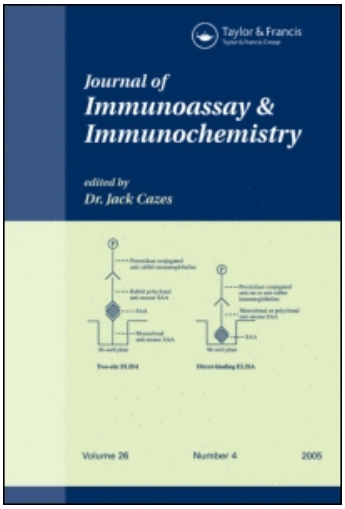

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RADIOIMMUNOASSAY FOR THE TESTOSTERONE 5α-REDUCTASE INHIBITOR TUROSTERIDE IN BIOLOGICAL FLUIDS

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ABSTRACT

An antiserum against turosteride (code name FCE 26073), a potent testosterone 5α-reductase inhibitor, has been raised in rabbits by immunization with an immunogen produced by conjugation of a derivative of FCE 26073 (FCE 27424) to bovine serum albumin. The antiserum was able to distinguish FCE 26073 from its derivatives modified at the 17β position and from all the endogenous steroids tested.

A radioimmunoassay for the determination of FCE 26073 in human plasma and urine was developed using this antiserum and tritium labeled turosteride. FCE 26073 was extracted from 50 μl of plasma or 25 μl of urine using ethyl-ether with a recovery greater than 90%.

Using this procedure it was possible to achieve a final limit of quantitation of 142 pg/ml in plasma and 284 pg/ml in urine.

The assay was validated in terms of reproducibility, accuracy and precision in the range 3.9 - 250 pg/50 μl of plasma and 25 μl of urine.

The plasma concentration of FCE 26073 in a healthy male volunteer who received 0.2 mg of the drug was measured using the radioimmunoassay.

(KEY WORDS: Turosteride, Radioimmunoassay, Testosterone, 5α-reductase inhibitors, Steroids)

INTRODUCTION

Turosteride (1-[4-methyl-3-oxo-4-aza-5α-androstane-17β-carbonyl]-1,3-diisopropylurea), code name FCE 26073, is a potent testosterone 5α-reductase inhibitor

active on both human and rat prostate enzymes (1). Inhibition of 5α-reductase provides a pharmacological tool for the treatment of benign prostatic hyperplasia (2) as well as for other conditions such as acne, female hirsutism and male pattern baldness (3-5). A compound of this class, finasteride, has been recently placed on the market for the treatment of benign prostatic hyperplasia (6).

FCE 26073 is highly specific since it interferes with the rat androgen receptor only at a concentration approximately 200-fold that inhibiting rat prostate 5α-reductase. Therefore turosteride's potent inhibition of testosterone propionate induced prostate hypertrophy in rats is more likely to be due to inhibition of 5α-reductase than to antagonism at the androgen receptor levels (1).

In the search for an analytical method for monitoring plasma levels of FCE 26073 in clinical trials, and in the absence of information about the therapeutic dosage, an HPLC method might not have sufficient sensitivity. In fact FCE 26073 has a low UV absorption and its lack of chemical reactivity does not allow derivatization or oxidation at the low potentials necessary for electrochemical detection. Therefore we chose to develop a radioimmunoassay since this procedure is highly sensitive.

In this paper we describe the development of the radioimmunoassay for FCE 26073 and the results obtained during its validation.

EXPERIMENTAL

Substances

Bovine serum albumin fraction V (BSA), N-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCC) and trinitrobenzensulfonic acid were from Sigma

Chemical Co. St. Louis, USA; Freund's complete and incomplete adjuvants were from Difco Detroit, USA; dextran T10 and T70 were from Pharmacia Uppsala, Sweden.

FCE 26073, FCE 27425, FCE 26729, FCE 27951, FCE 27244, FCE 27853, FCE 27854, FCE 27855, FCE 27856, FCE 26287 and FCE 27424 were from Farmitalia Carlo Erba, Chemical Department (see Fig. 4 for structural formulas of FCE 26073 and its related compounds).

Cholesterol, pregnenolone, progesterone, dehydroepiandrosterone, androstenedione, androsterone and testosterone were from Sigma Chemical Co., St. Louis, U.S.A. Charcoal Norit A was from Fisher Scientific Fair Lawn, USA.

All other chemicals were Farmitalia Carlo Erba analytical grade.

The 0.1 M phosphate buffer, pH 7.2, was obtained as follow: 13.61 g KH_2PO_4, 30 mg dextran T-10, 9 g NaCl, 1 g gelatine and 1 g NaN_3 were dissolved in 1 liter of bidistilled water and the pH was adjusted to 7.2 with 20% NaOH.

The charcoal suspension was prepared from 2 g charcoal and 20 mg dextran T-70 dissolved in 100 ml of 0.1 M phosphate buffer, pH 7.2.

Animals

Five New Zealand male rabbits weighing about 2 kg and aged two months at the beginning of the study were supplied by Charles River, Italy.

Preparation of the immunogen

The immunogen preparation scheme is shown in Fig.1. FCE 27424 (0.1 mmol) was dissolved in 5 ml of dry dioxane. To this solution 0.1 mmol of N-hydroxysuccinimide (NHS) and 0.1 mmol mg of dicyclohexylcarbodiimide (DCC) were added and the reaction was carried out for 5 h at 4°C under stirring and then for 15 h

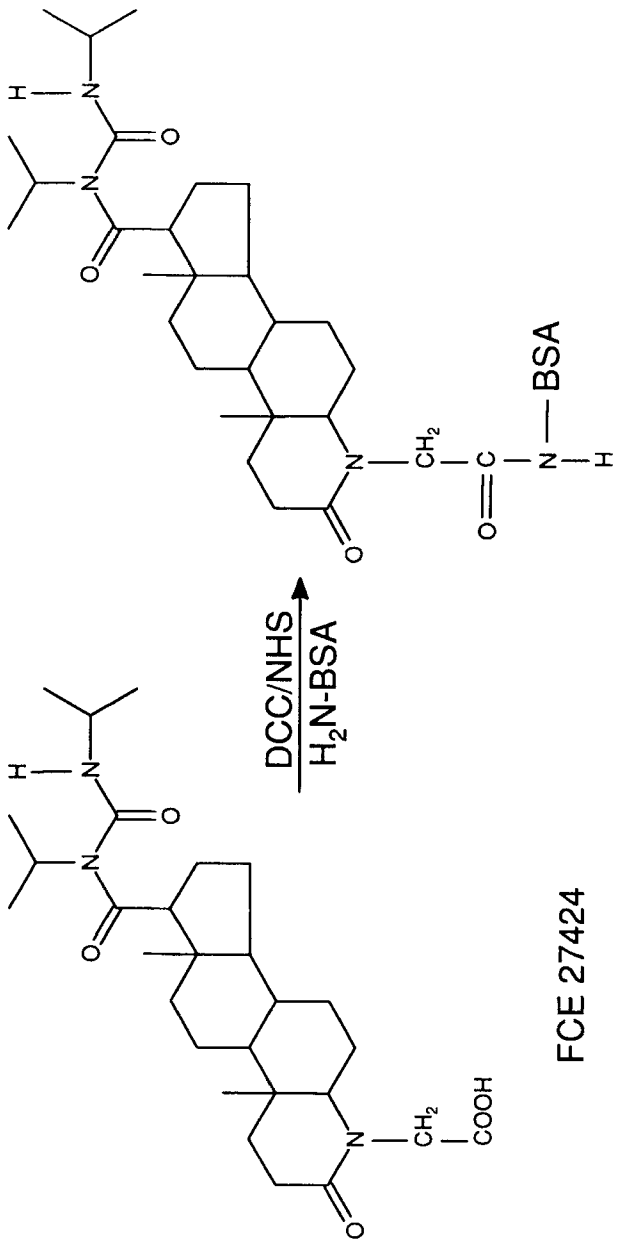

Figure 1: Synthesis of the immunogen (NHS), N-hydroxy Succinimide; DCC, Dicyclohexylcarbodiimide.

at room temperature. After separation of the white precipitate of dicyclohexylurea by paper filtration, the solution containing the activated ester of FCE 27424 was slowly added to 10 ml of 0.1 M phosphate buffer, pH 8.8, containing 0.09 g of NaCl and 140 mg of BSA. The reaction was carried out for 3 h at 4°C under stirring, maintaining the pH at about 8 with NaOH, then for 16 h at room temperature. To remove the free hapten not covalently bound to the carrier protein, the reaction mixture was then dialysed for three days against 2 l of distilled water which was replaced every 24 h. The number of molecules of FCE 27424 bound to BSA was calculated by determining the free amino groups still present on the BSA molecule after the conjugation procedure using the method described by Habeeb (7).

We measured about 23 free amino groups per mol of conjugate, and since there are about 61 amino groups on the BSA molecule (7) the number of molecules of FCE 27424 conjugated to BSA was 38.

Immunisation

The lyophilized immunogen (30 mg) was dissolved in 6 ml of saline buffer, the solution was divided into 0.7 ml aliquots and stored at -20°C. At the immunization time a 0.7 ml aliquot of the frozen immunogen solution was thawed, homogenized with 2.8 ml of saline and 3.5 ml of Freund's complete adjuvant and administered to five rabbits. Each animal received about 0.5 mg of the immunogen in 1 ml of the final solution injected intradermaly and subcutaneously at 20 different dorsal sites.

Two booster injections of the immunogen, emulsified with Freund's incomplete adjuvant, were given after 1 and 5 months and the blood was collected ten days after the last booster from the central ear vein of the animals. The blood was allowed to clot

at 37°C and centrifuged at 1100 g for 15 min to separate serum. The antiserum divided into small fractions was frozen at -20°C untill used in the RIA.

Labeled hapten

FCE 26287 was catalytically reduced with tritium gas at the Radiochemical Center, Amersham, U.K. The radiolabeled hapten, [1,2(n)-^{3}H] FCE 26073, was obtained with a specific activity of 48 Ci/mmol and a radiochemical purity of 99.3% measured by HPLC (Fig. 2).

The compound was stored in phosphate buffer pH 7.2 at -20°C in the dark and in these conditions was stable for at least 3 months.

Assay procedure

To obtain a standard curve, samples of FCE 26073 in duplicate at concentrations ranging from 3.9 to 250 pg/50 μl of plasma or 25 μl of urine were prepared. FCE 26073 from each sample was extracted with 1.5 ml of diethyl-ether. The tubes were vortexed for 2 min, centrifuged at 1200 g for 5 min and stored at -20°C in the dark. After 2 h the organic supernatant containing FCE 26073 was separated from the frozen aqueous fraction and poured into separate tubes. The organic extracts were evaporated to dryness under a nitrogen gas stream and reconstituted in 0.1 ml of phosphate buffer, pH 7.2. The recovery of this extraction procedure, calculated using tritium labeled FCE 26073, was identical in plasma and urine and was $93 \pm 3.7\%$ (n=20). To each reconstituted extract 0.1 ml of radiolabeled hapten (about 6000 dpm) and 0.1 ml of antiserum diluted in phosphate buffer were added and the final volume was adjusted to 550 μl with phosphate buffer. The tubes were vortexed and left to incubate for 16 h at 4°C in the dark. At the end of the incubation period 0.1 ml of charcoal suspension was

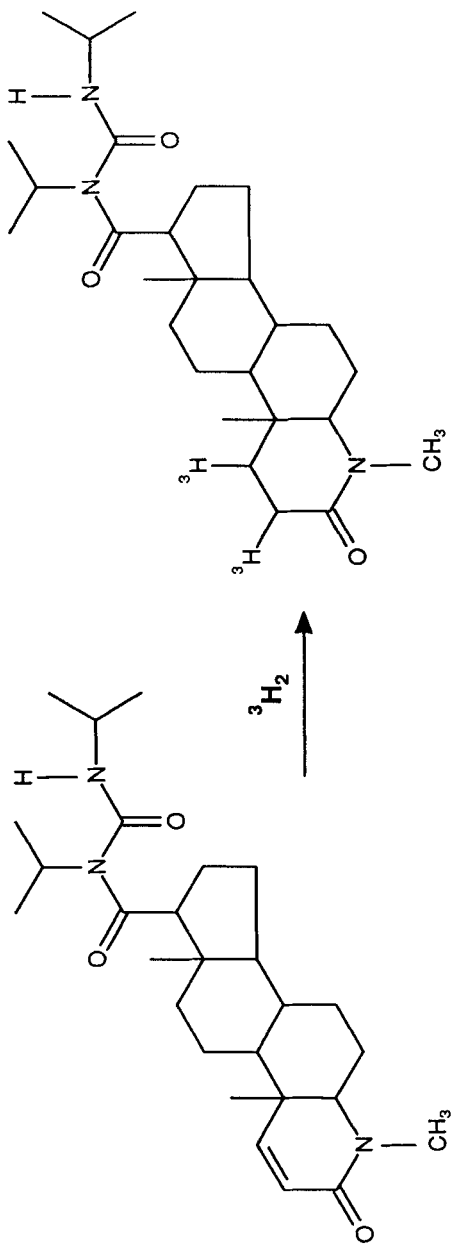

Figure 2: Synthesis of the radiolabeled FCE 26073.

added to each tube. The tubes were vortexed, left at 4°C for 15 min, and centrifuged at 1200 g for 20 min at 4°C. A 0.5 ml aliquot of the supernatant of each tube, containing the labeled hapten bound to the antiserum, was counted in a liquid scintillation counter for 10 min. The non-specific binding (NSB) was measured by incubating the radiolabeled hapten with 0.1 ml of reconstituted plasma or urine extract with no antiserum. The extraction of FCE 26073 from unknown samples was performed as described above on two plasma samples (50 μl each) or on two urine samples (25 μl each).

Validation of the method and data analysis

The antibody bound fraction was expressed as percentage B/B_0, the amount bound (B) relative to amount bound in the absence of analyte (Bo). The logit of the percentage bound was plotted against the log of FCE 26073 concentrations and computer-fitted to a straight line. Quality control and unknown sample concentrations were calculated from the standard curve obtained in presence of the corresponding biological fluid extract. To determine the precision and accuracy of the assay in plasma and urine, three quality control samples containing FCE 26073 at concentrations of 7.1, 25 and 130 pg/50 μl of plasma and 7.1, 25 and 110 pg/25 μl of urine (obtained by spiking blank plasma and urine samples with a standard solution of FCE 26073) were analyzed in quadruplicate on six different days over a one-month period. The coefficient of variation (C.V.) of the measurements was used as index of precision. The percentage of recovered FCE 26073 relative to the amount of FCE 26073 added was used as an index of accuracy.

The sensitivity limit of the assay in plasma and urine was defined as the concentration obtained from the standard curve at three times the S.D. of the Bo value.

The affinity constant of the antiserum for FCE 26073 was measured using the Scatchard analysis (8): briefly, samples containing different amounts of labeled hapten were incubated, in buffer, with the antiserum at a constant dilution under the conditions described in the paragraph "assay procedure". For each sample the fractions of total radioactivity added, specifically bound to the antiserum (B) and free (F) were measured. The ratio B/F was plotted against B expressed as mol/l calculated according to the specific activity of the labeled hapten (48 Ci/mmol). The slope of the straight line, obtained by least-square fitting of the points, was the affinity constant of the antiserum for the radiolabeled hapten.

Cross-reactivity

The ability of several FCE 26073 analogs (see Fig.4 for structural formulas of FCE 26073 and its analogs) to compete with the tritium labeled FCE 26073 for antibody binding sites was tested in buffer, pH 7.2, without extraction. We also tested the cross-reactivity of the antiserum with cholesterol, pregnenolone, progesterone, dehydroepiandrosterone, androstenedione, androsterone and testosterone. The cross-reaction of the antiserum with a given compound was defined as the amount of FCE 26073 causing 50% binding expressed as a percentage of the amount of compound which produced the same binding.

Plasma samples

Blood samples were obtained from a 21-year-old healthy male volunteer weighing 60.5 kg, treated with a single oral dose of 0.2 mg of FCE 26073 at the Institute Aster, Hôpital Cognacq-Jay, Paris, France. The institutional Ethics Committee approved the study and the volunteer gave written informed consent.

Blood samples (10 ml) were collected in heparinized tubes immediately before treatment and 1, 2, 4, 8, 12, 24 and 48 h after drug intake. The collected blood samples were immediately centrifuged at 1550 g for 10 min and the plasma was divided into two plastic tubes and stored at -20°C untill assayed.

RESULTS

Three out of five rabbits raised antibodies against FCE 26073 but only one with a good titer. This rabbit produced an antiserum (code number C23-2) that, diluted 1:10.000, bound about 30% of the labeled hapten added (about 0.06 pmol) in the absence of FCE 26073 and was used to develop the radioimmunoassay. A linear relationship between logit B/Bo% and log of the amount of FCE 26073 added was found in the range 3.9-250 pg/tube.

Fig. 3 shows the mean standard curves obtained by averaging the results obtained on 6 different days over a one-month period, in presence of 50 μl of extracted plasma (A) or 25 μl of extracted urine (B). Non-specific displacement of the tracer by plasma and urine constituents was assayed by extracting increasing amounts of blank human plasma or urine (pool of three subjects) in the assay. Up to 50 μl of plasma/tube could be extracted without any significant change of the Bo or NSB value. In the presence of human plasma extract the mean values obtained for Bo and NSB were 33% and 2.4% respectively. The mean curve was defined by the equation: logit B/Bo% = -2.52 log C (pg/50 μl) +4.2 (r was $>$ 0.99 in all cases).

Under equilibrium assay conditions, Scatchard analysis gave an affinity constant of 6.5×10^{10} l/mol.

The sensitivity limit of the assay in plasma, defined as the concentration obtained from the standard curve at three times the S.D. of the Bo value, was

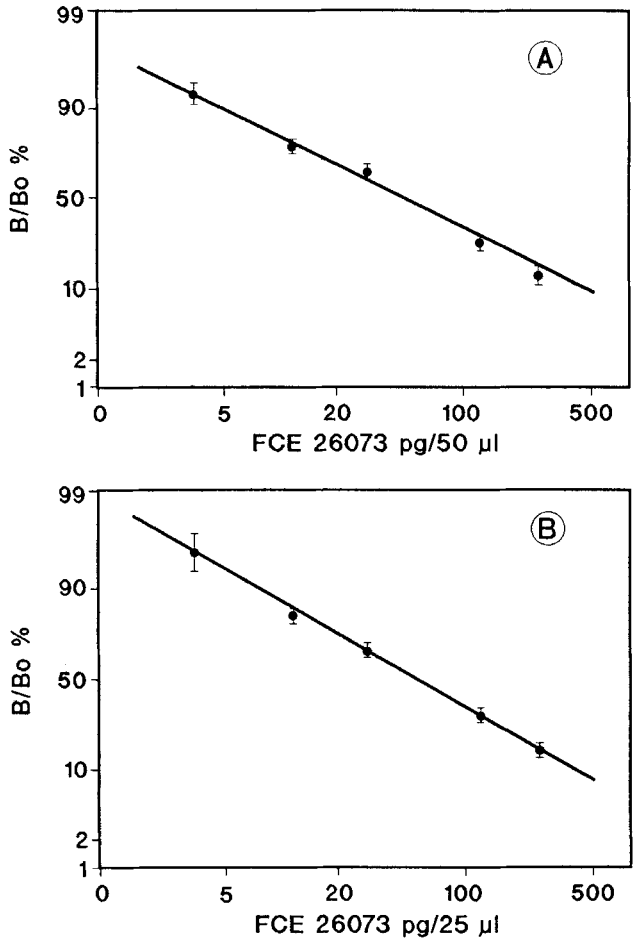

Figure 3: Mean standard curve obtained averaging six standard curves from six different days after extraction of 50 µl of human plasma (A) or 25 µl of human urine (B).

approximately 40 pg/ml whereas the limit of quantitation was 142 pg/ml (minimum measurable concentration with a coefficient of variation below 20%).

Intra- and inter-day precision expressed as C.V. ranged from 6.6% to 10.6% and from 7.1% to 17.4%, respectively (Table 1). Recovery ranged from 90.8% to 96.6% (Table 1).

Up to 25 μl of blank human urine per tube could be extracted without any significant change in Bo or NSB. Intra- and inter-day precision ranged from 3.3% to 12.3% and from 10.3% to 16.3%, respectively (Table 1). Recovery ranged from 90.9% to 96.5%, respectively (Table 1). The sensitivity limit of the assay in urine was 170 pg/ml whereas the limit of quantitation was 284 pg/ml.

The cross-reaction of the antiserum with the FCE 26073 related compounds tested is shown in Table 2. FCE 27244 and FCE 26729 gave the highest cross-reaction, 109% and 76% respectively, whereas no other FCE 26073 analogs cross-reacted with the antiserum C23-2. None of the endogenous steroids tested cross-reacted with our antiserum, the cross-reaction percentage always being below $2 \cdot 10^{-3}$%.

Plasma concentrations of FCE 26073 in a young healthy male volunteer who received the drug at the single oral dose of 0.2 mg are shown in Fig. 5. FCE 26073 was absorbed rapidly with the peak plasma concentration occurring 1 h after intake. The drug was detected in plasma up to the last time of blood collection.

DISCUSSION

This paper reports the production of an antiserum against FCE 26073. The antiserum was employed to develop a sensitive, convenient, and specific radioimmunoassay for the determination of FCE 26073 in human plasma.

Table 1

Accuracy and precision of the RIA method for the determination of FCE 26073 in plasma and urine

PLASMA				
Amount added (pg/50 μl)	Amount found (pg/50 μl)	% Found/added	Inter-assay CV %	Intra-assay CV %
7.1 (n=24)	6.6	92.9	17.4	10.6 (n=4)
25 (n=24)	22.7	90.8	8.2	3.0 (n=4)
130 (n=24)	125.6	96.6	7.1	6.6 (n=4)
URINE				
Amount added (pg/25 μl)	Amount found (pg/25 μl)	% Found/added	Inter-assay CV %	Intra-assay CV %
7.1 (n=20)	6.5	90.9	16.3	12.3 (n=4)
25 (n=24)	24.1	96.5	14.7	4.3 (n=4)
110 (n=20)	104.1	94.6	10.3	3.3 (n=4)

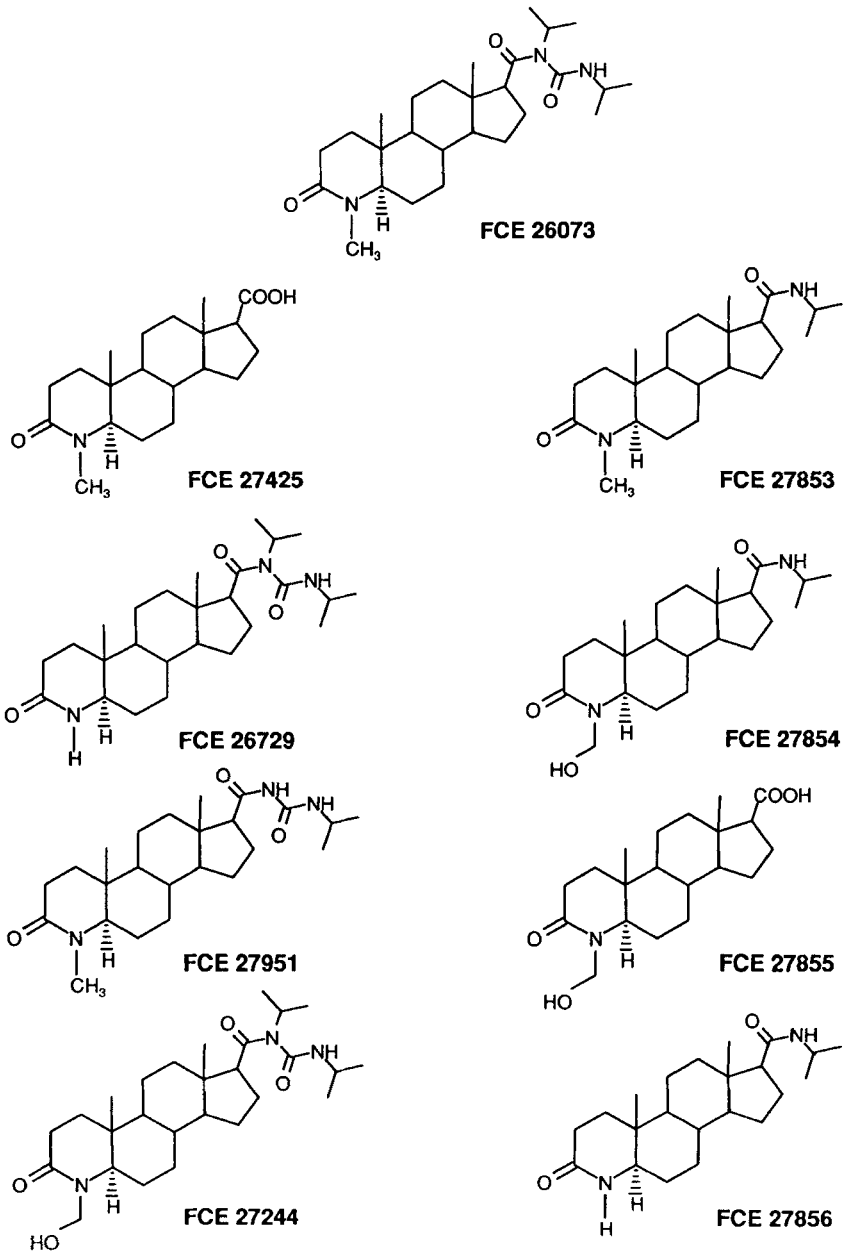

Figure 4: Structural formulas of FCE 26073 and its analogs tested for cross-reactivity with the antiserum C 23-2.

Table 2

Cross-reactivity of some FCE 26073 related compounds with the C23-2 antiserum

Compound	% Cross-reaction
FCE 26073	100
FCE 27244	109
FCE 26729	76
FCE 27853	0.04
FCE 27854	0.01
FCE 27856	$7.6 \cdot 10^{-3}$
FCE 27855	$3.6 \cdot 10^{-3}$
FCE 27425	$< 8 \cdot 10^{-4}$
FCE 27951	$< 8 \cdot 10^{-4}$

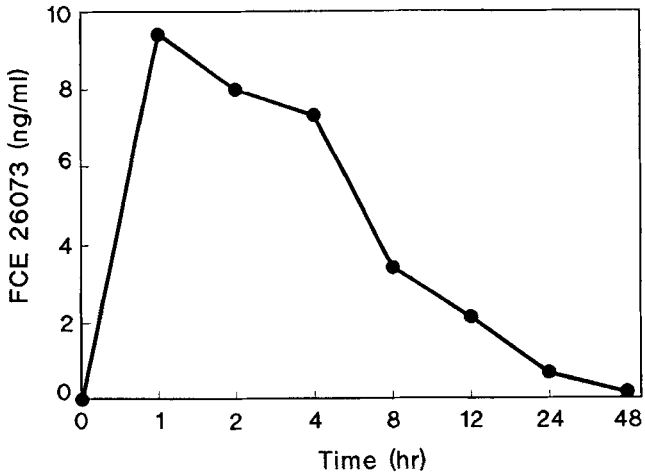

Figure 5: Plasma levels of FCE 26073 in a healthy male volunteer receiving the drug at a single oral dose of 0.2 mg.

Costanzer et al. reported the development of an HPLC method for the determination of another 5α-reductase inhibitor, finasteride, in human plasma with a limit of quantitation of 1 ng/ml (9). The radioimmunoassay described here is about 25 times more sensitive and allows the analysis of 200 plasma samples per day compared to 50 for the HPLC method.

The results of cross-reaction experiments show that modification on the 17β side chain of FCE 26073 resulted in almost complete loss of immunoreactivity. On the contrary alterations on the methyl group on the nitrogen in position 4 of FCE 26073 (FCE 27244 and FCE 26729) were not recognized by the antiserum (Fig. 4 and Table 2). These findings were expected since the immunogen was prepared by conjugation of FCE 27424 to BSA, through its carboxylic group. As a result of this conjugation, the 17β side chain was exposed for immunological recognition. This also explains the excellent specificity of the antiserum vs the principal endogenous steroids since they all lack the 17β side chain.

At the present time no information is available regarding the metabolic pattern of FCE 26073 in humans. If FCE 27244 and/or FCE 26729 are found in relevant amounts among FCE 26073 plasma or urinary metabolites, then a chromatographic purification procedure, already available in our laboratory (10), will be performed on the samples to assure the specificity of the determinations.

The radioimmunoassay described was used to measure the plasma levels of FCE 26073 in a healthy male volunteer who received 0.2 mg of FCE 26073 as a single oral dose. The drug appeared to be absorbed rapidly since the maximum plasma concentration occurred within 1 h after dosing. Even at a dose level of 0.2 mg, the

lowest currently being investigated in clinical trials, this radioimmunoassay was able to measure the plasma levels of FCE 26073 up to the last collection time 48 h after dosing.

In conclusion the radioimmunoassay here described should provide a highly sensitive analytical method for the determination of FCE 26073 in biological fluids.

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