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High-performance liquid chromatographic method for the determination of finasteride in human plasma at therapeutic doses

M. L. CONSTANZER*, B. K. MATUSZEWSKI and W. F. BAYNE

Merck Sharp & Dohme Research Laboratories, West Point, PA 19486 (U.S.A.)

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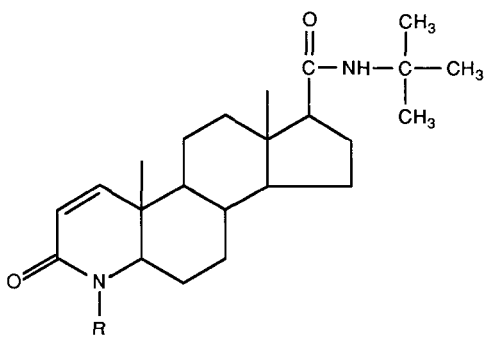
ABSTRACT

A high-performance liquid chromatographic (HPLC) method with ultraviolet detection for the determination of a novel 4-aza-steroidal inhibitor of 5α-reductase in human plasma has been developed. The assay is based on a single solid-phase extraction and an efficient HPLC separation on two analytical columns in series. The assay has been fully validated and used to support Phase II and III clinical pharmacokinetic studies. The lowest limit of quantification was found to be at 1 ng/ml and allowed pharmacokinetic evaluation of the drug at doses down to 5 mg.

INTRODUCTION

Finasteride [Proscar, N-(1,1-dimethylethyl)-3-oxo-4-aza-5α-androst-1-ene-17β-carboxamide (drug I, Fig. 1)] is a 4-aza-3-oxosteroidal inhibitor of human 5α-reductase. Its synthesis has been described [1]. Since the development of be-



R = H - Drug I

R = CH_3 - I.S.

Fig. 1. Structure of finasteride (I) and the internal standard (I.S.).

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nign prostatic hypertrophy (BPH) is androgen-dependent and the inhibition of 5α-reductase results in the decrease of dihydrotestosterone levels in the prostate, the potential exists for I and other similar 4-aza-steroidal inhibitors to be used in the BPH treatment [2,3]. Compound I was chosen from about 200 analogues for clinical evaluation. Clinical effects of finasteride, which is now in late Phase III clinical trials, have been described [4,5].

A method was needed with a limit of reliable quantification (LQ) of at least 1 ng/ml to support clinical studies employing 1–10 mg oral doses. In addition, in order to support large-scale pharmacokinetic studies, an efficient method capable of analyzing more than 50 plasma samples a day was required. A previously developed high-performance liquid chromatographic (HPLC) method [6] with an LQ of 25 ng/ml could not support clinical studies using low therapeutic doses of I. An assay, with the 1 ng/ml LQ, has been successfully developed and utilized routinely to support all major human biopharmaceutic studies. More than 3000 plasma samples from various clinical studies were analyzed using the procedure described in this paper.

EXPERIMENTAL

Materials and reagents

Acetonitrile, methanol and water (all HPLC grade, Fisher Scientific, Fair Lawn, NJ, U.S.A.), drug-free human control plasma (Sera-Tec Biologicals, Harrisburg, PA, U.S.A.), Baker solid-phase extraction system and Baker 1-ml nitrile extraction columns (J. T. Baker, Phillipsburg, NJ, U.S.A.) were purchased from their respective suppliers. Aza-steroids (I) and the internal standard (I.S.) were obtained from Merck Sharp & Dohme Research Labs. (Rahway, NJ, U.S.A.).

Apparatus

A Waters Assoc. 703 HPLC system equipped with a 730 data module, a 720 system controller, a WISP 710B automatic injector and a 6000A chromatographic pump (Waters-Millipore, Milford, MA, U.S.A.) were used for all analyses. As a detector, a variable-wavelength ultraviolet (UV) absorbance detector (Spectroflow 733, Kratos, Ramsey, NJ, U.S.A.) was employed. The detector output signal was interfaced to a Hewlett-Packard laboratory automation system (HP 3357 LAS, Palo Alto, CA, U.S.A.) for data collection, peak integrations and analyses. The chromatographic column consisted of an Altex RP-8, (15 cm × 0.46 cm, 5 μm) column coupled with a short Analytichem RP-18 (5 cm × 0.46 cm, 3 μm) column. A Brownlee Labs. (Santa Clara, CA, U.S.A.) RP-8, 5 μm (2.5 cm × 0.46 cm) column was utilized as a guard column. For optimum chromatographic performance, the guard column had to be replaced after each 100–110 injections of plasma extracts. Analytical columns were kept at room temperature.

The UV spectra of I were taken using either a diode-array spectrophotometer (Hewlett-Packard, Model 8451) or a HPLC photodiode-array UV detector (Varian, Model Polychrom 9060, Palo Alto CA, U.S.A.).

Chromatographic conditions

The mobile phase consisted of methanol–acetonitrile–water (6:5:7, v/v) delivered at a flow-rate of 1 ml/min. The mobile phase was filtered through a 0.2-μm Nylon 66 filter (Rainin Instruments, Woburn, MA, U.S.A.).

The UV detector was set at 210 nm, with a rise time of 2 s and an absorbance setting of 0.002 a.u.f.s. The slope sensitivity setting for peak detection was 0.1 mV/min. The autoinjector had a 23-min run time and injected a 200-μl volume. The retention times of I and I.S. under the conditions described above were 13.1 and 20.8 min, respectively.

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 of I. The concentrations were 2.0, 1.0, 0.5, 0.2, 0.1, 0.05, 0.02 and 0.01 μg/ml.

The I.S. was also prepared as a stock solution (1 mg/ml) in methanol. A working internal standard of 2 μg/ml was used for all analysis.

Stock solutions were prepared once per week and stored at ~5°C.

Samples preparation

The standard curve for I in plasma was constructed by spiking blank human plasma (1 ml) with known concentration of I in the range 1–200 ng/ml plus 100 ng/ml I.S. For each standard sample, 100 μl of the appropriate working standard and 50 μl of the internal standard solution were added to the plasma. The samples were vortex-mixed (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 plasma, the cartridge was washed with 2 ml of a 10% acetone–water mixture and 2 ml of water. Drug and I.S. were eluted with 250 μl of methanol. After adjusting the eluent strength to that of the mobile phase with 100 μl of water, 200 μl of this mixture were injected directly onto the HPLC system.

Presicion, accuracy, linearity and recovery

The precision of the method was determined by replicate analyses ($n = 5$) of human plasma containing I at concentrations 1, 2, 5, 10, 20, 50, 100 and 200 ng/ml. The accuracy of the assays was checked by preparing quality control samples at the start of the clinical studies. Plasma samples with known concentrations of I (5 and 150 ng/ml) were prepared and frozen at −15°C. These quality control standards were assayed along with the unknown samples each day analyses were performed. The calculated concentrations of the quality control samples were compared on a day-to-day basis. Recovery was calculated by comparison of the peak areas of I extracted from plasma to that of the injected standards.

The linearity of each standard curve was confirmed by plotting the ratio of drug to internal standard peak heights *versus* drug concentration. The 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 various patient's pre-dose plasma samples. No endogenous interferences were encountered.

RESULTS AND DISCUSSION

Initial studies at doses of 50–400 mg suggested that 24 h after oral administration of 5 mg of I to human subjects the plasma concentrations would be in the 1–10 ng/ml range; thus, a reliable and efficient method to quantify I at these concentrations was required. Several approaches for the detection of I after HPLC separation were initially considered. In addition to the UV absorption, fluorescence and electrochemical detection (ED) were explored, both directly and after an appropriate derivatization of I. Compound I was not easily oxidized at low potentials necessary for ED. Also, it was practically non-fluorescent. The straigthforward derivatization of I was not feasible, due to the lack of a functionality in the molecule of I which could be easily exploited for this purpose. Therefore, UV detection was utilized for the assay similar to that employed in the previously developed HPLC method [6]. In order to achieve the desired 25-fold improvement in assay sensitivity and to decrease sample preparation and analysis time, a new, and more efficient extraction procedure from plasma along with the appropriate chromatographic conditions were developed.

The UV spectrum of I in methanol indicated the presence of a strong absorption band with the maximum at 204 nm (ε = 15 900 M^{-1} cm^{-1}) and a shoulder between 220 and 270 nm (ε = 2400–600) of much lower intensity than expected for the unperturbed by nitrogen α,β-unsaturated ketone functionality (ε = 10 000–18 000, λ_{max} = 230–270 nm [7]). The maximum of absorption of various aza-steroids is also known to be shifted hypsochromically when compared with their carbocyclic counterparts. This shift is highly dependent on the relative position (distance) between the chromophore (α,β-unsaturated ketone) and electronegative substituent (nitrogen) [8]. The absorption of I at longer wavelength was not of sufficient intensity for sensitive UV detection. Therefore, the detection wavelength of 210 nm (ε = 14 700 M^{-1} cm^{-1}) had to be chosen for the assay of I.

In order to successfully run a multi-sample assay at this short wavelength and at high sensitivities, an efficient and highly selective sample preparation procedure had to be developed. The use of a single nitrile cartridge (1 ml) washed with a 10% acetone–water mixture (2 ml), effectively removed major plasma endogenous interferences. Only 1 ml of plasma was required to achieve the 1 ng/ml LQ. The numerous solvent evaporation steps required in the original method were all eliminated at the same time reducing the LQ from 25 to 1 ng/ml. Peak shape was improved, analysis and retention times were decreased, and minor interfering plasma component was separated by shortening the C_8 (5 μm) column from 25 to 15 cm, the addition of a short (5 cm) C_{18} (3 μm) column in series, and some

changes in the mobile phase composition. All these changes were necessary for high detection sensitivity.

An extensive method validation required to support human pharmacokinetic studies was performed. The within-day precision of the assay was less than 10% for all concentrations within the standard curve range (Table I).

TABLE I

INTRA-DAY PRECISION, ACCURACY AND RECOVERY FOR THE ANALYSIS OF I IN PLASMA

Concentration (ng/ml)	C.V. [a] (%)	Accuracy[b] (mean ± R.S.D.) (%)	Recovery (%)
1	3.2	100 ± 7.1	98.4
2	4.3	105 ± 4.1	96.9
5	5.3	100 ± 2.8	93.0
10	2.6	98 ± 3.8	92.9
20	2.3	97 ± 4.5	91.0
50	3.1	97 ± 2.5	97.9
100	1.9	98 ± 2.7	97.1
200	1.1	102 ± 1.4	94.8

[a] Coefficients of variation of replicate analyses (n = 5).

[b] Expressed as (mean observed concentration)/(spiked concentration) × 100, calculated from daily standard curves over a period of twenty days (n = 16).

Recovery of the drug was very high (91.0–98.4%) and was practically independent of concentration (Table I). In addition, recovery from plasma was compared with recovery from the buffer used in protein-binding experiments (0.07 *M* sodium chloride, 0.05 *M* phosphate buffer, pH 7.4). Standard curves obtained after extracting I from both plasma and buffer were compared. The slopes of the standard curves were practically the same. Based on the comparison of these slopes (0.0179) from plasma and 0.0177 from buffer), the recovery from plasma *versus* that from buffer was 101.1% within the concentration range 1–200 ng/ml.

The inter-day variability of the assay was determined by analyzing quality control samples in blank human plasma at high and low concentrations within the standard calibration curve. The quality control samples were stored at −15°C under the same conditions as subject samples (Table II).

The data in Table II indicate excellent stability of I in plasma for at least three months. In addition, the same quality control samples were reanalyzed, in five replicates, six months later, giving the mean values of 4.8 ng/ml (coefficient of variation, C.V. = 9.4%) and 147.4 ng/ml (C.V. = 4.0%). These data confirm the long-term (more than nine months) storage stability of I in plasma. Also, inter-day assay precision and accuracy were within 10% of the initial values.

TABLE II

INTER-DAY VARIABILITY FOR THE ASSAY OF QUALITY CONTROL PLASMA SAMPLES SPIKED WITH I

Spiked concentration (ng/ml)	n^a	Mean calculated concentration (ng/ml)	Coefficient of variation (%)
5	40	5.0	4.6
150	40	150.1	4.3

[a] Assayed over a period of three months.

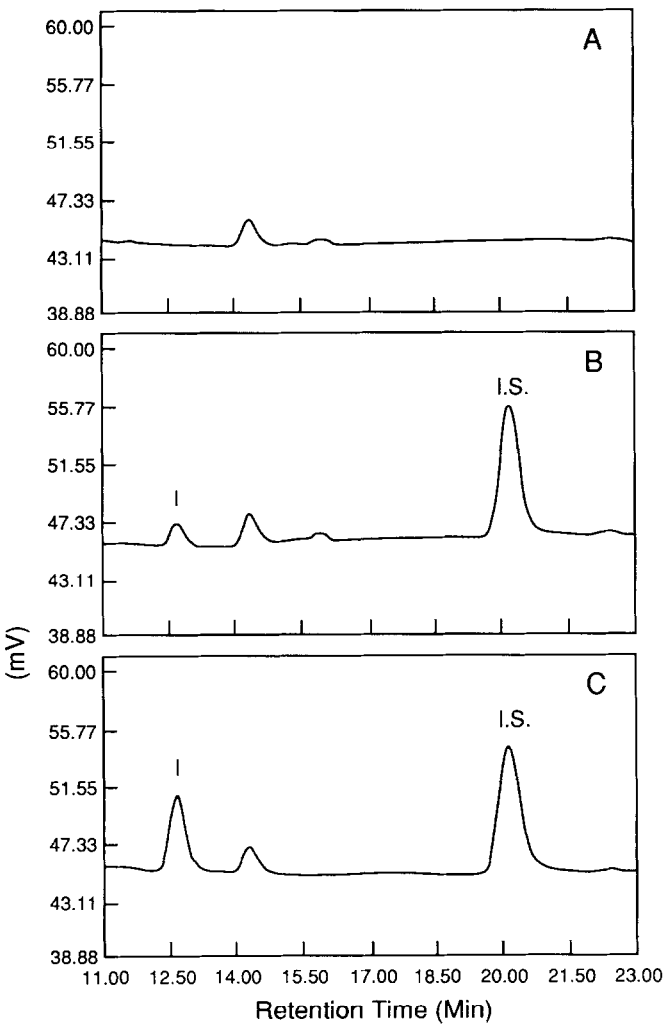

Fig. 2. Representative chromatograms of I in human plasma. (A) Blank control plasma; (B) control plasma spiked with 10 ng/ml I and 100 ng/ml internal standard (I.S.); (C) post-dose plasma sample of human subject 1.5 h after oral dosing with 5 mg of I; the concentrations of I and I.S. are equivalent to 34.5 and 100 ng/ml, respectively.

The specificity of the assay was confirmed by assaying blank human plasma and pre-dose, 0-h collections of subjects' plasma. No endogenous interference was encountered. The retention times for the metabolites of I (side-chain monohydroxylated and ring-hydroxylated analogues) under assay conditions were 2.4 and 2.1 min, respectively. Representative expanded chromatograms of the blank, control plasma spiked with I and I.S. and post-dose plasma samples are presented in Fig. 2.

The assay was utilized for analysis of plasma samples from all major human biopharmaceutic studies including bioavailability, bioequivalence and multiple-dose pharmacokinetic studies. Typical plasma concentration–time data for human subjects after a 5-mg single oral dose are presented in Table III.

In summary, a simple and efficient method of assaying I based on a single solid-phase extraction and an efficient HPLC separation was developed. The method was used routinely for a period of almost three years and had proven to be highly reliable and practical. Good laboratory practice, good maintenance of the chromatographic system components (pump, UV detector), and utilization of the computerized data acquisition system are essential to achieve high sensitivity and run the assay routinely at a detector wavelength of 210 nm. More than 50 plasma samples per day have been assayed, with the lowest reliable limit of quantification of 1 ng/ml. The method allows pharmacokinetic evaluation of the drug (I) at doses down to 5 mg.

TABLE III

CONCENTRATION OF FINASTERIDE IN HUMAN SUBJECTS FOLLOWING A SINGLE 5-mg TABLET

From bioavailability studies; the mean values of sixteen patients and respective standard deviations (S.D.) are included.

Time (h)	Concentration (mean ± S.D.) (ng/ml)
0	0.0 ± 0.0
0.5	17.2 ± 11.2
1.0	31.5 ± 11.6
1.5	33.6 ± 8.3
2.0	31.4 ± 5.6
3.0	28.0 ± 5.5
4.0	24.9 ± 4.6
6.0	20.2 ± 4.8
8.0	16.9 ± 3.8
10	13.4 ± 3.5
12	10.5 ± 3.7
14	7.4 ± 3.6
24	3.2 ± 2.5

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