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THE ISOLATION OF PROGESTERONE FROM
HUMAN PREGNANCY URINE

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ABSTRACT

Progestrone was isolated from the «freely extractable» fraction of
approximately 50 litres of pregnancy urine. Chemical and physical pro-
properties showed that the compound isolated was indeed progestrone.
The isolation procedure involved a modified form of the Girard reaction
in which a «conjugated ketone» fraction containing progestrone was
separated from both the non-ketonic components and the major bulk of
the saturated ketones. This was followed by paper and thin-layer chroma-
tography. The final separation was achieved using preparative gas-liquid
chromatography.
It was estimated from the gas-chromatographic peak heights that there
was no more than 250 μg of progestrone in the pooled urine residues.

The isolation of progestrone (pregn-4-ene-3,20-dione) from corpora lutea was
first reported over 30 years ago by four groups of workers (Butenandt et al.
1934; Hartmann & Wettstein 1934; Wintersteiner & Allen 1934; Slotta et al.
1934 a, b). In 1938 the hormone was found in ox adrenals (Beall 1938) and
later in human placental tissue (Pearlman & CerCEO 1952; Salhanick et al.
1952); the presence of progestrone has also been demonstrated in human

On the other hand, successive attempts to isolate and identify progestrone
in urine have previously been unsuccessful. Marker et al. (1937) extracted

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272
10,000 gallons of human pregnancy urine but failed to demonstrate the presence of the hormone. More recently, Drosdowsky et al. (1965) showed that approximately 0.01% of a dose of radioactive progesterone appeared in the urine as a fraction which behaved chromatographically like progesterone; this was in agreement with the results of Harkness (1962). However, no definite evidence of identity was obtained in both these studies. If 0.01% of the secreted hormone was present in urine then it should have been possible to obtain sufficient material for identification by physical methods. The further development of a modification of the Girard reaction in which a fraction containing »conjugated ketones« could be separated from both non-ketonic fraction and the major bulk of the saturated ketones, made it possible to re-investigate this problem. The aim of the present communication, a preliminary account of which has been given elsewhere (Ismail & Harkness 1966 a), is to report the isolation and identification of progesterone from the »freely extractable« fraction of human pregnancy urine.

MATERIALS AND METHODS

Subject
The subject was a normal woman, in good health and in the last three months of pregnancy. Complete urine collections were made for approximately five weeks; urine was stored at 4° C for no longer than one week before extraction.

Reagents and Solvents
The solvents and reagents used were of analytical grade quality in all cases in which this grade was available. The solvents were re-distilled before use. The Girard reagent T and cation exchange resin Amberlite IRC-50 (H) were treated as described by Ismail & Harkness (1966 b). Consistently good recoveries of progesterone were obtained by using resin of particle size about 0.5 mm diameter. Fine particle resin was found to give incomplete coupling under these conditions.

Paper and Thin-layer Chromatography
Paper chromatography was performed on Whatman No. 42 paper in the system light petroleum (b.p. 60–80° C) -methanol-water (100:80:20 by volume). The systems used for developing thin-layer plates were hexane-ethyl acetate (5:1.5, v/v); benzene-ethyl acetate (4:1, v/v) and chloroform. Details of the preparation and methods of detection have been described previously.

Gas-liquid Chromatography
A Pye panchromatograph with a flame ionization detector was used (see Ismail & Harkness 1966 b). In addition, separation of the components in the final extract was performed using a Perkin-Elmer model number 801 with a stream splitter of ratio 4:1. The column used in the latter apparatus was packed with silicone treated Gas-Chrom P (100–120 mesh) coated with 3% of SE-30. Nitrogen flow was 25 ml/min, and column temperature 240° C. The larger stream was passed directly to the exterior and the
peak corresponding to the progesterone was trapped in a glass tube at room temperature. The glass tube had a diameter of 4 mm, was 20 cm in length, and had two constrictions near the end.

**Spectroscopy**

The visible and ultraviolet absorption spectrum was recorded in a Unicam SP. 800 spectrophotometer with 1 cm light-path. For infrared spectroscopy a Unicam SP. 200 was used, and a micro-technique (Sykes & Kelly, personal communication) was employed. The residue was dissolved in chloroform and deposited as a spot on a KBr disc. The latter was then placed in a beam condenser and the infrared spectrum recorded.

**ISOLATION PROCEDURE AND RESULTS**

1. **Extraction**

The unhydrolysed urine was extracted in batches of 10 litres with two volumes of ether. The combined ether extract was washed twice with 0.05 volume n sodium hydroxide saturated with sodium chloride; this was usually sufficient to remove the yellow pigments. The extract was then washed three times with 0.05 volume of distilled water. The ether extract was dried with anhydrous sodium sulphate and evaporated to dryness in a waterbath.

2. **Girard separation**

The residue was dissolved in a suitable volume of 97% aqueous ethanol and transferred to a tube. The solution was reduced to a volume of 1.0 ml or to dryness in a current of air. The Girard separation was then performed by the modified method of Ismail & Harkness (1966 b).

3. **Paper chromatography**

The «conjugated ketone» fraction obtained was chromatographed on paper in the system previously described. Authentic progesterone was chromatographed in parallel with the extracts, and located by its absorption of ultraviolet light and by its reaction with a saturated solution of dinitrophenyl-hydrazine in ethanol. The area of the chromatograms of the extracts corresponding to progesterone was eluted with methanol. The eluate was then filtered through sintered glass funnels and the filtrate was evaporated to dryness.

4. **Thin-layer chromatography**

The residue from paper was then chromatographed on a thin-layer of silica gel G in the system hexane-ethyl acetate (5:1.5 v/v). The plates were removed from the tank, dried in the air for 15 minutes and then rechromatographed in
the same system. Progesterone has a low $R_F$ value in this system and this "over-running" procedure increased the separation considerably. The progesterone area was located by spraying with dinitrophenylhydrazine in ethanol. The area from the extract corresponding to progesterone was eluted with methanol, filtered through a sintered glass funnel and evaporated to dryness.

5. Gas-liquid chromatography
An aliquot of the residue was subjected to gas-liquid chromatography in the Pye panchromatograph. The stationary phases 1 % SE-30 (w/w) and 1 % QF-1 (w/w) were used. Three components were generally found, the last of which had the same retention time as progesterone. Attempts to separate these components from progesterone using repeated chromatography on thin-layers of silica gel were unsuccessful. Furthermore, neither mild oxidation with 2.5 % chromium trioxide (w/v) in 50 % aqueous acetic acid (v/v) nor acetylation altered markedly the chromatographic mobilities of the other components travelling with progesterone.

In order to obtain a pure progesterone fraction, gas-liquid chromatography was used and the component corresponding to progesterone was trapped as previously described.

6. Spectroscopy and chemical reactions
The infrared absorption spectrum of the trapped component shown in Fig. 1 is virtually identical with that of authentic progesterone; the only significant differences at 13 μ being due to a small amount of chloroform trapped in the spot. It should be noted that authentic progesterone was recovered unchanged when it was subjected to this procedure. The ultraviolet absorption spectrum in ethanol was also recorded as previously described and the spectrum was identical with that of authentic progesterone. Further gas-liquid chromatography of the trapped component on SE-30 and QF-1 phases confirmed that it behaved as a single compound corresponding to progesterone. In addition, its chromatographic behaviour was unaltered by mild chromium trioxide oxidation and by acetylation. Reduction with potassium borohydride as described by Bush (1961) gave products behaving similarly to those obtained from authentic progesterone on gas-liquid chromatography and in thin-layer chromatography. In addition, when the trapped component was reduced with potassium borohydride and the reaction products were acetylated with acetic anhydride and pyridine the thin-layer and gas-liquid chromatographic patterns of the reaction products were identical with those of authentic progesterone.

On the basis of this cumulative evidence it was concluded that the component obtained from the urine was indeed progesterone. It was estimated from the gas chromatographic peak heights that the pooled urine (approx. 50 litres) residue contained approximately 250 μg of progesterone.
DISCUSSION

The modified Girard procedure described by Ismail & Harkness (1966 b) for the separation of a »conjugated ketone« fraction, separates progesterone from cholesterol and related compounds, these contaminants have similar properties to progesterone on paper, thin-layer and gas-liquid chromatography (see Drosdowsky et al. 1965). In the present study, subsequent chromatography on paper and thin-layer gave a fraction containing progesterone together with two other components. Neither repeated chromatography on thin-layer using different systems, nor mild oxidation and acetylation markedly improved the separation. However, gas-liquid chromatography using columns coated with 1 % SE-30 (w/w), 3 % SE-30 (w/w) or 1 % QF-1 (w/w) showed complete separation of the progesterone peak from other compounds.

The present investigation has shown that progesterone is present in small amounts in urine. The evidence from paper, thin-layer and gas-liquid chromatography, together with the confirmation by spectroscopy and chemical behaviour, has established the identity of the compound.
The isolation of testosterone from urine by Schubert & Wehrberger (1960) and the subsequent measurement of the small amounts present in human urine has proved of value in the study of patients with endocrine disorders. Such measurements do provide a reasonably satisfactory index of androgen production in men (Vander Wiele et al. 1963; Horton et al. 1965); this is due to the fact that a good correlation exists between the amount of the hormone produced in the body and that excreted in the urine. If a relatively constant quantity of the progesterone secreted were converted to urinary progesterone, and if other steroids produced in the body were not converted in significant amounts into this steroid, the estimation of progesterone in urine should provide an index of the secretion of the hormone by the placenta, corpus luteum and to a lesser extent the adrenal cortex. The value of the isolation reported herein would therefore appear to be the possibility it holds out for a more direct serial urinary measurement related to the secretion of progesterone in human subjects.

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