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High-performance liquid chromatographic determination of finasteride in human plasma using direct injection with column switching

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Abstract

A fully automated column-switching high-performance liquid chromatographic (HPLC) method was developed for the quantification of finasteride [N-(1,1-dimethylethyl)-3-oxo-4-aza-5α-androst-1-ene-17β-carboxamide] in human plasma. Plasma samples were diluted with an equal volume of ethylene glycol–water (40:60, v/v), then the diluted sample (150 μl) was injected into the HPLC system without clean-up. The analyte was retained on a pretreatment column, whereas plasma proteins and other endogenous components were washed out to waste. The analyte was transferred to the analytical column in the heart-cut mode and then detected at 210 nm. A quantification limit of 1 ng/ml was attained. There was a linear relationship between peak height and drug concentration in plasma in the range 1–50 ng/ml. This method was validated and applied to the assay of plasma samples to characterize pharmacokinetic parameters in clinical studies.

Keywords: Finasteride

1. Introduction

Finasteride [N-(1,1-dimethylethyl)-3-oxo-4-aza-5α-androst-1-ene-17β-carboxamide] (**I**, Fig. 1) is a 4-aza-3-oxosteroid compound which inhibits the activity of human 5α-reductase and thus lowers dihydrotestosterone levels in the prostate [1,2]. Since the development of benign prostatic hypertrophy (BPH) is androgen-dependent, **I** was developed as an agent for the treatment of BPH.

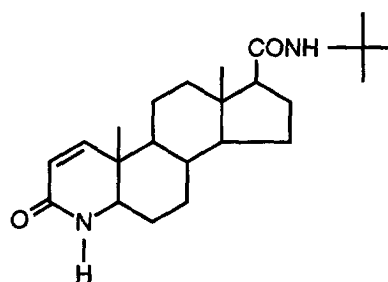

Fig. 1. Structure of finasteride (**I**).

For the quantification of **I** in biological samples, high-performance liquid chromatography

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(HPLC) [3,4] and liquid chromatography–tandem mass spectrometry (LC–MS–MS) [5] have been reported. The LC–MS–MS method is highly sensitive, but an expensive instrument is required for the assay. One of the HPLC methods provides sufficient sensitivity and selectivity for human plasma assay, where sample clean-up is essential and successfully performed by off-line solid-phase extraction [4]. For sensitive quantification of drugs in biological fluids, however, much recent research has been directed towards the use of column-switching systems [6,7]. The system makes it possible to avoid cumbersome and time-consuming procedures for off-line sample clean-up and to achieve high efficiency in the enrichment of analyte compounds.

This investigation was undertaken to examine whether a column-switching technique could be developed for the determination of **I** in human plasma. A fully automated HPLC method that allows direct injection of plasma samples has been developed using a heart-cut technique for extraction of the analyte. Owing to the use of on-line extraction, this method requires minimal sample preparation. In addition, the method provides an accurate assay resulting from the heart-cut technique.

2. Experimental

2.1. Reagents and materials

N-(1,1-Dimethylethyl)-3-oxo-4-aza-5α-androst-1-ene-17β-carboxamide (**I**) was supplied by Merck (Rahway, NJ, USA). Acetonitrile (HPLC grade), methanol and ethylene glycol (both analytical-reagent grade) were purchased from Wako (Osaka, Japan). Ultrafree filters (5 and 0.22 μm) were obtained from Millipore-Japan (Tokyo, Japan). Water was purified with a Millipore Milli-Q system.

2.2. Instrumentation

The HPLC system was constructed using Shimadzu (Kyoto, Japan) components, unless specified otherwise. The system consisted of four pumps (LC-10AD), a column oven (CTO-10A), an autosampler (SIL-10A) with a sample cooler, a degasser (DGU-3A), a six-port, two-position valve (FCV-12AH), a UV detector (SPD-10A), a data processor (C-R7A) and a system controller (SCL-10A). The detector output signal, passed through an SC77 signal cleaner (System Instruments, Tokyo, Japan), was interfaced to the data processor. The pumps, switching valve, autosampler and data processor were controlled by the system controller. The system included two preclean columns, a pretreatment and an analytical column.

2.3. Chromatographic conditions

The mobile phases (MP1–MP4) were acetonitrile–water mixtures [10:90 (v/v) for MP1, 25:75 (v/v) for MP2, 70:30 (v/v) for MP3 and 45:55 (v/v) for MP4]. The preclean columns (C1 and C2) were Capcell Pak C_{18} (35×4.6 mm I.D., 5 μm, SG-120) purchased from Shiseido (Tokyo, Japan). These columns were effective in eliminating impurities contained in MP1 and MP2, and were washed with MP3 every day before use. The pretreatment column (C3) was a Capcell Pak CN (35×4.6 mm I.D., 5 μm, SG-120) from Shiseido. The analytical column (C4) was an Inertsil ODS-2 (250×4.6 mm I.D., 5 μm) from GL Science (Tokyo, Japan). Columns C1 and C2 were operated at ambient temperature and C3 and C4 at 40°C. The autosampler was set at 2°C and had a run time of 38 min. The flow-rate of the mobile phase for C3 was 1.0 ml/min and for C4 1.1 ml/min. The detector was operated at 210 nm. The signal cleaner was set at a delay time of 5.1 s.

Fig. 2 shows a schematic diagram of the fully automated HPLC system. The system was operated according to the following procedure, where valve positions (V) and switchover times are given in parentheses. Throughout the procedure, except for step 4, MP4 was delivered to C4 to keep the chromatographic baseline steady.

Step 1 (V=0, 0 min): a sample is injected and the data processor starts to acquire signals from the detector. Step 2 (V=0, 0–5 min): the sample

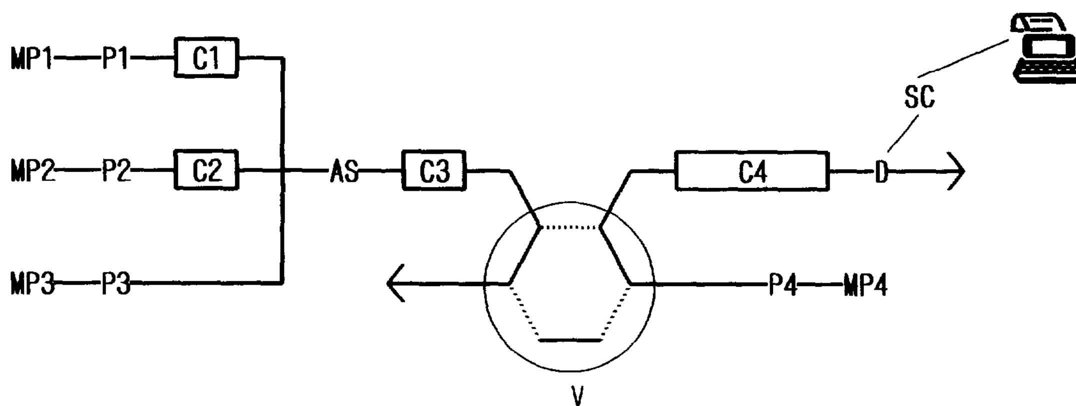

Fig. 2. Automated HPLC system with column switching used for the separation and quantification of **I** in plasma. MP1–MP4 = mobile phases 1–4; P1–P4 = pumps 1–4; C1–C4 = columns 1–4; AS = autosampler; V = six-port, two-position valve; D = UV detector; SC = signal cleaner. The solid and dotted lines in V indicate valve positions 0 and 1, respectively.

is swept into C3 with MP1. The analyte is retained, while proteins and polar substances are eluted to waste. Step 3 (V = 0, 5–13 min): mobile phase MP2 is delivered to C3, where the analyte is still retained and less polar substances are discarded with the effluent. Step 4 (V = 1, 13–14.5 min): the direction of flow of MP2 is changed. The fraction containing the analyte is transferred from C3 to C4 and the analyte is concentrated at the front of C4. Step 5 (V = 0, 14.5–34 min): the valve position is returned to the initial state. Mobile phase MP4 is pumped to C4 for chromatographic separation. Meanwhile, C3 is washed with MP3 to remove hydrophobic substances retained by the column. Step 6 (V = 0, 34–38 min): column C3 is reconditioned with MP1 to prepare for the next analysis.

2.4. Standard solutions

Compound **I** was dissolved in methanol at a concentration of 1 mg/ml. The solution was further diluted with ethylene glycol–water (40:60, v/v) to give standard solutions with concentrations of 1, 2, 5, 10, 30 and 50 ng/ml. Ethylene glycol was found to depress adsorption of **I** on membranes of the filters employed in this study. The solutions were stored at 4°C until use and were stable for at least 6 months.

2.5. Calibration graphs

The calibration graph for **I** was constructed by addition of standard solution (0.15 ml) to drug-free plasma (0.15 ml). The mixture was passed through a 5-μm filter and then a 0.22-μm filter by centrifugation at 1000 *g* for 5 min at 4°C. The filtrate (0.15 ml) was injected into the HPLC system. Plots of peak height against drug concentration were used to calculate the linear regression equation.

The same procedure was employed for the preparation of samples to assess the recovery, precision and accuracy of the assay.

2.6. Preparation of plasma samples

Plasma was separated from heparinized blood samples by centrifugation at 1500 *g* for 15 min at 4°C. After collection of plasma, the samples were stored at −30°C until analysis. After thawing, the sample was treated in the manner described for calibration graphs.

3. Results and discussion

An attempt to develop a GC–MS method for the quantification of **I** was unsuccessful. Compound **I**, containing two amide moieties, was

adsorbed on fused-silica capillary columns, and therefore to replace these active hydrogens, acylation and trimethylsilylation were carried out. Although the hydrogen at the N-4 position could be replaced, no reaction with the other amide took place because of the steric hindrance caused by the *tert.*-butyl group. Then, for an HPLC assay of **I**, introduction of chromophores in the N-4 position was attempted, but failed because the reactions tested were dependent on concentration.

Constanzer et al. [4] have reported that several detection methods do not allow the sensitive HPLC assay of **I**, except for UV detection at 210 nm. Compound **I** has only one intense absorbance at this wavelength, where a large number of matrix components absorb. Therefore, a heart-cut technique was employed for analyte extraction in the development of the column-switching system.

As we have previously reported, columns packed with silicone-coated silica are effective for on-line sample clean-up [7]. Among commercially available C_8, C_{18} and CN columns a CN column was selected, because it permitted the analyte to be moderately retained. Under these conditions, the analyte was moderately eluted and it could be preconcentrated on the head of the analytical column, thus preventing peak broadening in the chromatographic separation of drugs. The column maintained its efficiency without elevation of the back-pressure until 200 or more plasma samples had been directly injected, but was replaced routinely every 130–150 samples.

Together with the major metabolites of **I**, ω-hydroxylated and ω-carboxylated compounds, most endogeneous components were eluted from C3 before the heart-cut fractionation. However, additional washing of C3 with MP3 was necessary since the remaining plasma components interfered with the subsequent sample analysis.

Fig. 3 shows chromatograms of drug-free plasma, plasma spiked with **I** and a plasma sample from a clinical study. Compound **I** was eluted at 24–24.5 min. The signal cleaner was essential to produce low-noise background in chromatography. Without C1 and C2, unknown impurities contained in MP1 and MP2 were concentrated on C3 and eluted by MP4, giving a broad interference near the peak of interest.

The calibration graphs for **I** in human plasma were linear. A typical calibration graph is given by the equation $y = 133.3x + 143.4$ ($r = 0.9998$),

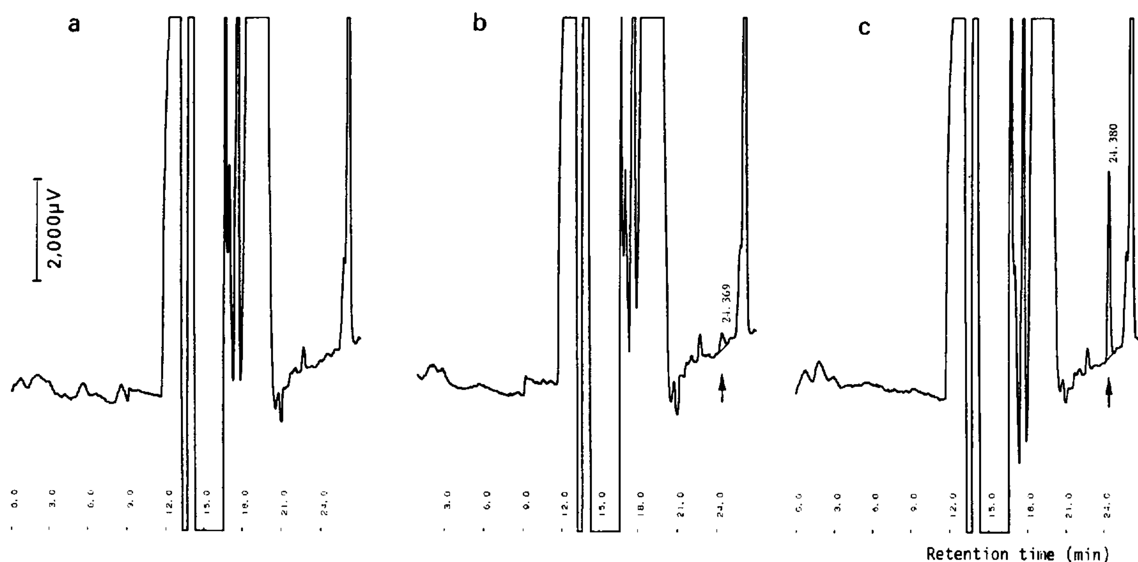

Fig. 3. Typical chromatograms of human plasma samples. (a) Drug-free plasma; (b) drug-free plasma spiked with 1 ng/ml **I**; (c) plasma collected from a volunteer 1 h after an oral dose with 5 mg of **I**.

Table 1
Within-day precision, accuracy and recovery for the assay of **I** in human plasma

Spiked concentration (ng/ml)	Measured concentration (mean ± S.D.) (ng/ml)	C.V. (%)	Accuracy[a] (%)	Recovery (%)
1.00	1.01 ± 0.0728	7.2	100.9	101.6
2.00	1.91 ± 0.134	7.0	95.3	110.3
5.00	5.06 ± 0.181	3.6	101.2	103.1
10.0	10.2 ± 0.122	1.2	102.1	109.5
30.0	30.1 ± 0.358	1.2	100.2	108.6
50.0	49.8 ± 1.14	2.3	99.5	106.9

[a] Accuracy = (measured plasma concentration/spiked plasma concentration) · 100.

where y is the peak height (μV), x is the drug concentration (ng/ml) and r is the correlation coefficient.

The on-line recovery of **I** from plasma was assessed using plasma samples spiked with concentrations of 1, 2, 5, 10, 30 and 50 ng/ml. The peak heights obtained after injection of the samples were compared with those produced by the same concentrations of **I** dissolved in ethylene glycol–water (20:80, v/v). The recovery for replicate analyses ($n = 5$) was high (101.6–110.3%) at the concentrations tested, as shown in Table 1, and was virtually independent of concentration.

Table 1 also shows the within-day precision, expressed by the coefficient of variation (C.V.), and the accuracy of the method. These were determined by replicate analyses ($n = 5$) of human plasma spiked with known amounts of **I** (1–50 ng/ml). All tested points had C.V.s of less than 7.2%, which was found at the lowest concentration, and accuracy between 95.3 and 102.1%, thus showing that the method provided good reproducibility without an internal standard.

The between-day precision was examined according to the following procedure. Spiked plasma samples with the same concentrations as above were analysed on three different days using a calibration graph for **I**. This was conducted to see if the number of samples to be measured could be maximized by minimizing the preparation of calibration graphs; direct injection shortens the lifetime of the pretreatment column. As shown in Table 2, however, the C.V.s for the between-day precision determined in this manner exceeded 10% at lower concentrations (20.1% at 1 ng/ml and 11.5% at 2 ng/ml). Hence the construction of a calibration graph was found to be necessary for every set of analyses of unknown samples.

In daily assays, the performance of the method

Table 2
Between-day variability for the assay of **I** in human plasma

Spiked concentration (ng/ml)	Measured concentration (ng/ml)				C.V. (%)
	Day 1	Day 2	Day 3	Mean ± S.D.	
1.00	0.828	1.21	1.20	1.08 ± 0.216	20.1
2.00	1.62	1.90	1.80	1.77 ± 0.142	11.5
5.00	5.04	5.29	5.52	5.28 ± 0.242	4.6
10.0	9.86	10.3	10.2	10.1 ± 0.242	2.4
30.0	29.9	30.2	29.8	30.0 ± 0.212	0.7
50.0	50.4	50.2	49.0	49.8 ± 0.726	1.5

was checked by the analysis of quality control (QC) samples, prepared by spiking drug-free plasma with **I** at low and high concentrations (5 and 30 ng/ml). The QC samples, stored under the same conditions as the clinical samples, were measured over a period of 1.5 months. The data in Table 3 show that the C.V.s were below 9.7%, indicating that the method was repeatable.

The drug concentrations in a few clinical samples exceeded the upper limit of the calibration graph. To these samples, an appropriate volume of a 1:1 mixture of drug-free plasma and the ethylene glycol–water mixture (40:60, v/v) was added, and the resultant sample was reassayed. Results of the assay and clinical pharmacokinetic study have been reported [8].

In summary, an automated direct-injection column-switching HPLC method was developed to avoid laborious off-line sample preparation.

Table 3
Between-day variability for the assay of quality control plasma samples spiked with **I**

Spiked concentration (ng/ml)	n[a]	Measured concentration (mean ±S.D.) (ng/ml)	C.V. (%)
5	26	5.55 ± 0.538	9.7
30	26	33.0 ± 2.33	7.1

[a] Measured over a period of 1.5 months.

Using absorption detection at 210 nm, the method had sufficient sensitivity with an LOQ of 1 ng/ml, where a 150-μl aliquot containing 75 μl of plasma was used as an assay sample. The method provided accurate analyses of more than 30 plasma samples per day without an internal standard. Hence this HPLC method offers the selectivity and sensitivity required for the determination of **I** in plasma samples in clinical studies.

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