Journal of Steroid Biochemistry, Vol. 11, pp. 1613 to 1617
Pergamon Press Ltd 1979. Printed in Great Britain

CONJUGATED ANDROSTENEDIONE IN HUMAN URINE

A. B. GOODALL and V. H. T. JAMES
Department of Chemical Pathology, St. Mary's Hospital Medical School, London, W2 1PG, England

(*Received* 5 *March* 1979)

SUMMARY

A method for the measurement of the total amount of androstenedione in urine, both free and conjugated, has been devised. After mild acid hydrolysis and a paper chromatographic purification stage, the amount of androstenedione has been estimated by radioimmunoassay. The mean excretion of androstenedione for 12 male subjects was 198 nmol/24 h, and for 11 female subjects, 77.4 nmol/24 h. A good correlation was found between the total urinary excretion of androstenedione and the mean plasma androstenedione concentration during the same period, for a group of postmenopausal subjects, indicating that the measurement of total urinary androstenedione is a useful reflection of plasma androstenedione values and thus androstenedione secretion through a 24 h period.

INTRODUCTION

Androstenedione is present in human urine both in the free and the conjugated form. Andino *et al.*[1] have reported the amounts of unconjugated androstenedione in urine, and other workers [2–5] have reported the values of androstenedione in urine obtained after incubation with β-glucuronidase and acid hydrolysis.

Androstenedione is secreted by the ovaries and the adrenal cortex. Adrenocortical secretion is episodic, and thus plasma androstenedione levels fluctuate markedly and show a diurnal pattern closely similar to that which is observed for cortisol [6]. A single plasma measurement is thus a poor indication of the secretion rate of androstenedione over a 24 h period. In an attempt to overcome this problem and to obtain a more meaningful estimate of the amount of androstenedione produced in a given period, a method has been developed to estimate the total amount of androstenedione in a urine sample.

The conjugated androstenedione in an aliquot of a urine collection is hydrolysed by mild acid hydrolysis, using an adaptation of the method of Burstein and Lieberman[7]. After hydrolysis, the ethyl acetate extract is purified by Bush B3 paper chromatography, and the amount of androstenedione estimated by radioimmunoassay, after silica gel column chromatography. Normal ranges have been established for male and female subjects, and values of total urinary androstenedione have also been measured in eight hirsute subjects. To determine whether urinary androstenedione excretion is a useful index of androstenedione secretion, a comparison has been made, in a group of postmenopausal women, between the mean plasma androstenedione levels measured at 30 min intervals, and the amount of androstenedione excreted in the urine over the same period of time as the blood samples were taken.

EXPERIMENTAL

Materials

[1,2,6,7(n)^{3}H]-Androstenedione (104 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, England. Ethyl acetate (A.R.) was freshly redistilled prior to use. Phosphate buffer pH 6.4 was prepared from di-sodium hydrogen phosphate (2.37 g) and sodium di-hydrogen phosphate (2.87 g) made up to 250 mls with distilled water. Paper used for chromatography was Whatman No. 2 chromatography paper cut into 1.5 cm strips, washed by running for at least 24 h in a chromatography tank containing methanol. Glass columns for silica gel chromatography were 6 mm in diameter and contained 30 mm silica which was washed with 95% aqueous methanol (2 × 5 ml), hot methanol (approx 55°C) (2 × 5 ml), and methanol (3 × 5 ml) between assays.

Urine hydrolysis method

To an aliquot of a 24 h urine collection (0.1 ml or 0.5 ml) made up to 0.5 ml with phosphate buffer (pH 6.4) was added approximately 100,000 d.p.m. of [^{3}H]-androstenedione in methanol (0.1 ml). The pH of the solution was adjusted to one using 2 N sulphuric acid. 30% (w/v) aqueous sodium chloride (1 ml) and ethyl acetate (1.5 ml) were added and the mixture shaken on a mechanical shaker for 10 min. After incubating at 37°C, for 16 h, the mixture was shaken for a further 10 min, and the ethyl acetate layer (0.5 or 1.5 ml) separated and evaporated to dryness at 40°C, under a stream of nitrogen. The residue was redissolved in dichloromethane–methanol (1:1, v/v) and applied to paper for chromatographic separation in a Bush B3 system running for 3.5 h after equilibration. After location of the radioactive areas, the paper was eluted with methanol (6 ml), through the silica gel columns. From the eluate, 1 ml was transferred to a plastic counting vial, and counted after evaporation

under nitrogen and addition of scintillator (3.5 ml), as a recovery check. Further aliquots of the methanol eluate (2 × 0.5 ml) were pipetted into glass assay tubes for radioimmunoassay. Standard quantities of androstenedione (10, 20, 40, 80, 160, 320 pg) were pipetted into assay tubes from individual standard solutions in methanol. [^{3}H]-Androstenedione (10,000 d.p.m. in methanol 0.1 ml) was added to all tubes containing standards and two tubes for the estimation of non specific binding and two zero antigen tubes. The amount of tracer in each of the tubes containing samples was adjusted to that contained in each of the standard tubes, by calculation from the counts in the recovery check, by addition of further aliquots of labelled tracer in methanol. 10% propylene glycol in methanol (0.1 ml) was added to all tubes, and the methanol was evaporated from all tubes, in a vacuum oven at 45°C.

The estimation of the amount of androstenedione in the eluates was continued by incubating with antiserum prepared in borate buffer and separating the antibody bound and free fractions by precipitation with a solution of polyethylene glycol in borate buffer, as in the method for the radioimmunoassay of androstenedione in plasma [8]. The only difference being that no [^{3}H]-androstenedione is added to the antiserum solution in borate buffer as [^{3}H]-androstenedione had already been added to all assay tubes.

Calculation

The amount of androstenedione in the samples was determined from the standard curve and calculated in nmol/24 h after correcting for losses.

Determination of androstenedione in plasma samples

Plasma androstenedione was measured as described previously [8].

RESULTS

All samples were assayed in duplicate and the intra assay variation was assessed from 32 urine samples for which the C.V. was 10.3%. The inter assay variation was assessed from urine pools which were stored deep frozen, and assayed in successive assays. The C.V. for single assays was 15.0% ($N = 18$) at 77.6 nmol/24 h and was 14.0% ($N = 12$) at 59.7 nmol/24 h.

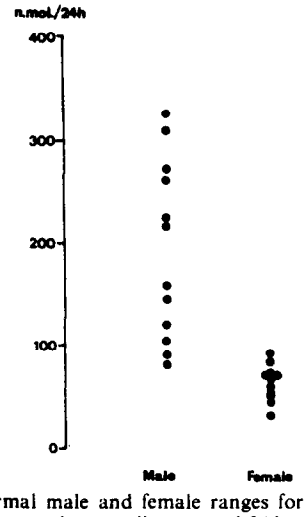

Fig. 1. Normal male and female ranges for total urinary androstenedione (nmol/24 h).

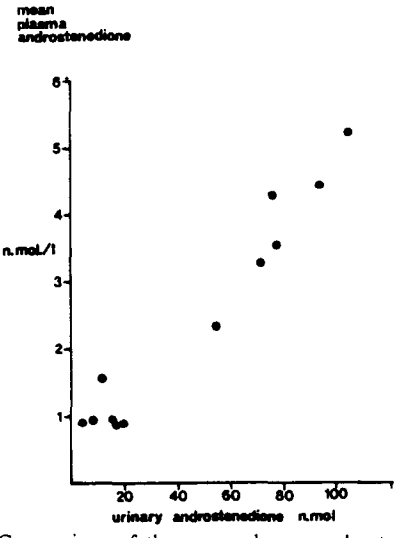

Fig. 2. Comparison of the mean plasma androstenedione value (nmol/l) with total urinary androstenedione (nmol) collected over the same period as the plasma samples were taken.

Water blanks were routinely run in the assay and were usually below the lowest standard. If the ethyl acetate was not freshly redistilled this could cause an increase in the water blank value.

Normal ranges for male and female subjects

The total urinary excretion of androstenedione in twenty-four hour urine collections have been measured for 12 normal men, aged 22–38, and for 11 normal women, aged 21–37. For the women the mean excretion was 77 nmol/24 h with a range of 45–108. For the men the mean was 198 nmol/24 h with a range of 81–327. The results are shown in Fig. 1.

Correlation between total urinary androstenedione and plasma androstenedione

For a group of postmenopausal subjects, the mean plasma value of androstenedione over a given number of hours has been correlated with total urinary androstenedione from a urine collection made over the same period of time. To obtain a good estimate of the mean plasma level blood samples were taken at half hourly intervals. The average number obtained for this purpose was 25 consecutive half hourly samples, with a minimum of 17 and a maximum of 47. As shown in Fig. 2 a good correlation (correlation coefficient of 0.97) has been obtained between these two parameters.

Suppression with dexamethazone

Plasma samples were taken at half hourly intervals from a postmenopausal patient for 4 h prior to the

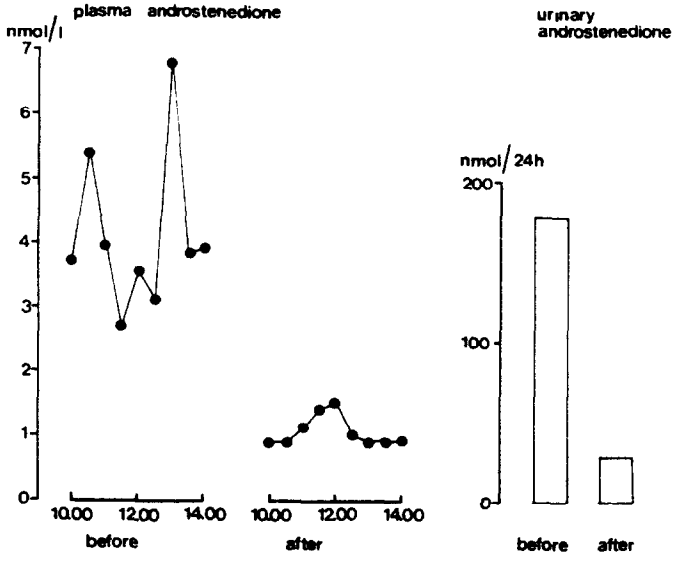

Fig. 3. Urinary and plasma androstenedione levels in a postmenopausal subject before and after treatment with dexamethazone.

start of 7 days treatment with dexamethazone (1.5 mg a day) and then again at the end of this treatment. Twenty four hour urine collections were made on the same two days as the blood collections. Figure 3 shows that the decrease in the values for plasma androstenedione after seven days are reflected by the decrease in the excretion of total urinary androstenedione, after the same period.

Hirsute subjects

Total urinary androstenedione has been measured in eight hirsute subjects. The results are shown in Fig. 4 compared with the normal female range. The mean value is 113 nmol/24 h with a range of 73 to 169.

DISCUSSION

Androstenedione is a major secretory product of the adrenal cortex and the gonads, and is an important prehormone for testosterone and oestrone, particularly in women. There is a need, in studying normal and abnormal androgen secretion, to be able to evaluate androstenedione secretion accurately. Single or occasional measurements of plasma androstenedione levels are not satisfactory for this purpose, since, as we have shown elsewhere [6, 9], both adrenal and ovarian secretion of androstenedione occur episodically. In the present study, we have investigated the value of urinary androstenedione for this purpose, on the assumption that it might integrate the fluctuating plasma levels and thus reflect the secretion rate of this hormone.

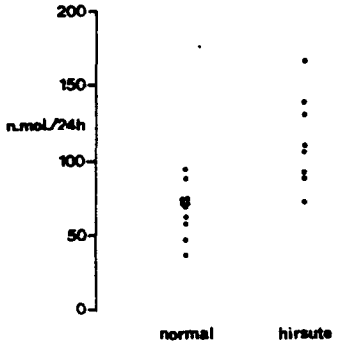

Fig. 4. Comparison of total urinary androstenedione in normal and hirsute female subjects.

Although we concluded from earlier work [1] that unconjugated or "free" androstenedione was a useful reflection of androstenedione secretion, it has been shown by others [2–5, 10–15] that human urine also contains conjugated androstenedione and spontaneous hydrolysis occurs under some conditions, with the risk of spuriously high values occurring. For this reason, the measurement of the total amount of androstenedione, free and conjugated, has been explored.

Evidence for the existence of conjugated androstenedione in urine has been produced by several authors. Schubert[10] found that β-glucuronidase released androstenedione from the urine of subjects to whom testosterone had been administered. He suggested that the androstenedione was conjugated to glucuronic acid through an enol linkage. Evidence for the existence of conjugated androstenedione has also been put forward by Matsui *et al.*[16] and Wotiz *et al.*[17] who have prepared by synthesis the 3-enol glucuronide of androstenedione. The sulphuric acid conjugate of androstenedione has been prepared by Oertel *et al.*[18].

Several groups of workers have estimated normal male and female ranges for androstenedione in urine

Table 1. Normal male and female ranges for androstenedione in urine (nmol/24 h)

	Male	Female
Longhino *et al.*[2]	46–213 (n = 10)	11–82 (n = 10)
Schubert *et al.*[3]	49–297 (n = 9)	45–108 (n = 15)
Schollberg *et al.*[4]	—	17–185 (n = 2)
Tajic[5]	21–64 (n = 10)	21–39 (n = 8)
This study	81–327 (n = 12)	45–108 (n = 11)

after incubation with β-glucuronidase and acid hydrolysis [2–5]. Tajic and Kovacic[11] reported the percentages of free, glucuronide and sulphate conjugated androstenedione in the urine of three healthy male and three female subjects. The amounts of androstenedione in the free forms and conjugated as glucuronide and sulphate have been measured [12, 13] during different phases of the menstrual cycle, and by Dalzell and Elatter[14] in the urine from pre-adolescent and adolescent children but only after incubation with β-glucuronidase and not acid hydrolysis.

In the present study, we found in agreement with others (Table 1), that the mean excretion of androstenedione is lower for women than for men although the ranges overlap. This sex difference is unexpected because the plasma levels [19] and metabolic clearance rate [20] of androstenedione in men and women are similar. The metabolism, rather than production rate, may thus differ between sexes.

Total urinary androstenedione has been measured in eight female patients presenting with hirsutism. The mean value for this group is 113 nmol/24 h compared to 77 nmol/24 h for normal females, with an overlap of the ranges. Similar results have been reported for plasma levels [21].

The data thus illustrate that there is in human urine, both free and conjugated androstenedione with a preponderance of the latter, but so far there is little additional information to relate urinary excretion to plasma levels and thus indirectly to the secretion rate of this hormone. The correlation between the excretion of total androstenedione in urine, and the mean plasma level, measured over extended periods of time up to 24 h has therefore been examined to see if urinary androstenedione may reflect the mean plasma level. The data reported here illustrate that in the group of postmenopausal subjects studied, the urinary levels were indeed a good index of mean plasma levels. In addition, the study in which adrenocortical secretion was depressed by administration of dexamethazone also illustrated the close relationship between blood and urine levels. Comparison between male and female subjects may be inappropriate, because of possible difference in metabolism, but this needs further investigation.

We conclude therefore that the total amount of androstenedione excreted in urine reflects the integrated plasma levels measured over a period of time. Since androstenedione secretion is episodic, it would appear that urinary excretion is a better index of androstenedione secretion than can be obtained from random plasma levels, and estimation of urinary androstenedione may be a useful means of investigating normal and abnormal aspects of androgen production.

Acknowledgements—We are grateful to Dr. W. Schopman for his generous gift of antiserum and to Mr. J. D. Hutton for his assistance in obtaining the plasma samples and urine collections from the postmenopausal subjects.

REFERENCES

1. Andino N., James V. H. T., Parker V. and Rippon A. E.: Excretion of non-conjugated androstenedione and testosterone in human urine. *Steroids* **28** (1976) 837–846.
2. Longhino N., Tajic M., Vedris M., Jankovic D. and Drobnjak P.: Urinary excretion of androstenedione, testosterone, epitestosterone and dehydroepiandrosterone during the menstrual cycle. *Acta endocr. Copenh.* **59** (1968) 644–651.
3. Schubert K. and Frankenberg G.: Die Bestimmung von Testosteron und Δ^4-Androstendion-(3,17) im Harn. *Hoppe-Seyler's Z. physiol. Chemie* **336** (1964) 91–95.
4. Schollberg Von K. and Hubl W.: Die Bestimmung von Epitestosteron, Testosteron und Δ^4-Androstendion-(3,17) in Harn gesunder Frauen. *Endokrinologie* **53** (1968) 222–229.
5. Tajic M.: Metoda za odredivanje testosterona i androstendiona u urine. Vrijednosti kod normalnih osoba i bolesnika. *Acta Medica Jugoslav.* **19** (1965) 89–106.
6. Tunbridge D., Rippon A. E. and James V. H. T.: Circadian relationships of plasma androgens and cortisol levels. *J. Endocr.* **59** (1973) xxi.
7. Burstein S. and Lieberman S.: Hydrolysis of ketosteroid hydrogen sulphates by solvolysis procedures. *J. biol. Chem.* **233** (1958) 331–335.
8. Goodall A. B., Rippon A. E. and James V. H. T.: A simple non-chromatographic radioimmunoassay for plasma androstenedione. *J. steroid Biochem.* **11** (1979) 1181–1184.
9. James V. H. T., Rippon A. E. and Jacobs H. S.: Plasma androgens in patients with hirsutism. In *The Endocrine Function of the Human Ovary* (Edited by V. H. T. James, M. Serio and G. Giusti). Academic Press, London (1976) pp. 457–470.
10. Schubert K.: Steroidstoffwechsel bei Brustkrebs. *Acta endocr., Copenh.* **27** (1958) 36–44.
11. Tajic M. and Kovacic E.: Free, glucuronic and sulphate testosterone, epitestosterone and androstenedione in the urine. *Die Naturwissenschaften* **55** (1968) 394.
12. Longhino N., Drobnjak P., Krk C. and Vedris M.: Urinary excretion of testosterone, epitestosterone and androstenedione, their glucuronides and sulfates during different phases of the menstrual cycle. *Acta Pharm. Jugoslav.* **22** (1972) 65–68.

13. Schollberg Von K., Seiler E. and Wagner S.: Testosteron, Epitestosteron und Δ^4-Androsten-3, 17-dion im Harn bei Frauen wahrend des Zyklus. *Endokrinologie* **55** (1969) 162–167.
14. Dalzell D. P. and Elattar T. M. A.: Gas chromatographic determination of urinary excretion of testosterone, epitestosterone and androstenedione in pre-adolescent and adolescent children. *J. clin. Endocr. Metab.* **36** (1973) 1237–1243.
15. Kjeld J. M., Puah C. M. and Joplin G. F.: Rise of unconjugated sex hormones in human urine on storage. *Clin. Chem. Acta* **80** (1977) 285–291.
16. Matsui M., Kawase M. and Okada M.: Preparation and stability in solution of androstenedione 3-enol glucosiduronate. *Chem. Pharm. Bull.* **22** (1974) 2530–2537.
17. Wotiz H. H., Smakula E., Lichtin N. N. and Leftin J. H.: D-Glucopyranosiduronates I. Steroidyl-β-D-glucopyranuronosides. *J. Am. Chem. Soc.* **81** (1959) 1704–1708.
18. Oertel G. W., Treiber L. and Reidt W.: Direct aromatization of C_{19}-Steroid sulphates. *Experienta* **23** (1967) 97–98.
19. Friedrich E., Jaeger-Whitegiver E. R., Bieder M., Keller E. and Schindler A.: Radioimmunoassay of plasma androstenedione. In *Radioimmunoassay of Steroid Hormones* (Edited by D. Gupta). Verlag Chemie, Weinheim (1975) pp. 73–82.
20. Horton R. and Tait J. F.: Androstenedione production and interconversion rates measured in peripheral blood and studies on the possible site of its conversion to testosterone. *J. clin. Invest.* **43** (1966) 301–313.
21. Andre C. M. and James V. H. T.: Plasma androgens in idiopathic hirsutism. *Steroids* **24** (1974) 295–309.