*Journal of Chromatography*, 105 (1975) 201–205


CHROM. 7999

## Note

# Application of a histochemical reaction to the thin-layer chromatography of steroids

LUIZ BIELLA SOUZA VALLE and RICARDO MARTINS OLIVEIRA-FILHO

*Department of Physiology and Pharmacology, Institute of Biomedical Sciences, University of São Paulo, São Paulo (Brazil)*

(Received September 6th, 1974)

The characterization of steroids by thin-layer chromatography (TLC) is achieved by means of analytical procedures such as the differential migration of the substances, the formation of derivatives and colour reactions. Several histochemical reactions[1–3] have been applied to TLC. Hadler and co-workers[4,5] proposed a histochemical reaction for the detection of cholesterol and related substances, based on oxidation of $\Delta^5$-3-OH steroids using permanganate–sulphuric acid or peracetic acid solutions. This reaction is terminated by sodium hydrogen sulphite. The colours that develop after aldehyde-fuchsin, toluidine blue or alcian blue treatment might be due to the formation of keto-acids; furthermore, a C-5 double bond and a hydroxyl group at the C-3 position seems to be essential for the reaction to occur. The sensitivity of this reaction was even greater than that of the Schultz test[6] and the specificity was similar to that of the Liebermann–Burchard reaction[7–9]. Spot tests for some steroids gave negative results with the Hadler reaction.

In this work, we describe some modifications of the Hadler reaction for the TLC of estrane, androstane and pregnane steroids.

## METHOD

One-dimensional TLC on silica gel G (Merck, Darmstadt, G.F.R.) was carried out essentially as described by Stahl[10], using chloroform as the mobile phase. Samples of 19 standard solutions of steroids (Sigma, St.-Louis, Mo., U.S.A.) (see Table I) were applied on to 20 × 20 cm plates (1, 2, 4 and 8 $\mu$g for each spot). After each run, the Hadler reaction was employed as follows:

(1) permanganate–sulphuric acid oxidative solution spray (0.125 *N* $H_2SO_4$ + 0.038 *N* $KMnO_4$, 1:1, pH 1.30);

(2) heating in a drying cabinet at 110° for 2 min, followed immediately by recording the results;

(3) sodium hydrogen sulphite solution spray (5% aqueous solution) and re-heating until the plates were completely dry and discoloured;

(4) colour-developing spray (toluidine blue or alcian blue, 0.5% solution in 1 *N* $H_2SO_4$, pH 0.50–0.60) followed immediately by recording the results;

(5) heating in a drying cabinet at 110° until the best colour development was

achieved (in most instances not more than 20 min were needed; see also Table II), followed by recording of the results;

(6) exposure of the plates to ultra violet (UV) light (254 and 366 nm) in order to detect some fluorescence activity.

## RESULTS AND DISCUSSION

Table I shows the results obtained after the treatment of the plates as described above. All of natural $C_{18}$ steroids gave good positive reactions to oxidation (steps 1 and 2), although estrone and estradiol were less sensitive than estriol. In the androstane series, androsterone and its epimer epiandrosterone gave no detectable reaction. It therefore appears that the $\Delta^4$-3-one structure gave a greater sensitivity to the oxidative reaction (see testosterone and androstenedione), whereas the absence of a double bond inhibited it independent of the presence of an $\alpha$-OH group (androsterone) or $\beta$-OH group (epiandrosterone) in the C-3 position. In this series, a double bond in the C-5 position (androstenediol and dehydroepiandrosterone sulphate) did not prevent a positive reaction from occurring, with either a hydroxyl group or even with a sulphate group in the C-3 position.

TABLE I

SENSITIVITY OF STEROIDS OF THE ESTRANE, ANDROSTANE AND PREGNANE SERIES TO THE HADLER HISTOCHEMICAL REACTION AS APPLIED TO THIN-LAYER CHROMATOGRAMS

Strength of staining: −, none; ~, weak; +, moderate; ++, strong; +++, very strong. Abbreviations: r = reddish; r(p) = reddish (pale); p = pale; g = greenish; y = yellowish.

| *Steroid* | *Oxidation* | | | | *Alcian blue* | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | *Immediately* | | | | *After heating* | | | |
| | *1 µg* | *2 µg* | *4 µg* | *8 µg* | *1 µg* | *2 µg* | *4 µg* | *8 µg* | *1 µg* | *2 µg* | *4 µg* | *8 µg* |
| Estradiol | + | + | ++ | +++ | − | ~ | + | ++ | + | ++ | ++ | +++ |
| Estrone | + | ++ | ++ | +++ | ~ | ~ | ~ | ++ | ~ | ~ | ++ | +++ |
| Estriol | ++ | +++ | +++ | +++ | ~ | + | + | + | ~ | ++ | ++ | +++ |
| Androstenedione | + | ++ | +++ | +++ | − | − | ~ | ~ | − | − | ~ | + |
| Testosterone | ++ | ++ | +++ | +++ | − | − | − | − | + | + | ++ | +++ |
| Androstenediol | − | ~ | ~ | + | − | − | − | − | + | + | ++ | +++ |
| Androsterone | − | − | − | − | − | − | − | − | − | − | ~ | + |
| Epiandrosterone | − | − | − | − | − | − | − | ~ | − | − | + | ++ |
| Dehydroepiandrosterone sulphate | ~ | ~ | + | + | − | − | − | − | ~ | + | + | + |
| Progesterone | ++ | ++ | +++ | +++ | − | − | − | ~ | − | − | − | − |
| 17$\alpha$-Hydroxyprogesterone | ++ | ++ | +++ | +++ | − | − | − | − | ~ | ~ | ~ | + |
| Cortisone | ++ | ++ | +++ | +++ | − | − | − | − | − | − | − | − |
| Corticosterone | ++ | ++ | +++ | +++ | − | − | + | + | − | ~ | + | ++ |
| Desoxycorticosterone | ++ | ++ | ++ | +++ | − | − | − | − | − | − | + | + |
| Pregnenolone | ~ | ~ | ~ | + | − | − | − | − | − | − | ~ | + |
| Pregnenolone acetate | − | ~ | + | + | − | − | − | − | − | − | + | + |
| 5$\beta$-Pregnan-3$\alpha$,17$\alpha$-diol-11,20-dione | − | − | − | − | − | − | − | − | − | ~ | + | ++ |
| 5$\beta$-Pregnan-3$\alpha$,17$\alpha$-diol-20-one | − | − | − | − | − | − | − | − | − | − | − | − |
| 5$\alpha$-Pregnan-3$\beta$,20$\beta$-diol | − | − | − | − | − | − | − | − | ~ | ~ | + | ++ |

Regarding the $C_{21}$ steroids, the $\Delta^4$-3-one structure (progesterone, 17$\alpha$-hydroxyprogesterone, cortisone, corticosterone and desoxycorticosterone) was the most active towards the oxidizing agent; the same occurred with $C_{19}$ steroids with a $\Delta^4$-3-one structure (androstenedione and testosterone). The $\Delta^4$-3-one structure (progesterone) was more reactive than the $\Delta^5$-3$\beta$-ol (pregnenolone) or another $\beta$-derivative (pregnenolone acetate). In a similar manner to the results with cholestanol[11], the absence of a double bond led to unreactivity, even though a hydroxyl group was present in the position C-3 (see Table I, pregnanes: 5$\beta$-pregnan-3$\alpha$,17$\alpha$-diol-11,20-dione; 5$\beta$-pregnan-3$\alpha$,17$\alpha$-diol-20-one; 5$\alpha$-pregnan-3$\beta$,20$\beta$-diol).

Before heating (step 4), the alcian blue reaction permitted estrogens to be distinguished from the other $C_{19}$ and $C_{21}$ steroids. Of the latter, only corticosterone showed some sensitivity, beginning at 4 $\mu$g. After heating (step 5), all of the estrogens gave only slightly different positive reactions, thus differing from the androgen and pregnane groups. The following sequence of decreasing sensitivity was found: testosterone or androstenediol > dehydroepiandrosterone sulphate > epiandrosterone > androsterone or androstenedione. For the $C_{21}$ steroids, the reaction was in general positive, beginning at a 4-$\mu$g spot concentration, but progesterone, cortisone and 5$\beta$-pregnan-3$\alpha$,17$\alpha$-diol-20-one did not react.

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

Before heating (step 4), toluidine blue gave no positive reaction with any of the steroids. After heating (step 5), most of them gave positive reactions for 4-$\mu$g spots. 5$\beta$-Pregnan-3$\alpha$,17$\alpha$-diol-11,20-dione was the more sensitive steroid under such conditions.

No direct relationship was observed between the sensitivity to the oxidizing reaction and the staining affinity, except for steroids with aromatic nuclei when alcian blue was used.

When exposed to UV light at 366 nm (step 6), alcian blue permitted progesterone and cortisone, which did not exhibit fluorescence, to be distinguished from dehydroepiandrosterone sulphate, androstenedione, androstenediol and desoxycorticosterone, which showed a pale fluorescence. Furthermore, all of the steroids showed reddish spots, except for corticosterone and 17$\alpha$-hydroxyprogesterone, which gave yellowish and greenish fluorescence, respectively. Estriol was revealed only poorly.

Under 366-nm UV light, toluidine blue showed reddish spots for estrogen,

TABLE II

COLOUR REACTIONS OF 19 STEROIDS ON THIN-LAYER CHROMATOGRAMS AFTER TREATMENT WITH ALCIAN BLUE OR TOLUIDINE BLUE REAGENT

Amount of steroid = 8 $\mu$g. Whenever two colours are reported, they occurred at different heating times; the second colour is the definitive one. Figures indicate the time required for the definitive colour development.

Abbreviations: b. = brown; bit. = bitumen; bl. = blue; br. = brilliant; bu. = burnt; ca. = cadmium; car. = carmine; ch. = chrome; cin. = cinnabar; co. = cobalt; d. = deep; Eng. = English; g. = green; gh. = greenish; In. = Indian; l. = light; la. = lake; oc. = ochre; or. = orange; p. = pale; Pr. = Prussian; r. = red; sc. = scarlet; Sien. = sienna; ter. = terra; umb. = umber; ver. = vermillion; vet. = verte; vi. = violet; y. = yellow.

| *Steroid* | *Alcian blue* | | *Toluidine blue* | |
|---|---|---|---|---|
| | *Colour* | *Time (min)* | *Colour* | *Time (min)* |
| Estradiol* | y.oc.d.-Eng.r.l. | 7 | ca.y.or.-ca.r.sc. | 13 |
| Estrone* | b.-b.oc. | 8 | ver.r.d. | 30 |
| Estriol* | umb.gh. | 30 | car. | 30 |
| Androstenedione* | ca.r.sc.-ch.g.d. | 10 | ca.y.or.-ca.r.sc. | 20 |
| Testosterone | Pr.bl. | 9 | co.vi.d. | 10 |
| Androstenediol | car.d.-bit. | 7 | Eng.r.l. | 30 |
| Androsterone | Eng.r.d.-b. | 20 | ca.y.or.-sc.la. | 17 |
| Epiandrosterone* | y.oc.d.-Eng.r.l. | 20 | ver.r.p. | 25 |
| Dehydroepiandrosterone sulphate | co.g.l.-b. | 15 | Ind.y.-br.y.d. | 10 |
| Progesterone* | — | — | Ind.y. | 30 |
| 17$\alpha$-Hydroxyprogesterone | y.oc.d. | 8 | ver.r.p. | 5 |
| Cortisone | — | — | Ind.y. | 30 |
| Corticosterone* | ch.g.l.-cin.g.l. | 10 | ca.y.or.-Eng.r.l. | 12 |
| Desoxycorticosterone | ter.vet.-b. | 12 | ca.y.or.-bu.Sien. | 13 |
| Pregnenolone | b.oc. | 7 | Eng.r.l.-ca.r.sc. | 10 |
| Pregnenolone acetate | b.-b.oc. | 5 | br.y.d.-Eng.r.l. | 10 |
| 5$\beta$-Pregnan-3$\alpha$,17$\alpha$-diol-11,20-dione | ch.g.d.-y.oc.d. | 15 | ca.y.or.-Eng.r.l. | 13 |
| 5$\beta$-Pregnan-3$\alpha$,17$\alpha$-diol-20-one | y.oc.d. | 7 | ca.r.p.-ca.y.or. | 5 |
| 5$\alpha$-Pregnan-3$\beta$,20$\beta$-diol | y.oc.d. | 5 | br.y.d.-ca.r.sc. | 5 |

* Showed "cobalt blue light" reaction immediately after alcian blue spray.

androgen and in most instances for the pregnane steroid series. However, progesterone, 17$\alpha$-hydroxyprogesterone, cortisone and corticosterone exhibited pale fluorescence reactions. Cortisone and progesterone were revealed only poorly. Thus it can be postulated that progesterone, 17$\alpha$-hydroxyprogesterone, cortisone and corticosterone could be easily distinguished from all of the similar steroids when observed under 366-nm UV light, no matter which stain (alcian blue or toluidine blue) is used.

As the variety of colours obtained after using the staining reagents (step 6) was very wide, it becomes very difficult to establish any basic staining pattern for a given structure (see Table II). Furthermore, a small structural difference (progesterone and 17$\alpha$-hydroxyprogesterone) can induce changes in UV sensitivity (Table I).

Although a reasonable colour stability was observed, it can vary in accordance to the heating time, exposure to air and sample concentration in the spot. The oxidative reaction itself can be employed successfully as a "differential detection method" for steroids. It is often sensitive below 1 $\mu$g per spot, and is rapid and easy to carry out in the laboratory.

## REFERENCES

1 I. Grundland, H. Bulliard and M. Maillet, *C.R. Soc. Biol.*, 143 (1949) 771.
2 K. Okamoto, M. Ueda and A. Kato, *Jap. J. Const. Med.*, 13 (1944) 102.
3 L. Lison, *Histochimie et Cytochimie Animales*, Gauthier-Villars, Paris, 1960, pp. 504–506 and 752.
4 W. A. Hadler, L. M. Ziti, O. de Luca, A. S. Patelli and J. A. Vozza, *Ciênc. Cult.*, 17 (1965) 245.
5 W. A. Hadler, L. M. Ziti, O. de Luca and A. S. Patelli, *Acta Histochem.*, 30 (1968) 70.
6 A. Schultz, *Zentralbl. Allg. Path. Path. Anat.*, 35 (1924) 314.
7 C. Liebermann, *Chem. Ber.*, 18 (1885) 1803.
8 H. Burchard, *Chem. Zentralbl.*, 61 (1890) 25.
9 J. S. Matthews, *Biochim. Biophys. Acta*, 69 (1963) 163.
10 E. Stahl, in E. Stahl (Editor), *Thin-Layer Chromatography*, Springer, New York, 1965, pp. 5–29.
11 L. B. S. Valle, R. M. Oliveira-Filho and S. A. Camara, to be published.