CHROM. 3613

Gas chromatographic estimation of urinary pregnane-3α,17,20α-triol and 11-keto-pregnane-3α,17,20α-triol*

ROSENFELD *et al.*[1] used paper and gas chromatography for estimating urinary pregnane-3α,17,20α-triol (PT). CHATTORAJ AND SCOMMEGNA[2] precipitated out the steroid conjugates with ammonium sulphate and estimated PT as the diacetate. Alumina column chromatography with estimation of PT and 11-keto-pregnane-3α,17,20α-triol (11-keto PT) as the trimethylsilyl ethers was used by KINOSHITA AND ISURUGI[3]. This communication presents a method using alumina column chromatography and estimation of PT and 11-keto PT as the diacetates by gas chromatography. 11-Keto PT was also estimated as the free steroid. Increased excretion of the urinary PT and the presence of 11-keto PT in congenital adrenal hyperplasia with the C_{21} hydroxylating deficiency is well established. 11-Keto PT is a particularly useful diagnostic aid, since it is absent in urine of normal subjects and in subjects with adrenal tumours where PT may be raised[4].

Experimental

Isolation. PT and 11-keto PT were hydrolysed and extracted by the extraction method of COX AND FINKELSTEIN[5]. Alumina chromatography was carried out with 3 g alumina dried at 105° and deactivated with 3–4 % v/w distilled water (Savory and Moore, Ltd.) or 6–7 % v/w distilled water (British Drug Houses, Ltd.) in benzene in a 10 cm column with a 25 ml reservoir and 0.3 porosity disk.

The elution system used was similar to that of LEON AND BULBROOK[6] with an additional step used by SHAND[7].

(1) 0.8 % Ethanol in benzene, 30 ml.
(2) 3.0 % Ethanol in benzene, 22 ml.
(3) 6.0 % Ethanol in benzene, 20 ml (pregnanetriol, some pregnanetriolone).
(4) 10.0 % Ethanol in benzene, 25 ml (remainder of pregnanetriolone).

Complete separation of 11-keto PT from PT was found with one batch of alumina as shown in Figs. 1 and 2 but not other batches. As both PT and 11-keto PT were generally estimated, fractions 3 and 4 were pooled (see Fig. 3). After evaporation acetylation was carried out with 0.2 ml pyridine and 0.2 ml acetic anhydride at 56° for 1 h. Evaporation was under nitrogen.

Gas chromatography. Analysis was with a 1.2 m glass column (I.D. 3 mm) packed with 80–100 mesh diaport S coated with SE30 (3.8 % by weight). The column was maintained at 230°–238° with nitrogen 40 p.s.i. An F and M 400 Biomedial model gas chromatograph with a flame ionizing detector was used. Relative retention times as compared with cholestane (8 min) were 1.97 for PT diacetate, 1.38 for 11-keto PT and 2.25 for 11-keto PT diacetate. For quantitation the peak weights were compared with reference standards. A linear relationship of peak weights to concentration over a range of 0.1–5 μg for PT diacetate and 11-keto PT as the free steroid and as the diacetate were observed. Cholesterol acetate was used as the internal standard.

The I.R. spectra of material collected in the 10 % ethanol in benzene fraction

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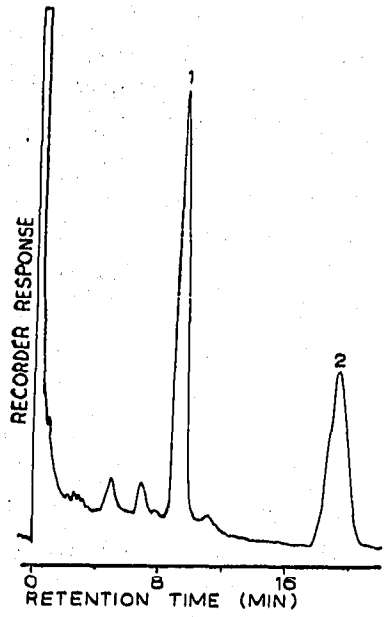

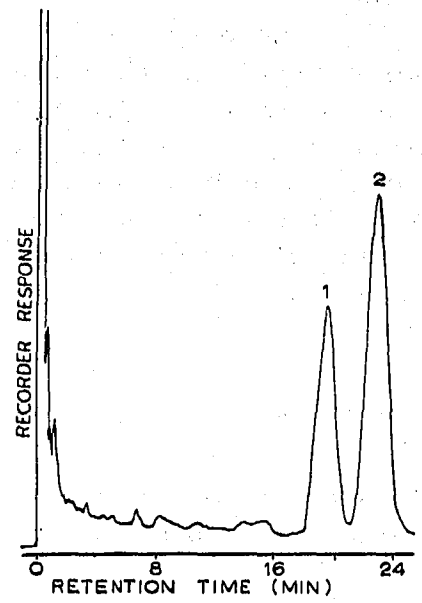

Fig. 1. Gas chromatographic analysis of 11-keto-pregnanetriol (1). Cholesterol acetate (2) was added as the internal standard. The extract is equivalent to 1/1,100 of a 24 h urine from a subject with congenital adrenal hyperplasia.

Fig. 2. Gas chromatographic analysis of 11-keto-pregnanetriol as the diacetate (1). Cholesterol acetate (2) was added as the internal standard. The extract is equivalent to 1/1,100 of the urine from a subject with congenital adrenal hyperplasia.

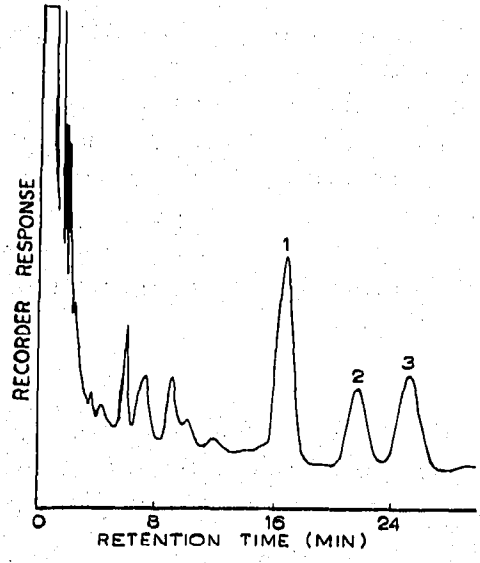

Fig. 3. Gas chromatographic analysis of pregnanetriol diacetate (1) and 11-keto-pregnanetriol diacetate (2). Cholesterol acetate (3) was added as the internal standard. The extract is equivalent to 1/7,000 of a 24 h urine extract from a subject with congenital adrenal hyperplasia.

from a urinary extract was acetylated and kindly compared by Dr. A. E. KELLIE, Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, with pregnanetriolone 3,20-diacetate (carbon disulphide 1800–700 cm^{-1}) and found to be similar.

Results and discussion

Values of 24 h urine excretion of PT were 1.5–15.2 mg and of 11-keto PT 348–5000 μg in four subjects with the C_{21} hydroxylating deficiency type of congenital adrenal hyperplasia who were on prednisone (5–10 mg daily). No 11-keto PT was detected in six normal subjects and three subjects with the Stein-Leventhal syndrome had values of 15–202 μg, which are similar to those published by SHEARMAN *et al.*[8]

Comparison of PT with the estimation as chromogens with sulphuric acid[9] on seven urine collections were within an average difference of 1 % over a wide range of values as shown in Table I.

TABLE I

QUANTITATION OF URINARY PREGNANETRIOL (mg/24 h)

Urine	*Gas chromatography*	*Sulphuric acid chromogens*
1	0.35	0.46
2	0.46	0.48
3	0.66	0.62
4	0.70	0.59
5	0.78	0.80
6	1.56	1.44
7	14.8	15.6

Comparison of 11-keto PT before and after acetylation in nine comparisons agreed within a maximum error of 25 %. The 11-keto PT values averaged 12 % lower than the acetylated 11-keto PT. 11-Keto PT from a subject with congenital adrenal hyperplasia before acetylation is shown in Fig. 1 and after acetylation in Fig. 2. Fourteen duplicate determinations were within a maximum error of 14% of each other. Recoveries of added PT and 11-keto PT (5–25 μg) gave a mean average of 77 % (63–112 %). The sensitivity of the method was of the order of 10 μg/24 h urine for both steroids. A relatively rapid and convenient method for determining pregnanetriol and pregnanetriolone by gas chromatography has been developed.

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Mise en évidence et dosage de la glutéthimide et de l'α-phénylglutarimide dans les milieux biologiques par chromatographie en phase gazeuse

Aucune des techniques colorimétriques[1,2], spectrophotométriques[3–7], fluorimétriques[8] ou chromatographiques en phase gazeuse[9,10] jusqu'ici préconisées ne permettait de caractériser et de doser séparément la glutéthimide* et son métabolite le plus important, l'α-phénylglutarimide, sur un même échantillon et au cours d'une même manipulation. Nous rapportons ici les premiers résultats d'une étude portant sur la mise au point d'une telle technique et cela selon des modalités originales.

Experimentation

Extraction. Nous avons utilisé la technique préconisée par GOLDBAUM et coll.[6].

Une partie aliquote de la solution ou de l'homogénat à examiner est extraite par agitation avec un volume déterminé de chloroforme. La phase chloroformique est ensuite lavée avec de petits volumes de NaOH 0.5 *N* et de H_2SO_4 0.5 *N* et évaporée à sec.

Le résidu est repris par 2 ml d'alcool absolu et 5 ml d'hexane. Après addition de 0.5 ml d'eau distillée, la glutéthimide et l'α-phénylglutarimide passent dans la phase alcool–eau. Une partie aliquote de cette phase alcool–eau est ensuite évaporée à sec.

Chromatographie en phase gazeuse

Les conditions opératoires mises au point sont les suivantes:
Appareil: Aerograph HyFi 600 D
Détecteur: Ionisation de flamme
Colonne: verre 8 ft. × 1/8 in.
Phase stationnaire: XF 1112 8% sur Chromosorb W HMDS 60–80 mesh
Température de la colonne: 195° C
Température injecteur: 250° C
Gaz vecteur: N_2, 25 ml/min.
Étalon interne: 3,3-diéthyl-2,4-dioxypipéridine (Sedulon®)
Volume injecté: 1–5 µl

* 3-Éthyl-3-phényl-2,6-dioxypipéridine.