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Note

Separation of 5α-reduced androgens by reversed-phase high-performance liquid chromatography

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5α-Reduction of testosterone results in the formation of 17β-hydroxy-5α-androstan-3-one (Dht), which is believed to be the active androgen at the cellular level. Dht can be further metabolized by most mammalian cells to several 5α-reduced androgens, including 5α-androstane-3α,17β-diol (3α-A-diol) and 5α-androstane-3β,17β-diol (3β-A-diol). It is not clear whether such metabolic transformations of Dht generally represent biological inactivation, or in fact biological activation in specific target cells. Studies on the metabolism of these steroids *in vivo* and *in vitro* must be carried out in order to resolve this question.

Methods for the separation of androgens are based mainly on partition chromatography on paper[1] or adsorption chromatography on thin layers[2,3]. Several liquid chromatographic systems for the separation of androgens have been devised[4–9]. These systems, however, are not optimized for the separation of 5α-reduced androgens. We have devised a chromatographic system for the simultaneous separation of all the biologically relevant 5α-reduced androgens by reversed-phase high-performance liquid chromatography (HPLC).

EXPERIMENTAL

All solvents were of HPLC-grade (Rathburn Chemicals, Walkerburn, Great Britain). Ultra-pure water was obtained by filtering distilled water through a Gelman Water-I filtration unit (Gelman Sciences, Ann Arbor, MI, U.S.A.). All steroids were obtained from Steraloids (Pawling, NJ, U.S.A.).

The chromatograph consisted of a Constametric III reciprocal pump [Laboratory Data Control (LDC), Riviera Beach, FL, U.S.A.], a Spectromonitor-III variable-wavelength UV detector (LDC) and a Refractomonitor III refractive index (RI) monitor (LDC). The column system consisted of a 50 × 4.6 mm I.D.guard column, dry-packed with 40-μm pellicular packing (Pelliguard-LC-18; Supelco, Bellefonte, PA, U.S.A.) and a 250 × 4.6 mm I.D. reversed-phase analytical column (Supelcosil-LC-18, 5 μm spherical packing; Supelco). Reversed-phase columns (250 × 4.6 mm I.D., 10-μm spherical packing) were also purchased from Gene Tec (Kungsbacka, Sweden) (Nucleosil-ODS) and Supelco (Chromosorb-LC-7). The injections were performed through a fixed-loop (20 μl) Rheodyne injector (Rheodyne, Cotati, U.S.A.).

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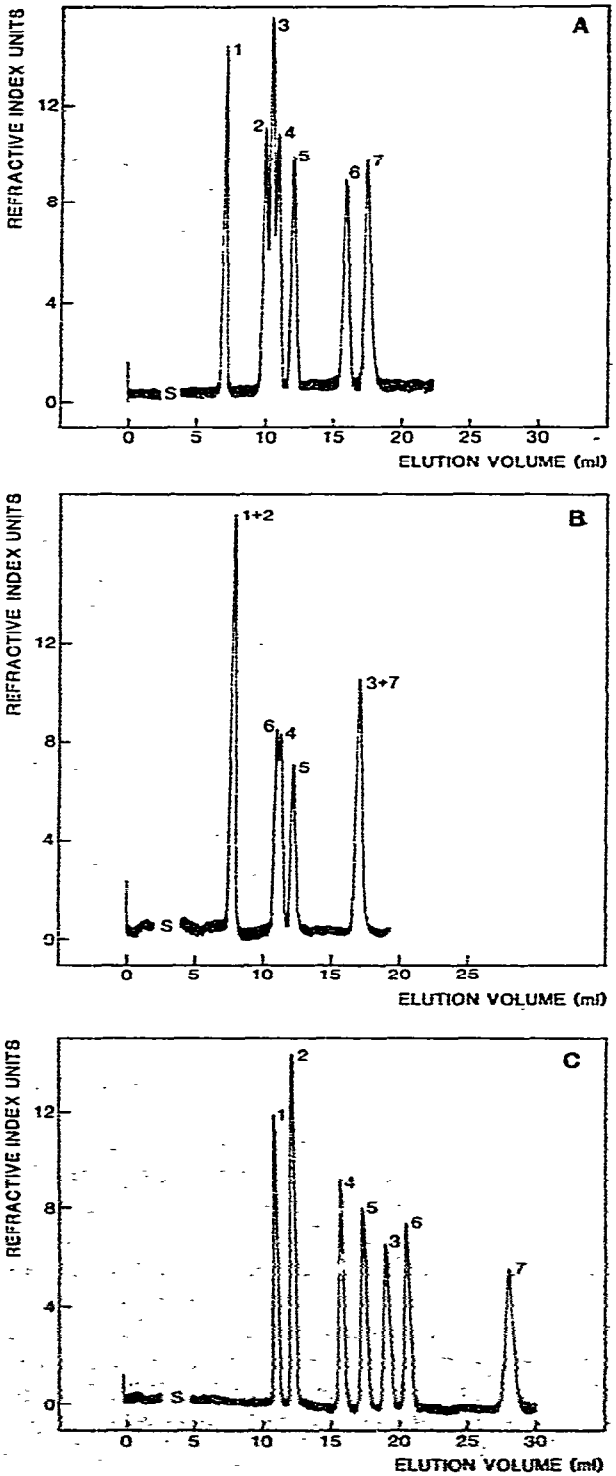

Fig. 1. HPLC of androgens on a 250 × 4.6 mm I.D. Supelcosil-LC-18 column. A 49 × 4.6 mm I.D. guard column, dry-packed with 40-μm pellicular reversed-phase material (Pelliguard LC-18) was placed in front of the analytical column. Flow-rate, 1.0 ml/min. A mixture of 30 μg of each of the steroids in the figure was injected. S = Solvent peak (omitted). For solvent systems and peak identifications, see Table I.

RESULTS AND DISCUSSION

Different reversed-phase columns and eluent mixtures were tested. Only the results obtained with the Supelcosil-LC-18 column are depicted in Fig. 1, as for our purpose this column generally displayed superior chromatographic properties to the other columns tested (Nucleosil-ODS and Chromosorb-LC-7). We employed a dual-piston reciprocal pump. Such pumps generate small fluctuations in eluent flow. This will appear in the RI-based chromatograms as a smeared baseline and jagged peaks (Fig. 1).

Reversed-phase chromatography of 5α-reduced androgens on a Supelcosil-LC-18 column with mixtures of methanol and water as eluents did not result in a satisfactory separation of 3β-A-diol, epiandrosterone and 5α-androstane-3,17-dione (5α-A-dione) (Fig. 1A). However, Dht, 3α-A-diol, androsterone and testosterone, were adequately separated from each other (Fig. 1A). Testosterone, 3β-A-diol, 5α-A-dione and androsterone were eluted as two peaks when mixtures of acetonitrile and water were used as eluents (Fig. 1B). Epiandrosterone and 3α-A-diol were incompletely resolved in this system (Fig. 1B). Mixtures of dioxane and water were also tested as eluents. They displayed chromatographic properties similar to those of acetonitrile–water mixtures.

3α/β-Hydroxysteroids appear relatively more polar and 17-ketosteroids less polar when eluted with acetonitrile–water compared with elution with methanol–water (Fig. 1). The relative polarities of 3-keto and 17β-hydroxy steroids are similar in acetonitrile–water and methanol–water (Fig. 1). Mixtures of acetonitrile, methanol and water were then tested as eluents. The specific solvent/steroid interactions seemed to be additive. Thus, it was possible to find a composition of the eluent which gave an optimal separation of all of the biologically relevant 5α-reduced androgens (Fig. 1C). Different columns of the selected type (Supelcosil-LC-18) tested displayed identical chromatographic properties. The method described is reproducible and relatively fast. By continously monitoring the radioactivity in the eluent, our method has been successfully applied to measurements of androgen metabolism *in vitro*[10].

TABLE I

IDENTIFICATION NUMBER, SYSTEMATIC AND TRIVIAL NAMES AND ELUTION VOLUMES OF THE STEROIDS USED

Elution volumes were obtained with a Supelcosil-LC-18 reversed-phase column at a constant flow-rate of 1 ml/min. Eluent systems: A, methanol–water (69:31), pressure 1400 p.s.i.; B, acetonitrile–water (49:51), pressure 900 p.s.i.; C, methanol–acetonitrile–water (33:26:41), pressure 1100 p.s.i.

Steroid No.	*Systematic name*	*Trivial name*	*Elution volume (ml)*		
			A	*B*	*C*
1	17β-Hydroxy-4-androsten-3-one	Testosterone	7.0	7.6	11.0
2	5α-Androstane-3β,17β-diol	3β-A-diol	9.8	7.6	12.5
3	5α-Androstane-3,17-dione	5α-A-dione	10.4	16.8	19.4
4	3β-Hydroxy-5α-androstan-17-one	Epiandrosterone	11.0	11.2	16.0
5	17β-Hydroxy-5α-androstan-3-one	Dht	12.0	12.2	17.6
6	5α-Androstane-3α,17β-diol	3α-A-diol	15.8	10.8	20.8
7	3α-Hydroxy-5α-androstan-17-one	Androsterone	17.4	16.8	28.5

This study demonstrates the usefulness of a three-component eluent in the reversed-phase chromatography of steroids. Our laboratory has also developed a new reversed-phase chromatographic system for the separation of C_{14}–C_{20} fatty acids based on a three-component eluent[11]. Thus, three-component eluents in reversed-phase chromatography may offer increased chromatographic selectivity and are also applicable to the separation of other classes of compounds.

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