CHROM. 5397

A chromatogram staining technique useful for 3β-hydroxy-5-ene steroids and certain steroid drugs

Steroids containing the 3β-hydroxy-5-ene grouping are of some general interest, because they include starting materials and intermediates for many industrial steroid syntheses[1], the intermediates of steroid biosyntheses[2,3], and they are produced in great variety and relatively large quantity by the human foetus[4,5]. They can be detected on thin-layer chromatograms by procedures such as spraying and heating with phosphomolybdic acid, or with reagents containing various strong acids[6,7]. Such methods have low specificity, and in the case of the strong acid spray reagents, their corrosive nature causes handling difficulties. A convenient technique is now described which enables 3β-hydroxy-5-ene steroids to be detected with considerable specificity, and with sensitivity similar to that of many less specific or less convenient methods.

The technique consists of exposing the thin-layer chromatogram to hydrochloric acid vapour. In the case of silica gel layers, regions where 3β-hydroxy-5-ene steroids are present rapidly become coloured (generally pink or violet) and at first show little or no fluorescence in UV light (366 nm or 254 nm). The initial colour fades over a period of many minutes (generally going grey and finally pale yellow), and as the visible colour fades the region becomes fluorescent in UV light (366 nm and 254 nm). The yellow fluorescence in shortwave UV (254 nm) can usually be seen for several days. A few other types of steroid give colour or fluorescent reactions (which are easily distinguished from those of the 3β-hydroxy-5-enes) and the technique is useful for detecting certain steroid drugs. The technique is potentially applicable to other adsorbents (*e.g.* alumina) and some colour and fluorescence changes can even be produced on paper. However, the changes are not identical with those on silica gel, and are generally less useful (less sensitive and less easily reproduced). The following applies to silica gel layers.

The conditions for the reaction are relatively uncritical. The chromatogram may be at room temperature (22°) or at 70°, and may be exposed to the acid vapour for 5 or 15 min, giving, in most cases, similar results. Nevertheless, some standardisation is desirable, particularly if negative results are to be relied upon as evidence for the absence of compounds expected to react.

The following scheme has proved very satisfactory for 250-μ-thick layers of silica gel $HF_{254+366}$ (E. Merck, Darmstadt, G.F.R.). The plate is removed from the solvent system in which it was developed, and the solvent is allowed to evaporate. The plate is first examined in shortwave UV light (254 nm), when 3-oxo-4-ene steroids, and other compounds with suitable chromophores, appear dark against the yellow-green fluorescence of the indicator dye present in the absorbent, and their locations are marked. The plate is next examined in longwave UV light (366 nm) and a variety of saturated and unsaturated steroids, including 3β-hydroxy-5-enes, can be seen as light regions against the light blue background and their positions marked. The plate is then warmed to 40° and put into a glass tank containing a little concentrated hydrochloric acid (3 ml per l tank capacity: hydrochloric acid specific gravity 1.18 g/ml). The plate is allowed to stand in the tank for 10 min, is then removed, coloured zones are marked and the plate is examined under shortwave and longwave UV light.

TABLE I

COMPOUNDS THAT SHOWED COLOUR AND FLUORESCENCE REACTIONS DIFFERENT FROM THE 3β-HYDROXY-5-ENE COMPOUNDS

Compound	*A distinguishing feature of its reaction*
17β-Acetoxy-19-nor-17α-pregn-4-en-20-yn-3-one	Red fluorescence in UV immediately after acid treatment
Androst-5-en-17-on-3β-yl sulphate (Na or NH_4 salt)	Appearance in UV
3β,17β-Diacetoxy-19-nor-17α-pregn-4-en-20-yne	Orange-red fluorescence in UV immediately after acid treatment
3-Hydroxyoestra-1,3,5(10)-trien-17-one	Slight reaction; yellow colour
17β-Hydroxy-18-methyl-19-nor-17α-pregn-4-en-20-yn-3-one	Red fluorescence in UV immediately after acid treatment
17β-Hydroxy-19-nor-17α-pregn-5(10)-en-20-yn-3-one	Red fluorescence in UV immediately after acid treatment
17β-Hydroxy-19-nor-5α,17α-pregn-20-yn-3-one	Transient pale yellow colour
3-Methoxy-19-nor-17α-pregna-1,3,5(10)-trien-20-yn-17β-ol	Fluorescent in UV immediately after acid treatment
3-Methoxyoestra-1,3,5(10)-trien-17β-ol	Slight yellow colour
3-Methoxyoestra-1,3,5(10)-trien-17-one	Very slight yellow colour
19-Nor-17α-pregna-1,3,5(10)-trien-20-yne-3,17β-diol	Fluorescent in UV immediately after acid treatment
Oestra-1,3,5(10)-triene-3,17β-diol	Yellow colour
Oestra-1,3,5(10)-triene-3,16α,17β-triol	Pink, fading rapidly; little fluorescence in UV
9β,10α-Pregna-4,6-diene-3,20-dione	No colour; yellow fluorescence in UV
Pregn-5-en-20-on-3β-yl sulphate (Na or NH_4 salt)	Appearance in UV

The yellow-green fluorescence normally given under shortwave UV by the indicator dye present in the absorbent is no longer visible, so the background is dark in shortwave UV, but it is still light in longwave UV. Any fluorescent spots are noted. The plate is re-examined after 10–20 min, and again after 1–2 h.

The sequence of colour and fluorescence changes regarded as characteristic of 3β-hydroxy-5-enes is as follows. A colour, generally pink, develops within a minute or two of exposure to the acid vapour. The colour depends upon the compound, ranging from orange-pink to purple-pink for the compounds tested, except for 3β,11β,17α,21-tetrahydroxypregn-5-en-20-one, which gave a blue colour. The time from exposure to acid vapour required for the colour to become easily visible depends upon the amount of material present, and was usually 1–2 min for 10–2 μg of steroid per cm^2. Immediately after the standard 10 min exposure to acid vapour, the coloured spots show little or no fluorescence in shortwave or longwave UV light. The coloured spots can be seen to be fading a few minutes after removal from the acid vapour, and when examined after 10–20 min are seen to be greyer (visible light), slightly fluorescent in longwave UV light, and weakly but clearly fluorescent (yellow) in shortwave UV light. After 1–2 h the coloured spots have largely faded, and may be yellow, yellow-grey or a pale brown colour. When less than about 2 μg of steroid per cm^2 is present, these faded colours generally cannot be seen. In shortwave UV light, the faded spots are fluorescent (yellow) against the dark background; a similar fluorescence in longwave UV often appears somewhat different against the light background and may be less distinct. A slight colour (esp. yellow) in visible light and yellow fluorescence in shortwave UV light are usually still present after several days.

The smallest amounts of steroid that can be clearly distinguished are in the

region of 1 μg/cm^2, *e.g.* about 0.6 μg/cm^2 for 3β-hydroxycholest-5-ene, about 0.8 μg/cm^2 for 3β-hydroxypregn-5-en-20-one, about 1 μg/cm^2 for 3β-hydroxyandrost-5-en-17-one, and about 1.5 μg/cm^2 for 3β,17α-dihydroxypregn-5-en-20-one.

Some steroids other than 3β-hydroxy-5-enes give coloured and/or fluorescent products when treated as described above (see Table I). A detailed description of the changes for each compound is unnecessary. The compounds include various oestrogens, and it is seen that a free hydroxyl group at the 3 position is not essential. The striking reactions of 19-nor-17α-pregna-1,3,5(10)-trien-20-yne-3β,17β-diol (ethynyloestradiol) and its 3-methyl ether (mestranol) no doubt involve participation of the 17α-ethynyl group. Several compounds containing this group gave marked reactions, while the corresponding de-ethynyl compounds did not. However, the one 17α-ethynyl compound tested which had a saturated steroid ring system, namely 17β-hydroxy-19-nor-5α,17α-pregn-20-yn-3-one, gave only a transient yellow colour, so the production of the more striking colour changes in this series involved reactive groups in the A ring as well as the 17α-ethynyl group. In this connection, 3β,17β-diacetoxy-19-nor-17α-pregn-4-en-20-yne (ethynodiol diacetate) was interesting because it contained the acetate ester of the allylic alcohol derivable from a 3β-hydroxy-5-ene (by migration of the double bond from 5 to 4) and a 17α-ethynyl group. In fact it gave a spectacular sequence of colour and fluorescence changes, and was detectable down to about 0.4 μg/cm^2. Reaction of 9β,10α-pregna-4,6-diene-3,20-dione (dydrogesterone) to give a colourless but fluorescent product was interesting because the other three 3-oxo-4,6-dienes (chlormadinone acetate, megestrol acetate and medrogestone) and the other 9β,10α compound (retrotestosterone) tested did not behave similarly.

Steroids found not to give any clear colour or fluorescence reaction under the standard test conditions include most of the naturally occurring structural types other than the 3β-hydroxy-5-enes, and oestrogens. They also include various drugs and synthetic intermediates. In many cases, these compounds probably remained unchanged. This was tested in selected cases as follows. The steroid was applied to a chromatogram, and exposed to acid vapour under the standardised conditions before the chromatogram was developed with solvent. The chromatogram was allowed to stand for several minutes (about 20 min) in the draught of a fume-hood and a further specimen of the same steroid was then applied in an adjacent lane, the chromatogram was immediately developed with solvent, and subsequently sprayed with dinitrophenylhydrazine reagent (saturated 2,4-dinitrophenylhydrazine in propan-2-ol, freshly acidified with concentrated hydrochloric acid, 0.2 ml per 20 ml). The spots obtained from the steroid exposed to acid vapour and that applied after acid treatment were then compared. In this way it appeared that 17β-hydroxyandrost-4-en-3-one, its propionate, the corresponding *retro* compound (17β-hydroxy-9β,10α-androst-4-en-3-one), and the related reduced compounds 17β-hydroxy-5α-androstan-3-one, 3β-hydroxy-5α-androstan-17-one, and 3α-hydroxy-5β-androstan-17-one remained substantially unchanged. So too did 21-hydroxypregn-4-ene-3,20-dione, its acetate, and 17α-acetoxy-6-methylpregna-4,6-diene-3,20-dione. However, 11β,17α,21-trihydroxypregn-4-ene-3,20-dione (cortisol) and 17α,21-dihydroxypregn-4-ene-3,11,20-trione (cortisone) evidently underwent some decomposition. It is interesting to note that the ester groups of a 21-acetate (primary hydroxyl), a 17β-propionate (secondary hydroxyl), and a 17α-acetate (a tertiary hydroxyl in this case), were not appreciably hydrolysed. Thus, although 3β-acetoxypregn-5-en-20-one gave a colour and fluo-

rescence reaction closely similar to 3β-hydroxypregn-5-en-20-one, it is unlikely that reaction of the acetate depended upon formation of the free 3β-hydroxy compound sa an intermediate. Pregn-5-en-20-on-3β-yl sulphate and androst-5-en-17-on-3β-yl sulphate (as sodium or ammonium salts in either case) gave reactions similar to but distinguishable from the corresponding free steroids.

An advantage of the staining technique described is that it allows many common spray reagents to be used subsequently on the same plate. In this way much information is obtained for little expenditure of laboratory time or valuable specimens.

The present findings may be contrasted with the report (ref. 7, p. 27) that when plates are sprayed intensively with concentrated hydrochloric acid and heated at 80–100° for 5 min, a blue colour is obtained from 3,7-dihydroxy-5-ene steroids, but no colour is obtained with other 3-hydroxy-5-ene or 3-hydroxy-4-ene steroids. The opportunity has not yet arisen to test any 3,7-dihydroxy-5-enes using the new technique.

Thanks are due to Professor S. C. FRAZER and Professor I. MACGILLIVRAY for their interest and encouragement; to Professor W. KLYNE and Dr. D. N. KIRK for gifts from the Medical Research Council Steroid Reference Collection; and to friends in many pharmaceutical companies for kindly giving pure specimens of steroid drugs and related compounds.

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Received March 15th, 1971