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Note

High-pressure liquid chromatography of androgens

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In connection with our work on the conversion of androstenedione to testosterone by growing pea plants[1], we perceived the need for an efficient method of separating adequate amounts of various metabolites of the androgen series for radiochemical assays. Although much research has gone into chromatographic methods for accomplishing this[2], very few liquid systems capable of resolving the numerous stereoisomers in this class of steroids have been described[3]. High-pressure liquid chromatography (HPLC)[4], particularly on the basis of adsorption, is eminently suitable for this application. For the detection of the androgens, which have a carbonyl group at C-3 and/or C-17, we have used a dual detector, registering the UV absorption at 254 and 280 nm[5].

EXPERIMENTAL*

The HPLC apparatus was assembled from commercially available components. A solvent reservoir, containing a mixture of 89.5 ml dichloromethane, 10 ml acetonitrile, and 0.5 ml 2-propanol (all "Distilled-in-Glass" quality; Burdick & Jackson, Muskegon, Mich., U.S.A.) was connected to the inlet of a dual-piston reciprocating pump (Tracor Model 990; Tracor, Austin, Texas, U.S.A.). The pump outlet was connected to a length of stainless-steel (SS) tubing, 0.02 in. I.D., having a capacity of *ca.* 30 μl, through a SS union. For sample application, this union was disconnected, the solvent in the tubing was removed by aspiration, and the sample was injected directly into the tubing, which formed the column inlet.

The column consisted of two SS chromatographic tubes (Alltech, Arlington Heights, Ill., U.S.A.), each 30 cm × 4.6 mm I.D., packed with Partisil 5 (Whatman, Clifton, N.J., U.S.A.), and connected in series. The column was prepared in our laboratory from a balanced-density slurry of the silica gel in a mixture of tetrabromoethane and tetrachloroethane. The slurry was packed into the tubes with a Haskel HPLC slurry-packing unit (Model 29426; Haskel, Burbank, Calif., U.S.A.). The efficiency of the two sections was determined by using nitrobenzene as the test material and hexane as the eluent. Plate counts were 3200 for each section.

* Reference to a company and/or product named by the Department is only for purpose of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

The outlet of the column was connected to the inlet of a Tracor Model 970 variable-wavelength detector, set at 254 nm. The latter had its outlet connected to the inlet of an Altex–Hitachi Model 100-30 detector (Altex, Berkeley, Calif., U.S.A.), set at 280 nm. The effluent from the latter was returned to the solvent reservoir. A dual-channel recorder (Model 385; Linear, Irvine, Calif., U.S.A.) was attached to the output of both detectors.

RESULTS AND DISCUSSION

Table I lists the eleven androgens separated in a single chromatogram. The chromatogram itself, developed at a flow-rate of 0.4 ml/min, is shown in Fig. 1. The Δ^4-3-ketones (III, VIII, IX) show a strong absorption at 254 nm. As little as 30 ng of testosterone (IX) can be detected. The 17-ketosteroids are detectable by their absorption at 280 nm, but the sensitivity is only about one-fiftieth of that for the Δ^4-3-ketones. The load capacity of our column, tested with etiocholanolone (X), is 12 mg. The amounts of each steroid in the mixture and other details of chromatographic conditions are given in the legend of Fig. 1.

TABLE I

ANDROGENS SEPARATED BY HPLC

Sample No.	*Systematic name*	*Trivial name*
I	5α-Androstane-3,17-dione	Androstanedione
II	5β-Androstane-3,17-dione	Etiocholanedione
III	4-Androstene-3,17-dione	Androstenedione
IV	3β-Hydroxy-5β-androstan-17-one	Epietiocholanolone
V	3β-Hydroxy-5-androsten-17-one	Dehydroepiandrosterone
VI	3β-Hydroxy-5α-androstan-17-one	Epiandrosterone
VII	3α-Hydroxy-5α-androstan-17-one	Androsterone
VIII	1,4-Androstadiene-3,17-dione	Androstadienedione
IX	17β-Hydroxy-4-androsten-3-one	Testosterone
X	3α-Hydroxy-5β-androstan-17-one	Etiocholanolone
XI	17α-Hydroxy-4-androsten-3-one	Epitestosterone

As a rule, the resolution of epimeric steroids by adsorption chromatography is better than that afforded by reversed-phase partition chromatography, except in the case of 17-epimers[3,4]. The C_{19} steroids in our chromatogram fall into three groups: (1) diketones, (2) 3-hydroxy-17-ketones, and (3) 17-hydroxy-3-ketones. As expected, group I (I–III) is first to emerge from the column, followed generally by group 2 (IV–VII) and then group 3 (IX and XI). However, there is some overlap between the three groups: the dienedione VIII, which is much more polar than the monoenedione III, falls between groups 2 and 3, and etiocholanolone (X), a member of group 2, falls between the two 17-hydroxy-3-ketones.

The elution of the A/B *trans*-steroid I before the A/B *cis*-steroid II is contrary to our experience with the 5α- and 5β-cholestan-3-ones[5]. Although the A/B *cis*-steroid with the axial hydroxyl group (IV) is the first compound in group 2 to be eluted, as one would predict[3], the steroids with equatorial hydroxyl groups (V and VI) are less strongly adsorbed than the axial epimer (VII), contrary to expectation. How-

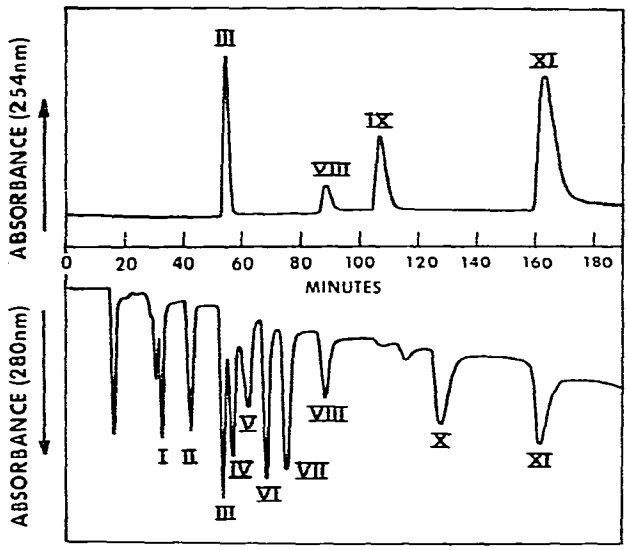

Fig. 1. Elution pattern of androgens. A mixture of 37 μg I, 39 μg II, 34 μg III, 196 μg IV, 188 μg V, 210 μg VI, 214 μg VII, 6 μg VIII, 42 μg IX, 297 μg X, and 99 μg XI was applied by stop-flow to a Partisil 5 column, consisting of two sections (300 × 4.6 mm I.D.). For the identity of I–XI, see Table I. Eluent; dichloromethane–acetonitrile–2-propanol (179:20:1); flow-rate, 0.4 ml/min; pressure, 800 p.s.i. The effluent passed through two detectors in series, the first one set at 280 nm, range 0.1, and the second one at 254 nm, range 1.28. The dual-pen recorder was set at 20 mV for the 254-nm range (top) and at 5 mV for the 280-nm range (bottom). Chart speed, 10 cm/min.

ever, this sequence agrees with the one obtained by triple development of a thin-layer chromatogram on alumina with benzene–diethyl ether (1:1)[6].

The separation of 17-epimers by thin-layer chromatography is difficult[7], but it can be accomplished by partition chromatography on paper[8]. In contrast to paper chromatography, where the 17β-epimer is invariably more polar than the 17α-epimer[3], we find in adsorption HPLC that the 17β-hydroxysteroid testosterone (IX) is eluted before epitestosterone (XI).

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