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# Picogram determination of finasteride in human plasma and semen by high-performance liquid chromatography with atmospheric-pressure chemical-ionization tandem mass spectrometry

M.L. Constanzer, C.M. Chavez, B.K. Matuszewski*

*Merck Research Laboratories, West Point, PA 19486, USA*

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**Abstract**

A method based on high-performance liquid chromatography (HPLC) with atmospheric-pressure positive-ion chemical ionization (APCI)–tandem mass spectrometric (MS–MS) detection for the determination of finasteride (MK-906, **I**) in human plasma and semen has been developed. The drug and internal standard (**II**) were extracted from biological matrices using a single solid-phase cyano cartridge. The eluent from the cartridge was injected directly onto the a 33 × 4.6 mm I.D. $C_{18}$, 3-$\mu$m column coupled with a base deactivated $C_{18}$ 20 × 4.6 mm I.D., 5-$\mu$m guard column. The column eluate was passed into the corona discharge APCI source by means of a heated nebulizer interface. The analyte and its internal standard were detected using multiple reaction monitoring (MRM) mode for enhanced selectivity and sensitivity. The chromatographic run time was 3 min, and the method had sufficient sensitivity, precision, accuracy and selectivity for the analysis of clinical samples containing finasteride at concentration of 0.2 ng/ml. The assay methodology confirms the versatility of APCI–MS–MS detection, combined with HPLC, for the quantitation of selected drugs in the sub-ng/ml range in biological fluids.

## 1. Introduction

Finasteride [N-(1,1-dimethylethyl)-3-oxo-4-aza-5$\alpha$-androst-1-ene-17$\beta$-carboxamide (**I**, Fig. 1)] belongs to a group of 4-aza-steroidal compounds which are potent inhibitors of human $\Delta^4$-3-ketosteroid 5$\alpha$-reductase [1–6], an enzyme that converts testosterone (T) to dihydrotestosterone (DHT) [7–10]. The increased levels of DHT are mainly responsible for enlargement of the prostate [7,11,12], and the inhibition of the 5$\alpha$-reductase with **I** was shown to have a beneficial therapeutic effect in the treatment of benign prostatic hyperplasia (BPH) by decreasing the size of prostate gland [13]. Finasteride has been recently approved for use in the treatment of BPH.

A high-performance liquid chromatographic (HPLC) method with ultraviolet (UV) detection for determination of **I** was previously developed [14] to support human pharmacokinetic studies based upon a 5-mg dosage regimen. Clinical

* Corresponding author.

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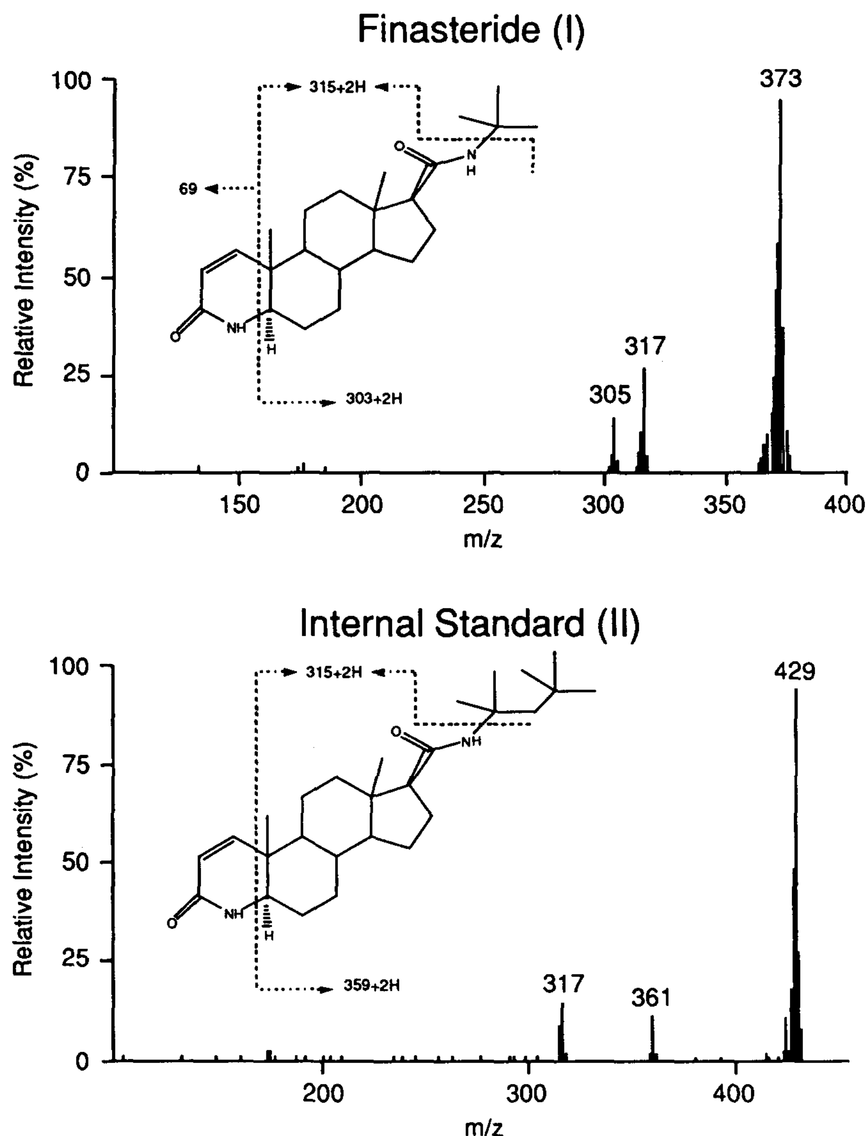


Fig. 1. Positive product ion mass spectra of the protonated molecular ions of finasteride (I) ($m/z$ 373) and internal standard (II) ($m/z$ 429); 50 ng of each compound analyzed by LC-MS-MS.

studies demonstrated that after a 5-mg oral dose, plasma concentrations of finasteride were in the range of 1 to 10 ng/ml 24 h after drug administration to human subjects. The HPLC-UV method had a limit of quantitation (LOQ) of 1 ng per ml of biological sample, a throughput of 50 samples a day, and required an efficient and highly selective sample preparation procedure to achieve the specificity at the low (210 nm) wavelength of detection. In order to completely map the plasma concentration time courses following oral administration of finasteride at 0.2 and 1-mg doses, and to monitor **I** at pg/ml concentrations in semen, it was necessary to

develop a more sensitive assay. The development of such an assay based on liquid chromatography (LC) with atmospheric pressure chemical ionization (APCI) tandem mass spectrometric (MS–MS) detection is the subject of this paper. The LC–MS–MS method has a LOQ of 200 pg/ml in both human semen and plasma, with a short run time of 3 min and sample throughput of more than 100 samples per day.

The combination of HPLC with APCI–MS–MS is becoming increasingly popular as an effective and convenient method for the quantitation of drugs in biological fluids [15–21]. The application of this technique for quantitative analysis of small molecules was pioneered by Henion and his group [15,16]. Use of the APCI-MS-MS instrument in the heated nebulizer mode with corona discharge results in high ionization efficiencies, and is compatible with HPLC mobile phases at flow rates of up to 2 ml/min. Under these conditions, the APCI–MS–MS instrument serves as a very sensitive and specific detector for the quantitative determination of drugs in biological fluids. The application of this methodology for the sub-ng/ml determination of **I** in human plasma and semen is described.

## 2. Experimental

### 2.1. Materials and reagents

Finasteride (**I**) and internal standard (**II**, Fig. 1) were obtained from Merck Research Laboratories (Rahway, NJ, USA). Acetonitrile, methanol, and water (all HPLC grade, Fisher Scientific, Fair Lawn, NJ, USA), ammonium acetate (reagent grade, Sigma, St. Louis, MO, USA) formic acid (Aldrich Chemical Co., Milwaukee, WI, USA) drug free human plasma and semen (Biological Specialties, Lansdale, PA, USA), J.T. Baker solid-phase extraction system and J.T. Baker 1-ml nitrile extraction cartridges (J.T. Baker, Phillipsburg, NJ, USA) were purchased from their respective suppliers.

Other reagents were of either HPLC or analytical grade and were used without further purification.

### 2.2. Apparatus

A Perkin-Elmer Biocompatible Binary pump 250 and ISS 200 autoinjector were used for all HPLC analyses. The chromatographic system consisted of a Perkin-Elmer (Danbury, CT, USA) $C_{18}$ 33 × 4.6 mm 3 $\mu$m analytical column coupled with a Keystone Scientific (Bellefonte, PA, USA) $C_{18}$ base deactivated 20 × 4.6 mm 5 $\mu$m guard column. For optimum performance the guard column was changed daily or after injection of 100 samples. The columns were heated to 70°C in a Jones Chromatography column oven (Alltech Chromatography, Deerfield, IL, USA). The mobile phase consisted of a 70:30 mixture of acetonitrile–water containing 0.1% formic acid delivered at a flow rate of 1 ml per minute. The effluent from the HPLC column was introduced directly into the ionization region of the mass spectrometer without flow splitting. The mobile phase was filtered though a 0.2-$\mu$m Nylon 66 filter (Rainin Instruments, Woburn, MA, USA). The HPLC autoinjector was set to a 3-minute runtime and the volume of extract injected was 100 $\mu$l.

Mass spectrometric detection was carried out using a PE-SCIEX API III triple-quadrupole instrument (PE-SCIEX, Thornhill, Toronto, Canada) operating in the heated nebulizer mode with corona discharge (+5 $\mu$A), using positive ion APCI. The temperature of the nebulizer probe was set at 500°C. Multiple reaction monitoring was employed using argon as collision gas at a thickness of $350 \cdot 10^{12}$ molecules $cm^{-2}$. The nebulizing gas (nitrogen) pressure and auxiliary flow were set at 80 p.s.i. (1 p.s.i. = 6894.76 Pa) and 2 l/min, respectively. The curtain gas was nitrogen at 0.9 l/min. The orifice potential and electron multiplier settings were +50 V and −3.8 kV. The dwell time was 400 ms, and the temperature of the interface heater was set at 60°C. The mass spectrometer was programmed to admit the protonated molecular ions $[M+H]^+$ at $m/z$ 373 (drug) and 429 (internal standard) via the first quadrupole filter (Q1) with collision induced fragmentation in Q2, and monitoring, via Q3, the production of $m/z$ 317 for both drug and internal standard. The output signal from

the mass spectrometer was interfaced to a Macintosh computer operating RAD and MacQuan Software (PE-SCIEX,) for data collection, peak integration and analysis. Peak-area ratios obtained from multiple reaction monitoring of analyte ($m/z$ 373→317)/internal standard ($m/z$ 429→317) were utilized for the construction of calibration curves, using weighted ($1/y$) linear least-squares regression of the plasma or semen concentrations and the measured area ratios.

### 2.3. Standard solutions

A stock standard solution of **I** (1 mg/ml) was prepared in methanol. This solution was further diluted with water to give a series of working standards with the concentrations of 1.0, 0.75, 0.50, 0.25, 0.10, 0.075, 0.05 and 0.02 μg/ml.

The internal standard **II** was also prepared as a stock solution (1 mg/ml) in methanol. A working standard of 0.1 μg/ml was used for all analysis. Stock standards and working standards of **I** and **II** were prepared once monthly.

### 2.4. Sample preparation

The standard curve for **I** in plasma was constructed by spiking blank human plasma or semen (1 ml) with known concentrations of **I** in the range of 0.2 to 100 ng/ml plus 10 ng/ml **II**.

For each standard sample, 100 μl of the appropriate working standards of **I** and **II** were added to plasma or semen. The samples were vortex-mixed for 10 s and transferred to a 1-ml nitrile extraction column under vacuum. The cartridge was pre-treated with 1 ml of methanol and 1 ml of water. After the addition of biological fluid, the cartridge was washed with 2 ml of water. Drug and I.S. were eluted with 300 μl of acetonitrile, and 100 μl of this mixture was injected directly onto the LC–MS–MS system.

### 2.5. Precision, accuracy, linearity and recovery

The precision of the method was determined by replicate analyses ($n = 5$) of human plasma and semen containing **I** at concentrations 0.2, 0.5, 0.75, 1.0, 2.5, 5.0 10.0, 25.0, 50.0 and 100.0 ng/ml. The accuracy of the assays was checked by preparing quality control (QC) samples at the start of the clinical study. QC samples with known concentrations of 0.75 and 75.0 ng per ml of biological matrix were prepared and frozen at −15°C. These QC standards were assayed along with clinical samples each day the analyses were performed. The calculated concentrations of the QC samples were compared on a day-to-day basis. Recovery was calculated by comparison of the peak areas of **I** extracted from biological samples to that of injected standards.

The linearity of each standard curve was confirmed by plotting the ratio of the drug and internal standard peak areas versus drug concentration. A standard curve was prepared and assayed daily with quality control and the unknown samples. The specificity of the assay was checked by analyzing blank and subjects' predose plasma samples. No endogenous interferences were encountered.

## 3. Results and discussion

Full-scan positive-ion spectra of both finasteride (**I**) and internal standard (**II**) yielded predominantly the protonated molecular ions at $m/z$ 373 and 429, respectively. The product ion mass spectra of these protonated molecular ions (Fig. 1) indicated the presence of the most intense daughter ion at $m/z$ 317, confirming similar fragmentation pattern as observed earlier for other 4-azasteroids subjected to ionization conditions used with the Sciex instrument [21].

The parent→daughter combinations of $m/z$ 373/317 and 429/317 were chosen for quantitation of I. By monitoring these parent→daughter pairs in the MS–MS mode, 20 pg of **I** or **II** injected into the LC–MS–MS system was detected with a signal-to-noise ratio of 5:1. This high sensitivity of detection allowed development of an assay for **I** in plasma or semen with the LOQ of 200 pg per ml of biological fluid. The LOQ was defined here as the lowest point on the standard curve for which the precision and accuracy were ≤10%. In addition to high specificity of MS–MS detection, parent com-

pound and **II** were chromatographically separated within the short (3 min) HPLC runtime required for high throughput multi-sample analyses. Although such chromatographic separation may not be always required in LC–MS–MS based assays, any additional degree of specificity was highly desirable in light of the possibility of the presence of metabolites in post-dose samples interfering with the quantitation of parent compound and/or internal standard.

Drug and internal standard were isolated from both semen and plasma using solid-phase extraction similar to that described earlier from plasma [14]. The recovery of **I** and **II** was ≥90% at all concentrations within the standard curve range and was practically the same from plasma and semen. The sample throughput was about 100 clinical samples per day, and was limited by the clean-up procedure, since the LC–MS–MS system controlled by routine acquisition and

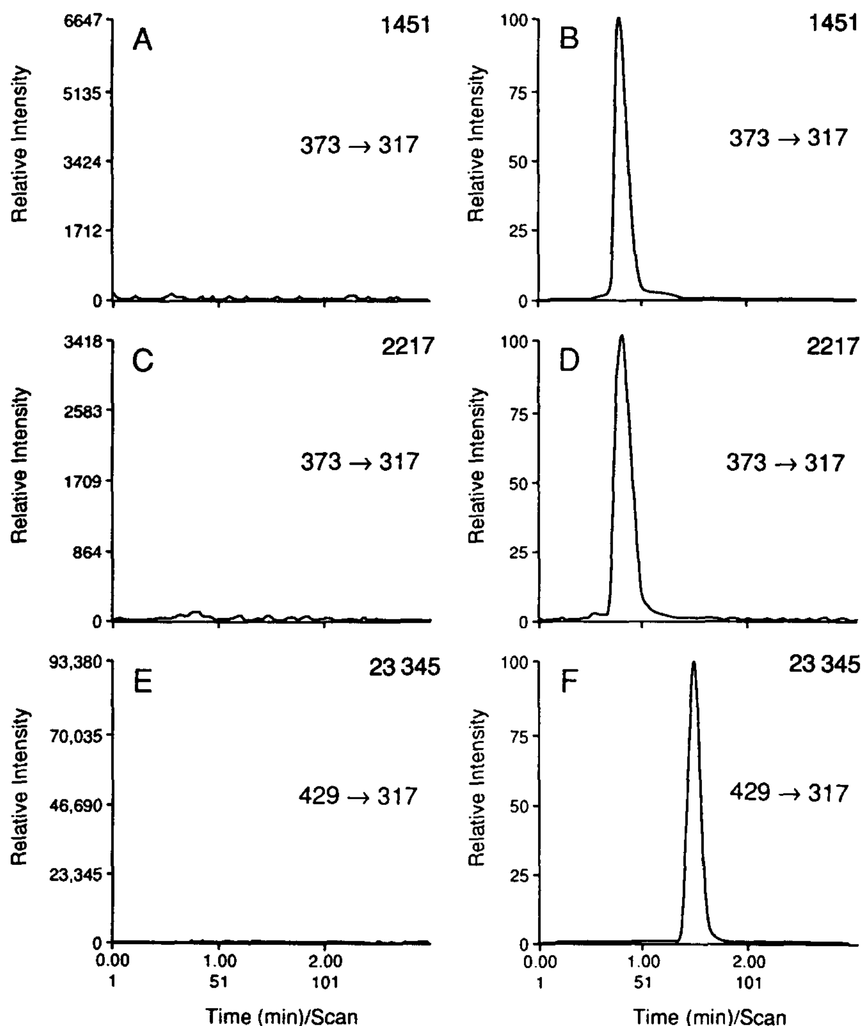


Fig. 2. Representative LC–MS–MS chromatograms of the extracts of semen obtained by multiple reaction monitoring (MRM) at $m/z$ 373→317 for finasteride and 429→317 for internal standard. A and C: extracts of control semen and predose semen of a subject in clinical study; B and D: extracts of control semen spiked with 1 ng/ml of **I** and semen sample of a subject after receiving 5 mg of finasteride daily for 42 days (concentration of **I** equivalent to 1.6 ng/ml); E and F: extracts of control semen and control semen spiked with 10 ng/ml of **II**. The numbers in upper right hand corner correspond to the peak heights expressed in arbitrary units.

Table 1
Intra-day precision, accuracy and recovery data for the analyses of finasteride in human plasma and semen

| Spiking level (ng/ml) | Plasma | | | Semen | | |
|---|---|---|---|---|---|---|
| | Found[a] (ng/ml) | Accuracy[b] | Precision[c] | Found[a] (ng/ml) | Accuracy[b] | Precision[c] |
| 0.20 | 0.20 | 100 | 3.5 | 0.20 | 100 | 4.4 |
| 0.50 | 0.48 | 96 | 7.2 | 0.48 | 96 | 7.5 |
| 0.75 | 0.79 | 105 | 3.5 | 0.75 | 100 | 6.3 |
| 1.00 | 0.99 | 99 | 5.3 | 1.03 | 103 | 9.1 |
| 2.50 | 2.55 | 102 | 4.4 | 2.45 | 98 | 7.8 |
| 5.00 | 5.50 | 110 | 3.1 | 4.70 | 94 | 8.1 |
| 10.00 | 10.53 | 105 | 3.4 | 9.82 | 98 | 7.2 |
| 25.00 | 26.84 | 107 | 4.6 | 23.53 | 94 | 9.9 |
| 50.00 | 52.13 | 104 | 7.9 | 49.38 | 99 | 8.1 |
| 100.00 | 101.42 | 101 | 3.9 | 95.43 | 95 | 7.1 |

[a] Mean values, $n = 5$.
[b] Calculated as (mean calculated concentration/nominal concentration) × 100.
[c] Expressed as coefficient of variation (%).

display software could analyze 100 biological fluid extracts in about 5 to 6 h.

The MRM chromatograms of extracts of control semen and from a subject following 5 mg daily dose of finasteride for 42 days are shown in Fig. 2.

The method was validated in both human plasma and semen. The within-day precision of the assay was less than 10% at all concentrations within the standard curve range (Table 1). The assay accuracy at all concentrations both in plasma and semen varied between 94–110% (Table 1). Inter-day precision, as measured by the concentration of QC standards, was below 13% (Table 2).

The method has been applied successfully to the determination of **I** in semen of subjects dosed with 0.2, 1.0 and 5.0 mg of finasteride, and is currently used to support other clinical studies with finasteride. It was found to be rugged and, due to short chromatographic run time and high specificity of MS–MS detection, highly efficient, allowing analysis of 100 clinical samples in 8–10 h.

The described technique confirms the versatility of atmospheric pressure ionization–tandem

Table 2
Inter-day variability for the assay of quality control semen samples spiked with finasteride

| Nominal concentration (ng/ml) | Initial mean concentration[a] (ng/ml) | Mean concentration[b] (ng/ml) | Coefficient of variation[c] |
|---|---|---|---|
| 0.75 | 0.71 | 0.68 | 7.2 |
| 75.00 | 69.47 | 68.86 | 12.8 |

[a] $n = 5$.
[b] Mean of seven analyses performed over a period of ten weeks.
[c] $n = 7$.

mass spectrometry, in conjunction with HPLC, for the quantitation of drugs in biological matrices in the sub-ng/ml range.

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