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Note

The detection of A-ring-hydroxylated estrogens and their methyl ethers on ascorbic acid-impregnated paper and thin-layer chromatograms

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"Reducing chromatography" on ascorbic acid-impregnated stationary phases has been developed in order to prevent the autoxidative decomposition of labile steroids[1–3]. This procedure has proved to be a valuable means for the quantitative determination of 2-hydroxyestrogens in human urine[4–7] and for the isolation of various catechol[8] and pyrogallolestrogens[9] in the course of incubation studies. However, it was found that these estrogens could not be localized on ascorbic acid-impregnated paper and thin-layer chromatograms by standard staining procedures, such as azo coupling or redox reactions (*e.g.*, Folin–Ciocalteu reagent). Therefore, radioactive reference compounds were generally required in "reducing chromatography".

As A-ring substituted estrogens are of major importance in the metabolism of estrogens in mammals, a colour reaction was developed for the detection of A-ring hydroxylated estrogens and their methyl ethers on ascorbic acid-impregnated paper and thin-layer chromatograms.

MATERIALS AND METHODS

Steroids

The following estrogens derived from estrone, estradiol, estriol and ethynylestradiol with different substitution patterns of the A-ring were prepared as described elsewhere: 2-hydroxyestrogens[10], 2-hydroxyestrogen mono- and dimethyl ethers[11], 4-hydroxyestrogens[10], 4-hydroxyestrogen mono- and dimethyl ethers[10], 2,4-dihydroxyestrogens[10] and 2,4-dihydroxyestrogen mono-, di- and trimethyl ethers[10].

Chromatography

Paper chromatograms (Schleicher & Schüll, Dassel, G.F.R., 2043 b Mg l) were prepared by impregnation with methanolic solutions of ascorbic acid, formamide–ascorbic acid or ethylene glycol–ascorbic acid[1]. Thin-layer plates (Polygram Sil G/UV_{254}, Macherey, Nagel & Co., Duren, G.F.R.) were immersed in a methanolic solution of ascorbic acid and the methanol was allowed to evaporate at room temperature[1]. Chromatograms of the different estrogens were developed in appropriate systems as described by Knuppen[12] and Lisboa[13]. After chromatography, the papers and thin-layer plates were allowed to stand at room temperature for *ca.*

15 min. The papers were then stained by dipping them into and the plates by spraying them with aqueous sodium metaperiodate solutions of different concentrations.

RESULTS

Thin-layer chromatography

The colours observed for different phenolic steroids after spraying the thin-layer plates with an aqueous solution of sodium periodate are listed in Table I. There was a marked difference in the chromogenic reaction of the catechol- (yellow) and pyrogallolestrogen (orange-brown) derivatives. The colours faded within a few minutes when using a 1% solution of sodium periodate, but they were stable for at least 1 h and more intense after staining with a saturated solution. The lower detection limits were about 2.5 μg for the *o*-dihydroxy compounds (1, 4, 8 and 10 in Table I) and about 5 μg for the monophenolic derivatives of the pyrogallol- and catecholestrogens (2, 3, 5, 6 and 11–13 in Table I).

TABLE I

A-RING SUBSTITUTION PATTERN OF THE DIFFERENT ESTROGENS TESTED AND THE COLOURS OBSERVED AFTER STAINING WITH SODIUM PERIODATE SOLUTION

A saturated aqueous solution of sodium periodate was used for staining ascorbic acid-impregnated silica gel thin-layer plates and paper chromatograms of the Zaffaroni type, while this solution was diluted with three parts of water for Bush-type paper chromatograms.

No.	*Substitution pattern*	*Colour reaction*
1	2,3-Dihydroxyestra-1,3,5(10)-trienes	Yellow
2	2-Hydroxy-3-methoxyestra-1,3,5(10)-trienes	Yellow
3	2-Methoxy-3-hydroxyestra-1,3,5(10)-trienes	Yellow
4	3,4-Dihydroxyestra-1,3,5(10)-trienes	Yellow
5	3-Hydroxy-4-methoxyestra-1,3,5(10)-trienes	Yellow
6	3-Methoxy-4-hydroxyestra-1,3,5(10)-trienes	Yellow
7	2,3,4-Trihydroxyestra-1,3,5(10)-trienes	No colour
8	2,3-Dihydroxy-4-methoxyestra-1,3,5(10)-trienes	Orange-brown
9	2,4-Dihydroxy-3-methoxyestra-1,3,5(10)-trienes	No colour
10	2-Methoxy-3,4-dihydroxyestra-1,3,5(10)-trienes	Orange-brown
11	2,3-Dimethoxy-4-hydroxyestra-1,3,5(10)-trienes	Orange-brown
12	2,4-Dimethoxy-3-hydroxyestra-1,3,5(10)-trienes	Orange-brown
13	2-Hydroxy-3,4-dimethoxyestra-1,3,5(10)-trienes	Orange-brown
14	2,3-Dimethoxyestra-1,3,5(10)-trienes	No colour
15	3,4-Dimethoxyestra-1,3,5(10)-trienes	No colour
16	2,3,4-Trimethoxyestra-1,3,5(10)-trienes	No colour
17	3-Hydroxyestra-1,3,5(10)-trienes	No colour

Strikingly, no colour reactions were observed when up to 30 μg of 2,3,4-trihydroxy- or 2,4-dihydroxy-3-methoxyestra-1,3,5(10)-trienes (7 and 9 in Table I) were treated on the chromatograms with sodium periodate solutions. According to the specificity of the *o*-quinone formation[14–16], neither the 3-hydroxyestra-1,3,5(10)-trienes (17 in Table I) nor the permethylated catechol- and pyrogallolestrogen derivatives (14–16 in Table I) were stained by treatment with sodium periodate solutions.

Paper chromatography

Similar observations were made after chromatography on papers impregnated with formamide–ascorbic acid or ethylene glycol–ascorbic acid (Zaffaroni type). Dipping the chromatograms into a saturated aqueous solution of sodium periodate led to intense colours that were visible for *ca.* 5 min, while the spots observed when a 1% solution of sodium periodate was used were considerably paler and more unstable.

Ascorbic acid-impregnated paper chromatograms developed with water-saturated organic solvents (Bush type) turned deep brown when treated with a saturated solution of sodium periodate. The brown background colour vanished rapidly within *ca.* 1 min, while the stained phenolic steroids remained visible for several minutes. As tested with sodium periodate solutions of different concentrations, fading of these spots could be delayed when the saturated solution was diluted with three parts of water.

The colours on the papers resembled those observed on thin-layer plates. Compounds with a catechol structure became visible considerably faster than those with a guaiacol substitution pattern. Although the colours were intense and fairly stable, the spots should be observed on a sheet of white paper and be marked immediately after staining. These precautions permitted the detection of less than 10 μg of the steroids on paper chromatograms of the Zaffaroni type and less than 5 μg on those of the Bush type. It should be stressed that ascorbic acid-impregnated paper chromatograms must not be dried at elevated temperatures prior to colour development, as the ascorbic acid was thereby decomposed to yellow or deep red products, which prevented the detection of the estrogens after staining.

DISCUSSION

In the course of pilot experiments, it was found that many chromogenic reagents for phenolic compounds could not be used on ascorbic acid-impregnated paper and thin-layer chromatograms. Detection of estrogens was obviously prevented by coloured decomposition products of the ascorbic acid formed by drying of the paper chromatograms at elevated temperatures. When heating was omitted, standard staining reactions were impeded by the ascorbic acid and or by the solvents used for impregnating the paper chromatograms of the Zaffaroni type. Removal of the ascorbic acid prior to drying was therefore attempted by dipping the chromatograms into water, but this procedure proved to be incomplete.

Thus, oxidative decomposition of the ascorbic acid to colourless products seemed to be the only possibility for the location of estrogens after "reducing chromatography". When ascorbic acid-impregnated thin-layer plates were sprayed with 3 *M* sulphuric acid and subsequently heated, all of the estrogens investigated exhibited intensely coloured spots on a white background, thereby indicating complete degradation of the ascorbic acid. As this reagent cannot be used for paper chromatograms and lacks any structural specificity, other oxidising reagents were tested.

Based on the work of Adler and co-workers[15,16], Clifford and Wight[14] used metaperiodate as a structure-specific locating reagent for polyhydric phenols and their partially methylated derivatives on paper strips which, however, were impregnated neither with ascorbic acid nor with stationary phases of the Zaffaroni type. In the

present work it was demonstrated that sodium periodate permits the specific staining of catechol- and pyrogallolestrogen derivatives by the formation of the corresponding *o*-quinones with simultaneous oxidative decomposition of the ascorbic acid to colourless products. For the chromogenic reaction of A-ring-substituted estrogens, a saturated solution of sodium periodate was found to be superior to the dilute solution used by Clifford and Wight[14].

Staining with sodium periodate is the first method so far described for the detection of unlabelled catechol- and pyrogallolestrogen derivatives on thin-layer and paper chromatograms impregnated with ascorbic acid. This procedure is therefore expected to facilitate the application of "reducing chromatography" to investigations on other autoxidizable catechol compounds of biological importance.

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