QUANTITATION OF URINARY ESTROGENS BY GAS CHROMATOGRAPHY*

J. FISHMAN AND J. B. BROWN

Sloan-Kettering Institute for Cancer Research, New York, N.Y. (U.S.A.)

(Received November 20th, 1961)

Although the method of BROWN (Method A)[1] for measurement of estrogens has been shown to be accurate and reliable, a simplified and more rapid method of analysis would prove of great value. The feasibility of separating steroids by gas chromatography has been recently shown and a large variety of pure steroids have been effectively separated on several different columns[2]. However, little information is available on steroid estimations by gas chromatography in crude extracts from natural sources such as urine. The main problem in this application is the nature and extent of purification necessary to obtain extracts adequate for the sensitive gas chromatography procedure. Obviously, a lengthy purification sequence would be self-defeating and no advantage over established methods. Our initial attempts to use gas chromatography in the estimation of the urinary estrogens were with late pregnancy urine, where the amounts of estrogens present are relatively large. For purposes of comparison values for estrone, estradiol and estriol only are reported since these are the metabolites estimated by Method A.

EXPERIMENTAL

Gas chromatography

The instrument was the Barber Coleman Model 10. Columns were 1.8 m × 5 mm i.d. packed with 100–140 mesh Gas Chrom P coated with SE-30 (3 % by weight**). The columns were kept at 235°, while the detection cell and flash heater were at 260° and 265° respectively. The gas was argon at 30 p.s.i. and an ionization detection system was used. Voltage was 600.

Calibration curves

Estrone acetate, estradiol diacetate and estriol triacetate were crystallized to constant melting point and dissolved in ethanol for calibration standards. The area of the peaks was obtained from the half-band width. The relationship to the amount present was not completely linear particularly at the lower values, but was sufficiently accurate for the problem at hand. On the same column under the same conditions there were only minor changes in the calibration plots with time. The retention times

* This investigation was supported in part by a grant from the American Cancer Society and a research grant (CY-3207) from the National Cancer Institute of the National Institutes of Health, United States Public Health Service.

** General Electric Company, Silicone Divison, Waterford, N.Y.

(estrone acetate – 9.3 min, estradiol diacetate – 13.5 min and estriol triacetate – 24.9 min), did not change if the same column was used. The limits of useful sensitivity were 0.5 μg for estrone and estradiol and 1.5 μg for estriol. A calibration plot is shown in Fig. 1.

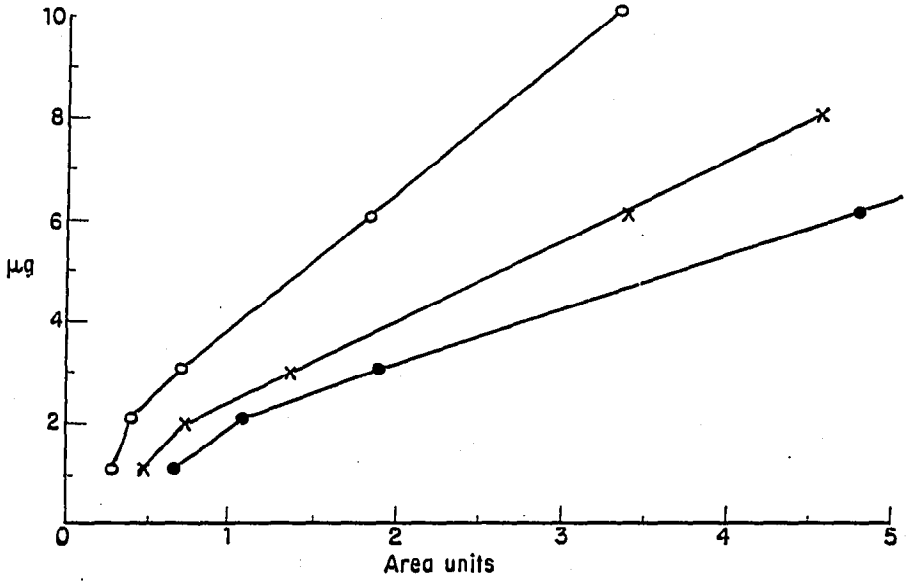

Fig. 1. Calibration curves for estrogen metabolites. ○ = estriol triacetate; × = estradiol diacetate; ● = estrone acetate.

Preparation of samples

Different samples of late pregnancy urine were incubated with β-glucuronidase* and then extracted with ether. The ether extract was washed with 9 % sodium bicarbonate solution saturated with sodium chloride and the solvent was removed. A portion of this extract was analyzed by Method A by one of the authors in the laboratories at Edinburgh. Another portion was acetylated by standing overnight in acetic anhydride and pyridine at room temperature and then being heated at 60° for 0.5 hours. Ethanol was added and the excess reagents were removed under a nitrogen stream on a water bath. The residue was dissolved in ethyl ether and filtered through 5 cm × 2 cm column of neutral alumina. The filtered extract was dried and dissolved in acetone for gas chromatography.

RESULTS AND DISCUSSION

It was found that the best results for estrone and estradiol were obtained when 0.5–1.0 % of a urine extract from a single day was injected onto the column. For estriol which is present in much larger amounts, 0.1–0.2 % of the extract from a single day had to be used. Despite the presence of numerous other materials the pertinent peaks were readily identified, and their areas could be determined. A typical chromatogram is shown in Fig. 2. The results obtained are listed in Table I (V.P.C.) and are compared with those obtained by Method A.

* β-Glucuronidase, known as Ketodase, was obtained from the Warner-Chilcott Laboratories, a division of Warner-Lambert Pharmaceutical Company, New York, N.Y.

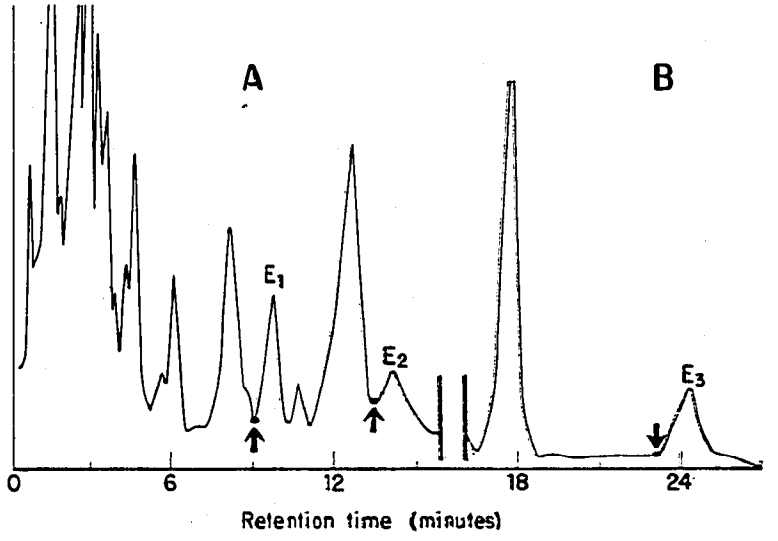

Fig. 2. Representative gas chromatogram of acetylated extract from late pregnancy urine. (A) represents 1 % of one day extract; (B) represents 0.1 % of one day extract. E_1 = estrone acetate; E_2 = estradiol diacetate; E_3 = estriol triacetate. → point from which a line parallel to the ordinate was drawn for calculation of peak height.

TABLE I

COMPARISON OF ANALYTICAL METHODS FOR ESTROGEN METABOLITES

*Sample**	*Steroid*	*% Sample used for V.P.C.*	*μg/24 h***	
			V.P.C.	*Method A*
1	E 1	1	640	600
	E 2	1	200	180
	E 3	0.1	9,800	9,500
2	E 1	1	920	1,050
	E 2	1	260	280
	E 3	0.1	10,000	10,250
3	E 1	1	200	200
	E 2	1	100	130
	E 3	0.1	8,200	7,800
4	E 1	0.5	300	280
	E 2	0.5	—	150
	E 3	0.1	10,800	11,000
5	E 1	1	570	620
	E 2	1	200	190
	E 3	0.1	9,400	9,700
6	E 1	1	350	400
	E 2	1	60	90
	E 3	0.2	5,750	6,000

* Samples represent different 24 hour collections from 3 different subjects.
** Values expressed as free steroids.

The results obtained by the two methods were quite comparable. The widest variations observed were in the estradiol values. This is quite likely due to both the relatively small quantities of this substance present as well as the fact that the peak for estradiol in the chromatograms was the least well defined. It should be pointed out that no correction has been applied to the gas chromatography results, while the figures from Method A are corrected by 20 % for manipulative losses. The purification of the samples as described above was minimal. It has been found that further separation into phenolic and neutral components was not necessary with late pregnancy urine. The acetylation is not *per se* a purification step but is carried out to make the estrogens more thermostable and introduce a substantial molecular weight distinction between the three compounds. This is necessary to permit separation on the non-polar phase employed, as discussed by WOTIZ AND MARTIN[3]. The quantity of material that can be placed on the column is of importance in this problem. With the minimal purification employed by us, 2 % of a day's extract still gives a background that does not obscure the estrogen peaks but the quantities of estrogens present are such that less than 0.5 % of a 24 hour collection will not give measurable peaks for estrone and estradiol. Therefore, in extracts with less estrogen, larger portions of extract would have to be chromatographed and more extensive purification would be necessary.

There is little doubt from the results obtained that gas chromatography is a useful analytical method for estrogens in late pregnancy urine. It is considerably faster and simpler than other available methods and gives results of comparable accuracy. However, further application of this method to estrogen analysis in non-gravid female and male urine, particularly to the other metabolites present, will require substantial refinement. It is hoped that current work on both instrumentation and sample preparation will permit the eventual rapid and uncomplicated quantitation of estrogens in physiological extracts.

SUMMARY

A method for the quantitative estimation of estrogens in late pregnancy urine by gas chromatography is described. The values obtained are compared with those obtained by an alternative chemical procedure (Method A).

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