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Note

Dns derivatization of anabolic agents with high-performance liquid chromatographic separation and fluorescence detection

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Anabolic agents are natural or synthetic chemical compounds which have properties of the sex hormones. A major and often controversial use of such agents is in meat production, because of the rapid weight gain produced in treated animals[1]. Samples of some of the more important anabolic agents were supplied to our laboratory by the Institute for Research on Animal Diseases with the goal of developing a sensitive, general method for their detection[2]. Although these were quite different chemical groups of compounds all of them, except, trenbolone, have one or more phenolic OH groups present. Therefore all of these phenolic compounds should react with Dns chloride (5-dimethylaminonaphthalene-1-sulphonyl chloride or Dns-Cl) to form fluorescent derivatives[3].

Various thin-layer chromatographic (TLC) methods have been published for the analysis of steroids and other anabolics by sulphuric acid-induced fluorescence[4,5] or the fluorescence of Dns derivatives[6,7]. High-performance liquid chromatography (HPLC) has also been used for the separation of selected anabolics with UV detection[8] and for the specific analysis of zenone based on its natural fluorescence[9,10]. Dns derivatives have been prepared of phenols[11] and of hydroxybiphenyls[12], separated by HPLC and TLC, respectively, and detected by their fluorescence.

The versatility and selectivity of HPLC separation, coupled with the great sensitivity of fluorescence detection, provides the analyst with an analytical system that is difficult to rival for speed, simplicity, reproducibility and sensitivity[13]. Pre-column Dns derivatization, followed by HPLC separation of the crude reaction mixture with fluorescence detection, was used to determine low nanogram (ppb, 10^9) levels of phenolic estrogenic steroids (estrone, estriol, and estradiol) by Schmidt and co-workers[13,14].

In this paper, we describe our successful modification and extension of the work of Schmidt *et al.*[14] for the detection and quantitation of nanogram levels of anabolic agents of the resorcylic acid lactones (RAL) and stilbene groups by a general procedure of pre-column Dns derivatization, HPLC separation, and fluorescence detection. Total reaction and chromatography time is less than 1 h. Partly successful attempts at analysing these compounds in samples of body fluids (serum, plasma, urine and bile of

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cows and sheep) are described. Finally, unsuccessful attempts to use the same chromatographic system for the detection of Dns hydrazone derivatives of anabolics with a keto group are reported.

EXPERIMENTAL

Solvents and reagents

Water was distilled and deionized and the pH adjusted to 3.5 with phosphoric acid. It was filtered through a Millipore 0.45-μm filter. Acetonitrile (Fisons HPLC grade) was used without further purification. HPLC solvents were thoroughly degassed before use. Dns-Cl was from BDH biochemicals. A 1.5 mg/ml-standard solution of Dns-Cl in acetone was used[14]. This solution was stored in a regrigerator. A buffer solution of $NaHCO_3$ in water (4 g/l) adjusted to pH 10.5 with 5 *N* sodium hydroxide was used[14]. Samples of the anabolic agents were donated by Dr. S. Dixon of the Institute for Research on Animal Diseases[2] and were determined to be pure by melting point determinations. Standard solutions of these anabolic agents were made up by dissolving 2 mg of each in 100 ml of ethanol (BDH, AnalaR, 99.7–100%).

Dns derivatization procedure

The appropriate amount (usually 100 μl or less) of the anabolic agent solution was transferred to a Pierce 1-ml Reacti-Vial. The solvent was evaporated with a stream of dry nitrogen at room temperature. To the residue in the vial was then added 40 μl of buffer solution and 100 μl of the Dns-Cl solution. The Dns-Cl is therefore present in large excess. The vial was vigourously shaken for 30 sec and then placed in a Pierce Reacti-Therm Heating Module at 100°C for 5 min. The vial contents changed from a pale yellow before reaction to colourless after reaction. The yield of Dns derivatized product was not changed by longer reaction times of 10 and 20 min. The vial was stored in a refrigerator after reaction.

Separation and detection procedures

The crude Dns derivatization product mixtures were injected via a 20-μl Rheodyne injector directly into a Perkin-Elmer Series 3-B liquid chromatograph. The column used was a 25 × 0.26 cm PAH-10 (reversed-phase C_{18}) LC column, Perkin-Elmer 258-0082, Serial No. 1155. Gradient elution was performed from acetonitrile–water (60:40) to acetonitrile–water (95:5) over 15 minutes (curve 1) at a flow of 1 ml/min. The solvent compensation was held at acetonitrile–water (95:5) over a 10-min period. An overnight flush with pure acetonitrile (0.1 ml/min) was employed. The column effluent was allowed to pass through a 20-μl flowcell in a Perkin-Elmer Model LS-3 fluorescence spectrometer. Excitation and emission wavelengths of 335 nm and 540 nm, respectively, (both slits 10 nm) were chosen after an examination of the spectra of several of the Dns derivatized anabolics using the stop-flow feature of the Series 3-B chromatograph. Chromatograms were recorded on a Perkin-Elmer Model 56 recorder.

Animal samples

Samples of serum, plasma, urine, and bile from cows and sheep were supplied to our laboratory[2]. After a hydrolysis with acid or with β-glucuronidase and sul-

phatase, samples were extracted with diethyl ether. The ether layer was dried and then evaporated to dryness in a Reacti-Vial. Dns derivatization was carried out in the manner described above. Body fluid samples were spiked with the anabolic agent when appropriate.

RESULTS AND DISCUSSION

Dns derivatization and chromatography

Fig. 1 is the chromatogram of a blank of the buffer plus Dns-Cl solution. Fig. 2 is the chromatogram which results from the Dns derivatization of a mixture of estriol,

Fig. 1. Chromatogram of a blank of the buffer plus Dns-Cl solution.

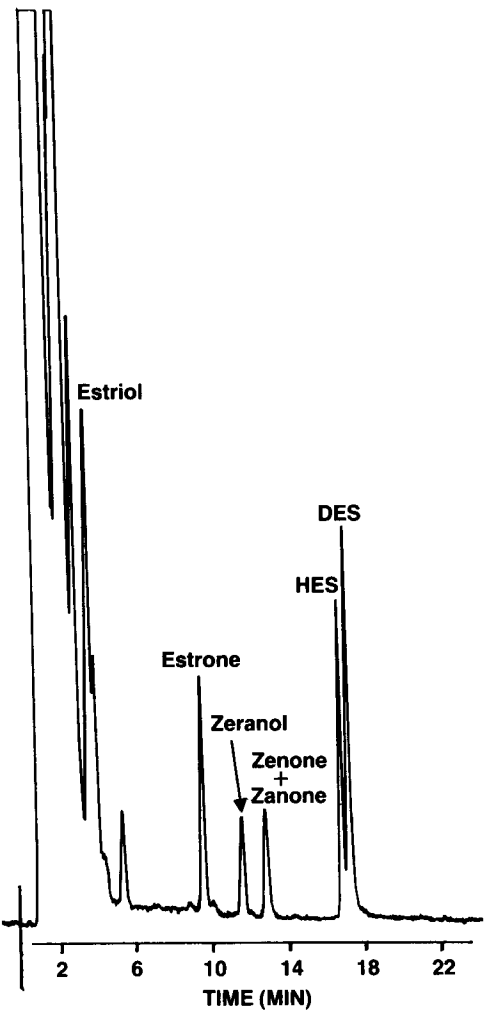

Fig. 2. Chromatogram after Dns derivatization of estriol, estrone, zenanol, zanone, zenone, HES and DES.

estrone, zenanol, zenone, zanone, hexestrol (HES) and diethylstilbestrol (DES). The amounts of each compound were not the same, as will be discussed later. The presence or absence of a double bond is a sufficient enough structural difference to allow HES and DES to be separated. The double bond is a less significant feature of the zenone and zanone structure and these two compounds are not separated from each other, although they do separate from zeranol. Retention times for each compound were confirmed by separate individual Dns derivatization and chromatography. Dns derivatives of both the RAL and stilbene compounds were shown to be stable for at least four days in the dark at 4°C (refrigerator).

Sensitivity

Separate experiments showed that the Dns derivatization of identical amounts of anabolic agent gave a peak height for the RALs which was only about 1/10 of that for the steroids and 1/15 of that for the stilbene. Therefore, the peaks in Fig. 2 are those which result from the Dns derivatization of several μg of the RALs but several hundred μg of the other anabolic. By separate experiments, the lower limit of detection of Dns derivatized DES was determined to be 5 ng, whereas that for zeranol was found to be 80 ng. Time of reaction, product stability, wavelength of excitation and emission, and air oxidation were all experimentally excluded as possible reasons for the smaller peak size for the RALs. The cleavage of the lactone ring under the basic buffer conditions is a possibility which requires further exploration. The basic conditions required for the Dns derivatization of phenols may reduce yields when there is a lactone ring present in the same structure.

Quantitation of Dns derivatization

Triplicate samples of DES ranging from 50 to 150 ng were Dns derivatized and the resulting peak heights averaged to construct a calibration curve. Each separate Dns derivatization gave a peak height within $\pm 15\%$ of the average value shown. Dns derivatization is a chemical reaction whose yield would not be expected to be exactly the same each time it is performed. Given that the amounts derivatized were very small and that the reactions and analyses were not all done on the same day, we were pleased with the stability of the analytical system and with the reproducibility of the data.

Dns-hydrazone experiments

Trenbolone does not have a phenolic OH group but it does have a keto group as do two of the RALs, zenone and zanone. DNS-hydrazones of these compounds were prepared[3] and subjected to the chromatographic conditions given above for the Dns derivatization of phenolic OH groups. Despite the similarity of structures of these derivatives (an –N=N– group replaces an –O–), no peaks, other than those in a blank, were observed.

Animal samples

Dns derivatization of cow and sheep serum and plasma gave very few peaks which might interfere with the identification of the anabolic agents. Unfortunately, these agents do not appear at any appreciable levels in serum and plasma but are found in much higher levels in urine and bile[2]. Fig. 3 shows the chromatogram that

results from the dansylation of the extract from 0.5 ml of sheep urine to which 100 ng (200 ppb) had been added. Therefore, only the anabolics with long retention times, HES and DES, can be detected and the lower limit of detection of these is rather high because of the very large number and high concentration of materials in urine which are extracted and Dns derivatized.

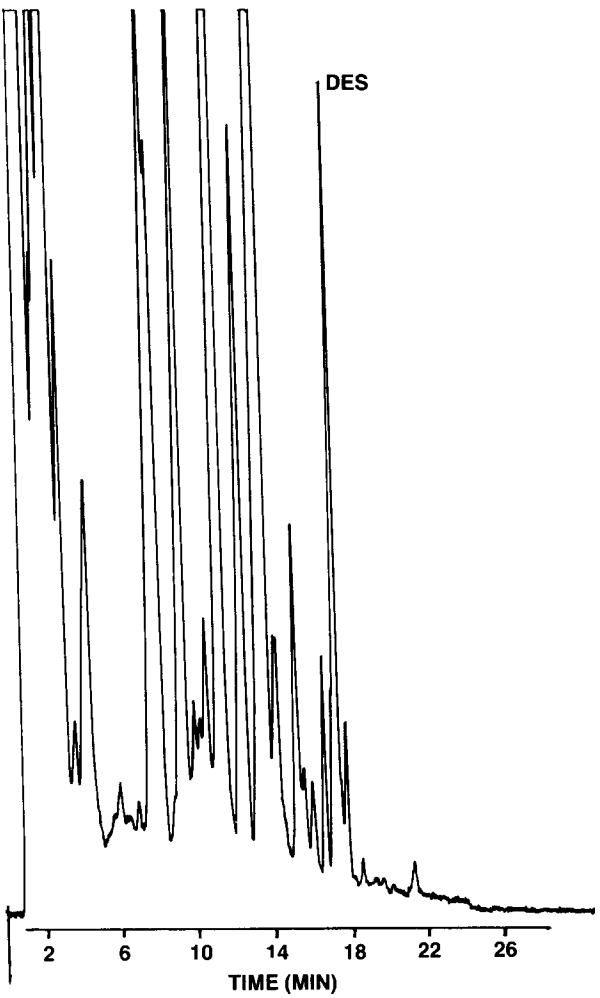

Fig. 3. Chromatogram after Dns derivatization of sheep urine plus DES (200 ppb).

Hydrolysis and ether extraction of cow bile gave large amounts of residual material. Unsuccessful attempts at Dns derivatizing spiked (DES) samples of cow bile led us to conclude that unacceptably high concentrations or amounts of Dns-Cl would be required to detect anabolic agents in bile by this method.

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Dr. S. Dixon of the Institute for Research on Animal Diseases, Longston, Newbury, Great Britain, provided us with samples of anabolic agents and with animal samples of plasma, serum, urine, and bile.

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