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## A simple staining technique useful for distinguishing 3-hydroxy-4-ene from 3-hydroxy-5-ene steroids on silica gel thin layers, and for other purposes

Various steroids on silica gel thin layers give coloured and/or fluorescent products when exposed to hydrochloric acid vapour[1]. The usefulness of this reaction for detecting the important class of 3$\beta$-hydroxy-5-ene steroids was recently pointed out[1]. The related 3-hydroxy-4-ene steroids are of some interest as intermediates and side-products in chemical syntheses, and it is useful to be able to detect them easily and to distinguish them from the 3-hydroxy-5-enes. It is now reported that, while both the 3-hydroxy-4-ene and 3-hydroxy-5-ene steroids react similarly on exposure to hydrochloric acid vapour at room temperature (23°) or a little above (*e.g.*, 40°), various 3-hydroxy-5-enes are not stained appreciably at somewhat lower temperatures, though the 3-hydroxy-4-enes still react. The technique is easily varied so as best to suit particular compounds which it is wished to distinguish. The following illustrates a convenient general procedure.

*Experimental*

The thin-layer plate (Kieselgel $HF_{254+366}$ E. Merck, Darmstadt, G.F.R.) is dried to remove the solvent in which the chromatogram was developed, and is then cooled to + 4° (refrigerator or cold room). It is then exposed for 10 min to hydrochloric acid vapour in a glass tank containing a little hydrochloric acid (sp.gr. 1.18 $g \cdot ml^{-1}$, 3 ml per litre tank capacity) at + 4°. Under these conditions, simple 3-hydroxy-4-ene steroids such as cholest-4-en-3$\beta$-ol and androst-4-ene-3$\beta$,17$\beta$-diol give pink spots and the corresponding 3$\beta$-hydroxy-5-enes do not.

If, after exposure to cold hydrochloric acid vapour, as above, the plate is warmed to 40°, 3$\beta$-hydroxy-5-enes (*e.g.*, cholest-5-en-3$\beta$-ol and 3$\beta$-hydroxypregn-5-en-20-one) often give pink spots during the warming, but this is somewhat variable (*e.g.*, 3$\beta$-hydroxyandrost-5-en-17-one and 3$\beta$-hydroxy-6-methyl-pregn-5-en-20-one do not always give visible coloured spots). Re-exposure of the warmed plate (40°) to hydrochloric acid vapour at room temperature gives more reproducible staining of the 3$\beta$-hydroxy-5-enes, but the method of choice is to run duplicate thin-layer plates and to expose one plate at 4° as described above, and the other at 40° as previously described[1].

The 3$\beta$-hydroxy-4-ene steroids were made by reducing 4-en-3-ones with sodium borohydride in propan-2-ol at 23°, and the reaction products before purification contained in each case small amounts of another compound, which, from the method of preparation and the $R_F$ values, were thought to be the corresponding 3$\alpha$-hydroxy-4-enes. They stained in the same way as the 3$\beta$-hydroxy-4-ene main products.

3$\beta$-Hydroxy-9$\beta$,10$\alpha$-pregn-5-en-20-one (retropregnenolone) did not stain at 4° and did stain at 40° giving, however, a grey colour (not pink).

Reduction of a small amount of 17$\beta$-hydroxy-9$\beta$,10$\alpha$-androst-4-en-3-one (retrotestosterone) with sodium borohydride in propan-2-ol at 23° gave a product which was not isolated, but was presumably 9$\beta$,10$\alpha$-androst-4-ene-3$\beta$,17$\beta$-diol. This gave a pink spot at 4°, similar to but not the same as that from androst-4-

ene-3β,17β-diol: the pink material from the retrocompound faded to a grey colour within minutes at room temperature. 3β-Hydroxy-6-methylpregn-5-en-20-one did not stain at 4°, and stained at 40°, giving a pink colour which was not exactly the same colour as, and which faded more quickly than the pink product from 3β-hydroxypregn-5-en-20-one. Thus, there is a general consistency in the behaviour of structurally similar steroids, but the reaction is certainly influenced by the shape and substituents in various parts of the molecule. The only 3β-hydroxy-5-ene so far encountered which did not give a coloured product at 40° was 3β-hydroxypregn-5-ene-7,20-dione (the only 7-oxo compound so far tested). The likely explanation is that conjugation of the 5-ene with a 7-oxo group alters its reactivity. Pregn-4-ene-3,6,20-trione and 6β-hydroxypregn-4-ene-3,20-dione, like pregn-4-ene-3,20-dione itself, did not stain at 4° or 40°. The opportunity has not yet arisen to test a 3-hydroxy-4,4-dimethyl-5-ene or 3-hydroxy-4-methyl-4-ene which would clearly be interesting apropos the reaction mechanism.

3,7-Dihydroxy-5-enes are of some interest as natural products and in the present context have interesting structures, being both allylic alcohols (like the 3-hydroxy-4-enes) and homoallylic alcohols (like the 3-hydroxy-5-enes). 3β, 7β-Dihydroxyandrost-5-en-17-one (a human metabolic product[2]) gave a strong blue colour at 4°, which rapidly turned turquoise on exposure to air at room temperature. When the colour faded (several hours) an orange fluorescence in short-wave UV light developed.

Although 3β,7β-dihydroxyandrost-5-en-17-one is the only fully characterized 3,7-dihydroxy-5-ene so far tested, other compounds of this type probably react similarly. Thus, when a solution of 3β-hydroxypregn-5-ene-7,20-dione in propan-2-ol was allowed to react with a little sodium borohydride at room temperature for 3 h, and an aliquot of the reaction mixture run on silica gel, three blue spots appeared on exposure of the plate to hydrochloric acid vapour at 4°. These were probably mainly 3β,7α-dihydroxypregn-5-en-20-one, and the corresponding 3β,7α,20β- and 3β,7β,20β-triols. The production in this way of a product or products giving a blue reaction on exposure to hydrochloric acid vapour at 4°, from an original compound that absorbed in short-wave UV light and did not give a colour reaction with hydrochloric acid vapour at 40°, distinguishes 3β-hydroxy-5-en-7-ones from a very large number of other compounds; and it requires only micrograms of material (which need not be pure).

When exposure to hydrochloric acid vapour is carried out at —15° to —20°, the 3-hydroxy-4-enes and 3,7-dihydroxy-5-enes generally still give colour reactions. There are some differences in the colours from those obtained at higher temperatures, and certain differences in the reaction rates for molecules of different structural types. Although these could be made use of by running appropriate standards on the same plate, they depend on the amount of material, the thickness of the thin layer, and other ill-defined factors, and are, in general, not sufficiently reproducible to be of much value.

The techniques described are very simple to carry out, and are easily adapted to specialized purposes. They should not be confused with the Hammarsten–Yamasaki reaction[3,4] which uses hot liquid hydrochloric acid, and has quite different specificity[5].

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