Finasteride Quantification in Human Plasma by High-Performance Liquid Chromatography Coupled to Electrospray Ionization Tandem Mass Spectrometry. Application to a Comparative Pharmacokinetics Study

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Key words
- clinical pharmacology
- estrogen
- bioequivalence

Abstract

A specific, fast and sensitive LC-MS/MS assay was developed for the determination of finasteride in human plasma using betamethsone dipropionate as the internal standard (IS). The limit of quantification was 1.0 ng/ml and the method was linear in the range of 1.0–25.0 ng/ml. The retention times were 0.75 min for finasteride and 0.85 min for IS. Method intra-batch precision and accuracy ranged from 3.6 to 7.1%, and 96.6 to 103.9%, respectively. Inter-batch precision ranged from 2.5 to 3.4%, while Inter-batch accuracy ranged from 100.3 to 103.5%.

The analytical method was applied to evaluate the pharmacokinetic and relative bioavailability of 2 different pharmaceutical formulations containing 1.0 mg of finasteride. This study evaluated 38 volunteers in a randomized, 2-period crossover study with 7 days washout period between doses. The geometric mean and respective 90% CI of finasteride test/reference percent ratios were 95.68% (91.2 – 104.6%) for C_{max}, 97.5% (92.1 – 103.3%) for AUC_{0-t} and 98.1 (92.67 – 103.8) for AUC_{0-inf}. Based on the 90% confidence interval of the individual ratios (test formulation/reference formulation) for C_{max} and AUC_{0-inf}, it was concluded that the test formulation is bioequivalent to the reference one with respect to the rate and extent of absorption of finasteride.

Introduction

Androgens are essential for prostatic development, growth and function. However, later in life they have a significant role in the pathogenesis of Benign prostatic hyperplasia (BPH) and prostate cancer [1]. The enzyme 5a-reductase (5aR) converts testosterone (TS) into a more potent androgen, dihydrotestosterone (DHT). Therefore, tissues with a high concentration of these enzymes, such as the prostate, act to concentrate the effects of circulating testosterone. Thus, the inhibition of DHT conversion offers the potential to prevent, delay or possibly treat BPH or prostate cancer, providing a different approach for their management. Finasteride, a synthetic 4-azasteroid, inhibits the action of the 5α-reductase enzyme [2, 3]. Treatment with finasteride results in a significant reduction of prostatic and circulating DHT levels compared with baseline [4] and is widely used for the treatment of BPH. Finasteride may also be used to prevent hair loss in younger man [5].

Finasteride is well absorbed and widely distributed after oral administration. It undergoes extensive hepatic metabolism to a series of 5 metabolites, of which 2 are active and possess less than 20% of the 5 alpha-reductase activity of finasteride, which are eliminated through the bile and urine [6]. Its absolute bioavailability is 63%, (range from 33 to 108%) [7]. Maximum plasma concentration is reached at 1–2 h [7], and it is approximately 90% bound to plasma protein. The elimination half-life averages 6–8 h.

Several liquid chromatographic methods with UV detection have been developed [8–13]. However, HPLC methods for finasteride measurement in plasma suffer from limitations such as low sensitivity, poor selectivity and being time-consuming due to complex sample preparation procedures.

Analytical methods describing the quantification of finasteride using a liquid chromatography coupled to a tandem mass spectrometry analysis have also been described [14–19]. Although the HPLC coupled to the triple-quadrupole mass spectrometry analysis (LC-MS/MS) is not always affordable for routine measurements in many laboratories, LC-MS/MS has become the widely used analytical tool for the pharmacokinetic

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study and quantification of drugs and its metabolites in biological samples due to its high sensitivity and selectivity.

To allow for the correct comparison of newer finasteride formulation with a reference one, we validated a LC-MS/MS method with good selectivity, linearity range, precision, accuracy, and lower limit of quantification (LLOQ) for the quantification of finasteride in plasma samples using betamethasone dipropionate as internal standard. This method was applied to evaluate the comparative bioavailability in 38 healthy volunteers. All volunteers received a single 1.0 mg tablet orally corresponding to the test finasteride or reference formulation, following an open, 2-period, balanced randomized, crossover protocol.

Experimental

Chemicals and reagents

Finasteride (batch # F1E139) and the internal standard betamethasone dipropionate (batch # L0G377) were obtained from USP, Rockville, MD. Ultrapure water was obtained from a Milli-Q system (Millipore, Sao Paulo, Brazil). The HPLC grade methanol and acetonitrila were obtained from Tedia Brasil (Rio de Janeiro, RJ, Brazil). Ethyl acetate and hexane were purchased from Quemis (Joinville, SC, Brazil). Formic acid was purchased from Merck Brazil (Rio de Janeiro, RJ, Brazil Sodium hydroxide was purchased from Haloquímica (São Paulo, SP, Brazil). Blank human blood was collected from healthy, drug-free volunteers. Plasma was obtained by centrifugation of blood treated with the anticoagulant Heparin (BD Vacutainer®, BD, Franklin Lakes, NJ USA) and stored at −20 °C until needed for preparation of calibration standards, quality control (QC) samples, and plasma blanks. Human Plasma samples (normal, hyperlipemic and hemolyzed) came from distinct drug free subjects.

Instrumentation and detection

The HPLC system used was an Agilent 1200 series (Agilent technologies, Waldbronn, Germany). A mass spectrometer Agilent 6460 triple quadrupole instrument (Agilent technologies, Waldbronn, Germany) was used. Electrospray positive mode with Multiple Reaction Monitoring (MRM) was used for detection. For finasteride, $[M+H]^+$ (m/z 373.5) was monitored as the precursor ion and a fragment at m/z 305.2 was chosen as the product ion. For Betamethasone dipropionate, the $[M+H]^+$ (m/z 505.3) was monitored as the precursor ion and a fragment at m/z 411.2 was monitored as the product ion. Mass parameters were optimized as gas temperature 325 °C, nebulizer gas 15 psi, capillar voltage 2 500 V, cone voltage 150 V, gas flow rate 11 L/mi, cell accelerator 7 V, declustering potential 40 V, dwell time 0.2 s for each transition, collision energy 28 eV for finasteride and 4 eV for Betamethasone dipropionate.

Preparation of standards and quality control (QC) samples

Standard stock solutions at 1.0 mg/mL of finasteride and Betamethasone dipropionate were prepared in acetoniltrila-water (70:30, v/v). Spiking solutions were then prepared by diluting the stock solution in acetoniltrila-water (70:30, v/v) to form 8 solutions 10 times concentrated. Standard and spiking solutions were stored under refrigerator conditions (2–8 °C) until analysis. Plasma standards were prepared by adding 200 µL of each of the 12 spiking solutions to 200 µL samples of human plasma. This process yielded calibration standards with nominal concentrations of 0.1, 0.3, 1.0, 5.0, 10.0, 15.0, 20.0 and 25.0 ng/mL and control samples of 0.3, 10.0, 20.0 ng/mL of finasteride in human plasma. Both calibrators and quality control samples were freshly prepared at the day of analysis and used within the stability period determined. Quality control samples for stability testing were stored as indicated in section "Stability".

Sample preparation

Liquid-liquid extraction was used to isolate finasteride and Betamethasone dipropionate from human plasma. A volume of 100 µl of Betamethasone dipropionate (5.0 ng/mL), 200 µL of plasma sample (respective concentration) and were added to polypropylene tubes, vortexed briefly and placed in an ice bath. This process was followed by addition of 50 µL of 0.1 M sodium hydroxide, 1.5 mL of extraction solvent (ethyl acetate-hexane, 50:50, v/v) and vortexed for approximately 10 s. Further samples were centrifuged at 3 000 rpm for 1 min at 4 °C. Samples were then frozen in dry ice bath and the supernatant from each sample was transferred into polypropylene tubes and evaporated to dryness under nitrogen flow at 40 °C. Finally, dried residue from each tube was reconstituted with 100 µL of reconstitution solution (acetonitrila-water with 0.1 % of formic acid, 90:10, v/v) and vortexed briefly. Subsequently, 20 µL of sample from each tube was transferred into auto sampler vials and injected into the LC-MS/MS.

Chromatographic conditions

A Eclipse XDB-C8, 100 × 2.1 mm, 3.5 µm, was selected as the analytical column. The mobile phase composition was 0.1 % formic acid: acetonitrile (20:80 v/v). The flow rate of the mobile phase was set at 0.5 mL/min. The column temperature was set at 40 °C. Betamethasone dipropionate was used as the appropriate internal standard. The retention time of finasteride and Betamethasone dipropionate were found to be 0.75 ± 0.1 min and 0.85 ± 0.1 min, respectively with a total run-time of 2 min for each sample.

Selectivity and lower limit of quantification

To evaluate method specificity, blank human plasma obtained from 6 different subjects was prepared, analyzed, and examined for response of the analyte and IS chromatographic profiles. The LLOQ was established at a level for which the response was greater than 5 times the blank response. The accuracy and precision criteria required that the LLOQ calibration standard accuracy and precision average within 20 % of target with a CV of ≤ 20 % for 5 validation batches.

The calculations were based on 6 replicates of LLOQ sample, whose precision and accuracy were determined intra-day and inter-day.

Linear dynamic range and over-range samples

Method linearity was investigated by analyzing a set of calibration standards with each of the 3 validation batches. Calibration curves were constructed by plotting area ratios (analyte/IS) vs. analyte concentrations for the 8 calibration standards and performing a weighted, $1/x^2$ linear regression analysis. Linearity was evaluated by examining the correlation coefficients of the calibration curves and by determining the accuracy of the calibration standards when calculating their concentrations using the regression curve parameters. Accuracy was determined by dividing the measured concentration by the theoretical concen-

tration and multiplying by 100 to express the value as a percentage.

Accuracy and precision

During method validation, QC samples were prepared by spiking known amounts of finasteride into normal human plasma at 4 distinct levels. These levels were: LLOQ-QC (QC at lower level of concentration), 0.1; QCL (QC at low level), 0.3; QCM (QC at medium level of concentration), 10.0; and QCH (QC at high level of concentration), 20.0 ng/mL plasma for finasteride. Accuracy and precision were determined during analysis of 3 validation batches, with each batch containing 6 replicates at each QC level. The accuracy and precision criteria required that for QCs above the LLOQ, accuracy and precision must average within 15 % of target with a CV of ≤ 15 % for 3 validation batches. As described in section "Selectivity and lower limit of quantification", the criteria for LLOQ calibration standard accuracy and precision must average within 20 % of target with a CV of ≤ 20 %.

Throughout the course of clinical sample analysis, accuracy and precision of the assay were continually monitored with QC samples prepared at 3 levels. For each analytical run of the study samples, QC samples were included at the low, medium, and high levels previously described, with at least 3 replicates at each level per run.

Autosampler carry-over

Immediately following injection of the highest calibration standard containing 25.0 ng/mL finasteride, three 20 µL injections of a 1 % formic acid blank or freshly extracted blank plasma samples were sequentially performed. All analyte and internal standard peak windows were examined for the presence of measurable response due to carry-over. Peaks were integrated and the percent carry-over was computed by dividing these areas by the corresponding peak area produced upon injection of the LLOQ.

Extraction efficiency and ionization suppression

Analyte and internal standard extraction efficiencies from human plasma were determined by comparing the LC-MS/MS response for each analyte and internal standard when analyzing plasma samples spiked with the compounds prior to extraction vs. the response obtained when the compounds were added to a prepared blank plasma matrix after extraction and immediately prior to reconstitution. 3 replicates were spiked at the QCL, QCM, and QCH levels before and after LLE.

Signal loss from all sources, including ionization suppression and extraction from plasma, was determined by directly comparing the LC-MS/MS responses for analyte and internal standard produced from the analysis when the compounds were added to a prepared blank plasma matrix after extraction with the corresponding responses obtained from injection of the compounds diluted in solvent. The mean response for each compound in plasma samples spiked at QCL, QCM, and QCH levels was determined from 3 replicates.

Stability

Stability of finasteride and Betamethasone dipropionate was determined under a variety of storage conditions chosen to simulate those expected to be encountered during the collection, storage, and analysis of study samples. For stability studies, QCL and QCH concentrations were used for evaluation of the storage conditions. A minimum of 3 replicate measurements were performed at each level and recoveries were determined vs. a freshly prepared calibration curve and expressed in percentage of degradation.

Initially, stability was evaluated after 3 freeze-thaw cycles at −20 °C. In each cycle, frozen samples were allowed to thaw at controlled ambient temperature (22 °C) and were subsequently refrozen for 24 h. Aliquots of all samples were quantified at the end of the third freeze-thaw cycle. The post processing stability was assessed for a 25-h period. In this assay, plasma samples spiked with QCs concentration were subjected to processing and stored after liquid-liquid extraction at room temperature prior to analyze by LC-MS/MS.

To evaluate the short term stability samples were initially thawed at room temperature (22 °C) and remained on the bench top for a time exceeding the maximum period of time expected for routine sample preparation (23 h). Long-term stability was evaluated in spiked human plasma stored at −20 °C for 185 days. Finstareide stock solutions were prepared as described, stored at 4 °C and 5 replicates were evaluated after 3 days. In addition, analite and IS work solutions were stored at room temperature and evaluated after 9 h.

Pharmacokinetic study

The analytical method developed here was applied to evaluate plasma pharmacokinetic of finasteride from 2 tablet formulations of 1.0 mg in healthy volunteers. 38 healthy male volunteers aged between 18 and 45 years and index of corporal mass within 18.72 and 29.73 were selected for the study after assessment of their health status by clinical evaluation (physical examination, ECG) and the following laboratory tests: blood glucose, urea, creatinine, AST, ALT, alkaline phosphatase, γ-GT, total bilirubin, albumin and total protein, triglyceride, total cholesterol, uric acid, hemoglobin, hematocrit, total and differential white cell counts and routine urinalysis. All subjects were negative for HIV, HCV and HBV. All subjects gave written informed consent and the study was conducted in accordance with the revised Declaration of Helsinki, the rules of Good Clinical Practice (ICH-GCP) and the Resolutions No.196/96 and 251/97 of National Health Council – Health Ministry, Brazil [20]. The clinical protocol was approved by the Research Ethics Committee of University of Campinas – Unicamp, São Paulo, Brazil.

The study was a single-dose, 2-way randomized crossover design with a 7 days washout period between the doses. The volunteers entered the Clinical Pharmacology Unit 10 h before drug administration and left the Unit 14 h after the beginning of sampling. For further sampling the volunteers returned to the clinical facility following the 24 h interval until the last time defined in the clinical protocol. After the predose sampling, each volunteer received a single dose of finasteride (1.0 mg of either tablet formulation) with 200 mL of water. The volunteers were then fasted for 4 h, after which period a standard lunch was served. No other food was permitted during the 'in-house" period and liquid consumption was allowed *ad libitum* after lunch (with the exception of xanthine-containing drinks, including tea, coffee, and soft drinks). The subjects were monitored throughout the study and the formulations were considered to be well tolerated. Blood samples were collected by indwelling catheter into heparin containing tubes before dosing and 20, 40 min and also 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10, 12, 16, 20, 24 and 36 h post-dosing. The blood samples were centrifuged at 2 000 × *g* for 10 min at 4 °C and the plasma separated and stored in a polypropylene cryogenic screw capped tubes at −20 °C until analyzed for finasteride content.

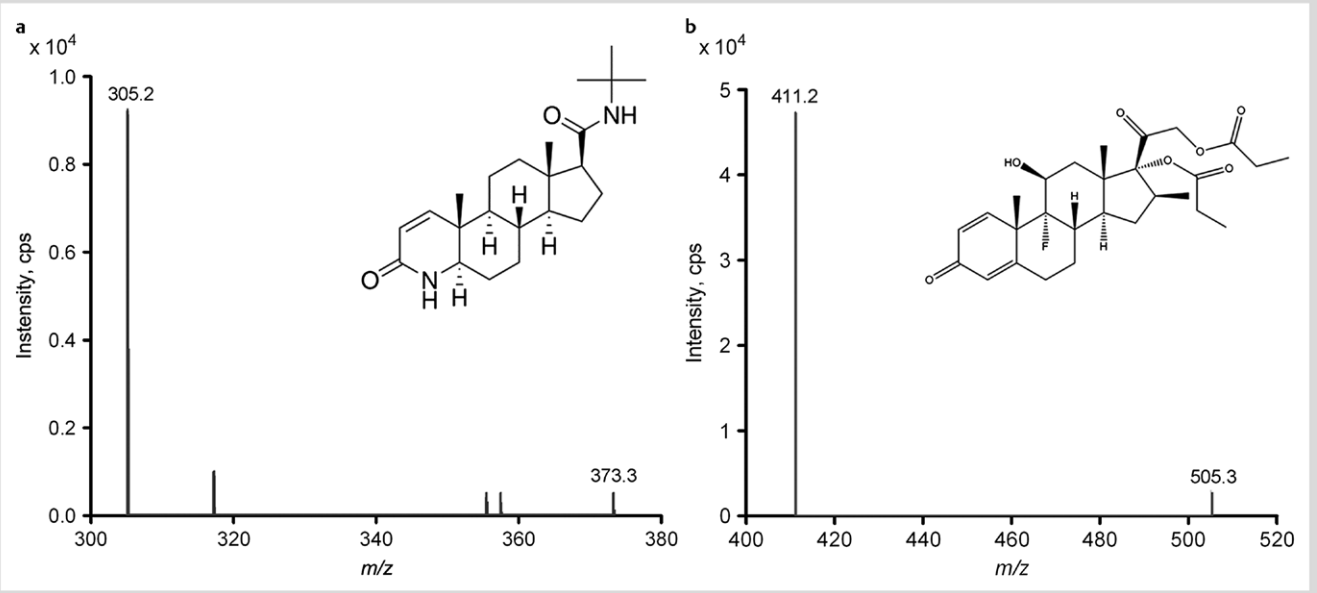

Fig. 1 Scan mass spectra of finasteride **a** and betamethasone **b**.

Statistical analysis

Bioequivalence between the 2 formulations was assessed by calculating individual test/reference ratios for the peak of concentration (C_{max}), area under the curve (AUC) of plasma concentration until the last concentration observed (AUC_{last}) and the area under the curve between the first sample (pre-dosage) and the area under the curve extrapolated to infinity (AUC_{0-inf}). C_{max} and the time taken to achieve this concentration (T_{max}) were obtained directly from the curves. The areas under the finasteride plasma concentration vs. time curves from 0 to the last detectable concentration (AUC_{last}) were calculated by applying the linear trapezoid rule.

The elimination rate constant (kel) was obtained as the slope of the linear regression of the log-transformed concentration values vs. time data in the terminal phase. The elimination half-life (t1/2) was calculated as 0.693/kel. The AUC_{0-inf} was calculated as AUC_{last} + Ct/kel, where Ct was the last measurable concentration.

Statistical calculations were defined at the level of $P \leq 0.10$ and bioequivalence was reached when the 90.0% confidence interval for C_{max}, AUC_{last} and AUC_{0-inf} felt within the range of 80.0–125.0% defined by both the Food and Drug Administration (FDA) and the National Sanitary Surveillance Agency (ANVISA). The software used included Equivtest® 2.0, MS Excel® 97, Tinn-R1.1, WinEdit® 2.0 and Scientific Work Place® 5.0.

Results and Discussion

The method for quantification of finasteride in human plasma was validated using the described procedures based on the Federal Guidance for Industrial Bioanalytical Method Validation [21] and the Brazilian National Sanitary Surveillance Agency (ANVISA) [22], prior to analysis of study samples as a requirement for drug quantification in pharmacokinetic studies.

To allow for the correct comparison of newer finasteride formulation with reference one, we validated a LC-MS/MS method with good selectivity, linearity range, precision, accuracy, and lower limit of quantification for the quantification of finasteride in plasma samples using betamethasone dipropionate as internal standard.

Product ion spectra and chromatography

The chemical structures of finasteride and the internal standard betamethasone dipropionate are displayed in **Fig. 1** along with the respective product ion mass spectra. These spectra were collected during infusion of the molecules under collision conditions used for quantification and the selected precursor to product ion transitions monitored with the assay is indicated.

For the determination of finasteride, the isocratic chromatographic condition was developed using a combination of solvent mixture in the mobile phase along with others parameters that provided good specificity to the method and eluted the analyte and IS with good peak shapes in a reasonably short time scale. The LC-MS/MS data were collected through 2.0 min but both compounds eluted close to 1.0 min after injection (0.75 min for finasteride and 0.85 min for betamethsone dipropionate). Typical peak shapes and retention times of the SRM chromatograms obtained from individual samples spiked with finasteride or the IS are shown in **Fig. 2**. The chromatography described here is the fastest compared to the similar methods described in the literature. The closest run time (3 min) was described by Constanzer et al. using HPLC coupled to atmospheric-pressure chemical-ionization tandem mass spectrometry [15] and Phapale [18] using reversed-phase ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS).

Extraction efficiency and ionization suppression

To achieve a level of ruggedness required for consistently reliable analysis of sequential batches, several important parameters were carefully optimized in the LLE procedure. Even after centrifugation, removal and usage of the ethyl acetate-hexane layer near the plasma interface was shown to be detrimental to assay ruggedness and to increase ionization suppression. A careful removal of the solvent layer produced a very clean extracts that resulted in no significant ionization suppression and a rugged analysis method. A back extraction step was not used, but rather

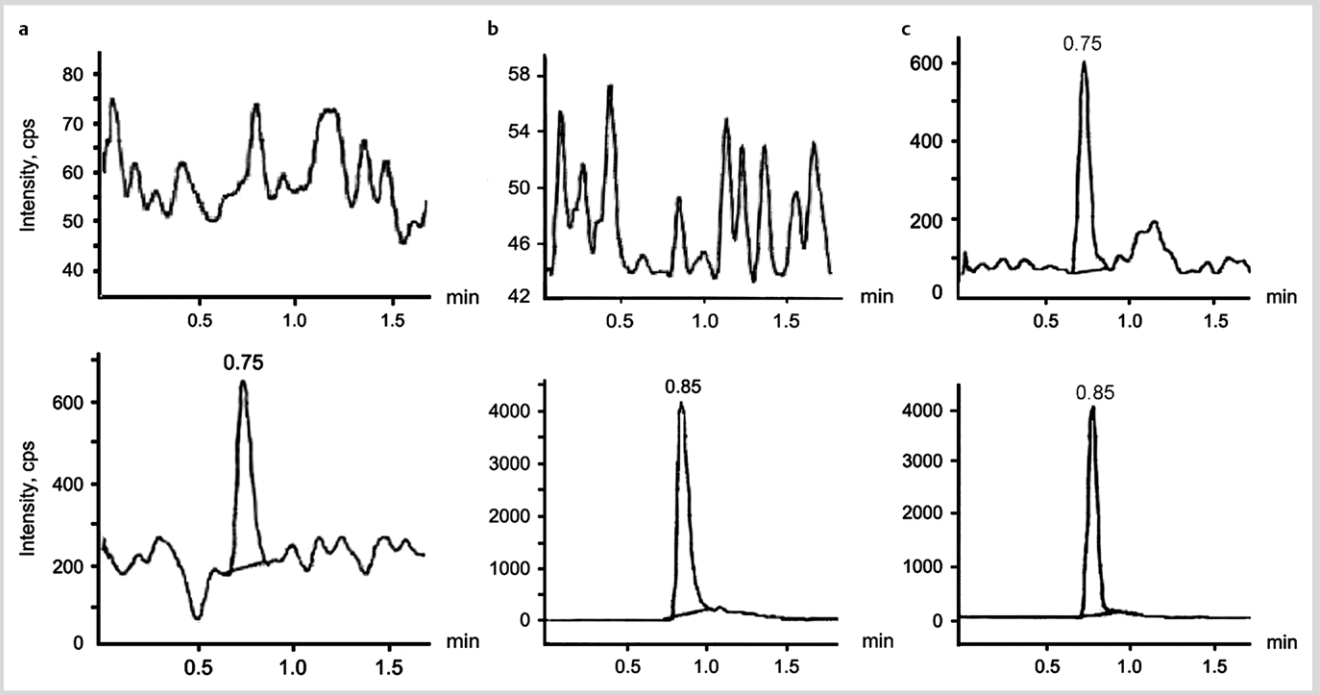

Fig. 2 MRM chromatograms of human plasma samples spiked with finasteride and/or betamethasone. The monitoring channels were *m/z* 373.5 > 305.2 for finasteride transition and *m/z* 505.3 > 411.2 for betamethasone transition. **a** Comparison of blank plasma samples without (upper) or with finasteride spiked at LLOQ concentration (0.1 ng/mL); **b** Comparison of blank plasma samples without (upper) or with (lower) betamethasone; **c** blank plasma samples simultaneously spiked with finasteride (upper panel) and betamethasone (lower panel).

the ethyl acetate-hexane was evaporated and the extract was reconstituted in acetonitrila-water (90:10, v/v) with 0.1 % of formic acid. These conditions increased sample throughput and proved to be very rugged and reliable, with thousands of plasma extracts typically injected on a single HPLC column.

Methods for high rates of bioanalytical sample analysis of finasteride using a liquid chromatography coupled to a tandem mass spectrometry analysis have also been reported in the literature [14–19]. Our method showed the best relation between LLOQ and chromatographic run time. The work of Phapale et al. [18] presented exactly the same LLOQ of 0.1 ng/mL, but the chromatographic run time was 3 min. Less sensitive analytical methods describing the quantification of finasteride in human plasma using mainly UV detection methods have also been reported [8–13].

Finasteride showed the recoveries (values ± CV(%), n = 5) for QCL, QCM and QCH in normal human plasma as follows: 74.2 ± 17.7 %, 95.8 ± 13.1 % and 80.6 ± 14.8 %, respectively. The recovery of the IS was 61.8 ± 19.5 %. Similar recovery values were obtained for hemolized and lipemic plasma.

These efficiencies may also include losses due to the extract solvent lost after freezing and transferring steps. However, method optimization revealed that attempting removal of the residual extraction solvent introduced contaminants that resulted in increasing HPLC back pressure and degradation of analyte peak shapes after a few hundred injections. However, with stable-labeled internal standards to compensate for analyte losses, the accuracy and precision remained excellent and sensitivity was not significantly compromised.

The ionization suppression was assessed in an attempt to evaluate additional potential sources of signal loss. In this assays, the MS/MS responses from the analysis of spiked, blank plasma subjected to the preparation procedure were compared with the analogous responses obtained from neat solutions. In all cases, the ratio of the peak areas measured from the plasma analysis compared to those obtained from the solvent diluted compounds (values ± CV(%), n = 5) for QCL, QCM and QCH in normal human plasma were as follows: 96.1 ± 13.3 %, 97.7 ± 13.2 % and 98.8 ± 10.2 %, respectively. The result for the IS was 94.5 ± 6.4 %.

Since the current experiment measures extraction efficiency plus all additional sources of signal loss, these results indicate that after accounting for losses due to recovery from plasma, there are no other significant sources of response loss, including ionization suppression. These data also provide additional evidence that the sample preparation conditions produce very clean sample extracts.

Selectivity and lower limit of quantification

During validation, analysis of 6 individual sources of normal human plasma produced no response for finasteride or betamethasone dipropionate. None of the 6 plasma sources resulted in quantifiable responses for both molecules. In addition, throughout clinical study sample analysis, pre-dose samples did not generate a significant response for the analyte or the internal standard, showing no contamination or insufficient wash-out period from previous dosage of medication.

The LLOQ for the method was established at 0.1 ng/mL and the signal-to-noise ratio was higher than 7. The SRM chromatograms obtained from extracted blank plasma simultaneously spiked with the LLOQ calibration standard and the internal standard are shown in **Fig. 2**, showing typical analyte signal-to-noise ratios achieved at this concentration. Analysis of study samples produced similar chromatographic results with no additional peaks observed.

There was no interfering peak when the analysis was performed using two other batches of hyperlipemic and hemolyzed plasma

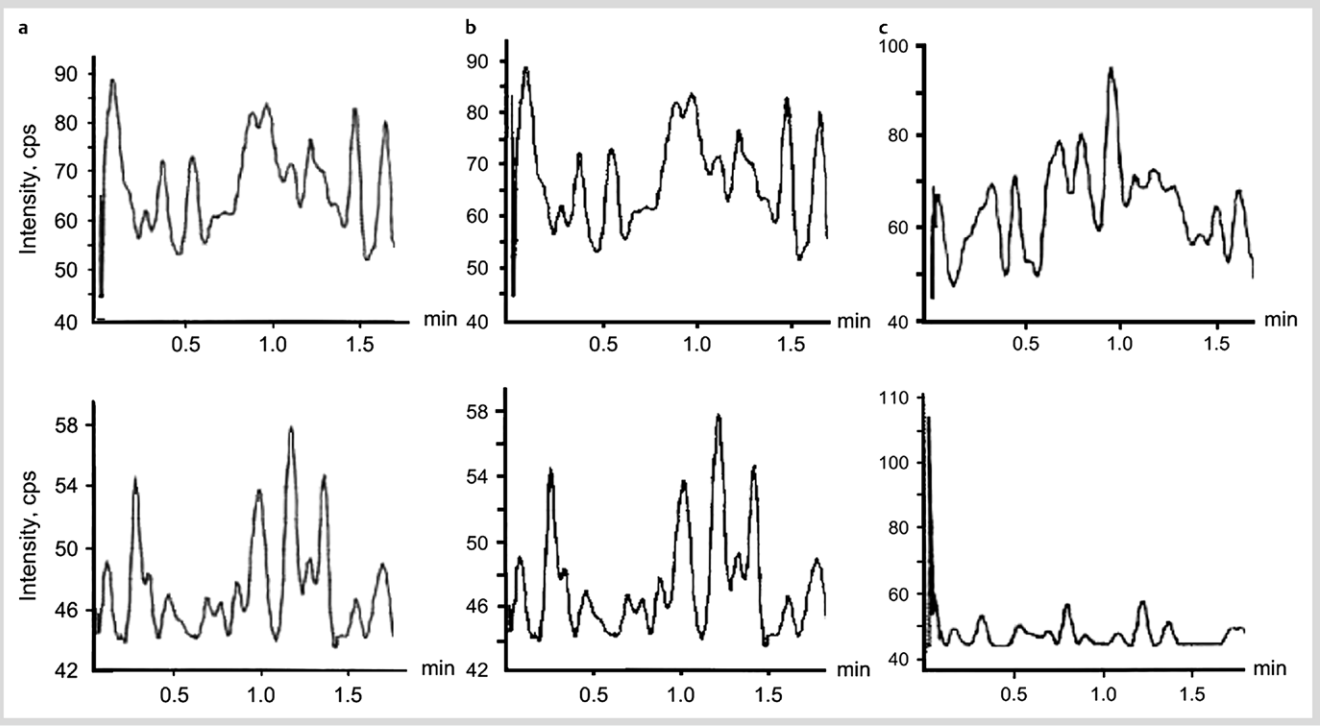

Fig. 3 Specificity of the response for the interfering peaks at the retention time for finasteride and betamethasone after the UPLC. MRM Chromatograms of blank plasma samples at both finasteride (upper panel) and betamethasone (lower panel) channels: **a** normal plasma; **b** hyperlipemic and **c** hemolyzed plasma sample.

Table 1 Accuracy and precision data for finasteride quantification in human plasma. Results were obtained during the validation of QC samples, including the LLOQ in human plasma.

QC samples	Nominal concentration (ng/mL)	Intra-run accuracy [a] (%)	Inter-run accuracy [b] (%)	Intra-run precision [c] (%CV)	Inter-run precision [b] (%CV)
QC-LLOQ	0.1	96.6	112.9	7.1	5.8
QCL	0.3	102.1	103.5	5.5	2.7
QCM	10.0	101.4	100.3	6.3	2.5
QCH	20.0	103.9	102.4	3.6	3.4

[a] (n = 6), expressed as (found concentration/nominal concentration) × 100
[b] Values obtained from all 3 runs (n = 18)
[c] n = 6

(**Fig. 3**). In addition, there was no significant ion suppression in the region where the analyte and internal standard are eluted.

Linear dynamic range and over-range samples

The simplest regression method for the calibration curves for finasteride quantification was $Y = a + bx(1/x^2)$ from 0.1 to 25.0 ng/mL. Including all the calibration curves from the validation process and the quantification of samples from the pharmacokinetic study, the correlation coefficient ranged from 0.9958 to 0.9989. To fit the calibration data, a $1/x^2$ linear function was used during analysis.

Accuracy and precision

The intra- and inter-batch precision (expressed as percent CV) and accuracy were established from validation runs performed at QCL, QCM and QCH (**Table 1**) and throughout analysis of all clinical study batches. The intra-batch precision and accuracy were also determined for the LLOQ levels. The intra-batch precision ranged from 3.6 to 7.1 % and the accuracy was within 96.6–103.9 %. For the inter-batch experiments, the precision ranged from 2.5 to 3.4 % and the accuracy was within 100.3–103.5 %. These results indicate excellent accuracy and precision at all quantification levels, as these data easily exceeded the guidance established accuracy criteria of ± 15 % for the QC levels (± 20 % at the LLOQ) with target CV values of ≤ 15 % (≤ 20 % at the LLOQ).

Autosampler carry-over

Injection of a diluent blank immediately following analysis of the highest calibration standard resulted in no observed response for finasteride.

Stability

Analyte stability was investigated for a variety of conditions that were utilized for handling and storage of both standards and samples. Stability was confirmed if after exposure to a given condition for a period of time, average measured analyte concentrations were below 15 % of variation compared to their respective spiked concentrations. Results of the stability studies are summarized in **Table 2**.

Table 2 Stability tests of finasteride in human plasma.

	Initial mean conc. (ng/mL)	% CV	Final mean conc. (ng/mL)	% CV	Variation (%)
Freeze and thaw stability test (3 cicles)					
QCL	0.303	11.6	0.323	11.6	6.51
QCH	18.9	13.3	20.9	8.74	10.88
Short Term stability test (23 h)					
QCL	0.303	11.6	0.307	6.54	1.21
QCH	18.9	13.3	20.6	6.11	9.21
Post Processing stability test (25 h)					
QCL	0.303	11.6	0.314	12.0	−2.9
QCH	18.9	13.3	19.1	12.8	0.2

n = 3 for each test
QCL = 1.5 ng/mL; QCH = 20 ng/mL

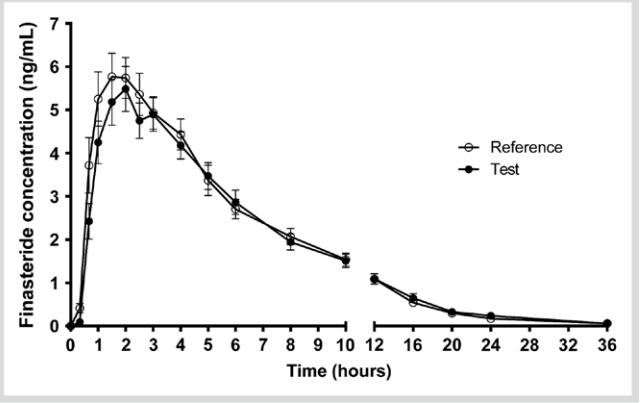

Fig. 4 Finasteride plasma mean concentration vs. time profiles obtained after the administration of 1.0 mg finasteride test or reference formulations. Results are expressed as mean ± SEM.

Freeze/thaw cycles were investigated to assure that no analyte losses occurred during the sample freezing and thawing that were required for analysis and possible sample retests. Both the analyte and the IS were shown to be stable for as many as 3 freeze/thaw cycles with temperature changes from ambient to −20 °C followed by a return to room temperature. Short-term storage of plasma at room temperature on the bench top was evaluated to simulate worst case exposure to room temperature after thawing and during possible delays in the sampling process prior to refreezing. Analyte stability was demonstrated for upto 23 h under these conditions. For prepared samples, stability was established to insure that during the time of analyses of one analytical batch stored in the auto injector, prepared samples could remain stable under the equipment conditions upto 25 h, defined as the maximum time to analyze the longest analytical batch in this study. Long-term storage stability was evaluated after 185 days to cover the total period that samples remained frozen at −20 °C during the complete study. Plasma samples spiked with finasteride at QCL and QCH concentrations showed a variation of −10.9 % and −2.4 %, respectively.

These studies also demonstrated that finasteride and the IS are stable in spiking solutions of acetonitrile-water (70:30, v/v) when stored in polypropylene cryovials at room temperature for 10 h or 3 days at 4 °C. The finasteride work solutions showed only −1.5 % variation between fresh and stored samples at room temperature after 10 h. Similarly, the finasteride work solutions stored at 4 °C showed a variation of 1.3 % after 3 days. The IS work solutions showed variation of −2.1 % after 10 h at room temperature and −5.1 % after 3 days at 4 °C.

Table 3 Arithmetic mean pharmacokinetic parameters obtained from 38 volunteers after administration of each 1.0 mg finasteride tablet formulation.

	Test formulation		Reference formulation	
	Mean	SD	Mean	SD
C_{max} (ng/mL)	6.97	3.19	7.32	3.87
T_{max} (h)	2.37	0.98	1.86	0.82
$T_{1/2}$ (h)	4.75	1.92	4.14	1.24
AUC_{last} ([ng × h]/mL)	41.46	22.67	42.77	22.22
AUC_{inf} ([ng × h]/mL)	43.58	23.46	44.56	22.58

Table 4 Geometric mean of the individual AUC_{last}, AUC_{0-inf} and C_{max} ratios (test/reference formulation) and the respective 90 % CIs.

	Parametric (n = 57)			
Parameters	Geometric mean (%)	90 % CI	Power (%)	CV (%)
C_{max} % ratio	97.68	91.23; 104.60	99 %	17.79
AUC_{last} % ratio	97.51	92.08; 103.27	99 %	14.88
AUC_{inf} % ratio	98.07	92.67; 103.78	99 %	14.69

Pharmacokinetic results

This method was applied to evaluate the comparative bioavailability in 78 healthy volunteers. All volunteers received a single 1.0 mg tablet orally corresponding to the test finasteride or reference formulation, following an open, 2-period, balanced randomized, crossover protocol.

Mean plasma concentration vs. time profiles for finasteride following oral administration of 2 formulations are displayed in **Fig. 4**. The plasma concentration of finasteride did not differ significantly after administration of both formulations.

Finasteride was well tolerated at the administered doses and no significant adverse reactions were observed or reported. No clinically relevant change was observed in any measured biochemical parameter. A total of 38 volunteers finished the study.

Table 3 shows the values of the pharmacokinetic parameters and **Table 4** summarizes the bioequivalence analysis for finasteride formulations. Briefly, the geometric mean and respective 90 % CI of finasteride test/reference percent ratios were 97.68 % (91.23; 104.60 %) for C_{max} and 97.51 % (92.08; 103.27 %) for AUC_{0-t}.

Conclusions

This work describes a fast, sensitive and robust method to quantify finasteride in human plasma using betamethasone as the internal standard. The sample preparation described in this

work produces very clean extracts and provides excellent ruggedness of the LC-MS/MS analysis with virtually no ionization suppression. The analytical method described in this work offers the advantage over those previously reported using LC-MS/MS showing a very low validated LLOQ (0.1 ng/mL) associated with a faster chromatographic run time (2.0 min).

The described method for finasteride quantification in human plasma was successfully applied in a bioequivalence study of two finasteride 1.0 mg tablet formulations using an open, randomized, 2-period crossover design. Since the 90 % CI for C_{max} and AUC ratios were all inside the 80–125 % interval, it was concluded that the test formulation of finasteride is bioequivalent to the reference formulation with respect to both the rate and the extent of absorption.

This example of PK results demonstrate the utility of this methodology, and bioanalytical measurements in general, for designing superior products and confirming their performance by measuring drug delivery and correlating with biological efficacy.

Conflict of Interest

We have no conflict of interest to declare in connection with the contents of this manuscript.

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