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CELITE COLUMN CHROMATOGRAPHY FOLLOWED BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY: A SIMPLE, TWO-STEP METHOD FOR SEPARATING 14 TESTICULAR STEROIDS

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SUMMARY

The separation of 14 major testicular steroids has been effected by a simple, two-step procedure involving Celite column chromatography followed by reversed-phase high-performance liquid chromatography. The technique does not alter the resolved steroids, which are then available for subsequent analysis.

INTRODUCTION

Mammalian testes produce and release a variety of C_{21}, C_{19} and C_{18} steroids[1]. Some, such as T*[1], DHT and 3α-DIOL are potent androgens involved in differentiation and growth of the male reproductive tract[2], as well as the process of spermatogenesis[3]. Several Δ^4-3-ketosteroids and Δ^5-3β-hydroxysteroids (*e.g.*, DIONE, Δ^5-DIOL, DHEA, 17α-PREG, PREG, PRO) are biosynthetic intermediates of the biologically active androgens[4]. The role of C_{18} testicular estrogens (*e.g.*, estriol, estrone, estradiol) remains unclear. Efforts to elucidate the biological function of these and other testicular steroids have been hampered by the tedious, time-consuming procedures involved in the separation and subsequent purification of each steroid. It occurred to us that Celite column partition chromatography in conjunction with reversed-phase high-performance liquid chromatography (HPLC) might be able to resolve these steroids. In this paper, we present a simple two-step process which clearly separates 14 important C_{21}, C_{19} and C_{18} testicular steroids.

* The following abbreviations and common names for steroids are used: 17β-hydroxy-4-androsten-3-one, testosterone (T); 17β-hydroxy-5α-androstan-3-one, dihydrotestosterone (DHT); 5α-androstane-3α,17β-diol (3α-DIOL); 5α-androstane-3β,17β-diol (3β-DIOL); 4-androstene-3,17-dione, 4-androstenedione (DIONE); Δ^5-androstene-3β,17β-diol, Δ^5-androstenediol (Δ^5-DIOL); 3β-hydroxy-5-pregnen-20-one, pregnenolone (PREG); 4-pregnene-3,20-dione, progesterone (PRO); 3β-hydroxy-5-androsten-17-one, dehydroepiandrosterone (DHEA); 3β,17α-dihydroxy-5-pregnen-20-one, 17α-hydroxypregnenolone (17α-PREG); 17α-hydroxy-4-pregnen-3,20-dione, 17α-hydroxyprogesterone (17α-PRO); 3-hydroxy-1,3,5(10)-estratrien-17-one (estrone); 1,3,5(10)-estratriene-3,17β-diol (estradiol); 1,3,5(10)-estratriene-3,16α,17β-triol (estriol).

MATERIALS AND METHODS

Apparatus

A Waters Assoc. 6000A liquid chromatograph equipped with a U6K injector was used. Commercially prepared 30 cm × 4 mm I.D. μBondapak C_{18} (Waters Assoc., Milford, Mass., U.S.A.) and 25 cm × 4.6 mm I.D. Partisil 10 ODS 2 (Whatman) columns were employed. A 10 cm × 7 mm I.D. pre-column (Whatman) was used to protect the main columns from impurities in the samples and mobile phase. The solvents used were acetonitrile–water (2:3) and methanol–water (7:3 and 2:3) at flow-rates of 0.5 or 1.0 ml/min and pressures of 300, 500 and 1400 p.s.i., respectively. Effluent from the high-performance liquid chromatograph was collected at 1-min intervals with an ISCO Model 820 fraction collector into mini-scintillation vials (New England Nuclear). Radioactivity in the vials was counted in a Hewlett-Packard Model 3320 liquid scintillation counter, the results being simultaneously printed on punched tape and teletype.

Punched tapes were read on a Hewlett-Packard Model 9883A tape reader, analyzed by a Hewlett-Packard Model 9821A calculator, then grapically illustrated with a Hewlett-Packard 9862A calculator/plotter.

Chemicals

Water was double-distilled in glass. Acetonitrile and methanol (spectrophotometric grade) were obtained from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.). Ethylene glycol, isooctane, benzene and ethyl acetate (all Nanograde) were purchased from Mallinckrodt (St. Louis, Mo., U.S.A.) and distilled in glass prior to use.

T, DHT, 3α-DIOL, 3β-DIOL, Δ^5-DIOL, 17α-PRO, DIONE, 17α-PREG, PREG, PRO, estriol, estrone, estradiol and DHEA were purchased from Steraloids (Pawling, N.J., U.S.A.) and recrystallized to constant melting point. ^{3}H-Labelled isotopes of these compounds were obtained from New England Nuclear and purified by thin-layer chromatography (TLC) prior to use. TLC was performed on pre-coated (250-μm) silica gel GF glass plates (Analtech, Newark, N.J., U.S.A.) using benzene–ethyl acetate (2:1) for development. The scintillation cocktail consisted of 4 g of 2,5-diphenyloxazole and 0.04 g of 1,4-bis-[2-(5-phenyloxazolyl)]benzene per liter of toluene.

Celite columns

Celite columns for partition chromatography were prepared with modifications in the technique originally developed by Siiteri[5]. Celite Analytical Filter (Fisher Scientific, Pittsburgh, Pa., U.S.A.) was heated to 1000 °F for at least 18 h before use, then cooled for 1 h in a desiccator. One gram of Celite was weighed out for each column to be used. The Celite was placed in a plastic bag immediately after weighing, and anhydrous ethylene glycol (0.5 ml per gram of Celite) was added. The bag was squeezed to remove air, then sealed. The Celite and ethylene glycol were mixed for 15 min by kneading the bag. Next, the bag was inflated with dry nitrogen, re-sealed, shaken for 5 min, then rolled flat with a pipette. This treatment under nitrogen was repeated twice more.

After mixing, 0.9 g of the impregnated Celite was weighed out for each

column, and immediately placed in a desiccator. Each 0.9-g aliquot of Celite mixture was removed from the desiccator at the time of packing and placed in a 5-ml disposable graduated glass pipette (Corning, Corning, N.Y., U.S.A.) on top of a 5-mm glass bead. The mixture was tamped down to the 3.5-ml mark with a plastic rod. Each column was rinsed twice with 3.5 ml of anhydrous isooctane, using nitrogen under pressure to achieve a flow-rate of 0.5 ml/min.

Sample preparation

Radioactively labelled steroids were combined with unlabelled steroids to obtain a specific activity of approximately $7.5 \cdot 10^6$ cpm/mg. The steroids (20 μg) were combined in a silanized conical 12-ml centrifuge tube and concentrated at the bottom. The steroids were then dissolved in a mixture of 5 μl of methanol and 200 μl of isooctane and placed on a Celite column with a pasteur pipette. Two subsequent washes of the tube with the solvent mixture were used to transfer the remainder of the steroids. The steroids were eluted from Celite columns with five different solvent steps at a flow-rate of 0.5 ml/min: (1) 3.5 ml of isooctane; (2) 6 ml of 5% benzene in isooctane; (3) 15 ml of 20% benzene in isooctane; (4) 10 ml of 35% benzene in isooctane; (5) 3.5 ml of ethyl acetate. The eluent from each of the five different solvent steps was collected in individual silanized, 15-ml glass centrifuge tubes. The steroids were dried under nitrogen, and concentrated at the bottom of the tube with three washes of diethyl ether. A small volume of ethylene glycol was removed from the Celite columns during elution with ethyl acetate. Therefore, tubes containing steroids from elution step 5 were further dried by lyophilization.

The steroids in each fraction from the Celite columns were resolved by reversed-phase HPLC. Using a 20-μl Pederson pipette, ethanol was placed into the bottom of the tubes containing the fractions from Celite chromatography. The tube was swirled on a Vortex mixer for 10 sec, then 10 μl of the ethanol solution were taken up in a micro-syringe (Precision Sampling, Baton Rouge, La., U.S.A.) and injected into the chromatograph. The remainder was counted in a scintillation vial to determine recoveries. Fractions 2 and 5 were eluted from a μBondapak C_{18} column with acetonitrile–water (2:3) as the mobile phase (0.5 ml/min). Fraction 1 was eluted from the same column with methanol–water (7:3). A Partisil 10 ODS 2 column with methanol–water (7:3) as the mobile phase (0.5 ml/min) was used to resolve the steroids in fraction 4. Steroids in fraction 3 were eluted from the Partisil 10 ODS 2 column with methanol–water (2:3) at 1.0 ml/min.

RESULTS

Celite column chromatography

Stepwise elution from Celite columns distributed the 14 testicular steroids between five different fractions (Fig. 1). PRO, PREG and DIONE appeared in the first eluate (isooctane) from Celite. Within this isooctane fraction, PRO was eluted first, followed by PREG and DIONE. The predominant steroids in the second fraction (benzene–isooctane, 1:19) were DHT and DHEA, which were eluted in that sequence. T, 17α-PRO, 3α-DIOL and estrone were included in fraction 3 (benzene–isooctane, 1:4). T was eluted first, but was nearly confluent with the 17α-PRO, which was followed by 3α-DIOL and estrone. Fraction 4 (benzene–isooctane, 7:13)

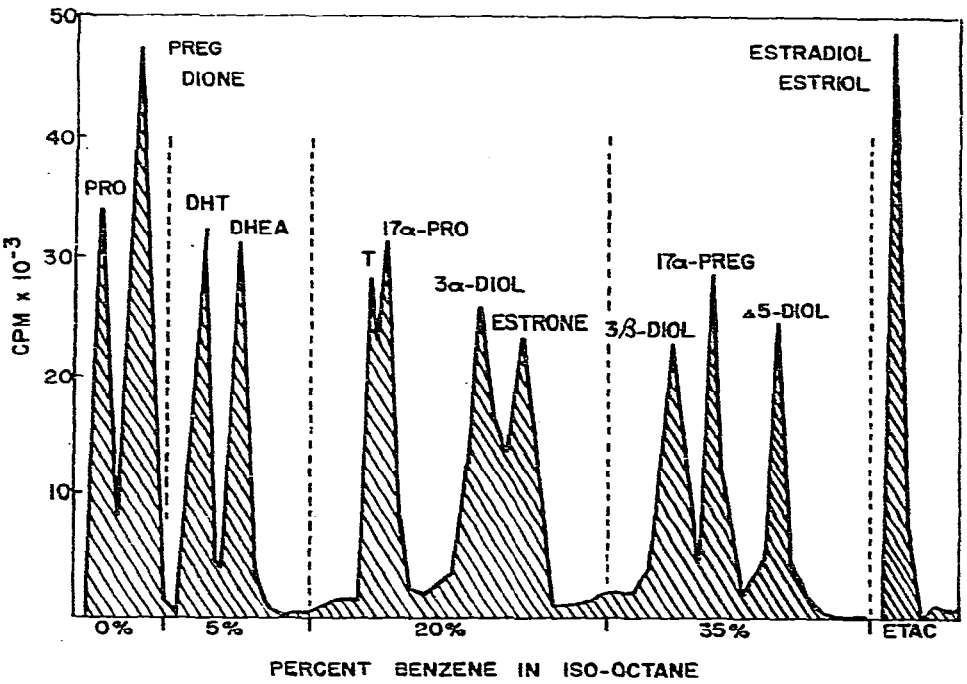

Fig. 1. Profile of radioactively labelled steroids eluted stepwise from Celite columns (0.9 g packed in a 5-ml pipette) impregnated with ethylene glycol. A flow-rate of 0.5 ml/min was obtained by applying dry nitrogen under pressure. Fractions (0.5 ml) of each eluent were collected in scintillation vials, evaporated, then counted.

contained 3β-DIOL, 17α-PREG and Δ^5-DIOL, which were eluted in that order. The highly polar estrogens estriol and estradiol were stripped from the Celite columns with ethyl acetate as a single peak in fraction 5.

Steroid recoveries from Celite columns ranged from 90 to 100%. However, the transfer of steroids from the sample tubes to the Celite columns was less quantitative. More polar steroids were less soluble in the methanol–isooctane mixture. Total recoveries from both the sample tubes and Celite columns thus ranged from 75% for estriol to 100% for PRO.

High-performance liquid chromatography

Steroids in each of the fractions from Celite columns were completely resolved by reversed-phase HPLC (Fig. 2). The peaks from unlabelled steroids detected by the differential refractometer corresponded with the peaks of radioactivity, allowing for a 5-min delay caused by the dead volume between the detector and the fraction collector. The steroids from fraction 1 eluted from the μBondapak C_{18} column with methanol–water (7:3) had the following elution times: DIONE, 19 min; PRO, 31 min; and PREG, 41 min. With a mobile phase of acetonitrile–water (2:3) the DHEA and DHT in fraction 2 were eluted from the same column with retention times of 46 and 59 min, respectively. Estriol and estradiol in fraction 5 had retention times of 14 and 35 min, respectively, when eluted from the same column with the same mobile phase. Steroids in fractions 3 and 4 could not be resolved by elution

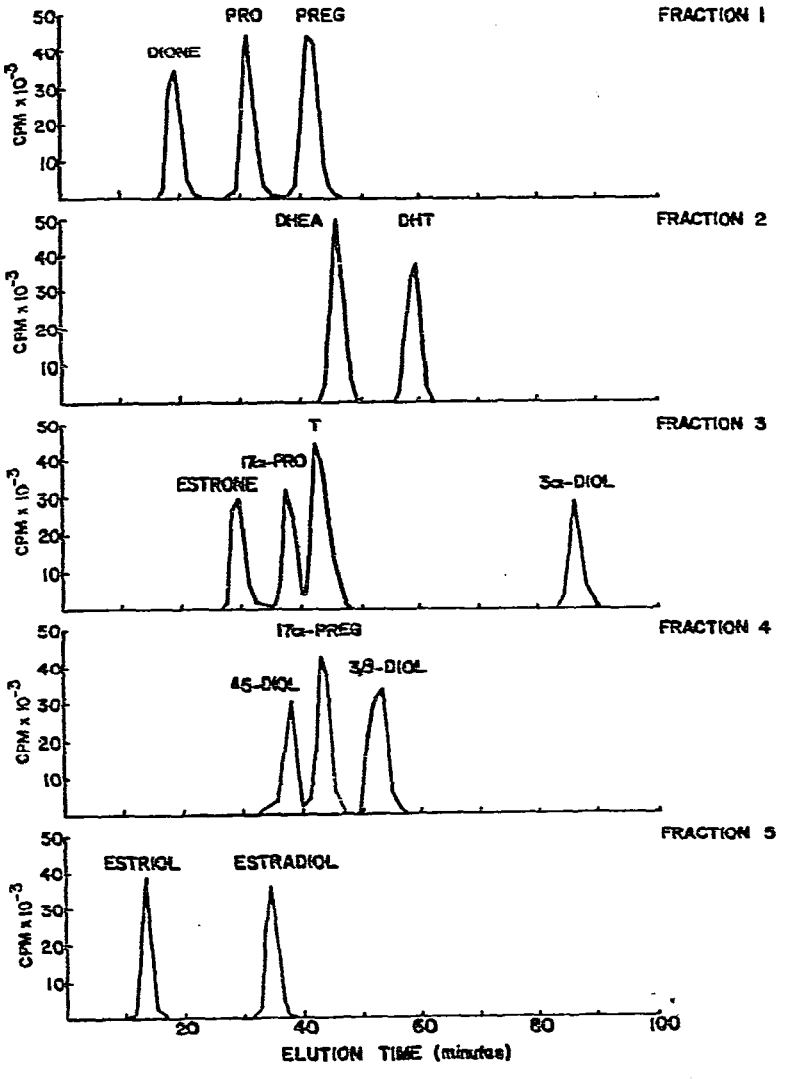

Fig. 2. Reversed-phase HPLC of steroids in the solvent fractions from Celite column chromatography. Fractions 2 and 5 were eluted from a μBondapak C_{18} column (void volume 2.5 ml) with acetonitrile–water (2:3). The flow-rate was 0.5 ml/min with a pressure of 350–400 p.s.i. Fraction 1 was eluted from the same column with methanol–water (7:3). The flow-rate was 0.5 ml/min at a pressure of 500–550 p.s.i. Celite fraction 4 was eluted from a Partisil 10 ODS 2 column (void volume 2.76 ml) with methanol–water (7:3). The flow-rate was 0.5 ml/min at a pressure of 700 p.s.i. Celite fraction 3 was eluted from the same Partisil column with methanol–water (2:3) at a flow-rate of 10 ml/min (1400 p.s.i.).

from the μBondapak C_{18} column; consequently, a Partisil 10 ODS 2 column was used with methanol–water (7:3 or 2:3) as the mobile phase. With methanol–water (7:3), Δ^5-DIOL, 17α-PREG and 3β-DIOL were eluted with retention volumes of 46, 53 and 64 min, respectively. Celite fraction 3 steroids, eluted from the same column

with methanol–water (2:3) at 1.0 ml/min, had the following retention times: estrone, 29 min; 17α-PRO, 37 min; T, 42 min; and 3α-DIOL, 86 min. Recoveries by HPLC were 100% in all instances.

DISCUSSION

Celite column chromatography of steroids is a simple means of separating steroids without chemical alteration[6]. The separated steroids were thus available for subsequent procedures, such as radioimmunoassay[7,8], or for scintillation counting when the separated steroids were derived from radioactive precursors[9]. More recently, resolution of testicular steroids has been attempted with HPLC[10–12]. Both HPLC and Celite columns rely on partition chromatography, but neither is sufficient by itself to resolve all of the major testicular steroids completely. Combination of the two techniques does accomplish this (Figs. 1 and 2).

Celite columns and reversed-phase HPLC were used in a two-step separation procedure. In the first step, the sample was divided into five groups of steroids by Celite column chromatography (Fig. 1). This was usually reproducible, with virtually no overlap of steroids between groups. Recoveries from Celite columns were good provided that the solvent (5 μl of methanol in 200 μl of isooctane) used to transfer the steroids from the sample tube was placed in the bottom of tube. Care must also be taken to avoid exposure of the Celite columns to moisture. High humidity causes the retention volumes of steroids to vary, resulting in the elution of some steroids in more than one fraction.

In the second step of the separation procedure, the groups of steroids obtained from Celite column chromatography were subjected to reversed-phase HPLC for further resolution. The absolute retention times of steroids eluted from an HPLC column varies slightly as a consequence of changes in the flow-rate or differences in the amount of water in the mobile phase. Thus, the relative retention times were determined by using unlabelled steroids as markers. Relative retention times of the steroids did not change significantly with alterations in the flow-rate or the amount of water in the mobile phase. However, different organic components (acetonitrile or methanol) in the mobile phase had a marked effect on the relative retention volumes. For example, it was possible to separate the steroids in fraction 4 (Fig. 2) on the μBondapak C_{18} column by using two different solvent systems. In the presence of acetonitrile–water (2:3) at 0.5 ml/min, the retention time of Δ^5-DIOL was 35 min, while both 17α-PREG and 3β-DIOL were eluted in 43 min. With methanol–water (7:3) at 0.5 ml/min the Δ^5-DIOL and 3β-DIOL were eluted together (23 min), but they were separated from 17α-PREG (28 min). Thus, the confluent steroid peak in one solvent system could be resolved in the other. On the Partisil 10 ODS 2 column (Fig. 2), however, only a single eluent (methanol–water, 7:3) was required to separate all three steroids.

The advantages of this steroid separation technique are simplicity, rapidity, high resolving power and availability of resolved steroids for further treatment. Thus, subsequent detection of the steroids may be accomplished by radioimmunoassay, competitive binding, UV absorption, refractive index measurement, flame ionization or counting of radioactivity. Resolved radioactively labelled steroids in the eluate may be recrystallized to constant specific activity to confirm their identity or, if the

steroids are unlabelled, they may be subjected to structural confirmation by mass spectrometry. Thus the combination of Celite chromatography with reversed-phase HPLC provides a powerful tool for studying testicular steroidogenesis.

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