Thin-layer chromatography of steroids: Specificity of two location reagents

Although there are many location reagents that can be used for detecting steroids, there are few of known specificity. When the specificity has been determined, it has been found that several of these reagents are either of limited application, *e.g.* isonicotinic acid hydrazide for Δ^4- and $\Delta^{1:4}$-3-keto steroids, or of low sensitivity, *e.g.* 2,4-dinitrophenyl-hydrazine (+ hydrochloric acid) for ketones.

During routine examination by thin-layer chromatography of compounds produced during the synthesis of corticosteroids from sapogenins and of synthetic C_{21} and C_{22} intermediates (the latter containing a 16-methyl or -methylene group), we have found three location reagents adequate to deal with almost all samples, namely, 2,5-diphenyl-3-(4-styrylphenyl)-tetrazolium chloride (T.P.T.Z.) (M & B 1767), effective on alumina, *p*-hydroxybenzaldehyde–sulphuric acid (Komarowsky's reagent)[1] and methanolic zinc chloride; the last two were studied mainly on silica gel, but were effective also on alumina. A brief note mentioning this work has already been published[6].

The tetrazolium salts are well known for use in the detection of reducing steroids; we mention the styryl derivative because in our view it is an improvement on the more usual anisole derivative. Its use has already been reported, but not perhaps sufficiently stressed[2]. This styryl phenyl derivative produces strong purple spots on a yellow ground, visible in daylight, compared with the weaker lilac spots given by the dianisole derivative ("blue tetrazolium") commonly used.

Use of Komarowsky's reagent was reported for location of sapogenins[3], but it was subsequently found that 23-substituted sapogenins would not react. The 23-position can be regarded as α to a potential C_{22} ketone, by considering the spiroketal group as the internal ketal of a 16,26-dihydroxy-22-ketone, and it is thought that *p*-hydroxybenzaldehyde condenses at the 23-position in the presence of sulphuric acid. Komarowsky's reagent has also been used for the detection of several corticosteroids[5]. More detailed examination has shown that it will react with all 3-keto steroids having an unsubstituted C_2 position, which is the active site α to the 3-ketone*. The reaction is thus analogous to that for sapogenins, both requiring a methylene group α to a ketone. Spots are visible in daylight and are generally yellow on a white ground, which will gradually turn pink if the plate has been heated at 100°.

Zinc chloride is, in our view, greatly to be preferred to the most commonly used metal halide, antimony trichloride, especially since the latter gives best results when used in conjunction with both thionyl chloride and chlorine[4]. Zinc chloride is non-corrosive and virtually non-toxic, the only precaution required in handling it being to protect it from moisture, as it is highly deliquescent. There are no unpleasant fumes produced by it during either spraying or heating of the plate.

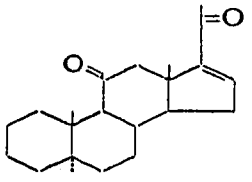
The specificity of zinc chloride for C_{21} and C_{22} steroids can be summarised as follows. All 3-hydroxy or 11-hydroxy derivatives are located, but for 3-ketones some form of activation is required, notably by an epoxide or a double bond. The activating effect of more distant groups, such as a 16,17 epoxide, is observed only when there is

* Substitution of the 3-ketone does not affect sensitivity, but this is diminished by substitution of the C_4 position, for example by a double bond or bromine atom.

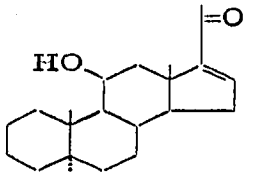
no 11-ketone in ring C. In the absence of an oxygen function at C_3, only 11-hydroxy derivatives will react.

Location of 3-ketones was achieved in the presence of 9,11- and 16,17-epoxides, of 11-hydroxyl groups, and of $\Delta^{1:4}$-, $\Delta^{4:6}$-, and $\Delta^{9:16}$- double bonds. The only effective monoenes were the $\Delta^{9(11)}$-derivatives, whereas Δ^{1}-, Δ^{4}- and Δ^{16}-3-keto steroids showed weak reactions.

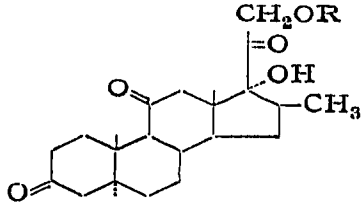
A double bond or an epoxide grouping can presumably produce reactivity at the 3-keto position because the steroid ring structure is under strain. In 3-keto-11-hydroxy compounds there is no distortion of the rings, and the reaction occurs at the 11 position. This was demonstrated by converting 5α-pregnan-16-ene-11,20-dione (I), a 3-desoxy compound not located by zinc chloride, to the corresponding 11-hydroxy derivative (II), which was then easily detectable.



I



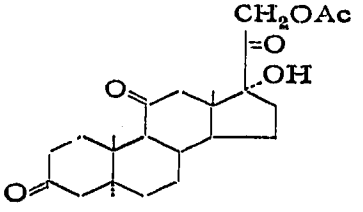
II



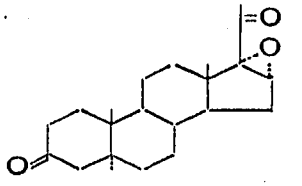
III (a) R=H
(b) R=CH_3CO

The presence of a 16-methyl group does not improve reactivity, since zinc chloride will not locate 16β-methyl-4,5α-dihydrocortisone 21-alcohol or acetate (III a + b), two C_{22} 3-keto steroids that do not contain a double bond or an epoxide grouping. The corresponding C_{21} steroid, 4,5α-dihydrocortisone acetate (IV), also gives weak spots with zinc chloride.

The deactivating effect of an 11-ketone is shown by 16α,17-oxido-5α-pregnane-3,20-dione (V), which gives strong spots with zinc chloride, whereas the introduction of an 11-ketone produces a nonreacting compound.



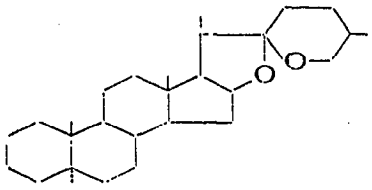
IV



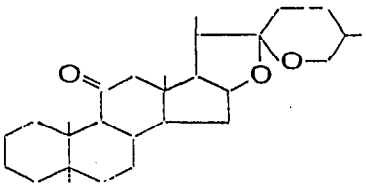
V

In steroidal sapogenins there is no specific group that appears necessary, and the reactivity could well be intrinsic to the spirostane system, since a compound containing no substituent groups, 5α,25D-spirostane (VI), is still located with zinc chloride. All sapogenins examined were found to react, including 3-desoxy compounds, such as 5α,25D-spirostan-11-one (VII), and 3-ketones, such as 5α,25D-spirostan-3-one (VIII) ("tigogenone").

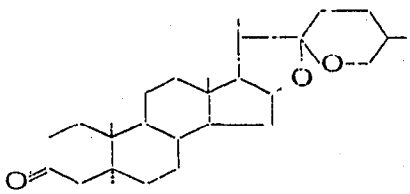
The variety of colours produced makes this reagent particularly useful for the examination of mixtures of close-running spots. Some spots are visible in daylight, but all reacting steroids give vivid spots of various colours when seen under U.V. light of 366 mμ wavelength.



VI

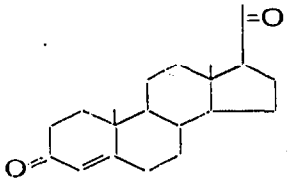


VII

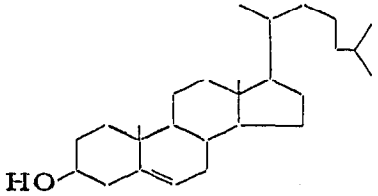


VIII

It should be stressed that the presence of a 3-hydroxy group does not apparently guarantee the location of all classes of steroid, for example sterols. Thus the only reference to the use of methanolic zinc chloride[5], which relates to its application for paper chromatography, claims positive detection of several oestrogens and androgens but not of progesterone (IX), a 3-keto steroid, or of cholesterol (X), a 3β-hydroxy steroid. We have not had the opportunity to examine such compounds by thin-layer chromatography.

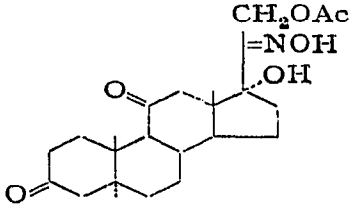


IX

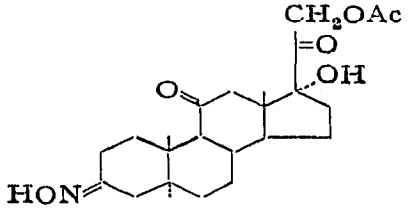


X

In the course of examining over 250 different steroids restricted to the classes mentioned initially we have only come across one anomalous reaction, that of the 3- and 20-oxime groups. The 20-oxime of 4,5α-dihydrocortisone acetate (XI) gives positive results with zinc chloride, and the 3-oxime (XII) is not detected. Neither would have been expected to react, since the parent compound (IV) does not. It would appear that the 20-oxime group is reacting as an hydroxyl compound rather than as a substituted ketone, but there is no obvious reason why only the 20 position should react in this way, except that the side chain is more easily able than ring A to undergo rearrangements.



XI



XII

It must be emphasised both for Komarowsky's reagent and for zinc chloride that faint colours are produced with a number of so-called unreacting compounds. However, the sensitivity is greatly diminished for these steroids, whereas for all three location reagents the detection limit for reacting steroids is 0.1 μg or less. It is therefore possible, with a 1 % solution and 2.5 λ loading, to detect 1 % or less of an impurity.

Preparation of location reagents

2,5-Diphenyl-3-(4-styrylphenyl)-tetrazolium chloride. Mix 1 part of a 1% solution of

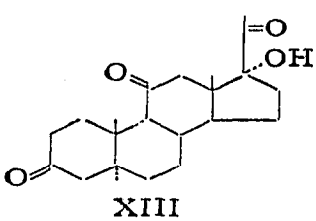
T.P.T.Z. salt in methanol with 10 parts of a 3% solution of sodium hydroxide in distilled water, and apply immediately.

Komarowsky's reagent. Mix 1 part of a 50% v/v solution of concentrated sulphuric acid in distilled water with 10 parts of a 2% solution of *p*-hydroxybenzaldehyde in methanol. Apply immediately, and heat the sprayed plate for 3–4 min at 105° or 10 min at 60° (less background colour develops).

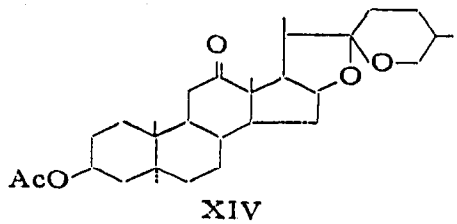
Zinc chloride. Prepare a 30% solution of technical grade zinc chloride in analytical reagent grade methanol, and filter the cloudy solution through a sterimat. After spraying, heat the plate for 60 min at 105°, and cover immediately with a second plate on removing the chromatoplate from the oven (the intensity and colour of spots is rapidly affected by atmospheric moisture). Examine under U.V. light of 366 mμ wavelength.

TABLE I

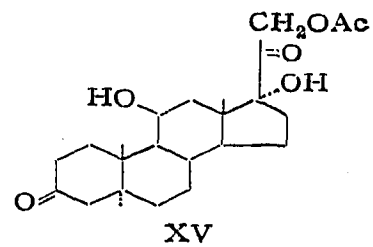
Formula	Basic no. of C atoms	Steroid (trival name)	Characteristic groups				Location reagent		
			3	11	17	21	Komarowsky's	Zinc-chlor.	T.P.T.Z.
XIII	21	21-Desoxydihydrocortisone	CO	CO	OH	CH_3	+	—	—
XIV	27	Hecogenin acetate	OAc	—	—	—	+	+	—
XV	21	Dihydrocortisol acetate	CO	OH	OH	OAc	+	+	+
IV	21	Dihydrocortisone acetate	CO	CO	OH	OAc	+	—	+
XVI	21	Prednisone acetate	CO	CO	OH	OAc	—	+	+
XVII	21	3,17-Desoxydihydrocortisone	—	CO	—	OH	—	—	+
XVIII	21	3,17,21-Desoxydihydrocortisone	—	CO	—	CH_3	—	—	—
XIX	27	11,23-Dibromohecogenin acetate	OAc	Br	—	—	—	+	—



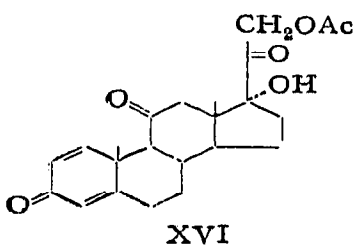
XIII



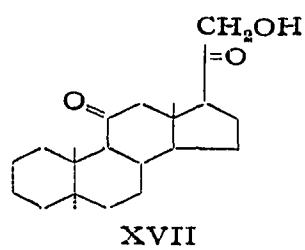
XIV



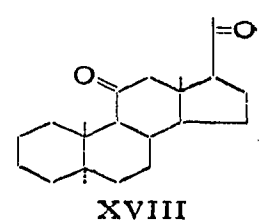
XV



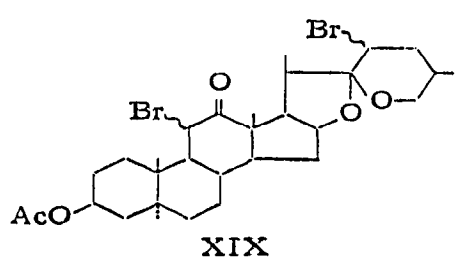
XVI



XVII



XVIII



XIX

Examples of differently reacting steroids

Some examples of the use of the above location reagents are given in Table I.

Note added in proof

The tetrazolium reagent is also effective on silica gel, provided that an 8% (2 *N*) solution of sodium hydroxide is used to ensure alkaline conditions on the adsorbent.

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Reverse phase thin-layer chromatography of 2,4-dinitrophenylhydrazones of *n*-alkanals and *n*-alkan-2-ones*

URBACH[1] of C.S.I.R.O. (Melbourne) has recently published a comprehensive thin-layer chromatographic (TLC) study of the 2,4-dinitrophenylhydrazones (DNPH's) of the aliphatic monocarbonyls. The author briefly reviewed the pertinent literature, and presented methods for both class and homolog separations. Our purpose in this communication is to briefly describe an alternate method for the separation by TLC of an homologous series of the DNPH's of aliphatic aldehydes and ketones. In URBACH's system, chain-length separation is achieved on Kieselguhr G plates impregnated with 2-phenoxyethanol; the impregnated plates are spotted with derivatives, and developed several times with 4% diethyl ether in light petroleum. Resolution of the DNPH's of C_1–C_{14} *n*-alkanals and C_3–C_{13} *n*-alkan-2-ones was achieved; the higher members of the series moved the fastest.

In our method we have adapted the KLEIN AND DE JONG[2] paper chromatographic procedure for TLC. Glass plates 5 × 20 cm were coated with a 250 μ layer of silica gel G using the Brinkmann apparatus. After allowing about 15 min for the adsorbent to set, the plates were placed in an oven and heated at 110° for at least 1 h. The plates were then cooled to room temperature and very slowly immersed in petroleum ether (boiling range 30–60°) containing 10% (v/v) of Shell Ondina 27 mineral oil. After impregnation, the petroleum ether was allowed to evaporate at room temperature, and the plates were then spotted. The plates were given a single development with

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