CHROM. 4334

A CHROMATOGRAPHIC AND FLUORIMETRIC METHOD FOR THE DETERMINATION OF OESTRIOL IN PREGNANCY URINE

RUTH DVIR AND R. CHAYEN
Endocrine Laboratory, Ichilov Hospital, Tel-Aviv (Israel)

(First received February 25th, 1969; revised manuscript received August 20th, 1969)

SUMMARY

A method is described for the measurement of oestriol in pregnancy urine. After hydrolysis of the urine, oestrogens are reacted with 1-dimethylaminonaphthalene-5-sulphonyl chloride (DANSYL-chloride) to give fluorescent derivatives. The oestriol derivative is separated by thin-layer chromatography on Kieselgel G in a solvent of ethanol–chloroform (5:95). It is located by viewing under UV light, eluted and measured in a spectrofluorimeter, using wavelengths of 346 mμ and 525 mμ, respectively, for activation and emission.

INTRODUCTION

Urinary oestriol is generally considered to be a valuable index of foetal viability. However, since the clinician requires results as soon as possible, most methods for determination of oestriol measure only total oestrogens and rely on the observation that oestriol is usually by far the major component in the later stages of pregnancy.

In order to measure oestriol specifically, BROWN AND COYLE[1] developed a method involving methylation and column chromatography. This procedure has become widely adopted but is still longer and more complicated than the less specific methods.

More recently, a note appeared[2] (without description of procedure) on thin-layer chromatographic separation of the fluorescent dimethylaminonaphthalene sulphonyl (DANSYL) derivative of oestrogens, followed by measurement of the fluorescence intensity. This procedure was applied to the determination of oestrogens in the plasma of the domestic fowl, where, however, there was much interference from other phenolic constituents.

In human pregnancy urine, the content of oestriol is considerably elevated, and we considered that the preparation, separation and measurement of the DANSYL derivative in this case should be a relatively simple matter.

MATERIALS AND METHODS

1-Dimethylaminonaphthalene-5-sulphonyl chloride (DANSYL-chloride) was

obtained from the Aldrich Chemical Company, Inc. Material obtained from three other sources was unsuitable as judged by melting point determination and solubility in acetone. The DANSYL-ation reagent was prepared as a solution of 2 mg/ml of DANSYL-chloride in acetone. The solution was kept in the refrigerator and was stable for at least one month. In practice, however, fresh solutions were prepared every week.

Acetone and ethanol were analytical-grade reagents, and the latter was redistilled. Diethyl ether was washed with ferrous sulphate solution and redistilled. Oestriol was obtained from Ikapharm, Ltd.

Buffer solution (pH 10.5) was prepared by mixing 100 ml of 8% sodium bicarbonate solution with 15 ml of 5 *N* sodium hydroxide, and by adjusting the pH exactly with the aid of a pH meter with an addition either of more sodium hydroxide or bicarbonate.

Final procedure for determination of oestriol

Samples of 1 ml of a 24-h urine collection were diluted to 10 ml with distilled water in a 100-ml round-bottomed flask fitted with a reflux condenser. Concentrated hydrochloric acid was added (1.5 ml), and the solution was boiled under reflux for 30 min, then 1.5 g of sodium chloride were added to prevent emulsification. The cooled solution was extracted with 20 ml of ether, and the ether layer was washed with 8 ml of 8% sodium bicarbonate.

The ether extract in a 25-ml conical centrifuge tube fitted with a ground-glass joint was evaporated to dryness by warming in a water bath at 40–45° under a stream of air. In order to aid evaporation of residual water, 0.2 ml of alcohol was added, and the evaporation was repeated. To the evaporated extract were added 0.2 ml of buffer solution (pH 10.5) and 0.2 ml of DANSYL-chloride reagent. The mixture was allowed to stand at room temperature for 15 min and then was heated in a beaker of water. The heating was continued until the water had reached boiling point, and for a subsequent period of 20 min. The mixture was cooled, 3 ml of 0.5 *N* aqueous sodium hydroxide solution were added and the DANSYL-oestriol was extracted into 6 ml of ether by shaking in the stoppered tube. The lower, aqueous layer was removed by suction and the ether layer was evaporated to dryness, as previously, in the same tube.

To the evaporated extract was added 0.5 ml of chloroform, and 0.1 ml of this solution was applied to a thin-layer chromatography plate of Kieselgel G (Merck), 250 μ thick, which had been activated at 120° for 30 min. Chromatography was performed in a solvent of ethanol–chloroform (5:95) and, after allowing the solvent to evaporate, the separated DANSYL-oestriol was identified by viewing the plate under a UV-lamp giving maximum transmission at about 366 mμ. The spot, having an R_F value of about 0.3, was scraped into a centrifuge tube and extracted by standing for 3 min in 3 ml of ethanol (after initial vibration on a vortex mixer). The centrifuged solution was decanted into a 1-cm-square quartz fluorimeter cuvette, and the fluorescence intensity was measured in a Farrand MK-1 spectrofluorimeter. Wavelength settings were 346 mμ for activation and 525 mμ for emission. Slit system number 20 was used and the sensitivity setting was 0.1.

A standard sample of 10 μg oestriol was run with each batch of urine samples, and oestriol values were estimated in terms of fluorescence intensity of the standard.

Variations of the method

Formation of DANSYL derivative. Variables in the DANSYL-ation procedure include:

(1) Solvent for preparing the solution of DANSYL-chloride and concentration of the solution.

(2) Composition and pH of the medium in which DANSYL-ation is carried out.

(3) Time and temperature of the reaction.

In the present study, methyl ethyl ketone was tried as a substitute for acetone in order to use a higher-boiling solvent. Concentrations of DANSYL-chloride were in the range of 1–5 mg/ml. In all cases, 0.2 ml of the DANSYL-chloride reagent was treated with 0.2 ml of an alkaline solution. For the latter, three different solutions were tried: 8% $NaHCO_3$, buffer (pH 10.5) and 0.5 *N* NaOH. Temperature was varied from ambient to 100°, and time of reaction from 1 min at 100° to several hours at room temperature.

Chromatography. Kieselgel G plates were used as described above. Although the DANSYL-oestriol is soluble in a number of solvents, chloroform was found to be most suitable for applying a compact spot.

Suitable solvents for chromatography were ethanol–benzene (5:95), ethanol–chloroform (5:95) and dioxan–chloroform (5:95). In these solvents, DANSYL-oestriol gave spots of low R_F, and DANSYL-oestradiol gave high R_F values. DANSYL-oestrone ran almost with the solvent front.

Cyclohexane–ethyl acetate (3:2) was also tried in order to lower the R_F value of DANSYL-oestrone, but with this solvent the R_F value of DANSYL-oestriol was too low.

RESULTS

Formation of DANSYL-oestriol

Change of concentration of DANSYL-chloride in acetone from 1–5 mg per ml gave no changes in results. However, in order to allow for a safety margin in the event of deterioration of the reagent, it was decided to adopt a concentration of 2 mg/ml.

Of the various alkaline solutions tried, there was no appreciable difference between them, but buffer solution (pH 10.5) gave the most constant results. Linear calibration graphs were obtained when the reaction mixture was left overnight at room temperature, but quantitative formation of DANSYL-oestriol could be attained much more quickly by heating in a boiling water bath. The time of heating was relatively unimportant within the range of 1–30 min, and a 20-min period was finally adopted.

Although, in practice, the results were reproducible, there appeared to be a disadvantage in using so volatile a solvent as acetone and heating it at 100°. Under these conditions, the acetone is volatilised very quickly, with evolution of large bubbles. This brings about two problems: firstly, the reaction no longer takes place in a medium that is 50% aqueous acetone and, secondly, some of the solute tends to be carried onto the sides of the tube above the bulk of the reaction mixture.

In an attempt to lessen the volatility of the solvent, the next higher homologue, methyl ethyl ketone, was tried. This is not entirely miscible with water and did not

give reproducible results. An additional disadvantage was the need for more vigorous conditions for volatilising the solvent at a later stage.

Finally, as a compromise measure, acetone was again used as solvent, but the reaction mixture was allowed to stand at room temperature for 15 min, and was then brought gradually to 100° by standing in a water bath that was heated from room temperature to boiling. The tube was kept in the water bath for a further 20 min. Under these conditions, constant results and maximum derivative formation were obtained.

Chromatography

Thin-layer chromatography of standard DANSYL-oestrogens and of DANSYL-ated urine extracts is shown in Fig. 1. The solvent was ethanol–chloroform (5:95).

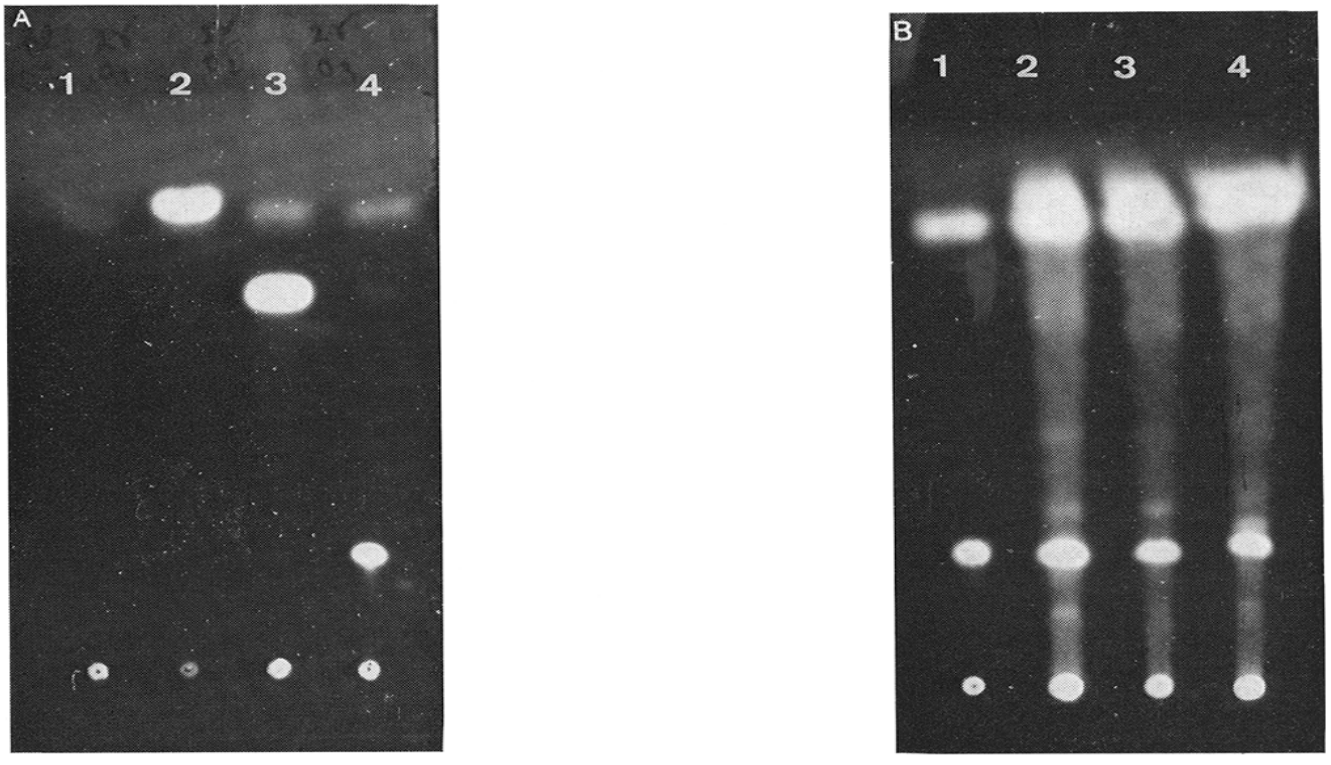

Fig. 1. Thin-layer chromatograms of DANSYL-ated oestrogens, photographed under UV light at 366 mμ. The support is Kieselgel G (Merck) applied to a thickness of 250 μ. Solvent is ethanol–chloroform (5:95). (A) Chromatogram of DANSYL-ated oestrogen standards. 1 = 'blank' (no oestrogen added); 2 = oestrone; 3 = oestradiol; 4 = oestriol. (B) Chromatogram of DANSYL-ated urinary oestrogen extracts. 1 = standard oestriol; 2, 3 and 4 = oestrogen extracts of pregnancy urines.

The R_F value of the oestriol derivative is about 0.3, and this allows separation both from more polar and less polar materials. This solvent was highly suitable for DANSYL-oestriol but less so for DANSYL-oestradiol; the oestrone derivative ran almost with the solvent front.

Sensitivity and reproducibility of the method

Linearity of the fluorescence intensity with quantity of chromatographed spots

of DANSYL-oestriol was obtained over a range of 0.1 to 50 μg oestriol in the original sample. So it can be said that measurements can be made on 1 ml of a 24-h urine that contains approximately 0.1–50 mg oestriol. The only adjustment required is change in the sensitivity setting of the spectrofluorimeter.

Reproducibility was studied on twenty urine samples. Duplicate samples were measured; then recovery experiments were performed with known amounts of standard oestriol added before and after hydrolysis of the urine. The standard deviation between duplicates was $\pm$ 4.4% of the quantity of oestriol. There was no significant difference between recovery of oestriol added before or after hydrolysis. Thus the average recoveries, with standard deviations, were 92.4 $\pm$ 7.7% and 92.9 $\pm$ 7.2%, respectively, for oestriol added before and after hydrolysis of the urine.

DISCUSSION

It is by no means certain that oestriol determination is the best oestrogenic measure of foetal distress. Thus COHEN[3,4] has shown that oestrogen fractions other than the three classical ones may be of importance, especially in complicated pregnancies, and that these compounds are acid labile. A further drawback in methods involving acid hydrolysis is the destruction of oestriol in the presence of large quantities of sugar, as may occur in diabetic pregnancy.

Despite these reservations, oestriol measurement is still a valuable aid in obstetric practice, and a need exists for a speedy and specific method for its determination. Not only does TLC satisfy this requirement for specificity, but presumably the method could also be applied to the labile oestrogens if a sufficiently mild, though speedy, procedure could be found for their release from the conjugates.

In the present method, the advantage of fluorescence has been added in order both to permit location of the separated oestriol and to provide great sensitivity. The latter factor allows for the dilution of the urine by a factor of 10 before hydrolysis, which means that any deleterious effect of sugar will be minimised. A further benefit of the fluorimetric method is that oestriol may be measured over the whole concentration range, without the need to dilute the sample or to change the standard.

Since derivative formation is performed on the crude ether extract of the hydrolysed urine and purification is effected by means of TLC, the whole procedure is speedy, and a complete analysis can easily be performed during one working day. It may also be noted that many samples may be analysed simultaneously since the time-consuming steps are hydrolysis, evaporation of solvent, derivative formation and chromatography. There is no theoretical limit to the number of samples that can be put simultaneously through these stages.

As may be seen in Fig. 1, DANSYL-ated urine extracts give rise to slight tailing in the chromatographic system used. In theory, therefore, it should be necessary to measure the background fluorescence and to subtract this 'blank' determination from the oestriol value. This could indeed be important when measuring samples in which the level of oestriol is much less than 1 mg/day. However, in pregnancy urine there is no significance in measuring quantities less than this. Inasmuch as 'blank' values in normal pregnancy excretion of oestriol are less than 1% of the measured value, it is unnecessary to measure the background fluorescence.

Similarly, in theory, it is advisable to use more than one standard sample, but

in practice the linearity of fluorescence with concentration over the entire range is so good that the one oestriol standard is sufficient. Nevertheless, when first introducing the method, it may be advisable to use two or more standards in order to check this linearity.

A final test of any clinical method is the comparison with an existing accepted method. Since most methods for determining oestriol in pregnancy are simply measurements of total oestrogens, we have preferred to compare our results with those obtained by the method of BROWN AND COYLE[1], which is also a chromatographic procedure. In general, results have been similar, but in cases of missed abortion we have occasionally noted complete absence of oestriol by the present method whilst obtaining positive values by the method of BROWN AND COYLE. In this condition, there is excretion of other oestrogenic material, which, however, is not oestriol. The nature of this material will be described in a future publication.

The method described in this communication is economical in terms of quantity of reagents and solvents required and can be performed by relatively unskilled technicians. It has been in routine use in our laboratory for nearly a year, and the results are in good accordance with clinical findings.

ACKNOWLEDGEMENTS

We wish to thank Dr. M. HARNIK, of Ikapharm, Ltd., for providing us with the DANSYL-chloride and advice. Thanks are also due to Mr. O. POLLACK for the UV photography.

REFERENCES

1 J. E. BROWN AND M. G. COYLE, *J. Obstet. Gynaecol. Brit. Commonwealth*, 70 (1963) 219.
2 J. W. WELLS, *Acta Endocrinol., Suppl.* 119 (1967) 119.
3 S. L. COHEN, *J. Clin. Endocrinol. Metab.*, 26 (1966) 994.
4 S. L. COHEN, *IIIrd Intern. Congr. Endocrinol.*, Excepta Med. Internat. Congr. Ser. No. 157, Abstr. No. 271, Excerpta Medica, Amsterdam, 1968.