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DEUTERIUM LABELLED STEROID HORMONES: TRACERS FOR THE MEASUREMENT OF ANDROGEN PLASMA CLEARANCE RATES IN WOMEN

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Summary—A method employing stable isotope-labelled tracers and gas chromatograph–mass spectrometry (GC–MS) analysis has been used to measure the plasma clearance rates (PCR's) of androstenedione (A) and testosterone (T) in normal women and women with androgen abnormalities including hirsutism and polycystic ovary syndrome. A solution of deuterium-labelled A and T is infused at a constant rate and blood samples taken at 2 and 2.25 h. Solvent extracts of the derived plasma samples, to which an internal standard has been added, are derivatized with pentafluoropropionic anhydride and the endogenous steroid and deuterated steroid are quantitated after an injection of the derivatization mixture into a capillary column GC–MS. The concentration of the deuterated steroid in the infusion mixture is measured and the PCR is calculated. In premenopausal normal women the PCR_A is 1950 ± 184 l/24 h ($n = 5$) and the PCR_T is 484 ± 82 l/24 h ($n = 7$).

INTRODUCTION

Hirsutism and related manifestations of virilism in women are linked to excess production of the androgens androstenedione and testosterone [1, 2]. Laboratory assessment generally involves measurement of the plasma levels of the hormones by radioimmunoassay but frequently in these disorders the plasma androgen content is within the range found in normal women and further study requires assessment of the plasma clearance rate (PCR) by inference from the basic equation: Production rate (PR) = plasma concentration × PCR [3].

The technique of measuring PCR's of steroid hormones by the constant infusion of a radioactive tracer method of Tait [4] has been used extensively for obtaining androstenedione and testosterone PCR's in normal women (Table 1) and in women with hirsutism [2, 5], polycystic ovary syndrome [2, 6], and hyper- and hypothyroidism [7, 8].

In a continuing study exploring the use of stable isotope-labelled tracers for clinical procedures [15, 16, 17] we have developed a similar method to that of Tait, by replacing the radioactive-labelled androstenedione and testosterone with deuterium-labelled analogues followed by quantitation on a gas chromatograph–mass spectrometer (GC–MS). Quantitation of both the deuterated and endogenous androstenedione and testosterone in the plasma samples by the GC–MS technique requires the use of internal standards. 19-Norandrostenedione and 19-nortestosterone were selected because they are not present in human plasma and they should exhibit identical chemical behaviour to their methyl analogues.

A significant advantage of the method is the capability of measuring the plasma circulating level and hence the production rate of the steroid at the same time as the PCR. This is especially important for steroids which exhibit a diurnal rhythm. A measurement of the plasma level of the steroid must be made at least 2 h prior to the PCR measurement when using Tait's method.

Other advantages include a considerably reduced analysis time as repeated recrystallizations of the steroid, required for the radioactive-labelled tracer method, are not required and the method is ethically acceptable [18].

EXPERIMENTAL

[7,7,15-$^2H_3$]Androstenedione (>96% triply deuterium-labelled) and [7,7-$^2H_2$]testosterone (>97% doubly deuterium-labelled) were prepared using the methods of Seamark *et al.* [19] and Blair *et al.* [20]. Mass spectral analysis was performed on a Hewlett–Packard 5992B operating under "single ion monitoring" mode (SIM). The instrument was fitted with an on-column injector (model OC1-3, Scientific Glass Engineering (SGE), Melbourne, Australia), a 12 m × 0.3 mm i.d. silica bonded phase column (BP1, SGE, Melbourne, Australia) and a glass jet separator.

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Solutions for infusion were prepared by dissolving the deuterated steroid (5 mg) in propylene glycol–water (3:1, 10 ml) and filtering through 0.2 μm Millipore filters under sterile conditions. The solutions were shown to be pyrogen free before use.

Infusion procedure

Aliquots (1 ml for [$^2H_3$]androstenedione and 0.6 ml for [$^2H_2$]testosterone) of the deuterated steroid in propylene glycol–water (3:1) were added to sterile saline (65 ml). This solution was infused from a glass syringe through a Teflon catheter at 20 ml/h for a period of 2.5 h into the antecubital vein of the subject. Blood samples (10 ml) were taken at 2 h and 2.25 h from a vein in the opposite arm. A sample of the infusion solution was collected at the end of the infusion period. A plasma fraction of each blood sample was prepared by centrifugation and stored frozen at −10°C.

Sample preparation for mass fragmentography

(a) *Androstenedione.* A plasma sample (2 ml), to which was added 19-norandrostenedione (20 ng) in ethanol (10 μl), was extracted with hexane (2 × 3.5 ml). The extract was evaporated under a stream of nitrogen and derivatized with a mixture of acetonitrile (50 μl), acetone (50 μl) and pentafluoropropionic anhydride (50 μl) at 20°C for 1 h. The derivatizing reagents were removed in a stream of nitrogen and the residue was dissolved in dodecane (10 μl) for injection on the GC–MS.

A sample of the infusion solution (25 μl) and 19-norandrostenedione (200 ng) in ethanol (10 μl) were added to saline (1 ml). This solution was extracted and derivatized in the same manner as the plasma samples.

(b) *Testosterone.* A plasma sample (2 ml), to which was added 19-nortestosterone (20 ng) in ethanol (10 μl), was extracted with ether (2 × 3.5 ml). The extract was evaporated under a stream of nitrogen and the residue, in a glass tube, was dissolved in hexane–chloroform (19:1, 3 ml) and added to the top of a Lipidex-5000 column (i.d., 8 cm × 4 mm), which had been equilibrated in hexane. The eluant was discarded and chloroform (3 ml) was added to the glass tube and transferred to the top of the Lipidex-5000 column. The resultant eluant was evaporated in a stream of nitrogen and derivatized with a mixture of tetrahydrofuran (100 μl), pentafluoropropionic anhydride (100 μl) and hexane (800 μl) at 60°C for 1 h. The derivatizing reagents were removed in a stream of nitrogen and the residue was dissolved in dodecane (10 μl) for injection on the GC–MS.

A sample of the infusion solution (50 μl) and 19-nortestosterone (200 ng) in ethanol (10 μl were added to saline (1 ml). This solution was extracted and derivatized in the same manner as for the plasma sample.

Calculation of PCR

For mass spectral analysis the ions monitored were *m/z* 418.3 (19-norandrostenedione derivative), 432.3 (androstenedione derivative), 435.3 (trideuterated androstenedione derivative) when measuring an androstenedione PCR and *m/z* 566.4 (19-nortestosterone derivative), 580.4 (testosterone derivative) and 582.4 (dideuterated testosterone derivative) for a testosterone PCR. The mass spectra

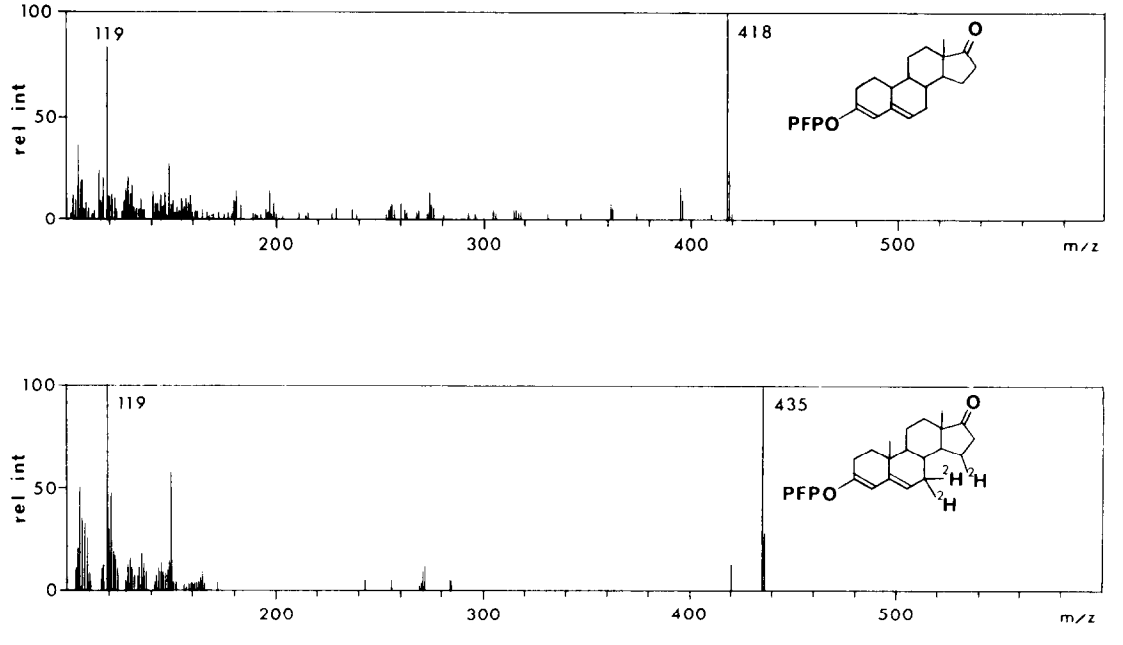

Fig. 1. EI mass spectra of the pentafluoropropionyl derivatives of 7,7,15-[$^2H_3$]androstenedione (bottom) and 19-norandrostenedione.

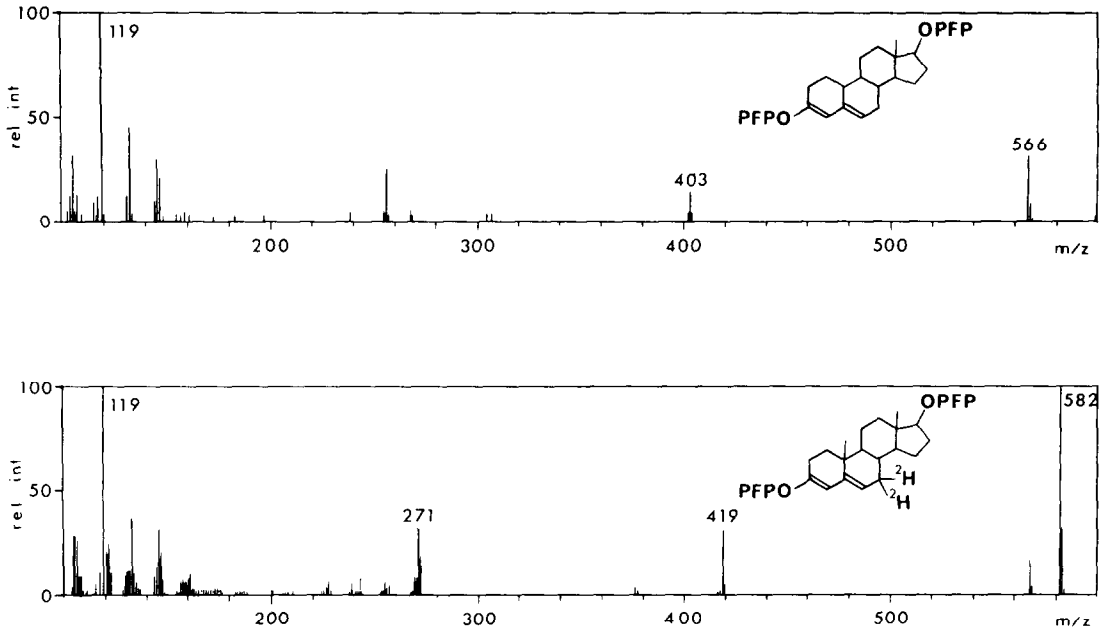

Fig. 2. EI mass spectra of the dipentafluoropropionyl derivatives of 7,7-[$^2H_2$]testosterone (bottom) and 19-nortestosterone.

of the pentafluoropropionate derivatives of 19-norandrostenedione and [7,7,15-$^2H_3$]androstenedione are shown in Fig. 1. Those of 19-nortestosterone and [7,7-$^2H_3$]testosterone are shown in Fig. 2.

Quantitation of both the labelled and endogenous androstenedione and testosterone in the plasma samples and the infusion mixture is achieved by substitution into a linear regression equation generated from the relative mass spectral responses of mixtures of the steroid and its corresponding internal standard (19-norandrostenedione or 19-nortestosterone).

The PCR is calculated from the product of the pump flow rate and the average concentration of deuterated androstenedione or testosterone in the plasma divided by the concentration of deuterated androstenedione or testosterone in the infusion mixture.

RESULTS

To demonstrate the validity of using the internal standards, 19-norandrostenedione and 19-nortestosterone to quantitate the corresponding deuterated steroids, standard curves were constructed by plotting ratios of the deuterated steroid (androstenedione or testosterone) to the internal standard against the mass spectral response ratio of the correspnding derivatives obtained after subjecting mixtures to standard conditions of extraction and derivatization (see Fig. 3). A linear response over two decades of range was observed in each case.

The levels of radioactive-labelled tracers used for measuring PCR's of androstenedione and testosterone reach a plateau in blood prior to 2 h infusion [9]. This was verified for deuterium-labelled androstenedione and testosterone when they were indi-

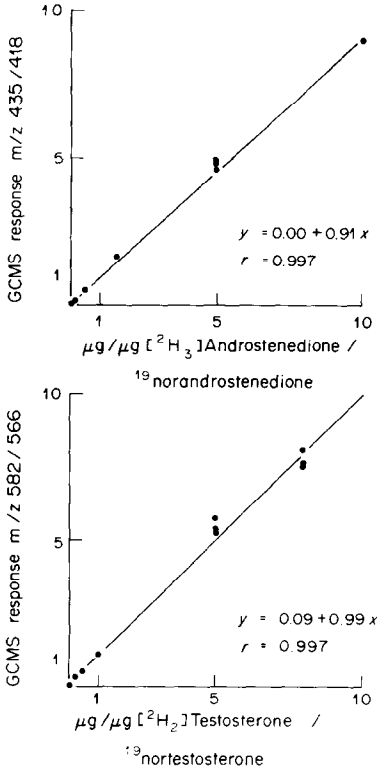

Fig. 3. Ratios of deuterium-labelled androstenedione and 19-norandrostenedione (top) and of deuterium-labelled testosterone and 19-nortestosterone plotted against the GCMS response of the corresponding mixtures of pentafluoropropionyl derivatives.

Table 1. PCR's of testosterone and androstenedione in premenopausal normal women measured by the radioactive-labelled tracer method

	Testosterone			Androstenedione			
Author	MCR_T	SD	*N*	MCR_A	SD	*N*	Ref.
Horton, 1966	894	47	5	2230	190	6	[9]
Southren, 1967	675	139	6				[10]
Bardin, 1967	590	44	5	2070	260	5	[2]
Southren, 1968	545	103	8				[11]
Abraham, 1969	828	45	6	1886	228	6	[12]
Gordon, 1969	525	75	4	2399	165	6	[7]
Longcope, 1969	440	50	6	2020	140	6	[13]
Saez, 1972	486*	85	7				[8]
Kirshner, 1976	590	45 (SE)	9	2070	260	9	[3]
Mowszowicz, 1984	600	150	10				[14]

*Assuming an average body surface area of 1.6 m^2.

Table 2. Androstenedione PCR's in normal women and in women with endocrine disorders measured by the deuterium-labelled tracer method

	PCR (litres/day)	SD	*N*
Normal premenopausal	1950	184	5
Postmenopausal*	1847	155	12
Idiopathic hirsutism†	2079	259	9
Polycystic ovarian syndrome†	2312	343	8
Obese	2177	370	6
Anorexia nervosa	1862	504	4

*Mean age 66.9 ± 5.7 years.
†Classification based on the criteria of intermenstrual interval, Ferriman Gallwey hair score and LH/FSH ratio.

vidually infused to achieve circulating levels at or below the plasma level of the endogenous steroid in normal women. In addition there was no significant change in the plasma level of the endogenous steroid during the infusion. After each infusion the levels of endogenous and deuterated steroid in the plasma samples obtained after 2 h and 2.25 h infusion were compared and the assay was rejected if the difference exceeded 10%.

Ideally tracer amounts of the deuterium-labelled steroid should be infused to avoid perturbation of the system but higher levels are required to satisfy the practicalities of reliable quantitation. Vermeulen *et al.* [21] have shown that the maintainence of circulating levels of free testosterone of up to 1 μg/100 ml in plasma of normal women has an insignificant effect on their testosterone PCR (measured by the method of Tait [4]). We demonstrated that during two sequential infusions of deuterium-labelled testosterone in a normal woman to achieve circulating plasma levels of 0.34 and 0.85 μg deuterated testosterone/100 ml plasma (i.e. 4 and 10 times higher than routinely used) the testosterone PCR's were 422 and 420 l/24 h respectively. The androstenedione PCR determination in women was affected when the circulating level of deuterated androstenedione exceeded 0.15 μg/100 ml which is two times higher than the level reached in the routine infusions.

For both androstenedione and testosterone, large volumes of plasma from a representative subject were collected and analyzed on six separate occasions. The interassay variation for androstenedione PCR determinations was 9.7% (mean 2209 ± 215 l/24 h), whilst that for the testosterone PCR determination was 8.2% (mean 750 ± 62 l/24 h).

Androstenedione and testosterone PCR's of pre- and postmenopausal women and women with various endocrine disorders were measured (see Tables 2 and 3). No attempt was made to standardize the time of PCR measurements as a consequence of the observations of Southren *et al.*, [10] that the PCR of testosterone does not vary significantly during the day. The PCR values were not corrected for variations in body surface area.

DISCUSSION

The mean androstenedione PCR of normal women was in good agreement with published values obtained by the method of Tait. Postmenopausal women showed a lower PCR (smaller body mass and reduced blood flow) while women with a higher than normal body mass, hirsutism and polycystic ovarian syndrome had a higher PCR. The differences in PCR values from those of normal women were, however, statistically insignificant.

The mean testosterone PCR of normal women was at the low end of the range of values obtained by

Table 3. Testosterone PCR's in normal women and in women with endocrine disorders measured by the deuterium-labelled tracer method

	PCR (litres/day)	SD	*N*
Normal premenopausal	484	82	7
Idiopathic hirsutism	694	79	7
Polycystic ovarian syndrome	640	163	4

various workers using Tait's method (Table 1). Hirsute women had a statistically significant elevation in testosterone PCR, as observed by Casey [5].

The interassay variations of the PCR's of both androstenedione and testosterone, determined by the deuterium-labelled tracer method, are smaller. Since the plasma concentration of the endogenous hormone is determined at the same time as that of the tracer the procedure is both simpler and faster and a complete PCR and PR determination (including the infusion) can be completed in one working day.

As no hazardous (radioactive) materials are used this procedure can be safely considered, for example, to elucidate problems such as the large drop in binding capacity of the testosterone binding globulin in pregnant women at delivery observed by Vermeulen *et al.* [21], but not by others using slightly different methods, a dispute which for ethical reasons can no longer be resolved using radioactive-labelled tracers.

Isotope-dilution methods with deuterium-labelled steroids are reference methods for the quantitation of steroid hormones. By analogy the method presented in this paper for measuring the PCR's of steroid hormones, androstenedione and testosterone in particular should be considered at least as a more practical alternative if not a reference method for others.

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