CHROM. 6142

A NEW METHOD FOR PREVENTING OXIDATIVE DECOMPOSITION OF CATECHOL ESTROGENS DURING CHROMATOGRAPHY

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(First received March 27th, 1972; revised manuscript received May 5th, 1972)

SUMMARY

Methods are described for the chromatography of the very labile 2-hydroxyestradiol-17β on paper, thin layers and columns without oxidative decomposition. This is accomplished by impregnating the stationary phases with ascorbic acid as reductant, which does not alter the R_F values of the several estrogens tested on paper and silica gel. The recoveries of radioactive 2-hydroxyestradiol-17β are markedly improved by chromatography in the presence of ascorbic acid as compared with standard chromatographic procedures. Amounts as small as 2 μg of 2-hydroxyestradiol-17β are easily and quantitatively recovered when using these new reducing chromatographic systems.

INTRODUCTION

In the last decade, the biogenesis and metabolism of 2-substituted phenolic steroids have been extensively studied by *in vitro* and *in vivo* experiments. The results have suggested that the 2-hydroxyestrogens belong to the major metabolites of estrone and estradiol-17β in man[1–8].

Recently KNUPPEN and his colleagues[9–12] were able to demonstrate that the enzymic inactivation of catechol amines is strongly inhibited by 2-hydroxylated estrogens. To obtain more information on the physiological significance of this observation, which might be relevant to the occasional occurrence of hypertension of unknown origin during pregnancy, it is necessary to investigate the quantitative correlation between urinary excretion of catechol amines and catechol estrogens during pregnancy.

Although 2-hydroxyestrogens have been closely studied for several years, their handling is still extremely difficult, because they readily undergo oxidative decomposition. Thus, no satisfactory quantitative assays of the catechol estrogens are available today, apart from a radioimmunoassay for 2-hydroxyestrone in plasma recently described by YOSHIZAWA AND FISHMAN[13]. Furthermore, the preparation of labelled 2-

hydroxyestrogens with very high specific activities for internal yield correction is impeded by degradation during chromatographic purification, especially when very small amounts are used[14,15].

When biological material containing 2-hydroxyestrogens is submitted to the usual chromatographic separation procedures, analytical losses of the catechol estrogens approach 100% in some instances. It was therefore clearly necessary to establish experimental conditions under which no decomposition of catechol estrogens would take place during the chromatographic procedures. The experiments reported in this paper were carried out with 2-hydroxyestradiol-17β as a representative of the catechol estrogens.

MATERIALS AND METHODS

Steroids

2-Hydroxyestradiol-17β (2-OHE$_2$; 1,3,5(10)estratriene-2,3,17β-triol) was generously donated by Schering (Berlin, G.F.R.). [6,7-^{3}H$_2$]2-Hydroxyestradiol-17β-triacetate[16] (specific radioactivity 115 mCi/mmole) was synthesized as follows: 2-Hydroxyestradiol-17β-triacetate[17] was oxidized with CrO_3 in glacial acetic acid according to the method of LONGWELL AND WINTERSTEINER[18]. The resulting 6-oxo compound was reduced with $NaBH_4$ in methanol at room temperature to the corresponding 6α-hydroxy steroid. Treatment of the latter compound with methanolic HC yielded 6-dehydro-2-hydroxyestradiol-17β (*cf.* ref. 19), which was acetylated with acetic anhydride in pyridine and then tritiated in ethyl acetate with Pd–charcoal. The [6,7-^{3}H$_2$]2-hydroxyestradiol-17β-triacetate thus obtained was hydrolysed with methanol–5 *N* H_2SO_4 (3:1; v/v), containing 1% (w/v) of ascorbic acid, at room temperature for 3 days. The resulting material was purified by paper chromatography on ascorbic acid-impregnated papers in system A_R as described below. The final [6,7-^{3}H$_2$]2-hydroxyestradiol-17β had a radiochemical purity of more than 95%. In general, stock solutions were prepared by dissolving 100 μCi of the tritiated 2-OHE in 50 ml of methanol and adding 0.3 g of ascorbic acid; they were stable for at least 3 months when stored at 2° in the dark. Usually, 0.5 ml of this stock solution was used for one chromatogram without removing the ascorbic acid.

Other materials

All chemicals used were of p.a. grade and were purchased from E. Merck (Darmstadt, G.F.R.). Organic solvents were redistilled before use. The standard solution of ascorbic acid contained 15 g of ascorbic acid and 4 ml of glacial acetic acid in 400 ml of methanol.

Measurement of radioactivity

An Intertechnique liquid scintillation spectrometer, Model SL 36, was used for the measurements of the radioactivity. The scintillation fluid contained 4 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis-2-(5-phenyloxazolyl)benzene in 1 l of dry sulphur-free toluene.

On paper chromatograms the radioactivity was counted directly by a paper strip scanner (Berthold paper chromatogram scanner, Model LB 280) with a dua

rate meter integrator, LB 242 K. The radioactivity on thin-layer plates was located with a Berthold Dünnschicht Scanner II, Model LB 2723.

Paper chromatographic methods

General. Strips of Schleicher and Schüll 2043 b Mgl paper were immersed for impregnation in the appropriate solutions, blotted with filter-paper and dried in a horizontal position at room temperature for at least 30 min (ref. 20). Unless otherwise stated, the chromatograms were equilibrated for at least 60 min at 22 ± 2°. After development and drying, the radioactivity on the chromatograms was located; the appropriate areas were cut into small pieces and these were thoroughly extracted with a mixture of methanol and the standard ascorbic acid solution (10:1; v/v) at room temperature. The combined methanolic extracts were evaporated to dryness *in vacuo* at room temperature and the residue was distributed between 10% (v/v) aqueous acetic acid and dichloromethane–ethyl acetate (1:1; v/v) to remove the material used for impregnating the paper chromatograms. The combined organic phases were evaporated to dryness after the addition of 0.2 ml of the standard ascorbic acid solution.

System A_R (with addition of ascorbic acid). Impregnation was carried out with standard ascorbic acid solution. The solvent system was 1,2-dichloroethane–methylcyclohexane–acetic acid–water (125:75:140:60, by vol.); the upper, mobile phase was saturated with ascorbic acid.

System A (without addition of ascorbic acid). No impregnation was carried out. The solvent system was 1,2-dichloroethane–methylcyclohexane–acetic acid–water (125:75:140:60, by vol.).

System B_R (with addition of ascorbic acid). Impregnation was carried out with formamide–methanol (1:1, v/v), containing 1.9% (w/v) of ascorbic acid. The solvent system was chlorobenzene–ethyl acetate (1:1; v/v), saturated with formamide and ascorbic acid. As ascorbic acid is slowly decomposed in the presence of formamide, these solutions were prepared shortly before use.

System B (without addition of ascorbic acid). Impregnation was carried out with formamide–methanol (1:1; v/v). The solvent system was chlorobenzene–ethyl acetate (1:1; v/v), saturated with formamide.

System C_R (with addition of ascorbic acid). Impregnation was carried out with ethylene glycol–methanol (1:2; v/v), containing 2.5% (w/v) of ascorbic acid. The solvent system was dichloromethane–ethyl acetate (3:1; v/v), saturated with ethylene glycol and ascorbic acid.

System C (without addition of ascorbic acid). Impregnation was carried out with ethylene glycol–methanol (1:2; v/v). The solvent system was dichloromethane–ethyl acetate (3:1; v/v), saturated with ethylene glycol.

Thin-layer chromatography methods

General. Thin-layer plates (MN-Polygram Alox N/UV_{254}, Macherey-Nagel, Düren, G.F.R., and DC-Fertigplatten, Kieselgel F_{254}, E. Merck, Darmstadt, G.F.R.) were immersed for impregnation in the appropriate solutions, blotted with filter-paper and dried in a horizontal position at room temperature for at least 30 min. After development of the chromatograms and drying at room temperature, the radioactivity

was located; the radioactive zones were scraped off and eluted with formic acid–standard ascorbic acid solution (1:1; v/v). The eluates were evaporated and the residues treated as described in the paper chromatographic section above.

Silica gel plates with addition of ascorbic acid. Impregnation was carried out with standard ascorbic acid solution. The solvent system was acetic acid–chloroform–methylcyclohexane (1:2:2, by vol.), saturated with ascorbic acid.

Silica gel plates without addition of ascorbic acid. No impregnation was carried out. The solvent system was acetic acid–chloroform–methylcyclohexane (1:2:2, by vol.) (ref. 14).

Alumina plates with addition of ascorbic acid. Impregnation was carried out with standard ascorbic acid solution. The solvent system was 2-butanone–acetic acid (1:1, v/v), saturated with ascorbic acid.

Alumina plates without addition of ascorbic acid. No impregnation was carried out. The solvent system was ethanol–acetic acid (3:1, v/v).

Column chromatography methods

Silica gel columns. Silica gel (Kieselgel, E. Merck, 0.05–0.2 mm) was impregnated with ascorbic acid by stirring 30 g in a solution of 7.5 g of ascorbic acid in 200 ml of methanol. After 30 min, the silica gel was collected and dried overnight at room temperature. The ascorbic acid-impregnated silica gel thus obtained showed a yellowish colour, which became more intense after several days. Therefore, the impregnated silica gel was used within the following 2 or 3 days. The columns (0.9 × 8 cm) were prepared by suspending the impregnated silica gel in the solvent system acetic acid–chloroform–cyclohexane (1:2:2, by vol.), which was saturated with ascorbic acid.

Chromatography on non-impregnated silica gel was carried out in the same way except that the solvent system was not saturated with ascorbic acid.

Alumina columns. Protection of the steroid by ascorbic acid during chromatography was achieved as follows. Columns (0.9 × 6 cm) were prepared with a slurry of 5 g of alumina (Aluminum Oxide Woelm acid, activity grade I; Woelm, Eschwege, G.F.R.) in methanol containing 1.9% (w/v) of ascorbic acid. After passage of a further 20 ml of the same solution, the radioactive 2-OHE_2 was applied to the column, elution of the steroid being carried out afterwards with another 15 ml of 1.9% (w/v) ascorbic acid in methanol.

For chromatography without ascorbic acid, the columns were filled with a slurry of 5 g of alumina in methanol. The radioactive 2-OHE_2 was applied to the column and after the passage of 20 ml of methanol it was subsequently eluted with 15 ml of methanol–formic acid (1:1, v/v).

Calculation of recovery

To determine the total radioactivity recovered after the different chromatographic procedures and after elution, portions (1%) of the resulting material were submitted to liquid scintillation counting. In order to calculate the extent of degradation of the radioactive 2-OHE_2 the residue was subjected to paper chromatography in system A_R. After scanning, the radioactivity at the position of the 2-OHE_2 was related to the total scanned radioactivity on the paper chromatograms[9].

RESULTS AND DISCUSSION

In order to protect 2-hydroxyestradiol-17β from oxidation, attempts were made to carry out chromatography under reducing conditions by simply impregnating the stationary phases with a reductant; for this purpose ascorbic acid and $SnCl_2$ were tested. While impregnation with $SnCl_2$ had no stabilizing effect (Table II), ascorbic acid gave excellent results.

Strikingly, the R_F values of 2-OHE_2 were not influenced by the impregnation of paper chromatograms with ascorbic acid. This was also valid for the paper chromatography of some other estrogens tested in several systems, and it therefore seems possible that standard paper chromatographic procedures can generally be modified by impregnation with ascorbic acid, which should guarantee the stability of 2-hydroxyestrogens without affecting the properties of the chromatographic system. Moreover, the chromatographic behaviour of 2-OHE_2 on silica gel (column and thin-layer chromatography (TLC)) was not influenced by impregnation with ascorbic acid, whereas the R_F values were changed markedly when alumina was treated with ascorbic acid.

For this investigation, it was necessary to establish experimental conditions under which 2-OHE_2 is quantitatively removed from paper and thin-layer plates. As a result of pilot experiments with different solvents, a methanol–ascorbic acid solution was chosen for the elution of 2-OHE_2 from paper, and a methanol–formic acid–ascorbic acid solution for elution after alumina and silica gel TLC. Hence, the recovery of total radioactivity from the appropriate areas of the paper chromatograms varied between 90 and 100%, depending on the previous impregnation, whereas about 90% of the radioactivity was eluted from silica gel and alumina thin-layer plates.

In order to check the reliability of the results, the standard deviation for the final recovery of 2-OHE_2 was calculated in a series of experiments. For this purpose, various amounts (2.5, 10 and 40 μg) of 2-OHE_2 were chromatographed in system A_R, eluted from the paper and submitted to solvent distribution. The standard deviation of the final recovery, as calculated by the combination of liquid scintillation counting and a second paper chromatography in the system A_R, varied between ± 3.5 and $\pm 5.4\%$ (Table I).

As Table I clearly demonstrates, the stability of 2-OHE_2 could be markedly increased in a number of typical paper chromatographic systems used for the separation of phenolic steroids[20]. Chromatography on ascorbic acid-impregnated papers led to considerably increased recoveries, of as little as 2 μg, compared with standard procedures. Under the conditions of "reducing chromatography" in the systems A_R, B_R and C_R, the recoveries of undecomposed 2-OHE_2 were 93, 88 and 87%, respectively, whereas the standard systems A, B and C gave only 57, 0 and 46% recoveries, respectively. It was possible to dry paper chromatograms impregnated with ascorbic acid at elevated temperatures and to store them under normal laboratory conditions for more than a week without decomposition of 2-OHE_2. The pronounced variation of the stability of 2-OHE_2 in the three paper chromatographic systems used without ascorbic acid can be explained by the increasing instability of the catechol estrogens in alkaline media. Thus there is complete decomposition of 2-OHE_2 after using formamide-impregnated paper, as formamide easily becomes alkaline by decomposition, whereas chromatography with the very acidic Bush system (system A) still

TABLE I

PERCENTAGE RECOVERY OF 2-OHE_2 AFTER PAPER CHROMATOGRAPHY IN DIFFERENT SYSTEMS

Comparison between standard chromatographic procedures in the systems A, B and C, and "reducing chromatography" in the systems A_R, B_R and C_R; further details are given in the text. Each figure represents the mean of four independent measurements, and the highest and the lowest values are given in parentheses.

Applied amount of 2-OHE_2 (μg)	*Chromatographic systems*						*Treatment of the chromatograms before elution*
	A	*A_R*	*B*	*B_R*	*C*	*C_R*	
40	51 (44–57)	91 ±4.1[8][a]	—	—	—	—	Drying at room temperature for 3 h
10	58 (51–64)	94 ±5.4[8][a]	0	86 (83–88)	34 (30–39)	86 (85–87)	
2.5	57 (52–62)	93 ±3.5[16][a]	—	—	—	—	
10	60 (56–62)	91 (89–93)	—	88 (85–91)	—	84 (81–86)	Drying at room temperature and exposure to normal laboratory conditions for 1 week
40	—	—	—	81 (80–82)	—	88 (84–91)	Drying at 80° for 2 h
10	28 (26–31)	89 (86–92)	0	88 (84–91)	43 (40–46)	87 (86–89)	
2.5	—	—	—	84 (80–87)	—	89 (88–90)	
10	—	—	—	82 (80–84)	—	83 (80–87)	Drying at 80° for 2 h and exposure to normal laboratory conditions for 1 week

[a] Calculation of the standard deviation. The number of independent measurements is given in square brackets.

TABLE II

PERCENTAGE RECOVERY OF 2-OHE_2 AFTER TLC USING DIFFERENT STATIONARY PHASES

Comparison between chromatography on non-impregnated and ascorbic acid-impregnated stationary phases; further details are given in the text. Each figure represents the mean of four independent measurements, and the highest and the lowest values are given in parentheses.

Applied amount of 2-OHE_2 (μg)	*Stationary phases*				
	Non-impregnated alumina	*Alumina impregnated with ascorbic acid*	*Non-impregnated silica gel*	*Silica gel impregnated with ascorbic acid*	*Silica gel impregnated with $SnCl_2$*
40	47 (46–48)	70 (67–73)	39 (36–42)	65 (62–68)	21 (17–24)
2.5	<10	72 (67–76)	23 (19–29)	64 (61–66)	17 (15–19)

resulted in recoveries of nearly 60%. System C (ethylene glycol impregnation) resulted in recoveries that varied between those of systems A and B.

Although standard TLC was performed under acidic conditions, which were expected to minimize the oxidative decomposition of the catechol estrogens, the recovery of undecomposed 2-OHE_2 was poor (Table II). Only by using "reducing chromatography" on ascorbic acid-impregnated silica gel and alumina were adequate recoveries of 2-OHE_2 obtained. However, compared with paper chromatography, TLC gave inferior recoveries.

TABLE III

PERCENTAGE RECOVERY OF 2-OHE$_2$ AFTER COLUMN CHROMATOGRAPHY USING DIFFERENT STATIONARY PHASES

Comparison between chromatography on non-impregnated and ascorbic acid-impregnated stationary phases; further details are given in the text. Each figure represents the mean of four independent measurements, and the highest and the lowest values are given in parentheses.

Applied amount of 2-OHE$_2$ (μg)	*Stationary phases*			
	Non-impregnated alumina	*Alumina impregnated with ascorbic acid*	*Non-impregnated silica gel*	*Silica gel impregnated with ascorbic acid*
40	53 (47–60)	83 (78–87)	79 (76–84)	91 (88–93)
2.5	55 (53–59)	85 (83–86)	81 (77–82)	90 (88–92)

Applying the principle of "reducing chromatography" to column chromatography improved the recovery of undecomposed 2-OHE$_2$ again in comparison with non-impregnated silica gel and alumina (Table III).

Finally, it should be mentioned that 2-OHE$_2$ should be transferred in a solution containing ascorbic acid to chromatograms and columns. This precaution is taken to avoid oxidative decomposition of the steroid before the start of chromatography.

The results obtained in this work should facilitate further investigations concerning the physiological significance of catechol estrogens in man.

ACKNOWLEDGEMENTS

This investigation was supported by the Deutsche Forschungsgemeinschaft. The authors wish to express their gratitude to Mr. E. R. SMITH, London, for reading the manuscript.

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