Short Communication

Gas chromatography in the analysis of urinary pregnanediol*

Separation of a wide range of pure steroids by gas chromatography has been accomplished recently (reviewed by HORNING *et al.*[1]). Rapidity is a great advantage of this technique over other chromatographic methods used in steroid analysis. Little work has yet been reported on the application of gas chromatography to the analysis of urinary steroids as distinct from pure steroids. In this study the gas chromatographic technique was evaluated as a method which could give rapid and accurate results in the analysis of pregnanediol in pregnancy urine.

Apparatus

The gas chromatograph used was a Pye Argon Model 12001 with a 20 mC ^{90}Sr β-ionization detector. A septum-injector and pre-heater stage were added to the standard equipment. The pre-heater temperature was 250°. Injections of 2 μl of solutions of steroids or extracts in absolute ethanol were made onto 4 ft. long columns, 5 mm internal diameter, packed to a length of approximately 110 cm.

Suitable column packings and liquid phases were selected from the behaviour of pure pregnanediol and other steroids, and re-investigated to determine their applicability to urine extracts. Two selected for work with extracts were (a) the non-polar 3 % "Embaphase" silicone oil, (May & Baker Ltd., London) on Gas Chrom Z, (Applied Science Laboratories) used at 240°, and (b) the polar phase 0.5 % neopentylglycol adipate (NGA) on Gas Chrom Z, 100/140 mesh used at 210–220°.

Chromatography of pure steroids

The behaviour of the columns with pure steroids is shown in Table I.

All these major urinary neutral steroids are well separated from pregnanediol on the adipate column. Amounts of 1–10 μg of pregnanediol were normally applied to the columns. In actual running, pregnanolone and pregnanediol were not completely separated on the 3 % embaphase but were readily separated on 1 % or 0.5 % neopentylglycol adipate columns. The total of pregnanolone and pregnanedione was found to be less than 20 % of the pregnanediol levels in the 50 urines examined. Further results refer to adipate columns.

Chromatography of urine extracts

Extracts were prepared as in the first stage of the procedure of KLOPPER *et al.*[2], using an acid hydrolysis, toluene extraction, alkali partition and permanganate oxidation of impurities (extract B, see below). For gas chromatography, extract residues were dissolved in 50 μl of ethanol. From these solutions, 2 μl aliquots were injected into

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the chromatograph. Solutions in ethanol gave sharper peaks than those in chloroform or tetrahydrofuran.

With pregnancy urine extracts a clear separation of a peak corresponding to pregnanediol has been obtained with no interference from other peaks. Typical results

TABLE I

GAS CHROMATOGRAPHY OF PURE STEROIDS

Steroid	*Retention times relative to cholestane*	
	(a) on 3% NGA at 220°*	*(b) on 0.5% embaphase at 240°*
Cholestane**	1.00	1.00
5β-Pregnane-3α,20α-diol	4.31	0.68
5β-Pregnan-3α-ol-20-one	3.52	0.66
5β-Pregnane-3,20-dione	3.64	0.65
Androsterone	2.39	0.39
Aetiocholanolone	2.39	0.38
Dehydroepiandrosterone	2.64	0.38
11-Oxo-androsterone	5.75	0.48
11-Oxo-aetiocholanolone	5.36	0.44
11-Hydroxy-aetiocholanolone	10.1	0.73

* Neopentylglycol adipate

** *Cholestane retention time* was (a) 2.0 and (b) 7.9 min with the above columns at an outlet argon gas flow rate of 60 ml/min.

are illustrated in Fig. 1. A pregnanediol peak was observed to be one of the main peaks in extracts from 50 urines of the 2nd to 8th month of pregnancy. For late pregnancy urines the pregnanediol peak was the major one observed. For early pregnancy, peaks corresponding to mixtures of androsterone, aetiocholanolone and dehydroepiandrosterone were similar in magnitude to that of pregnanediol but well separated rom it as would be expected from the behaviour of pure steroids (Table I). Six

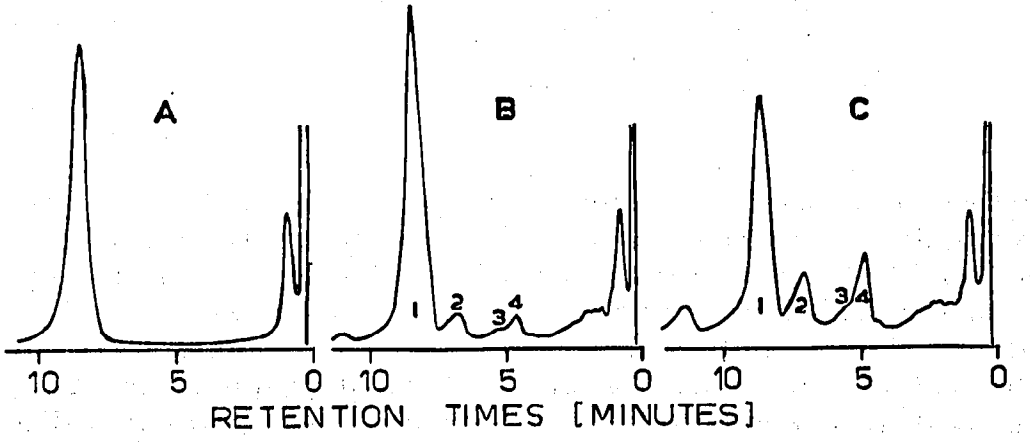

Fig. 1. Gas chromatography on 0.5% NGA at 220°; Argon flow 60 ml/min. Pure pregnanediol 6.3 μg (A); urine extracts from a patient 25 weeks pregnant, pregnanediol 32 mg/24 h (B); and from a patient 16 weeks pregnant, pregnanediol 11 mg/24 h (C). Steroids indicated as present: (1) pregnanediol; (2) pregnanolone and pregnanedione; (3) dehydroepiandrosterone; (4) aetiocholanolone and androsterone.

recovery experiments from urine gave 92–103 % recoveries for pregnanediol. For 20 extracts examined as neutral toluene extracts without a permanganate wash, the recording obtained was practically the same as with the wash (extract B).

For non-pregnancy urine, exploratory experiments with 4 urines containing from 0.8–2 mg/24 h. pregnanediol showed that a pregnanediol peak could be observed, but it was a relatively minor one in the presence of other strong peaks, such as those of 17-oxosteroids. For such extracts, good chromatograms with pregnanediol as a major peak were obtained following a prior purification that occurs on an alumina chromatogram as in the KLOPPER procedure. An alternative for non-pregnancy urine may be the formation of the trimethylsilyl derivatives as noted by HORNING *et al.*[1].

Identity of the "pregnanediol" peak

Firstly a comparison was made of the quantitative results by gas chromatography on extracts at stage B and the full sulphuric acid chromogen procedure of KLOPPER *et al.*[2]. For quantitation by gas chromatography the area of peaks was determined by triangulation and compared to standard calibration values run on the same day. Results obtained by the two methods are shown in Table II. Good agreement was obtained between the methods for the pregnancy urines, each of which was from a different subject.

Secondly, portions of the extracts from various later stages in the KLOPPER procedure were taken for gas chromatography to observe the number of other peaks appearing and the resolution of pregnanediol and pregnanediol diacetate from them

TABLE II

QUANTITATIVE ESTIMATION OF URINARY PREGNANEDIOL

Sample	*Pregnanediol found (mg/24 h)*	
	(a) by gas chromatography	*(b) by sulphuric acid chromogens*
Pregnancy urine 1	37	38
Pregnancy urine 2	28	30
Pregnancy urine 3	8.9	9.0
Pregnancy urine 4	7.5	8.3
Pregnancy urine 5	4.2	3.8
Follicular phase urine	0.5	0.8

as extracts were purified. An alumina chromatogram of the urine extracts (stage B) removes a number of relatively strong peaks including those corresponding to androsterone, dehydroepiandrosterone, aetiocholanolone and most of the pregnanolone. The pregnanediol peak was practically the only one present after this alumina purification. It was significant that the other alumina chromatogram fractions gave no peak in the region of pregnanediol on gas chromatography. After acetylation, the only strong peak that was observed, corresponded to pregnanediol diacetate. After further alumina chromatography of the acetylated material, only a single peak was eluted on gas chromatography, with a retention time which corresponded to that of pregnanediol diacetate. This indicates the homogeneity of the pregnanediol peak observed on gas chromatography of extracts at stage B.

Using cyanoethylpolysiloxane on Gas Chrom P, TURNER *et al.*[3] also reported promising results in the estimation of pregnanediol in pregnancy urine. Their conditions did not separate pregnanediol from pregnanolone however. Some preliminary data on urinary pregnanediol separations have been given by PATTI *et al.*[4] with SE 52 columns.

The results presented above indicate that gas chromatography can be used for rapid and accurate analysis of pregnanediol in pregnancy urine, following a very simple hydrolysis and extraction procedure. Further work is in progress to establish fully the specificity, accuracy and reproducibility of the procedure as a standard analytical technique for pregnanediol estimation.

Department of Obstetrics and Gynaecology, University of Adelaide, Adelaide (Australia) R. I. COX

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Notes

Devices for continuous development and sample application in preparative thin-layer chromatography

The resolution of closely related substances by thin-layer chromatography may often be improved by decreasing the polarity of the solvent system to a point where the R_F values are less than 0.1. Either repeated or continuous development is then required to obtain appreciable mobilities and complete separation. Repeated development has the advantage of being technically simple and, because the lower parts of the zones are readied first by the new solvent front, tailing effects are reduced. However, a disadvantage is the long drying time required between developments when relatively thick layers are used for preparative work.

In paper chromatography, continuous development is usually carried out by the descending overflow technique. Methods for descending development of thin-layer chromatograms have been described[1,2], but the zones are broader than those obtained by ascending development[2] and special apparatus is required. Two methods of continuous development based on evaporation of the solvent from the terminal edge of the plate have been reported[2,3]. BRENNER AND NIEDERWIESER[3] used a hori-