Research Article

A Validated Method Development of Dutasteride in Human Plasma Using LC-MS/MS

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ABSTRACT
This paper presents a rapid, reliable and specific LC-MS/MS developed and validated method for quantification of dutasteride a potent inhibitor of 5α-reductase in human plasma. The analyte and internal standard dutasteride-d6 were extracted by solid phase with methanol/water/formic acid using glas-col multipulse vortexer. Detection of analyte was performed by Agilent,Zorbax Eclipse, C18,4.6*50mm, 5µm column with a mobile phase of 10mM ammonium acetate/methanol (15/85v/v) with flow rate of 0.85ml/min and injection volume 20µl. The mass transition optimized was m/z 529.3→535.3 and m/z 461.2→467.2 to measure I and II respectively. Lower limits of quantification were found to be 0.100 ng/ml for dutasteride. Acceptable precision and accuracy were obtained for linear concentration range. The average percentage range recovery of the dutasteride was 61.8% and that of the ISTD was found to be 50.95%. The present method was applied to quantify the drug in the human plasma samples and used in the bioavailability and bioequivalence studies.

Keywords: Liquid chromatography, Mass Spectrophotometry, method development, Validation.

1. INTRODUCTION

Dutasteride is a 5 α -reductase inhibitor that inhibits both type 1 and type 2 isoenzymes of 5 α –reductase, the enzyme responsible for converting testosterone to dihydrotestosterone in the prostate and other tissues. Dihydrotestosterone[1] is the primary cause of prostate growth and has been proven to play a key role in the development and progression of benign prostatic hyperplasia. Dutasteride is being investigated for its efficacy in reducing the risk of prostate cancer in at risk men in the 4 year REDUCE[2] study and as treatment to extend the time to progression in men with low risk localized prostate cancer in the 3 year REDEEM[3] study. Dutasteride is an effective treatment option in patients with moderate to severe symptomatic BPH and demonstrable prostatic enlargement and may have potential to reduce the risk of developing biopsy-detectable prostatic cancer in at risk individuals (or) extending the time to progression in low risk localized prostate cancer. Chemically DTS is (5 α, 17 β) –N-{2,,5 bis (trifluoromethyl)phenyl }-3- α o-4-azandrost-1-ene -17-azaandrost-1-ene- 17-carboxamide with an empirical formula $C_{12}H_{30}F_6N_2O_2$, representing a molecular weight of 528.5 g/mol. It is bound to 99% to albumin[4], 97% to acid glycoprotein > 96% to serum protein. Literature survey revealed LC-MS, HPLC methods for estimation of DTS in biological samples. RP HPLC[5] method developed for the estimation of Dutasteride in tablet dosage form (patel et al.2010,).DTS was determined in bulk drug and pharmaceutical formulations by application of spectrophotometer (kamila et al., 2010). Stability indicating TLC method was developed in pharmaceutical dosage forms [6](vish et al.,2009). It is also estimated in combined form with other drugs like Alfuzosin Noel et al, 2009), Tamsulosin Aagarwal et al ,2008) by using LC- MS /MS with different extraction methods. There was one report which deals estimation of DTS[7] in plasma by LC-MS. The paper presents a rapid solid phase extraction and liquid chromatography, mass spectrophotometer and validation for Dutasteride in human plasma.

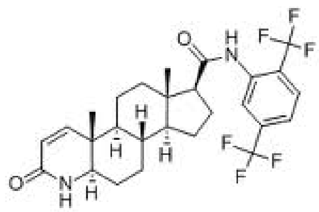

Fig. 1: Structure of Dutasteride

2. EXPERIMENTAL

2.1Chemicals

Dutasteride drug substance was obtained from Dr.Reddy's and Dutasteride $^{13}C_6$ working standard was obtained from Syncan laboratories. Concomitant drugs were obtained from USP, Varda Biotech .reagents like formic acid SQ grade , liquor ammonia Excel R grade were from Qualigens, methanol Lichrosolv grade and HPLC – grade water from Milli Q system (Millipore, Bedford, MA,USA was used. All other chemicals were of analytical grade.

2.2 Chromatography

Agilenti 1100 series HPLC was equipped with a quaternary pump, a degasser, an auto sampler, an injector with a 20 µl loop. The separation of compounds was made on a Zorbax Eclipse, C18 column (5µm, 4.6 × 50 mm i.d) at 25°c . The mobile phase contain 10 mM ammonium acetate, methanol (15:85 V/V) pumped at a flow rate of 0.85 ml/min. MS/MS contains MDS SCIEX API 4000.

2.3 BIOANALYTICAL METHOD DEVELOPMENT

Sample extraction technique

Step 1: Blank samples, calibration curve standards, quality control samples were withdrawn from the deep freezer and allowed to thaw, vortexed to ensure complete mixing of contents. 50 µl of 50% methanol in water solution was added to a vial which is labelled as plasma blank. 50 µl of ISTD8 was added to pre labelled vials. 0.500ml of plasma was transferred to all the above vials from the respective samples. Then 0.500ml of 2% formic acid in water solution was added to all samples, vortexed to ensure complete mixing of contents.

Step 2: water MCX OASIS 30 mg, 1 CC Catridges were taken on to a positive processor and the below procedure is followed.

2.3.1 Conditions

1.000 ml of methanol followed by 1.000 ml of water followed by 1.000 ml of 2% formic acid in water solution was added.

Application

The sample was applied and allowed to dry for about 2 min under positive pressure for about 2 minutes.

Elution

Drug was eluted with 1 ml of 1% liquor ammonia[9]in methanol solution. The samples were evaporated under a stream of nitrogen at 50°c. The residue was reconstituted with 0.250 ml of mobile phase. The samples were loaded into auto injector vials. 20 µl of the sample was injected into LC-MS/MS system[10].

2.3.2. Preparation of reference standard solution

The mixture of 5 ng/ ml concentration of Dutasteride and 5 ng/ ml concentration of ISTD in mobile phase was prepared.

2.3.3 Preparation of calibration curve standards

Preparation of stock dilutions of standard Dutasteride solution

Stock dilutions of DTS ranging from 2.000ng/ml to160.000ng/ml were prepared with 50 % methanol in water using dilutions of main stock solution prepared for calibration curve standards are shown in table 1.

Spiking of plasma for calibration curve standard

Different concentrations of DTS ranging from 0.1000 ng/ml to 8.0000ng/ml with K_2EDTA human plasma were prepared and labelled as CC1 to CCL, which are mentioned in table 2.

2.4 Bioanalytical method validation parameters

2.4.1. System suitability

Six sets of known concentrations of reference standard solution of analyte and ISTD (Levonorgestrel-d6) in mobile phase were injected. The CV% for retention time and area ratio (analyte area/ ISTD area) was calculated.

2.4.2. Selectivity

Blank samples from the K_2EDTA[11] human plasma obtained from eight donors of which one from haemolytic K_2EDTA human plasma and other from lipemic K_2EDTA human plasma were analyzed.

Six samples at LLOQ[12] concentration spiked using the K_2EDTA human plasma of any one donor was analysed and the mean of the peak response was compared with blank samples.

2.4.3. Specificity /Selectivity
The interference at analyte retention time caused due to ISTD by injecting six replicates of matrix blank with ISTD was evaluated. The response of analyte , if any, obtained with the mean response of analyte obtained with LLOQ concentration injected was compared.
The interference at ISTD retention time caused due to analyte by injecting six replicates of matrix blank with analyte was evaluated.

2.4.5. Carryover effect
One blank plasma from the biological matrix for seven injections and one sample at ULOQ concentration for six injections was prepared. These samples were processed alternatively to check if there is any carryover in the blank samples due to ULOQ[13] samples.

2.4.6. Sensitivity
It is determined by lower limit of quantitation. LOQ-QC is the lower limit of quantitation quality control sample that can be measured with acceptable accuracy and precision.

2.4.7. Ruggedness
It is performed for changes in the following parameters that were studied during precision and accuracy batches[14].
Column (minimum two columns with same make and lot or if possible different lot numbers) and Analyst (this was studied during precision and accuracy batches with two different analysts)

2.4.8. Recovery
Analytical results of six replicates of analyte along with ISTD for extracted samples at three concentrations were compared. % recovery of analyte[15] and ISTD using appropriate chromatographic conditions was assessed.

2.4.9. Linearity
Minimum four calibration curves were performed for linearity. The number of standards used in constructing a calibration curve is a function of the anticipated range of analytical values. Concentration of standards were chosen on the bases of the concentration range expected in a particular study.

2.4.10. Accuracy
Under each calibration curve, six replicate of each of the low concentration (LQC), geometric mean concentration (GMQC), medium concentration (MQC) and high concentration (HQC) quality control samples were analyzed.
Under any one calibration curve, six replicate each of haemolytic LQC, lipemic LQC[16], haemolytic HQC and lipemic HQC samples spiked in haemolytic and lipemic plasma were analyzed.

3.0. RESULTS AND DISCUSSION
3.1. Validation parameters
3.1.1. System suitability
The results of system suitability have been tabulated in table 3. The results were within the acceptance criteria.

3.1.2. Selectivity
The selectivity of the present method was established by checking the blank K_2EDTA human plasma, K_2EDTA lipemic plasma and K_2EDTA haemolytic plasma obtained from 8 different donors. As these blood samples were collected from eight different people, all possible K_2EDTA human plasma profiles which may contain any interfering compounds that elute along with Dutasteride and ISTD (Dutasteride $^{13}C_6$)
Also spiked six samples at LLOQ concentration of Dutasteride and ISTD (Dutasteride 13 C6) in plasma except haemolytic and lipemic plasma. By comparing the response of analyte and ISTD, if any with the mean response of LLOQ injected. Hence there were no significant interfering peaks found at Dutasteride retention time and ISTD (Dutasteride$^{13}C_6$) retention time in plasma blanks, which was mentioned in table 4 and chromatograms were represented in fig2 and fig3.

3.1.3. Specificity
The specificity of the present method was established by checking the interference at Dutasteride retention caused due to ISTD by injecting six replicates of matrix blank with ISTD and interfering at ISTD retention time caused due to DTS by injecting 6 replicates of matrix blank with MQC of Dutasteride.
The response of analyte and ISTD was compared, if any with the mean response of LLOQ concentration injected. Hence there was no significant interference peaks obtained at DTS retention time due to ISTD and there was no peaks obtained at Dutasteride –d6 (ISTD) retention times caused due to DTS and chromatogram was represented in fig4.

3.1.4. **Carry over effect**
The carry over effect of the present method was established by processing two blank

samples from biological matrix (plasma) for seven injections and two samples at ULOQ concentration six injections. These samples were analysed alternatively to check if there is any carry over in the blank sample. There was no carry over effect observed[17].

3.1.5. Sensitivity
The lower limits of quantification (LLOQ) was found to be 0.100 ng/ml for DTS. The % accuracy was 101.67% and precision denoted by CV% was 7.48%.

3.1.6. Ruggedness
Ruggedness was studied along with precision and accuracy batches where effect of column and analyte change was observed.

The effect of column variation has been shown in precision and accuracy batch II and III and the effect of analyte variation has been shown in precision and accuracy batch I and II Results of column variation and analyte variation obtained for precision and accuracy were within the acceptance criteria.

3.1.7. Recovery
The average percentage recovery of the Dutasteride was 61.81% and that of the ISTD was found to be 50.95 %. Results were mentioned in table 5 (a) and 5 (b).

3.1.8. Linearity
The method was found to be linear between the ranges of 0.100 ng/ ml to 7.993 ng/ml for Dutasteride. A straight line fit was made through the data points by $1/X^2$ weighed method. The observed correlation co efficient was greater than 0.99 in all the cases.

Hence the method is linear in the stated range. Calibration curve was mentioned in fig 5.

3.1.9. Accuracy
The % accuracy observed for inter batch QC samples was 97.333% , 96.89%,96.19 % and 98.28% for LQC,GMQC,MQC and HQC respectively. The % accuracy for the inter batch QC samples was ranged from 97% to 97.56%, 95.50% to 97.7%, 94.63% to 98.06% and 96.68% to 99.52 % for LQC, GMQC ,MQC and HQC respectively.

4. CONCLUSION
In the present work, an attempt was made to provide a newer, sensitive, simple, accurate LC-MS/MS method. It was validated the developed method that results good sensitivity, less interferences, rugged and rapid with good recovery. The validated method can imply in the Bioavailability and Bioequivalence studies for the approval of drug in the market.

Table 1: Preparation of calibration curve standard

Stock concentration (ug/ml)	Volume of stock (ml)	Volume of diluents (ml)	Final volume (ml)	Final concentration (ug/ml)
10.000	0.080	4.920	5.000	160.000
10.000	0.060	4.940	5.000	120.000
1.000	0.300	4.700	5.000	60.000
1.000	0.200	4.800	5.000	40.000
1.000	0.100	4.900	5.000	20.000
0.100	0.500	4.500	5.000	10.000
0.100	0.200	4.800	5.000	4.000
0.100	0.100	4.900	5.000	2.000

Table 2: Preparation of spiked calibration curve standards

Stock concentration (ug/ml)	Volume of stock (ml)	Volume of plasma (ml)	Final volume (ml)	Final concentration (ug/ml)	Label
160.000	0.500	9.500	10.000	8.000	cc8
120.000	0.500	9.500	10.000	6.000	cc7
60.000	0.500	9.500	10.000	3.000	cc6
40.000	0.500	9.500	10.000	2.000	cc5
20.000	0.500	9.500	10.000	1.000	cc4
10.000	0.500	9.500	10.000	0.500	cc3
4.000	0.500	9.500	10.000	0.200	cc2
2.000	0.500	9.500	10.000	0.100	cc1

Table 3: System Suitability of Dutasteride

s.no	RT(min)		Peak response		
	Analyte	Internal std	Analyte	Internal std	Analyte/Internal std
1	1.19	1.19	115568	75880	1.52
2	1.19	1.18	120423	77559	1.55
3	1.19	1.19	115388	75685	1.52
4	1.19	1.19	110917	72506	1.53
5	1.19	1.19	116096	75545	1.54
6	1.19	1.18	110920	74045	1.50
Mean	1.19	1.19			1.53
SD	0.0000	0.0052			0.0180
CV%	0.00	0.44			1.18

Table 4: Selectivity of Dutasteride

ID	Dutasteride peak area	ISTD peak area
LLOQ-1	3798	113073
LLOQ-II	3479	111616
LLOQ-III	3669	110380
LLOQ-IV	3584	111467
LLOQ-V	3442	108205
LLOQ-VI	3539	109568
Mean	3585	110718

Table 5: Recovery of Dutasteride

Standard	ID	Un extracted std peak area	ID	Extracted std peak area	% recovery
HQC	Rc Aqs HQC-01	274457	RcSpHQC-01	160597	
	Rc Aqs HQC-02	267720	RcSpHQC-02	162794	
	Rc Aqs HQC-03	270508	RcSpHQC-03	160692	
	Rc Aqs HQC-04	271614	RcSpHQC-04	167121	
	Rc Aqs HQC-05	272403	RcSpHQC-05	164347	
	Rc Aqs HQC-06	271756	RcSpHQC-06	162637	
Mean		271410		163031	
		2229.8562		2452.2087	60.07
CV %		0.82		1.50	
Mean (Avg of 3 means)					61.81

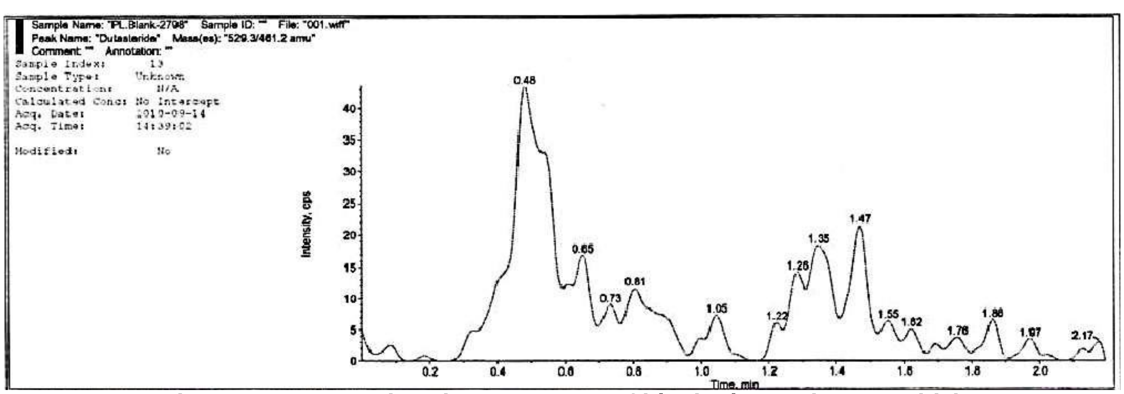

Fig. 2: Representative chromatogram of blank plasma (Dutasteride)

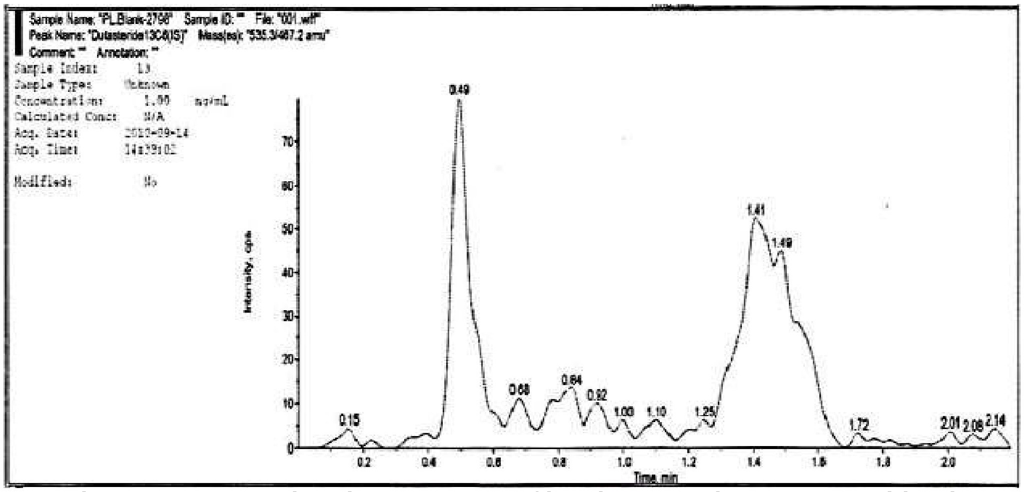

Fig. 3: Representative chromatogram of blank plasma (ISTD-Dutasteride13)

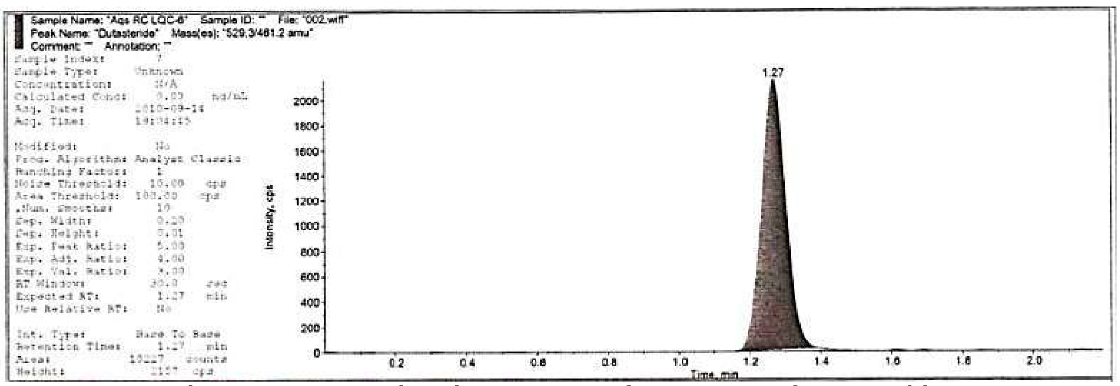

Fig. 4: Representative chromatogram for recovery of Dutasteride

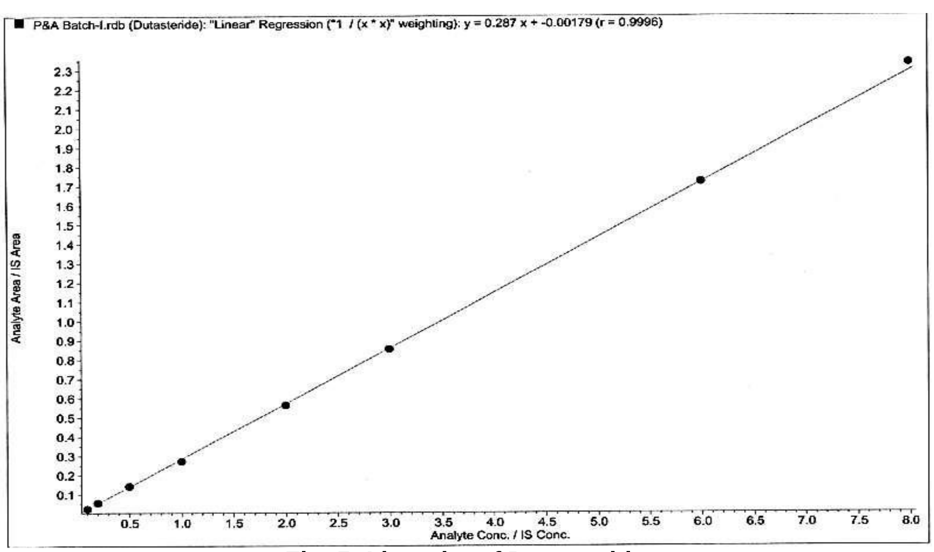

Fig. 5: Linearity of Dutasteride

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