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STUDIES ON TESTOSTERONE-17β-GLUCOSIDURONATE IN HUMAN PLASMA

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SUMMARY

A specific radioimmunoassay (RIA) for the determination of testosterone-17β-glucosiduronate (TG) in human plasma is described. A sensitivity of 25 pg was obtained in the standard curve with an intra-assay coefficient of variation (CV) of 8.5% and an inter-assay CV of 12.5%. Plasma TG concentrations were found as follows (mean ± S.D., ng/ml): Normal males 2.1 ± 0.45 (n = 20); normal females 0.47 ± 0.18 (n = 17); hirsute females 1.36 ± 0.68 (n = 14); *hypogonadic males* 1.08 ± 0.3 (n = 10). TG concentration in the testicular vein of a normal male was 1.91 ng/ml, 30 min after 5000 IU HCG iv 3.0 ng/ml. The infusion of 2 mg of TG for 2 h to normal men induced a 16-fold increase in plasma TG levels, reaching a steady state after 1 h. Plasma concentrations of unconjugated testosterone (T) and 5α-dihydrotestosterone (DHT) estimated simultaneously were not altered. Intravenous infusions of 1000 IU HCG caused a significant elevation of plasma T but no change in plasma TG levels.

INTRODUCTION

Testosterone-17β-glucosiduronate (TG) has been isolated from human plasma in 1965 by Hadd and Rhamy[1]. Quantitative determinations of TG in the plasma of two subjects have been reported by Burger *et al.*[2]. In this paper the estimation of TG in human plasma by means of a radioimmunoassay (RIA) is described.

MATERIAL AND METHODS

Solvents and reagents

Absolute ethanol and diethyl-ether (reagent grade) were purchased from Merck (Darmstadt) and the scintillation fluid Insta-Gel from Packard (Zürich). Bovine serum albumin (BSA) and γ-globulin were obtained from Behring-Werke (Marburg) and β-glucuronidase from Institut Pasteur (Paris). Polyethyleneglycol 6000 (PEG) was purchased from Riedel-de-Haen (Seelze-Hannover). [1,2 ^{3}H]-TG (specific radioactivity 40 Ci/mmol) was obtained from New England Nuclear (Dreieichenhain-Frankfurt) and highly purified TG ("A" grade) from Calbiochem (Lucerne).

Antiserum

The raising and characterization of the specific antibody against TG have been described previously [3]. The antiserum showed cross reactions only with free testosterone (T) (27%) and with 5α-dihydrotestosterone-17β-glucosiduronate (DHTG) (20%).

Assay procedure

(Table 1) Male or female plasma (4 ml) containing 2200 d.p.m. [1,2 ^{3}H]-TG was extracted twice with 40 ml of diethylether. The organic phase was used to measure non conjugated T and DHT [4]. The remaining plasma was extracted again twice with 6 ml ethanol and left at room temperature for 4 h. After removal of proteins by centrifugation the ethanolic phase was evaporated to dryness under nitrogen in a water bath at 50°. The residue was redissolved in 2 ml double-distilled water. Fifty μl were used in duplicate assays and 200 μl for recovery control. A standard curve was prepared over a range of concentrations between 25 and 1600 pg[3]. The residues of both standards and samples were redissolved in 200 μl of a 1:8000 dilution of the antiserum in 0.9% NaCl with 0.1% BSA. About 20,000 d.p.m. [^{3}H]-TG in 50 μl of the same buffer were added and shaken on a Circomix®. After an incubation period of 2 h at 4°C 100 μl of 1% γ-globulin in 0.9% NaCl were added, followed by 500 μl of 20% PEG. The tubes were thoroughly mixed and immediately centrifuged at 4°. The supernatant free fraction was mixed with 8 ml Insta-Gel®, and the radioactivity measured as described previously[3].

Separation of bound and free [^{3}H]-TG

RIA tubes with the amounts of antiserum and [^{3}H]-TG indicated above (total amount bound = B_0) were incubated for 2 h at 4° and submitted to increasing concentrations of γ-globulin and PEG. The procedure was repeated with tubes containing only [^{3}H]-TG and buffer instead of antiserum (blank values = N). Optimal concentrations of γ-globulin and PEG are those which achieve the highest B_0 value together with a low N value.

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Abbreviations and trivial names DHT: Dihydrotestosterone = 17β-hydroxy-5α-androstan-3-one. T: Testosterone = 17β-hydroxy-4-androsten-3-one. TG: Testosterone-17β-glucosiduronate = 3-oxo-4-androsten-17β-yl-βD-glucopyranosiduronate.

Table 1. Flow sheet

4 ml Plasma
Diethylether extraction (10 v/v)

→ Ether phase (determination of unconjugated steroids)

Water phase
Shake with 2 × 4 v/v ethanol
4 h Room temperature
Centrifugation

→ Denatured proteins—withdrawn

Ethanolic extract evaporated under N_2 in a waterbath at 50°
Redissolved in 0.5 v/v dist. Water
2 × 50 µl, Evaporated at 50°C in RIA Tubes

200 µl Antibody K 74 dissolved (1:8000) in 0.9% saline with 0.1% BSA
+50 µl [^{3}H]-TG in the same buffer (20.000 d.p.m.)
2 h Incubation at 4°
+100 µl 1% γ-Globulin in 0.9% saline
+500 µl 20% PEG in 0.9% saline
Centrifugation
Supernatant (free Fraction) mixed with 10 ml Instagel®
β-Scintillation counting (B & F 5000)
RIA (Calculation by means of a Diehl Alphatronic on-line desk computer. Tolerated error less than 8%).

Urinary excretion of TG was estimated as described previously[3].

Experimental design

1. *TG infusions.* Six normal male volunteers (20–28 years of age) arrived at the hospital at 7 am. and remained recumbent throughout the experimental period. Two mg TG dissolved in 200 ml 0.9% NaCl were continuously infused into the cubital vein by means of a peristaltic pump for 2 h. The timing of blood sampling is demonstrated in Fig. 4. Samples were heparinized, immediately centrifuged and frozen until used. Urine was collected on two days before the experiment, on the day of infusion and one day after.

2. *HCG infusions.* To 6 normal male volunteers 1000 IU HCG (Predalon®) were infused intravenously under similar conditions. HCG was dissolved in 300 ml 0.9% NaCl and infused by means of a peristaltic pump for 3 h. Blood samples were taken before the beginning of the infusion and at 90 and 180 min. Blood was processed as above. Unconjugated T and DHT were also determined[4].

Informed consent was obtained from all subjects.

RESULTS

Figure 1 demonstrates that 20–25% of PEG and 0.3–0.5 mg of γ-globulin are necessary to achieve a good separation of B_0 from N. Increasing amounts of both reagents induced higher unspecific binding, which is not shown in the graph.

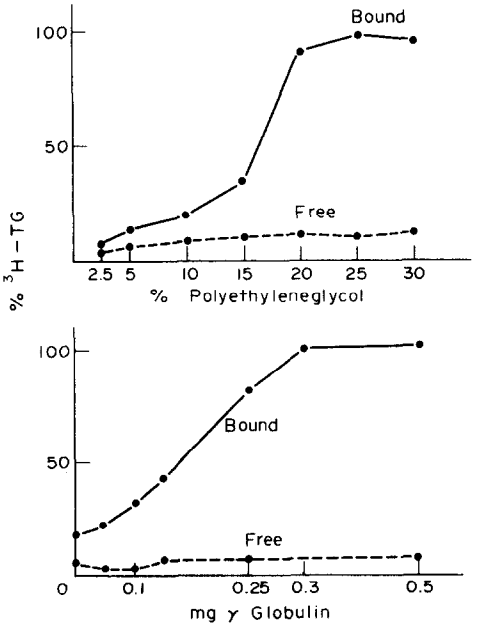

Fig. 1. Precipitation of free and antibody-bound [^{3}H]-testosterone-17β-glucosiduronate (TG) as a function of polyethyleneglycol and γ-globulin concentrations.

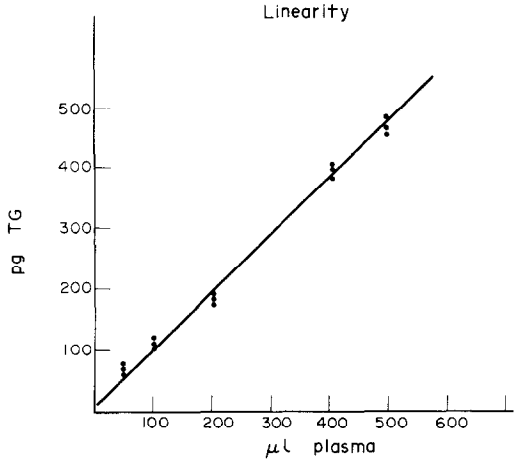

Fig. 2. Linearity of TG estimations in different amounts of plasma.

Figure 2 shows that a linear response in TG determinations was obtained when the plasma vols were enlarged stepwise. The addition of non-labelled TG to male or female plasma resulted in recovery values of 91 ± 5%. The recovery following the extraction procedure was found to be 93 ± 6%. It is important to obtain a protein free extract because the PEG separation can be affected by protein residues. The intra-assay and inter-assay coefficients of variation were found to be 8.5 and 12.5%, respectively.

In 11 plasma samples (Table 2) TG was estimated by direct RIA and after β-glucuronidase hydrolysis with ether extraction and purification by t.l.c.[4] using the antibody of Nieschlag and Loriaux[5]. The ratio TG/T (1.77 ± 0.30) was found to be close to the molecular weight ratio of TG/T (1.64).

Plasma TG values are compiled in Fig. 3. In 20 normal male and 17 female subjects 2.1 ± 0.45 ng/ml and 0.47 ± 0.18 ng/ml, respectively, were found. Fourteen female patients with hirsutism exhibited a significantly increased mean value of 1.36 ± 0.68 ng/ml. Male patients with hypogonadism of different aetiology showed significantly lower TG concentrations (1.08 ± 0.3 ng/ml) compared with normal male subjects. The corresponding mean values of plasma T were 6.32 ± 2.27 ng/ml, 0.43 ± 0.07 ng/ml, 1.28 ± 0.55 ng/ml and 1.82 ± 0.68 ng/ml, respectively. In a male patient (36 years of age), undergoing catheterization of the renal veins for hypertension, blood was drawn from the left testicular vein and from the cubital vein before and 30 min after the infusion of 5000 IU HCG. The following results have been obtained (ng/ml): Before: *V. testic.* T 11.42; TG 1.91; *V. cubit.* T 9.7; TG 2.8; HCG: *V. testic.* T 77.32; TG 3.0; *V. cubit.* T 11.58; TG 2.48. Determinations of T and TG in two charcoal stripped plasma samples from normal males revealed zero values.

The infusion of 2 mg TG produced a marked increase in plasma TG concentrations, reaching a mean of 32 ng/ml 60 min after starting the experiment (Fig. 4). At this time a steady state is maintained. After the end of the infusion a steep decrease of plasma TG was observed. The plasma concentrations of unconjugated T and DHT remained unchanged during the whole period. The urinary excretion of TG showed an increase of 658 ± 168 μg/24 h over the control values on the day of TG infusion. This figure corresponds to about 33% of the total amount of TG infused.

The intravenous infusion of 1000 IU HCG resulted in a significant elevation of plasma T levels. However, no influence on the plasma TG concentrations could be detected (Fig. 5).

DISCUSSION

The above presented results show that the previously described antibody against TG[3] can be used also for the estimation of this steroid conjugate in human plasma. The specificity of the method is warranted by removal of unconjugated T, as only T and DHTG exhibited cross-reactions with the antiserum [3]. The occurrence of DHTG in human plasma and urine has not been described. Even after the infusion of a high dose of DHT, no interfering amounts of

Table 2. Simultaneous determinations of testosterone-17β-glucosiduronate (TG) by direct RIA and following β-glucuronidase hydrolysis and t.l.c. (T), respectively

TG (ng/ml)	T	Ratio TG/T
0.94	0.69	1.36
1.10	0.67	1.67
1.14	0.93	1.23
1.20	0.71	1.69
1.74	1.00	1.74
3.00	1.71	1.75
10.00*	5.50	1.82
12.00*	6.40	1.88
12.20*	6.29	1.94
14.00*	6.43	2.18
27.00*	12.14	2.22
		$\bar{x}$ 1.77 ± 0.30

* Plasma samples obtained during TG-infusion.

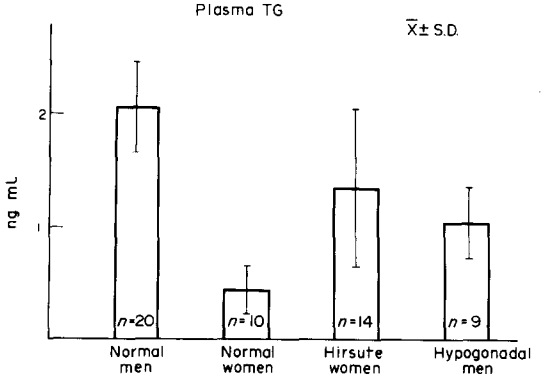

Fig. 3. Plasma TG concentrations in normal males and females as well as in patients with different forms of hypogonadism and hirsutism.

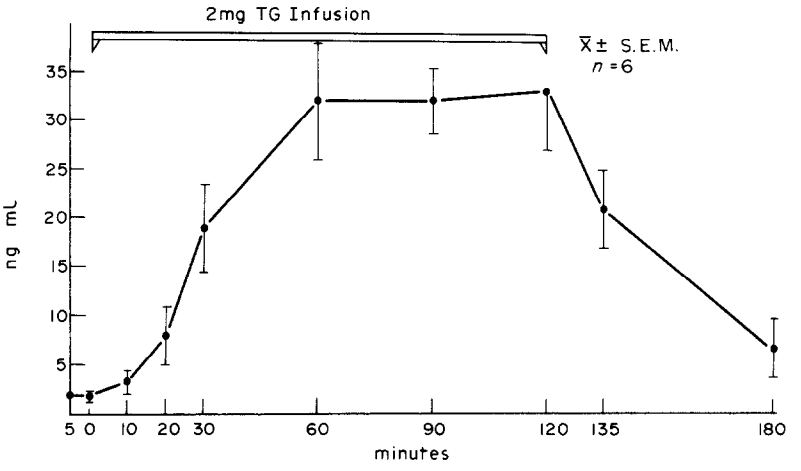

Fig. 4. Plasma TG values during an infusion of 2 mg TG in 6 normal males.

DHTG could be observed[3]. The simultaneous determination of TG by RIA and following β-glucuronidase hydrolysis with purification of the liberated T by t.l.c. revealed a TG/T ratio very similar to the molecular weight ratio of TG/T. The specificity of the antibody together with the utilization of PEG to separate bound and free fractions in the assay provided a very good precision. PEG selectively precipitates antibody bound activity[6] with no stripping at all.

Burger *et al.*[2] were the first who reported on T concentrations in the plasma of a male (13.1 ng/ml) and of a female (0.5 ng/ml) following β-glucuronidase hydrolysis. Additional data could not be retrieved from the pertinent literature. The difference in plasma TG concentrations between normal males and females reported in this paper has been found to be markedly lower than that of the corresponding T levels, the T/TG ratios being 3:1 and 0.8:1, respectively. These findings are not unexpected because it has been described earlier[7] that in females the greatest part of androstenedione and dehydroepiandrosterone, which is converted into TG, does not enter the plasma pool of unconjugated T. Furthermore it is known, that in human beings TG is not a metabolic end product, but is metabolized without hydrolysis[8]. From data published earlier[8, 9] it can be concluded that the transformation of proandrogens into TG as well as the direct metabolism of TG exhibit sex specific differences.

The determination of TG in plasma samples obtained from the testicular vein revealed similar concentrations compared with those in peripheral plasma. Thereby the assumption that the testicles do not contribute to plasma TG[7] is confirmed. However, recent studies on human seminal plasma[10] yielded TG concentrations 6–9 times above those found in blood plasma. The physiological meaning of this concentration gradient deserves further investigation.

Infusions of high amounts of unlabelled TG lead to a rapid increase in plasma TG values, which maintained a steady state from 60 min after starting the experiment until the end at 120 min. The rapid decay of plasma TG after cessation of TG application is due to a great deal to a rapid metabolism of the conjugate. Within 24 h after beginning of the TG infusion only 33% of the administered amount is excreted as TG in the urine. This is in accordance with earlier reported results obtained with small amounts of labelled TG[8].

The observation that intravenous infusions of HCG to normal males lead only to a significant increase in plasma T but not in TG concentrations, is surprising and cannot be explained sufficiently. However, preliminary observations in females infused with different amounts of T showed that the administration of 2000 μg resulted in a significant increase in plasma TG, whereas the infusion of 1000 μg did not.

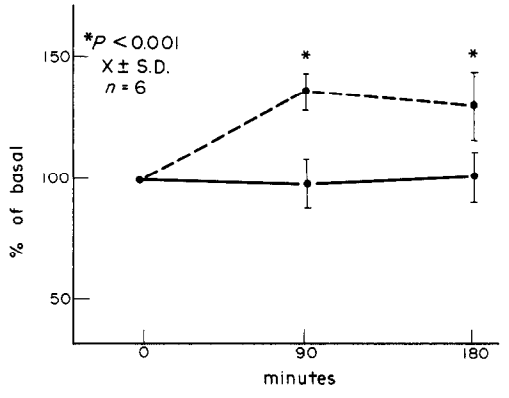

Fig. 5. Plasma testosterone (dotted line) and TG (full line), expressed in percentage of the basal values, during an infusion of 1000 IU HCG.

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