

# Determination of Testosterone Production Rates in Men and Women Using Stable Isotope/Dilution and Mass Spectrometry\*

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## ABSTRACT

Production rates of testosterone were determined in healthy men and women during the follicular phase of their menstrual cycle using the stable isotope dilution technique and analysis by gas chromatography/mass spectrometry. In an initial series of experiments, 0.07 mg/h (men) or 0.01 mg/h (women) 1,2-*d*-testosterone was infused for 36 h. After an equilibration period of 12 h, blood samples were obtained at 20-min intervals throughout 24 h. In men, no diurnal rhythmicity of testosterone production was observed, whereas in women, testosterone production rates were largest from 0400–1200 h. In a second series of experiments, the infused dose of 1,2-*d*-testosterone was reduced to 0.015 mg/h (men) and 0.0001 mg/h (women), respec-

tively. Blood samples were obtained at 20-min intervals during the last 12 h (0800–2000 h) of the observation period. In accordance with results obtained by others using radioactive tracers, estimated production rates were  $147 \pm 31 \mu\text{g/h}$  ( $3.7 \pm 2.2 \text{ mg/day}$  in men) and  $1.8 \pm 0.6 \mu\text{g/h}$  ( $0.4 \pm 0.1 \text{ mg/day}$  in women). To reduce both the duration of the experiment and the number of samples to be processed, we subsequently demonstrated that similar production rates may be obtained when the equilibration period before blood sampling is reduced to 6 h and the period of blood sampling is reduced to 4 h. This protocol is suitable for clinical use in a routine setting to obtain analytically correct estimates of testosterone production *in vivo*. (*J Clin Endocrinol Metab* 82: 1492–1496, 1997)

**G**AS CHROMATOGRAPHY/MASS spectrometry (GC/MS) and stable isotope-labeled analogs of testosterone are used to determine concentrations (1–3) and MCRs of testosterone and its conversion rates into its main metabolic products (4–6). This technique is superior to the use of radioactive tracers, both analytically (7) and ethically. In the present series of experiments we used infusions of deuterium-labeled testosterone and analysis by GC/MS to determine the production rates of testosterone in healthy men and women and to establish a protocol suitable for clinical use in patients with potentially abnormal testosterone production rates.

## Materials and Methods

### Experimental protocol

Twelve healthy nonobese men, aged 22–34 yr, and 10 healthy nonobese women, aged 19–32 yr (in the follicular phase of the menstrual cycle), who had been carefully informed about the aims and the possible risks of the study gave their written consent to participate in 1 or several parts of this investigation. On the day of the experiments, an indwelling catheter was inserted into an antecubital vein, and 1,2-*d*-testosterone (in 500 mL 0.9% saline also containing 2 mL of the individual's own blood) was infused iv and continuously (Infusomat, Braun-Melsungen, Germany) until the end of the respective experimental protocol. At the beginning and end of each infusion, a sample of the infusate was obtained from the end of the infusion line to permit for correction of losses

by adsorption and determination of actual infusion rates by GC/MS analysis. After an equilibration period of 12 h (Exp 1 and 2) or 6 h (Exp 3), a second indwelling catheter was inserted into the contralateral arm, and blood samples (5 mL) were obtained at 20-min intervals until the end of the respective experimental protocol. Blood samples were subsequently pooled for periods of 4 h. In addition, one sample was pooled for the entire period of blood sampling (24, 12, or 4 h, respectively).

### Exp 1

Six healthy men and five healthy women participated in this protocol. The infusion of 1,2-*d*-testosterone was started at 0800 h (13 mL/h) to provide a theoretical infusion rate of 0.2 mg/h (men) or 0.04 mg/h (women), respectively. However, due to losses by adsorption, mean actual infusion rates during this protocol were only 0.07 mg/h (men) and 0.01 mg/h (women). Starting at 2000 h, blood samples (5.0 mL) were obtained at 20-min intervals for the next 24 h.

### Exp 2

Seven healthy men and seven healthy women participated in this protocol. The infusion of 1,2-*d*-testosterone (20 mL/h) was started at 2000 h to provide a theoretical infusion rate of 0.02 mg/h (men) or 0.0004 mg/h (women). However, due to losses by adsorption, mean actual infusion rates during this protocol were only 0.015 mg/h in six of the seven men. The seventh male volunteer received only one tenth of this dose, *i.e.* 0.0015 mg/h. In five of the seven healthy women, the mean actual infusion rate was 0.0001 mg/h. The remaining two women received an approximately 7-fold smaller dose (17 and 14 ng/h, respectively). Starting at 0800 h on the following morning, blood samples were obtained at 20-min intervals for the next 12 h.

### Exp 3

The five healthy men and five healthy women participating in this protocol had been part of Exp 2. The infusion of 1,2-*d*-testosterone (40 mL/h) was started at 0800 h with the attempted infusion rates as in Exp 2 (men, 0.02 mg/h; women, 0.0004 mg/h). Starting at 1400 h, blood samples were obtained at 20-min intervals for one 4-h period.

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**TABLE 1A.** Plasma concentrations of native T as determined by GC-MS and calculated production rates of T (2000–2000 h) in six healthy men**Exp 1**

Infusion rate of 1, 2-*d*-T (36 h; 13 mL/h):  
 Theoretical: 0.2 mg/h  
 Actual: 0.07 mg/h (mean), equivalent to 168 µg/24 h

	1	2	3	4	5	6	Mean ± SD
2000–2400 h							
T (ng/mL)	3.74	3.74	2.63	4.08	5.19	7.70	4.51 ± 1.76
PR T (µg/h)	232	47	84	105	63	72	101 ± 67
0000–0400 h							
T (ng/mL)	4.59	3.04	2.89	4.15	4.51	6.69	4.31 ± 1.37
PR T (µg/h)	130	51	84	71	63	61	76 ± 28
0400–0800 h							
T (ng/mL)	3.84	3.91	5.42	5.53	3.72	6.72	4.86 ± 1.22
PR T (µg/h)	89	51	95	76	63	69	74 ± 16
0800–1200 h							
T (ng/mL)	3.90	2.48	4.71	4.63	3.48	3.92	3.85 ± 0.82
PR T (µg/h)	111	42	108	71	62	55	75 ± 28
1200–1600 h							
T (ng/mL)	4.05	2.45	3.20	2.23	3.95	4.24	3.35 ± 0.86
PR T (µg/h)	114	43	83	95	50	54	73 ± 28
1600–2000 h							
T (ng/mL)	3.01	2.23	3.22	2.99	3.35	3.51	3.05 ± 0.45
PR T (µg/h)	88	40	99	63	43	49	64 ± 25
2000–2000 h (mean values calculated from above figures)							
T (ng/mL)	3.86	2.98	3.67	3.94	4.03	5.46	3.99 ± 0.81
PR T (µg/h)	127	46	92	80	57	60	77 ± 30
2000–2000 h (estimated from additional sample by GC-MS analysis)							
T (ng/mL)	3.80	2.95	3.72	3.95	3.97	5.36	3.96 ± 0.78
PR T (µg/h)	119	50	97	80	58	60	77 ± 33

**TABLE 1B.** Plasma concentrations of native T as determined by GC-MS and calculated production rates of T in seven healthy men**Exp 2**

Infusion rate of 1, 2-*d*-T (24 h; 20 mL/h)  
 Theoretical: 0.02 mg/h  
 Actual: 0.015 mg/h (mean of subjects 1–6; subject 7, 0.0015 mg/h)

	1	2	3	4	5	6	7	Mean ± SD
0800–1200 h								
T (ng/mL)	7.81	6.81	6.19	9.21	5.84	5.87	13.93	7.96 ± 2.90
PR T (µg/h)	149	115	161	329	56	266	76	165 ± 100
1200–1600 h								
T (ng/mL)	9.71	6.94	7.45	9.59	8.07	3.98	12.86	8.37 ± 2.76
PR T (µg/h)	91	108	164	247	72	336	63	154 ± 39
1600–2000 h								
T (ng/mL)	9.55	6.81	5.55	4.85	10.79	5.62	9.66	7.54 ± 2.40
PR T (µg/h)	95	92	160	188	86	328	67	145 ± 92
0800–2000 h (mean values calculated from above figures)								
T (ng/mL)	9.02	6.85	6.40	7.88	8.23	5.16	12.15	7.96 ± 2.24
PR T (µg/h)	112	105	162	255	71	310	69	155 ± 94
0800–2000 h (estimated by GC-MS from additional pooled plasma sample)								
T (ng/mL)	8.72	7.10	6.48	8.06	8.19	4.93	12.7	8.03 ± 2.43
PR T (µg/h)	111	103	150	241	70	281	71	147 ± 31

**TABLE 1C.** Plasma concentrations of native T as determined by GC-MS and calculated production rates of T in five healthy men during a 12-h infusion of 1,2-*d*

	1	2	3	4	5	6	7	Mean ± SD
1400–1800 h								
T (ng/mL)			8.61	5.91	9.77	5.78	9.76	7.97 ± 1.99
PR T (µg/h)			160	188	86	328	67	166 ± 103

**Materials**

All organic solvents were of high performance liquid chromatography grade and purchased from Baker Chemicals (Phillipsburg, NJ). Nonactive testosterone (17β-hydroxy-4-androsten-3-one) was obtained from Steraloids (Wilton, NH). Radioactive [<sup>3</sup>H]1,2,6,7-testosterone (SA, 100 Ci/mmol) and stable labeled 1,2-*d*-testosterone (isotopic enrich-

ment, 99.0%) were purchased from New England Nuclear (Boston, MA) and CIL (Andover, MA), respectively.

**Sample preparation and analysis by GC-MS**

Plasma samples (5.0 mL) supplemented with 20,000 dpm [<sup>3</sup>H]testosterone for later control of recovery and with 20 mL 0.5% trifluoroacetic

**TABLE 2A.** Plasma concentrations of native T as determined by GC-MS and calculated production rates of T in 5 healthy women  
**Exp 1**

Infusion rate of 1,2-D-T (36 h: 13 mL/H):  
Theoretical: 0.4 mg/h  
Actual: 0.01 mg/h (mean), equivalent to 0.24 mg/24 h

	1	2	3	4	5	Mean ± SD
2000–2400 h						
T (ng/mL)	0.184	0.100	0.150	0.124	0.124	0.136 ± 0.032
PR T (μg/h)	6	4	7	3	2	4.4 ± 2.1
0000–0400 h						
T (ng/mL)	0.164	0.110	0.147	0.125	0.162	0.142 ± 0.024
PR T (μg/h)	5	5	3	2	3	3.6 ± 1.3
0400–0800 h						
T (ng/mL)	0.146	0.106	0.138	0.160	0.175	0.144 ± 0.026
PR T (μg/h)	8	6	9	2	4	5.8 ± 2.9
0800–1200 h						
T (ng/mL)	0.208	0.097	0.142	0.090	0.145	0.136 ± 0.047
PR T (μg/h)	10	3	7	2	8	6.0 ± 3.4
1200–1600 h						
T (ng/mL)	0.117	0.107	0.107	0.126	0.176	0.127 ± 0.029
PR T (μg/h)	4	5	5	2	4	4.0 ± 1.2
1600–2000 h						
T (ng/mL)	0.174	0.076	0.089	0.110	0.186	0.127 ± 0.050
PR T (μg/h)	6	3	5	2	4	4.0 ± 1.6
0800–2000 h (mean values calculated from above figures)						
T (ng/mL)	0.166	0.099	0.129	0.123	0.161	0.136 ± 0.028
PR T (μg/h)	7	4	6	2	4	4.6 ± 1.9
0800–2000 h (estimated by GC/MS from additional pooled plasma sample)						
T (ng/mL)	0.185	0.102	0.129	0.121	0.161	0.140 ± 0.033
PR T (μg/h)	14	11	9	5	9	9.6 ± 1.5

**TABLE 2B.** Plasma concentrations of native T as determined by GC-MS and calculated production rates of T in seven healthy women  
**Exp 2**

Infusion rate of 1,2-d-T (24 h; 20 mL/h)  
Theoretical: 0.0004 mg/h  
Actual: 0.0001 mg/h (mean of patients 1–5, patient 6, 14 ng/h; patient 7, 14 ng/h)

	1	2	3	4	5	6	7	Mean ± SD
0800–1200 h								
T (ng/mL)	0.136	0.138	0.276	0.186	0.197	0.206	0.128	0.181 ± 0.053
PR T (μg/h)	1.0	2.1	2.3	2.8	2.5	2.0	1.3	2.0 ± 0.6
1200–1600 h								
T (ng/mL)	0.127	0.130	0.203	0.147	0.150	0.157	0.136	0.150 ± 0.026
PR T (μg/h)	0.9	2.9	2.1	2.4	1.6	1.6	1.0	1.8 ± 0.7
1600–2000 h								
T (ng/mL)	0.121	0.099	0.218	0.126	0.118	0.138	0.109	0.133 ± 0.040
PR T (μg/h)	1.0	2.1	2.3	2.0	1.8	1.5	0.9	1.7 ± 0.6
0800–2000 h (mean values calculated from above figures)								
T (ng/mL)	0.128	0.122	0.232	0.153	0.155	0.167	0.124	0.154 ± 0.038
PR T (μg/h)	1.0	2.2	2.2	2.5	2.0	1.7	1.0	1.8 ± 0.6
0800–2000 h (estimated by GC-MS from additional pooled plasma sample)								
T (ng/mL)	0.127	0.121	0.235	0.160	0.161	0.171	0.118	0.156 ± 0.041
PR T (μg/h)	1.0	2.2	2.2	2.5	2.0	1.7	1.0	1.8 ± 0.6

**TABLE 2C.** Plasma concentrations of native T as determined by GC-MS and calculated production rates of T in five healthy women during a 12-h infusion of 1,2-d-T

	1	2	3	4	5	6	7	Mean ± SD
1400–1800 h								
T (ng/mL)			0.185	0.202	0.224	0.221	0.135	0.193 ± 0.036
PR T (μg/h)			4.9	4.3	4.2	5.1	1.2	3.9 ± 1.6

acid (TFA) were applied to Sep-Pak C<sub>18</sub> cartridges (500 mg; Waters/Millipore, Milford, MA) pretreated with successive application of 5.0 mL methanol, 5.0 mL ethyl acetate, 20 mL water, and 5.0 mL TFA (0.5%, wt/vol). After sample application, the cartridges were first treated with three doses of 5.0 mL TFA (0.5%, wt/vol). Testosterone was subsequently eluted by ethyl acetate (two doses, 1.0 mL), dried under a stream of nitrogen at 37 °C, reconstituted in 100 μL CH<sub>2</sub>Cl<sub>2</sub>, and further prepu-

rified by thin layer chromatography (chloroform-acetone, 70:30). The zone containing testosterone was eluted (twice, 2.5 mL methanol) and supplemented with 10 ng dehydrotestosterone (1,4-androstadien-17β-ol-3-one) as an internal standard for GC/MS analysis. Derivatization was subsequently performed with heptafluorobutyric anhydride-acetone (1:4; 60 min) at room temperature. Recovery of [<sup>3</sup>H]testosterone from the derivatized samples was 38.5 ± 5.0% (n = 40). Analysis by

GC-MS (Finnigan MAT95 equipped with a 25-m CB5 fused silica column, San Jose, CA) was then performed using the selected ion monitoring mode and electric ionization (resolution, 6000). The tracer ions were [m/e 678 (dehydrotestosterone; internal standard), m/e 680 (native testosterone), and m/e 682 (1,2-*d*-testosterone)]. The sensitivity at a peak to noise ratio of 10:1 was less than 100 fg.

#### Calculation of testosterone production rate

Production rates of testosterone (PR[T]) were calculated from the product of the known infusion rate (Rt) and the ratio of tracer infusate enrichment (Et) to tracer dilution in the plasma (Es): (PR[T] = Rt × (Et/Es - 1) (8).

### Results

The plasma concentrations of unlabeled (native) testosterone and the testosterone production rates for Exp 1, 2, and 3 are summarized in Tables 1 and 2 for men and women, respectively. In men, production rates of testosterone ranged from  $64 \pm 25$  to  $101 \pm 67$   $\mu\text{g}/\text{h}$  during a 24-h period when 1,2-*d*-testosterone was infused in a comparatively large dose (Exp 1). No diurnal rhythmicity was apparent. The average production rate, determined as the mean of six individual samples obtained in each volunteer, was  $77 \pm 30$   $\mu\text{g}/\text{h}$ , which was identical ( $77 \pm 33$   $\mu\text{g}/\text{h}$ ) to the value obtained by analysis of an additional sample pooled throughout the 24 h. Thus, daily testosterone production based on this experiment was  $1.8 \pm 0.7$  mg/day, a quantity roughly 10-fold larger than the amount of infused 1,2-*d*-testosterone during this period (Table 1A).

In healthy women, testosterone production rates during this initial series of experiments ranged from  $3.6 \pm 1.3$  to  $6.0 \pm 3.4$   $\mu\text{g}/\text{h}$ . The largest production rates were seen from 0400–0800 h and from 0800–1200 h. The average production rate, determined as the mean of six individual samples obtained in each female volunteer, was  $4.6 \pm 1.9$   $\mu\text{g}/\text{h}$ . Analyzing an additional sample pooled throughout the 24 h resulted in a value of  $9.6 \pm 1.5$   $\mu\text{g}/\text{h}$ . Thus, daily testosterone production rates during this experiment ranged from 0.1–0.2 mg/day, a quantity in the same order as the amount of infused 1,2-*d*-testosterone during this period (Table 2A).

By reducing the dose of infused 1,2-*d*-testosterone by a factor of 5 to 0.015 mg/h in men and by a factor of 100 to 0.0001 mg/h in women (Exp 2), we ascertained the total infused amount of 1,2-*d*-testosterone to be far below the expected production rate of the hormone, thus excluding any potential interference by exogenous testosterone with its endogenous production. In this setting, the calculated mean production rates of testosterone were  $155 \pm 94$   $\mu\text{g}/\text{h}$  ( $3.7 \pm 2.2$  mg/day in men; Table 1B) and  $1.8 \pm 0.6$   $\mu\text{g}/\text{h}$  ( $0.4 \pm 0.1$  mg/day in women; Table 2B). Similar mean production rates of testosterone were found using an identical infusion rate of 1,2-*d*-testosterone, but reducing the equilibration period from 12 to 6 h (Exp 3; men,  $166 \pm 103$   $\mu\text{g}/\text{h}$ ; women,  $3.9 \pm 1.6$   $\mu\text{g}/\text{h}$ ; Tables 1C and 2C).

### Discussion

Using short (70-min) infusions of a radioactive tracer, Rivarola *et al.* (9) 30 yr ago found the production rates of testosterone in two healthy men to be 6.4 and 5.5 mg/day, respectively. In two healthy women, testosterone production

rates were 0.37 and 0.27 mg/day, respectively. Using the same technology, later investigators reported testosterone production rates ranging from 3.0–7.0 mg/day (10, 11) in healthy men and around 0.3 mg/day (12) in healthy women.

The advantages of stable labeled radioactive tracers compared to radioactive materials include (13) the ability of long term infusions to achieve steady state conditions, the avoidance of incomplete recovery of the tracer and the tracee from biological materials, and the fact that both labeled and endogenous materials are simultaneously analyzed using an identical technology. In addition, ethical considerations preclude infusions of radioactive materials causing some countries, including Austria, to prohibit by law the use of radioactive tracers in healthy volunteers.

The aim of this study was first to determine the production rates of testosterone in healthy men and women throughout 24 h for 4-h periods using a stable labeled compound and GC/MS analysis under steady state conditions, *i.e.* after a 12-h preequilibration period. The analytical precision of the method used is demonstrated by the fact that average testosterone production rates throughout 24 h, mathematically calculated as the mean value of these six individual samples, were similar to an analytically obtained value obtained from an additional plasma sample pooled for 24 h.

During 1,2-*d*-testosterone infusions of 0.07 and 0.01 mg/h in men and women, respectively, mean estimated production rates were 77  $\mu\text{g}/\text{h}$  (1.8 mg/day) in men and 4.6  $\mu\text{g}/\text{h}$  (0.1 mg/day) in women. For both sexes, these values are below those previously reported by others (10–12) and uncomfortably close to the testosterone infusion rates employed. The question, therefore, arose whether such a comparatively large amount of exogenous testosterone could have suppressed its endogenous production rate.

Therefore, in a second series of experiments the infused tracer dose was reduced to 0.015 mg/h in men and 0.0001 mg/h in women, amounts equivalent to about 10% (men) or 5% (women) of the potential testosterone production rates. Mean production rates of testosterone during this second series of experiments were 147  $\mu\text{g}/\text{h}$  in men and 1.8  $\mu\text{g}/\text{h}$  in women. One man (no. 7) and two women (no. 5 and 6) received even smaller tracer doses (1.5 and 0.015  $\mu\text{g}/\text{h}$ , respectively), stretching the currently available limits of detection to their extreme. Nevertheless, production rates of testosterone in these three individuals were in the same range as those in the remaining subjects. Based on these results, it is unlikely that the smaller doses of testosterone employed have some impact on its endogenous production rate, although we cannot exclude this possibility with certainty. Rather, we feel justified to conclude that the mean production rates of testosterone determined during this second series of experiments (men,  $3.7 \pm 2.2$  mg/day; women,  $0.43 \pm 0.14$  mg/day) represent accurate endogenous testosterone production rates, in line with estimates in young healthy men reported by researchers using radioactive tracers (10, 11), whereas those in healthy women were slightly lower than rates previously reported (12). Additional individually conducted experiments, described below, led us to suggest that testosterone production rates in females up to at least  $0.3 \pm 0.4$  mg/day must be regarded as normal, as previously described by Samoljik *et al.* (12) for healthy nono-

bese women. Production rates of cortisol obtained by GC/MS analysis appear to be lower than hitherto assumed (13). In regard to testosterone production rates, however, results obtained using this more advanced technology are in keeping with data obtained by means of the radiotracer technique.

Production rates of testosterone in men failed to show any diurnal variation during either series 1 (24-h observation period) or series 2 (12-h observation period). In women, however, testosterone production was higher after 0400 h. As both male and female volunteers were studied under the same experimental conditions in the largely stress-free setting of a metabolic ward, it is unlikely that methodological differences should account for these sex-specific differences. Apparently, the diurnal rhythm of ACTH is of inadequate importance in healthy men, in whom the main share of testosterone is of testicular origin, but influences adrenal testosterone secretion in women.

The results discussed above were supposed to provide the basis for future studies in patients with disorders of androgen secretion. Thus, to permit routine estimation of testosterone production rates 1,2-*d*-testosterone infusion was reduced to 6 h and subsequent blood sampling to 4 h, as a 36-h or even a 24-h experimental protocol represents a logistical challenge for any semiroutine investigation and *de facto* precludes its use on an out-patient basis. This also reduced the number of samples to be analyzed by GC/MS to one per individual. To demonstrate the validity of this approach, experiments were performed in the same group of volunteers who had taken part in protocol 2, employing identical 1,2-*d*-testosterone infusion rates (men, 0.015 mg/h; women, 0.0001 mg/h). Using this approach in healthy men, both plasma concentrations of endogenous testosterone and its estimated production rates were in the same range as those determined after a more prolonged tracer infusion. In regard to healthy women, the production rates of testosterone during this series were higher, although in a comparable range. This protocol, therefore, provides a comparatively simple

way to evaluate testosterone secretion rates in various pathological situations.

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