Journal of Chromatography, 395 (1987) 563–568
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1206

RADIO GAS CHROMATOGRAPHY FOR EVALUATION OF SUB-CELLULAR HORMONE SYNTHESIS IN THE ANDROGEN INSENSITIVITY SYNDROME

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SUMMARY

This paper gives a description of a radio gas–liquid chromatographic method for the evaluation of androgen hormone synthesis patterns in the testicular tissue of a patient suffering from the androgen insensitivity syndrome (AIS). A modified dual-column gas chromatography system, equipped with column switching facilities and a radioactivity monitor run parallel to a flame ionisation detector, enables the monitoring of radioactive intermediates of testosterone anabolism and catabolism, generated from a labelled precursor. Tissue preparations were incubated with tritiated pregnenolone for 45 min at 37°C. Steroid hormones were stripped from the aqueous phase by solvent extraction and analysed by gas chromatography as methoxitrimethylsilyl (MO-TMS) derivatives on a 15 m × 0.32 mm I.D. fused-silica capillary column coated with DB-5. The results reveal abnormal enzyme kinetics due to accelerated precursor utilisation. The findings reflect the pathophysiology of AIS at the sub-cellular level of the androgen hormone target organ.

INTRODUCTION

AIS is an inherited androgen target organ insufficiency, mainly in the genital region, caused by androgen receptor and/or 5α-reductase defects. Since serum androgens and urinary 17-ketosteroids are within the normal range for males[1], these female individuals appear biochemically as normal males. A correct diagnosis can only be made by analysing the receptor and enzyme situation in genital tissue.

It was the aim of this study to evaluate the patterns of intermediates of testosterone (T) anabolism and catabolism in the testicular tissue of a patient with the complete form of AIS. Under normal conditions, both $\Delta 5$ and $\Delta 4$ paths can be used for androgen synthesis by the tissue. The $\Delta 5$ path is more physiological for the conversion of cholesterol and pregnenolone to T[2]. Other authors[3] predict a shift towards the $\Delta 5$ pathway in patients with different types of androgen hormone disorders. Many different methods have been used to evaluate these pathways, mainly by analysing single hormone intermediates by radioimmunoassay or by multistep paper or thin-layer chromatographic (TLC) methods[4–7]. Thus, these methods do not provide a total and interdependent reflection of the hormonal patterns. In order to overcome this

0021-9673/87/$03.50

disadvantage gas chromatography (GC) on capillary columns proved to be the best analytical tool.

Therefore, radio gas chromatography (RGC) was used for the evaluation of T synthesis from a radiolabeled precursor in order to gain a precise picture of all intermediate steroids of the synthesis and metabolism pathways, respectively.

EXPERIMENTAL

Materials

Radioinert steroids were from Steraloids (Wilton, NH,U.S.A.) and tritiated compounds (specific activity *ca.* 1.85 TBq/mmol) were from Amersham (Buckinghamshire, U.K.). Reagents for hormone derivatisation were from Pierce (Rotterdam, The Netherlands). Other chemicals were purchased from Merck (Darmstadt, F.R.G.) Serva (Heidelberg, F.R.G.) and Sigma (Deisenhofen, F.R.G.) and were used without further purification.

Labelled compounds were tested for radiochemical purity by TLC prior to use.

A list of systematic and trivial names of the steroids as well as of abbreviations used in text, is given in Table I.

GAS CHROMATOGRAPHY

RGC was performed on a modified dual-column Packard (Delft, The Netherlands) instrument (Model 433), equipped with column switching facilities and a radioactivity monitor, *i.e.* a gas proportional counter for the continuous measure-

TABLE I

STEROID HORMONES USED FOR MONITORING ANDROGEN SYNTHESIS PATTERNS

Systematic and trivial names of steroid hormones used for identification of intermediates in testosterone synthesis and metabolism in testicular tissue. Abbreviations used in text are given in brackets. Labelled compounds (tritiated hormones) are marked with an asterisk.

5β-Androstane-3α,17β-diol	β-Diol	(β-DIOL)
5α-Androstane-3α,17β-diol	α-Diol	(α-DIOL)*
5β-Androstane-3α-ol-17-one	Etiocholanolone	(ETIO)
5-Androstene-3β-ol-17-one	Dehydroepiandrosterone	(DHEA)*
5-Androstene-3β,17β-diol	Androstenediol	(ANDIOL)
5α-Androstane-3α-ol-17-one	Androsterone	(A)
5α-Androstane-17β-ol-3-one	Dihydrotestosterone	(DHT)*
1,3,5-Estratriene-3,17β-diol	Estradiol	(E2)
4-Androstene-17β-ol-3-one	Testosterone	(T)*
4-Androstene-3,17-dione	Androstenedione	(ANDION)
5β-Androstane-3α,11β-diol-17-one	11OH-Etiocholanolone	(OHE)
5-Pregnen-3β-ol-20-one	Pregnenolone	(PRG)*
5α-Androstane-3α,11β-diol-17-one	11OH-Androsterone	(OHA)
5β-Pregnane-3α,17α,20α-triol	Pregnanetriol	(P3)
5-Pregnene-3α,17α-diol-20-one	17OH-Pregnenolone	(17PRG)
4-Pregnene-3,20-dione	Progesterone	(PO)*
4-Pregnene-17α-ol-3,20-dione	17OH-Progesterone	(17PO)*
5-Cholestene-3β-ol	Cholesterol	(CHOL)*

ment of tritiated compounds in gases (Packard, Model 894) by combustion technique. Parallel to this detector, a flame ionisation detector was operated for mass detection. The dual detector system, in combination with the modified column-switching device, allowed a variable split ratio of the carrier gas at the outlet of the column between both detectors, as well as the use of either detector separately. In combination with the "heart-cutting" technique this provided the possibility of alternating the choice of detector during GC, thus facilitating the identification of labelled peaks by using radio-intert reference substances (alkanes n-$C_{24}H_{50}$ and n-$C_{32}H_{66}$, respectively) for the calculation of relative retention times (RRT) or methylene units (MU)[8].

Steroids were analysed as their MO-TMS derivatives[9,10] on a J&W (Rancho Cordorva, CA, U.S.A.) fused-silica capillary column (DB-5; 15 m × 0.32 mm I.D.). Helium was employed as carrier gas at a flow-rate of 15 cm/s. Chromatograms were temperature-programmed, starting at 200°C. For injection, a solid-phase sampling device[11] was used. The temperature of the injector and detector blocks and of the transfer line to the radioactivity monitor was 300°C. The combustion furnaces of the radioactivity monitor were operated at 800°C. Propane was used as the quench gas for the counter tube. For the calculation and documentation of results computing integrators were used, which were coupled on-line to a laboratory data system.

Tissue incubation

Testicular tissue was obtained during orchidectomy from a 16 years old patient with the complete form of AIS. The tissue specimen was homogenised and incubated under aerobic conditions with tritiated PRG (100 pmol/mg homogenate protein) as precursor for the steroid synthesis in a modified Krebs-Ringer solution at 37°C for 45 min[12]. After incubation, the steroids were stripped from the aqueous phase by organic solvent extraction, derivatised and analysed by GC. Prior to GC, the recovery of radioactivity was measured in an aliquot sample by liquid scintillation counting.

RESULTS

Incubation of the tissue sample was performed in duplicate. A typical radiochromatogram showing 8 tritiated compounds is shown in Fig. 1. The results of the RGC analyses of the incubation mixtures are summarised in Table II, and a typical radiochromatogram of the patient is shown in Fig. 2. The values in Table II represent mean values calculated from the data of both incubation experiments (standard deviations were less than 10%).

DISCUSSION

Earlier incubation experiments[13,7] are hardly comparable, as there are great differences in the quality of steroid separation methods[4–7]. Our experiments with intact systems suggest a linear relationship between the enzymatic actions that are responsible for the formation of hormone intermediates in T biosynthesis (linear kinetics), mainly following the $\Delta 5$ pathway. The results of the present study show a fast degradation of PRG (97%) within the incubation period, in contast to the normal concentrations of the main androgens T (3%) and DHT (7%) and their subsequent

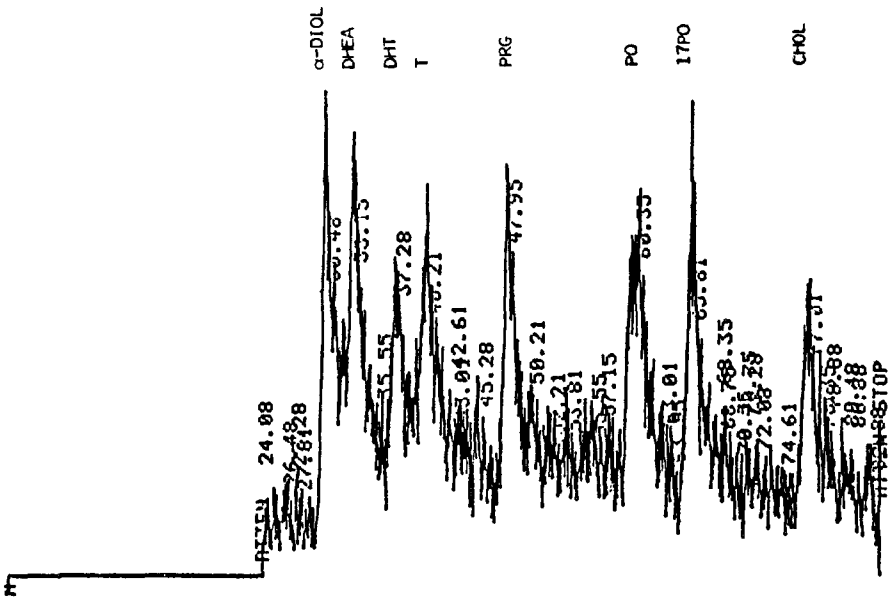

Fig. 1. Typical radiochromatogram of eight tritiated steroid standards analysed as MO-TMS ethers. Radioactivities of component peaks ranged from 30 to 60 kBq (abs.). Column, 15 m × 0.32 mm I.D. DB-5 fused-silica capillary; detection radioactivity monitor (gas proportional counter); carrier gas helium (15 cm/s); temperature programme from 200°C. Peaks were identified by their RRT and MU, relative to alkanes n-$C_{24}H_{50}$ and n-$C_{32}H_{66}$, respectively. Reference alkanes were traced on FID by using the "heart-cutting" technique.

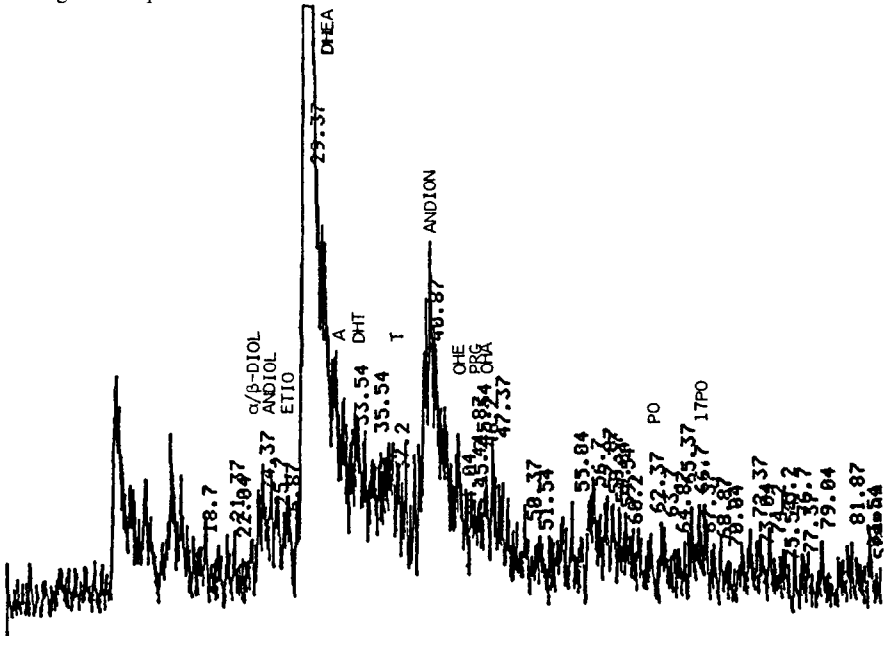

Fig. 2. Radiochromatogram of the steroid pattern in testicular tissue in a patient with AIS. Steroids were extracted from incubation mixtures and converted to MO-TMS ethers before GC. A radioactivity monitor was used for detection of labelled compounds (metabolites) derived from the tritiated precursor (PRG). GC conditions and identification procedures as given in Fig. 1.

TABLE II

PRECURSOR (PRG) UTILISATION IN TESTICULAR TISSUE

Results of RGC analysis of testicular tissue preparation after incubation with tritiated PRG (100 pmol/mg homogenate protein). Data represent amounts of steroids synthesized from PRG by the tissue. For conditions see text.

*Steroid**	*(pmol/mg)***
PRG (residual)	2.85
17PRG	3.03
PO	0.65
17PO	1.26
ANDION	19.20
DHEA	32.79
ANDIOL	2.94
T	2.91
DHT	6.67
DIOL(S)	1.01
A+OHA	24.86
ETIO+OHE	1.83

* For abbreviations see Table I.

** Mean values calculated from two incubations.

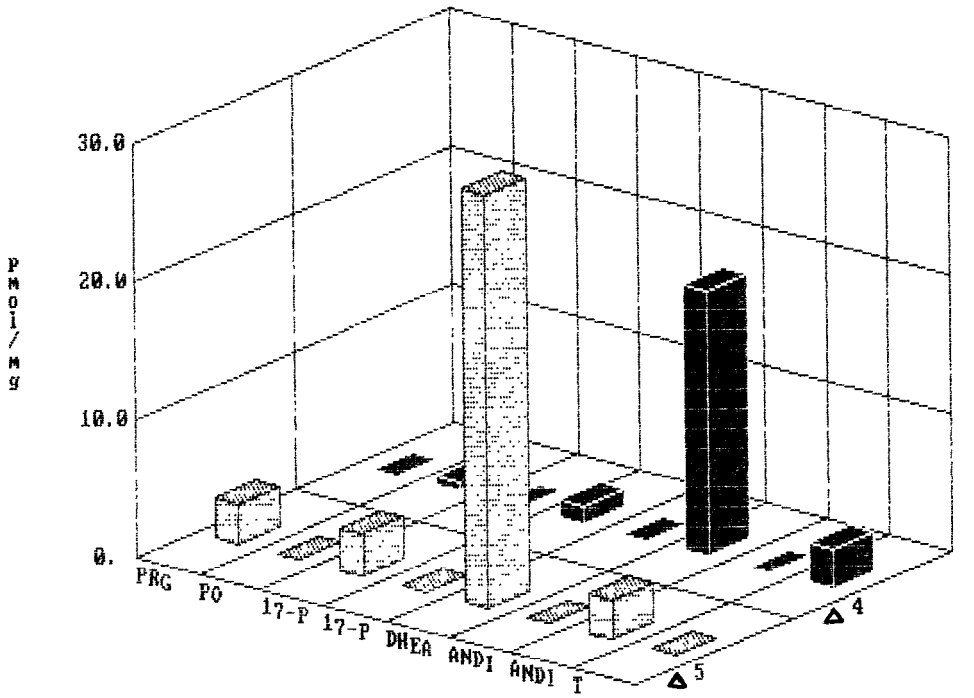

Fig. 3. Graphic illustration of results of RGC analyses of incubated testicular tissue (see Table II). showing the anabolic intermediates. Bars represent steroids (pmoles) generated from the precursor (PRG) within the incubation period, following the $\Delta 4$ and $\Delta 5$ pathways, respectively, as indicated on the abscissa.

diol metabolites (1%), respectively. The rapid start of the synthesis is followed by a rapid turn-over of the intermediates until the direct T precursors are reached.

DHEA, part of the $\Delta 4$ path, showed a synthesis yield of 33% of the precursor concentration. For $\Delta 5$-ANDION a markedly increased formation (19 pmol/mg homogenate) could also be observed. This indicates a significant slowdown of the synthesis just before T is formed, resulting in normal end product concentrations (T/DHT), but the overall linearity of the kinetics of the androgen synthesis (PRG → T) was disturbed. The predicted $\Delta 5$-shift[3] could be confirmed, but in order to normalise the hormonal situation, the contribution of $\Delta 4$-ANDION seemed to be necessary. A comparative illustration of the distribution of the anabolic intermediates between $\Delta 4$ and $\Delta 5$ compounds is given in Fig. 3. The activities of the enzymes isomerase, 3β-OH-steroiddehydrogenase and 17β-reductase appear to be decreased at that stage of synthesis.

RGC has proved to be the optimal analytical tool for this kind of biochemical investigation, because only capillary GC, in combination with a radioactivity monitor, has the capacity to reflect the total hormonal pattern, thus providing a comprehensive picture of the hormonal situation on the cellular and sub-cellular levels.

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