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An improved and validated LC method for resolution of bicalutamide enantiomers using amylose tris-(3,5-dimethylphenylcarbamate) as a chiral stationary phase

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Abstract

An improved HPLC method for determination of enantiomeric purity of bicalutamide in drugs and pharmaceuticals was developed and validated. Baseline separation with resolution ≥6.0 was achieved within 10 min on Chiralpak AD-H (250 mm × 4.6 mm; particle size 5 μm) column using *n*-hexane:2-propanol (65:35 v/v) as mobile phase at a flow rate of 1.0 ml/min at 25 °C. The detection was made at 270 nm using UV detector while a polarimetric detector connected in series was used for identification of enantiomers. The effects of 2-propanol, ethanol and temperature on enantioselectivity and resolution of enantiomers were evaluated. The method was validated in terms of accuracy, precision and linearity in the range of 10–250 μg/ml and the r^2 was >0.9999. The recoveries were 99.68–100.25% with <1% R.S.D. The limits of detection (LOD) and quantification (LOQ) of enantiomers were (2.4, 3.0 and 7.6, 9.3) × 10^{-8} g/ml for (*S*)-(+)-BCT and (*R*)-(−)-BCT enantiomers, respectively. The method was found to be suitable for rapid determination of enantiomeric purity of bicalutamide in bulk drugs and pharmaceutical formulations.
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Keywords: Bicalutamide; Anti-androgen; Chiralpak AD-H; Enantioselective; Amylose tris-(3,5-dimethylphenylcarbamate); Polarimetric detection

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 the treatment of prostate cancer. It competes with testosterone and dihydrotestosterone for binding sites on the prostate and other androgen-sensitive tissues. It does not bind as tightly to the receptors as do testosterone and dihydrotestosterone. However, it binds preferentially to receptors located outside the central nervous system and causes little increase in testosterone levels with little agonist activity [1–3]. BCT is a racemic mixture with most of its activity residing in the (*R*)-enantiomer. AstraZeneca, Sweden has patented the formulation of (*R*)-(−)-BCT quite recently [4]. The pharmacokinetics of both the enantiomers was studied thoroughly [5,6]. Owing to the existence of pharmacological and toxicological differences between stereoisomers, regulatory authorities demand experimental proof for enantiomeric ratio and purity of bulk drugs and pharmaceutical formulations. Chiral chromatography is an important analytical tool for separation and determination of inactive enantiomers present in enantiomerically pure drugs and finished products.

A thorough literature search has revealed that, there are a very few HPLC methods were reported for determination of BCT in plasma [7–9]. Cockshott et al. have used HPLC for determining the enantiomers of BCT in plasma. The undifferentiated enantiomers in the human plasma were first collected on ODS column and then separated by Ultron ES-OVM column [10]. Kenneth James and Nnochiri Ekwuribe [11] have synthesized enantiomerically pure (*S*)-(+)-BCT and (*R*)-(−)-BCT and separated them on a Chiralcel OJ column without reporting the chromatographic conditions. Bargmann-Leyder et al. [12] have compared LC and SFC separations on cellulose-derived Chiralcel OD and amylose-derived Chiralpak AD chiral stationary phases using BCT and several other chiral compounds. Tucker and Chesterson [13] have resolved the racemic mixture

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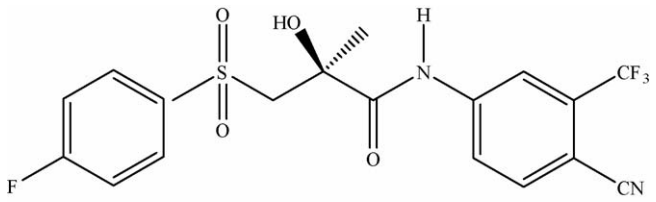
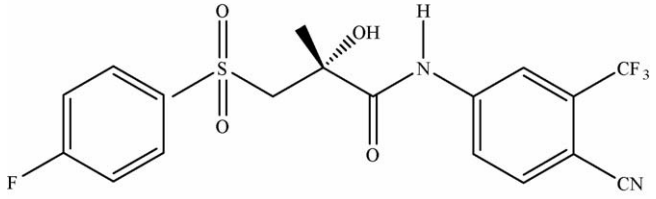


(*S*)-(+)-BCT



(*R*)-(-)-BCT

Fig. 1. Structural representation of BCT enantiomers.

of BCT by forming diasteriomers on a Spherisorb-NH_2 column. Recently Torok et al. [14] have evaluated different CSPs for separation of enantiomers of BCT and its impurities. A protein-based Chirobiotic-T column with 90% of 2-propanol as an organic modifier was also used. However, the protein-based columns are generally less rugged and the use of high content of organic modifier may reduce the column life significantly due to high backpressures. On Chiralcel OD-H column, a maximum resolution of 1.2 was reported but it may not be acceptable for quantitative determinations according to USP, which normally requires a minimum of 1.5. Like all other anti-cancer drugs (*R*)-(−)-BCT is cyto-toxic and its *S*-enantiomer equally so. In such cases, the old rule to detect and estimate impurities at 0.1% levels could not be applied and the cyto-toxic components have to be estimated at levels of 0.01% or even less [15]. Under these conditions, the resolution obtained by Torok et al. may not be sufficient enough for trace level estimation of (*S*)-(+)-BCT. Further, the method was not validated for determination of enantiomeric purity of BCT. To the best of our knowledge, a complete validated method for direct separation and determination of BCT enantiomers is not available in the literature. In the present investigation, an improved and rapid HPLC method was developed and validated using a more rugged polysaccharide, Chiralpak AD-H column and less amount of organic modifier (35%) for determining the enantiomeric purity of BCT in bulk drugs and pharmaceuticals. The method is quite rapid and more sensitive with high resolution ($R_s \geq 6.0$) and run time less than 10 min. It was validated in terms of accuracy, precision and linearity as per ICH guidelines. The effects of organic modifier and temperature on resolution and retention were thoroughly studied. Further, it was found to be suitable for isolation and purification of individual enantiomers by semi preparative HPLC.

2. Experimental

2.1. Materials and reagents

All reagents were of analytical-reagent grade unless stated otherwise. HPLC-grade *n*-hexane, 2-propanol and ethanol were purchased from S.D. Fine Chem. (Mumbai, India). (*RS*)-BCT and (*R*)-(−)-BCT were gifted from Hygro Chemical Pharmtek Pvt. Ltd. (Hyderabad, India). All solutions were filtered through 0.45 μm membrane filters procured from Pall Pharmalab Filtration Pvt. Ltd. (Mumbai, India).

2.2. Apparatus

The HPLC system composed of LC-10AT VP pump, SPD-10A VP UV detector and SIL-10AD VP auto injector, and SCL-10A VP system controller attached with thermostat (all from Shimadzu, Kyoto, Japan). Polarimetric detector (IBZ Messtechnik GmbH, Hannover, Germany) was connected to UV detector in series for identification of the enantiomers. Chiralcel OD-H (250 mm × 4.6 mm; particle size 5 μm) with Chiralcel OD-H (1 cm × 4.6 mm) guard column, Chiralpak AD-H (250 mm × 4.6 mm; particle size 5 μm) with Chiralpak AD-H (1 cm × 4.6 mm) guard column (Daicel Chemical Industries, Tokyo, Japan) were used for separation. The chromatographic and the integrated data were recorded using HP-Vectra (Hewlett Packard, Waldron, Germany) computer system.

2.3. Chromatographic conditions

Chromatographic separation was achieved on Chiralpak AD-H (250 mm × 4.6 mm; particle size 5 μm) column attached with Chiralpak AD-H (1 cm × 4.6 mm) guard column with mobile phase consisting of *n*-hexane:2-propanol (65:35 v/v) at 25 °C. The flow rate was 1.0 ml/min and the UV detector was kept at 270 nm for monitoring of the eluents. Injection volume was 20 μl and total run time was 10 min.

2.4. Preparation of stock and standard solutions

Stock solutions of (*RS*)-BCT and (*R*)-(−)-BCT were prepared by dissolving 200.1 mg of (*RS*)-BCT and 100.2 mg of (*R*)-(−)-BCT precisely weighed in respective 100 ml volumetric flasks, dissolved in 25 ml methanol and made up to the mark with the mobile phase. The stock solutions were wrapped with aluminum foil and kept in the refrigerator at 5 °C. The specified concentration of each enantiomer was taken as 50 μg/ml for the analysis. Solutions of samples and standards in the range 10–250 μg/ml were prepared by diluting appropriate volumes of stock solutions in 25 ml volumetric flasks with the mobile phase.

3. Results and discussion

The selectivity of two different columns, viz., Chiralcel OD-H and Chiralpak AD-H as well as the effects of mobile phase characteristics on resolution and retention of BCT enantiomers

were studied. The parameters studied were the type of column, concentration of organic modifier and temperature.

3.1. Column selectivity

Two different polysaccharide-based stationary phases were evaluated. Chiralcel OD-H (cellulose tris-(3,5-dimethylphenylcarbamate)) and Chiralpak AD-H (amylose tris-(3,5-dimethylphenylcarbamate)) columns were tried using 2-propanol and ethanol as organic modifiers in *n*-hexane. The chiral recognition mechanism on these CSPs is generally due to the formation of solute–CSP complexes through inclusion of the enantiomers in to the chiral cavities in the higher order structures of the CSPs [16–18]. In case of CSPs with carbamate derivatives, the binding of the solutes to the CSPs is through interactions between the solutes and the polar carbamate groups on the CSPs [19,20]. The carbamate groups can interact with solutes through hydrogen bonding using C=O and NH groups, and through dipole-dipole interaction using C=O moiety. In the present study the available functional groups on the solutes are −OH, and −NH which can form hydrogen bonds with the C=O group on the CSPs. Wainer et al. [19] have reported that the solutes having aromatic functionalities could provide additional stabilizing effect to the solute–CSP complex by insertion of the aromatic ring into the chiral cavity. In the present case, this type of stabilization effect may be possible due to the presence of the aromatic functionality on the solutes. Chiralcel OD-H column did not show any selectivity for the BCT enantiomers with 2-propanol, while it showed some selectivity with ethanol. A resolution of 1.56 with higher retention was obtained using 10% of ethanol on this column (Fig. 2). Whereas Chiralpak AD-H column has shown excellent selectivity for the BCT enantiomers with decreased retention (Tables 1 and 2). These differences in chiral recognition mechanism could be attributed to the different configurations of the glucose residues (β and α linkages) and higher order structures of chiral stationary phases

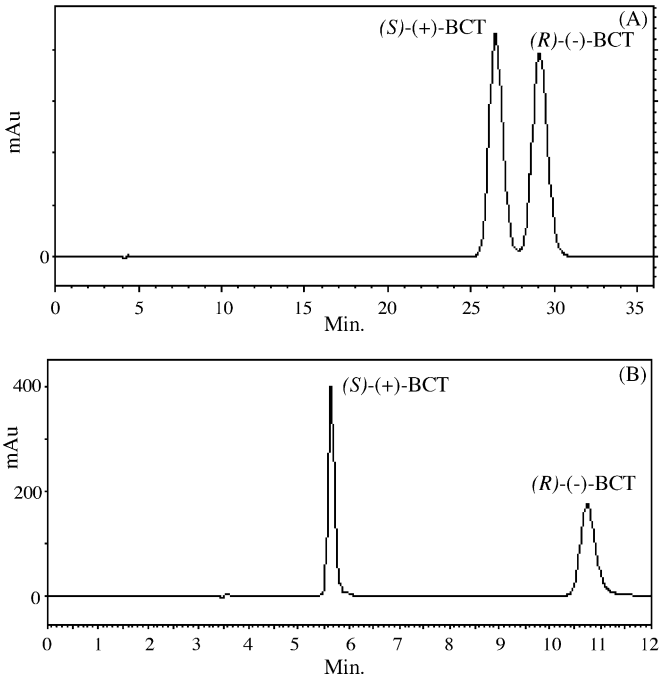


Fig. 2. Typical chromatograms showing the separation of BCT enantiomers at 25 °C using UV detector at 270 nm on (A) Chiralcel OD-H column with *n*-hexane:ethanol (90:10 v/v) as a mobile phase; (B) Chiralpak AD-H column with *n*-hexane:ethanol (60:40 v/v) as a mobile phase.

of OD-H and AD-H columns [18]. Finally, Chiralpak AD-H was chosen for further development.

3.2. Effect of organic modifier

The type and concentration of organic modifier was found to influence the retention and resolution of BCT enantiomers. The selectivity and resolution of the BCT enantiomers with 2-propanol and ethanol on Chiralpak AD-H column are shown in Tables 1 and 2, respectively. Both the organic modifiers have

Table 1
The effect of temperature and 2-propanol on selectivity and resolution of BCT enantiomers on Chiralpak AD-H column

Temperature (°C)	*n*-Hexane:2-propanol (v/v)	k_1	k_2	α	R_s
25	75:25	1.64	3.16	1.93	9.08
25	70:30	1.15	2.21	1.92	7.94
25	65:35	0.84	1.58	1.88	6.32
25	60:40	0.68	1.27	1.87	6.04
30	75:25	1.53	2.87	1.88	9.02
30	70:30	1.03	1.91	1.85	7.72
30	65:35	0.76	1.41	1.86	6.57
30	60:40	0.61	1.12	1.84	5.76
35	75:25	1.44	2.67	1.85	8.90
35	70:30	0.99	1.82	1.84	7.52
35	65:35	0.74	1.35	1.82	6.48
35	60:40	0.58	1.05	1.81	5.58
40	75:25	1.35	2.47	1.83	8.65
40	70:30	0.95	1.71	1.80	7.31
40	65:35	0.69	1.23	1.78	6.22
40	60:40	0.56	0.99	1.77	5.38

k_1: retention factor of (*S*)-(+)-BCT; k_2: retention factor of (*R*)-(−)-BCT; α: selectivity; R_s: resolution.

Table 2
The effect of temperature and ethanol on selectivity and resolution of BCT enantiomers on Chiralpak AD-H column

Temperature (°C)	*n*-Hexane:ethanol (v/v)	k_1	k_2	α	R_s
25	75:25	2.02	6.36	3.15	17.11
25	70:30	1.65	5.04	3.05	15.76
25	65:35	1.11	3.31	2.98	14.18
25	60:40	0.88	2.58	2.93	12.55
30	75:25	1.87	5.76	3.08	17.58
30	70:30	1.51	4.51	2.99	16.08
30	65:35	1.02	2.98	2.92	14.55
30	60:40	0.81	2.30	2.84	12.83
35	75:25	1.73	5.27	3.05	18.14
35	70:30	1.43	4.26	2.98	16.51
35	65:35	0.94	2.70	2.87	14.96
35	60:40	0.76	2.14	2.82	13.07
40	75:25	1.61	4.81	2.99	19.21
40	70:30	1.37	4.14	3.02	17.10
40	65:35	0.87	2.59	2.98	15.39
40	60:40	0.72	1.95	2.71	13.52

k_1: retention factor of (*S*)-(+)-BCT; k_2: retention factor of (*R*)-(−)-BCT; α: selectivity; R_s: resolution.

shown good selectivity for BCT enantiomers. However, ethanol has shown better selectivity when compared to 2-propanol. The (*R*)-(−)-BCT was retained longer with ethanol compared to 2-propanol. This phenomenon could be explained by the difference in the steric bulkiness around the hydroxyl moiety of the mobile phase modifier. The lower alcohols could be inserted in to the cavity of the CSP more easily than bulkier alcohols. The insertion of the mobile phase modifier into the cavities of the CSP could induce changes in the dominant chiral recognition mechanism leading to formation of more stable diastereomeric complexes of the enantiomers causing higher retention with better selectivity. The separation of BCT enantiomers using ethanol as organic modifier is shown in Fig. 2. The effect of concentration of ethanol and 2-propanol was studied. On decreasing the concentration of organic modifier, the capacity factors as well as resolutions were increased. Using 2-propanol, sharp peaks with higher sensitivity (higher detections limits) and lower retention were observed. Thus, 2-propanol was chosen as an organic modifier. As a compromise between resolution and retention, 35% of 2-propanol in *n*-hexane was found to be an optimum composition of the mobile phase for analysis.

3.3. Effect of temperature

The effect of column temperature on resolution and retention factors of BCT enantiomers was studied in the range 298–313 K (25–40 °C) on Chiralpak AD-H column. On increasing the temperature, the retentions were decreased with both 2-propanol and ethanol (Tables 1 and 2). These results could be attributed to the fact that the analytes on molecular level have small adsorption as temperature increased and therefore migrates fast through the column. On increasing the temperature, resolutions were increased with ethanol and decreased with 2-propanol. Under thermodynamically equilibrium conditions, free energy (ΔG°) accompanying the separation of two enantiomers was related to the retention factors by the following equation:

$$\Delta G^\circ = -RT \ln k \quad (1)$$

where k is a retention factor, R the gas constant and T is the temperature in K. An expansion of Eq. (1) to involve the enthalpy (ΔH°) and entropy (ΔS°) terms yields:

$$\ln k = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} \quad (2)$$

Van't Hoff plots were drawn for logarithm of retention factor ($\ln k$) versus inverted temperature ($1/T$) in K for the two isomers, which yielded straight lines (Fig. 3). ΔH° and ΔS° for the two enantiomers were obtained from slope and intercept of the straight lines, respectively. The change in free energy accompanying the separation of two enantiomers was given by

$$\Delta\Delta G^\circ = \Delta\Delta H^\circ - T\Delta\Delta S^\circ \quad (3)$$

The enthalpy change ($\Delta\Delta H^\circ$), entropy change ($\Delta\Delta S^\circ$) and Gibb's free energy change ($\Delta\Delta G^\circ$) accompanying the separation on Chiralpak AD-H column with *n*-hexane:2-propanol

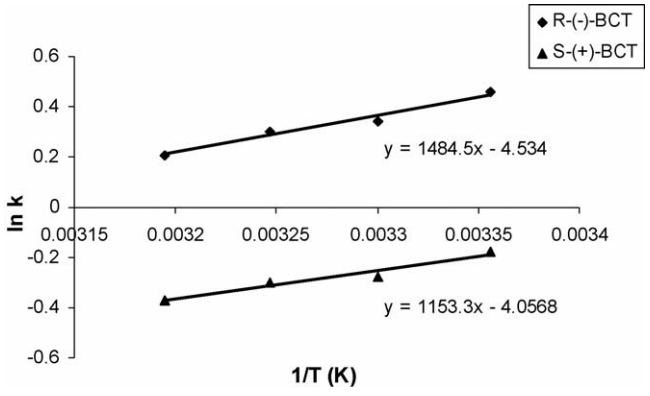


Fig. 3. Van't Hoff plots of the BCT enantiomers at 35% of 2-propanol on Chiralpak AD-H column.

Table 3
Thermodynamic data calculated from the Van't Hoff plots of BCT enantiomers

Enantiomer	ΔH° (kJ mol^{-1})	$\Delta\Delta H^\circ$ (kJ mol^{-1})	ΔS° (J K^{-1} mol^{-1})	$\Delta\Delta S^\circ$ (J K^{-1} mol^{-1})	$\Delta\Delta G^\circ$ (kJ mol^{-1})
(*S*)-(+)-BCT	−9.589	−2.753	−33.73	−3.97	−1.570 (298 K)
(*R*)-(−)-BCT	−12.342		−37.70		

ΔH° = slope × R; ΔS° = intercept × R; $\Delta\Delta G^\circ = \Delta\Delta H^\circ - T\Delta\Delta S^\circ$.

(65:35 v/v) are recorded in Table 3. The data indicated that the separation of BCT enantiomers was an enthalpy driven process.

Thus, a mobile phase containing *n*-hexane:2-propanol (65:35 v/v) was optimized for the separation of BCT enantiomers on Chiralpak AD-H column maintained at 25 °C. The flow rate was kept at 1.0 ml/min throughout the analysis. This method was rapid, reliable and more sensitive compared to the one reported by Torok et al. [14]. Torok et al. reported a resolution of 1.20 with higher retentions on a similar Chiralcel OD-H column but it may not be acceptable for quantification according to USP, which normally requires a minimum of 1.5. They have found a maximum selectivity of 1.40 and resolution of 2.25 on Chirobiotic T column using 90% of 2-propanol as an organic modifier, which could damage the column on long run. Bargmann-Leyder et al. have compared LC and SFC separations on cellulose-derived Chiralcel OD and amylose-derived Chiralpak AD chiral stationary phases using various chiral compounds, including BCT [12]. They have got a selectivity of 1.24 and 1.13 at higher retention times on Chiralcel OD column by LC and SFC, respectively. The present method was superior as higher selectivity (1.88) and resolution ($R_s \geq 6.0$) was obtained with in 10 min using only 35% of 2-propanol. The novelty of the method lies in the absolute identification of BCT enantiomers using a polarimetric detector connected to UV in series. Improvement in terms of higher sensitivity (detection limits) and resolution were obtained due to higher selectivity of the column and mobile phase. The chromatographic separation of (*RS*)-BCT and *R*-(−)-BCT in the optimized conditions using UV and polarimetric detectors is shown in Fig. 4. The method was validated in terms of accuracy, precision and linearity as per ICH guidelines.

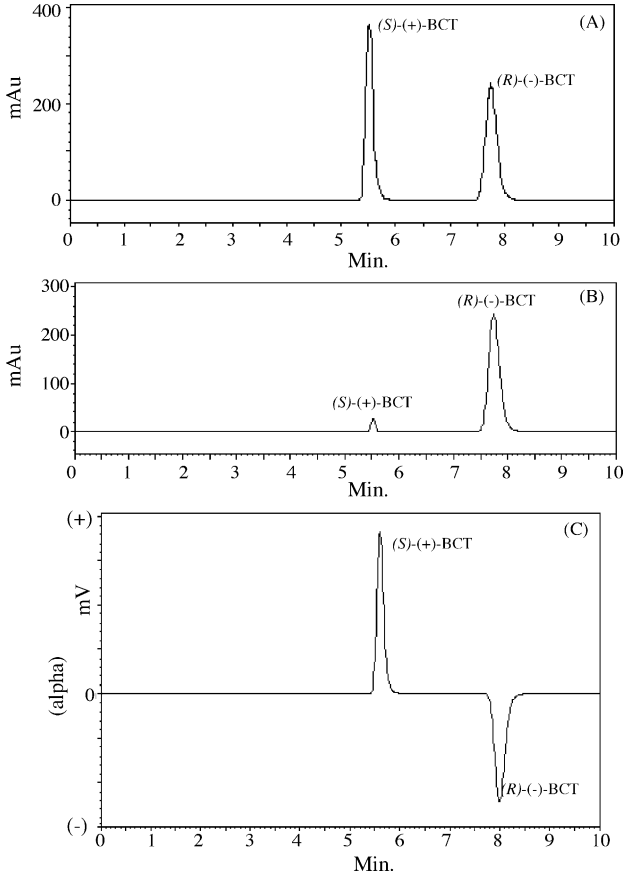


Fig. 4. Typical chromatograms showing the separation of BCT enantiomers on Chiralpak AD-H column with *n*-hexane:2-propanol (65:35 v/v) as a mobile phase at 25 °C: (A) (*RS*)-BCT and (B) (*R*)-(−)-BCT using UV detector at 270 nm and (C) (*RS*)-BCT using polarimetric detector.

4. Method validation

4.1. System suitability

(*RS*)-BCT solution (10 μg/ml) was prepared by taking appropriate volume of stock solution in 25 ml volumetric flask and making up to the mark with the mobile phase. It was used as a standard solution for system suitability. An aliquot of 20 μl was injected into the system three times. The system was deemed to be suitable if the resolution between the BCT enantiomers was not less than 6.0. The number of theoretical plates for *S*-(+)-BCT and *R*-(−)-BCT were 2800 and 2500 and tailing factors were 1.15 and 1.18 (at 10% base), respectively.

4.2. Precision

Precision of the method was tested by preparing six individual solutions of (*RS*)-BCT and making triplicate injections for each solution. The %R.S.D. of the assay was less than 0.32%. Inter- and intra-day assay precision was performed by analyzing the solutions for five times in a day for 3 days. The %R.S.D. of the assay was less than 0.41% for both the isomers.

4.3. Linearity

Calibration graphs were drawn in the range of 10–250 μg/ml of BCT enantiomers by preparing fresh solutions every time for 3 days. The curves were linear with $r^2 \geq 0.9999$ and the regression equations for *S*-(+)-BCT and *R*-(−)-BCT were $75\,121x - 5023$ and $75\,896x - 1398$, respectively.

4.4. Accuracy

Accuracy studies were performed by spiking BCT solution at five levels in the range 50–150% with respective to specified

Table 4
Results of analysis of bulk drugs and pharmaceutical formulations

Formulation	Claimed value (mg)	(*S*)-(+)-BCT		(*R*)-(−)-BCT	
		Found (mg)	Assay (%)	Found (mg)	Assay (%)
Bulk-I	50	24.85	49.70	25.15	50.30
Bulk-II	50	24.83	49.66	25.17	50.34
Bulk-III	50	0.35	0.70	49.65	99.30
Bulk-IV	50	0.26	0.52	49.74	99.48
Form-I	50	24.79	49.58	25.21	50.42
Form-II	50	24.91	49.82	25.09	50.18
Form-III	50	24.88	49.76	25.12	50.24

level (50 μg/ml) and analyzing each solution in triplicate ($n = 3$) for 3 days. The percentage recoveries were between 99.68 and 100.25% with <1% R.S.D.

4.5. Robustness

Robustness of the method was studied by making small deliberate changes in the method parameters. A variation of 1% of 2-propanol in the composition of the mobile phase did not effect the resolution except that retentions were changed. The effect of temperature has been studied by analyzing sample at $25 \pm 2\,^{\circ}C$. Again retention times were changed but the resolution remained above 6.0. The effect of flow rate was studied by analyzing the samples with 0.9 and 1.1 ml/min flow rates. In both the cases the resolution was found to be above 6.0.

4.6. LOD and LOQ

Limits of detection (LOD) and quantification (LOQ) were calculated using signal/noise (S/N) ratio method. LOD was taken as a concentration of the analyte where S/N was 3 and found to be (2.4 and 3.0) $\times 10^{-8}$ g/ml for *S*-(+)-BCT and *R*-(−)-BCT, respectively. LOQ was taken as a concentration of the analyte where S/N was 10. It was found to be (7.6 and 9.3) $\times 10^{-8}$ g/ml for *S*-(+)-BCT and *R*-(−)-BCT, respectively.

4.7. Assay of bulk drugs and pharmaceutical dosage forms

Ten weighed tablets of BCT (equivalent to 50 mg of BCT) were ground to powder and an equivalent of 100 mg of active ingredient dissolved in methanol was taken in 100 ml volumetric flask, ultra sonicated for about 10 min, made up to the mark with methanol and the supernant liquid was collected. Sample solutions were prepared by diluting appropriate volumes of supernant solution in 25 ml volumetric flasks with the mobile phase. The proposed LC method was applied to analyze different formulations of BCT. The two enantiomers were very well separated under the developed conditions and there was no interference from the excipients in determining the enantiomeric purity. Three pharmaceutical formulations and four bulk drugs were analyzed. The results are given in Table 4. From these results, it could be seen that the developed method is quite simple, rapid and reliable for determination of enantiomeric purity of BCT in bulk drugs and pharmaceutical formulations.

5. Conclusions

Separation and determination of bicalutamide enantiomers on two different polysaccharide-based chiral stationary phases, viz., Chiracel OD-H and Chialpak AD-H was studied. Chiralpak AD-H column has shown excellent selectivity for BCT enantiomers. Baseline separation with $R_s \geq 6.0$ was achieved between the two enantiomers within 10 min. The effect of organic modifiers and temperature on resolution and retention of enantiomers have been evaluated to optimize the mobile phase composition. The enantiomeric separation was found to be an enthalpy driven process. The method was validated with respect to accuracy, precision, linearity, LOD, LOQ and robustness. The developed method is quite simple, rapid, sensitive and enantioselective and could be of use for determination of enantiomeric purity of BCT in bulk drugs and pharmaceuticals. Improvement in terms of rapidity, resolution, sensitivity and ruggedness compared to that of Torok et al. [14] was achieved on Chiralpack AD with 35% of 2-propanol as an organic modifier. Further, it is suitable for isolation and purification of individual enantiomers by semi preparative HPLC.

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