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A RADIOIMMUNO—CHROMATOGRAPHIC SCANNING METHOD FOR THE ANALYSIS OF TESTOSTERONE CONJUGATES IN URINE AND SERUM

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SUMMARY

A method for the analysis of testosterone (and 5α-dihydrotestosterone) conjugates in human serum and urine samples is described. The samples were brought to pH 1 and extracted with a diethyl ether—methanol mixture. After evaporation the residues were run in a thin-layer chromatography system, individual samples' paths were cut into 1-cm long pieces and eluted with methanol. The methanol was evaporated and the residue subjected to acid hydrolysis. The released steroid was extracted by diethyl ether and measured by radioimmunoassay. The methodology described represents a new approach to the qualitative and quantitative study of steroid conjugates in serum and urine, and can easily be applied to the study of steroid conjugates in other biological material.

INTRODUCTION

A large number and wide variety of steroid conjugates isolated from animal and human sources have been described [1]. The majority are in the form of glucuronides and sulphates though the presence of steroid phosphates has been reported in serum [2] and in urine [3]. Testosterone sulphate (TS) in human urine and serum has been reviewed by Dessypris [4]. According to this review only two papers reported on the concentration of TS in human serum [5, 6]. Testosterone glucuronide (TG) in urine and serum has been studied by various methods, including double isotope derivative [7, 8], gas chromatography [9] and more recently radioimmunoassay [10, 11]. Purvis et al. [12] in a recent study reported the presence of dihydrotestosterone sulphate in human seminal plasma.

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Thin-layer chromatography (TLC) has been used by workers in the past to separate steroid glucuronides and sulphates from the free steroids [13, 14]. In this paper a simple and sensitive method is described which provides means of studying profiles of testosterone (T) conjugates in biological material.

EXPERIMENTAL

Materials and instruments

The solvents used were of analar grade and obtained from BDH (Poole, Great Britain) or James Burrough (London, Great Britain). The TLC aluminium sheets pre-coated with silica gel (Art. No. 5554) were obtained from Merck (Darmstadt, G.F.R.). Sulphur trioxide—pyridine complex was obtained from Aldrich (Middlesex, Great Britain).

Unlabelled steroids: testosterone glucuronide, testosterone and 5α-dihydrotestosterone were supplied by Sigma (London, Great Britain). Tritium labelled steroids; 5α-dihydrotestosterone (TRK 395) and testosterone (TRK 402) were purchased from the Radiochemical Centre (Amersham, Great Britain). Tritiated testosterone glucuronide (NET-291) was purchased from New England Nuclear (Dreieichenhain, G.F.R.). Labelled and unlabelled testosterone sulphates were synthesized by a modification of the method of Sobel and Spoerri [15] and separated by TLC according to Klein and Giroud [16]. The testosterone sulphate produced was further identified as described elsewhere [17]. All labelled steroids including conjugated steroids were checked on TLC for purity before use. A Shandon Universal Chromatank (17.5 × 12.5 × 12 cm) was used for all TLC work. Scintillation counting was performed in an Intertechnique Model SL 36 liquid scintillation spectrometer. The scintillation fluid consisted of toluene—Triton (2:1, v/v) with 0.4% PPO (Intertechnique) and 0.05% α-NPO (Nuclear Enterprises). The counting sample (1.0—1.5 ml) was mixed with 10 ml scintillation fluid and this system had a counting efficiency for tritium of approximately 35%.

Samples

Serum and urine samples were from normal males and females aged 21—39 years and were either freshly obtained or had been stored at —20°.

Extraction of samples

The method used for extraction of T conjugates from urine and serum is modified from that of Kornel [18]. To 250 μl of serum or 500 μl of urine in a conical glass centrifuge tube a few drops of 6 *M* H_2SO_4 were added to bring the pH down to about 1 and the samples were then saturated with 50% (w/v) ammonium sulphate. The tube was shaken and allowed to stand for 5 min before extraction with 4 volumes of diethyl ether—methanol (3:1) followed by centrifugation at 2000 *g* for 5 min. The upper phase was transferred to disposable glass tubes containing 15—30 mg of sodium bicarbonate to neutralise any acid. The solvents were evaporated in a heating block at 40° under gentle air stream.

Chromatography of conjugates (System A)

The pre-coated TLC sheets were kept in an air-tight chamber containing silica gel granules before use. The chromatography tank was lined with filter papers at both ends and saturated at room temperature with chloroform—methanol—water (70:30:2), the system used to develop the sheets, for at least 30 min before use.

The residue from the ether—methanol extracts were dissolved in 100 μl methanol which was applied onto the TLC sheets 2 cm from the lower edge. Each tube was washed a second time with 100 μl methanol to secure satisfactory recovery. Samples were spaced 2 cm apart. Appropriate markers (10—20 μg) were applied on the margin of each sheet. The plates were developed by ascending chromatography and allowed to run until the solvent front had reached the upper edge (2.2 h). After drying, the zones for TG, TS and T were located by UV light. Each sample path was now cut into eighteen 1-cm long pieces, which were bent and put into conical glass centrifuge tubes where they were eluted with 1.5 ml of methanol. Centrifugation at about 2000 *g* for 3 min brought down suspended silica gel particles. The methanol was transferred into disposable glass tubes where it was evaporated in a heating block at 40° under gentle air stream.

Acid hydrolysis

The residues from the TLC pieces above were dissolved in 1.0 ml working phosphate buffer (0.1 *M*, pH 7.5 containing 0.2% bovine serum albumin and 0.01% thiomersal) or water and subjected to acid hydrolysis by 3 *M* H_2SO_4 for 20 h at 40° as described elsewhere. The working buffer was used to facilitate dissolution of non-polar compounds when the residue was transferred to other tubes for hydrolysis. The acid hydrolysate in each tube was extracted with 5 volumes of diethyl ether. The ether was evaporated at 40° in disposable glass tubes containing 15—30 mg sodium bicarbonate. The residue was dissolved in working phosphate buffer of which aliquots were taken for T assay or extracted for a run in system B.

Chromatography of androgenic steroids (System B)

The steroids liberated by the acid hydrolysis in the TG and TS peaks of System A were extracted by diethyl ether. After evaporation of the ether the residues were applied on the TLC sheets (Merck, Art. No. 5554) which were developed in a system of toluene—acetone (4:1). The mean R_F values for a few structurally related androgenic steroids were as follows: T 0.44, DHT 0.64; 5α-androstane-3β, 17β-diol 0.24; 5β-androstane-3α, 17β-diol 0.13; 5α-androstane-3α, 17β-diol 0.28. Further processing of the TLC sheets was similar to that of System A. The 1-cm pieces cut out of the sheets were eluted with diethyl ether and the residue, after evaporation, dissolved in working buffer and assayed in the immunoassay for T.

Assay of T and DHT

The radioimmunoassay has been described elsewhere [19] but was used here without prior chromatography to separate T and DHT. The antiserum

used was raised in rabbits against testosterone-3-carboxymethyloxime—bovine serum albumin and was specific for T (100%) and DHT (66%), other related steroids crossreacting 3% (5α-androstane-3β, 17β-diol), 2% (5α-androstane-3α, 17β-diol), 0.7% (androstenedione), 0.5% (epitestosterone), 0.3% (5β-androstane-3α, 17β-diol), <0.1% (androsterone, testosterone acetate, dehydroepiandrosterone) and <0.01% (testosterone sulphate, testosterone glucuronide).

RESULTS

Chromatography of conjugates

The TLC of the testosterone conjugates (System A) was highly reproducible. The R_F values (mean ± standard deviation) of the markers (TG, TS and T) in six consecutive runs were 0.13 ± 0.02, 0.48 ± 0.01 and 0.87 ± 0.02, respectively. The saturation of the tank before each run was found to be essential for securing good reproducibility.

Profiles in urine

The chromatographic (System A) profiles of T and T-containing conjugates in urine are shown in Figs. 1 and 2. Fig. 1 shows a representative profile in a male urine and Fig. 2 the same type of profile in two female urine samples. TG ran the shortest distance (4 cm) from the origin, TS ran about twice as fast (8 cm) and T itself ran fastest, close to the solvent front (16—17 cm).

In order to find out whether any urinary steroids other than T and DHT might be causing the readings in the peaks corresponding to the glucuronide and the sulphate, an aliquot of the hydrolysate from the respective peak frac-

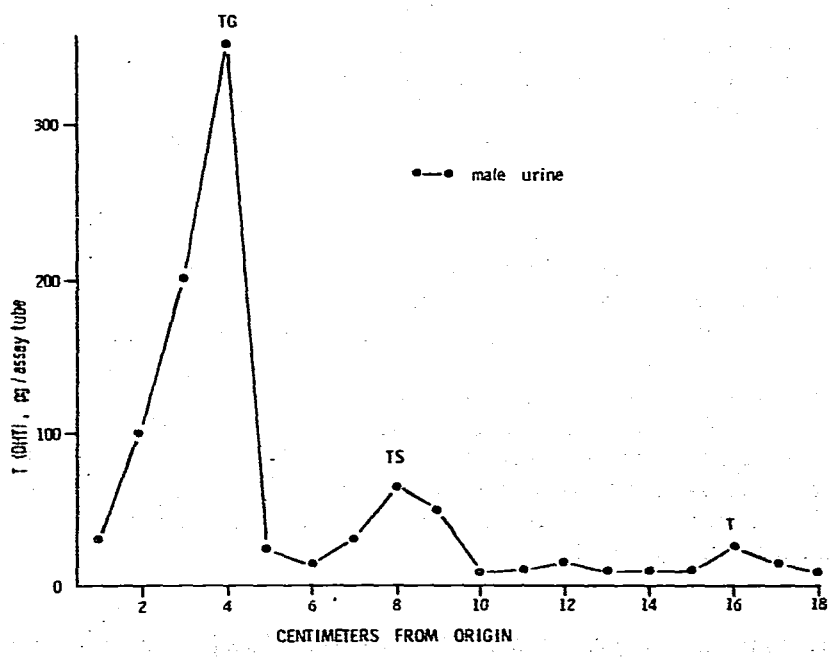

Fig. 1. TLC of a male urine using System A (Figs. 1—6 are all based on extractions of 1-cm sections of the chromatogram and radioimmunoassay of the extracts).

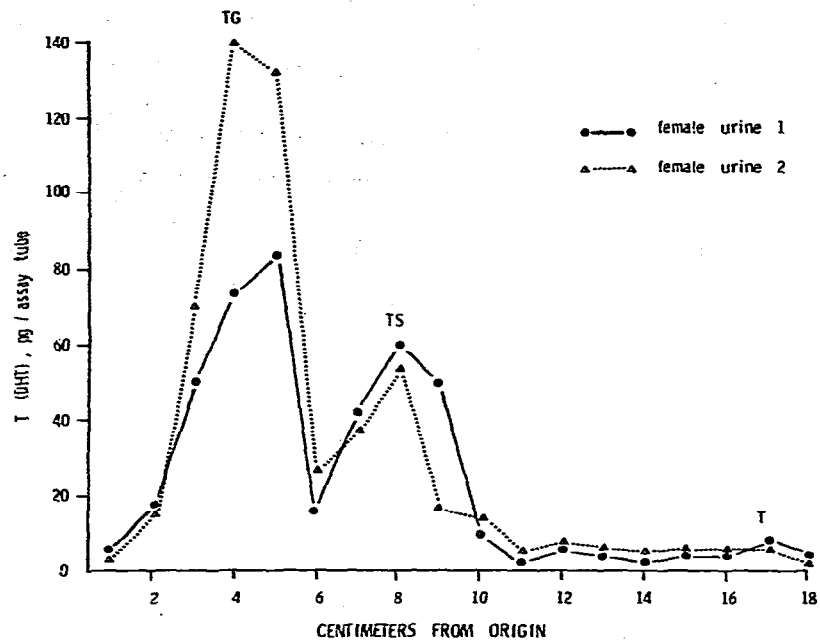

Fig. 2. TLC of female urine in System A.

tions (TG and TS) was run in System B. These results are shown in Fig. 3 (TG) and Fig. 4 (TS). It may be seen that practically all the readings are caused by T and DHT. The TG fraction is made up mostly by T (Fig. 3) whereas in the TS fraction DHT contributes about 30% to the peak.

Profiles in serum

Profiles of the T-containing compounds from two male serum samples are shown in Fig. 5. In male serum the proportional size of the individual peaks is the reverse of that in male urine. Unconjugated T has the highest concentration in male serum and TG the lowest. In female serum the TS peaks were slightly higher than the TG peaks and the unconjugated T peaks were the smallest.

Aliquots from serum TG and TS peaks are also run in System B. Again almost all of the conjugate peaks consisted of T and DHT. Also, as for the urine conjugates, the sulphate peak contained relatively more of DHT than did the glucuronide peak. Fig. 6 shows representative androgen profiles from the TS peak of a male and a female serum.

Recovery studies and quantitation

Unlabelled and labelled conjugates as well as unconjugated T were added to two urine and two serum samples and the recovery through the whole procedure assessed. The recovery ranged from 60 to 89% being similar for both the labelled and unlabelled compounds and no obvious difference was found between serum and urine samples. Based on an average recovery of

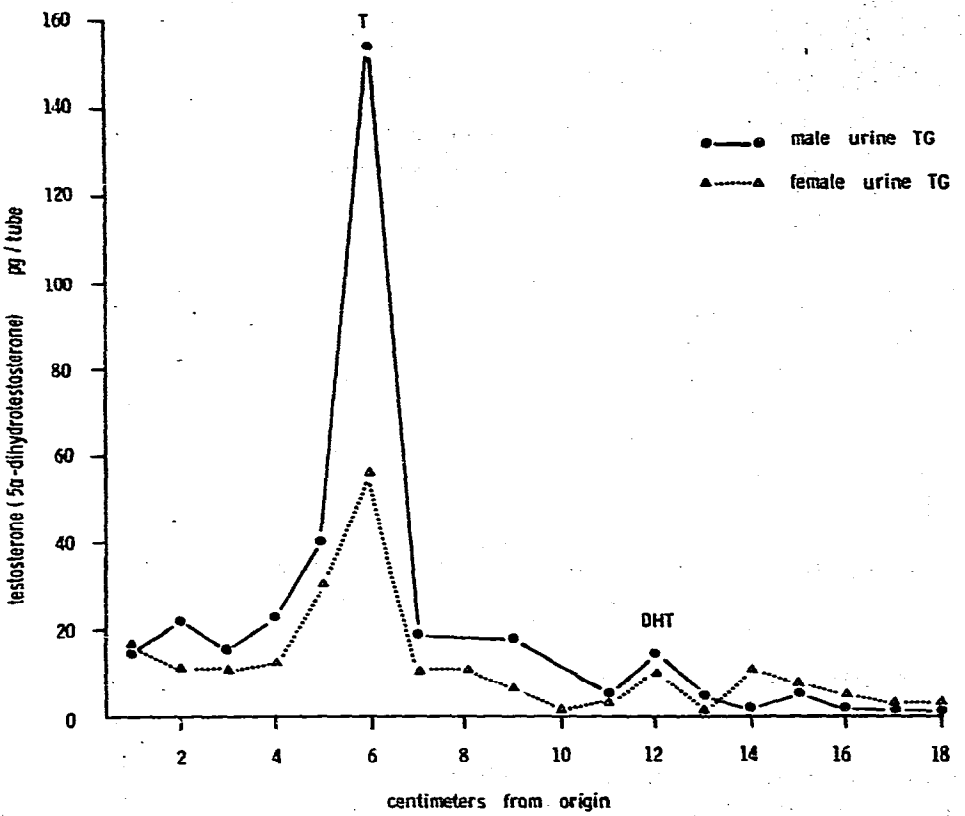

Fig. 3. TLC in System B of urinary TG hydrolysate from System A.

72% we estimated the amount of TG and TS in 4 urines and sera from both sexes. The ranges of TG values for males and females were 80—163 and 92—271 ng per 100 ml in serum and 17—54 and 12—60 μg per 24 h in urine, respectively. The ranges of TS values for males and females were 200—308 and 80—430 ng per 100 ml in serum and 0.3—13.6 and 0.1—2.9 μg per 24 h in urine, respectively. These values are expressed as the T-conjugates but include both the T and DHT conjugates.

DISCUSSION

The method presented for the study of T and DHT conjugates, which we have called a radioimmuno—chromatographic scanning (RICS) method, represents a new approach towards the study of steroid conjugates in biological material. It gives simultaneously the relative amounts and number of the individual steroid containing compounds. The correct detection hinges on the hydrolysis procedure used. We have studied the acid hydrolysis of the T con-

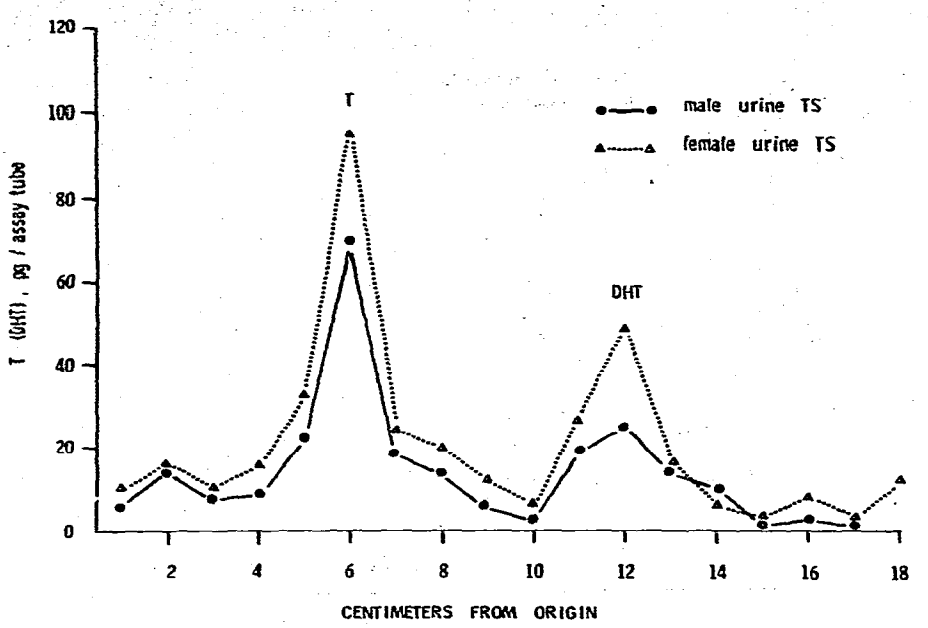

Fig. 4. TLC in System B of urinary TS hydrolysate from System A.

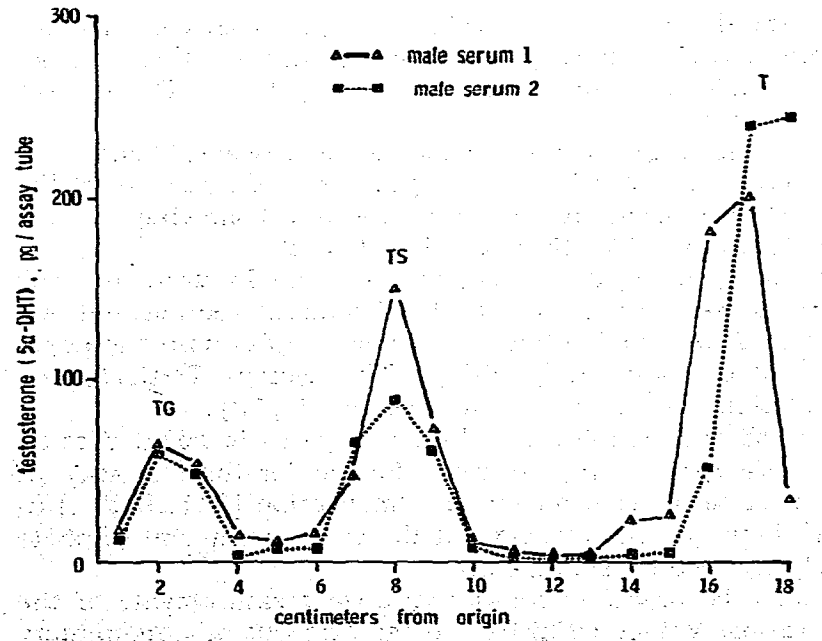

Fig. 5. TLC of male serum in System A.

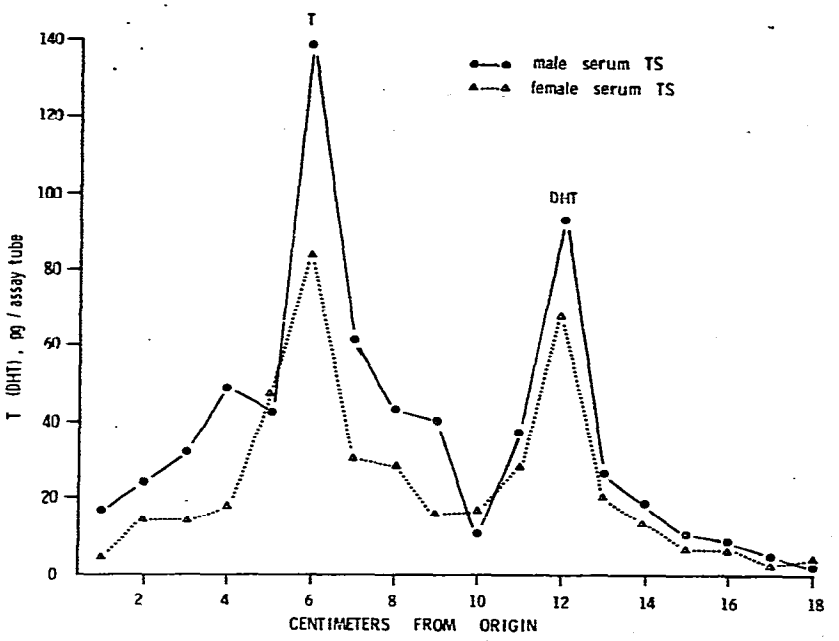

Fig. 6. TLC of serum TS hydrolysate in System B.

jugates specially [17] and did not find any evidence that it destroyed testosterone itself. Furthermore, besides a very acid-labile conjugate, these studies suggested that only the sulphate and glucuronide conjugates of T and DHT existed in normal human urine.

Our antiserum was sufficiently specific for the two androgens, T and DHT to allow us to use an ether extract of the acid hydrolysate directly for radioimmunoassay. This is clearly borne out of Figs. 3, 4 and 6 showing that the antiserum "sees" little if any steroids other than T and DHT.

The concentration of unconjugated (free) testosterone in urine is small [19, 20] and constitutes less than 1% of the total amount (conjugated and unconjugated). Because of the small volumes of urine samples used the peaks of unconjugated testosterone are barely visible. The average T—DHT ratio in the unconjugated fraction in urine from both sexes is 2.5 [19].

The acid extraction of the samples is a disadvantage as it might destroy acid-labile conjugates. We have in fact found evidence for the existence of such an acid-labile conjugate of testosterone in human urine [21]. If the pH-adjustment is omitted, however, the recovery of the glucuronide and sulphate conjugates is drastically reduced to less than 30%.

The method can easily be modified for quantitative measurements of the glucuronides and sulphates. Values published so far vary but our preliminary values in serum and urine are in reasonable agreement with those of other workers [4—7, 9—11]. Our values for TG in male urine are however somewhat lower and our TS values in serum higher than those reported. The later discrepancy can be explained to some extent by the relative amount of DHT

included in our measurements. It is conceivable that our conjugate peaks are not homogenous, consisting of more than one conjugate of similar polarity. Our studies with a different technique involving anion-exchange columns [22] do, however, agree with the present findings of only two conjugate peaks in human urine.

The higher T—DHT ratio in the sulphate peaks as compared to the glucuronide peaks is interesting and may prove to be of biological significance.

The method described here for T (and DHT) should be easily adopted for the study of conjugates of other steroids, provided relatively specific antisera are available and favourable hydrolysing properties of conjugates. Besides studying steroid conjugates in biological fluids it should be of interest to apply these techniques to the study of steroid conjugation in tissues.

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