TEMPORAL VARIATIONS OF TESTOSTERONE LEVELS IN THE PERIPHERAL BLOOD PLASMA OF MEN


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SUMMARY

Testosterone was measured in the peripheral blood plasma of normal men by radioimmunoassay. The results were analysed to test for the possible existence of a circadian rhythm, for fluctuations superimposed on any such rhythm, and for day-to-day variations. Unequivocal evidence of a circadian rhythm was found in all but one of the subjects studied and the cycle appeared to be accompanied by a series of fluctuations of lower amplitude lasting for 1–2 h. Samples taken from the same subjects on consecutive days showed marked variation between days, but no regular cyclic pattern. The possible existence of such a cycle is not, however, eliminated. On the basis of all the data obtained a regime is suggested for the collection of blood samples from individuals whose androgenic status is to be assessed.

INTRODUCTION

A study of the temporal variations in plasma testosterone concentrations in apparently normal healthy men is a necessary preliminary to a study of testosterone secretion under experimental conditions, and the following specific aspects would appear to be significant and were investigated: (i) the average concentration of testosterone in the plasma in any individual throughout the day, and the degree of variation between individuals, (ii) the possible occurrence of a circadian rhythm of plasma testosterone concentration, and if it exists its degree of reproducibility between individuals in terms of both its timing and amplitude, (iii) the possible occurrence of short-term fluctuations in plasma testosterone concentration and (iv) the degree of day-to-day variation within individuals. Determination of these variations is not only of clinical and experimental significance, but is also important to an understanding of the pituitary control of testicular function.

MATERIALS AND METHODS

All the subjects were apparently normal men between 20 and 30 years of age. A normal pattern of activity was maintained throughout the day, and the subjects went to bed and arose at their own accustomed times (approximately 24.00 and 08.00 h, respectively), usually sleeping normally. The few occasions when sleep
was disturbed are noted. In one study, subject GAL had been living on a remote island for 2½ weeks and had established a complete shift of 5 h in his rhythm of activity, sleeping between approximately 05.00 and 13.00 h.

When only one blood sample was to be taken, venipuncture of the antecubital vein was employed. For more regular sampling a cannula (‘Butterfly’, 19G, Abbott Laboratories) was implanted under local anaesthesia (Lignocaine, Pharmaceutical Manufacturing Co., U.K.) into a forearm vein. Samples (10 ml) were removed through a 19 g needle and the cannula immediately irrigated with heparinized normal saline. The dead space of the cannula was 0.2 ml, and was not taken into account. Daily, blood samples were taken at the following times: SFD, 09.00 h; NM, 19.00 h; PHR, 12.00 h.

All glassware was soaked overnight in detergent (Decon), scrubbed and thoroughly washed in tap water followed by acetone. Water was de-ionized and double distilled. General reagents were, unless otherwise stated, of Analar grade obtained from BDH Chemicals, Poole, and solvents were redistilled. Freund’s complete adjuvant was obtained from Difco Laboratories, Detroit 1, Michigan, U.S.A. and 2-ethoxy-6,9-diaminoacridine lactate (Rivanol) was purchased from Koch–Light Laboratories, Colnbrook. Phosphate-buffered saline with Merthiolate (PBSM) consisted of 0.1 m-phosphate buffer (pH 7.0) containing sodium chloride (0.9 %, w/v) and Merthiolate (0.1 %, w/v); 0.1 % and 0.5 % bovine serum albumin (BSA) consisted of PBSM containing BSA (0.1 % or 0.5 %, w/v) respectively. Charcoal (0.5 g) (Norit A, Sigma, Kingston-upon-Thames) was suspended in PBSM (200 ml) containing dextran T-40 (5 mg) (Pharmacia, Uppsala, Sweden). Testosterone-1,2,6,7-tetratritiated (sp. act. 100 Ci/mmol) was obtained from The Radiochemical Centre, Amersham. Toluene scintillant consisted of 2,5-diphenyloxazole (PPO) (3 g) and 1,4-di[2-(5-phenyloxazolyl)] benzene (300 mg) in toluene (1 l), and dioxan scintillant, PPO (5 g) and naphthalene (100 g) dissolved in dioxan (1 l). The dioxan was previously purified by refluxing for 2 h with sodium metal followed by redistillation.

**Testosterone antibody**

Testosterone-3-O-carboxymethoxyloxime–bovine serum albumin conjugate (T-BSA) was prepared by the method of Erlanger, Beiser, Borek, Edel & Lieberman (1967). The conjugate was injected into five adult male Californian rabbits as follows. An initial dose of T-BSA (2 mg/rabbit) was dissolved in 1 ml saline (0.9 %, w/v) and emulsified with 1 ml Freund’s complete adjuvant. This was further emulsified in 1 ml aqueous ‘Tween 80’ (1 %, w/v) and the resultant 3 ml emulsion divided between two injections (1 ml) into the thigh musculature and four dorsal subcutaneous injections (0.25 ml). Booster doses (1 mg) were given similarly at monthly intervals thereafter. Blood was removed from the marginal ear vein 10 days after the second and subsequent booster doses. The antiserum used in this study was coded B3R3FT meaning that this was the blood obtained after the third injection (B3) into rabbit 3 (R3), was fully treated (F), i.e. with BSA and Rivanol, as described by Exley, Johnson & Dean (1971), and was raised against testosterone (T). The cross-reactions of various steroids which might compete with testosterone for a binding site on the antibody were calculated according to the method of Abraham (1969) and are shown in Table 1.
Radioimmunoassay

Duplicated plasma samples (100 µl), sodium hydroxide (0.1 M, 100 µl), tritiated testosterone recovery standard (2400 d.p.m. in 50 µl methanol) and water (750 µl) were all extracted with ether (2 ml) in stoppered test-tubes (B 10, Quickfit). Duplicate blanks, containing an equivalent volume of water, were extracted in parallel with the plasma samples. The aqueous phase was then discarded, and the ether phase washed with water (2 x 1 ml). The ether was evaporated to dryness under a stream of nitrogen in a water-bath at 40 °C. The efficiency of extraction was assessed by reconstituting the extracts in 500 µl methanol, 100 µl of which were removed, dried and counted in toluene scintillant. A standard curve consisting of 0, 0.25, 0.5 and 1.0 ng testosterone in duplicate was also prepared, after which tritiated testosterone

Table 1. Cross-reactions of various steroids with antiserum B3R3FT

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Cross-reaction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>100.0</td>
</tr>
<tr>
<td>5α-Androstan-3α,17β-diol</td>
<td>0.1</td>
</tr>
<tr>
<td>5α-Androstan-3α-ol-17-one</td>
<td>0.01</td>
</tr>
<tr>
<td>5α-Androstan-3β-ol-17-one</td>
<td>0.05</td>
</tr>
<tr>
<td>5α-Androstan-17β-ol-3-one</td>
<td>27.0</td>
</tr>
<tr>
<td>5β-Androstan-17β-ol-3-one</td>
<td>9.0</td>
</tr>
<tr>
<td>4-Androstene-3,17-dione</td>
<td>0.01</td>
</tr>
<tr>
<td>5-Androstene-3β,17β-diol</td>
<td>0.1</td>
</tr>
<tr>
<td>5-Androstene-3β-ol-17-one</td>
<td>0.05</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Cyproterone acetate</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Oestrone</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Oestradiol</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Oestriol</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Progesterone</td>
<td>0.015</td>
</tr>
</tbody>
</table>

(15000 d.p.m. = 30 pg in 50 µl methanol) was added to all the tubes which were then blown down to dryness again. Antibody diluted 2500 : 1 in 200 µl 0.1 % BSA was added to each tube which was then equilibrated overnight at 4 °C. After equilibration, the tubes were placed in an ice bath and 100 µl 0.5 % BSA were added, followed by 1 ml charcoal. After a 10 min equilibration, the tubes were centrifuged (2000 g for 10 min at 4 °C) and 1 ml supernatant was removed and counted in dioxan scintillant. The reciprocal of the fraction of the tritiated testosterone bound was then plotted against the mass of non-radioactive testosterone present. The same factor was also calculated for the unknown samples and the mass of testosterone present read from the standard curve. The results were corrected for the blank and the efficiency of extraction and expressed as ng testosterone/100 ml plasma.

RESULTS

Testosterone assay

Assay blanks ranged from 5 to 45 pg (mean 30 pg). The intra-assay precision was assessed by measuring each of various samples ten times within a single assay and the inter-assay precision was assessed by measuring similar samples in duplicate in five separate assays. The results are shown in Table 2. The specificity was tested by
comparing the results from ten samples measured by the assay with and without the inclusion of a chromatographic purification. This consisted of silica gel thin-layer chromatography (CT, Reeves Angel, 0·25 mm thickness) using the solvent system ether:chloroform (1:1, v/v), which separates testosterone from 5z-dihydrotestosterone. Specificity, expressed as the ratio of the results with and without chromatography, is also shown in Table 2; specificity generally ranged between 90 and 100% with a mean of 95%.

Table 2. The precision, accuracy and specificity of radioimmunoassay used in the present study

<table>
<thead>
<tr>
<th>Precision</th>
<th>Inter-assay precision</th>
</tr>
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<tbody>
<tr>
<td>Sample number</td>
<td>Testosterone in sample (pg)</td>
</tr>
<tr>
<td>1</td>
<td>44</td>
</tr>
<tr>
<td>2</td>
<td>122</td>
</tr>
<tr>
<td>3</td>
<td>481</td>
</tr>
<tr>
<td>4</td>
<td>610</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Accuracy</th>
<th>Testosterone added (pg)</th>
<th>Testosterone measured (pg)</th>
<th>Increment (pg)</th>
<th>Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>250</td>
<td>75</td>
<td>335</td>
<td>260</td>
<td>1·04</td>
</tr>
<tr>
<td>500</td>
<td>610</td>
<td>535</td>
<td>1·07</td>
<td></td>
</tr>
<tr>
<td>750</td>
<td>880</td>
<td>805</td>
<td>1·07</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td></td>
<td>1·06</td>
</tr>
</tbody>
</table>

Specificity:

Mean: 95 ± 4 (± 1 s.d.)%  Range: 89–103%

Circadian cycle and diurnal mean

Hourly levels of plasma testosterone in seven men over a 24 h period are shown in Fig. 1. The diurnal means based on these data are also shown, together with averages for the group illustrated in 2 h blocks. It can be seen that all but one of the men showed similar cycles, and that the averages demonstrate a simple cycle, with a peak at between 06.00 and 08.00 h and a low point between 20.00 and 22.00 h.

Statistical analysis of the untransformed data may be biased towards the individuals with relatively high testosterone levels. Accordingly the data were first expressed as percentages of each individual’s own diurnal mean, and the sets of values obtained were then pooled into 2 h blocks to give one average 24 h pattern. All the data in Fig. 1 were included in this calculation, except those obtained when GAL was under the influence of a 5 h time shift, and the blocks were compared by t-tests.

The results showed first that the lowest period of the cycle, namely that starting at 20.00 h, differs significantly from each of the three periods constituting the peak of the cycle, that is 04.00, 06.00, 08.00 h (P < 0.001). Secondly, the other two points which constitute the low part of the cycle, 18.00 and 22.00 h, also differ significantly.
Plasma testosterone levels in men

Fig. 1. Plasma testosterone concentration, measured at hourly intervals throughout the day in seven normal men. The broken line indicates the diurnal mean; downward pointing arrows, the evening minimum and upward pointing arrows, the morning maximum.
from each of the three high periods ($P < 0.05$). There were no statistically significant differences ($P > 0.05$) within the high or within the low periods.

**Short-term fluctuations**

Fig. 2 shows testosterone levels in three subjects, measured at 20 min intervals over a period of 6 h. A number of irregular fluctuations are demonstrated including some particularly large fluctuations (equivalent to 30–40 % of the individual’s diurnal mean) lasting for 1–2 h.

![Graph of plasma testosterone concentrations](image)

Fig. 2. Plasma testosterone concentrations, measured at 20 min intervals over a period of 6 h, in three men.

**Day-to-day variations**

From the results of daily blood samples taken from three subjects (Fig. 3) it was apparent in all four investigations that considerable day-to-day variations occurred. The coefficient of variability within each investigation was PHR1 15 %, PHR2 14 %, SFD 14 %, NM 23 %.
DISCUSSION

Methodology

Since large numbers of testosterone analyses were needed in the present study, an assay was required which was rapid, precise, accurate and reasonably specific. Chromatographic purification was omitted since speed was essential and any form of chromatography is time-consuming. Problems of specificity were not anticipated since the only significantly cross-reacting steroid, 5α-dihydrotestosterone, is present in human male plasma in much lower concentrations than those of testosterone (Tremblay, Foley, Corvol, Park, Kowarski, Blizzard, Jones & Migeon, 1972). Since the variability was inversely proportional to the quantity of testosterone being measured, the volume of plasma was increased for samples containing very low concentrations of testosterone. Results of the assessment of specificity, as well as those of precision and accuracy, were considered to be adequate for the kind of work being undertaken.

Diurnal mean

The term ‘diurnal mean’ is used to indicate the average concentration of testosterone in a group of plasma samples taken at regular intervals throughout a 24 h period in a single subject. A number of investigations have reported values for plasma testosterone concentrations throughout the day in groups of men of varying age, using different types of assay (Dray, Reinberg & Sebaoun, 1965; Resko & Eik-Nes, 1966; Crafts, Llerena, Guevara, Lobotsky & Lloyd, 1968; Gordon, Spinks, Dulmanis,
The results of a number of studies on testosterone concentrations in blood samples taken at regular intervals throughout the day would appear to be consistent with a circadian cycle of plasma testosterone concentrations, showing a maximum in the early morning and a minimum in the late evening (Dray et al. 1965; Resko & Eik-Nes, 1966; Crafts et al. 1968; Saxena et al. 1969; Neischlag & Ismail, 1970; Faiman & Winter 1971; Okamoto et al. 1971; Rose et al. 1972). However, the poor synchronization of these cycles, together with other reports that there is no cyclic variation in plasma testosterone (Gordon et al. 1968; Boon et al. 1972; Alford et al. 1973), have previously left the matter open to doubt.

There are two main difficulties to be avoided when planning experiments designed to investigate the existence of such a rhythm. The first is that if short-term fluctuations of lower amplitude are imposed on an overall testosterone cycle, samples taken at intervals of 3 h or more may reflect these short-term changes and obscure an overall diurnal cycle, thus possibly explaining the inconsistent data reviewed above. It has been shown by Murray & Corker (1972) and Naftolin, Judd & Yen (1973) and by the present report that brief fluctuations of this kind do occur. And it is noteworthy that out of the three reports in which no cycle was found, two (Gordon et al. 1968; Boon et al. 1972) used sampling periods of 3 and 6 h respectively. By contrast, when samples were taken at 1.5 h intervals (Rose et al. 1972) a clear 24 h cycle was revealed. In the present work, by taking samples at hourly intervals throughout the day, it has been possible to distinguish between short-term fluctuations and overall changes in testosterone levels.

The second problem arises from the influence of disturbance to the sleep pattern of the subjects. It seems that plasma testosterone concentration is influenced by periods of sleep (Evans, Maclean, Ismail & Love, 1971), and in some of the earlier studies (Boon et al. 1972; Alford et al. 1973) where no diurnal rhythm was shown, sleep was disturbed.
Plasma testosterone levels in men

As shown in Fig. 1 all but one of the subjects in the present study showed a circadian cycle of plasma testosterone concentration. However, two of these subjects, GAL and NM, were unavoidably deprived of sleep during part of the night and both of these occasions were associated with a further minimum in their testosterone levels. During the study in which GAL had established a 5 h time shift in his pattern of waking and sleeping, his minimum at 03.00 h and maximum at 12.00 h are comparable with a minimum at 10.00 h and a maximum at 07.00 h in the other subjects. The timing of the cycles is very reproducible, all the cycles showing maxima and minima within approximately 2 h of the average times for the group. The amplitude of the cycles is, however, more variable.

Short-term fluctuations

From the results shown in Fig. 2, it would appear that short-term fluctuations in the level of plasma testosterone are a normal feature in men. The extent of these fluctuations is considerable, and samples taken at long intervals may give misleading results. The extent and duration of the fluctuations demonstrated are consistent with those found by Murray & Corker (1972) and Naftolin et al. (1973). It would appear therefore that in any investigation of factors which may affect plasma testosterone concentrations it is clearly inadequate to demonstrate the occurrence of fluctuations in testosterone concentration alone. The extent and timing of such fluctuations must be distinguishable from spontaneous changes.

Day-to-day variations

A report (Fox, Ismail, Love, Kirkham & Loraine, 1972) on a single subject from whom samples were taken at the same time every day for two periods of 7 weeks, showed considerable variation between days, with little evidence of any regular multi-day cycle. Changes in plasma testosterone concentration in this subject were, however, associated with sexual activity rather than being entirely intrinsic. Accordingly, a study was carried out in three unmarried men who, although most probably exposed to some erotic stimuli, were without sexual partners at the time of the study. The results (Fig. 3) showed marked changes in plasma testosterone concentrations from day-to-day in all three subjects.

Although a regular multi-day cycle has been demonstrated for urinary oestrone and 17-oxosteroid excretion in men (Exley & Corker, 1966), no such cycle was found for urinary testosterone excretion (Corker & Exley, 1968) and again in the present study no consistent multi-day cycles were apparent in plasma testosterone concentrations. However, it is possible that even if such a cycle did exist, it might not be apparent in the relatively small series of samples presented here and, furthermore, single daily plasma testosterone values may not give an accurate impression of that day’s overall testosterone secretion.

Regime for future blood sampling

In order to assess a subject’s overall androgenic status it is essential to take blood samples on several days since no one day can be relied upon to be typical. Samples must also be taken at more than one time of the day since the circadian cycle is of variable amplitude and androgen levels may be unusually high or low at one time.
of the day although the diurnal mean value may be normal. In practice, the number of samples that can be taken and analysed is limited and a compromise solution is unavoidable. For our own work we have selected a regime in which three samples were taken at 10.00 h and three at 16.00 h, all on different but not widely separated days.

In conclusion, it has been demonstrated that in the majority of men who are fully active during the day and who enjoy a normal night’s sleep, there is a circadian cycle in plasma testosterone levels with a maximum in the early morning and a minimum in the late evening. Although the timing of these cycles is very similar in different subjects, their amplitude is very variable and the cycle has a series of brief and relatively small fluctuations superimposed upon it. Testosterone levels in blood samples taken daily at the same time of day showed marked variability, but no cyclic character.

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REFERENCES


