SPECIFIC DENSITOMETRIC METHOD OF DETERMINING DIETHYLSTILBESTROL IN MUSCLE, LIVER, AND KIDNEY TISSUES

L. F. Adigamov and M. F. Nesterin

UDC 615.256.51.07

A densitometric method of determining diethylstilbestrol in extracts of muscles, liver, and kidneys is described. The compound can be determined in 250-750 g of tissue.

Diethylstilbestrol (DES) is used in medicine for the treatment of various diseases and also in experimental research to study physiological and biochemical effects induced by estrogens. Its possible use as a growth stimulator for farm animals has recently been discussed. Meanwhile, before the mechanism of action of DES can be finally explained, its distribution and conversions in the tissues must be studied. For this purpose specific methods of determination of DES and its metabolic products must be worked out.

The object of the present investigation was to develop a densitometric method of determination of DES whose sensitivity and specificity would be greater than those of the spectrophotometric methods described in the literature [7]. An increase in specificity can be obtained by using methods of chromatographic separation of DES from various components contained in the tissue extracts.

EXPERIMENTAL METHOD

Extraction of DES on columns with 96% ethanol, hydrolysis with 2 N HCl, and purification in a $CHCl_3$–H_2O–Na_2CO_3–NaOH system were carried out by Umberger's method [9], and fluorometric investigation on the MPF-2A spectrofluorometer (Hitachi, Japan) was carried out by Bane's method [5]. For chromatography on a column (0.6 × 8.0 cm) silicagel L, 200 mesh (Chemapol, Czechoslovakia) containing 10% water was used. Before use the column was washed with 30 ml chloroform. The sample for testing was applied to the column in 1-2 ml chloroform. The elution rate was 0.5 ml/min. For chromatography on a thin layer of silicagel KSK-2, 160 mesh, discs measuring 14 × 18 cm and 20 × 20 cm were used. (The thickness of the unattached layer was 0.5 mm, and of the attached layer 0.25.) Chromatography on paper (No. 7, Filtrak, East Germany) was carried out in a solvent system of benzene–methanol–water (5:7:3).

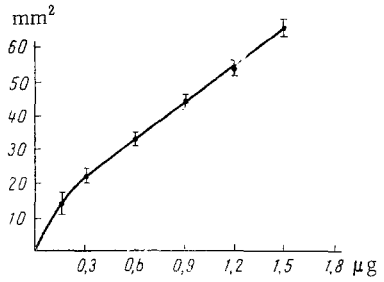

Fig. 1. Determination of diethylstilbestrol by a densitometric method (calibration curve). Abscissa, content of diethylstilbestrol (in μg); ordinate, area of peak (in mm^2).

EXPERIMENTAL RESULTS AND DISCUSSION

Three samples (each 250 g) of bovine tissue (muscle, liver, or kidney) were used for extaction. The minced tissue was twice extracted with 96% ethanol (250 ml each time). After filtration the tissue was mixed with an equal weight of Celite 545 (Ferak, East Germany) and further extracted with 1000 ml 96% ethanol on a column measuring 6.5 × 40 cm. To 1500 ml of the ethanol extract of each sample 50 ml of 2 N HCl was added, the volume was

Laboratory of Hormonal Research, Institute of Nutrition, Academy of Medical Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR N. A. Yudaev.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 75, No. 6, pp. 123-125, June, 1973. Original article submitted April 14, 1972.

TABLE 1. Separation of DES from Various Substances by Thin-Layer Chromatography (twice) in a Solvent System of Ether–Benzene (1 : 4)

Substance	R_f
Diethylstilbestrol	0,5
Estrone	0,57
Cholesterol, cholesteryl caproate, triolein, tributyrin	>0,6
Estradiol, androstenedione, testosterone, epitestosterone, androsterone, etiocholanolone, dehydroepiandrosterone	<0,4
Estriol, cortisol, cortisone, corticosterone, aldosterone	<0,2

reduced by evaporation to 100-150 ml, and purification was carried out in a system of $CHCl_3$–H_2O–Na_2CO_3–NaOH.

The extracts of the three parallel samples (of the corresponding tissue) were then pooled and evaporated to dryness in vacuo. The dry residue was dissolved in 30 ml 70% methanol and shaken vigorously with 10 ml hexane. The hexane layer was twice washed with an equal volume of 70% methanol and the hexane discarded. The methanol extracts were pooled and evaporated to dryness in vacuo; after acidification with 2 N HCl the aqueous residue was twice extracted with chloroform (30 ml each time). The chloroform was dehydrated, evaporated down to a volume of 1-2 ml, and applied to a silicagel L column. Elution with 20 ml chloroform (fraction No. 1, not containing DES) and with 30 ml 1% ethanol in chloroform (fraction No. 2, containing DES) was carried out. Fraction No. 2 was evaporated down to minimal volume and applied to a silufol disc (Kavalier, Czechoslovakia). Chromatography was carried out in a solvent system of ether–benzene (1 : 4). The silufol disc was irradiated in ultraviolet light for 30 min, the strip of the chromatogram (4 × 15 cm) with developed yellow stains of the conversion product of DES was cut out, and densitometry carried out in reflected light on the ERI-65 densitometer. The content of DES was determined from a calibration curve (Fig. 1).

If the conditions are such that densitometry is impossible, DES can be estimated quantitatively by other methods, using this same method of purification of the extracts: either spectrophotometrically or fluorometrically. For this purpose, after chromatography on a silicagel L column, the fraction containing DES was evaporated down in vacuo and chromatography again carried out in a thin layer of silicagel KSK-2 or on paper. After chromatography in a thin layer of silicagel, in order to localize the DES, the chromatogram was irradiated in ultraviolet light (0.5-2 min) until the appearance of yellow stains of the photochemical reaction product. Elution was carried out with 8 ml 96% ethanol, 8 ml chloroform, and 8 ml methanol. The silicagel was separated and the eluates evaporated to dryness in vacuo. The dry residue was dissolved in 2 ml 96% ethanol, 2 ml 1.8% K_2HPO_4 was added, and after further irradiation in ultraviolet light (15 min) the optical density was determined at $\lambda = 410$ nm. After chromatography on paper and elution with 5 ml ethanol, 5 ml chloroform, and 5 ml methanol, the fluorescent product 3,6-dihydroxy-9,10-diethylphenanthrene [5] was obtained and estimated quantitatively.

The purification method described above enables the quantity of tissue used for extraction to be increased from the 20-200 g used in methods described previously [6, 7, 9] to 250-750 g. The photochemical reaction lying at the basis of the densitometric determinations of DES, and consisting of the formation of a tricyclic diketone (3, 4, 5, 6, 12, 13-hexahydro-3,4-dioxo-9,10-diethylphenanthrene) under the influence of ultraviolet light, according to several workers [5-8] is specific for the determination of DES in biological material.

Another advantage of the densitometric determination is that the photochemical conversion of DES and its subsequent quantitative determination were carried out directly on the chromatogram. By means of this method about 0.2 μg DES can be detected. Because of the difficulty of obtaining a quantitative yield for the conversion of the diketone into the diol and purification of the latter, the more complex fluorometric method has no significant advantages over the densitometric method.

The method of purification of the extracts from lipids (within the limits of the method described) is sufficiently effective. By chromatography on a silicagel M column standard preparations of DES can be separated from lipids of low polarity (cholesterol esters and triglycerides) and also from more highly polar fractions (certain fatty acids, phospholipids). As Table 1 shows, thin-layer chromatography is another convenient method of purifying DES from various substances. A sufficiently high degree of purity is essential because some lipid fractions inhibit the photochemical conversion of DES and may lead to "incomplete discovery" of the compound [3].

The method described above was used by the writers for an experimental investigation of the distribution of DES in tissues of muscles, liver, kidneys, and intestinal mucosa of rats, and also for the determination of residual quantities of the substance in muscle tissue and organs of stimulated bulls [2]. In the last

series of investigations residual amounts of DES were successfully detected in specimens of meat products in which they could not be detected by spectrophotometric methods [2].

The method of purification of the extracts described above and the methods of quantitative analysis of DES can be used in biochemical investigations to study the distribution and metabolism of the compound in animal tissues, in clinical practice, and also in agricultural experiments as a means of verifying the absence of the substance in meat products when the optimal method of stimulating growth of animals is sought.

LITERATURE CITED

1. L. F. Adigamov and K. A. Perevoshchikova, Vopr. Med. Khimii, No. 5, 552 (1971).
2. L. F. Adigamov, L. Ya. Solov'eva, and M. F. Nesterin, Vopr. Pitaniya, No. 3, 76 (1972).
3. K. A. Perevoshchikova, V. Ya. Parinov, et al., Vopr. Med. Khimii, No. 4, 439 (1971).
4. A. A. Pokrovskii, M. F. Nesterin, et al., Vestn. Akad. Med. Nauk SSSR, No. 2, 3 (1972).
5. D. Banes, J. Ass. Offic. Agric. Chem., 44, 323 (1961).
6. I. M. Goodeyer and L. S. Hatfield, J. Am. Pharm. Ass. Sci. Ed., 43, 605 (1954).
7. Official Methods of Analysis, Section 33039, Washington (1965).
8. P. L. Schuller, J. Chromatogr., 31, 237 (1967).
9. E. I. Umberger, D. Banes, et al., J. Ass. Offic. Agric. Chem., 46, 471 (1963).