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A Non-Radioactive FPLC Method for Measuring Steroid 5α-Reductase Activity in Cultured Cells

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Abstract

In this paper we describe a non-radioactive assay for a tota ofl 5α - reductase activity in cultured human skin fibroblasts. Androstenedione and dihydrotestosterone were detected by FPLC chromatography as the metabolites of testosterone in these cells. The values of the kinetic constants K_M: 1.39 x10^{-6} M and v_{max} : 0.51 x 10^{-9} mols dihydrotestosterone /mg protein/min are in agreement with those obtained by more sophisticated radioactive assays, suggesting that this method could be used for measuring 5α-reductase activity.

Keywords: steroid 5α-reductase, dihydrotestosterone

Introduction

The steroid 5α-reductase (3-oxo-5alpha-steroid 4-dehydrogenase, E.C. 1.3.99.5) is a NADPH dependent enzyme that catalyses the conversion of a variety of substrates with a 3-oxo-$\Delta^{4,5}$ structure in the steroid A ring into 5α reduced compounds. From this reduced steroids, 17-β-hydroxy-5α-androstan-3-one (dihydrotestosterone, DHT) is of particular interest being a more potent androgen hormone than testosterone. The enzyme occurs in two isoforms: type I, composed of 259 amino acids and with an optimal pH of 6-9 and type II composed of 254 amino acids and with an optimal pH around 5,5. The genes encoding type I and type II isozymes are found in chromosomes 5p and 2p, respectively, and each consists of 5 exons and 4 introns (Russell and Wilson, 1994).

Testosterone and DHT exerts their genomic action after binding to the androgen receptor, member of the nuclear receptors family. The activated steroid-receptor complex enters into the nucleus, recognizes specific DNA sequences (SRE – steroid response elements), recruits the basal transcription machinery and initiates the transcription of regulated genes.

There are several androgen – dependent disorders determined by an increased activity of 5α -reductase, reflected into an exces of DHT: hirsutism (Mestayer *et al*., 1996), polychystic ovary syndrome (Jakimiuk *et al*., 1999), male pattern baldness, benign prostatic hyperplasia (Thigpen *et al*., 1992). Drugs capable of inhibition of one or both isoforms (finasteride, epristeride, MK-386) are used in the treatment of these disorders (Chen et al., 1996). Male pseudohermaphroditism is, by contrary, characterised by a partial or total loss of 5α -reductase tipe II activity. Deletions or point mutation in the corresponding gene are

responsible for altered activity and abnormal differentiation of external genitalia occurences, caused by insufficient DHT during fetal life (Russell and Wilson, 1994).

In dermal fibroblasts, testosterone is metabolized mainly to 5α - dihydrotestosterone (an irevesible reaction *in vitro* and *in vivo*) and to a lesser content to androstenedione (a partial revesibile reaction catalized by 17β - hydroxy steroid dehydrogenase). DHT is further metabolized to 3α- and 3β - androstanediols by 3α and respectivly 3β - hydroxy steroid dehydrogenases (NADPH dependent enzymes) (Maudeldone et al., 1986; Wilson, 1975).

Several methods were developed for measuring 5α-reductase activities. Tritiated testosterone is incubated with tissue homogenates or with cultured cells and the radiolabeled steroid metabolites are separated by thin layer chromatography or reverse phase chromatography (Maudeldonde et al., 1986; Tamura et al., 1996). Radioactive assays are preferred because the low activity of the enzyme, near the limit of detection of classic spectrophotometric or spectrofluorimetric NADPH monitoring techniques. Here we describe a nonradioactive assay for measuring 5α - reductase activities in cultured cells by a high-resolution chromatography separation of testosterone metabolites and their detection on line, in UV light (214nm).

Materials and Methods

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine, tripsine, antibiotics (amphotericin, penicillin, streptomycin) were from Sigma. Standard steroids: testosterone, androstenedione, DHT and dexamethasone were from Fluka. HPLC grade acetonitril and ethyl acetate were purchased from Merck.

Skin fibroblast culture

Primary culture fibroblasts were obtained from pubic dermal biopsies from patients with polichystic ovary sindrome, presented at "C.I. Parhon" Hospital, Bucharest (Mowszowicz et al., 1980). Cells were grown in DMEM suplemented with 10% FBS, antibiotics (50U/ ml penicillin, 50 μg/ml streptomycin, 0.5μg/ml amphotericin), L-glutamin (2 mM) and sodium bicarbonate (22%) at 37^{o} C in a humidified atmosphere with 5% CO_2.

Enzymatic assay for 5α-reductase

When cells arrived at confluency (1-2 x 10 ^{6} cells /plate) they were serum depleted for 24 h. After that, they were incubated with testosterone (0.5-10μM) in 10 ml of serum free medium, at 37^{o} C. The reactions were stopped on ice, at various times (0, 15, 30, 45, 60 minutes). Dexamethasone (5μM), a testosterone structural analog, was added as a recovery standard. Steroids were extracted from the culture medium with ethyl acetate (Baltes and Hanocq, 1998), followed by vacuum evaporation of the organic phase. The dry sec was dissolved in a minimum volume of 100% acetonitrile. Samples diluted in 10% acetonitrile were subjected to reverse phase chromatography on an AKTA FPLC system (Amersham Pharmacia Biotech): PepRPC HR 5/5 porous silica C_2/C_{18} column, 25 – 50% acetonitrile in water continuous gradient, 1.2ml/min flow rate and UV 214 nm detection. Separate samples containing fixed amounts of steroids (0.5-10 μM) dissolved in medium without cells were assayed for determining the recovery yield for each steroid. The AKTA FPLC Unicorn software, using authentic steroids standards, did automatic quantification of testosterone metabolites. Cells treated only with dexamethasone were used as blank.

Protein content was assayed by a modification of Lowry method (Shopsis and Mackay, 1980).

Results and Discussions

Non-metabolized testosterone, androstenedione and DHT were separated and detected by FPLC in the cell culture medium (**Figure 1**).

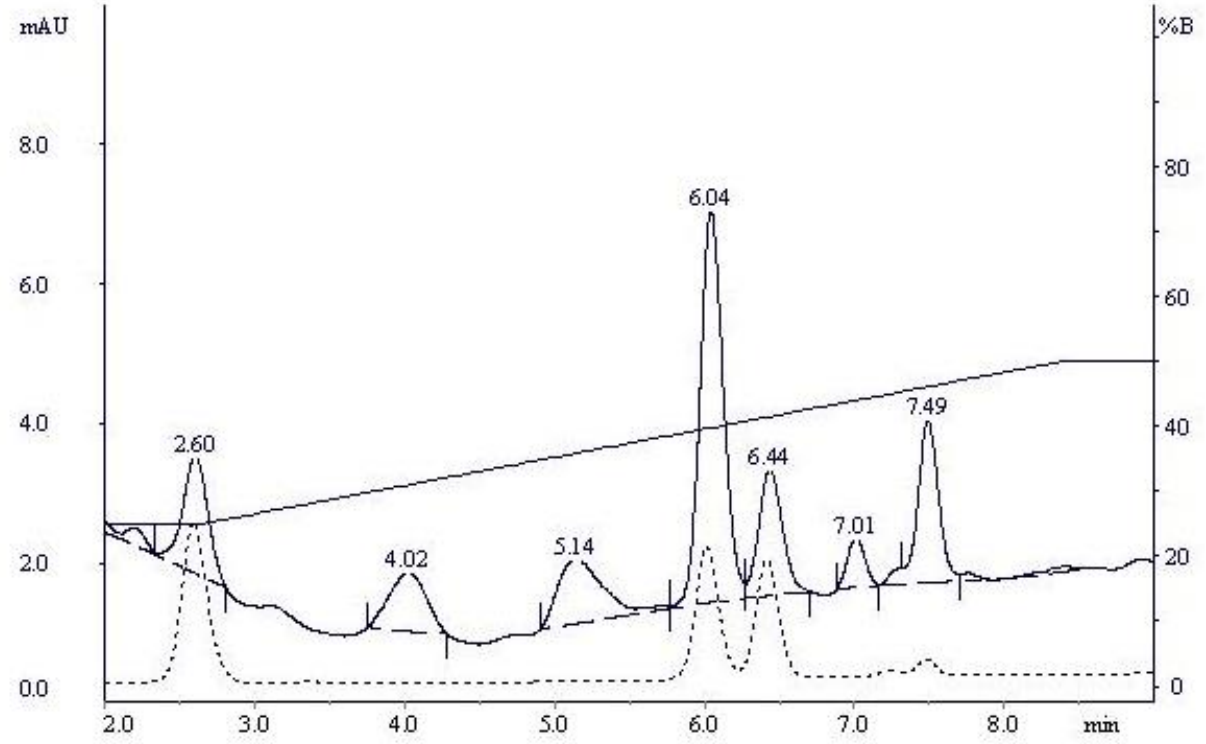

Figure 1. Chromatographic profile of testosterone metabolites separated from skin fibroblast culture medium
Testosterone 2μM, 30 min incubation, 37 °C;
PepRPC HR5/5 column; 25% - 50% acetonitrile (B) in 7column volume elution; 1,2ml/min flow rate, 214nm detection; Standards (dash line): dexamethasone - 2.60min, testosterone - 6.04min, androstenedione - 6.44min and dihydrotestosterone - 7.49 min.

We were not able to detect androstanediols because they were under the limit of detection due to a very low absorbtion coefficient in UV light. Similar chromatographic assays but with radiolabeld steroids showed that the formation of androstanediols is slowly and DHT is the mainly 5α-reduced metabolite of testosterone in the first 45-60 minutes of the reaction (Mowszowicz et al., 1986; Luu-The et al., 1994). According to these data, androstenedione and DHT formed in the first 30 minutes of the reaction and detected with our method accounts for 85-92% from the reacted testosterone (**Figure 2**). The amounts of detected steroids were corrected by their recovery yields.

The kinetic constants were assayed for the first 30 minutes after the testosterone was added. The specific activity was expressed as nmols of formed DHT/min/mg protein. After Hanes plot of Michaelis – Menten equation, we determined a specific activity of 0.51 x 10^{-9} mols DHT/mg protein/min and an apparent constant K_M = 1, 39 x 10^{-6}M (**Figure 3**).

Our data are in agreement with those obtained by more sophisticated radioactive methods (Jakimiuk et al., 1999; Luu-The et al., 1994) and is faster. This non-radioactive method could be used for 5α -reductase assays both in cell culture and in tissue homogenates (skin, prostate) for rapid screening of drugs capable of inhibiting these enzymes.

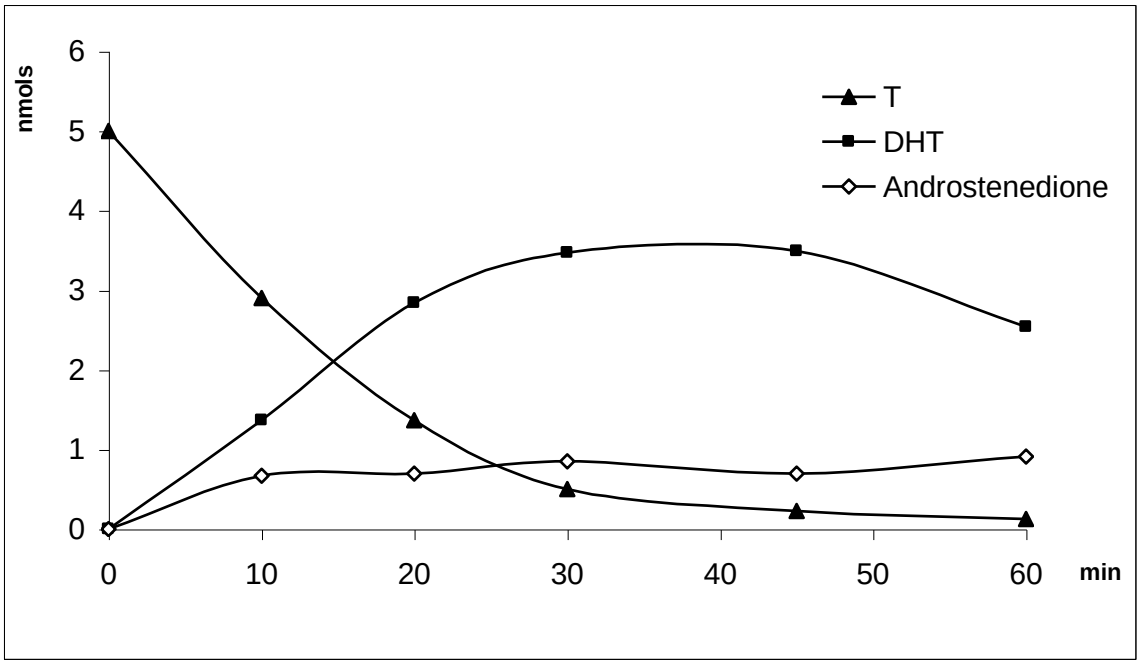

Figure 2. Testosterone metabolites formation in cell culture medium after incubation of fibroblast with T 5μM.

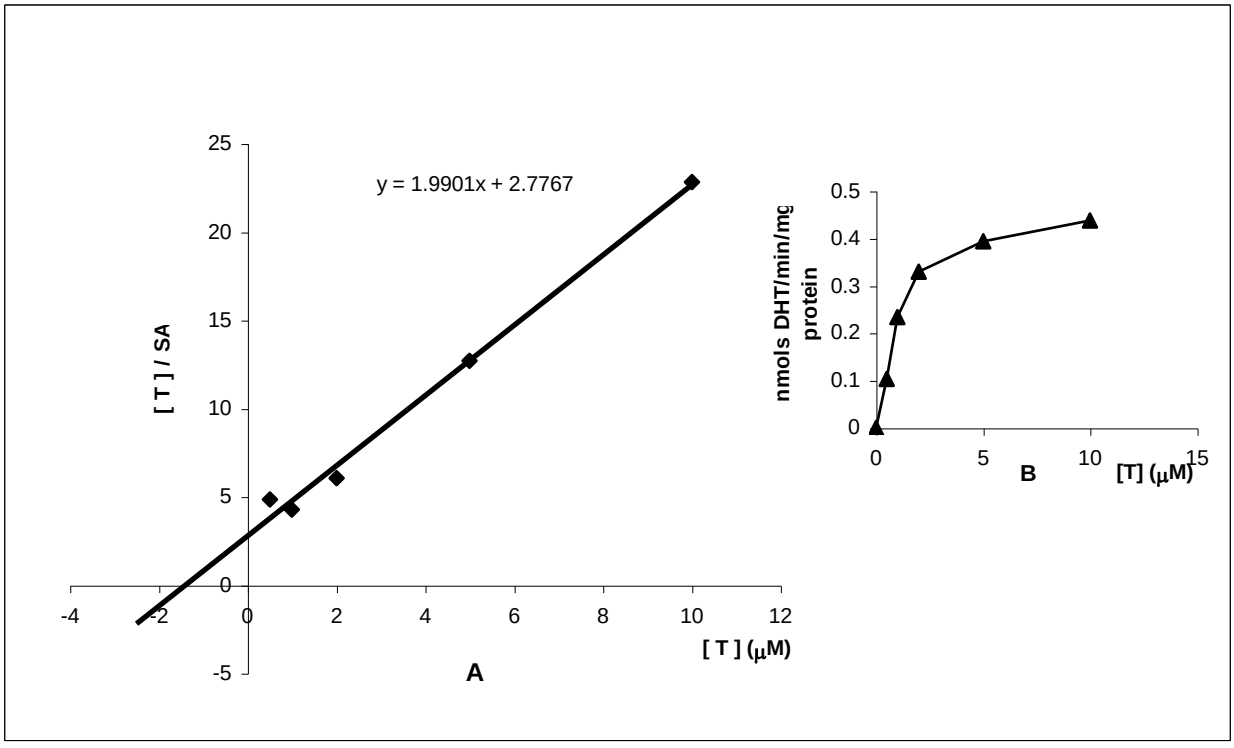

Figure 3. Hanes plot (A) and graphic representation of Michaelis-Menten (B) equation.
The deducted kinetic constants are: K_M = 1, 39 x 10^{-6}M; v_{max} = 0.51 x 10^{-9} mols DHT/mg protein/min.

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