THE APPLICATION OF PARTITION CHROMATOGRAPHY TO THE SEPARATION AND CHARACTERISATION OF OESTROGENS BY ISOTOPE DILUTION

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INTRODUCTION

The criteria of classical chemistry can seldom be applied to the identification of metabolites particularly of steroid hormones, isolated from tissues or body fluids after experiments with precursors at physiological levels. However, isotope dilution methods, pursued with sufficient rigour, yield information which leaves little doubt of the identity of the material isolated. Chromatography, in conjunction with methods for the measurement of radioactive and unlabelled compounds, plays an important role in the separation and identification of metabolites isolated in experiments of this kind.

We report here chromatographic procedures developed for the separation of mixtures of oestrogens of widely differing polarity and of closely related epimers. Techniques for recording permanently the position of oestrogens on paper chromatograms by autopositive photography and for the determination of ^{14}C on paper by liquid scintillation counting are also described.

MATERIALS AND APPARATUS

Materials

(*a*) *Solvents*

Ethanol was absolute alcohol B.P.C. grade (J. Burrough Ltd., London, S.E. 11). Methanol, ethanol, benzene and light petroleum (b.p. 80–100°) were of A.R. grade. Ligroin, hexane (free from aromatic hydrocarbons) and ethylene dichloride were laboratory reagent grade (B.D.H. Ltd., Poole, Dorset). Solvents used for column chromatography and fluorimetry were redistilled before use, but solvents for paper chromatography were used without further purification.

(*b*) *Reagents*

Potassium ferricyanide and phosphomolybdic acid were of A.R. grade. Sulphuric acid for fluorimetry was either A.R. grade (for dilution to 65 %) or M.A.R. grade (B.D.H., for dilution to 90 %). Ferric chloride was laboratory reagent grade (Hopkin & Williams Ltd., Chadwell Heath, Essex). *m*-Dinitrobenzene was B.D.H. laboratory reagent purified according to CALLOW, CALLOW AND EMMENS[1]. The solution for liquid scintillation counting of ^{14}C was prepared as described by STITCH[2].

(c) Steroids

Oestrone (3-hydroxyoestra-1,3,5(10)-trien-17-one) was supplied by L. Light and Co. Ltd., Colnbrook, Bucks. Oestradiol-17α (3,17α-dihydroxyoestra-1,3,5(10)-triene) and oestradiol-17β (3,17β-dihydroxyoestra-1,3,5(10)-triene) were supplied by Schering A.G., Berlin. Oestriol-16α,17β (3,16α,17β-trihydroxyoestra-1,3,5(10)-triene) was supplied by Sigma Chemical Corporation, St. Louis, Miss., U.S.A. Dr. T. F. GALLAGHER (Sloan-Kettering Institute, New York, U.S.A.) supplied samples of oestriol-16β,17β (3,16β,17β-trihydroxyoestra-1,3,5(10)-triene) and oestriol-3,16α,17α (3,16α,17α-trihydroxyoestra-1,3,5(10)-triene). Oestradiol-17β 3-methyl ether (3-methoxy-17β-hydroxyoestra-1,3,5(10)-triene) was prepared as described by BROWN[3]. Oestrone 3-methyl ether (3-methoxyoestra-1,3,5(10)-trien-17-one) and oestriol-16α,17β 3-methyl ether (3-methoxy-16α,17β-dihydroxyoestra-1,3,5(10)-triene) were prepared as described by LEVITZ[4]. The infra-red spectra and chromatographic behaviour of the oestrogen methyl ethers were identical with those of authentic specimens supplied by Professor W. KLYNE (M.R.C. Steroid Reference Collection, Westfield College, London, N.W. 3).

Testosterone (17β-hydroxyandrost-4-en-3-one) was supplied by Paines and Byrne Ltd., Greenford, Middlesex. Androst-4-ene-3,17-dione was supplied by Dr. R. I. DORFMAN (Worcester Foundation for Experimental Biology, Shrewsbury, Mass., U.S.A.).

(d) Supporting phases

Paper chromatography was carried out on Whatman No. 1 filter paper selected for chromatography. Sheets (20 cm × 50 cm) with the grain of the paper parallel to the longer side, were used without purification. If the mobile phase was required to overrun the paper, the end of the paper was cut to form ten equally spaced points.

Celite 535 (Johns Mansville & Co., 20, Albert Embankment, London, S.E. 11), purified according to BAULD[5], was used for column chromatography.

(e) Photographic materials

Photography was carried out on autopositive paper (Extra light weight 42, Kodak Ltd., Kodak House, Kingsway, London, W.C. 2). Photographic developer and fixer were Kodak Duostat brand.

Apparatus

(a) Tanks for paper chromatography

Glass tanks (19 cm × 30 cm × 57 cm, Aimer Products Ltd., 56–58 Rochester Place, Camden Road, London, N.W. 1), lagged all round with expanded polystyrene (sheets, 3.5 cm thick), were used for descending chromatography. The walls were covered inside with sheets of filter paper moistened with mobile and stationary phases. The temperature in the tanks was maintained at 19–21°.

(b) Tubes for column chromatography

Glass tubes (22 cm long, 10–11 mm internal diameter) fitted at one end with a perforated glass disc, were used for column chromatography.

(*c*) *Printing box for photographic recording of paper chromatograms*

After location of carrier steroids by chemical reactions on strips cut from paper chromatograms, each strip was photographed in a printing box (20 cm × 30 cm × 60 cm). Eight 60 W bulbs on the floor of the box in two lines parallel to the longer sides provided the light source. The inside of the box was aluminium painted to aid reflection. Flashed opal glass (25 cm × 45 cm) was mounted above the bulbs as a light diffuser. Above this a sheet of plate glass (27 cm × 50 cm × 5 mm) was mounted. The box was fitted with a lid hinged on one longer edge. On the lower side of this lid a second sheet of plate glass (27 cm × 50 cm × 5 mm) was mounted.

(*d*) *Fluorimeter*

Fluorimetric assay of oestrogens was carried out with an E.I.L. Direct Reading Fluorimeter Model 27A (Electronic Instruments Ltd., Lower Mortlake Road, Richmond, Surrey). An Ilford 601 filter was used as primary filter. In this position it was found to be unstable and was replaced when necessary. The secondary filter was a combination of Chance OY13, Ilford 108 and Chance OB2 filters.

(*e*) *Liquid scintillation counter*

^{14}C was assayed with an Ekco N612 liquid scintillation counter (Ekco Electronics Ltd., Southend-on-Sea, Essex).

METHODS

Methods for paper chromatography

(*a*) *Solvent systems*

Solvents were mixed in the following volumes and allowed to stand for at least 3 h at laboratory temperature (19–22°) before use:

(i) to separate oestrone, oestradiol-17β, oestriol-16β,17β and oestriol-16α,17β: benzene 200 ml, methanol 140 ml, water 60 ml;

(ii) to separate oestradiol-17α and oestradiol-17β: light petroleum (b.p. 80–100°) 200 ml, methanol 160 ml, water 40 ml, (BUSH system A[6]);

(iii) to separate oestrogen 3-methyl ethers: ligroin 200 ml, methanol 192 ml, water 8 ml[7].

The upper layer was used as mobile phase, the lower layer was the stationary phase.

(*b*) *Loading and running*

Material was dissolved in methanol–chloroform (1:1 v/v) and applied along a line 10 cm from, and parallel to, the shorter edge of the paper. 5–25 μg of each oestrogen per cm gave the best separations. After equilibration of the paper in the tank for at least 3 h, descending chromatography was carried out.

(*c*) *Detection of steroids on paper chromatograms*

The steroids were located by passing a strip (0.5 cm × 45 cm), cut from the centre of the chromatogram, through the reagents.

(i) Free oestrogens were detected at a level of 5 μg/sq. cm, with a mixture of equal volumes of 1 % aq. $FeCl_3$ and 1 % aq. $K_3Fe(CN)_6$[8].

(ii) Oestrogen 3-methyl ethers were detected at a level of 5 μg/sq. cm, with a 10 % solution of phosphomolybdic acid in ethanol[9].

(iii) C_{19} steroids were located with Zimmermann reagent, as described by BUSH[10]. Testosterone gave a blue band whereas 17-oxo-steroids gave a purple band.

(d) Photographic recording of paper chromatograms

The dried coloured strip with the position of the origin and solvent front (if present) marked clearly, was covered with a sheet of autopositive photographic paper and placed between the sheets of plate glass in the printing box. After exposure to the light source for 15–75 sec the photographic paper was developed, washed, fixed, washed and dried.

(e) Determination of ^{14}C after paper chromatography

After removal of a strip for detection of carrier steroids, an area of the paper, from 0.6 cm behind the origin to at least 0.6 cm beyond the solvent front (or to the edge of the paper) and 2.5 cm on either side of the original loading area, was cut into segments (1.2 cm) with this dimension parallel to the solvent flow. Each segment was immersed in liquid scintillator (3 ml) and ^{14}C was assayed with the Ekco N612 counter[11].

Methods for column chromatography

(a) Solvent system

Hexane (110 ml), benzene (90 ml), methanol (140 ml) and water (60 ml) were shaken together and maintained at laboratory temperature (19–22°) for at least 3 h. Celite for the column was treated with the lower layer. The upper layer was used as the first mobile phase. The second mobile phase was prepared by mixing hexane (20 ml) with benzene (80 ml). Ethylene dichloride was the third mobile phase.

(b) Column preparation

Stationary phase (5 ml, see above) was stirred slowly into purified Celite (5 g). The glass tube was filled with mobile phase. Celite-stationary phase was added in small portions and packed to form a column 15 cm high with a MARTIN packer[12]. The flow rate was 24–33 ml/h, with the solvent head at the top of the tube.

(c) Application of oestrogens to the column

The steroid mixture, dissolved in chloroform–methanol (1:1 v/v), acetone or methylene dichloride was transferred to four circular paper discs (8 mm diameter, Whatman No. 1 filter paper). After evaporation of the solvent, the discs were placed on top of the Celite column.

(d) Elution of oestrogens from the column

The mobile phase was run out from the column until the Celite was covered with approximately 0.2 mm. The loaded paper discs were placed on top of the Celite and mobile phase (0.5 ml) was added and allowed to run into the column. A further two volumes (0.5 ml each) were added and allowed to soak in. When the level of solvent was approximately 0.2 mm above the discs the tube was filled with mobile phase No. 1.

After twenty fractions (3 ml each) had been collected the eluting solvent was

changed to mobile phase No. 2. After a further 25 fractions had been collected mobile phase No. 2 was replaced by mobile phase No. 3. In all 60 fractions were collected.

(*e*) *Determination of oestrogens after column chromatography*

The eluted carrier oestrogens were detected and quantitatively estimated by fluorimetry with sulphuric acid. The method of BATES AND COHEN[13] (modified in our laboratory by using 45 min heating at 80° with 90 % H_2SO_4, addition of 65 % H_2SO_4 and fluorescence stabilisation for 1 h at 4°) was used.

(*f*) *Determination of ^{14}C after column chromatography*

^{14}C was assayed in each fraction by liquid scintillation counting by the procedure described by STITCH[2].

RESULTS

Paper chromatography

(*a*) *Separation of oestrone, oestradiol-17β, oestriol-16β,17β and oestriol-16α,17β*

Oestrone (R_F 0.88 ± 0.05; 19 observations), oestradiol-17β (R_F 0.72 ± 0.05; 19 observations), oestriol-16β,17β (R_F 0.40 ± 0.03; 3 observations) and oestriol-16α,17β (R_F 0.11 ± 0.02; 19 observations) were separated by chromatography in benzene–methanol–water (10:7:3 by vol.), for 3 h. Oestradiol-17α and oestradiol-17β were not separated in this system.

(*b*) *Separation of oestradiol-17α and oestradiol-17β*

These epimeric oestrogens were separated by chromatography in light petroleum (b.p. 80–100°)–methanol–water (10:8:2 by vol.), BUSH system A[6], for 30–50 h. The mobility of oestradiol-17α (0.21–0.35 cm/h) relative to that of oestradiol-17β (0.15–0.24 cm/h) was found to be 1.4. Thus in practice clear separations were achieved (Fig. 1) although the absolute mobilities were variable.

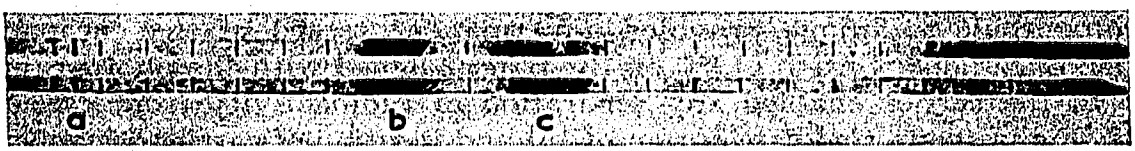

Fig. 1. Paper chromatography of oestradiol-17β (b) and oestradiol-17α (c) in light petroleum (b.p. 80–100°)–methanol–water (10:8:2 by vol.). Photographic record of two different chromatograms. The dark line (a) is the origin.

(*c*) *Separation of oestrogen 3-methyl ethers*

We have examined the behaviour of some oestrogen 3-methyl ethers in ligroin–96 % methanol[7], and report the following R_F values: oestrone 3-methyl ether 0.75 ± 0.05 (6 observations); oestradiol-17β 3-methyl ether 0.57 ± 0.05 (9 observations); oestriol-16α,17β 3-methyl ether 0.10 (3 observations). In addition the R_F values of the free oestrogens were oestrone 0.12 (4 observations), oestradiol-17β 0.05 (4 observations), oestriol-16α,17β 0 (2 observations). The R_F values of testosterone (0.13) and androst-4-ene-3,17-dione (0.26) were also recorded.

Column chromatography

Separation of oestrone, oestradiol-17α, oestradiol-17β, oestriol-16β,17β and oestriol-16α,17β

These compounds were readily separated, but oestriol-16β,17β and oestriol-16α,17α were not fully resolved. Fig. 2 shows a typical elution pattern.

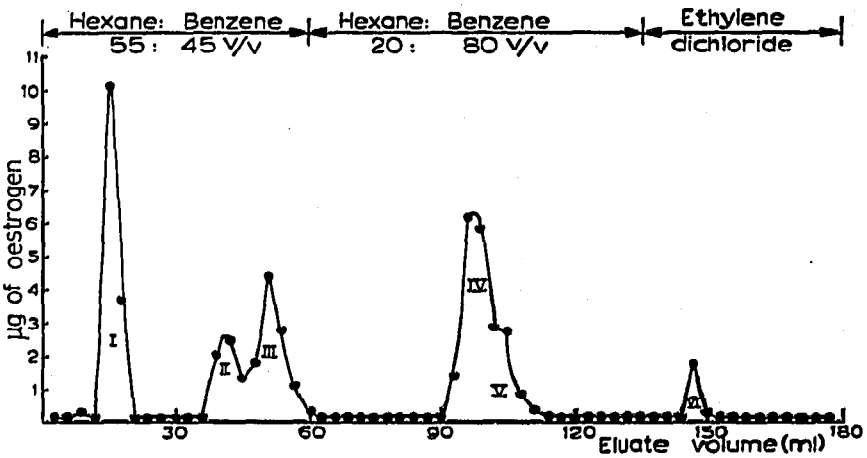

Fig. 2. Elution pattern after partition column chromatography of oestrone (I), oestradiol-17α (II), oestradiol-17β (III), oestriol-16α,17α (IV), oestriol-16β,17β (V) and oestriol-16α,17β (VI). The stationary phase was 70 % aq. methanol. The composition of the mobile phase is indicated on the diagram.

Although 2 mg oestradiol-17β chromatographed on this column gave a single recognisable peak, best results were achieved when mixtures containing 50 μg or less of each oestrogen were chromatographed. 71–88 % of oestrone, oestradiol-17α, oestradiol-17β and oestriol-16β,17β and 48 % oestriol-16α,17β were recovered in the eluate.

Photographic recording of paper chromatograms

The photographic technique provided a permanent record of the chromatogram. Inspection of the distribution of ^{14}C on the paper aligned with the photograph of the position of the carrier enabled any separation of radioactivity from carrier to be observed. Fig. 3 shows the distribution of ^{14}C and location of carrier oestrone, oestradiol-17β and oestriol-16α,17β after incubation of ^{14}C testosterone with rat ovarian tissue[14]. The distribution of 0.3 mμC ^{14}C after chromatography of a mixture of radioactive oestrogens could be determined by this method.

DISCUSSION

To improve the reliability of identification of oestrogens by reverse isotope dilution with chromatographic techniques, we have developed methods for separating oestrogens on paper and on partition columns, for recording permanently the position of oestrogens on paper, and for measuring ^{14}C on paper chromatograms.

The separation of oestrone, oestradiol-17β, oestriol-16β,17β and oestriol-16α,17β on one paper chromatogram in 3 h reported here, obviates the need for two separate

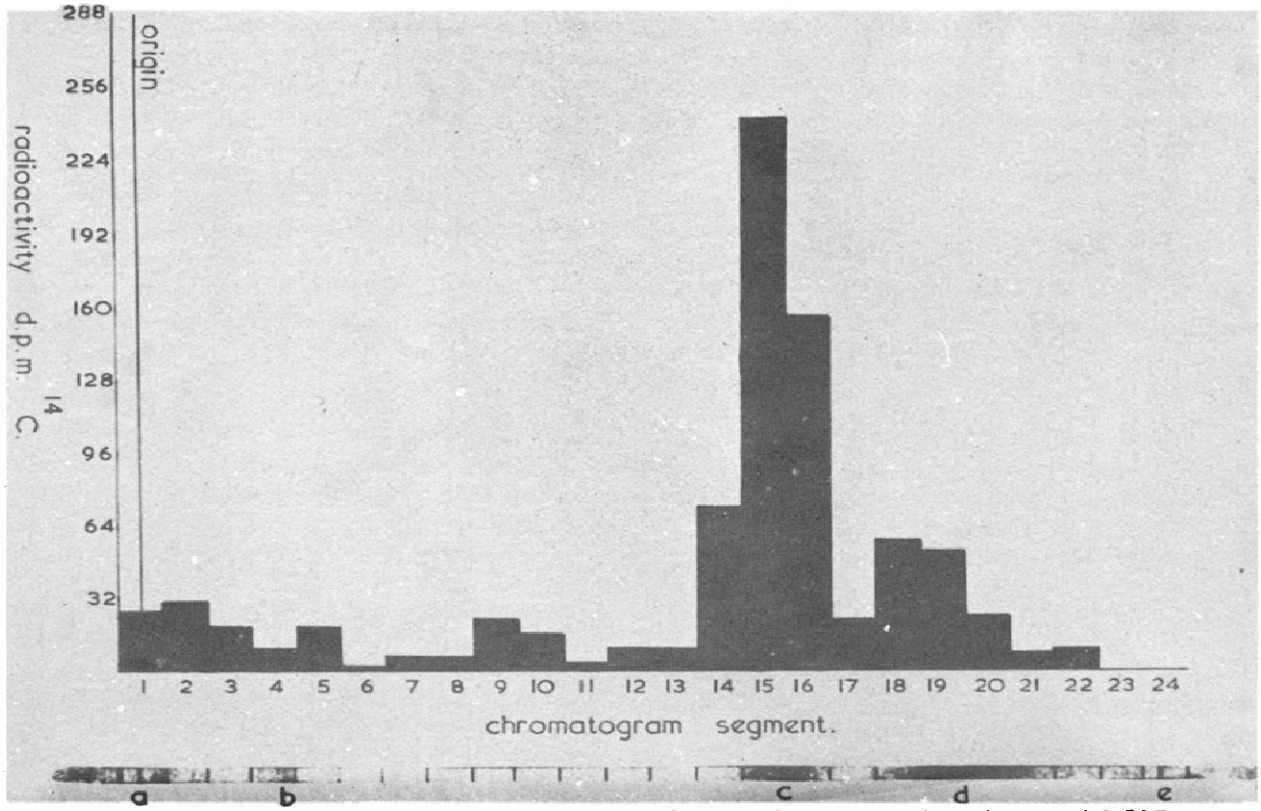

Fig. 3. Paper chromatography of an extract from an incubation of ovarian tissue with ^{14}C-testosterone[14]. Distribution of ^{14}C aligned with the photographic record of the position of carrier oestriol-16α,17β (b), oestradiol-17β (c) and oestrone (d). (a) marks the origin and (e) the solvent front.

chromatograms[15]. The application of BUSH system A to the separation of the oestradiol epimers should be of value as an additional method, although solvent systems have been described for this separation[16–19]. SMITH[7] observed that the 3-methyl ethers of oestrone, oestradiol-17β and oestriol-16α,17β were separated by paper chromatography in ligroin: 96% aqueous methanol but did not report R_F values. We confirmed that these methyl ethers were separated from each other and found, furthermore, that they were separated from the corresponding parent oestrogens in this system. Oestriol-16α,17β 3-methyl ether and oestrone however, were not separated. The R_F values obtained were recorded.

A major difficulty in the partition column chromatography of oestrogens has been to devise methods capable of handling compounds of widely differing polarity (*e.g.* 2-methoxyoestrone and oestriol-16α,17β). This problem was overcome by loading the column from paper discs and by changing the eluting solvent in two discrete stages.

Several methods utilising partition column chromatography for the resolution of oestrogen mixtures extracted from urine[5,20,21] and blood[22] have been described. The separation of the epimeric oestradiols, however, was not reported in these papers.

Eluates from the column after chromatography of extracts of blood and ovarian tissue appeared sufficiently clean for the determination of carrier oestrogen by H_2SO_4 fluorimetry and of ^{14}C by liquid scintillation counting. However, before strictly quantitative measurements are carried out, it is essential to investigate the possibility of quenching of fluorescence[23] and of phosphorescence[2] by material from the tissue extract.

Comparison of the elution patterns of carrier oestrogens and ^{14}C, together with the determination of specific activity, provided the basis for isotope dilution assay and for the identification of metabolites.

The autopositive photographic process gave a permanent record of paper chromatograms free from subjective error in drawing the position of steroid zones. A major advantage of this technique is that the photographic processing can be carried out in daylight.

A basic requirement for the identification of unknown compounds by isotope dilution techniques is the determination of specific activity at each stage of a serial purification process[24]. Inspection of the distribution of radioactivity and carrier material in eluates from chromatography columns readily allows any separation to be observed. Moreover, direct determinations of specific activity can be made. Measurement of specific activity after paper chromatography, however, is more difficult and usually involves elution of material from the paper. The combination of photography and ^{14}C assay on paper, reported here, although not yielding a direct measurement of specific activity, enables separation of radioactivity and the carrier (equivalent to a change in specific activity) to be detected.

This photographic technique should therefore be of value whenever isotope dilution studies are combined with paper chromatography.

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SUMMARY

Methods are described for separating oestrone, oestradiol-17α, oestradiol-17β, oestriol-16β,17β and oestriol-16α,17β by partition column chromatography and for separating oestrone, oestradiol-17β, oestriol-16β,17β and oestriol-16α,17β on paper.

A separation of oestradiol-17α and oestradiol-17β was achieved on paper in a system originally described[6] for the chromatography of androgens.

Mixtures of oestrone, oestradiol-17β, oestriol-16α,17β and their 3-methyl ethers were resolved by paper chromatography in the system described by SMITH[7].

Autopositive photography was used to record permanently the position of steroids on paper chromatograms.

The application of this technique, combined with liquid scintillation counting of ^{14}C on paper chromatograms, to the identification of radioactive oestrogens is discussed.

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