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PLASMA TESTOSTERONE IN HEALTHY MEN: STUDY OF NYCTEROHEMERAL AND DAILY VARIATION USING GAS–LIQUID CHROMATOGRAPHY AND RADIOIMMUNOASSAY

M. D. MANSFIELD, I. F. SOMMERVILLE and
N. J. BLACKLOCK

Department of Biochemical Endocrinology, Chelsea Hospital for Women, Institute of Obstetrics and Gynaecology, London, S.W.3, and Department of Urology, Royal Naval Hospital, Haslar, Gosport, Hampshire, England

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SUMMARY

Plasma testosterone has been determined by gas–liquid chromatography or radioimmunoassay in 557 plasma samples from 167 healthy men. Samples were collected at 4 h intervals over a period of 24 h or between 09.00 and 10.00 h on twelve consecutive days. The results obtained establish the nature and extent of the nycterohemeral variation in healthy men and exhibit a maximum at about 06.00 h and a low plateau from 14.00 until 22.00 h. The results indicate that more than one sample should be taken in attempts to establish the concentration of plasma testosterone for diagnostic purposes or for monitoring the effect of therapy. Furthermore, these samples should be taken at the same time each day and preferably during the plateau.

INTRODUCTION

Determination of the blood production rate of testosterone in terms of the metabolic clearance rate and the mean plasma concentration in systemic blood, indicates that the production of the androgen is more than 20-fold higher in men than women and that the secretion rate is more than 50-fold higher in men [1, 2]. This direct approach to the study of androgen secretion is not suitable for clinical application and, as an index of androgen production, the determination of the hormone in peripheral venous plasma appears to offer the most practical alternative, although the difference between the mean values in men and women are considerably lower than the difference in the secretion rates [3]. However, before using this approach to the study of androgen production in pathological conditions, it was essential to establish physiological variations in plasma testosterone concentrations and a review of the literature indicates that the nature and extent of such variations has not been entirely established by the application of an acceptable technique to an adequate sample population. Thus, the application of a number of different analytical methods and experimental designs has produced conflicting evidence as to whether there is circadian or random variation or as to whether such variations can be minimized by a collection of blood samples at the same time each day [4–16]. The present study is an attempt to resolve some of these reported differences by the application of a gas–liquid chromatographic method and a radioimmunological technique to a large group of healthy volunteers.

MATERIALS AND METHODS

Subjects studied and timing of samples

Samples from 167 healthy men have been studied by either method as shown (Table 1). The subjects were members of the Royal Navy with a mean ± standard deviation for age of 33 ± 6 y, height of 69 ± 2 inches and weight of 176 ± 28 lbs. In the study of 4 h samples by gas–liquid chromatography, the samples were drawn at 11.00, 15.00, 19.00, 23.00, 03.00, 07.00, 11.00 h and for the larger series determined by radioimmunoassay the samples were drawn at 10.00, 14.00, 18.00, 22.00, 02.00, 06.00, 10.00 h. For the study of daily variations, the samples were collected between 09.00 and 10.00 h daily for 12 days.

Approximately 10 ml peripheral venous blood was withdrawn into a sterile syringe; transferred into a blood collection tube containing lithium heparin and plastic beads; mixed and centrifuged immediately. The plasma was decanted into a plastic tube; deep frozen and transported from the Naval Establishment to the

Table 1. Comparative study of plasma testosterone in normal male subjects

No. of subjects	No. of samples	Time of sampling	Assay
41	41	Random	g.l.c.
47	47	Random	g.l.c.
9	63	Every four hours	g.l.c.
12	12	Random	RIA
12	12	Random	RIA
34	238	Every four hours	RIA
12	144	Every day for twelve days	RIA
Total No. of Subjects	Total No. of Assays		
167	557		

department in London over dry-ice in a metal vacuum container.

Analytical Procedures

a. Gas–liquid chromatography. The procedure involves the addition of testosterone-4-14C as labelled internal standard, mild saponification with sodium hydroxide, extraction with diethyl ether and preliminary purification on thin-layer chromatography. After formation of the heptafluorobutyrate derivative, the extracts are rechromatographed on silica gel, followed by gas–liquid chromatography using a solid injection technique and Nickle-63 electron-capture detection. A second internal standard (20α-dihydroprogesterone heptafluorobutyrate) is added before taking the aliquot for gas chromatography and the remainder of the sample is used for liquid scintillation counting [17].

b. Radioimmunoassay. This method is more rapid and a much larger sample work-load can be processed at one time. The plasma is extracted with diethyl ether, after the addition of a [1, 2, 6, 7-^{3}H]-testosterone internal standard and the extract chromatographed on a micro-column of Sephadex-LH20. Standards and unknowns are equilibrated with antiserum to testosterone-3-oxime–bovine serum albumin and the unbound steroid removed with Dextran–coated charcoal. The supernatant containing the bound fraction is then decanted into a vial for liquid scintillation counting [18]. The technical details and evaluation of the method have been reported elsewhere [17, 18].

In an attempt to overcome the problem of inter-assay variation and to establish a quality control, samples were repeated in subsequent batches to check on variation in results. The samples from individual subjects were all assayed at the same time and compared with the same set of standards. In the case of the daily variation all the samples were assayed simultaneously and compared with the same set of standards. This point is emphasised in view of the individual variations observed (Table 5) despite collection of the samples at the same time each day. The inter-assay coefficients of variation for the two methods are 12·4% for gas–liquid chromatography and 7·1% for radioimmunoassay.

RESULTS

The results obtained in a preliminary series of random samples are summarized (Table 2). There is no statistical difference between the values obtained by gas-chromatography or radioimmunoassay ($P = > 0{\cdot}5$).

The results obtained by gas-chromatography in 4 h samples collected from 9 volunteers are shown (Table 3). The lowest values were those observed at 19.00 and 23.00 h with the higher values at 07.00 and 11.00 h. In view of the individual variations in concentration between subjects these results were also expressed as a percentage of the subject mean when the two values (mean ± S.D.) of the 11.00 h samples were 111·0 ± 13·6 and 117·0 ± 17·9 respectively and the 07.00 h value was 108·7 ± 22·7 whereas the lower values, to which reference has been made, were 83·8 ± 14·0 at 19.00 and 80·8 ± 23·0 at 23.00 h.

The results obtained in the more extensive study by radioimmunoassay are shown (Table 4). The lowest values were those at 14.00, 18.00 and 22.00 h and there was again evidence of a sharp rise overnight to a maximum value at 06.00 h. When these results were expressed as percentages of the subject mean, the 10.00 h samples gave values of 103 ± 19 and 107 ± 15 respectively and the value at 06.00 h was 119 ± 20. In

Table 2. Comparative study of plasma testosterone in random samples by gas–liquid chromatography and radioimmunoassay

	Testosterone ng/100 ml plasma		
No. of subjects	Mean ± S.D.	Range	Assay
41	528 ± 261	238–1195	g.l.c.
47	573 ± 245	174–1282	g.l.c.
12	573 ± 190	339–975	RIA
12	576 ± 208	300–890	RIA

Table 3. Nycterohemeral variation of plasma testosterone—absolute values using gas–liquid chromatography

Subject	Subject mean	Testosterone ng/100 ml plasma: g.l.c. Time 11.00	15.00	19.00	23.00	03.00	07.00	11.00
1	164	181	—	121	63	252	179	190
2	328	361	258	189	233	342	458	455
3	253	348	174	175	202	283	269	319
4	366	444	684	311	257	166	214	483
5	636	734	676	608	643	530	665	599
6	513	509	304	437	566	682	595	500
7	521	566	516	513	472	478	603	501
8	478	516	382	454	495	448	473	579
9	330	293	366	311	206	248	428	457
Mean ± S.D.		439 ± 164	420 ± 189	347 ± 167	349 ± 199	381 ± 165	432 ± 177	454 ± 127

Table 4. Nycterohemeral variation of plasma testosterone—absolute values using radioimmunoassay

Subject	Subject mean	Testosterone ng/100 ml plasma: RIA Time 10.00	14.00	18.00	22.00	02.00	06.00	10.00
1	275	265	200	240	195	330	395	300
2	247	230	165	250	200	320	250	315
3	350	330	410	315	260	440	385	310
4	296	300	280	210	245	250	420	365
5	336	300	350	365	265	355	335	385
6	484	485	390	310	410	520	685	590
7	549	580	430	505	485	625	580	640
8	304	350	305	300	295	320	220	335
9	265	245	245	265	230	290	335	245
10	219	160	125	200	245	235	300	265
11	201	230	170	160	185	175	270	220
12	267	275	160	295	190	235	355	360
13	94	110	85	115	100	90	75	85
14	245	215	290	160	135	365	310	240
15	281	440	230	235	340	220	250	255
16	404	395	290	380	380	415	525	440
17	213	140	225	170	125	315	260	255
18	423	305	295	345	525	440	630	420
19	269	270	225	250	230	300	340	270
20	281	260	165	260	255	405	340	—
21	364	375	285	310	350	395	435	400
22	461	615	325	280	360	570	615	460
23	376	400	280	320	320	420	450	440
24	380	415	285	310	315	395	470	470
25	240	225	300	240	205	205	225	280
26	231	280	180	225	230	135	365	205
27	214	215	200	235	185	185	250	230
28	306	300	435	285	210	235	330	345
29	465	475	485	395	300	635	475	490
30	197	240	170	245	170	195	190	170
31	209	170	125	210	190	235	265	265
32	190	240	155	100	105	350	265	115
33	259	296	175	190	165	355	345	285
34	128	185	160	85	90	140	110	—
Mean ± S.D.		303 ± 118	253 ± 99	258 ± 87	250 ± 104	326 ± 135	354 ± 141	327 ± 125

Table 5. Daily variation of plasma testosterone—absolute values using radioimmunoassay

Subject	Subject mean	Testosterone ng/100 ml: RIA Day 1	2	3	4	5	6	7	8	9	10	11	12
1	780	835	825	730	775	805	710	845	750	795	785	750	760
2	608	625	515	700	600	525	760	420	545	515	695	710	695
3	748	875	720	805	800	890	870	755	610	740	675	535	710
4	507	595	580	620	695	475	455	580	440	370	455	415	405
5	388	345	415	290	410	335	405	405	375	430	445	435	375
6	636	760	725	555	555	595	435	725	725	725	800	490	550
7	406	365	300	400	535	440	380	405	435	460	460	380	315
8	297	440	410	340	340	395	230	315	265	185	175	245	230
9	360	315	355	385	410	340	360	325	410	375	365	340	340
10	312	330	325	305	350	305	290	290	290	285	345	295	340
11	362	395	455	485	370	360	410	270	300	220	390	350	350
12	226	300	165	155	170	290	210	290	245	245	250	250	145
Mean ± SD		515 ± 213	482 ± 197	481 ± 202	501 ± 195	480 ± 195	460 ± 211	469 ± 204	449 ± 174	445 ± 211	487 ± 206	433 ± 164	435 ± 198

contrast, the subject mean percentage values at 14.00, 18.00 and 22.00 h were 86 ± 20; 88 ± 16; and 84 ± 16 respectively. When the values in the evening (20.00 h) were compared by the "*t*" test with those in the morning (06.00 h) the difference was highly significant ($P = <0{\cdot}001$).

The results of samples collected daily for 12 days are shown (Table 5). When these are expressed as percentages of the subject mean, the results of the 12 consecutive days were as follows: 111 ± 18; 103 ± 19; 102 ± 19; 107 ± 17; 104 ± 16; 97 ± 16; 100 ± 17; 97 ± 12; 94 ± 19; 103 ± 17; 94 ± 14; 91 ± 14. Thus the over-all mean values show a fall in plasma concentration over the 12 days of study but it will be apparent from the individual results that this is due, in part, to a marked fall in 2 men (Subjects 4 and 8) and to the degree of variation which occurred from day to day in most men.

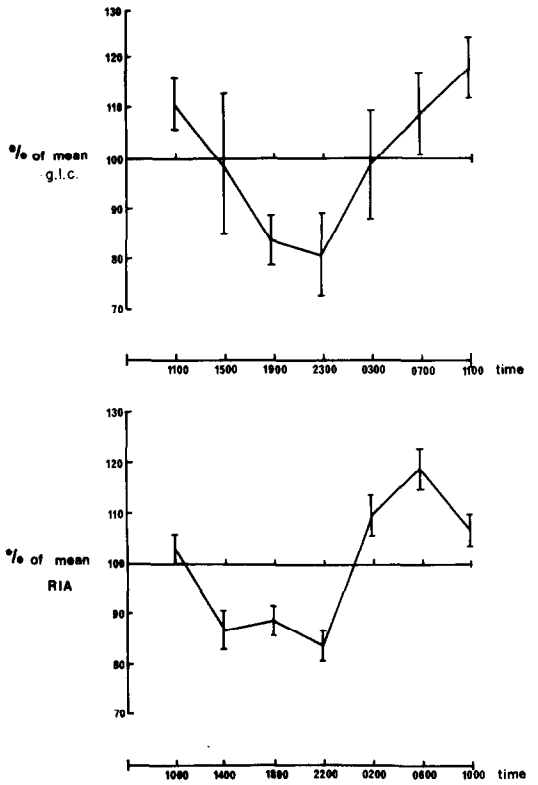

Fig. 1. Analysis of 4 h samples by g.l.c. (63 assays in 9 men) and R.I.A. (238 assays in 34 men). The concentration of plasma testosterone is expressed as a % of the mean value for each man.

DISCUSSION

Close correlation between the results obtained in random samples by either gas–liquid chromatography or radioimmunoassay and a previous comparison of the two methods when applied to the same samples of male plasma [18] yields indirect evidence in favour of the specificity of the radioimmunological method. However, a small over-estimation is anticipated due to the fact that 5α-dihydrotestosterone is not removed by the column of Sephadex LH20 and there is an appreciable cross-reaction with this compound using the antiserum to testosterone-3-oxime–BSA. The mean values obtained by either method agree with those reported by other workers using techniques based upon the principles of double isotope derivative formation, gas–liquid chromatography and competitive protein binding [3].

The results of 4 h sampling are summarized in Fig. 1 and the frequency distribution of the results is illustrated in Fig. 2. This figure shows the number of samples with their highest (drawn above the line) and lowest (below) value, at each sampling time: e.g. 16 men had their highest plasma testosterone concentration at 06.00 as shown by the radioimmunoassay study. Some of these results have been the subject of a preliminary report [19].

It is apparent that the changes are more significant in the study by radioimmunoassay of the 34 subjects. Thus applying the "*t*" test the difference between the evening and early morning samples in the smaller series is expressed by a *P* value of $<0{\cdot}5$ whereas the corresponding difference in the larger series gives a *P* value of $<0{\cdot}001$. Furthermore, the nycterohemeral variation is clearly demonstrable in the frequency distribution of the large series but is not apparent for the smaller series (Fig. 2). This emphasizes the importance of studying an adequate number of samples and it should be noted that the most extensive studies previously reported in the literature have been limited to 4 h samples in 12 subjects [10] and a recent study involving the investigation of 13 healthy men with 8 h samples in 10 subjects and 2 h in the remainder [15]. The nycterohemeral variation in the 34 men involves significantly low values between 14.00 and 22.00 h (75% of the maximum) and the coefficients of variation between the mean values at 4 h intervals range from 7·7 to 51·1% (mean 23·4%). With regard to the degree of variation observed in samples collected at the same time each day over 12 days, the coefficients of variation from day to day range from 5·4 to 30·0% (mean 16·3%). Thus, the mean coefficient is considerably lower than that for 4 h sampling and approximately twice the inter-assay coefficient of the radioimmunological method. Furthermore, the variations observed from day to day between samples taken between 09.00 and 10.00 h, almost certainly reflect the fact that the

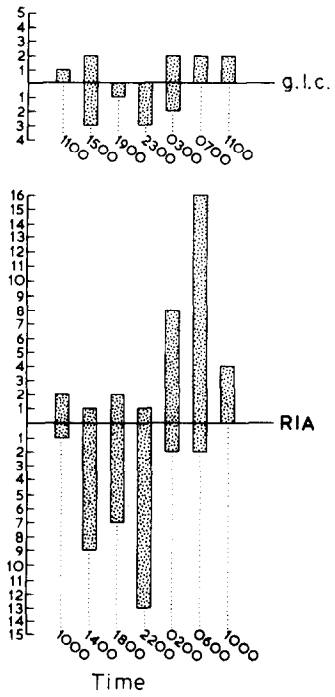

Fig. 2. Frequency distribution of the concentration of plasma testosterone in the two groups illustrated in Fig. 1. (9 subjects by g.l.c. and 34 subjects by RIA).

plasma concentration is usually rising at that time and at a variable rate from one individual to another.

On the other hand, reference has already been made to the considerable differences in the trend of concentrations in individual subjects and this is illustrated in Fig. 3. It is probable that these reflect relatively long-term changes in androgen production associated with such factors as the frequency of intercourse but it may be concluded that a more valid assessment of plasma testosterone concentration can be based upon several samples taken at the same time each day than upon random samples taken at different times of the day. For the investigation of patients with suspected pathology or in evaluating the effect of therapy, it would appear to be advisable to take blood samples during the plateau which lasts throughout the afternoon and early evening.

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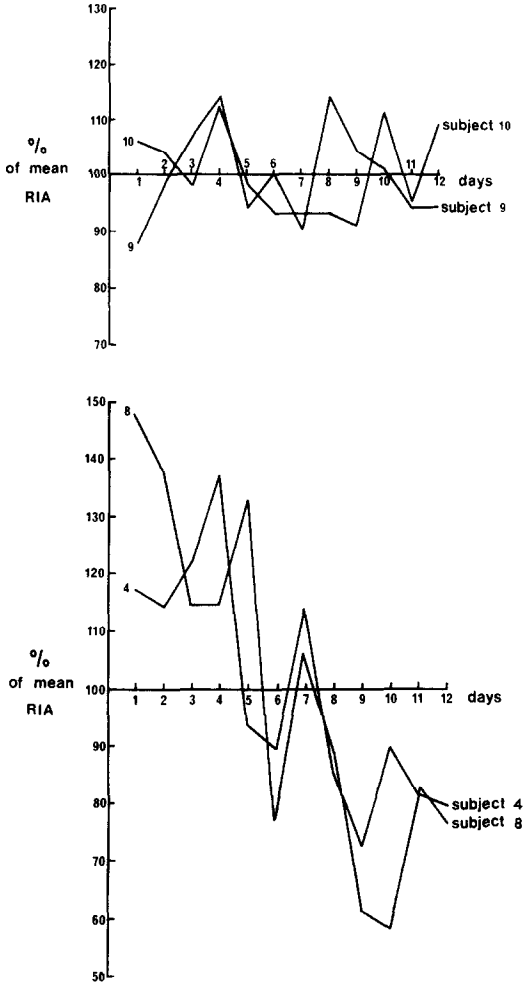

Fig. 3. Plasma testosterone concentrations (expressed as % of subject mean) in samples taken at same time each day for 12 days. Four samples from Table 5 are illustrated.

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