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# FURTHER DATA ON A HISTOCHEMICAL REACTION AS APPLIED TO THE THIN-LAYER CHROMATOGRAPHY OF STEROLS

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## SUMMARY

A method for the characterization of sterols on silica gel G layers is proposed. After the chromatograms have been sprayed with a permanganate–sulphuric acid oxidative reagent and the reaction has been terminated with sodium hydrogen sulphite, the plates are sprayed with the colour-developing reagent (an acid solution of alcian blue or toluidine blue). The plates are also viewed under UV radiation (254 and 366 nm). Only 3 of the 28 sterol samples assayed (spot content 8 $\mu$g) did not show positive reactions after oxidation, which suggests that this step can be used as a "universal" detection method for sterols. After staining the plate, several sterols are shown to be easily differentiated from one another. The exposure of the plates to UV radiation assists characterization. In general, the reaction exhibits satisfactory sensitivity for the qualitative and differentiating detection of sterols; it is also rapid and easy to carry out.

## INTRODUCTION

In previous work[1], we have shown that the histochemical reaction of Hadler and co-workers[2,3] can be applied to the thin-layer chromatography (TLC) of estrane, androstane and pregnane steroids. Sterol oxidation by permanganate–sulphuric acid or peroxyacetic acid solutions would lead to the formation of keto-acid groups[4], which would be deeply stained by either toluidine blue or alcian blue reagents. Spot tests demonstrated that the reaction was specific for $\Delta^5$-3-ol steroids when oxidation was carried out with peroxyacetic acid, and that the pH of the oxidizing agent was important for differentiation between the sterols[2].

In this work, cholesterol, its natural and synthetic esters and sterols with several structural modifications have been assayed by using the modified Hadler reaction. Two effective *in vivo* blocking agents of desmosterol-$\Delta^{24}$-reductase[5,6] were also assayed.

TABLE I

SENSITIVITIES OF STEROLS TO THE HADLER HISTOCHEMICAL REACTION AS APPLIED TO THIN-LAYER CHROMATOGRAMS

Strength of staining: —, none; ~, weak; +, moderate; ++, strong; +++, very strong; •, "water-mark". Abbreviations: g = green; p = pale; r = red; y = yellow.

| *Sterol* | *Oxidation* | | | | *Alcian blue* *Immediately* | | | |
|---|---|---|---|---|---|---|---|---|
| | *1 µg* | *2 µg* | *4 µg* | *8 µg* | *1 µg* | *2 µg* | *4 µg* | *8 µg* |
| *Group A* | | | | | | | | |
| 5-Cholesten-3β-yl stearate (cholesterol stearate) | + | + | ++ | +++ | — | — | — | — |
| 5-Cholesten-3β-yl oleate (cholesterol oleate) | ~ | ~ | + | ++ | — | — | — | — |
| 5-Cholesten-3β-yl palmitate (cholesterol palmitate) | + | ++ | ++ | +++ | — | — | — | — |
| 5-Cholesten-3β-yl myristate (cholesterol myristate) | + | ++ | ++ | +++ | — | — | — | — |
| 5-Cholesten-3β-yl benzoate (cholesterol benzoate) | ~ | + | + | ++ | — | — | — | — |
| 5-Cholesten-3β-yl propionate (cholesterol propionate) | ~ | + | + | + | — | — | — | — |
| 3α,7α,12α-Trihydroxy-5β-cholan-24-oic acid (cholic acid) | • | • | • | • | — | — | — | — |
| 3α,12α-Dihydroxy-5β-cholan-24-oic acid (deoxycholic acid) | • | • | • | • | — | — | — | — |
| 3,7,12-Trioxo-5β-cholan-24-oic acid (dehydrocholic acid) | • | • | • | • | — | — | — | — |
| *Group B* | | | | | | | | |
| 5-Cholesten-3β-ol (cholesterol) | + | ++ | +++ | +++ | — | — | — | — |
| 5-Cholesten-3β,7α-diol (7α-hydroxycholesterol) | + | + | ++ | ++ | — | — | — | — |
| 5-Cholesten-3β,7β-diol (7β-hydroxycholesterol) | + | + | ++ | ++ | — | — | — | — |
| 3β-Hydroxy-5-cholesten-7-one (7-ketocholesterol) | ~ | + | + | + | + | + | + | + |
| 5,7-Cholestadien-3β-ol (7-dehydrocholesterol) | ~ | + | + | + | — | + | + | + |
| *Group C* | | | | | | | | |
| 1,4-Cholestadien-3-one | ++ | ++ | ++ | ++ | + | + | + | + |
| 4,6-Cholestadien-3-one | ++ | ++ | ++ | ++ | — | — | ~ | ~ |
| 3,5-Cholestadien-7-one | + | + | ++ | +++ | — | — | — | — |
| 5-Cholesten-3-one | ++ | ++ | ++ | +++ | + | + | + | + |
| 4-Cholesten-3-one (cholestenone) | + | + | ++ | +++ | — | + | + | + |
| 5α-Cholestan-3-one (cholestanone) | — | — | — | — | — | — | — | — |
| 5α-Cholestan-3β-ol (cholestanol) | — | — | — | — | — | — | — | — |
| 5α,6α-Epoxy-5α-cholestan-3β-ol (epoxycholesterol) | — | — | — | — | — | — | — | — |
| 5α-Cholestane-3β,5α,6β-triol (cholestanetriol) | — | ~ | + | + | — | — | — | — |
| *Group D* | | | | | | | | |
| 5,24-Cholestadien-3β-ol (desmosterol) | + | ++ | +++ | +++ | — | — | — | — |
| 24β-Methyl-5,7,22-ergostatrien-3β-ol (ergosterol) | + | ++ | ++ | ++ | ~ | + | + | + |
| 24β-Ethyl-5-cholesten-3β-ol (β-sitosterol) | — | + | + | + | — | — | + | + |
| 20α-Amino-2-dimethylaminoethyl-5α-pregnen-3β-ol (SC-11952) | + | + | ++ | +++ | — | — | + | + |
| N-Methyl-N-(3-dimethylamino-propyl)-17β-amino-5-androsten-3β-ol (SC-12937) | + | + | + | + | — | — | + | + |

| | | | | Toluidine blue | | | | | | | | Ultraviolet radiation | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| After heating | | | | Immediately | | | | After heating | | | | Alcian blue | | Toluidine blue | |
| 1 µg | 2 µg | 4 µg | 8 µg | 1 µg | 2 µg | 4 µg | 8 µg | 1 µg | 2 µg | 4 µg | 8 µg | 254 nm | 366 nm | 254 nm | 366 nm |
| ~ | + | + | + + | – | – | – | – | ~ | ~ | + | + + | y | y | y | y |
| – | ~ | + | + + | – | – | – | – | – | ~ | + | + + | r | r | p | p |
| ~ | ~ | + | + + | – | – | – | – | ~ | + | + + | + + | y | y | y | y |
| ~ | + | + + | + + + | – | – | – | – | ~ | + | + | + + | y | y | y | y |
| ~ | ~ | + | + | – | – | – | – | – | – | + | + | p | p | r | r |
| + | + | + + | + + + | – | – | – | – | ~ | + | + + | + + + | r | r | r | r |
| – | – | ~ | ~ | – | – | – | – | ~ | ~ | ~ | + | g | g | – | b |
| – | – | ~ | ~ | – | – | – | – | – | – | ~ | ~ | g | g | – | b |
| – | – | ~ | ~ | – | – | – | – | – | – | ~ | ~ | b | b | – | ~ |
| ~ | + | + + + | + + + | – | – | – | – | + | + + | + + | + + + | r | r | r(p) | r(p) |
| ~ | + | + | + + | – | – | – | – | ~ | ~ | + + | + + | r | r | r | r |
| ~ | + | + | + + | – | – | – | – | ~ | + | + + | + + | r | r | ~ | ~ |
| ~ | ~ | ~ | + | – | – | – | – | – | – | – | ~ | p | p | – | – |
| ~ | ~ | ~ | ~ | – | – | – | – | – | – | ~ | ~ | p | p | – | – |
| ~ | + | + | + + | – | – | – | – | ~ | ~ | + | + | – | r | – | – |
| ~ | + | + + | + + | – | – | – | – | ~ | ~ | ~ | + | p | p | y | y |
| ~ | + | + | + + | – | – | – | – | ~ | ~ | + | + + | y | y | – | r |
| – | ~ | ~ | + | – | – | – | – | – | ~ | ~ | + | b | b | – | – |
| ~ | + | + | + | – | – | – | – | ~ | + | + | + | p | p | p | p |
| – | ~ | ~ | + | – | – | – | – | ~ | + | + | – | y | y | y | y |
| ~ | ~ | + | + + | – | – | – | – | ~ | + | + | + | r | r | p | p |
| + | + + | + + | + + | – | – | – | – | + | + | + | + + | y | y | – | y |
| – | + | + + | + + | – | – | – | – | – | + | + | + + | r | r | r | r |
| ~ | + | + | + + + | – | – | – | – | ~ | ~ | + – | + + + | r | r | r | r |
| ~ | + | + | + | – | – | – | – | – | – | + | – | r | r | r | r |
| ~ | + | + | + + | – | – | – | – | ~ | ~ | + | + | r | r | – | r |
| ~ | ~ | ~ | + | – | – | – | – | ~ | ~ | – | + + | y | y | r | p |
| ~ | ~ | + | + + | – | – | – | – | ~ | ~ | + | + | y | y | r | p |

## METHOD

One-dimensional TLC on silica gel G (Merck, Darmstadt, G.F.R.) was carried out as described by Stahl[7]. Samples of 26 sterols (Sigma, St. Louis, Mo., U.S.A., and Steraloids, Pawling, N.Y., U.S.A.) and of two synthetic nitrogen-containing sterols, inhibitors of cholesterol biosynthesis (G.D. Searle, Chicago, Ill., U.S.A.), dissolved in chloroform–methanol (1:1, v/v) were applied to 20 × 20 cm plates (1, 2, 4 and 8 $\mu$g for each spot), and the chromatograms were developed with chloroform.

We have examined four groups of sterols, as follows (see also Table I):

Group A: natural and synthetic esters of cholesterol, and bile acids;

Group B: cholesterol and sterols with modifications in the B ring;

Group C: sterols with modifications in the A or A and B rings, or at the decalin junction;

Group D: sterols with modifications in the side chain.

After each run, the Hadler reaction was applied as previously reported[1]. Briefly, the process includes (1) spraying with permanganate–sulphuric acid solution, (2) heating and recording the results, (3) interrupting the oxidation reaction by spraying with sodium hydrogen sulphite solution, (4) spraying with colour-developing reagent (an acid solution of toluidine blue or alcian blue) with immediate recording of the results, (5) heating until the best colour is attained, followed by recording the results, and (6) exposing the plates to UV radiation in order to detect fluorescence.

## RESULTS AND DISCUSSION

Table I shows the results obtained after treatment of the chromatograms as indicated above. The natural esters of cholesterol (group A) showed better sensitivity in the oxidative reaction than did the synthetic ones (propionate and benzoate), whereas all the bile acids tested exhibited only "water-marks" (translucent areas). Of the sterols in group B, the most sensitive to the oxidizing reagent was cholesterol, as had been noted by Hadler *et al.*[4]. Those sterols in group C with a *trans*-junction between rings A and B (cholestanol, cholestanone and epoxycholesterol) were unreactive to the oxidant, despite the hydroxyl or oxo function at C-3. All the sterols in group D gave positive reactions with the oxidizing agent. The sensitivity was better with desmosterol ($\Delta^{5,24}$) and ergosterol ($\Delta^{5,7,22}$-24$\beta$-methyl), indicating that an increase in the number of double bonds could be responsible for the increased reactivity. Surprisingly, SC-11952, with a 5$\alpha$ structure, was more reactive than SC-12937, with a $\Delta^5$ structure. On the whole, however, the results obtained in TLC with oxidative reaction at low pH agreed with those of Hadler *et al.*[3] and confirmed that the absence or blockage of double bonds inhibits the oxidative reaction (see, for example, bile acids and those sterols of group C with a 5$\alpha$ structure in Table I). Further, several of the unsaturated sterols have different sensitivities.

Staining with alcian blue at room temperature did not reveal cholesterol esters and bile acids (which supports the spot tests of Hadler *et al.*[3]), whereas, in group B, only 7-keto- and 7-dehydrocholesterol gave positive responses. Positive results obtained with $\Delta^4$, $\Delta^5$ or $\Delta^{1,4}$ unsaturated 3-oxo-steroids in group C, and with most of the group D sterols (but not desmosterol), made it difficult to establish any relationship between chemical structure and the affinity for the stain. Over-all consideration

of the reaction with alcian blue at room temperature makes it possible to differentiate sterols of great physiological significance (*e.g.*, cholesterol, 7-hydroxycholesterol, desmosterol and cholestanol) from 7-keto- and 7-dehydrocholesterol. After heating, no clear-cut relationship was observed between sensitivity to oxidation and affinity for the stain (see Table I). The reaction was less sensitive for the bile acids, as might be expected, since some substances in this category (*e.g.*, cholic and dehydrocholic acids) are resistant to several colour reagents[8]. However, in TLC, the results seem to offer better sensitivity than those obtained by Hadler *et al.*[3], despite the relatively long period of heating. It is noteworthy that 7-ketocholesterol, a sterol difficult to render visible, is detectable both by the oxidative reaction and by staining with alcian blue. The alcian blue reaction at room temperature permits differentiation of 7-keto-cholesterol from either 7-hydroxycholesterol or 3,5-cholestadien-7-one, without the need for prior dehydration or reduction[9]. This reaction offers interesting possibilities for differentiation between cholestanone, epoxycholesterol and cholestanol and other sterols. Under UV radiation (366 nm), chromatograms stained with alcian blue permitted excellent visual distinction of cholesterol oleate and propionate (red) from cholesterol benzoate (pale) and other esters (yellow). With regard to the bile acids, substitution in positions C-3 and C-12 seems to affect their fluorescence reactions, since dehydrocholic acid (bluish) can be distinguished from cholic and deoxycholic acids (greenish). Among the group B sterols, it was possible to differentiate 7-keto- and 7-dehydrocholesterol from the others; this is advantageous in chromatographic studies of the autoxidation products of cholesterol. Further, the 7$\alpha$-epimer was detected with better sensitivity than 7$\beta$-hydroxycholesterol. The results in Table I for group C steroids show that, under UV radiation, it is difficult to establish a relationship between the chemical structure and the fluorescence developed. Small differences in structure seem to be critical (see, for example, 5-cholesten-3-one and cholestenone). It is difficult to explain exactly what makes compounds as different as, say, cholestanone and 3,5-cholestadien-7-one show the same UV reaction (yellow); similar remarks apply to desmosterol and ergosterol (red) in group D.

No positive reaction was observed before heating when the chromatograms were sprayed with toluidine blue; even after heating, the sensitivity did not differ greatly from that attained with alcian blue. However, in group B, it was possible to differentiate 7-keto- and 7-dehydrocholesterol from cholesterol and the epimeric 7$\alpha$- and 7$\beta$-hydroxycholesterols. Under UV radiation (254 nm), some of the sterols did not show the sensitivity exhibited when alcian blue was used. In group A at 366 nm, however, cholesterol oleate (pale) could be distinguished from cholesterol benzoate and propionate (reddish), and these last-named two esters from all the others (yellowish). Dehydrocholic acid, which did not fluoresce, was easily differentiated from cholic and deoxycholic acids, both of which showed bluish reactions. In group B, sterols with a 7-keto or $\Delta^{5,7}$ grouping did not fluoresce, and so could be differentiated from cholesterol and 7$\alpha$-hydroxycholesterol, which gave red responses. 7$\beta$-Hydroxycholesterol was difficult to visualize. Among the sterols of group C, there was a wide variety of reactions, but the lack of fluorescence of 1,4-cholestadien-3-one and 5-cholesten-3-one at either 254 or 366 nm might be of value in distinguishing these from the other sterols in this group. Visualization of 3,5-cholestadien-7-one was difficult.

It can be seen that a number of the fluorescence reactions on the alcian blue-stained plates paralleled those on the toluidine blue-stained plates. In radiation of

TABLE II

COLOUR REACTIONS OF 28 STEROLS ON THIN-LAYER CHROMATOGRAMS AFTER TREATMENT WITH ALCIAN BLUE OR TOLUIDINE BLUE REAGENT

Amount of sterol = 8 μg. Whenever two colours are reported, they occurred at different heating times; the second colour is the final one. Figures indicate the time required for the final colour development. Abbreviations: b. = brown; bl. = blue; br. = brilliant; ca. = cadmium; ch. = chrome; co. = cobalt; d. = deep; ea. = earth; g. = green; gh. = greenish; go. = gold; In. = Indian; l. = light; la. = lake; le. = lemon; oc. = ochre; or. = orange; p. = pale; Poz. = Pozzuoli; r. = red; sc. = scarlet; ter. = terra-cotta; ul. = ultramarine; umb. = umber; ver. = vermilion; vet. = verte; vo. = violet; y. = yellow; z. = zinc.

| *Sterol* | *Alcian blue* | | *Toluidine blue* | |
|---|---|---|---|---|
| | *Colour* | *Time (min)* | *Colour* | *Time (min)* |
| *Group A* | | | | |
| Cholesterol stearate | ter. vet.–b. oc. | 7 | ca. y. or. | 13 |
| Cholesterol oleate | d. oc. | 5 | ca. r. sc. | 14 |
| Cholesterol palmitate | umb. gh.–y. oc. | 11 | ver. r. p. | 13 |
| Cholesterol myristate | umb. gh.–br. y. d. | 7 | ver. r. p. | 15 |
| Cholesterol benzoate | umb. gh. | 10 | ver. r. p. | 15 |
| Cholesterol propionate | umb. gh. | 3 | l. r. | 7 |
| Cholic acid | In. y.–z. y. | 25 | y. oc. p.–In. y. | 30 |
| Deoxycholic acid | In. y.–y. oc. | 15 | ca. y. oc.–ca. r. d. | 10 |
| Dehydrocholic acid | ch. y. p.–ch. y. le. | 30 | ch. y. d.–ca. y. d. | 30 |
| *Group B* | | | | |
| Cholesterol | y. oc.–b. oc. | 5 | Poz. ea. | 12 |
| 7α-Hydroxycholesterol | ul. d.–go. oc. | 5 | ul. d.–Poz. ea. | 7 |
| 7β-Hydroxycholesterol | ul. d.–go. oc. | 6 | ul. d.–Poz. ea. | 7 |
| 7-Ketocholesterol | br. y. d.–ca. r. sc. | 13 | Poz. ea. | 18 |
| 7-Dehydrocholesterol | br. y. d.–ca. r. d. | 13 | — | — |
| *Group C* | | | | |
| 1,4-Cholestadien-3-one | Poz. ea. | 20 | ver. r. p. | 30 |
| 4,6-Cholestadien-3-one | co. bl. l. | 15 | Poz. ea. | 7 |
| 3,5-Cholestadien-7-one | In. y. | 7 | ch. y. or. | 17 |
| 5-Cholesten-3-one | Poz. ea. | 30 | ca. y. or. | 20 |
| Cholestenone | ca. g. p. | 8 | ch. g. l.–Poz. ea. | 10 |
| Cholestanone | go. oc. | 30 | ch. y. d.–ca. r. sc. | 15 |
| Cholestanol | In. y.–b. oc. | 10 | ca. y. or.–ca. r. sc. | 20 |
| Epoxycholesterol | Poz. ea. | 6 | br. y. d.–In. y. | 7 |
| Cholestanetriol | y. oc. | 7 | br. y. d.–ch. y. or. | 5 |
| *Group D* | | | | |
| Desmosterol | In. y.–b. oc. | 6 | ver. r. d.–Poz. ea. | 5 |
| Ergosterol | umb. gh. | 10 | ver. r. p.–sc. la. | 15 |
| β-Sitosterol | go. oc. | 10 | ver. r. d.–ca. r. d. | 10 |
| SC-11952 | Poz. ea.–y. oc. | 12 | br. y. d. | 30 |
| SC-12937 | co. vo. d.–b. oc. | 8 | ver. r. d. | 30 |

366 nm, all the group D sterols except those containing nitrogen showed red spots. However, it should be noted that SC-11952 and SC-12937 viewed under 254-nm radiationappeared as reddish spots, but were pale in 366-nm radiation.

The results presented in Table II show that different colours can develop with

only slight structural differences. Further, the several colours reported can vary with temperature, time of heating, and the purity of the chemicals used during the chromatographic procedure. Although it was observed that the colours were reasonably stable (up to 24 h for most of the sterols), they could vary with time. On the other hand, spraying the chromatograms with a freshly prepared 5% solution of nitric acid in methanol helped to clarify the background, although it sometimes caused discoloration of the spots.

Our results seem to justify the conclusion that the Hadler histochemical reaction as adapted for use with TLC is a relatively simple and rapid technique, exhibiting satisfactory sensitivity for the qualitative and differentiating detection of sterols. We found that most of the negative results obtained in spot tests on paper became positive in TLC. Such improved sensitivity can be ascribed, as well as to the effect of heating, to the concentration of the samples on the surface of the fine granules of an inert adsorbent such as silica gel.

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REFERENCES

1 L. B. S. Valle and R. M. Oliveira-Filho, *J. Chromatogr.*, 105 (1975) 201.
2 W. A. Hadler, L. M. Ziti, O. de Luca, A. S. Patelli and J. A. Vozza, *Ciênc. Cult.*, 17 (1965) 245.
3 W. A. Hadler, L. M. Ziti, O. de Luca and A. S. Patelli, *Acta Histochem.*, 30 (1968) 70.
4 W. A. Hadler, L. M. Ziti, O. de Luca, A. S. Patelli and J. A. Vozza, *Ciênc. Cult.*, 17 (1965) 246.
5 L. B. S. Valle, S. Oga, R. M. Oliveira-Filho and W. J. Yasaka, *Proc. IV Latinoamerican Congr. Pharmacol. Therap.*, Caracas, Venezuela, July 1972, p. 47.
6 N. Tortamano, L. B. S. Valle, R. M. Oliveira-Filho, P. L. Armonia, S. Oga and W. J. Yasaka, *Ciênc. Cult.*, 27 (1974) 502.
7 E. Stahl, in E. Stahl (Editor), *Thin-layer Chromatography*, Springer, New York, 1965, p. 5.
8 D. Waldi, in E. Stahl (Editor), *Thin-layer Chromatography*, Springer, New York, 1965, p. 483.
9 L. L. Smith and J. C. Price, *J. Chromatogr.*, 26 (1967) 509.