CHROM. 4841

Determination of steroids by densitometry of derivatives

II. Direct fluorometry of DANSYL estrogens

In a preceding communication[1] the determination of estrogens by direct densitometry of their azobenzene-4-sulfonates has been reported. The sensitivity of this assay was found to approximate 50 ng. Since estrogens are known to react also with 1-dimethylaminonaphthyl-5-sulfonyl chloride (DANSYL chloride)[2], as evidenced by a recent publication on the estimation of estriol in pregnancy urine[3], it seemed of particular interest to attempt the direct fluorometry of DANSYL estrogens on chromatoplates for quantitation of ng amounts of estrogens.

Methods

Studies on the reaction between estrogens and DANSYL chloride have revealed, that in a 10^{-3} *N* sodium hydroxide solution in aq. acetone the velocity of derivative formation depends on the water content of the reaction mixture[4]. Higher concentrations of water not only increased the rate of formation of derivatives but also favored their hydrolysis within a certain period of time. Based on these experiences, the following procedure was adopted for the conversion of estrogens into their DANSYL derivatives.

The dry residue with up to 1000 ng of estrogen is dissolved in 0.9 ml of acetone. Then 0.1 ml of 0.01% DANSYL chloride in dry acetone and 0.01 ml of 0.1 *N* sodium hydroxide are added. The reaction mixture is kept at 50° for 30 min, diluted with 20 ml of benzene and extracted once with 5 ml of 0.1 *N* sodium hydroxide and twice with 5 ml of water before being filtered through anhydrous sodium sulfate and evaporated to dryness under nitrogen.

By means of chloroform or benzene the residue is quantitatively transferred onto chromatoplates with silica gel (No. 1500, acid stable, without binder; Schleicher & Schüll, Dassel, G.F.R.). Ascending chromatography is performed in chloroform–benzene–ethanol (18:2:1). Finally, the dry chromatogram is submitted to direct fluorometry in the TLD 100 (Vitatron, Dieren, The Netherlands), using the appropriate filter combination.

Results and discussion

Recovery experiments with 10–1000 ng of [6,7-^{3}H]estrone (3-hydroxy-1,3,5-estratrien-17-one) or [6,7-^{3}H]estriol (1,3,5-estratriene-3,16α,17β-triol) showed that the final benzene extracts contained 93.4–95.2% of original ^{3}H-activity, indicating an almost complete conversion of estrogens into their DANSYL derivatives. At concentrations between 1 and 10 μg, the recovery was found to range from 95.0 to 98.7%.

In the solvent system chloroform–benzene–ethanol (18:2:1), used for separation of DANSYL estrogens, the following R_F values were obtained: 0.65 $\pm$ 0.02 for DANSYL estrone, 0.50 $\pm$ 0.02 for DANSYL estradiol (1,3,5-estratriene-3,17β-diol) and 0.11 $\pm$ 0.01 for DANSYL estriol. The R_F values of the corresponding free estrogens amounted to 0.48 $\pm$ 0.2 for estrone, 0.34 $\pm$ 0.02 for estradiol and 0.05 $\pm$ 0.01 for

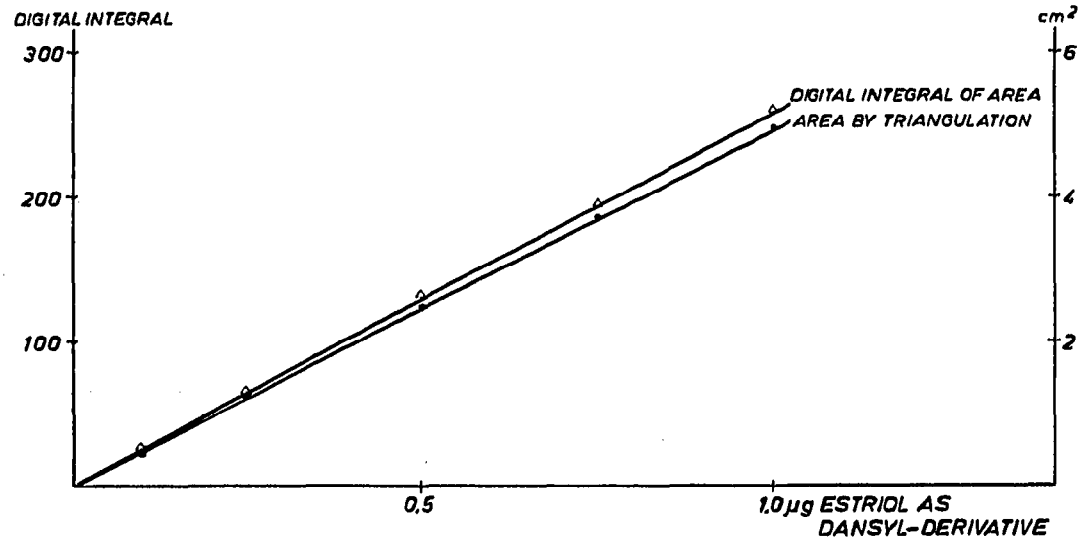

Fig. 1. Direct fluorometry of DANSYL estriol on chromatoplates.

estriol. Hence, the separation of these three estrogens and their DANSYL derivatives by ascending chromatography in the above solvent system can be considered satisfactory.

The absorption spectra of DANSYL estrone, DANSYL estradiol and DANSYL estriol exhibited maxima at 273 and 362 nm, the latter wavelength being used for excitation of fluorescence. In the emission spectra of the estrogen derivatives a common maximum near 513 nm was observed. By direct fluorometry of 100–1000 ng of estriol as DANSYL derivative, a linear relationship between the intensity of fluorescence, recorded in digital integrals of the peak area, and the amount of steroid could be established (Fig. 1). The multiple analysis of 250 ng of estriol and 750 ng of estrone by the DANSYL method revealed an accuracy of $\pm$ 5.3% for the lower concentration and $\pm$ 4.8% for the higher levels, n being 8 and 6 respectively. The evaluation of recorded peaks by triangulation led to comparable results, as demonstrated in Fig. 1. Since these measurements were performed at a rather low sensitivity of the instrument (settings: span = 78.6 and $c = 5$), the sensitivity of the foregoing assays did not exceed 50 ng. When the sensitivity of the instrument was increased (settings: span = 100 and $c = 6$), as little as 5 ng of either estrogen could be estimated (Fig. 2). At such low levels, however, the accuracy of the method still varied between 10.2 and 13.0%. According to preliminary findings, improvements in chromatographic techniques,

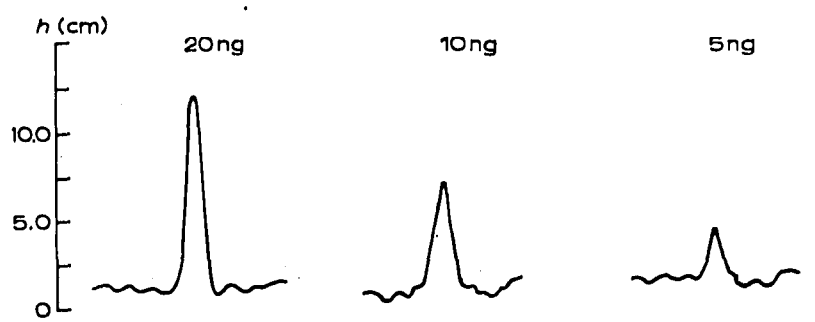

Fig. 2. Estimation of 5–20 ng of estriol as DANSYL derivative.

such as the use of uniform and extremely thin layers or the automated application of derivatives, appear to raise the accuracy considerably. A successful increase in the accuracy of the method eventually may extend its application to the analysis of estrogens in peripheral human plasma or non-pregnancy urine.

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Direct bioautography on thin-layer chromatograms as a method for detecting fungitoxic substances

A simple bioautographic technique according to WELTZIEN[7], and modified by DEKHUIJZEN[1] for detection of fungitoxic substances has been in use for many years in this laboratory. Chromatograms on Whatman No. 3MM paper are developed with propanol–water (85:15) and after drying are sprayed with a conidial suspension of *Glomerella cingulata*. After incubation, clearly visible inhibition zones indicate the presence of fungitoxic compounds. Chromatography thus permits not only the detection of fungitoxic substances *per se*, but also makes the study of the conversion reactions and of decomposition of such compounds possible.

Although this method is elegant for many reasons (high sensitivity, possibility of keeping records), it has the disadvantage of paper chromatography in general, the development being rather time consuming (16 h). This proved to be especially inconvenient in the study of fungitoxic compounds which are gradually converted non-enzymatically into other compounds either by oxidation (*e.g.* phenylthiosemicarbazide and derivatives[2]) or hydrolysis (*e.g.* benomyl[3]). The rather slow development did not give a satisfactory separation of the various components, confluent spots being obtained instead. Therefore, silica gel thin-layer chromatography on DC-Alufolie Kieselgel F_{254} plates (Merck) was considered as an alternative to the paper chromatographic technique.

With the widespread usage of TLC, many bioautographic methods have been introduced which make use of this technique for the more rapid separation of antimicrobial substances. On perusing the literature we did not really come across a very simple bioautographic technique based on TLC. Moreover several additional manip-