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VALIDATION OF CHIRAL LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRIC METHOD FOR THE ESTIMATION OF BICALUTAMIDE ENANTIOMERS IN HUMAN PLASMA: APPLICATION TO A BIOEQUIVALENCE STUDY

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Abstract: Bicalutamide (BCT) is a non-steroidal antiandrogen used for the treatment of prostate cancer. Its chemical name is (*RS*)-N-[4-cyano-3-(trifluoromethyl) phenyl]-3-[(4-fluorophenyl) sulfonyl]-2-hydroxy-2-methyl-propanamide. BCT as such is a racemic mixture, but most of its pharmacological activity is attributed to its *R* enantiomer. A single, simple and selective method for simultaneous estimation of (-)-BCT and (+)-BCT in human plasma was validated using (-)-d4-BCT and (+)-d4-BCT as internal standards. The compounds were separated on a Chiralpak AD-3R column under isocratic conditions consisting of 5mM ammonium acetate buffer and methanol (70:30, v/v), with a total run time of 6.5 minutes and detected by tandem mass spectrometry with negative ionization mode. The ion transitions recorded in multiple reaction monitoring mode were m/z 429.0→185.0 for BCT enantiomers and m/z 433.0→185.0 for d4-BCT enantiomers. Linearity in plasma was observed over the concentration range 10.0 – 3000.0 ng/mL for (-)-BCT and 2.0 – 200.0 ng/mL for (+)-BCT. The mean recovery was 81.2 % and 78.0 % for (-)-BCT and (+)-BCT respectively. The coefficient of variation of the assay was less than 4.2 % and 6.8 % for (-)-BCT and (+)-BCT with an accuracy of 99.3 % to 104.1 % and 98.6 % to 103.4 % for (-)-BCT and (+)-BCT respectively. Stability was evaluated under different conditions including bench top, processed sample, freeze and thaw, autosampler and long term. The validated method was applied for the determination of individual BCT enantiomers in human plasma samples from a bioequivalence study of 150mg fixed dose formulation in 12 healthy Indian subjects. Assay reproducibility was demonstrated by reanalysis of 10% incurred samples.

Keywords: Bicalutamide; Chiral; LC-MS/MS; Human Plasma; Enantiomers; Bioequivalence

INTRODUCTION

There are two main classes of antiandrogens that are clinically used [1-3]. A few steroidal ligands have been used as antiandrogens, including cyproterone, oxendolone and spironolactone. However, the clinical application of steroidal antiandrogens has been limited greatly by poor oral bioavailability, lack of tissue selectivity, poor pharmacokinetic properties and potential side effects like hepatotoxicity, androgenic effects and feminizing side effects like gynecomastia and loss of libido in men [4-6]. Moreover, the rigid steroid backbone does not allow wide structural modifications for new drug development. Non-steroidal antiandrogens are the current pharmacological treatment of choice for progressive androgen-dependent prostate cancer, either as monotherapy or with adjuvant castration or luteinizing hormone-releasing hormone (LHRH) superagonists to block the synthesis of endogenous testosterone. The non-steroidal ligands are more favorable for clinical and therapeutic applications because of the lack of cross-reactivity with other steroidal receptors which eliminates the unwanted side effects [4-6]. In addition, they demonstrate a highly improved oral bioavailability as compared to their steroidal counterparts and are also open to various structural modifications.

The propionanilide derivatives are the first developed non-steroidal antiandrogens and include drugs such as flutamide, hydroxyflutamide, nilutamide and bicalutamide [7].

Bicalutamide (BCT) under the trade name of Casodex® is one of the leading non-steroidal anti-androgens used for the treatment of prostate cancer [8]. This drug competes with testosterone and dihydro-testosterone for binding sites on the prostate and other androgen-sensitive tissues and has little or no agonist activity [9]. The drug is well tolerated and has very few side-effects [10]. BCT as such is a racemic mixture, but most of its pharmacological activity is attributed to its *R* enantiomer.

Due to the existence of pharmacological and toxicological differences between stereoisomers, regulatory authorities demand investigational proof for enantiomeric ratio and purity of bulk drugs and pharmaceutical formulations. Chiral liquid chromatography coupled with tandem mass spectrometry is an important analytical tool for separation and quantification of drug enantiomers.

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A thorough review of literature reveals that no validated liquid chromatography-tandem mass spectrometric analytical method for the quantification of BCT enantiomers in biological fluids has been published in the literature. However, the methods for the enantiomeric separation of BCT and its related compounds based on chiral HPLC have been published in the past by several authors. Analytical methods already reported on this drug comprise of analysis by UV – Visible Spectrophotometry [11, 12], HPLC method using the UV detector [13, 14] in plasma [15-18]. The undifferentiated enantiomers in the human plasma were first collected on ODS column and then separated them by ES-OVM column [18]. BCT was synthesized enantiomerically pure and separated by chiralcel OJ column without reporting the chromatographic conditions [19]. The comparison of LC and SFC separations on cellulose derived chiralcel OD and amylase derived chiralpak AD chiral stationary phases using BCT and several other chiral compounds [20] and racemic mixture of BCT forming diastereomers on a Spherisorb-NH2 column [21] have been published. Evaluation of different CSPs for separation of enantiomers of BCT and its impurities is also available [14]. Method to detect the component in formulation and biological fluids using fluorescent detection with or without β Cyclodextrin as enhancer has been reported [22-24]. A stability indicating HPLC method [25] and isolation of process related impurities and degradation products of BCT [26] have also been reported in the literature. Recently, LC-MS/MS method using electrospray ionization and simple protein precipitation step for the determination of racemic BCT has been published [27].

All the published methods had a long chromatographic run time and generally required complex extraction procedures to remove interferences, which were obviously impractical for high throughput analysis. Therefore, it was necessary to develop a simple, precise and sensitive method for the determination of BCT enantiomers in human plasma which will give high throughput analysis in short interval of time. The present work comes up with a rapid, simple, sensitive and precise isocratic reversed-phase HPLC-MS/MS method for the determination of BCT enantiomers in human plasma with a quantification limit sufficiently low to support stereo selective pharmacokinetic studies.

MATERIALS AND METHODS

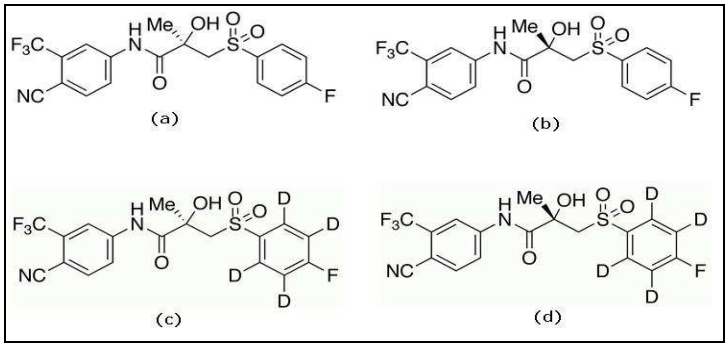


Figure 1: Chemical Structures of (a) (-)-BCT, (b) (+)-BCT, (c) (-)-d4-BCT, (d) (+)-d4-BCT.

Reference standards of BCT enantiomers and d4 BCT enantiomers (Fig. 1) with 99% purity were purchased from Syncom (Groningen, Netherlands). The HPLC grade solvents viz. methanol and acetonitrile were purchased from J.T. Baker INC. (Phillipsburg, NJ, USA). LC-MS grade ammonium acetate was procured from Sigma-Aldrich Co. (Bangalore, India). Strata-X 33µm polymeric reversed phase (30mg/1mL) solid phase extraction cartridges and HPLC grade water were procured from E. Merck Ltd. (Mumbai, India). Blank human blood was collected with Na Heparin as anticoagulant from healthy and drug free volunteers. Plasma was separated by centrifugation at 3000 RPM at 10°C, and stored at –20°C.

Instrumentation and Analytical Conditions:

The chromatographic separation and quantification was achieved by a liquid chromatography system, LC-10AD (Shimadzu, Kyoto, Japan) coupled with mass spectrometer, API-5000 (ABS Sciex, Canada). The chiral column, Chiralpak AD-3R (150 x 4.6 mm, 3µ) from Daicel Chemicals Ind. Ltd. (Hyderabad, India) was used for separation of BCT enantiomers and internal standards. Mobile phase of 5mM ammonium acetate with methanol in the ratio of 70:30 (*v/v*) was pumped isocratically at flow rate of 0.4 mL/min. Auto sampler temperature was set at 10°C and the injection volume was 2µL. The column oven temperature was maintained at 25°C and the total LC run time was 6.5 min.

The MS/MS system was operated in the multiple reaction monitoring (MRM) mode for monitoring the transition of the deprotonated molecular ion *m/z* 429.0 to the product ion *m/z* 185.0 for BCT enantiomers and the transition of the deprotonated molecular ion *m/z* 433.0 to the product ion *m/z* 185.0 for the d4 BCT enantiomers. The instrument response was optimized for BCT enantiomers and internal standards by infusing a constant flow of a solution of the drug dissolved in mobile phase.

Electrospray ionization (ESI) was performed in the negative ion mode. The source temperature was set to 450°C with ion spray voltage of -4500. Nitrogen gas was used as the CAD gas. The curtain gas was kept at 25 and the GAS1 and GAS2 optimized were 35 and 70 respectively. Compound dependant parameters set for (-)-BCT and its IS were Decluster Potential: -70 and -90 V; Entrance Potential: -10 V for both; collision energy: -30 and -56 eV; Cell Exit Potential: -25 V for both. The compound dependant parameters set for (+)-BCT and its IS were Decluster Potential: -90 and -95 V; Entrance Potential: -10 V for both; collision energy: -25 and -54 eV; Cell Exit Potential: -23 and -17 V respectively. Q1 and Q3 were maintained at unit resolution and the dwell time was kept at 150ms. The instrument was interfaced with computer running analyst version 1.4.2 software.

Preparation of standards and quality control samples:

Stock solutions of BCT enantiomers and IS were prepared by dissolving the test compounds in methanol to obtain 500 µg/mL concentration for each enantiomer. Stock solutions of BCT enantiomers prepared were serially diluted to prepare working solutions in required concentration range with diluent methanol: water (80:20, v/v). Two separate stock solutions of (-)-BCT and (+)-BCT were prepared for bulk spiking of calibration curve and quality control samples for the method validation experiment. The calibration standards and quality control (QC) samples were prepared by spiking 5% of the total plasma volume with working solutions. Calibration standards were prepared at concentration of 10.0, 20.0, 100.0, 300.0, 600.0, 1200.0, 1800.0, 2400.0, and 3000.0 ng/mL for (-)-BCT and at concentration 2.0, 4.0, 10.0, 20.0, 40.0, 80.0, 120.0, 160.0, and 200.0 ng/mL for (+)-BCT. Similarly, quality control samples (QC's) were prepared at four different concentrations namely, 10.0 (LLOQ), 30.0 (LQC), 1500.0 (MQC) and 2100.0 (HQC) ng /mL for (-)-BCT and at concentration 2.0 (LLOQ), 6.0 (LQC), 100.0 (MQC) and 140.00 (HQC) ng/mL for (+)-BCT. Sufficient calibration standards and quality control samples were prepared to validate the method. Aliquots of the standards and quality controls were stored at -70 °C until used for validation runs.

Sample preparation:

50 µL plasma sample was taken in polypropylene tube (Tarsons, India) and 25 µL of IS dilution (working solution of 0.100 µg/mL each of (-)-d4-BCT and (+)-d4-BCT) was added to it. The contents were vortexed to mix. 200µL of HPLC water was added to each sample. The contents were vortexed for 30 seconds and centrifuged for 5 minutes at 14000 RPM. After centrifugation the samples were loaded on Strata-X 30mg/1mL polymeric reversed phase SPE cartridges preconditioned with 1mL of methanol and equilibrated with 1mL of HPLC water. The plasma matrix was drained out from the extraction cartridges by applying positive nitrogen pressure. The sorbent bed was washed with 1 mL of HPLC water followed by 1 mL of 10% (v/v) methanol in water. The analytes and internal standards were eluted with 2 mL of mobile phase and transferred into autosampler vials for injection. 2 µL of the sample was injected into the LC-MS/MS system through the autosampler.

Method Validation:

Validation experiments of the method were carried out according to USFDA guidelines [28].

Selectivity:

Selectivity was performed using 10 different sources of blank plasma comprising of 6 normal, two hemolysed and two lipemic. These blank plasma samples were processed as per the extraction method and their response was assessed at the retention time of the analytes and the internal standards with six LLOQ samples for (-)-BCT and (+)-BCT (prepared from the screened blank plasma, which had the least interference).

Cross Selectivity:

Cross Selectivity was performed to check the possibility of cross contribution of one enantiomer at the retention time and MRM of the other enantiomer. To assess the cross selectivity blank matrix was spiked with concentration of (-)-BCT at LQC level in duplicate, similarly blank matrix was spiked with concentration of (+)-BCT at LQC level in duplicate. These spiked LQC samples were processed along with LLOQ samples which were spiked separately for both the analytes. The mean response of the interfering peak at the retention time of one enantiomer in the replicate LQC samples was compared against the processed LLOQ samples of the other enantiomer and vice versa, which should be $\leq$20% of the mean response of the LLOQ samples.

Carry Over:

Carryover effect was evaluated to ensure that the rinsing solution used to clean the injection needle and port is able to avoid any carry forward of injected sample in subsequent runs. The design of the experiment comprised blank plasma, LLOQ, upper limit of quantitation (ULOQ) followed by blank plasma to check for any possible interference due to carryover.

Linearity and lower limit of quantification:

The linearity of the method was determined by analyzing three standard plots associated with a nine-point standard calibration curve. The ratio of area response for analyte to IS was used for regression analysis. Each calibration curve was analyzed individually by using least square weighted ($1/X^2$) linear regression. The calculation was based on the peak area ratio of analyte versus the area of internal standard.

The concentration of the analyte were calculated from calibration curve (y = mx + c; where y is the peak area ratio) using linear regression analysis with reciprocate of the drug concentration as a weighing factor ($1/X^2$). Several regression types were tested and the linear regression (weighted with $1/concentration^2$) was found to be the simplest regression. The lowest standard on the calibration curve was accepted as the lower limit of quantitation (LLOQ), if the analyte response was at least five times more than that of drug free (blank) extracted plasma. The deviation of standards other than LLOQ from the nominal concentration should not be more than ±15.0% and for LLOQ it should not be more than ±20.0%.

Precision and Accuracy:

The intra-batch and inter-batch accuracy and precision were determined by replicate analysis of the four quality control levels on three different days. In each of the precision and accuracy batches, six replicates at each quality control level were analysed. Mean and standard deviation (SD) were obtained for calculated drug concentration over these batches. Accuracy and precision were calculated in terms of relative error (%RE) and coefficient of variation (% CV) respectively.

Matrix Effect:

The assessment of matrix effect (co-eluting, undetected endogenous matrix compounds that may influence the analyte ionization) was performed by processing six lots of different normal controlled plasma samples in replicate (*n*=4). LQC and HQC working solutions were spiked post extraction in duplicate for each lot. The results found should fall within the acceptable limit set i.e. the RSD of area ratio to be within ± 15% at each level tested.

Recovery:

Absolute recoveries of the analytes were determined at the three different quality control levels viz. LQC, MQC and HQC, by comparing the peak areas of the extracted plasma samples with those of the un-extracted standard mixtures (prepared in the elution solution at the same concentrations as the extracted samples) representing 100% recovery.

Dilution integrity:

The dilution integrity experiment was intended to validate the dilution test to be carried out on higher analyte concentrations (above ULOQ), which may be encountered during real subject samples analysis. It was performed at 1.6 times the ULOQ concentration. Six replicates samples of ½ and ¼ th concentration were prepared and the concentrations were calculated by applying the dilution factor of 2 and 4 respectively against the freshly prepared calibration curve.

Stabilities:

Stability experiments were conducted to evaluate different conditions that plasma samples may encounter during sample shipment as well as pre- and post-processing such as several freeze-thaw cycles and short term storage of plasma samples at room temperature. All stability results were evaluated by measuring the area response (analyte/IS) of stability samples against freshly prepared comparison samples with identical concentration. Stock solutions and working solutions of analyte and IS were checked for short term stability at room temperature and long term stability at 2–8°C. The solutions were considered stable if the deviation from nominal value was within ±10.0%. For extracted sample conditions such as Autosampler stability, processed sample stability (at room temperature), bench top stability (at room temperature), and freeze–thaw stability at 3 and 5 freezing (at -20°C) and thawing (not warming) at room temperature cycles were performed at LQC and HQC using six replicates at each level. Long term stability of spiked plasma samples stored at –20° C was also studied at both these levels. The samples were considered stable if the deviation from the mean calculated concentration of freshly thawed quality control samples was within ±15.0%.

Bioequivalence study design and incurred sample reanalysis:

The bioequivalence study was conducted with a single fixed dose of a test (150mg tablets from a Generic Company) and a reference Casodex® 150 mg Film tabletten of Astrazeneca GmbH, 22876 Wedel, Germany, in 12 normal, healthy, adult, male human subjects under fasting conditions. Each subject was judged to be in good health through medical history, physical examination and routine laboratory tests. Written consent was taken from all the subjects after informing them about the objectives and possible risks involved in the study. The study was conducted strictly in accordance with guidelines laid down by International Conference on Harmonization, E6 Good Clinical Practice [29]. The subjects were orally administered a single dose of test and reference formulations after recommended wash out period of 7 days with 200 mL of water. Blood samples were collected at 0.00 (pre-dose), 1.00, 2.00, 3.00, 4.00, 6.00, 9.00, 12.00, 16.00, 20.00, 24.00, 28.00, 32.00, 36.00, 40.00, 44.00, 48.00, 60.00, 72.00, 96.00 and 120.00 hours after oral administration of test and reference formulation. Samples at 60.00, 72.00, 96.00 and 120.00 hours were collected on ambulatory basis. Plasma was separated by centrifugation and kept frozen at –20°C until analysis. During study, subjects had a standard diet while water intake was unmonitored. The pharmacokinetic and statistical parameters of (-)-BCT and (+)-BCT were estimated by SAS® version 9.2 (SAS institute Inc. USA). ANOVA was

performed on log transformed pharmacokinetic parameters C_{max} and AUC_{0-120}. To assess bioequivalence, two one sided 90% confidence intervals were calculated for test by reference ratios of geometric least square means of C_{max} and AUC_{0-120} for *R*-enantiomer. T_{max} was calculated by nonparametric Wilkoxon test. An incurred sample reanalysis (assay reproducibility test) was also conducted by random selection of subject samples. The selection criteria included samples which were near the Cmax and the elimination phase in the pharmacokinetic profile of the drug. The results obtained were compared with the data obtained earlier for the same sample using the same procedure. The percent change in the values should not be more than ±20% [30].

RESULTS AND DISCUSSIONS

Enatio selective chromatographic conditions:

To resolve BCT enantiomers several types of chiral columns were tried including macrocyclic glycopeptide based, protein based and polysaccharide based. On macrocyclic glycopeptide based column, there was very slight resolution for BCT enantiomers. Protein based column (Ultron ES-OVM) showed good resolution and separation for BCT enantiomers (Fig. 2).

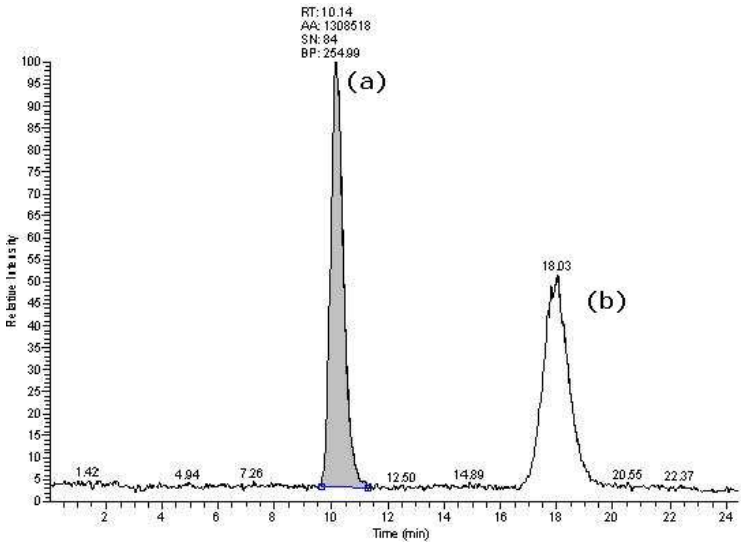
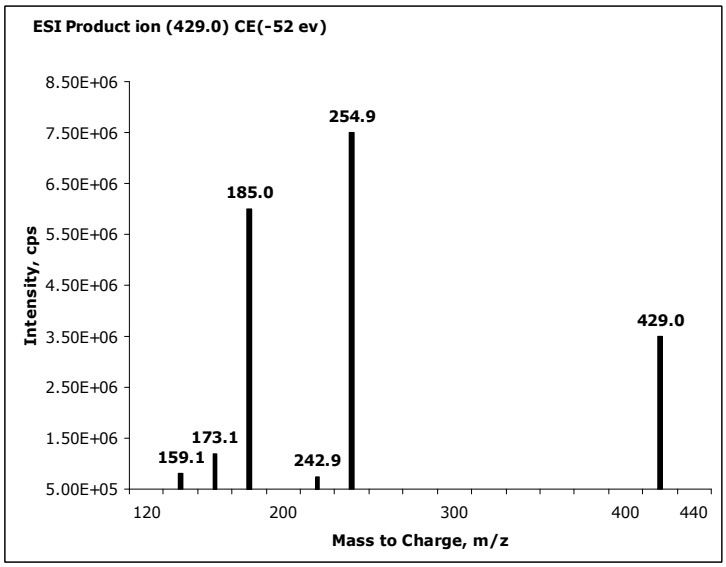


Figure 2: Typical Chromatograms of BCT enantiomers on Ultron ES OVM Column, (a) (-)-BCT and (b) (+)-BCT *(Note: Mobile phase, Ethanol: 12.5mM Ammonium formate buffer, 25:75% v/v)*

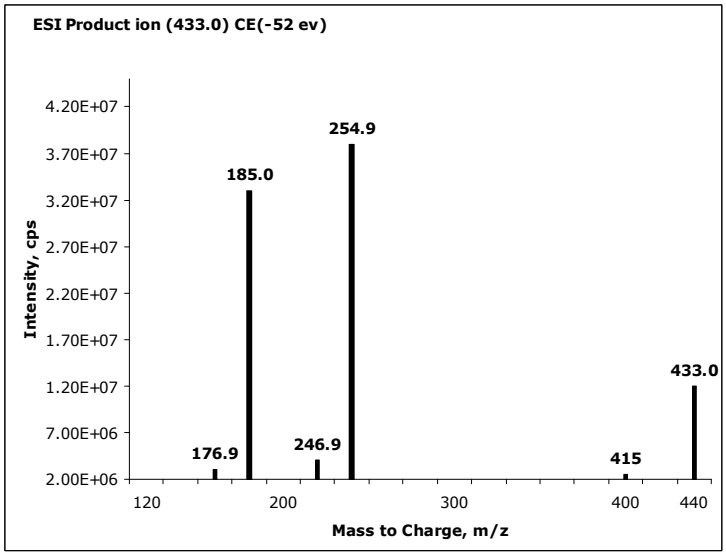
But this type of column can handle very low concentration of organic solvents in the mobile phase [31], which may reduce the sensitivity of MS detection. On the other hand, the normal phase solvents are mostly incompatible with electrospray ionization (ESI) techniques, due to poor ionization or would-be explosion of the mobile phase. Also, long run time and high back pressure on this column insisted us to go for polysaccharide based reverse phased column, Chiralpak AD-3R. The chiral selector's amylose tris (3, 5-dimethylphenyl carbamate) are physically coated on silica matrix in this type of columns, which enables chiral separation in reverse phase that is most suitable for LC-MS/MS detection. Most importantly, this column brought down the run time of enantio separation to 6.5 minutes, most appropriate for the rapid and high throughput analysis. To find the best eluting solvent system, various combinations of methanol/acetonitrile along with buffers (ammonium trifluoro acetate/acetic acid, ammonium formate/formic acid, ammonium acetate/acetic acid, ammonium bicarbonate/ ammonium hydroxide) having different ionic strengths (1–10mM) in the pH range of 3.0–10.0 and volume ratios were tested. With acetonitrile as organic solvent, moderate peak tailing was observed. For better peak shape and higher response, the buffer selected for this study was 5mM Ammonium acetate because of its volatilization and compatibility to MS. The buffer concentration of 50, 20, 10, 5, 2mM were investigated. No significant influence of these buffer concentrations was detected on the enantio selective separation, but the retention time of the enantiomers became shorter and signal response of MS became lower when increasing the buffer concentrations. Hence, 5mM was chosen as the optimum. The effect of pH was also studied in the range of 3.5 to 6.5, by adding acetic acid. The result displayed no notable changes on the separation, retention time and signal response on lowering the pH range. Different column temperatures were also tested from 25°C to 45°C, and concluded that the resolution improved with 25°C column temperature. Based upon these results, the mobile phase composition was set at 5mM ammonium acetate buffer-methanol, (70:30, v/v).

Method Validation:

Selectivity and Cross Selectivity: In the negative ESI mode, deprotonated molecules at *m/z* 429.0 and 433.0 were observed as the most abundant ions for BCT enantiomers and d4 BCT enantiomers, respectively. During daughter ion scan, fragment of 429.0 → 254.9 was observed as predominant fragment. During the developmental work fragment of 254.9 showed high baseline and interference in selectivity experiments. Hence, more stable fragment of 185.0 was chosen for further analysis. The transitions of *m/z* 429.0 → 185.0 for BCT enantiomers and 433.0 → 185.0 for d4 BCT enantiomers were chosen in MRM mode. The product ion spectra of $[M-H]^-$ ions of BCT enantiomers and d4 BCT enantiomers are shown in Fig. 03.



(a)



(b)

Figure.3: Product ion spectra of $[M-H]^-$ (a) BCT enantiomers and (b) d4-BCT enantiomers.

Selectivity of the method was assessed by comparing the chromatograms of blank plasma samples from 10 different sources with the corresponding LLOQ samples. Typical chromatograms of a blank plasma sample, a blank plasma sample spiked with (-)-BCT and (+)-BCT at LLOQ and respective IS are shown in Fig. 04 and Fig. 05. Percent interference observed was less than 4.95% and 0.03% at RT and MRM of BCT enantiomers and d4 BCT enantiomers, respectively.

Whereas, cross selectivity exercise showed 1.40% contribution of (+)-BCT at the retention time and MRM of (-)-BCT and 5.24% for the *vice versa.*

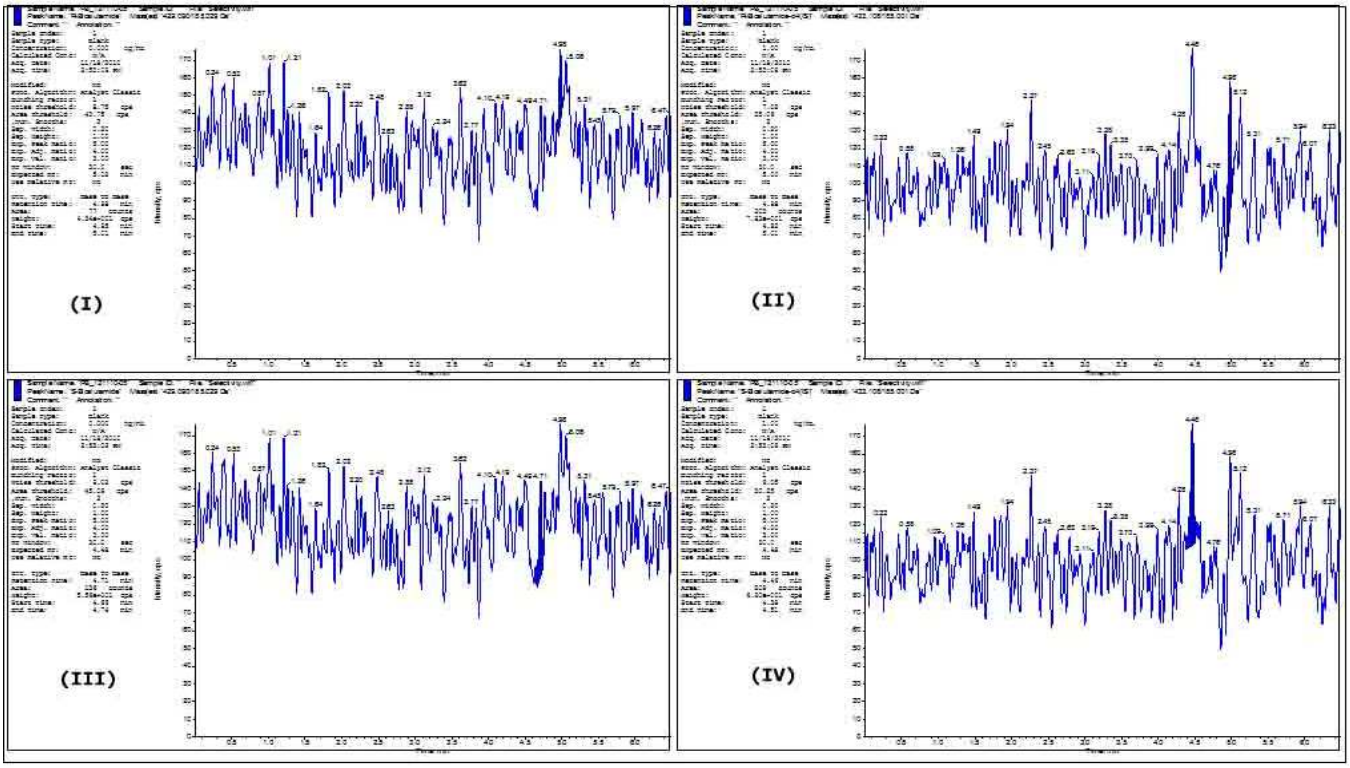


Figure.4: Representative MRM chromatograms of Blank plasma samples of (I) (-)-BCT, (II) (-)-d4-BCT (IS), (III) (+)-BCT and (IV) (+)-d4-BCT (IS) in human plasma.

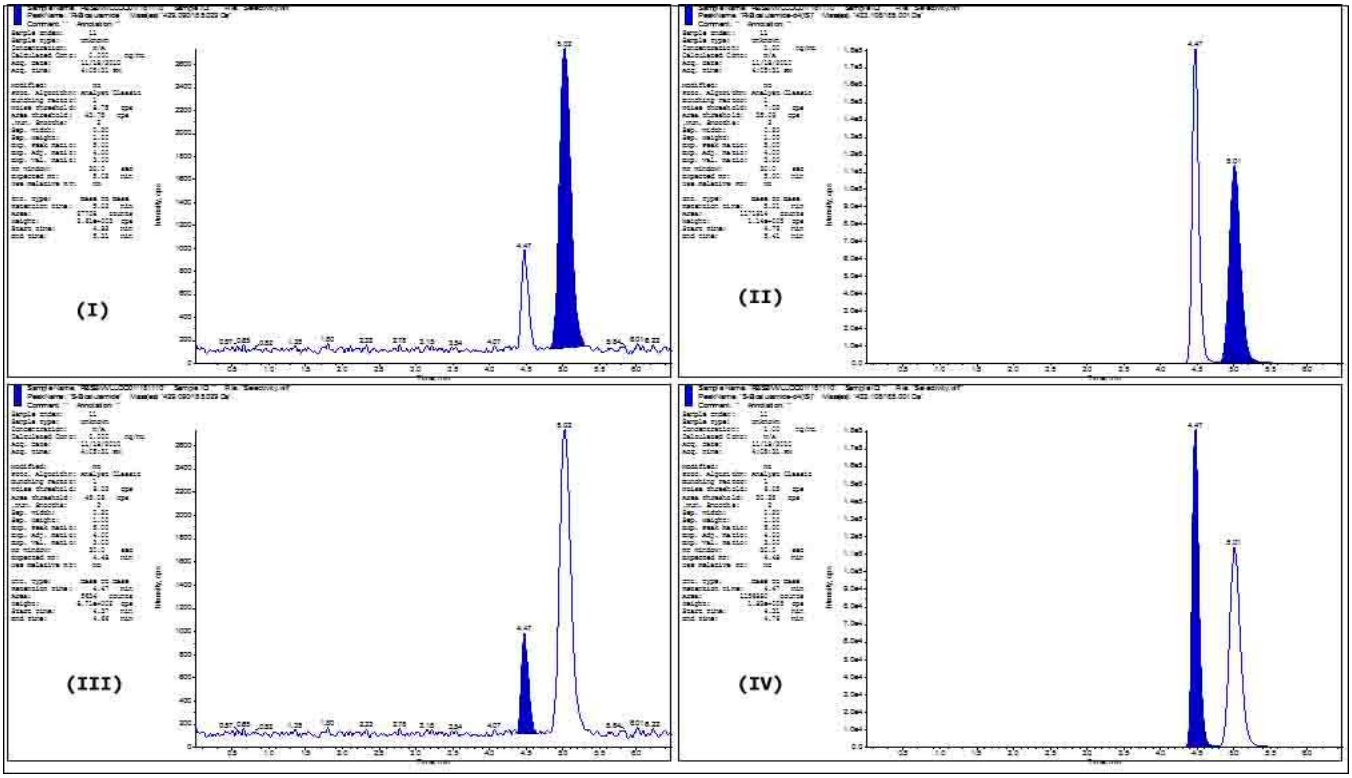


Figure.5: Representative MRM chromatograms of blank human plasma sample spiked with (I) (-)-BCT (10.0 ng/mL), (II) (-)-d4-BCT (0.100 µg/mL) (IS), (III) (+)-BCT (2.0 ng/mL) and (IV) (+)-d4-BCT (0.100 µg/mL) (IS).

Carry Over:

Carry over was evaluated at less than 2.47% and 0.02% at RT and MRM of BCT enantiomers and d4 BCT enantiomers, respectively, with 2µL injection volume, which shows that the rinsing solution of Acetonitrile-Water (40:60 v/v) is good enough to clean the injection needle and port.

Linearity and lower limit of quantification:

The linearity was evaluated based on the average of nine calibrators analyzed on three separate days. Acceptable linearity was achieved in the range of 10.0–3000.0 ng/ml for (-)-BCT and 2.0–200.0 ng/ml for (+)-BCT. For (-)-BCT, the slope was 0.0022 with an intercept of -0.00002. A slope of 0.00238 with an intercept of –0.00014 was determined for (+)-BCT. The correlation coefficients (R^2) for both enantiomers were greater than 0.9991 in all validation batches.

LLOQ samples (*N*=6) were analyzed in each validation run to evaluate sensitivity in a robust manner. The validated assay utilized an LLOQ of 10.0 ng/ml for (-)-BCT and 2.0 ng/ml for (+)-BCT and resulted in a signal-to-noise ratio (S/N) of approximately 150 and 70 for both, respectively. A typical LC–MS/MS chromatogram of the LLOQ sample is shown in Fig. 5. Reliable precision (RSD% ≤1.9) and accuracy (RE% ≤7.7) for (-)-BCT was obtained. Similarly, reliable precision (RSD% ≤3.1) and accuracy (RE% ≤4.8) were obtained for (+)-BCT (Table 1).

Table.1: Precision and accuracy of quality control samples of BCT enantiomers

	LLOQ		LQC		MQC		HQC	
	(-)-BCT	(+)-BCT	(-)-BCT	(+)-BCT	(-)-BCT	(+)-BCT	(-)-BCT	(+)-BCT
					Day 1			
N	6	6	6	6	6	6	6	6
Mean	10.770	2.095	32.098	6.185	1541.326	100.641	2110.769	139.093
RSD%	1.9	3.1	1.4	2.0	1.2	2.4	1.6	1.8
RE%	7.7	4.8	7.0	3.1	2.8	0.6	0.5	-0.6
					Day 2			
N	6	6	6	6	6	6	6	6
Mean	10.159	2.012	32.006	6.172	1590.816	104.717	2131.67	143.260
RSD%	3.4	10.4	2.0	2.7	1.2	1.8	1.4	1.8
RE%	1.6	0.6	6.7	2.9	6.1	4.7	1.5	2.3
					Day 3			
N	6	6	6	6	6	6	6	6
Mean	9.980	2.126	30.530	6.064	1513.760	99.861	2044.207	136.132
RSD%	2.7	5.1	2.2	3.3	0.8	0.8	4.2	5.3
RE%	-0.2	6.3	1.8	1.1	0.9	-0.1	-2.7	-2.8
					Interday			
N	18	18	18	18	18	18	18	18
Mean	10.303	2.078	31.544	6.140	1548.634	101.740	2095.549	139.495
RSD%	4.2	6.8	2.9	2.7	2.3	2.7	3.1	3.8
RE%	3.0	3.9	5.1	2.3	3.2	1.7	-0.2	-0.4

Precision and Accuracy:

The back-calculation results for all calibration standards showed ≤2.3% RSD and –0.5 to 4.1% RE for (-)-BCT and showed ≤3.8% RSD and –1.4 to 3.4% RE for (+)-BCT for all three validation curves as summarized in Table 2. The precision and accuracy of the method were determined by analyzing six replicates of QC samples at low (30.0 ng/ml, LQC), medium (1500.00 ng/ml, MQC), and high levels (2100.0 ng/ml, HQC) for (-)-BCT and low (6.0 ng/ml, LQC), medium (100.00 ng/ml, MQC), and high levels (140.0 ng/ml, HQC) for -(+)-BCT in three separate batches, Table 1. For (-)-BCT, the precision was in the range of 2.3–3.1% RSD and the accuracy was in the range of -0.2–5.1% RE over the three concentration levels evaluated in all the three batches. The precision and accuracy of the LQC, MQC, and HQC for (+)-BCT was in the range of 2.7–3.8% RSD and -0.4–2.3% RE, respectively, over these batches. These results demonstrate that the method provides excellent precision and accuracy.

Table.2: Precision and accuracy of calibration standards of BCT enantiomers

ng/mL	10.00	20.00	100.00	300.00	600.00	1200.00	1800.00	2400.00	3000.00
(-)-BCT									
N	3	3	3	3	3	3	3	3	3
Mean	9.928	20.131	104.141	308.290	615.101	1200.905	1790.389	2358.365	2890.298
RSD%	0.3	0.7	0.4	1.7	2.3	0.6	1.4	0.7	3.7
RE%	-0.7	0.7	4.1	2.8	2.5	0.1	-0.5	-1.7	-3.7

ng/mL	2.00	4.00	10.00	20.00	40.00	80.00	120.00	160.00	200.00
(+)-BCT									
N	3	3	3	3	3	3	3	3	3
Mean	1.993	4.046	9.860	19.990	41.365	81.866	119.933	162.289	199.584
RSD%	1.0	2.0	1.1	1.8	3.8	0.9	1.0	0.7	3.2
RE%	-0.3	1.1	-1.4	-0.1	3.4	2.3	-0.1	1.4	-0.2

Recovery and Matrix Effect

The mean absolute recoveries of (-)-BCT determined at 30.00, 1500.00 and 2100.00 ng/mL were 84.1% (RSD 0.9% & 5.3%), 79.8% (RSD 1.2% & 3.3%) and 79.7% (RSD 1.2% & 5.3%), respectively. The mean absolute recoveries of (+)-BCT determined at 6.00, 100.00 and 140.00 ng /mL were 81.2% (RSD 2.8% & 5.8%), 76.2% (RSD 1.4% & 3.2%) and 76.7% (RSD 2.2% & 5.2%), respectively. The mean absolute recovery of (-)-d4-BCT and (+)-d4-BCT were 91.0% (RSD≤4.9 %) and 90.1% (RSD≤5.1 %), respectively (Table 3).

Minimal matrix effect for (-)-BCT and (+)-BCT was observed from the six different plasma lots tested. The RSD of the area ratios of post spiked recovery samples at LQC and HQC levels were less than 1.80% for (-)-BCT and 2.61% for (+)-BCT. For the internal standard the RSD of the area ratios over both LQC and HQC levels was less than 1.04% and 1.15% for (-)-d4-BCT and (+)-d4-BCT, respectively. This indicated that the extracts were "clean" with no co-eluting compounds influencing the ionization of the analyte and the internal standard.

Table.3: Results for Recovery

Analyte	N	Mean (Peak Area)		SD		RSD		Recovery
		Samples[A]	Samples[B]	Samples[A]	Samples[B]	Samples[A]	Samples[B]	
LQC	6							
(-)-BCT		90863	76454	796.9	4042.8	0.9	5.3	84.1
(+)-BCT		19105	15513	538.8	901.7	2.8	5.8	81.2
(-)-d4-BCT		1164420	1095693	14155.2	49265.7	1.2	4.5	94.1
(+)-d4-BCT		1160511	1069877	16345.5	54351.5	1.4	5.1	92.2
MQC	6							
(-)-BCT		4549060	3629412	54131.8	118026.3	1.2	3.3	79.8
(+)-BCT		333514	254264	4802.8	8203.4	1.4	3.2	76.2
(-)-d4-BCT		1190784	1068426	18088.1	37276.9	1.5	3.5	89.8
(+)-d4-BCT		1194516	1067126	16210.3	37914.3	1.4	3.6	89.4
HQC	6							
(-)-BCT		6175642	4919066	71991.1	259531.6	1.2	5.3	79.7
(+)-BCT		454080	347763	10114.8	18249.0	2.2	5.2	76.7
(-)-d4-BCT		1186480	1057157	13833.1	52233.3	1.2	4.9	89.1
(+)-d4-BCT		1191808	1055139	24003.8	41289.6	2.0	3.9	88.6

Note: [A]Samples spiked with pure standards solutions of both enantiomeric BCT and d4 BCT pairs in mobile phase (neat samples). [B]Extracted human plasma samples spiked with pure standards solutions of both enantiomeric BCT and d4 BCT pairs.

Dilution integrity:

The dilution integrity of the method was determined by analyzing six replicates of DIQC samples (4800.00 ng/ml) for (-)-BCT and 320.00 ng/mL for (+)-BCT after diluting for ½ and ¼ times with blank plasma. For (-)-BCT, the precision was 1.14% and 2.71% RSD and the accuracy was 5.64% and 1.40% RE over the two dilution levels, respectively. For (+)-BCT, the precision was 1.10% and 2.92% RSD and the accuracy was -2.56% and -0.94% RE over the two dilution levels, respectively. Results show that samples with concentration greater than the upper limit of the standard curve could be quantified with reliable accuracy after being diluted with blank matrix.

Stabilities:

Plasma stability data is shown in Table 4. Stock solutions of BCT enantiomers were stable for 23 h at room temperature and 19 days at refrigerated temperature. Bench top, processed sample and autosampler stability for (-)-BCT and (+)-BCT revealed that BCT enantiomers were stable in plasma for at least 23 h at room temperature and processed samples were stable for at least 5 h at room temperature and 61 h in auto sampler at 10°C. It was confirmed that repeated freezing and thawing (five cycles) of spiked plasma samples at LQC and HQC level did not affect the stability of BCT enantiomers and were found stable for minimum five freeze and thaw cycles. The long term

stability results also indicated that BCT enantiomers were stable in human plasma for up to 31 days at a storage temperature of -20°C.

Table.4: Stability of BCT enantiomers under various conditions

	LQC		HQC	
	(-)-BCT	**(+)-BCT**	**(-)-BCT**	**(+)-BCT**
	(30.0 ng/mL)	**(6.0 ng/mL)**	**(2100.0 ng/mL)**	**(140.0 ng/mL)**
Bench top stabiliy (room temperature, 23 h), *N*=6				
Mean	31.061	6.136	2111.858	137.862
RSD%	3.4	2.8	1.7	1.4
RE%	3.5	2.3	0.6	-1.5
Freeze-Thaw stability (5 Cycles, -20°C), *N*=6				
Mean	30.478	6.064	2006.421	133.912
RSD%	0.6	2.0	4.9	5.2
RE%	1.6	1.1	-4.5	-4.3
Autosampler stability (10°C, 61 H), *N*=6				
Mean	30.613	6.163	2018.959	133.799
RSD%	1.1	0.2	4.6	5.2
RE%	2.0	2.7	-3.9	-4.4
Processed sample stability (room temperature, 5 H), *N*=6				
Mean	30.599	6.061	1986.237	133.775
RSD%	0.3	4.5	5.2	5.7
RE%	2.0	1.0	-5.4	-4.4
Long term stability (-20°C, 31 D), *N*=6				
Mean	32.098	6.185	2110.769	139.093
RSD%	1.4	2.0	1.6	1.8
RE%	7.0	3.1	0.5	-0.6

Application of the method in healthy human subjects and Incurred sample results:

The validated method was successfully applied for the assay of BCT enantiomers in healthy Indian male subjects. Fig. 6 shows the plasma concentration vs. time profile for BCT enantiomers under fasting condition. Table 5 summarizes the mean pharmacokinetic parameters after oral administration of 150mg tablets of Bicalutamide test and reference formulation. About 845 samples including the calibration and QC samples along with subject samples were analyzed during a period of 5 days and the precision and accuracy for calibration and QC samples were well within the acceptable limits. The C_{max}, T_{max} and AUC_{0-120} for both the enantiomers obtained in the present work were comparable with the available literature. No statistically significant differences were found between the two formulations in any parameter. The ratios of mean log-transformed parameters (C_{max} and AUC_{0-t},) and their 90% CIs were all within the defined bioequivalence range of 80–125%. These observations confirm the bioequivalence of the test sample with the reference product in terms of rate and extent of absorption. The % change for assay reproducibility in 10% incurred samples was within ±11.22% for both the enantiomers. This authenticates the reproducibility of the proposed method.

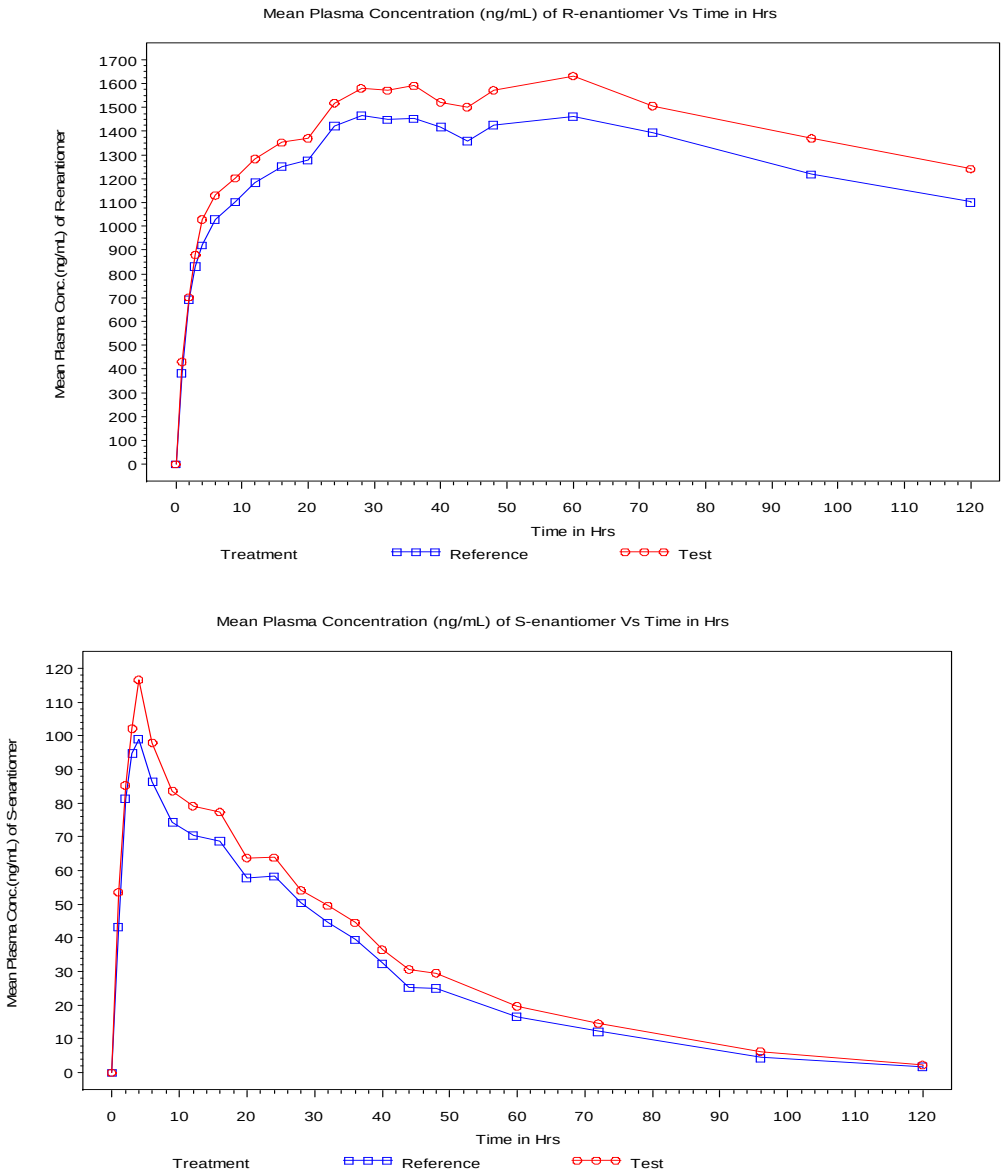


Figure.6: Mean plasma concentration-time profile of BCT enantiomers after oral administration of test (150mg of BCT tablet from a Generic company) and a reference (Casodex® 150 mg Filmtabletten of Astrazeneca GmbH, 22876 Wedel, Germany) formulation to 12 healthy volunteers.

Table.5: Mean pharmacokinetic parameters following 150mg oral dose of Bicalutamide test and reference formulation to 12 healthy Indian subjects under fasting condition.

	(-)-BCT		(+)-BCT	
	Test	**Reference**	**Test**	**Reference**
	Mean±SD	**Mean±SD**	**Mean±SD**	**Mean±SD**
C_{max} (ng/mL)	1670.35±435.53	1565.29±331.23	121.62±30.24	104.35±21.21
T_{max} (h)	41.08±16.22	40.02±18.52	5.33±6.12	4.19±2.47
AUC_{0-120} (h*ng/mL)	167623.62±41645.86	167623.62±41645.86	3781.65±1590.22	3301.07±1002.43

Where, Cmax: maximum plasma concentration; Tmax: time point of maximum plasma concentration; AUC0–t: area under the plasma concentration–time curve from 0 h to 120 h; SD: standard deviation.

CONCLUSION

A simple, selective and rapid method for the simultaneous estimation of BCT enantiomers in human plasma was developed and validated using high-performance liquid chromatographic separation and electrospray ionization tandem mass spectrometric detection in negative mode. The validated method can be applied to pharmacokinetic studies for simultaneous estimation of BCT enantiomers. This method is an excellent analytical option for rapid and simultaneous quantification of BCT enantiomers in human plasma.

A very simple and reliable bioanalytical assay using LC–MS/MS for quantitative determination of BCT

enantiomers in human plasma has been successfully developed and validated for the first time. The baseline separation of the enantiomers was achieved within 6.5 min using a Chiralpak AD-3R column in the reversed-phase mode at simple isocratic LC condition. The sample preparation using SPE was straightforward, simple, and easy for automation, thereby enabling a high throughput capability for analyzing BCT enantiomers while providing very clean samples for bioanalytical assays. The bioanalytical assay yields highly reproducible chromatographic and statistical results when quantifying enantiomeric BCT and provides an accurate and precise format for analyzing subject samples obtained from clinical studies.

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