## ACKNOWLEDGMENTS

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# Application of Glass Fiber Chromatography
# for Separation of $C_{19}$ Steroids[1]

In the conversion of androst-5-en-3$\beta$-ol-17-one (DHA) (1–3) and its sulfate ester (DHA-sulfate) (4) to androst-4-en-3-one-17$\beta$-ol (Testosterone), androst-5-ene-3$\beta$,17$\beta$-diol ($\Delta^5$-androstenediol) has been implicated as an intermediate. It is not known if this pathway also involves androst-4-ene-3$\beta$,17$\beta$-diol ($\Delta^4$-androstenediol). This question is not easily answered because the $\Delta^4$- and $\Delta^5$-androstenediols were not readily separated either by paper chromatography (3, 5) or by thin-layer chromatography (6). This communication describes a method for rapid resolution of $\Delta^5$-androstenediol and $\Delta^4$-androstenediol utilizing $AgNO_3$ treated silica gel impregnated glass fiber sheets (ITLC-SG, Gelman Instrument Company). In addition the chromatographic behavior on ITLC-SG and $AgNO_3$ impregnated ITLC-SG (Ag-ITLC-SG) of other $C_{19}$ steroids is presented. This procedure was developed for studies of the metabolism of isotopically labeled DHA and DHA-sulfate in tissue fractions.

The method of extraction of the steroids has been described (7). The extracts were first chromatographed on paper (Whatman 2) using the Zaffaroni system (8). The formamide impregnated paper was developed with descending cyclohexane/benzene 1/1. Allowing the solvent front to

TABLE 1

Chromatographic Behavior of Fifteen $C_{19}$ Steroids on Silica Gel Impreagnted Glass Fiber Sheets

| Areas[a] | Steroid | Solvent A[b] | | | | Solvent B[c] | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | ITLC-SG[d] | | Ag-ITLC-SG[e] | | ITLC-SG[d] | | Ag-ITLC-SG[e] | |
| | | $R_f$ | Mobility, cm | $R_f$ | Mobility, cm | $R_f$ | Mobility, cm | $R_f$ | Mobility, cm |
| I ($R_f$ 0.38–0.40) | 5α-Androstane-3β,17β-diol | 1.00 | 10.3 | 1.00 | 10.2 | 1.00 | 10.1 | 1.00 | 10.2 |
| | Androst-5-ene-3β,17β-diol | 0.98 | 10.1 | 0.68 | 7.0 | 1.01 | 1.02 | 0.74 | 7.6 |
| | Androst-4-ene-3β,17β-diol | 1.00 | 10.3 | 0.82 | 8.3 | 1.02 | 10.3 | 0.86 | 8.8 |
| | 5β-Androstane-3α,17β-diol | 0.68 | 7.0 | 0.64 | 6.5 | 0.56 | 5.7 | 0.60 | 6.1 |
| II ($R_f$ 0.48–0.54) | Androst-4-en-3-one,17β-ol | 1.02 | 10.5 | 1.02 | 10.4 | 0.86 | 8.7 | 0.85 | 8.7 |
| | 5α-Androstane-3α,17β-diol | 0.91 | 9.4 | 0.91 | 9.3 | 0.96 | 9.7 | 0.97 | 9.9 |
| | 5β,Androstane-3β,17β,diol | 1.05 | 10.8 | 1.07 | 10.9 | 1.14 | 11.5 | 1.15 | 11.7 |
| III ($R_f$ 0.69–0.72) | 5α-Androstane-3β-ol,17-one | 1.00 | 12.7 | 1.00 | 12.4 | 1.00 | 11.9 | 1.00 | 12.2 |
| | Androst-5-en-3β-ol,17-one | 1.00 | 12.7 | 0.85 | 10.6 | 1.02 | 12.1 | 0.84 | 10.3 |
| | 5α-Androstan-3-one,17β-ol | 1.03 | 13.1 | 1.04 | 13.0 | 1.06 | 12.6 | 1.05 | 12.8 |
| | 5β-Androstan-3α-ol,17-one | 0.84 | 10.7 | 0.82 | 10.2 | 0.80 | 9.5 | 0.82 | 10.0 |
| IV ($R_f$ 0.77–0.80) | Androst-4-ene-3,17-dione | 1.05 | 13.3 | 1.08 | 13.5 | 0.94 | 11.2 | 0.95 | 11.6 |
| | 5α-Androstan-3α-ol,17-one | 0.94 | 11.9 | 0.97 | 12.0 | 1.01 | 12.0 | 1.03 | 12.5 |
| | 5β-Androstan-3β-ol,17-one | 1.04 | 13.2 | 1.05 | 13.0 | 1.10 | 13.1 | 1.11 | 13.5 |
| ($R_f$ 0.84) | 5α-Androstane-3,17-dione | 1.18 | 15.0 | 1.19 | 14.8 | 1.20 | 14.3 | 1.19 | 14.5 |

[a] Roman numerals represent the four areas obtained with Zaffaroni system described in text.

[b] Solvent A = chloroform, one development.

[c] Solvent B = cyclohexane/ethyl acetate 7/1 (v/v), two developments.

[d] Silica gel impregnated glass fiber sheets, Gelman Instrument Co.

[e] Same as $d$ except for treatment with 10% $AgNO_3$ solution as described in text.

[f] I and II, reference compound = 5α-androstane-3β,17β-diol; III and IV, reference compound = 5α-androstan-3β-ol-17-one.

move 35 cm from the origin divided the $C_{19}$ steroids studied into four major areas. These areas were eluted with absolute methanol and then chromatographed either on ITLC-SG or on Ag-ITLC-SG.

The ITLC-SG sheets were impregnated with $AgNO_3$ by dipping them into a 10% aqueous solution of $AgNO_3$, were air-dried overnight in the dark, and were used the following morning. The sheets were developed ascending in unsaturated glass tanks ($25 \times 29 \times 10$ cm) to a distance of 16 cm from the origin (development time approximately 25 min). When two developments were used, the sheets were dried in a stream of air for 15 min before commencing the second development. Samples were applied as 0.05 ml aliquots in methanol at 0.5 cm intervals. Radioactive areas were located with a Vanguard Autoscanner 880 in the same manner as employed for scanning paper strips. Standards on ITLC-SG were detected either by exposing to iodine vapors or by spraying with concentrated sulfuric acid in absolute ethanol 1:1 (v/v) and heating for 15 min at 110°C. Standards on Ag-ITLC-SG were detected as dark spots after holding the sheet over a hot plate for 1 min or by using the sulfuric acid/ethanol reagent as described above.

For further studies, the radioactive areas were eluted with absolute methanol. To remove $AgNO_3$, the methanol was evaporated and the residue dissolved in water and extracted three times with equal volumes of methylene chloride.

The chromatographic behavior of fifteen $C_{19}$ steroids on ITLC-SG and Ag-ITLC-SG in chloroform (solvent A) and in cyclohexane/ethyl acetate 7/1 (solvent B) are presented in Table 1. With either solvent, Ag-ITLC-SG resolved $\Delta^5$-androstenediol and $\Delta^4$-androstenediol, and separated these two unsaturated diols from their 5α-saturated analog. It also resolved DHA and its saturated 5α analog. Solvent B, utilizing Ag-ITLC-SG in conjunction with prior chromatography in the Zaffaroni system, brought about the resolution of all fifteen $C_{19}$ steroids studied except for 5α-androstan-3β-ol-17-one vs. 5α-androstan-3-one-17β-ol and DHA vs. 5β-androstan-3α-ol-17-one, which were only partially resolved. However, each of these two mixtures could be resolved by acetylation followed by one development in cyclohexane/ethyl acetate 150/8 on ITLC-SG.

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# Application of Glass Fiber Chromatography for Separation of Some Neutral and Phenolic Steroid Sulfates[1]

Our continued interest in the metabolism of steroid sulfates led to the development of a rapid technique for the separation of dehydroepiandrosterone sulfate and androstenediol 3-sulfate on silica gel impregnated glass fiber sheets (ITLC-SG, Gelman Instrument Company). The chromatographic behavior on ITLC-SG of several other steroid sulfates was also determined.

Steroid sulfates were extracted from tissue fractions as described earlier (1). The final residue, dissolved in absolute methanol, was applied as 0.05 ml aliquots at 0.5 cm intervals. The chromatographic procedure was similar to that described for free steroids (2), using two developments with chloroform/acetone/acetic acid 110/35/6 (v/v). It took 25 min for the solvent front to travel 16 cm. Standards were detected either by spraying with methylene blue reagent as described by Crepy *et al.* (3), except that the reagent was not diluted with acetone, or by spraying with sulfuric acid/ethanol 1/1 (v/v) and heating for 15 min at 110°C. Detection and elution of radioactive samples were performed as described (2). Steroid sulfates could be solvolyzed directly on the ITLC-SG sheets by exposing them to an atmosphere of HCl/dioxane 90/10 (v/v) for 3 hr (4) and the resulting free steroids eluted for further studies.

As is shown in Table 1 all of the neutral 17-hydroxy-3-monosulfate