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EFFECT OF THE STEREOSPECIFIC VS NON-STEREOSPECIFIC ANALYTICAL METHOD ON THE DRUG BIOEQUIVALENCE: CASE STUDY OF BICALUTAMIDE TABLETS

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Abstract: Stereoselectivity is an important consideration in the risk assessment of pharmaceuticals. Chiral separation, enantioselective analysis and asymmetric synthesis technology is rapidly improving, principally driven by the pharmaceutical industry. Therefore it may be reasonable to expect manufacturers to carry out toxicological testing using the individual pure enantiomers of chiral compounds and to justify whenever chiral products are manufactured as the racemates. There are numerous examples in the scientific literature of individual enantiomers displaying different biological effects. Differential metabolism of chiral compounds has been demonstrated for a wide variety of pharmaceuticals; however, differential effects can also result from other factors. The most obvious difference between enantiomers is intrinsic toxicological effect (pharmacodynamics), where one enantiomer has a different mode of action from another or may even be non-toxic. A better understanding of when stereoselective pharmacokinetic and bio-availability investigations are needed or not needed can be obtained by exploring the relationships that exist between the separate pharmacokinetic parameters of enantiomers and racemates. The present work throws light on one of the most important aspect in bio-availability studies, i.e. the effect of the analytical method (stereospecific vs. non- stereospecific) on the statistical power of the bio-availability and bio-equivalence trials. Bicalutamide (BCT) is a non-steroidal antiandrogen used for the treatment of prostate cancer. BCT as such is a racemic mixture, but most of its pharmacological activity is attributed to its *R* enantiomer. 50mg dose of BCT was monitored using stereospecific as well as non-stereospecific liquid chromatography tandem mass spectrometric analytical methods. The data revealed that the absolute bio-availability of Bicalutamide tablets is primarily due to R enantiomer. S enatiomer contribution to the bio-availability is negligible, due to its rapid clearance. Interestingly, data revealed that pharmacokinetic parameters for Bicalutamide enantiomers do not follow dose proportion kinetics. Based on the above facts, it may be courageous enough, to state that single enantiomer drug based on R enantiomer for Bicalutamide is promising.

Key Words: Bicalutamide; Chiral; LC-MS/MS; Human Plasma; Enantiomers; Racemates; Stereospecific; Non-Stereospecific; Analytical method; Bioequivalence.

INTRODUCTION

Carcinoma of the prostate (CaP) is one of the most frequently diagnosed noncutaneous cancer in men (Veveris-Lowe *et al.*,2005, Li *et al.*,2005, Mohler *et al.*,2004, Jemal *et al.*,2002, Brinkmann *et al.*,2000, Visakorpi *et al.*,1995, Taplin *et al.*,1995, Jemal *et al.*,2003). The specific causes of prostate cancer remain unknown till date. Dihydrotestosterone and testosterone are the main androgenic hormones which are implicated in the initiation and promotion of the disease (Brawer, 2003). In vitro studies using the AR+ human prostate cancer cell line, LNCaP, provided the first evidence that structural alteration in the AR is responsible for prostate cancer (Veldscholte *et al.*, 1990, Benetl *et al.*, 1996).

In addition to the identification of AR gene mutations in hormone-relapsed prostate cancer cases, amplification of the AR gene in recurrent prostate tumors have also been reported (Ford *et al.*, 2003, Golias *et al.*, 2009). The elevated AR levels resulting from AR gene amplification probably increases sensitivity to residual circulating androgens facilitating tumor growth.

Currently, there are two main classes of antiandrogens that are clinically used (Fig. 1) (Kimura *et al.*,2001, Gao *et al.*,2005, Mohler *et al.*,2005). 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 (Ishak *et al.*, 1987, Gao *et al.*, 2006, Gao *et al.*, 2007). Moreover, the rigid steroid backbone does not allow wide structural modifications for newer 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

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which eliminates the unwanted side effects (Ishak *et al.,* 1987, Gao *et al.,* 2006, Gao *et al.,* 2007). Moreover, 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 (Eulexin), hydroxyflutamide, nilutamide and bicalutamide (Casodex).

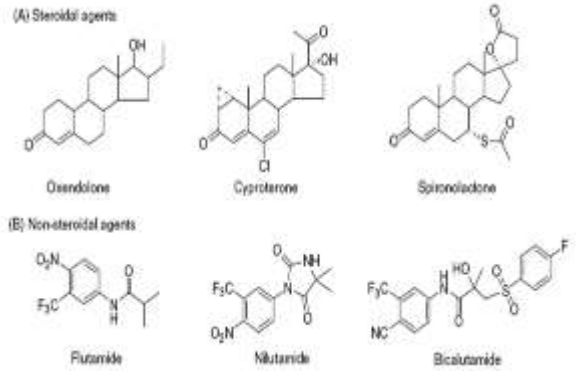


Figure 1: Steroidal (A) and Non-steroidal (B) anti-prostate cancer agents

The treatment of prostate cancer traditionally involves surgery, radiation therapy, and hormonal therapy with luteinizing hormone–releasing hormone agonists and antiandrogens (Heidenreich *et al.,* 2008). Bicalutamide is an oral, nonsteroidal, pure antiandrogen that acts competitively to block androgen receptors. Clinical trials have reported that Bicalutamide monotherapy or combination therapy improved overall survival in patients with early nonmetastatic or advanced prostate cancer (Furr BJ 1996, Wirth *et al.,* 2008, Wirth *et al.,* 2004).

Bicalutamide is a racemate of *R*- and *S*-bicalutamide. The *R*-enantiomer is reported to have a higher affinity for androgen receptors than does the *S*-enantiomer; therefore, antiandrogenic activity resides mainly in the *R*-enantiomer, with little or no antiandrogenic activity residing in the *S*-enantiomer (Mukherjee *et al.,* 1996). *R*-bicalutamide is absorbed slowly, with Cmax achieved between 15 and 48 hours after dosing, whereas *S*-bicalutamide is absorbed rapidly (McKillop *et al.,*1993). Bicalutamide is highly protein bound and is extensively metabolized by glucuronidation and hydroxylation. *R*-bicalutamide is metabolized predominantly by the cytochrome P450 system; the major metabolic pathway for *S*-bicalutamide is glucuronidation (McKillop *et al.,* 1993, Boyle *et al.,* 1993). The mean t1/2 of *R*- and *S*-bicalutamide is reported to be ~4.2 days and ~19 hours, respectively. Because *R*-bicalutamide is more slowly absorbed and cleared than *S*-bicalutamide >99% of circulating Bicalutamide is of the *R*-bicalutamide form at steady state after multiple dosing of bicalutamide racemate (Cockshott 2004).

In the present study, we have developed stereospecific analytical methods using Liquid chromatography tandem mass spectrometry in human plasma that distinguished between individual enantiomers of bicalutamide. The developed method was applied to assess the bio-availability of the test and reference formulations of Bicalutamide tablets in a bioequivalence study. To arrive at the absolute bioavailability of the enantiomers, it was decided to generate the bio-availability data of the racemate of Bicalutamide using non-stereospecific LC-MS/MS method. Accordingly, in the present work, we have studied stereospecific vs. non-stereospecific analytical methods for quantification of racemic as well as enantiomers of bicalutamide. Stereospecific analytical method for separation of BCT enantiomers was thoroughly discussed in our earlier paper (Pradhan VR *et al.,* 2013). In the present manuscript, we are summarizing non-stereospecific analytical method in more detail.

MATERIALS AND METHODS

Reference standards of Racemic BCT, Nimesulide, BCT enantiomers and d4 BCT enantiomers (Fig. 2) 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.

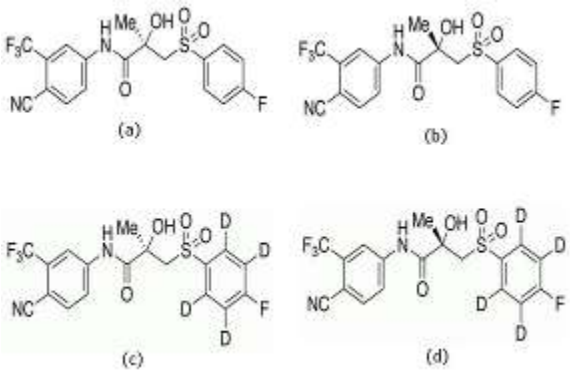


Figure 2: Chemical Structures of (a) (-)-BCT, (b) (+)-BCT, (c) (-)-d4-BCT, (d) (+)-d4-BCT.

Instrumentation and Analytical Conditions

The chromatographic separation and quantification of racemic BCT and its internal standard, Nimesulide was achieved by a liquid chromatography system, LC-10AD (Shimadzu, Kyoto, Japan) coupled with mass spectrometer, API-2000 (AB Sciex, Canada). Analytical column Hypersil Hypurity, C18, (4.6mm x 50mm) was used for the purpose. Mobile phase of 2mM ammonium acetate pH 5.50±0.05 with acetonitrile in the ratio of 20:80 (v/v) was pumped isocratically at flow rate of 0.4 mL/min. to resolve racemic BCT. Auto sampler temperature was set at 10°C and the injection volume was 5µL. The column oven temperature was maintained at 25°C and the total LC run time was 3.0 min.

Preparation of standards and quality control samples

Stock solutions of BCT, BCT enantiomers and their respective IS were prepared by dissolving the test compounds in methanol to obtain 500 µg/mL concentration for each. 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, (R)-(-)-BCT and S-(+)-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.

The calibration standards were prepared by bulk spiking of screened pooled plasma with corresponding working standard solutions to give concentrations of 10.004, 20.008, 100.040, 200.080, 500.200, 800.320, 1200.479 and 1500.599 ng/mL of BCT in plasma for non-stereospecific method on API-2000. Whereas, quality control samples were prepared at 10.008 (LLQC), 30.024 ng/mL (LQC), 700.560 ng/mL (MQC) and 1125.900 ng/mL (HQC) of BCT.

The calibration standards were prepared by bulk spiking of screened pooled plasma with corresponding working standard solutions (5%v/v of working standard solution spiked in plasma) to give concentrations of 10.078, 20.156, 50.390, 120.935, 241.870, 483.740, 725.610, 1027.947, 1208.342 ng/mL for (R)-(-)-Bicalutamide and 2.004, 4.008, 8.016, 10.020, 20.039, 40.078, 60.117, 85.166, 99.694 ng/mL for (S)-(+)-Bicalutamide in plasma for the stereospecific analytical method for quantitation of BCT enantiomers on API-3000. Whereas, quality control samples were prepared at 10.082 ng/mL (LLQC), 30.246 ng/mL (LQC), 403.276 ng/mL (MQC), 846.880 ng/mL (HQC) for (R)-(-)-Bicalutamide and 2.006 ng/mL (LLQC), 6.002 ng/mL (LQC), 33.012 ng/mL (MQC), 70.025 ng/mL (HQC) for (S)-(+)-Bicalutamide.

Sufficient calibration standards and quality control samples were prepared to validate both the methods. Aliquots of the standards and quality controls were stored at -20°C until used for validation runs. For stability studies fresh stock solutions were prepared at the time of evaluation.

A working solution of the internal standard was prepared everyday by diluting the respective internal standard stock solutions with diluent to prepare a suitable concentration for each method.

Sample preparation

Stereospecific analytical method: 50µL 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 auto sampler vials for injection. 2 µL of the sample was injected into the LC-MS/MS system through the auto sampler.

Non-stereospecific analytical method: Plasma samples to be processed were thawed at room temperature. Strata X (30mg/1mL) SPE cartridges were conditioned by passing through them 1mL of Methanol and equilibrated with 1 mL of Water. To prepare samples for loading, 200µL plasma was taken in polypropylene tube (Tarsons, India), to that 50 µL of internal standard (about 10.000 µg/mL of Nimesulide) was added. Samples were Vortexed to mix for 30 seconds, centrifuged for 5 minutes at 15000 RPM and loaded on cartridges. The samples were loaded on SPE cartridge and eluted at pressure not more than 2psi. Sorbent bed was washed with 1 mL of Water followed by 1 mL of 10% (v/v) methanol in water and the analyte was eluted with 1 mL of Mobile phase. The samples were transferred into autosampler vials for injection.

Method Validation

Validation experiments of the method were carried out according to USFDA guidelines (Guidance for Industry: Bioanalytical Method Validation, 2001).

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 racemic BCT, (-)-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 ≤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 unextracted 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 Auto sampler 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 (50mg tablets from a Generic Company) and a reference Casodex® 50 mg Filmtabletten 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 (Guidance for Industry, CBER, 1996). 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, 52.00, 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 racemic BCT, (-)-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 racemic BCT and *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% (Yadav M *et al.*, 2011).

RESULTS AND DISCUSSIONS

Method Validation

Results of Non-Stereospcific analytical method:

Table 01 provides the summary of method validation results for racemic BCT, whereas Fig.3 gives representative MRM chromatograms.

Table 1: Method Validation Summary of Non-Stereospcific analytical method

Validation Parameters	Results
Selectivity	% Interference in normal human plasma with heparin as an anticoagulant ≤ 8.33%, in Hemolysed plasma ≤ 0.65% and in Lipemic plasma ≤ 2.37%, % Interference of IS in normal human plasma with heparin as an anticoagulant 0.00%, In Hemolysed plasma 0.00 % and In Lipemic plasma 0.00 %, % CV of LLOQ analyte area response 10.47 % % CV of LLOQ IS area response 2.36%
Carryover Effect	Mean % Interference, For Analyte: 1.05%, For IS: 0.03%
Inter Day Precision	% CV: (LLQC: 7.95%), (LQC: 7.67%), (MQC: 4.91%), (HQC: 3.23%)
Inter Day Accuracy	% Nominal: (LLQC: 91.81%), (LQC: 99.01%), (MQC: 95.84%), (HQC: 91.02%)
Intra Day Precision	% CV: (LLQC: 6.75%), (LQC: 5.27%), (MQC: 4.95%), (HQC: 2.38%)
Intra Day Accuracy	% Nominal: (LLQC: 88.11%), (LQC: 98.69%), (MQC: 97.86%), (HQC: 90.55%)
Recovery	Mean Overall Recovery: (Analyte: 87.78%), (IS: 93.44%) Precision: (Analyte: 3.55%), (IS: 2.11%)
Bench Top Stability At Room Temperature (24 Hrs)	% Difference: (LQC: 14.60%), (HQC: 0.14%)
Freeze Thaw Stability	% Difference: (LQC: 13.04%), (HQC: 6.17%)
Autosampler Stability (29 Hrs)	%Difference: (LQC: 2.38%), HQC: 4.00%
Room Temperature Stock Solution Stability (6 Hrs)	% Difference: (Analyte: -1.81%), (IS: -1.90%)
Room Temperature Stock Solution Stability (24 Hrs)	% Difference: (Analyte: 0.09%), (IS: -0.80%)
Refrigerated Stock Solution Stability (5 Days)	% Difference: (Analyte: -0.71%), (IS: -3.87%)
Dilution Integrity	Four times dilution: (Precision: 1.69%), (Accuracy: 106.00%) Two times dilution: (Precision: 2.94%), (Accuracy: 102.30%)
Reinjection Reproducibility	% Difference: (LQC: 3.65%), (HQC: 0.63%)
Matrix Effect	%CV of Post spiked QCs: (LQC: 3.97%), (HQC: 3.01%) % CV for IS response 10.08%
Refrigerated Stock Solution Stability (36 Days)	% Difference: (Analyte: -7.39%), (IS: -3.77%)
Long Term Stability In Matrix (173 Days)	% Difference: (LQC: -0.25%), (HQC: 2.84%)

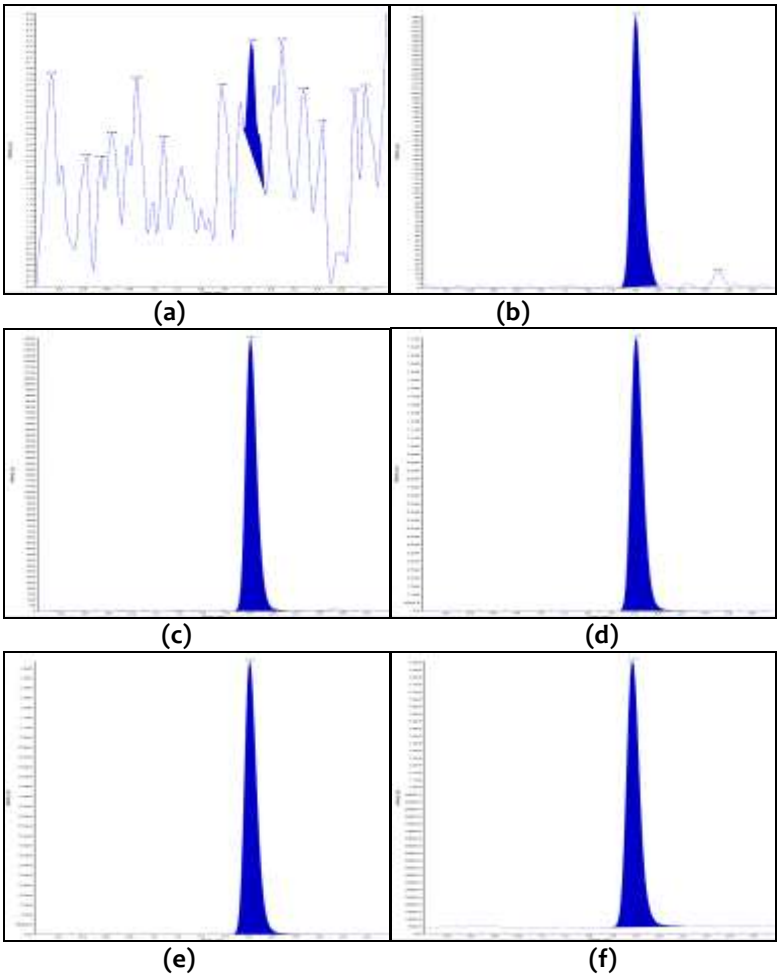


Figure 03: Representative MRM Chromatograms of human plasma samples a) Blank plasma sample, b) Bicalutamide at LLOQ level c) Bicalutamide at MQC level d) Bicalutamide at ULOQ level e) Unknown sample of Bicalutamide at Cmax level from Bio-equivalence study, f) Nimesulide, Internal standard.

Results of Stereospcific analytical method

Method validation results of stereospecific analytical method for separation of BCT enantiomers was thoroughly discussed in our earlier paper (Pradhan VR *et al.*, 2013).

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

The validated stereospecific as well as non-stereospecific analytical methods were successfully applied for the assay of racemic BCT and BCT enantiomers in healthy Indian male subjects. Fig. 4 shows the plasma concentration vs. time profile for racemic BCT under fasting condition. Fig. 5 shows the plasma concentration vs. time profile for BCT enantiomers under fasting condition. Table 2 summarizes the mean pharmacokinetic parameters after oral administration of 50mg tablets of Bicalutamide test and reference formulation for racemic BCT. Whereas, table 3 summarizes the mean pharmacokinetic parameters after oral administration of 50mg tablets of Bicalutamide test and reference formulation for BCT enantiomers. About 804 samples per study 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 for both the studies. The C_{max}, T_{max} and AUC_{0-120} for racemic BCT and 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 for both the studies. 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 from both the studies was within ±20% for racemic BCT as well as for both the enantiomers. This authenticates the reproducibility of the proposed methods.

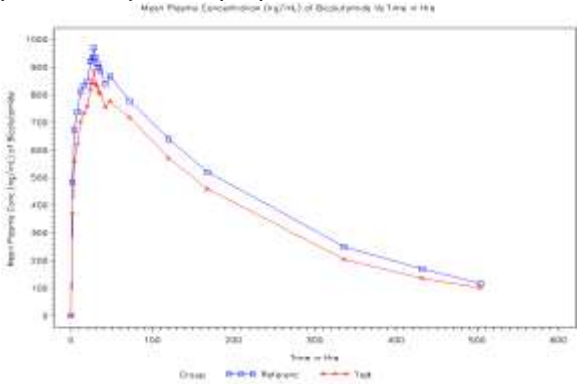
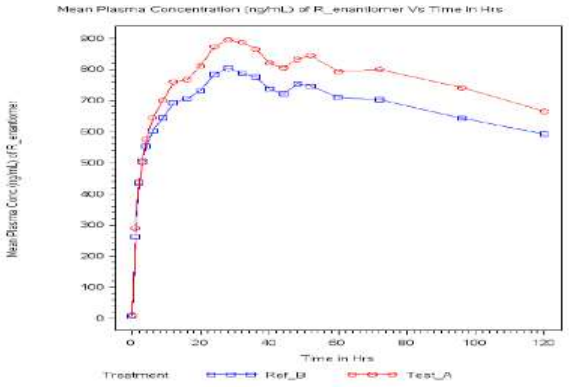


Figure 4: Mean plasma concentration-time profile of racemic BCT after oral administration of test (50mg of BCT tablet from a Generic company) and a reference (Casodex® 50 mg Filmtabletten of Astrazeneca GmbH, 22876 Wedel, Germany) formulation to 12 healthy volunteers.



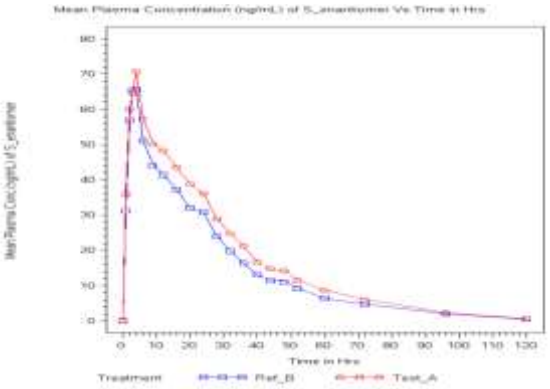


Figure 5: Mean plasma concentration-time profile of BCT enantiomers after oral administration of test (50mg of BCT tablet from a Generic company) and a reference (Casodex® 50 mg Filmtabletten of Astrazeneca GmbH, 22876 Wedel, Germany) formulation to 12 healthy volunteers.

Table 2: Comparison of Treatment A with Reference B for racemic BCT

						Strength	Intra – Subject CV	Confidence Intervals (CI) (90 %)	
Obs.	**Name**	**Geomean A**	**Geomean B**	**mse**	**Ratio**	**Power**	**intra_cv**	**Lower Limit**	**Upper Limit**
1	Log transformed AUC(0-120)	185155.20	208056.60	0.03	88.99	99.98	16.48	83.53	94.82
2	Log transformed AUC (0-inf)	210348.10	243790.70	0.03	86.28	99.96	17.34	80.72	92.23
3	Log transformed Cmax	879.92	972.89	0.02	90.44	100.00	13.49	85.86	95.27

Where, Geomean A = Geometric Mean of Test Product; Geomean B = Geometric Mean of Reference Product; mse= Mean square error
p= probability

Table 03: Mean pharmacokinetic parameters following 50mg 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)	927.11±208.69	832.41±181.41	75.83±23.92	69.66±19.09
T_{max} (h)	31.85±8.20	32.31±10.18	4.22±3.84	3.71±2.57
AUC_{0-120} (h*ng/mL)	92441.21±21237.63	82287.45±16960.09	1987.18±619.19	1671.57±509.32

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.

DISCUSSION

Stereoselectivity is an important consideration in the risk assessment of pharmaceuticals. Chiral separation, enantioselective analysis and asymmetric synthesis technology is rapidly improving, principally driven by the pharmaceutical industry. Therefore it may be reasonable to expect manufacturers to carry out toxicological testing using the individual pure enantiomers of chiral compounds and to justify whenever chiral products are manufactured as the racemate. There are numerous examples in the scientific literature of individual enantiomers displaying different biological effects. Differential metabolism of chiral compounds has been demonstrated for a wide variety of chemicals; however, differential effects can also result from other factors. The most obvious difference between enantiomers is intrinsic toxicological effect (pharmacodynamics), where one enantiomer has a different mode of action from another or may even be non-toxic. The contribution of pharmacokinetic factors towards differential effects of enantiomers have been recognized more recently and include an important role for metabolism, but also include protein or tissue binding and active transport processes that might occur during absorption or excretion of a chemical when interpreting data from in vivo studies.

The main objective of this work was to develop stereoselective as well as non-stereoselective assay for estimation of racemic BCT and BCT enantiomers in human plasma by high performance liquid chromatography tandem mass spectrometry. In case of BCT enantiomers, *R*-enantiomer is

pharmacologically active, whereas S-enantiomer is inactive.

A better understanding of when stereoselective pharmacokinetic and bio-availability investigations are needed or not needed can be obtained by exploring the relationships that exist between the separate pharmacokinetic parameters of enantiomers and racemates. It should be recognized that the parameters of clearance, extent of absorption, volume of distribution and half-life for a racemate are complex collections and composites of those of each stereoisomer. Assay of a racemate by non-stereospecific methods effectively results in the summation of the individual stereoisomer plasma concentration. Due to the possible differences in the rates of absorption and elimination, the concentration ratio of the stereoisomers may vary throughout the plasma concentration profile. The present work throws light on one of the most important aspect in bio-availability studies, i.e. the effect of the analytical method (stereospecific vs. non- stereospecific) on the statistical power of the bio-availability and bio-equivalence trials.

Bicalutamide, 50mg dose was monitored using stereospecific as well as non-stereospecific analytical methods. The data revealed that the absolute bio-availability of Bicalutamide tablets is primarily due to R enantiomer. S. enantiomer contribution to the bio-availability is negligible, due to its rapid clearance. Stereospecific analytical method for 150mg dose further confirmed this fact (Pradhan VR *et al.*, 2013). Interestingly, data revealed that pharmacokinetic parameters for Bicalutamide enantiomers do not follow dose proportion kinetics. Based on the above facts, it may be courageous enough, to state that single enantiomer drug based on R enantiomer for Bicalutamide is promising.

CONCLUSION

Simple, sensitive, rugged, high throughput and effective methods for determination of racemic BCT as well as BCT enantiomers in human plasma by LC-MS/MS using multiple reaction monitoring were developed and fully validated according to international regulatory guidelines. The greater advantages of the current methods are, short run time, simple method and very good sample processing technique. The sample preparation using SPE was straightforward, simple, and easy for automation, thereby enabling a high throughput capability for analyzing racemic BCT and BCT enantiomers while providing very clean samples for bioanalytical assays. The bioanalytical assays yields highly reproducible chromatographic and statistical results when quantifying racemates and enantiomers and provides an accurate and precise format for analyzing subject samples obtained from clinical studies. These methods were successfully adopted for the analysis of the samples received from different bio-equivalence studies. Incurred sample reanalysis confirmed the excellent reproducibility of both these methods.

We look forward to that this work will contribute to the current progress within the literature to support awareness in clinical pharmacology of enantio selectivity. The chiral separation of racemic drugs is a necessary operation in pharmaceutical industry as well as in clinical therapeutics. Therefore, the development of new chiral separation techniques is and will be a topic of interest in academic research as well as in industrial progress. However, the use of a single isomer must be seriously taken after long clinical assessments between racemate and single enantiomer actions because in some cases, racemates have more therapeutic advantages than single isomers. It is also important to give more information about chiral drugs especially racemic form to healthcare professionals in order to help them for finding an optimal treatment and a right therapeutic control.

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