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The detection of diethylstilboestrol (DES) in urine by thin-layer chromatography

The use of synthetic hormones, with both an anabolic and an oestrogenic action, such as diethylstilboestrol (*trans*-α,α'-diethyl-4,4'-stilbenediol) has gained in popularity during the last years, especially in connection with the fattening of calves and poultry. The fact that these compounds belong to the so-called biologically highly active compounds and the presumptions expressed in literature that they possess carcinogenic or cancer-stimulating activity made several governments decide to prohibit the use of compounds with a hormonal activity for other than therapeutic purposes. Hence the detection of DES is of special interest.

In The Netherlands DRYER's[1] bromimetric method and BANES AND UMBERGER's[2,3] U.V.-irradiation method, both in a modification of HUIS IN 'T VELD*, are used for quantitative determination of DES. Besides these, need was felt for a simple specific identification method. Thin-layer chromatography turned out to be suitable for this purpose.

The use of paper chromatography for the detection of DES has been described a few times. In accordance with the chemical character of DES a method based on so-called "reversed-phase" chromatography[3–6] had to be developed. In view of the time-consuming character of this technique it is surprising that more attention has not

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been paid to the application of thin-layer chromatography for the detection of DES. BONINO[7] was one of the first to point out the advantages of thin-layer chromatography for this subject. In his paper "Thin-layer chromatography and the detection of stilboestrol" at the symposium held in Rome 1963 he briefly described a working procedure. The use of thin-layer chromatography for the identification of DES is also mentioned by PONDER[8]. Both methods mentioned refer to the determination of DES in animal feeding stuffs; application of these methods to the extracts of urine as they were obtained following the isolation procedure of BROWN[9] as modified by HUIS IN 'T VELD gave less satisfactory results in our hands. The present communication describes a method for the rapid separation of DES from naturally occurring phenolic substances in urine by two-dimensional thin-layer chromatography using a single solvent system. The specific detection of DES in urine is based on the conversion of DES into its phenanthrene derivative. The method is suitable for the analysis of samples of urine containing 10 p.p.b. or more diethylstilboestrol.

Reagents

All the reagents used in this investigation are of "analytical reagent" grade.

Ether. This was redistilled through a fractionating column before use. The ether used must comply with the test described below: Shake 10 ml of the ether for one minute with 1 ml of starch solution (1 % w/v) and three drops of potassium iodide (20 % w/v). The aqueous phase should not be coloured blue.

Stock standard diethylstilboestrol solution. A solution in ethanol (abs.) is prepared so that it contains exactly 50 mg of DES per 50 ml. A working standard of diethylstilboestrol solution is prepared by diluting 5 ml of stock standard DES solution to 50 ml with ethanol (abs.) (1 μl $\equiv$ 0.1 μg of DES).

Apparatus

Preparation of chromatoplates. To prepare the chromatoplates 30 g Silica Gel G (Merck AG) were homogenized with 60 ml distilled water in a Waring Blendor for about 2 min and the slurry formed was applied to five glass plates (20 $\times$ 20 cm) to a thickness of 250 μ using the Desaga applicator. The plates were air-dried for several hours, then oven-dried overnight at 100°. The plates may be stored without any special precautions.

U.V.-lamps. For irradiation an exposure cabinet obtained from Camag (Muttenz Schweiz) type TL 900, equipped with a low pressure mercury resonance arc lamp type Sylvania G 8 T 5 Germicidal (254 nm) was used. The viewer was the Desaga UVIS multi-wavelength illuminator, equipped with Sylvania G 8 T 5 Germicidal (254 nm) and Sylvania F 8 T 5 BLB (366 nm) lamps; both were low pressure mercury resonance arc lamps. Each reflector was fitted with a 254 nm filter.

Chromatographic procedure

1 ml of the urine extract dissolved in ethanol (corresponding to about 20 ml urine) is transferred to a small flask and the solvent is evaporated off with a gentle current of nitrogen gas, by warming the flask on a water bath until only the last traces of solvent remain. The residue is dissolved in 200 μl of chloroform. A 100 μl aliquot of this solution (corresponding to about 10 ml of urine) is transferred with a Hamilton microsyringe to each of two chromatoplates and exactly 4 μl of the work-

ing standard DES is added to the spot on one plate. The starting-point is 2.5 cm above the lower edge and 2.5 cm from the left edge, care being taken to evaporate the solvent in a nitrogen stream between each application. As reference 4 μl of the working standard DES was applied on a starting-point 2.5 cm above the lower edge and 2.5 cm from the right edge, and at a second starting-point 2.5 cm below the upper edge and 2.5 cm from the left edge.

The chromatoplates are placed in chromatography jars, previously saturated with the solvent vapours. The wall of the jars is lined with filter-paper soaked in solvent to ensure uniform saturation of the enclosed space. The solvent is allowed to ascend to a height of 10 cm (from starting point). The eluting solvent used is a mixture of hexane–ether–dichloromethane (4:3:2), which is renewed twice a day. The plates, after developing, are irradiated with U.V. light (254 nm) for 15 min. The plates are examined and compared under U.V. light (254 nm) for absorbance and under U.V. light (366 nm) for fluorescence. DES is converted into the yellow-coloured 3,4,5,6,12,-13-hexahydro-3,6-dioxo-9,10-diethylphenanthrene, a conversion which may be considered rather specific. The limit of detection is 0.1 μg. If no DES is visible on the chromatoplate with the same R_F value as the standard, there may still be some doubt whether DES is present or not. This is a consequence of the background of the plate. To obtain a still better separation of DES from naturally occurring substances with the same R_F value as DES the following procedure is recommended:

The plates are dried in air after the first elution irradiated for 15 min and developed in the second dimension with the same solvent. The solvent is allowed to ascend to a height of 10 cm (from the starting point). The irradiation product has a much slower mobility than DES itself. A very successful separation has been obtained in this

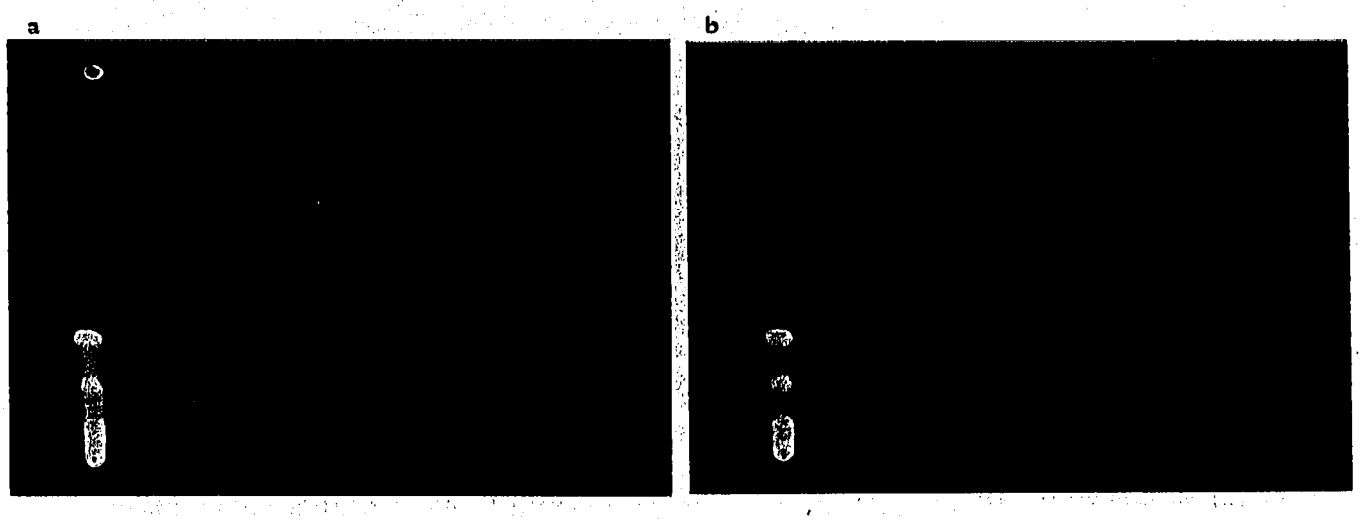

Fig. 1.(a) One dimensional thin-layer chromatogram on Silica Gel G of urine extract, reference DES 0.4 μg. Developing solvent: *n*-hexane–ether–dichloromethane (4:3:2). Photographed under 366 nm. Camera fitted with U.V. filter, Optochrome yellow filter G 2 and Optochrome green filter PO. (b) One dimensional thin-layer chromatogram on Silica Gel G of urine extract and 0.4 μg DES internal standard, reference DES 0.4 μg. Developing solvent: *n*-hexane–ether–dichloromethane (4:3:2). Photographed under 366 nm. Camera fitted with U.V. filter, Optochrome yellow filter G 2 and Optochrome green filter PO.

way from substances with the same R_F value as DES. The plates are examined* and photographed under 366 nm U.V. light (see Figs. 1 and 2).

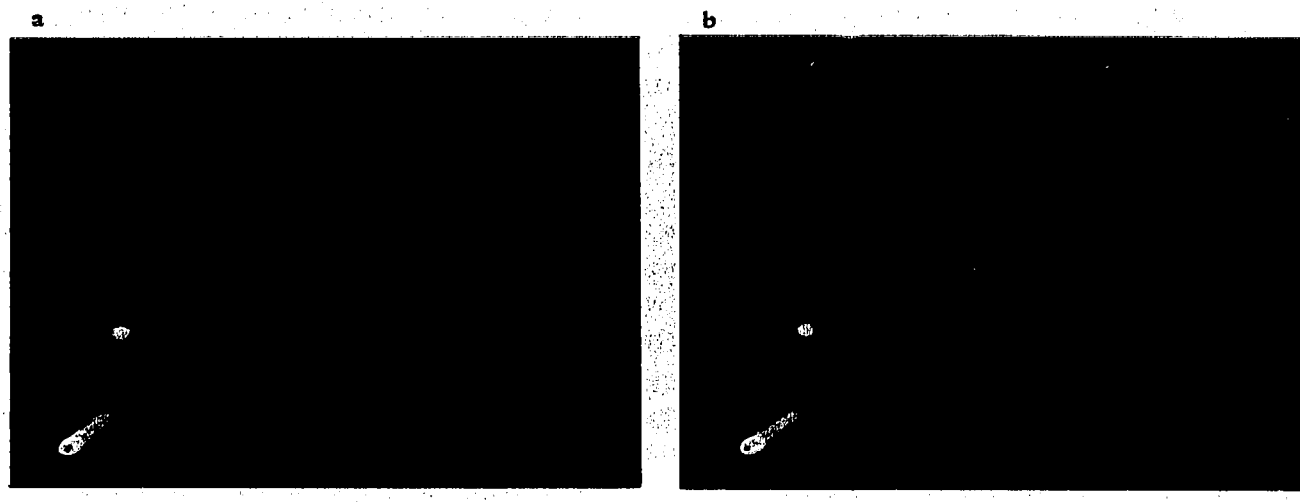

Fig. 2.(a) Two-dimensional thin-layer chromatogram on Silica Gel G of urine extract, reference DES 0.4 μg. Developing solvent: *n*-hexane–ether–dichloromethane (4:3:2). Photographed under 366 nm. Camera fitted with U.V. filter, Optochrome yellow filter G 2 and Optochrome green filter PO. (b) Two-dimensional thin-layer chromatogram on Silica Gel G of urine extract and 0.4 μg DES internal standard, reference DES 0.4 μg. Developing solvent: *n*-hexane–ether–dichloromethane (4:3:2). Photographed under 366 nm. Camera fitted with U.V. filter, Optochrome yellow filter G 2 and Optochrome green filter PO.

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* The second spot of the reference DES is in all probability the *cis*-configuration of α,α'-diethyl-4,4'-stilbenediol. This product is also converted into the phenanthrene derivative.