQ were determined by measuring the magnitude of analytical background by injecting blank samples and calculating the signal-to-noise ratio for each compound by injecting

Table 7
Recovery data

Sample	Recovery[a] (%)	R.S.D. (%)	Recovery[a] (%)	R.S.D. (%)	Recovery[a] (%)	R.S.D. (%)	Recovery[a] (%)	R.S.D. (%)	Recovery[a] (%)	R.S.D. (%)
Amount added (μg/ml)	0.5		0.7		1.0		1.5		2.0	
I	96.71	2.05	97.62	1.92	100.91	1.21	99.94	0.61	100.26	0.44
II	98.77	1.95	101.28	1.31	98.89	1.72	100.41	0.73	100.31	0.52
III	98.29	1.89	101.75	2.15	100.61	1.35	99.71	0.91	99.920.51	
IV	96.91	2.54	97.84	2.43	101.11	0.65	99.51	0.71	99.89	0.45
V	102.14	1.79	100.87	1.67	98.95	1.25	100.49	0.69	99.54	0.51
VI	97.42	2.38	101.61	1.61	100.71	0.82	98.55	1.55	99.33	1.45
VIII	96.31	2.87	103.54	2.25	98.75	1.75	97.67	1.95	100.57	1.61
Amount added (μg/ml)	100		150		200		250		300	
VII	99.75	0.35	100.29	0.25	99.87	0.51	100.11	0.28	99.97	0.21

R.S.D.: relative standard deviation.
[a] Mean of three determinations.

Table 8
Linearity data

Sample	Range[a] (μg/ml)	Regression equation	r^2	LOD (μg/ml)	LOQ (μg/ml)
I	0.5–5.0	$y = 43875x - 5790$	0.9838	0.088	0.26
II	0.5–5.0	$y = 56992x - 6283$	0.988	0.074	0.20
III	0.5–5.0	$y = 71936x + 4485$	0.9895	0.079	0.23
IV	0.5–5.0	$y = 61668x - 2753$	0.9976	0.108	0.32
V	0.5–5.0	$y = 55812x - 1654$	0.9983	0.114	0.34
VI	0.5–5.0	$y = 55569x - 5352$	0.9906	0.138	0.41
VII	10–300	$y = 54861x + 36257$	0.9998	0.146	0.43
VIII	0.5–5.0	$y = 59957x - 9849$	0.9841	0.165	0.48

[a] Ten calibration levels were used.

Table 9
Results of analysis of impurities in bulk drug and formulations

Sample	Impurities (±S.D.)[a] (%, w/w)						
	I	II	III	IV	V	VI	VIII
Bulk drug-1	–	0.04 ± 0.0030	–	–	0.04 ± 0.001	0.04 ± 0.0015	–
Bulk drug-2	–	–	–	0.06 ± 0.002	–	–	0.05 ± 0.0025
Bulk drug-3	–	–	–	–	0.05 ± 0.0011	0.06 ± 0.0025	–
Bulk drug-4	–	0.02 ± 0.0020	–	0.05 ± 0.0011	–	0.05 ± 0.0018	–
Formulation-1	–	–	–	0.04 ± 0.0010	–	0.05 ± 0.0021	0.04 ± 0.0021
Formulation-2	–	–	–	0.05 ± 0.0022	–	0.04 ± 0.0017	–

–: not detected; S.D.: standard deviation.
[a] Mean of three determinations.

a series of solutions until the S/N ratio 3 for LOD and 10 for LOQ. The results are given in Table 8.

4.7. Analysis of bulk drug and formulations

High-low chromatographic techniques were employed for detecting trace level impurities in bulk drug and tablet formulations of BCT. Ten tablet formulations were accurately weighed (equivalent to 500 mg of BCT), ground to powder and an equivalent of 200 mg of active ingredient dissolved in methanol was taken into a 100 ml volumetric flask, ultra sonicated for about 10 min and made up to the mark with the mobile phase. The supernatant liquid was collected. For determining the impurities, the same solution was used. The results are recorded in Table 9. The separation of impurities in formulations is shown in Fig. 11A. The peaks were identified by injecting and comparing with the retention times of the individual compounds and also by the absorption spectra recorded by PDA detector (Fig. 12). The concentrations of impurities relative to BCT were in the range 0.02–0.06% (Table 9). The assay for determining the BCT was carried out by diluting the above solutions to 200 μg/ml with the mobile phase. The results of assay for bulk drug and formulations are given in Table 10.

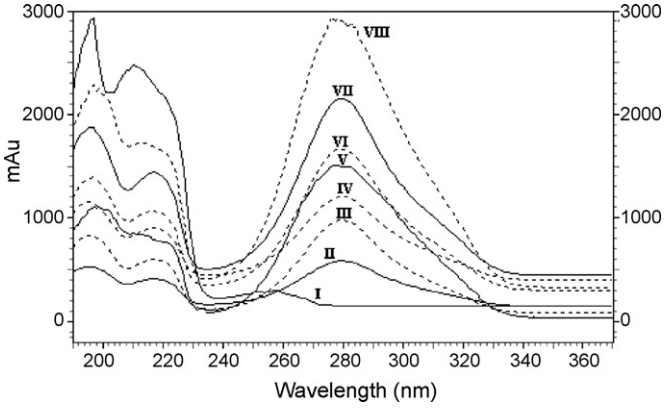

Fig. 12. Overly UV spectra of BCT (VII) and impurities (I–VI and VIII).

Table 10
Results of analysis of BCT in bulk drug and formulations

Sample	Taken (mg)	Found[a] (mg)	Recovery (%)	R.S.D. (%)
Bulk drug-1	50.39	50.45	100.12	0.23
Bulk drug-2	49.75	49.70	99.90	0.35
Bulk drug-3	49.82	49.97	100.30	0.25
Formulation-1	50.50	50.40	99.80	0.27
Formulation-1	49.85	50.12	100.54	0.39

[a] Mean of three determinations.

5. Conclusion

A simple and rapid stability indicating isocratic RP-HPLC method has been developed and validated for determining the process related substances and degradation products of BCT. Attempts were made to separate BCT and its process related impurities on different commercial C_{18} columns. Studying the effects of organic modifier and concentration and pH of buffer chromatographic conditions were optimized. The developed method was found to be selective, sensitive, precise, linear, accurate and reproducible in determining the BCT and its potential impurities, which may be present at trace level in the finished products. Forced degradation of bicalutamide was carried out

under thermal, photo, acidic, alkaline and peroxide conditions. Two unknown process impurities and the alkaline degradation products (I and III) were isolated and characterized by ESI-MS/MS, ^{1}H NMR and FT-IR spectral analysis. The degradation reaction is found to be a second order reaction. Thus, the developed method can be used for process development as well as quality assurance of BCT in bulk drug as well as pharmaceutical formulations.

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