ELSEVIER

Journal of Chromatography B, 719 (1998) 191–197

JOURNAL OF CHROMATOGRAPHY B

Determination of PNU 157706, a new dual inhibitor of 5α-reductase, in rat plasma by high-performance liquid chromatography with ultraviolet detection

G. Basileo, M. Breda*, C.A. James

Drug Metabolism Research, Pharmacia & Upjohn, Viale Pasteur 10, 20014 Nerviano, Milan, Italy

Received 11 May 1998; received in revised form 27 August 1998; accepted 28 August 1998

Abstract

A HPLC procedure was developed and validated for determining nanogram per milliliter concentrations of the dual 5α-reductase inhibitor PNU 157706 in rat plasma. The compound was extracted from plasma with diethyl ether followed by purification using a CN cartridge. The chromatographic separation was performed with a C_{18} column using a water–acetonitrile–methanol mixture as eluent. UV detection at 210 nm was used for the quantification of the compound over the concentration range 5–500 ng/ml plasma. The method has a lower limit of quantification of 5 ng/ml and good precision and accuracy. This method has performed well during analysis of several toxicokinetic and pharmacokinetic studies in the rat.

Keywords: PNU 157706; 5α-Reductase inhibitor

1. Introduction

5α-Reductase is a NADPH-dependent enzyme which catalyses the reduction of testosterone to the more potent androgen dihydrotestosterone [1]. PNU 157706 [*N*-(1,1,1,3,3,3-hexafluorophenylpropyl)-3-oxo-4-aza-5α-androst-1-ene-17β-carboxamide], (Fig. 1) is a novel dual inhibitor of 5α-reductase [2,3] which may be used for the treatment of benign prostate hyperplasia, prostatic cancer and male pattern baldness.

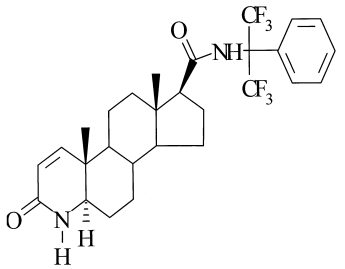

Fig. 1. Structural formula of PNU 157706.

In this study optimum conditions were developed for the determination of PNU 157706 in rat plasma by high-performance liquid chromatography (HPLC) with UV detection. The method was fully validated

*Corresponding author.

0378-4347/98/$19.00
PII: S0378-4347(98)00412-5

and used to analyze initial toxicokinetic and pharmacokinetic studies.

2. Materials and methods

2.1. Chemicals

PNU 157706 was synthesised at Pharmacia & Upjohn (Nerviano, Italy). All other chemicals and solvents were analytical grade from Carlo Erba Reagent (Milan, Italy).

2.2. Chromatographic equipment

The HPLC system used in this study consisted of a binary gradient pump (Model P2000, Thermo Separation Products (TSP), San José, CA, USA), an autosampler (Model 717 plus, Waters, Milford, MA, USA), a variable-wavelength UV detector (Model UV 975, Jasco, Hachioji, Japan) and a recorder-integrator (Model Chromject, TSP) connected to a Labnet network (TSP).

2.3. HPLC conditions

The chromatographic separation was performed with a 250×4 mm I.D. Nucleosil 120-3 C_{18} reversed-phase column (particle size 3 μm, Macherey-Nagel, Duren, Germany). The analysis was conducted under isocratic conditions, then a gradient step was employed to elute more highly retained compounds prior to the next injection. Two mobile phases were used: mobile phase A consisting of water–acetonitrile–methanol (34:41:25, v/v/v) and mobile phase B consisting of water–acetonitrile (20:80, v/v). At the start of the analysis mobile phase A was pumped for 25 min at a flow rate of 0.8 ml/min, then, over 2 min, the system was changed to 100% mobile phase B which was pumped for 9 min at a flow rate of 1 ml/min. The system was returned to 100% mobile phase A over 2 min at the flow rate of 0.8 ml/min, followed by an 8 min period of column stabilisation under the initial elution conditions. The total run time was 45 min. The UV detector was set at 210 nm and a 1 V signal was sent from the detector to the integrator.

2.4. Preparation of standards

A standard stock solution of PNU 157706 (strength 98.1%) was prepared by dissolving about 2 mg (exactly weighed) of the test compound in 50 ml of methanol. From this stock solution five working solutions were prepared weekly in a water–methanol mixture (80:20, v/v) to final concentrations of about 50, 150, 450, 1350 and 4500 ng/ml. Aliquots of these working standards (0.1 ml) were spiked into blank rat plasma (1 ml) and assayed to evaluate the linearity of the method. Five working solutions were prepared, as described above, to final concentrations of about 70, 700 and 3500 ng/ml for determination of the extraction recovery, precision and accuracy of the method and to concentrations of 200 and 2500 ng/ml for determination of the stability of the compound in plasma samples. When stored at +4°C, the stock and working solutions are stable for at least one month and one week, respectively.

2.5. Chromatographic performance

The suitability of the chromatographic system was checked before each series of analyses by evaluating the column efficiency, the peak symmetry and the reproducibility of the response. This evaluation was carried out according to USP [4] using the System Suitability Test software supplied by TSP. The column efficiency was expressed as the number of theoretical plates (N), the peak symmetry as the tailing factor (T), and the reproducibility as coefficient of variation (C.V.) of six replicated injections.

2.6. Analytical procedure

Plasma (1 ml) and 1 ml 1 *M* NaOH were placed in a glass stoppered test tube and extracted with 5 ml diethyl ether by vortex mixing for 1 min. After centrifugation at approximately 1200 *g* for 3 min, the organic phase was evaporated under a stream of N_2 at 37°C. The residue was redissolved with 0.2 ml ethyl acetate and 0.8 ml *n*-hexane, vortex mixed (1 min) and applied to a 100 mg Bakerbond-CN activated cartridge (Baker, Phillipsburg, NJ, USA). The cartridge, pre-conditioned with 2 ml methanol, 2 ml ethyl acetate and 2 ml *n*-hexane, was washed with 1 ml *n*-hexane–cyclohexane–chloroform (35:30:35,

v/v/v). The compound was eluted with 2 ml acetone. The extract was brought to dryness under N_2 at 37°C and the residue was dissolved with 0.25 ml of water–methanol (65:35, v/v). After vortex mixing (1 min) and centrifugation at approximately 1200 *g* for 1 min, 0.2 ml of the final solution was analysed by HPLC.

2.7. Evaluation of the extraction recovery

Blank rat plasma (1 ml) was spiked with three different concentrations of PNU 157706 (over the concentration range 7.15–363.2 ng/ml) in triplicate and assayed as described above. Peak areas obtained were compared to the peak areas obtained by direct injection of unextracted standards.

2.8. Evaluation of the lower limit of quantification (LLQ)

The LLQ was defined in terms of the lowest amount of the calibration curve that gave a precision (C.V.) and an accuracy better than 20%.

The LLQ at 5 ng/ml was assessed by analysis of three spiked plasma samples in one analytical run.

2.9. Evaluation of the stability

Three spiked samples/concentration/time were prepared by adding 0.1 ml of standard solutions, containing the compound of interest, to 1 ml of blank rat plasma. The samples were prepared in 10 ml polypropylene test tubes capped and labeled for identification, then stored at 37°C for 1 and 4 h and at −30°C for one and three months. For the evaluation of the effects of three freeze–thaw cycles on the analyte, each freeze cycle was performed by thawing at ambient temperature and refreezing at −30°C overnight. The plasma concentrations tested were about 20 ng/ml (about four times the LLQ) and 250 ng/ml.

2.10. Calculations

Calibration curves were obtained by plotting peak areas (y) against the analyte concentration in plasma (ng/ml) (x). Weighted linear regression (weighting factor $1/y$) was used to plot the line of best fit and consequently to calculate the PNU 157706 concentration in quality control (QC) and unknown samples. For the evaluation of PNU 157706 stability in rat plasma the response factor (RF=ratio between the area of the analyte and its concentration) obtained after the analysis of the stored samples was compared to the corresponding values measured in freshly prepared samples. The recovery was expressed as % amount found to added.

3. Results and discussion

Under the chromatographic conditions adopted for analysis, PNU 157706 gives a gaussian peak with retention time (t_R) of about 20 min. (Fig.2a). Due to the relatively poor selectivity of UV detection at 210 nm, a highly selective extraction procedure to isolate PNU 157706 from plasma was required. The effectiveness of both solid-phase (SPE) and liquid–liquid extraction (LLE) of PNU 157706 from plasma was carefully evaluated. Preliminary recovery data after SPE using different sorbents showed poor recovery of PNU 157706 from the plasma matrix. The most likely reason for loss of PNU 157706 recovery was protein binding as confirmed in a separate study [5]. Although protein precipitation is often used to overcome high protein binding prior the SPE, for this assay a LLE was adopted as the first step. This procedure gave high recovery of PNU 157706 in spite of the high protein binding, provided an initial clean-up of the sample, and transfered the analytes into non-polar solvent which was suitable for retention on a polar SPE cartridge.

LLE of plasma with diethyl ether under alkaline conditions reduced the extent of plasma interferences co-extracted with PNU 157706 compared with extraction under neutral or acid conditions. SPE using a CN sorbent proved the most selective among those assessed and, gave a quantitative recovery of PNU 157706. Overall, the complex extraction procedure developed for isolation of PNU 157706 from plasma, proved satisfactory in terms of reproducibility of the operation, percentage of the drug extracted and purity of the isolate from the subsequent chromatographic analysis. The mean extraction recovery from plasma evaluated at three different concentrations

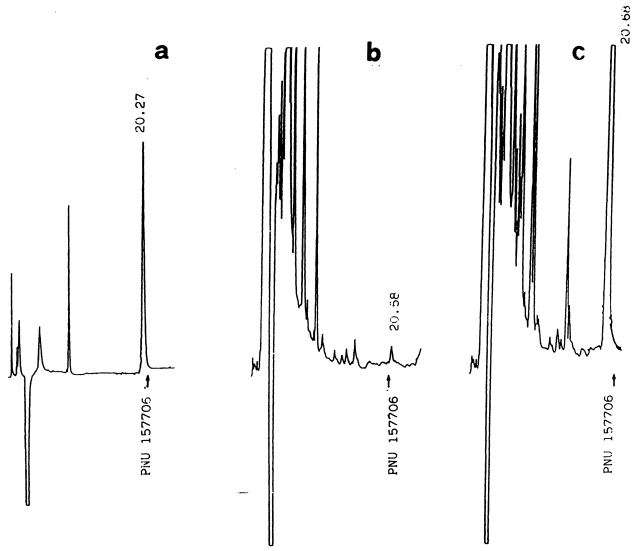

Fig. 2. Chromatograms obtained from a standard solution of PNU 157706 (a), from 1 ml of blank rat plasma spiked with 4.8 (b) and 454 ng/ml (c) of PNU 157706.

between 7.15 and 363.2 ng/ml ranged from 71.3 to 74.5% (Table 1).

A complication in the chromatographic analysis resulted from the presence of some late eluting peaks that were observed from the analysis of blank rat plasma. These peaks, attributable to endogenous components of the biological matrix, required long analysis times (about 90 min) to avoid peak overlapping during the sequential analysis of several biosamples. To reduce the analysis time a gradient procedure was introduced. A rapid change of mobile phase composition could be performed easily since no buffer was present in the two solvent mixtures (the presence of a buffer was of no value in the separation of PNU 157706). The effects of the change in the mobile phase composition were evaluated in terms of reproducibility and performance of the chromatographic separation (Table 2): the retention time of PNU 157706 showed very good reproducibility; tailing factor and theoretical plate number did not show appreciable variations. In addition, the life-span of the column proved acceptable since more than a thousand samples could generally be assayed using the same column.

Table 1
Evaluation of the extraction recovery of PNU 157706 from rat plasma

Concentration added (ng/ml)	Day	*n*	Recovery ±S.D. (%)	±C.V. (%)
7.15	1	3	75.5±7.5	10.0
7.15	2	3	69.2±4.4	6.4
7.15	3	3	71.6±7.1	9.9
Mean		9	72.1±6.3	8.7
68.1	1	3	70.9±3.6	5.1
68.1	2	3	77.1±1.1	1.4
68.1	3	3	65.8±0.8	1.2
Mean		9	71.3±5.3	7.4
363.2	1	3	73.9±8.4	11.4
363.2	2	3	85.5±0.6	0.8
363.2	3	3	64.0±6.9	10.7
Mean		9	74.5±10.8	14.5

The calibration was linear over the range of 5 to 500 ng/ml. The coefficient of linear regression (r) ranged from 0.9965 to 0.9999. The equation describing the mean calibration curve was $y=3132.02x-1390.39$ (slope C.V.=6%, $n=3$). Back-calculated values for the calibration standard points showed a C.V. ranging from 3.2 to 9.6%.

The precision and accuracy are summarised in Table 3. The intra-day precision (expressed as C.V.) ranged from 0.7 to 11.4%. The inter-day precision

Table 2
System suitability test parameters for the determination of PNU 157706

Amount injected (ng)	Retention time (min)	Area (mV·s)	Theoretical plate number (*N*)	Tailing factor (*T*)
45.4	19.37	242 899	23 354	1.18
45.4	19.40	248 679	23 519	1.16
45.4	19.36	236 758	23 797	1.15
45.4	19.30	244 852	23 277	1.19
45.4	19.27	244 970	23 390	1.20
45.4	19.30	243 867	23 556	1.19
Mean	19.34	243 632	23 467	1.18
S.D.	0.05	3914	186	0.02
C.V. (%)	0.3	1.6	0.8	1.6

evaluated at the same concentrations was better than 10.9%. The intra-day and the inter-day accuracy ranged from 94.3 to 111.7% and from 97.4 to 104.1%, respectively.

The instrumental limit of detection (LOD) was 2 ng while the LLQ for plasma samples was set at 5 ng/ml (Fig. 2b). At this concentration the intra-assay C.V. was 6.1% ($n=3$) and the mean±S.D. accuracy was 111.2±6.8% with a signal-to-noise ratio higher than 5.

No interfering peaks with PNU 157706 were found in reagent blanks and in extracts of rat plasma, from a separate sources. Pooled rat (male) plasma used for the calibration curve and for quality control samples was interference free. In addition, monkey and human plasma also were free of interferences, indicating the assay would be suitable for use with samples from these species.

The results of PNU 157706 stability in rat plasma are reported in Table 4. Mean response values for the stored samples were within +20% of responses for fresh samples, under all storage conditions. It can be concluded that PNU 157706 in rat plasma can be stored at 37°C for up to 4 h and at −30°C for up to three months prior to assay. In addition, no effects on the plasma concentrations of the compound were evident after three freeze–thaw cycles. These stability data were also confirmed in monkey and human plasma.

The method described here was employed for the determination of PNU 157706 in plasma samples in male rats after single and repeated (day 14) oral

Table 3
Accuracy and precision of the method for the determination of PNU 157706 in rat plasma

QC sample concentration (ng/ml)	Day	*n*	Accuracy			Precision	
			Mean found (ng/ml)	% Recovery (intra-day)	% Recovery (inter-day)	C.V. (%) (intra-day)	Pooled C.V. (%) (inter-day)
7.15	1	3	7.68	107.4		±9.1	
	2	3	7.29	101.9		±5.7	
	3	3	7.37	103.0	104.1	±10.2	±7.8
68.10	1	3	63.49	94.3		±5.1	
	2	3	69.39	101.2		±1.4	
	3	3	66.16	97.7	97.4	±1.2	±4.7
363.20	1	3	350.24	96.4		±11.4	
	2	3	405.71	111.7		±0.7	
	3	3	343.69	94.6	100.9	±10.7	±10.9

Table 4
Stability of PNU 157706 at two different concentrations in rat plasma

Storage	Time	20 ng/ml recovery found/added (%) mean±S.D. (n=3)	250 ng/ml recovery found/added (%) mean±S.D. (n=3)
37°C	1 h	104.4±6.5	107.8±3.4
	4 h	99.5±4.3	106.3±2.3
−30°C	1 month	96.4±4.4	100.9±2.0
	3 months	99.8±7.0	90.7±15.1
Freeze–thaw	3 cycles	108.3±2.3	98.6±3.7

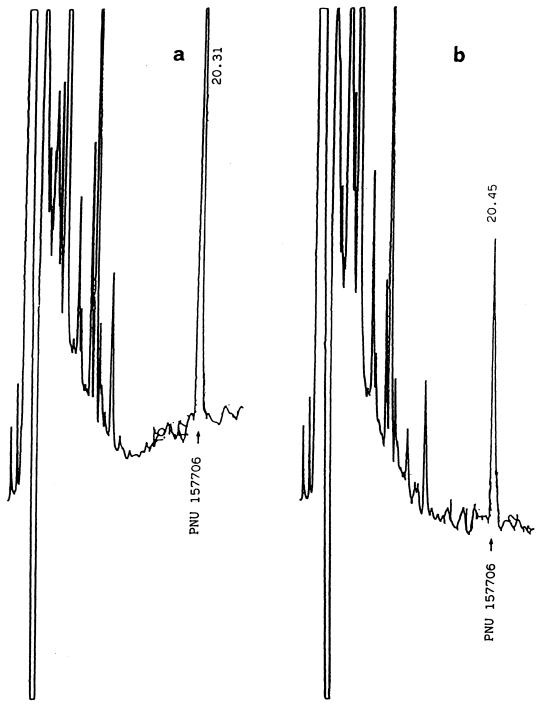

Fig. 3. Chromatograms of plasma obtained from male rats 8 h after one day (a) and 1 h after 14 days (b) of oral treatment with 3 mg/kg/day of PNU 157706.

administrations of 3, 30 and 300 mg/kg/day of PNU 157706. Typical chromatograms obtained from male rats treated with an oral dose of 3 mg/kg/day are shown in Fig. 3.

4. Conclusions

The method described here is selective for the determination of PNU 157706 in rat plasma. It is

linear, precise and capable of accurately determining this drug in the 5–500 ng/ml concentration range. The procedure is well suited to measuring plasma concentrations of PNU 157706 in toxicokinetic and pharmacokinetic studies in the rat.

References

[1] J.D. Wilson, in: R.O. Greep, E.B. Astwood (Eds.), Handbook of Physiology 5, American Physiology Society, Washington, DC, 1975, p. 491.

[2] E. Di Salle, D. Giudici, A. Radice, T. Zaccheo, G. Ornati, M. Nesi, A. Panzeri, S. Delos, P.M. Martin, 13th International Symposium of the Journal of Steroid Biochemistry and Molecular Biology, Monaco, May 1997.

[3] T. Zaccheo, D. Giudici, R. Vivaldi, E. Di Salle, 13th International Symposium of the Journal of Steroid Biochemistry and Molecular Biology, Monaco, May 1997.

[4] US Pharmacopoeia XXIII, US Pharmacopoeial Corporation, Rockville, MD, USA, 1995, p. 1774.

[5] M.G. Castelli, personal communication.