

Selective and rapid liquid chromatography–tandem mass spectrometry assay of dutasteride in human plasma

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Abstract

A simple, rapid, sensitive and specific liquid chromatography–tandem mass spectrometry method was developed and validated for quantification of dutasteride (I), a potent and the first specific dual inhibitor of 5 α -reductase, in human plasma. The analyte and internal standard (finasteride (II)) were extracted by liquid–liquid extraction with diethyl ether/dichloromethane (70/30, v/v) using a Glas-Col Multi-Pulse Vortexer. The chromatographic separation was performed on a reverse phase Xterra MS C₁₈ column with a mobile phase of 10 mM ammonium formate/acetonitrile (15/85, v/v, pH adjusted to 3.0 with formic acid). The protonated analyte was quantitated in positive ionization by multiple reaction monitoring with a mass spectrometer. The mass transitions m/z 529.5 \rightarrow 461.5 and m/z 373.3 \rightarrow 317.4 were used to measure I and II, respectively. The assay exhibited a linear dynamic range of 0.1–25.0 ng/mL for dutasteride in human plasma. The lower limit of quantitation was 100 pg/mL with a relative standard deviation of less than 15%. Acceptable precision and accuracy were obtained for concentrations over the standard curve ranges. A run time of 1.2 min for each sample made it possible to analyze a throughput of more than 400 human plasma samples/day. The validated method has been successfully used to analyze human plasma samples for application in pharmacokinetic, bioavailability or bioequivalence studies.

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1. Introduction

Dutasteride (Fig. 1), (17 β -*N*(2,5-bis (trifluoromethyl) phenylcarbamoyl)-4-aza-5-androst-1-en-3-one), is a potent and specific dual 5 α -reductase inhibitor for the treatment of benign prostatic hyperplasia (BPH) and lower urinary tract symptoms (LUTS) [1,2]. It was approved in October 2002 by USFDA and it has been approved in several countries [3,4].

Dutasteride inhibits the conversion of testosterone to 5 α -dihydrotestosterone (DHT) [2]. DHT is the androgen primarily responsible for the initial development and subsequent enlargement of the prostate gland. DHT is converted from testosterone by the enzyme 5 α -reductase, which exists as two isoforms, types 1 and 2. Type 1 5 α -reductase is found primarily in the skin and liver, but has also been

found in prostatic tissue in BPH. Type 2 5 α -reductase is found in the prostate [2].

Dutasteride reaches peak serum concentrations approximately 2–3 h after being taken orally. Single doses of dutasteride 0.1–40 mg resulted in C_{\max} values of 0.6–166 ng/mL [2,5]. DHT levels were significantly reduced from baseline by 72–95% following single-dose administration of dutasteride 1–40 mg in volunteers (all $P \leq 0.001$ versus placebo). It is about 60% bioavailable after oral administration, and is primarily metabolized in the liver (by CYP3A4 isoenzyme) and excreted in the feces. The drug has a large volume of distribution and is extensively distributed into central and peripheral compartments, including semen [1,5]. The mean semen dutasteride concentration was 3.4 ng/mL (range 0.4–14 ng/mL) following 12 months of dutasteride 0.5 mg/day. On average, 11.5% of the serum dutasteride concentration was found in the semen after 12 months [2]. The terminal elimination half-life is 3–5 weeks, and the drug remains detectable in serum for 4–6 months after treatment is discontinued [3].

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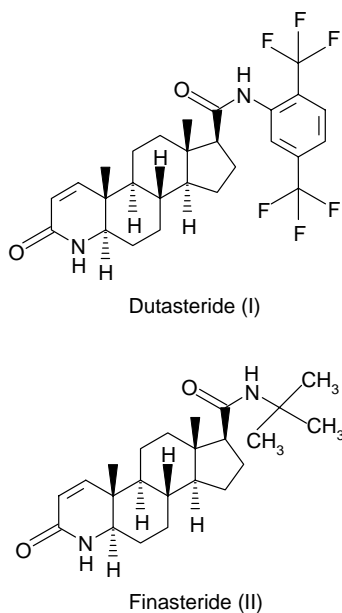


Fig. 1. Chemical structures of dutasteride (I) and internal standard (finasteride (II)).

Recently, Burinsky et al. [6] reported the mass spectral fragmentation reactions of dutasteride and related compounds, using various ionization conditions (EI, CI, APCI and ESI) in both positive and negative ion modes. The spectral differences are attributed to ion populations having either different structures or different internal energy distributions (as a consequence of the method of ionization). Irrespective of their origin, the protonated molecules undergo interesting fragmentation reactions when collisionally activated [6].

To date, no simple chromatographic method has been reported for dutasteride quantitation in plasma. Morris et al. [7] reported a LC/APCI/MS method for the analysis of dutasteride in serum. In order to quantify plasma concentrations of dutasteride in clinical trials, it was necessary to develop and validate an assay with appropriate sensitivity, selectivity, accuracy and precision. The most attractive approach for an assay which would be robust, sensitive, selective and would allow high throughput was a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method [8]. We now report for the first time an LC–MS/MS method developed and validated for the quantitative determination of dutasteride in human plasma. It was essential to establish an assay capable of quantifying dutasteride at concentration down to 100 pg/mL. At the same time, it was expected that this method would be efficient in analyzing large number of plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies after therapeutic doses of dutasteride.

In recent years, a number of laboratories have reported the use of high-throughput bioanalytical procedures using LC–MS/MS [8–11]. Our method is simple, rapid, robust, specific and sensitive that makes it an attractive procedure in high-throughput bioanalysis.

2. Experimental

2.1. Chemicals

The pure substances of dutasteride and finasteride (internal standard, I.S.) were from Sipra Laboratories Ltd. (Hyderabad, India). Chemical structures are presented in Fig. 1. Stock solutions of dutasteride (0.5 mg/mL) and I.S. (1 mg/mL) were separately prepared in 10 mL volumetric flasks with methanol. HPLC-grade LiChrosolv methanol, LiChrosolv acetonitrile, diethyl ether and dichloromethane were from Merck (Darmstadt, Germany). Ammonium formate, formic acid and sodium hydroxide pellets were from Merck (Worli, Mumbai, India). HPLC type I water from a Milli-Q system (Millipore, Bedford, MA, USA) was used. All other chemicals were of analytical grade.

2.2. LC–MS/MS apparatus and conditions

The HPLC Agilent 1100 Series (Agilent Technologies, Waldbronn, Germany) is equipped with G1312A binary pump, G1379A degasser, G1367A autosampler equipped with a G1330B thermostat, G1316A thermostatted column compartment and G1323B control module. The chromatography was on Waters Xterra MS C₁₈ column (3.5 μm, 50 mm × 3 mm i.d.) at 30 °C temperature. The mobile phase composition was a mixture of 10 mM ammonium formate buffer/acetonitrile (15/85, v/v, pH adjusted to 3.0 with formic acid), which was pumped at a flow-rate of 0.6 mL/min.

Mass spectrometric detection was performed on an API 4000 triple quadrupole instrument (ABI-SCIEX, Toronto, Canada) using MRM. A turbo electrospray interface in positive ionization mode was used. The main working parameters of the mass spectrometer are summarized in Table 1. Data processing was performed on Analyst 1.3 software package (SCIEX).

Table 1
Tandem mass-spectrometer main working parameters

Parameter	Value
Source temperature (°C)	150
Dwell time per transition (ms)	200
Ion source gas (gas 1) (psi)	30
Ion source gas (gas 2), psi	20
Curtain gas (psi)	12
Collision gas (psi)	8
Ion spray voltage (V)	5000
Entrance potential (V)	10
Declustering potential (DP) (V)	150 (Analyte) and 120 (I.S.)
Collision energy (V)	47 (Analyte) and 31 (I.S.)
Collision cell exit potential (V)	12 (Analyte) and 12 (I.S.)
Mode of analysis	Positive
Ion transition for dutasteride (<i>m/z</i>)	529.5/461.5
Ion transition for finasteride (<i>m/z</i>)	373.3/317.4

2.3. Sample processing

A 500- μ L volume of plasma sample was transferred to a 15-mL glass test tube, then 50 μ L of I.S. working solution (100 ng/mL) and 100 μ L of 0.1N sodium hydroxide solution were spiked. After vortexing for 30 s, 4 mL aliquot of extraction solvent, diethyl ether/dichloromethane (70/30) was added using Dispensette Organic (Brand GmbH, Postfach, Germany). The sample was vortex-mixed for 3 min using Multi-Pulse Vortexer (Glas-Col, Terre Haute, USA). The organic layer (3.5 mL) was transferred to a 5-mL glass tube and evaporated to dryness using TurboVap LV Evaporator (Zymark, Hopkinton, MA, USA) at 40 °C under a stream of nitrogen. Then the dried extract was reconstituted in 200 μ L of diluent (water-acetonitrile, 50:50, v/v) and a 20- μ L aliquot was injected into chromatographic system.

2.4. Bioanalytical method validation

2.4.1. Calibration and control samples

Working solutions for calibration and controls were prepared from the stock solution by dilution using water/methanol (1/1). The I.S. working solution (100 ng/mL) was prepared by diluting its stock solution with water/methanol (1/1). Twenty-five microliters of working solutions were added to 475 μ L drug-free plasma to obtain dutasteride concentration levels of 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 and 25.0 ng/mL. Quality control (QC) samples were prepared as a bulk, at concentrations of 0.1 ng/mL lower limit of quantification (LLOQ), 0.5 ng/mL (low), 10.0 ng/mL (medium) and 20.0 ng/mL (high).

2.4.2. Calibration curve

A calibration curve was constructed from a blank sample (a plasma sample processed without an I.S.), a zero sample (a plasma processed with I.S.) and eight non-zero samples covering the total range (0.1–25.0 ng/mL), including LLOQ. Such calibration curves were generated on six consecutive days. Linearity was assessed by a weighted ($1/x$) least squares regression analysis. The calibration curve had to have a correlation coefficient (r^2) of 0.99 or better. The acceptance criterion for each back-calculated standard concentration was 15% deviation from the nominal value except LLOQ, which was set at 20%.

2.4.3. Precision and accuracy

The within-batch precision and accuracy were determined by analyzing six sets of quality control samples in a batch. The between-batch precision and accuracy was determined by analyzing six sets of quality control samples on three different batches. The quality control samples were randomized daily, processed and analyzed in position either (a) immediately following the standard curve (b) in the middle of the batch, or (c) at the end of the batch. The acceptance criteria of within- and between-batch precision were 20% or better for LLOQ and 15% or better for the rest of concentrations

and the accuracy was $100 \pm 20\%$ or better for LLOQ and $100 \pm 15\%$ or better for the rest of concentrations. The precision of the method was expressed as relative standard deviation and accuracy of the method was expressed in terms of bias (percentage deviation from true value).

2.4.4. Recovery

Recovery of dutasteride was evaluated by comparing the mean peak areas of six extracted low and high quality control samples to mean peak areas of six neat reference solutions (unprocessed). Recovery of I.S. was evaluated by comparing the mean peak areas of ten extracted quality control samples to mean peak areas of ten neat reference solutions (unprocessed) of the same concentration.

3. Results and discussion

Electrospray MS/MS was used to analyze dutasteride, as it is beneficial in developing a selective and sensitive method. The positive ion TurboIonSpray Q1 mass spectrum and product ion mass spectrum of dutasteride and the I.S. are shown in Figs. 2 and 3, respectively. $[M + H]^+$ was the predominant ion in the Q1 spectrum and was used as the precursor ion to obtain product ion spectra. The most sensitive mass transition was from m/z 529.5 to m/z 461.5 for dutasteride and from m/z 373.3 to m/z 317.4 for the I.S. LC–MRM is a very powerful technique for pharmacokinetic studies since it provides sensitivity, selectivity and specificity requirements for analytical methods. Thus, the MRM technique was chosen for the assay development. The MRM state file parameters were optimized to maximize the response for the analyte. The parameters presented in Table 1 are the result of this optimization.

3.1. Method development

Different mobile phases consisting of water–methanol or water–acetonitrile were evaluated to improve HPLC separation and enhance sensitivity in MS. Modifiers such as formic acid and ammonium formate alone or in combination in different concentrations were added. The best signal was achieved using 10 mM ammonium formate buffer/acetonitrile (15/85, v/v), with pH adjusted to 3.0 with formic acid. The formic acid was found to be necessary in order to lower the pH to protonate the dutasteride and thus deliver good peak shape. The percentage of formic acid was optimized to maintain this peak shape whilst being consistent with good ionization and fragmentation in the mass spectrometer.

The tandem mass spectrometer allows the selective detection of substances with varying masses or fragments without chromatographic separation. The development of the chromatographic system was focused on short retention times, paying attention to matrix effects as well as good peak shapes. A high proportion of organic solvent [10 mM

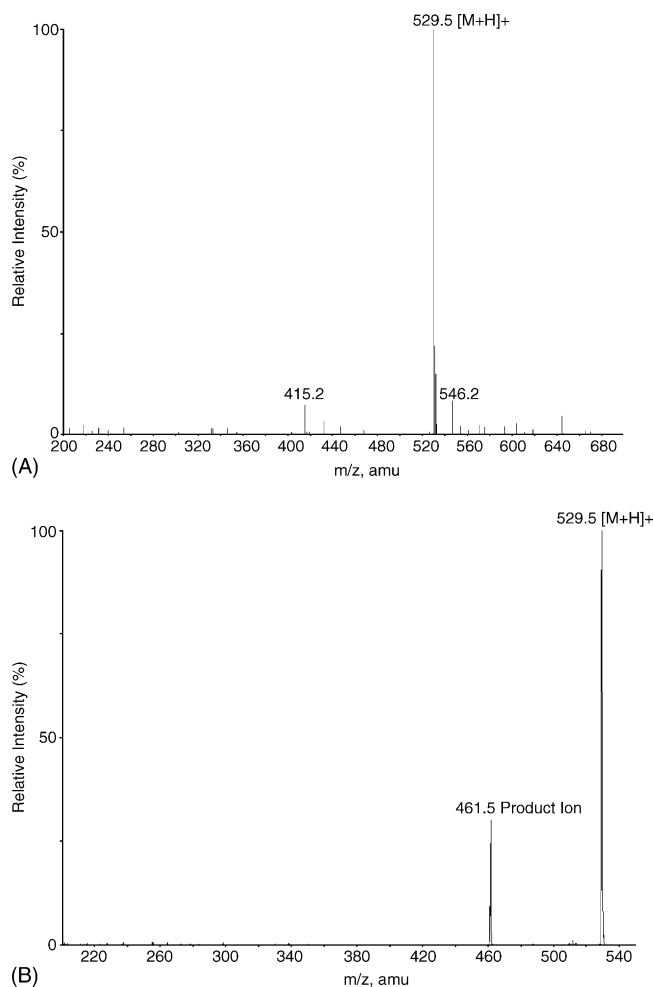


Fig. 2. Full scan positive ion TurboIonSpray (A) Q1 mass spectra and (B) product ion mass spectra of dutasteride.

ammonium formate/acetonitrile (15/85, v/v; pH 3.0)] was used to elute the analyte and the I.S. at retention times of 0.7 and 0.55 min, respectively. Flow rate of 0.6 mL/min produced a good peak shape and brought the runtime to 1.2 min.

3.2. Internal standard

A stable isotope labeled analyte has to be used as an internal standard to deal with sample matrix effects. Since such internal standard is not commercially available, an alternative approach has been used. Internal standard substance should match the chromatographic retention, recovery and ionization properties with the matrix of dutasteride. Finasteride (Fig. 1) was found to fulfill these criteria sufficiently. The matrix effects were similar to the matrix effects of dutasteride. Hence finasteride has been chosen as internal standard in the quantitative assay for dutasteride from plasma.

3.3. Calibration curves

The calibration curve was linear over the concentration range of 0.1–25.0 ng/mL for the analyte (Fig. 4). The

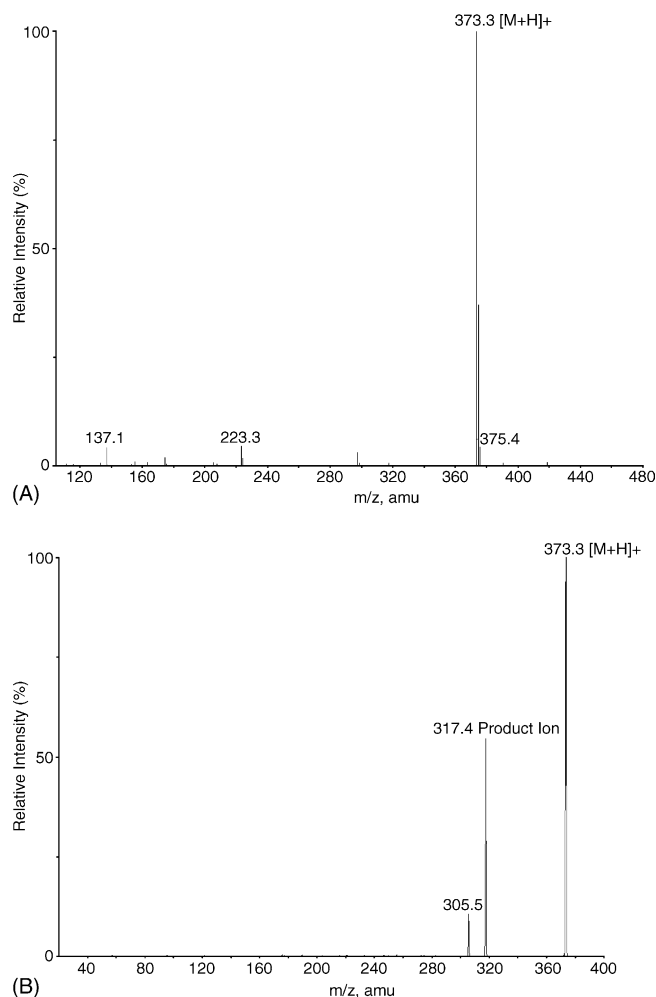


Fig. 3. Full scan positive ion TurboIonSpray (A) Q1 mass spectra and (B) product ion mass spectra of internal standard (finasteride).

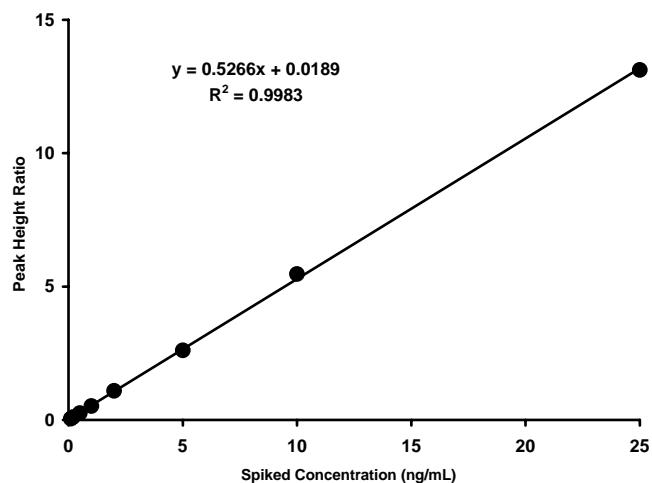


Fig. 4. Typical calibration curve for the assay of dutasteride plasma concentrations.

Table 2
Precision and accuracy data of back-calculated concentrations of calibration samples for dutasteride in human plasma

Concentration added (ng/mL)	Concentration found (mean \pm S.D. $n = 6$) (ng/mL)	Precision (%)	Accuracy (%)
0.1	0.10 \pm 0.006	6.380	101.050
0.2	0.21 \pm 0.018	8.705	102.667
0.5	0.51 \pm 0.024	4.729	102.300
1.0	1.03 \pm 0.056	5.468	103.300
2.0	1.79 \pm 0.091	5.077	89.333
5.0	4.86 \pm 0.309	6.356	97.233
10.0	9.84 \pm 0.845	8.594	98.367
25.0	25.37 \pm 0.753	2.968	101.467

eight-point calibration curve gave acceptable results for the analyte and was used for all the calculations. The mean correlation coefficient of the weighted calibration curve generated during the validation was 0.998 for the analyte. Table 2 summarizes the calibration curve results for the analyte. The precision and accuracy for the analyte covering the concentration of 0.1–25.0 ng/mL ranged from 2.968 to 8.705% and 89.333–103.300%, respectively. The calibration curve obtained as described above was suitable for generation of acceptable data for the concentrations of the analyte in the samples during the validations.

3.4. Specificity

The specificity of the method was examined by analyzing blank human plasma extract (Fig. 5) and an extract spiked only with the internal standard (Fig. 6). As shown in Fig. 5, no significant interference in the blank plasma traces was seen from endogenous substances in drug-free human plasma at the retention time of the analyte. Fig. 6 shows the absence of interference from the internal standard to the

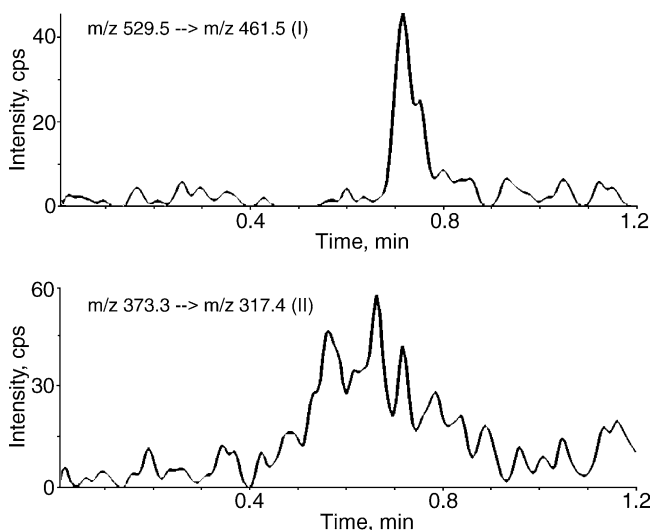


Fig. 5. MRM ion-chromatograms resulting from the analysis of blank (drug and internal standard free) human plasma for dutasteride and internal standard.

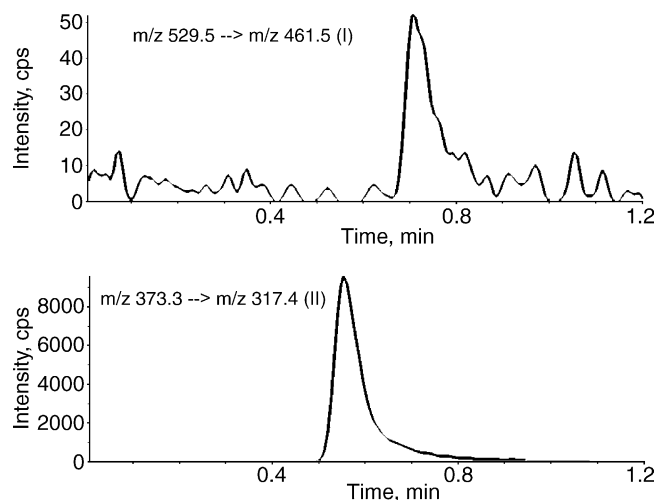


Fig. 6. MRM ion-chromatograms resulting from the analysis of blank (drug-free spiked with internal standard) human plasma for dutasteride and internal standard.

MRM channels of the analyte. Figs. 7 and 8 depict a representative ion-chromatogram for limit of detection (LOD) and for the lower limit of quantitation (LLOQ, 0.1 ng/mL) of the calibration curve, respectively. Excellent sensitivity was observed for 20- μ L injection volume (LLOQ) corresponding to ca. 2 pg on-column.

3.5. Matrix effect

Due to the components of the sample matrix, signal suppression or enhancement may occur. These matrix effects in the LC-MS/MS method were evaluated by spiking blank plasma extracts with low and high QC samples. The resulting chromatograms were compared with chromatograms of pure samples equally concentrated. Six independent plasma

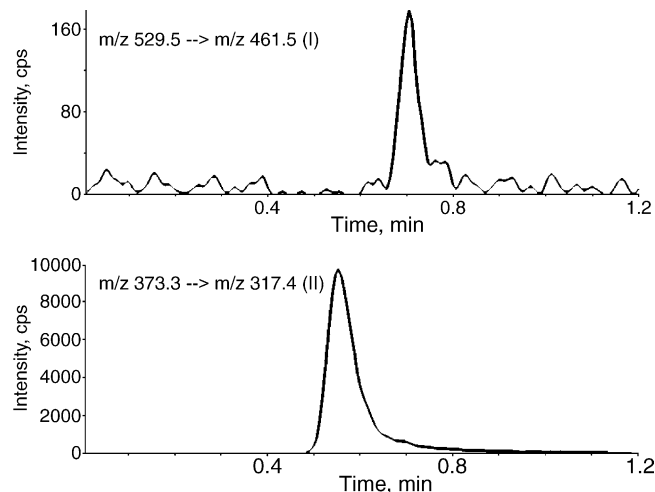


Fig. 7. Representative MRM ion-chromatograms resulting from the analysis of limit of detection (LOD) of dutasteride spiked with the internal standard.

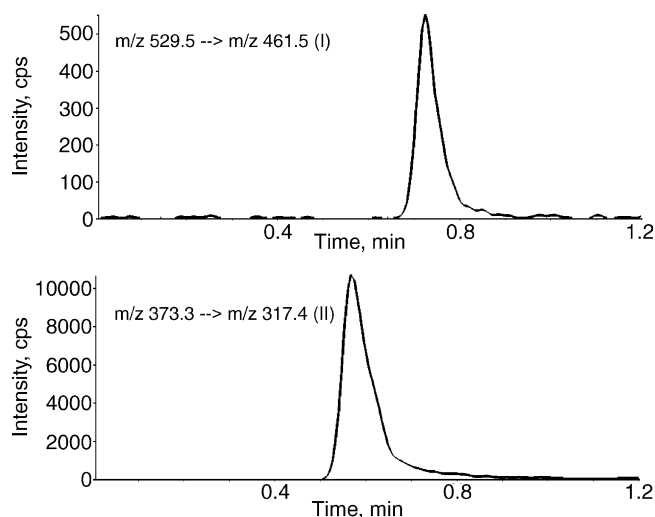


Fig. 8. Representative MRM ion-chromatograms resulting from the analysis of 100 pg/mL (LLOQ) of dutasteride spiked with the internal standard.

lot were used with six samples from each lot. The results (data was not shown) showed that there was no significant difference for peak responses between these samples. This effect is most likely due to the sample clean up with LLE.

3.6. Extraction recovery

The extraction recovery of dutasteride was 40.701% on average, and the dependence on concentration is negligible. The recovery of the I.S. was 64.039% at the concentration used in the assay (100 ng/mL). Recovery of the analyte and I.S. were low, but it was consistent, precise and reproducible. With the consistency in the recovery of dutasteride and I.S., the assay has proved to be robust in high throughput bioanalysis.

3.7. Lowest concentration

The lower limit of quantitation of dutasteride in human plasma assay was 0.1 ng/mL. The between-batch precision at the LLOQ was 4.577%. The between-batch accuracy was 106.606% (Table 3). The within-batch precision was 11.399% and the accuracy was 111.167% for dutasteride.

3.8. Middle and upper concentrations

The middle and upper quantitation levels of dutasteride ranged from 0.5 to 20.0 ng/mL in human plasma. For the between-batch experiment, the precision ranged from 5.921 to 7.437% and the accuracy ranged from 101.467 to 108.678% (Table 3). For the within-batch experiment, the precision and accuracy for the analyte met the acceptance criteria ($< \pm 15\%$) and precision was below 10% at all concentrations tested.

3.9. Stability

To examine the batch size that could be analyzed, two batches were prepared containing 144 samples and a system suitability sample (145 in total) and analyzed consecutively. The run was then examined for changes in retention time and sensitivity throughout. The retention time for dutasteride ranged from 0.70 to 0.72 min. There was no noticeable change in sensitivity for dutasteride or the internal standard. With the run time of 1.2 min and a batch size of approximately 150 samples, it was evident that the desired high throughput for the assay would be achievable.

The freeze–thaw stability of the analyte was determined by measuring the assay precision and accuracy for the samples which underwent three freeze–thaw cycles. The stability data were used to support repeat analysis. In each freeze–thaw cycle, the frozen plasma samples were thawed at room temperature for 2–3 h and refrozen for 12–24 h. After completion of each cycle the samples were analyzed and results were compared with that of zero cycle. The results showed that the analyte was stable in human plasma through three freeze–thaw cycles (Fig. 9). The results demonstrated that human plasma samples could be thawed and refrozen without compromising the integrity of the samples.

The storage time in long-term stability evaluation brackets the time between the first sample collection and the last sample analysis. The sample long-term storage stability at -72°C was evaluated to establish acceptable storage conditions for subject samples. Aliquots of human plasma samples spiked with analyte at concentrations of 0.5 and 20 ng/mL were analyzed on day 1. Then the samples from the same pools were analyzed against calibration curves from freshly prepared standards after storage at -72°C for 10 days. The precision and accuracy for the analyte on day 10 ranged

Table 3
Precision and accuracy of the LC–MS/MS method for determining dutasteride concentrations in plasma samples

Concentration added (ng/mL)	Within-batch precision ($n=6$)			Between-batch precision ($n=3$)		
	Concentration found (mean \pm S.D.) (ng/mL)	Precision (%)	Accuracy (%)	Concentration found (mean \pm S.D.) (ng/mL)	Precision (%)	Accuracy (%)
0.10	0.11 \pm 0.013	11.399	111.167	0.11 \pm 0.005	4.577	106.606
0.5	0.51 \pm 0.035	7.016	101.100	0.54 \pm 0.035	6.126	108.678
10.0	9.64 \pm 0.950	9.860	96.367	10.15 \pm 0.730	7.437	101.467
20.0	21.12 \pm 1.375	6.510	105.583	20.84 \pm 1.159	5.921	104.194

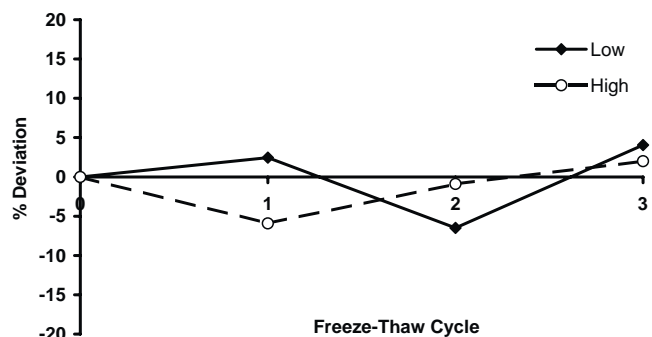


Fig. 9. Freeze–thaw cycle stability of dutasteride at two concentration levels (0.5 and 20 ng/mL). Deviations are less than 10% even after third freeze–thaw cycle.

from 10.154 to 12.561% and from 98.812 to 103.969%, respectively (Table 4).

Stability of the dutasteride and its internal standard after processing in the autosampler is important when determining a large number of plasma samples. Twelve sets of quality control samples were prepared as described in Section 2.3, and placed into the autosampler to +10 °C. Six sets were analyzed at once (controls) and six sets 24 h later. The results indicated that the analyte and I.S. were stable for at least 24 h. It took less than 10 h to run 400 samples with a sample turnover rate of 1.2 min/sample. This rapid assay method facilitates the analysis of several 100 samples in one working day.

There was no significant difference between the responses of spiked standards at time zero and after 24 h for dutasteride, indicating the stability of analyte at room temperature over 24 h. Moreover, the analyte was found to be stable after reconstitution in diluent for at least 12 h at 4 °C. The re-injection reproducibility was established to determine if an analytical batch could be reanalyzed in case of an unexpected delay in analyses. The same set of QC samples were repeated after the analysis with a 3 h gap between, during which the samples were stored at 4 °C, and in all cases the deviations were less than 15%. On similar lines, stability of the extracted dry residue was also determined to be over

Table 4
Stability of human plasma samples of dutasteride

Sample concentration (ng/mL) (<i>n</i> =6)	Concentration found (mean ± S.D.) (ng/mL)	Precision (%)	Accuracy (%)
Short-term stability (24 h)			
0.5	0.58 ± 0.056	9.685	101.333
20.0	18.45 ± 1.982	10.741	94.293
Long-term stability (10-days at –72 °C)			
0.5	0.57 ± 0.071	12.561	98.812
20.0	20.34 ± 2.066	10.154	103.969
Autosampler stability (24 h)			
0.5	0.55 ± 0.023	4.177	96.059
20.0	21.25 ± 1.476	6.947	91.397

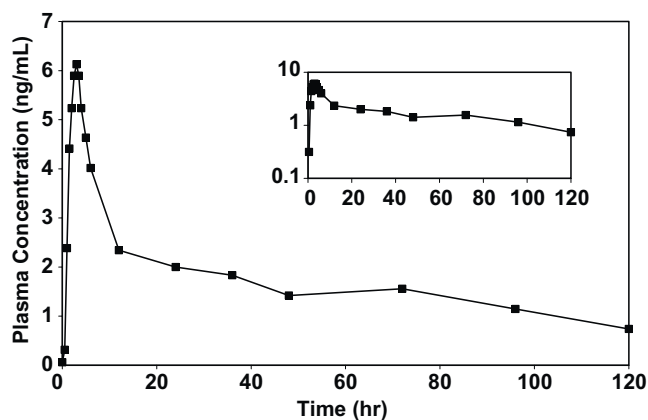


Fig. 10. Concentration vs. time profile over 120 h of dutasteride in human plasma from a subject receiving a single 0.5 mg dose of dutasteride.

24 h (deviations observed <10%). In addition, the stock solutions of dutasteride and I.S. were also found to be stable for at least 3 months at 4 °C.

3.10. Application

The validated method has been successfully used to quantify the dutasteride concentration in the human plasma samples after the administration of a single 0.5 mg oral dose of dutasteride. The concentration versus time profile of a subject receiving a single dose of dutasteride is presented in Fig. 10.

4. Conclusions

In summary, this is the first method described for the quantification of dutasteride from human plasma by LC–MS/MS in positive ionization mode using multiple reaction monitoring. The current method has shown acceptable precision and adequate sensitivity for the quantification of dutasteride in human plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies. Furthermore, it was utilized for the analysis of hundreds of subject samples. The method described is simple, rapid, sensitive, specific and fully validated as per FDA guidelines [12]. The cost-effectiveness, simplicity and speed of liquid–liquid extraction and sample turnover rate of 1.2 min per sample make it an attractive procedure in high-throughput bioanalysis of dutasteride. The validated method allows quantification of dutasteride in the 0.1–25.0 ng/mL range.

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