BBA 4007

STUDIES IN STEROID METABOLISM

XIV. MODIFICATION OF THE ANALYSIS OF URINARY ESTROGENS BY GAS CHROMATOGRAPHY

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SUMMARY

Modifications of the previously presented method of estrogen determination are reported. Use of an SE-30 phase gives sharper peaks and allows faster analyses, also, one further compound—16-*epi*estriol—may be analyzed simultaneously.

INTRODUCTION

The first application of gas chromatography to the analysis of urinary steroids[1,2] demonstrated the usefulness of this tool in attaining accurate and rapid evaluations of the classic estrogens. The procedure described in those reports utilized either a Dow–Corning Silicone grease column or mixed columns of Silicone grease–SE-30 elastomer (2:1).

Further investigation of this procedure, as reported below, allows a faster separation as well as measurement of another substance known to be present in pregnancy urine.

RESULTS

Fig. 1 shows a crude urine extract chromatographed on a 6-foot, 0.25-in outer diameter copper column of 3 % SE-30 (deposited on 80–100 mesh diatomaceous earth (Anakrom ABS)) at 240° and a pressure of 30 lb/in^2. Quantitative comparison of estrone and estriol content of six crude urine samples on the SE-30 column and Dow–Corning Silicone grease column showed a variation for estrone and estriol acetates within the limits of reproducibility of the original method, obviating a complete reassessment of the methodology. The use of crude urine extracts appeared to present the problem of detector fouling. It has been our experience that the argon ionization detector under such adverse conditions required frequent (weekly) cleaning and in our oldest instrument (18 months) the detector became so corroded that it had to be replaced.

Use of hydrogen flame ionization detector

In view of this corrosion problem and the fact that steroids may be chromatographed intact at the relatively high temperatures[3] necessary at lower flow rates it was decided to use a flame ionization detector (Research Specialties Company,

Richmond, Calif. (U.S.A.)). This type of detector has several advantages. It does not corrode and indeed, in four months continuous use, required no shutdown for cleanouts. It allowed the use of much higher temperatures which in turn permitted the use of longer and more highly resolving columns, limited only by the heat stability of the stationary phase. Furthermore, the specific detector used showed an average increase in sensitivity of one half a magnitude. As little as 0.003 μg of estrone acetate was detectable at a retention time of approx. 5 min. From the standpoint of economics it had the further advantage of being operated with nitrogen at greatly reduced cost.

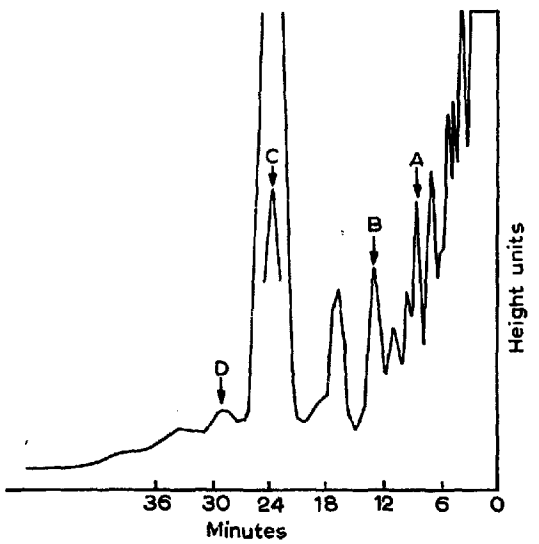

Fig. 1. Chromatogram of a 1/1030 aliquot of a ninth month pregnancy urine. The following compounds were identified: A, Estrone acetate; B, estradiol diacetate; C, estriol triacetate; D, 16-*epi*estriol triacetate. Column conditions: 6 foot, 3% SE-30 on Anakrom ABS, 80–100 mesh. Temperature, 245°; flow, 130 ml/min; pressure, 30 lb/in^2.

Ample linearity of response to estrone acetate and estradiol diacetate was obtained. A straight line was derived on plotting peak height *versus* concentration from 0.08 to 10.0 μg after correction for attenuation changes.

In general, chromatograms of urine extracts have been run, with the column described above, at 265° with 14 lb/in^2 of nitrogen pressure. The flame ionization detector was operated with 8 lb/in^2 of compressed air and 10 lb/in^2 of hydrogen pressure.

Irreversible adsorption

Over a period of months it was noted that whenever a steroid was injected into a column following a lay-over period of several hours or days, low detector response was observed with some increase after the second and third injection. The peak height measured usually after the third and subsequent injections remained constant. This suggested an essentially irreversible adsorption of steroid on the column, which after saturation (priming) of the binding sites did not further interfere with chromatography. Casual observation also suggested that the more heat labile steroids like aldosterone diacetate required priming more frequently than did the stable estrogen acetates.

In order to get some measure of this adsorption, estrone acetate was chromatographed on an SE-30 (3%) preparative column (0.5 in outer diameter) and the

effluent material was collected and quantified by ultraviolet absorption spectra and the Kober reaction. Collection of material was carried out by leading the exit tube from the gas chromatograph into the mouth of a large test tube which was nearly completely submerged in liquid nitrogen. Trapping of the aerosol, followed by argon evaporation, left behind the steroid residue. Mean recovery of estrone acetate (500 μg) in 20 experiments was 91 %. As shown in Table I, much of the material from the first injection was retained by the column. After the second injection less steroid was adsorbed and the third run passed through the column essentially complete.

TABLE I

RECOVERY OF ESTRONE ACETATE FROM AN UNPRIMED COLUMN

Total column packing: 58 g 3% SE-30 on Chromsorb (30–60 mesh). The column was cured at 250° for two weeks.

Injection	*Injected (mg)*	*Recovered (mg)*	*Adsorbed (%)*	*Steroid adsorbed/g of packing (µg)*
1	0.5	0.335	33	2.85
2	0.5	0.415	17	1.46
3	0.5	0.465	7	0.60

This type of adsorption has so far been observed with columns coated with SE-30 and SE-52 elastomers and Dow–Corning Silicone grease. A similar phenomenon with other phases may be anticipated.

Identification of peaks

Using the method of trapping individual components described previously, further identification of the peaks corresponding to the three classic estrogens as well as that corresponding to 16-*epi*estriol has been carried out. Paper chromatographic travel rates in a toluene–propylene glycol system, followed by infrared verification of the eluate as a micro KBr window allowed identification of the peaks marked A, B, C and D (Fig. 1) as estrone acetate, estradiol diacetate, estriol triacetate and 16-*epi*estriol triacetate respectively.

These identifications were further corroborated by comparison of the retention times of the extracted materials with authentic steroid acetates as shown in Table II.

TABLE II

SEPARATION OF SOME ESTROGEN ACETATES

Cloumn: 3% SE-30 elastomer on Anakrom ABS (80–100 mesh), 6 foot, 3/16 in outer diameter at 265° under 14 lb/in^2 N_2.

	Retention time (min)	*Relative retention time*	*Separation factor*
Estrone	9.7	1	
			1.34
Estradiol	13.0	1.34	
			1.04
2-Methoxyestrone	13.7	1.40	
			1.44
16-Ketoestradiol	19.8	2.03	
			1.04
16-Hydroxyestrone	20.7	2.12	
			1.19
Estriol	24.6	2.52	
			1.18
16-*Epi*-estriol	29.0	2.97	

Pregnancy urine data

In Fig. 2 are shown the concentrations of three estrogen acetates obtained during a single pregnancy over a span of 3.5 months immediately prior to parturition, using the modification of the method described above.

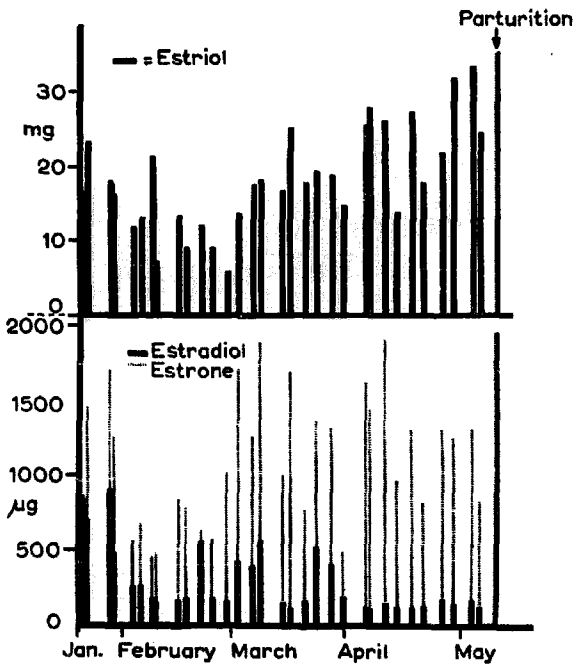

Fig. 2. Excretion of estrone, estradiol and estriol during the last trimester of a single pregnancy. Analyses were carried out as described using a flame ionization detector and a column temperature of 265°, with 14 lb/in^2 of N_2 pressure.

DISCUSSION

The modifications described here further decrease the working time necessary for an individual assay while introducing an analytical capability for the assay of four different estrogens. The use of pure SE-30 stationary phase and finer mesh support produces sharper peaks and greater peak resolution and slightly increases the sensitivity of the method. The heat stability of SE-30 is somewhat lower than that of Dow–Corning Silicone grease but up to 270° it was found to be acceptable, showing relatively little column bleeding.

A recent publication by FISHMAN *et al.*[4] using essentially the approach described previously[2] but using a straight SE-30 column further verifies the analytical reliability of this method, by comparing favorably the results obtained after gas chromatography of an acetylated crude extract with results obtained by the BROWN procedure[5].

The results obtained from the analysis of the pregnancy urines are essentially in keeping with those obtained for the measurement of these estrogens by other procedures. Of possible interest may be the observation that while estriol excretion tends to rise continuously toward parturition, estradiol and estrone excretion seems to decrease several weeks before. The significance of this disparity in excretion is obscure and needs verification by accumulation of further data.

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