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Urinary Unconjugated 5α-Androstane-3α, 17β-diol in Patients with Prostatic Tumours

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Summary. A sensitive and reliable radioimmunoassay (RIA) for urinary unconjugated 5α-androstane-3α, 17β-diol is described. The mean overall recovery of unconjugated 5α-androstane-3α, 17β-diol was found to be 57.4%. The sensitivity of the assay was 79 fmol per assay tube and the intra and inter-assay variations ranged between 7.2% and 11.4%. The mean ± SEM for the concentration of this androgen in the urine of normal men was 339.6 ± 66.8 nmol/24 h. The corresponding values for patients with benign prostatic hypertrophy (BPH) and carcinoma of the prostate (Ca) were 297.8 ± 44.7 and 1592.1 ± 622.7 respectively. The mean value for Ca patients was significantly higher than either BPH ($p < 0.05$) or normal subject ($p < 0.02$), suggesting a differential urinary excretion pattern for unconjugated 5α-androstane-3α, 17β-diol between BPH and Ca patients. It is concluded that the combined measurement of this androgen in the plasma and urine provides a more accurate assessment of the profile of this hormone than a single plasma estimation.

Key words: Urinary 5α-androstane-3α, 17β-diol, Unconjugated androgens, Urinary androgens, Benign prostatic hypertrophy, Carcinoma of the prostate, Normal men.

Introduction

Five alpha-androstane-3α, 17β-diol is a three keto reduced metabolite of 5α-dihydrotestosterone which exhibits a high androgen potency in bioassays [1] and has been reported to be capable of inducing canine prostatic hyperplasia [2]. The origin of this steroid in man is reported to derive from the peripheral conversion of secreted testosterone as well as from the liver [3, 4]. These studies have shown that an estimated 50% of the serum 5α-androstane-3α, 17β-diol originates from conversion of testosterone in peripheral tissues including the liver. Using the constant infusion technique, the production rate of 5α-androstane-3α, 17β-diol in blood has been estimated to be similar to its daily excretion rate [4, 5]. In contrast, a great proportion of the 5α-dihydrotestosterone, which is metabolised in either the liver or its target organs, such as the prostate is reduced *in situ* to 5α-androstane-3α, 17β-diol and only a minor fraction of 5α-dihydrotestosterone enters the blood [3]. All these observations would support the assumption that 5α-androstane-3α, 17β-diol including its isomers are the end products of androgen metabolism and are excreted into the urine. Furthermore, such findings have led to suggestions that the urinary estimation of 5α-androstane-3α, 17β-diol is a more reliable index than its serum estimation [6, 7] and subsequently, a radioimmunoassay for the total measurement of this steroid in urine has been developed which estimates both the unconjugated and the conjugated fractions of this steroid [8]. However, the concentrations of the steroids in the two fractions may not be equally related to pathological conditions involving this androgen metabolite. Indeed, it has been suggested that the unconjugated fraction may be derived from that portion of steroid in blood which is non-protein bound and is therefore more readily filtered by the kidney [9–11]. This had led to the assumption that unconjugated 5α-androstane-3α, 17β-diol might provide a simple measure of the active fraction (non-protein bound) in circulating blood. The lack of a reliable method to measure this unconjugated fraction in the urine, has prompted us to develop a radioimmunoassay for this steroid in normal urine, and to extend its application in the urine of patients with benign prostatic hypertrophy and carcinoma of the prostate.

Materials and Methods

Subjects

Twenty-two normal men aged between 25–57 years, 18 patients with benign prostatic hypertrophy (BPH) (59–83 years) and 13 with carcinoma of the prostate (Ca) (41–79 years) were studied.

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Patients with cancer of the prostate were classified according to the primary tumour site (T) and metastatic status (M) as described by Wallace et al. [12]. Based on this classification, the 13 Ca patients were as follows T0M0 (1), T0M1 (1), T1M0 (2), T2M0 (2), T2M1 (1), T3M0 (2), T3M1 (2) and T4M1 (2). None of the patients with prostatic tumours had received any hormonal therapy or undergone castration prior to this study. Twenty-four specimens of urine were collected and every specimen was refrigerated immediately after voiding and aliquots were kept at −20 °C prior to assay.

Chemicals

The following steroids were obtained from Sigma Chemicals Co. (Poole, Dorset UK): 5α-androstane-3α, 17β-diol: 5α-androstane-3β, 17β-diol; 5β-androstane-3α, 17β-diol; 5β-androstane-3β, 17β-diol, testosterone; 5α-dihydrotestosterone; progesterone and oestradiol. Other steroids used were: androsterone and 5-androsten-3β, 17β-diol (Koch Light, Colnbrook, Buckinghamshire UK) and 5α-androstane-3α, 17α-diol (Schering AG, Berlin) 5α [1α, 2α, (n) ^{3}H] androstane-3α, 17β-diol (S.A. 44 Ci/mmol) was purchased from the Radiochemical Centre, Amersham, UK. The purity of this steroid was checked by t.l.c. prior to use. All solvents used were analytical grade which was obtained from BDH Chemicals Ltd., Poole, Dorset, UK. Buffers, silica gel plates and scintillation fluids were similar to those described by Ghanadian et al. [13].

Antisera

Antisera for the radioimmunoassay of 5α-androstane-3α, 17β-diol was raised in rabbits against 15β carboxyethylmercapto 3α-androstane-diol bovine serum albumin. This antisera was a generous gift from Dr. Rao of the South West Foundation for Research and Education, San Antonio, Texas, USA. Additional specificity studies to those previously reported by Rao et al. [14] were described by Ghanadian and Puah [15].

Assay Procedure

Extraction of Urine. Three millilitres of urine from a 24 h collection were mixed with ≅ 1,000 cpm (≃ 31 fmol) of 5α-[^{3}H]androstane-3α, 17β-diol in order to assess the overall recovery of the assay. The mixture contained in a test tube (50 ml capacity) was allowed to stand at room temperature for 10 min before being extracted with 2 x 12 ml of diethyl ether. The ether phase was removed into a glass tube (75 mm x 12.5 mm) for drying in a water bath. The residue was reconstituted in acetone (2 x 100 μl) and applied onto a silica gel plate.

Thin Layer Chromatography. The plate was developed in universal chromatank (Shandon) in a system of toluene–acetone (4:1). The average Rf values in this system for the following steroids were: 5α-androstane-3α, 17β-diol (0.27) 5β-androstane-3α, 17β-diol (0.15) 5α-androstane-3α, 17α-diol (0.18) and androsterone (0.45). The plate was air dried and the area which corresponds to the 5α-androstane-3α, 17β-diol was cut out and eluted with diethyl ether (2 x 3 ml). Silica gel fines were removed by centrifuging the samples at 2,000 rpm for 5 min. The ether phase was decanted into glass tubes and dried in a water bath.

Radioimmunoassay. After evaporating the ether, the residue was reconstituted in tris buffer (0.5 ml). Portions were removed for assay (0.2 ml) and to assess the overall recovery (0.2 ml). The assay portions and duplicate sets of 5α-androstane-3α, 17β-diol standards (34.2–2,736 fmol) was equilibrated in Luckham LP3 plastic tubes (Burgess Hill, Sussex UK) with 0.1 ml of antisera at room temperature. After incubating for 15 min 0.1 ml of tritiated 5α-androstane-3α, 17β-diol (≃ 6,000 cpm) was added and the tubes were rotamixed and incubated overnight at 4 °C. The free steroid was separated from the bound form by adding 1.0 ml of dextran coated charcoal. After incubating at 4 °C for 15 min the tubes were then centrifuged at 2,000 g at 4 °C for 15 min and the resulting supernatant was transferred to 10 ml of scintillation fluid. The radioactivity was counted in a Packard Tri-Carb liquid scintillation spectrometer model 2660.

Effect of Storage. For this study urine was collected without preservatives and refrigerated after voiding. Aliquots were removed from samples collected from each of the following subjects: one healthy male, one untreated patient with carcinoma of the prostate and one untreated patient with benign prostatic hypertrophy. After storing these samples for up to 4 days at either room temperature (≃ 21 °C) or at 4 °C, the contents of unconjugated 5α-androstane-3α, 17β-diol were measured as described above.

Urinary Creatinine. The completeness of each urine collection was checked by measuring urinary creatinine. This was estimated by an autoanalyser (technicon) using the alkaline picrate reaction.

Results

Recovery and Accuracy

The recovery of unconjugated 5α-androstane-3α, 17β-diol from 3 ml of urine by using 2 x 4 volumes of diethyl ether was found to be 80.8 ± 1.5% (mean ± SEM, n = 10). However, the overall recovery for [^{3}H]5α-androstane-3α, 17β-diol which included a purification step and subsequent analysis of the steroid was 57.4 ± 0.8% (mean ± SEM, n = 10). The accuracy of the method was assessed by adding various amounts of authentic 5α-androstane-3α, 17β-diol to urine samples. When 342, 684, 1,368 and 2,736 fmol of steroids were added to a urine sample, which was assayed in triplicate the amounts recovered after correcting for individual lossess were 102.5 ± 18.0, 98.3 ± 13.0, 101. 3 ± 16.4 and 105.5 ± 10.7% respectively.

Specificity

The specificity of this assay depends on the specificity of the antisera and the resolving power of the t.l.c. in separating the cross-reacting steroids. Our specificity studies (Table 1) showed that the following steroids cross-reacted (measured at 50% binding) with this antisera: 5β-androstane-3α, 17β-diol (7.8%) androsterone (1.9%) and 5α-androstane-3α, 17α-diol (1.3%). However, these steroids were well separated from 5α-androstane-3α, 17β-diol in the t.l.c. system of toluene–acetone (4:1).

A check on this purification step was performed on seven urine samples by rerunning the extract after the standard t.l.c. run in another system consisting of methylene choride–dioxan (19:1). The average Rf values for the following steroids in latter system using pure steroid standards were: 5α-androstane-3α, 17β-diol (0.26), 5β-androstane-3α, 17β-diol (0.13) androsterone (0.54) and 5α-androstane-3α, 17α-diol (0.06). Results obtained following the double t.l.c.

Table 1. Cross reactivities of various steroids tested with anti-15β-carboxyethylmercapto-3α-androstanediol bovine serum albumin

Steroid	Cross-reactivity [%]
5α-Androstane-3α, 17β-diol	100
5β-Androstane-3α, 17β-diol	7.8
Androsterone	1.9
5α-Androstane-3α, 17α-diol	1.3
5α-Androstane-3β, 17β-diol	0
5β-Androstane-3β, 17β-diol	0
5-Androsten-3β, 17β-diol	0
5α-Dihydrotestosterone	0
Testosterone	0
Oestradiol-17β	0
Progesterone	0
Stilboestrol	0
Cyproterone acetate	0

Table 2. Urinary unconjugated 5α-androstane-3α, 17β-diol (pmol/l) after purifications in two t.l.c. systems. Values are Mean ± SD. Figures in parentheses indicate the number of estimations

Male Urine	Toluene-acetone (4:1)	Toluene-acetone (4:1) followed by methylene chloride-dioxan (19:1)
Normal		
EW	539.0 ± 59.2 (4)	519.8 ± 68.6 (4)
PM	419.9 ± 47.7 (3)	374.7 ± 15.9 (3)
BPH		
KP	255.8 ± 58.8 (4)	322.9 ± 28.4 (4)
ZA	406.6 ± 70.8 (4)	360.8 ± 106.7 (4)
CA		
WS	553.7 ± 93.0 (4)	539.3 ± 147.7 (4)
GH	3,492.8 ± 378.7 (3)	3,190.8 ± 49.1 (3)
JA	2,240.8 ± 142.3 (3)	2,316.7 ± 223.3 (3)

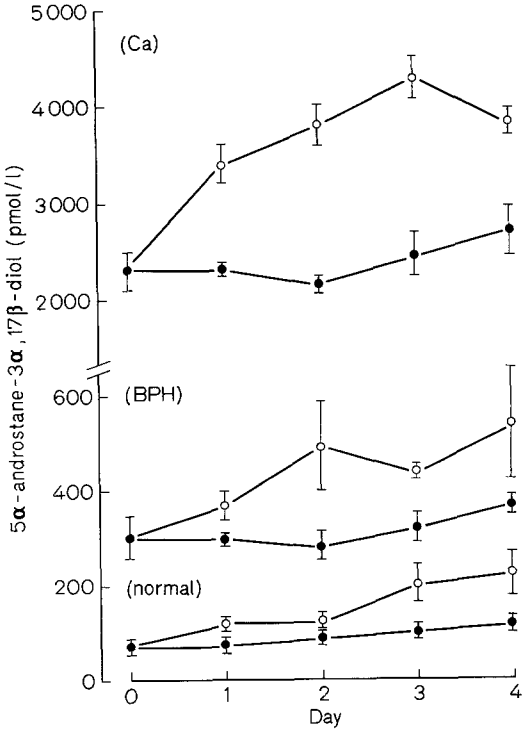

Table 3. Concentration of urinary unconjugated 5α-androstane-3α, 17β-diol (nmol/24 h) in normal men and in patients with benign prostatic hypertrophy (BPH) and carcinoma of the prostate (Ca). Values in brackets denote number of subjects

	Mean ± SEM	Range	Age [years]
Normal	339.6 ± 66.8[b] (22)	87.0–1,336.5	25–57
BPH	297.8 ± 44.7[a] (18)	87.6–723.0	59–83
Ca.	1,592.1 ± 662.7 (13)	195.3–6,822.9	41–79

[a] Significantly different from Ca, $p < 0.05$
[b] Significantly different from Ca, $p < 0.02$

systems were not significantly different from those obtained with the standard system (Table 2).

Sensitivity and Precision

The sensitivity of this assay was 79 fmol (23 pg) per assay tube based on the calculation procedure suggested previously [13]. Determination of the precision of the assay was accomplished by using urinary pools of low and high 5α-androstane-3α, 17β-diol concentrations. The intra-assay coefficients of variation for low and high urinary pools were 10.1% (n = 10) and 7.2% (n = 10) respectively. The inter-assay coefficient of variations for low and high urinary pools were 11.4% (n = 12) and 8.4% (n = 12) respectively.

Water Blanks

In four consecutive assays, the mean ± SEM of water blank readings was 31 ± 7 fmol (9 ± 2 pg).

Effect of Storage and Levels of Urinary Unconjugated 5α-androstane-3α, 17β-diol

The contents of unconjugated 5α-androstane-3α, 17β-diol did not change significantly in urine samples stored at 4 °C for up to 2 days. However, there were significant changes in the level of this steroid when urine samples were allowed to stand at room temperature (≃ 21 °C) for up to 24 h (Fig. 1). In contrast there were no significant changes in the concentrations of this steroid in either low or high urinary pooled samples stored at −20 °C for up to one year.

◀ **Fig. 1.** The effect of storage on the level of urinary unconjugated 5α-androstane-3α, 17β-diol from patients with benign prostatic hypertrophy (BPH), carcinoma of the prostate (Ca) and a normal male. Values are Mean ± SEM of three estimations. Temperatures are either at ≃ 21 °C (○–○) or 4 °C (●–●)

Urinary Unconjugated 5α-androstane-3α, 17β-diol

The results for the urinary unconjugated 5α-androstane-3α, 17β-diol in normal men and patients with prostatic tumours are shown in Table 3. There was a wide range of variation for the concentration of this androgen within each group. Analysis of the results (*t*-test) revealed no difference between the level of this steroid in the urine of patients with BPH and that of normal men. However, the concentration of this steroid in the urine of patients with Ca prostate was found to be significantly higher than either normal ($p < 0.02$) or patients with BPH ($p < 0.05$). There was no relationship between the different stages of the tumour in Ca patients and the level of this steroid in their urine. This may be due to the limited number of patients studied for each class of tumour.

Discussion

The present investigation describes a simple and reliable radioimmunoassay for the measurement of urinary unconjugated 5α-androstane-3α, 17β-diol. The assay is highly specific, in that, not only the antisera used was specific, but also those steroids which cross-reacted with the antisera to a small extent (< 8%) were eliminated from the assay by t.l.c. Other criteria of the assay in particular, accuracy and precision of the method were found to be statisfactory.

The effect of storage on the content of unconjugated 5α-androstane-3α, 17β-diol was assessed. It was demonstrated that there were no significant changes in the content of this androgen in urine samples stored either at 4 °C for up to 2 days or at −20 °C for up to one year.

The value for the total urinary 5α-androstane-3α, 17β-diol in normal men is reported to be 193 ± 79 μg/24 h; 660 ± 270 μmol/24 h [8], which is significantly higher than the concentration of the unconjugated fraction obtained in this study. It is important to note that whilst the level of circulating testosterone is approximately 10 times higher than 5α-dihydrotestosterone [16], and 20 times higher than 5α-androstane-3α, 17β-diol [15], the urinary unconjugated testosterone is only three times higher than 5α-dihydrotestosterone [11] and four times higher than 5α-androstane-3α, 17β-diol. This would suggest that the measurement of the urinary unconjugated 5α-androstane-3α, 17β-diol could provide a more reliable index than a similar estimation in the blood.

Our results clearly demonstrate a significantly higher level of this steroid in the urine of patients with Ca prostate than that of BPH, suggesting that in patients with Ca prostate a considerable amount of the circulating 5α-androstane-3α, 17β-diol is excreted by the kidney. The excretion pattern of this androgen appears to be different in the two types of prostatic tumours. Indeed, we found that the plasma level of this androgen was higher in BPH than in Ca patients [15]. This together with a relatively lower level of 5α-androstane-3α, 17β-diol in the plasma [15] would suggest that the measurement of this steroid in the urine in conjunction with the plasma values could provide a more accurate evaluation of the biological significance of this androgen in these patients.

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