REVIEW

THE PAPER CHROMATOGRAPHY OF OESTROGENS

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CONTENTS

1. INTRODUCTION

The progress made during the past fifteen years in many fields of biochemical investigation, and especially in the biochemistry of steroid hormones, could not have been achieved without paper chromatography. Reviews of the application of this technique to the separation of steroid hormones have already been made (*e.g.*[20,48,75,85]). However, the isolation of new oestrogens from urine[12,41,60,68] (for reviews see [44,67]), the discovery of new metabolic products of oestrogens[24,54,56,63,93] and the role of oestrogens in neoplasia[26] have accentuated interest in the biosynthesis, metabolism and mode of action of these compounds. Therefore, this review of the applications of paper chromatography to the separation of oestrogens may be of value. An attempt has been made to cover the literature published between 1950 and early 1961. Any omissions noted reflect solely the emphasis stressed by the reviewer.

In this review the term "development" of a chromatogram refers to the movement of the mobile phase down the paper and not to the production of coloured zones by a particular reagent. Also, the general term "oestrogen" is restricted to compounds which have the oestra-1,3,5(10)-trien-3-ol nucleus and does not imply that such a compound has any particular biological effect. Solvent systems are described in the text of this review without solvent ratios but full details of these are

given in the appropriate tables. No reference is made in these tables to the temperature at which chromatography was carried out. To include these temperatures would have made the tables unduly complicated and not all authors have recorded the temperature used.

2. THE SEPARATION OF SELECTED GROUPS OF OESTROGENS

(a) *Oestrone, oestradiol-17β and oestriol-3,16α,17β*

Until 1953 oestrone, oestradiol-17β and oestriol-3,16α,17β were the only oestrogenic hormones isolated from human urine, and proven chemical methods for the estimation of oestrogens in body fluids are available for these three compounds alone[8,25]. Consequently, methods for the separation of oestrone, oestradiol-17β and oestriol-3,16α,17β have received considerable attention.

Formamide-impregnated paper, first used for the paper chromatography of corticosteroids[27], was applied by AXELROD[1] to the separation of oestrogens. This author achieved a clear separation of oestrone, oestradiol-17β and oestriol-3,16α,17β with *o*-dichlorobenzene as mobile phase. Development of formamide-impregnated paper with chloroform followed by toluene was reported by PRUSIKOVA[83] to resolve a mixture of oestrone, oestradiol-17β, oestriol-3,16α,17β, cortisol and cortisone.

Oestrone, oestradiol-17β and oestriol-3,16α,17β were separated by ROSENKRANTZ[86] on paper impregnated with propylene glycol and developed with toluene, a system previously used for the separation of corticosteroids[27]. JELLINEK[50] has used this system for the chromatography of natural and synthetic oestrogens. Oestrone and oestradiol-17β were separated by MEYER[71] using either toluene–propylene glycol[86] or hydrocarbon–aqueous ammonia, a modification of an earlier method[49]. Ethylene glycol has been used as the stationary phase for the separation of oestrone and oestradiol-17β[96] with carbon tetrachloride as mobile phase. A variety of mobile phases for use with triethylene glycol as the stationary phase have also been described[95].

MITCHELL AND DAVIES[73,74], applied systems in which both mobile and stationary phases were volatile, to the paper chromatography of oestrogens. These workers used one solvent mixture to separate oestrone and oestradiol-17β and another to purify oestriol-3,16α,17β. The design by PUCK, of a single system of this type, in which all three oestrogens were separated in 3 hours[84], was a great improvement. A clear separation of the three oestrogens was obtained using heptane–methanol[32,82].

SHORT[92] reported that oestrone, oestradiol-17β, oestriol-3,16α,17β and progesterone were separated by the development of a single paper chromatogram in two distinct stages. Chromatography first in ligroin–aqueous methanol for 2 h and then in light petroleum–methanol for 16 h achieved a separation of these four steroids. Two hydrocarbon–aqueous methanol systems have been used by SMITH[94] to separate oestrogens extracted from ovarian fluids. Oestrone, oestradiol-17β and oestriol-3,16α, 17β have been separated by chromatography for 3 h in benzene–aqueous methanol[78].

A separation of oestrone and oestradiol-17β has been carried out using alumina-treated paper[28] whilst BOSCOTT[10,11] achieved a separation of the three oestrogens on paper treated with *p*-toluenesulphonate.

HEFTMANN[46] took advantage of the phenolic nature of the oestrogens and coupled them with a diazonium salt. The coloured derivatives produced were readily separated

by paper chromatography in a hydrocarbon–aqueous ethanol system, which was later modified[47,74] to give improved separations.

A variety of systems for the chromatography of oestrogens were described briefly by BOSCOTT[9], but no R_F values were published.

Summarising, many systems have been devised in which oestrone, oestradiol-17β and oestriol-3,16α,17β are readily separated. Those with formamide or propylene glycol as the stationary phase, in general, require a longer time for development and will handle larger quantities of material than those employing aqueous methanol. AXELROD[2] stated that a suitable load of oestrone on the origin when using *o*-dichlorobenzene–formamide was 100 μg per cm, whilst we regard 15 μg per cm an adequate load of oestriol-3,16α,17β in benzene–aqueous methanol[79].

(*b*) *Oestradiol-17α and oestradiol-17β*

Oestradiol-17α and oestradiol-17β are epimeric with respect to the hydroxyl group at C-17. Hydrocarbon–aqueous methanol solvent mixtures in which oestrone, oestradiol-17β and oestriol-3,16α,17β are separated on one chromatogram (see Section 2a) have insufficient resolving power to separate oestradiol-17β and oestradiol-17α. This limitation, if not appreciated, can lead to serious errors in the interpretation of experimental results.

Paper chromatography in *o*-dichlorobenzene has been used by AXELROD[1] to separate these epimers, whilst ROSENKRANTZ[86] achieved a separation with toluene–propylene glycol. Benzene–hexane (1:1)–formamide[91] and benzene–Skellysolv B (1:1)–formamide[100] have also been used.

A clear separation of oestradiol-17α and oestradiol-17β was achieved by paper chromatography in light petroleum (b.p. 80–100°)–aqueous methanol for 41 h[78]. VELLE and his colleagues[38,81] separated the epimers in a system previously described for the separation of pregnanetriols[34]. The mobility of oestradiol-17α was greater than that of oestradiol-17β, but no absolute mobilities were reported.

BOSCOTT[10] separated these compounds on paper treated with sodium *p*-toluenesulphonate and developed with toluene, but no separation was achieved if development was carried out with toluene–methanol (9:1)[11].

Chromatography on glass paper treated with potassium silicate and developed with iso-octane–benzene or with benzene has been described[45] for the separation of these epimers. It is of interest that the mobility of oestradiol-17β reported by these workers is higher than that of oestradiol-17α. In all other cases reviewed the mobility of oestradiol-17β was found to be lower than that of oestradiol-17α.

Only marginal separations of the epimers were achieved by paper chromatography in either butanol–aqueous K_3PO_4 solution[11] or chloroform–formamide[52]. Oestradiol-17α and oestradiol-17β have been separated by the chromatography of coloured derivatives in hydrocarbon–aqueous ethanol systems[47].

(*c*) *The epimeric 3,16,17-oestriols*

The work of BREUER and his colleagues[17] has resulted in two useful methods by which these four epimeric oestriols may be separated. The compounds were chromatographed for 6 h in chloroform–formamide. Oestriol-3,16β,17β (R_F 0.17) and oestriol-3,16α,17α (R_F 0.22) were resolved, although chromatography for a longer time would have improved the separation. Oestriol-3,16α,17β and oestriol-3,16β,17α had the

same R_F value (0.04). This pair of epimers was rechromatographed for 24 h in benzene–aqueous methanol when oestriol-3,16β,17α had the higher mobility. A variation of this method involves a partial separation of the oestriols through the formation of acetonides[76]. The *cis*-glycols oestriol-3,16α,17α and oestriol-3,16β,17β form acetonides. By chromatography of this fraction (as free oestrogens) in chloroform–formamide and the *trans*-glycol fraction (oestriol-3,16β,17α and oestriol-3,16α, 17β) in benzene–aqueous methanol a clear separation of the epimers was achieved. The separation of oestriol-3,16α,17β and oestriol-3,16β,17β has been carried out using both hydrocarbon–aqueous methanol[32,72,78,88] and chloroform–formamide[17,63] systems.

Derivatives of the four epimeric 3,16,17-oestriols prepared by reaction with diazonium salts have been separated by paper chromatography[12,13,19,20].

(d) 6-Hydroxy and 6-oxo-oestrogens

Oestriol-3,6,17β, and oestriol-3,16α,17β could not be separated by chromatography in chloroform–formamide[21]. However, the mobility of oestriol-3,6α,17β was found to be greater than that of oestriol-3,6β,17β in chloroform–ethyl acetate (5:1)–formamide and a separation was achieved[18]. In this system oestriol-3,16α,17β had a higher mobility than both the 3,6,17β-oestriols. In contrast, the mobility of oestriol-3,16α,17β was lower than that of oestriol-3,6'α',17β when chromatographed in benzene–aqueous methanol[30], but these mobilities were reversed on chromatography in toluene–acetic acid. This reversal of mobilities was also observed with the corresponding 3-methyl ethers.

The mobilities of 6α- and 6β-hydroxyoestrone have been determined in chloroform–formamide[16], but are not sufficiently different to permit a separation in this system. Monochlorobenzene–formamide has been used for the chromatography of 6-oxo-oestrone[14], whilst a mixture of aqueous acetic acid and ethylene dichloride has proved useful for the separation of 6-oxo-oestriol and oestetrol-3,6'α',16α,17β[54].

The separation of 6-oxo-oestriol, an oestetrol-3,6,16α,17β and oestriol-3,16α,17β by paper chromatography in benzene–aqueous methanol has been reported[58].

(e) 2-Hydroxyoestrogens

COOMBS[31] and KING[53,54] have described the separation of some 2-hydroxyoestrogens. Oestriol-2,3,17β had a slightly higher R_F value (0.59) than oestriol-3,16α,17β (R_F 0.54) when chromatographed in aqueous acetic acid–methylene dichloride[31]. Oestriol-3,16α,17β had a higher mobility than both oestetrol-2,3,16α,17β and oestetrol-3, 6'α',16α,17β in a similar system[54]. Oestriol-2,3,17β was reported[39] to be readily separated from oestradiol-17β in a hydrocarbon–aqueous methanol system.

(f) Oestrogens with an α-ketol in ring D and 16-oxo-oestrone

After the isolation from human urine of derivatives of oestrone hydroxylated at C-16[24,59,60] many reports of metabolic studies involving these oestrogens have been published. Paper chromatography has been used extensively in these studies.

LAYNE AND MARRIAN[59,60] used paper chromatography in chloroform–formamide to separate 16β-hydroxyoestrone from other urinary oestrogens. BREUER, KNUPPEN AND PANGELS[17] separated 16α-hydroxyoestrone, 16β-hydroxyoestrone and 16-oxo-oestradiol-17β in this system, but the latter compound had the same mobility as 16-oxo-oestradiol-17α. Data reported by LEVITZ, SPITZER AND TWOMBLY[62,63] indicate

that paper chromatography in this system would separate 16α-hydroxyoestrone, 16-oxo-oestradiol-17β, oestriol-3,16β,17β and oestriol-3,16α,17β. Confirmation of this was given, in part, by a report[65] that 16-oxo-oestradiol-17β had a greater mobility than oestriol-3,16β,17β and oestriol-3,16α,17β when chromatographed in chloroform–formamide.

Hydrocarbon–aqueous methanol mixtures have also been widely used. A broad separation of urinary metabolites of oestradiol-17β was carried out by BROWN, FISHMAN AND GALLAGHER[24]. A separation of 16β-hydroxyoestrone from 16-oxo-oestradiol-17β and 16α-hydroxyoestrone (which ran together) was achieved by chromatography in this type of system[36]. 16-Oxo-oestradiol-17β and 16-oxo-oestradiol-17α were separated by chromatography in benzene–light petroleum–aqueous methanol for 25 h[17]. Three different hydrocarbon–aqueous methanol systems capable of the separation of 16-oxo-oestrone from the less mobile 16-oxo-oestradiol-17β have been reported[72]. Other solvent systems of this kind have been used for the separation and purification of 16-oxo-oestrone[93] and in the investigation of urinary oestrogens after partition chromatography on columns[43].

(g) *2-Methoxyoestrogens*

Paper chromatography has been applied to the separation of 2-methoxyoestrogens from the parent compound after metabolic studies. KRAYCHY AND GALLAGHER[56] chromatographed oestrone and 2-methoxyoestrone on paper in chloroform–formamide and reported that the mobility of 2-methoxyoestrone was more than five times that of oestrone. KING[55] has described a system which will separate 2-methoxyoestrone from oestrone and also 2-methoxyoestradiol-17β from oestradiol-17β. Paper chromatography in methylcyclohexane–propylene glycol has been used to purify 2-methoxyoestrone[6] and 2-methoxyoestradiol-17β[3]. ENGEL, BAGGETT AND CARTER[37], FRANDSEN[41] and BREUER AND KNUPPEN[15] carried out purification of these compounds using mixtures of hydrocarbons and aqueous methanol.

3. THE SEPARATION OF OESTROGEN DERIVATIVES

(a) *Esters*

The paper chromatography of esters of steroid oestrogens has not been fully exploited. MARKWARDT[66] separated oestrogen esters by reverse phase paper chromatography on silicone treated paper. SHORT[91] recommended ligroin–aqueous methanol for the separation of the mono- and di-acetates of oestradiol-17β. Oestrone acetate was reported to have an R_F value of "about 0.65" when chromatographed in hexane–formamide[98]. Toluene–propylene glycol has been used for the paper chromatography of oestriol-3,16α,17β-triacetate[99].

The behaviour of esters of oestrone, oestradiol-17β and oestriol-3,16α,17β prepared by reaction with excess *p*-iodophenylsulphonyl chloride was studied by LEEGWATER[61] but the number of hydroxyl groups esterified in each steroid was not determined. These esters were chromatographed in either carbon tetrachloride–paraffin oil or cyclohexane–paraffin oil.

(b) *Conjugates with sulphuric acid or glucuronic acid*

DICZFALUSY and his colleagues have used paper chromatography, with other tech-

niques, to identify oestriol glucosiduronate[69] and oestriol-3-sulphate[70] isolated from human meconium. In each case paper chromatography in several systems was carried out and data for the material isolated and for an authentic specimen were tabulated.

(*c*) *3-Methyl ethers*

Ligroin–96% aqueous methanol has been reported as a system suitable for the paper chromatography of the 3-methyl ethers of oestrogens[94]. A good separation of the 3-methyl ethers of oestradiol-17β and of oestradiol-17α was obtained[81] by paper chromatography in Skellysolv B–formamide.

(*d*) *Derivatives obtained by reaction with diazonium salts*

The separation of the coloured derivatives prepared by coupling oestrogens with diazonium compounds was one of the first applications of paper chromatography to the general problem of the separation of oestrogens[46]. The coloured derivatives were easily located on the developed chromatogram without the necessity for a spray reagent. Data are now available on the behaviour of derivatives of oestrone, oestradiol-17β, oestriol-3,16α,17β[20,46,47,74], oestriol-3,16α,17α[12,13], oestriol-3,16β,17α[19], oestriol-3,16β,17β[13,20], oestradiol-17α[47], 16-oxo-oestradiol-17β, 16β-hydroxyoestrone and 16α-hydroxyoestrone[13]. The paper chromatography of these derivatives has a useful application in the identification of steroid oestrogens by allowing additional comparisons to be made between an unknown compound and authentic material.

4. THE DETECTION OF OESTROGENS ON PAPER CHROMATOGRAMS

The most widely used reagents for the detection of oestrogens are those which depend on the reducing properties of the phenol group.

A mixture of equal volumes of 1% aq. ferric chloride and 1% aq. potassium ferricyanide[7] has been used by many workers[32,78,82,94,99]. FOLIN AND CIOCALTEU'S reagent[40] has also been widely used[22,81]. The limit of detection of oestrogen by this reagent was reported to be 0.5 μg per sq.cm[74], but was afterwards[73] stated to be 5 μg per sq.cm. Neither of these reagents is specific for phenolic oestrogens and care should be taken in the interpretation of sprayed chromatograms. For example, a strong reaction is given with FOLIN AND CIOCALTEU'S reagent by α-ketols[74].

The detection of oestrogens by reaction with a diazonium salt has been reported[11,72]. Stable diazonium compounds are now available and solutions of these make very convenient reagents[60]. The colour can be formed by exposing the paper to ammonia vapour after spraying with diazonium salt. With a solution of Fast Black Salt K, 5 μg per sq.cm of oestrone can be detected[79].

Oestrogens fluoresce when treated with concentrated sulphuric or phosphoric acid. This property has been made the basis of a method of detection by AXELROD[2] and by BOSCOTT[11] whereas other workers have used phosphomolybdic acid[66,84]. AXELROD[2] has reported that 3–5 μg of oestrogen per sq.cm can be detected by the sulphuric acid technique.

The colours produced by oestrogens upon treatment with a variety of reagents, *e.g.* antimony pentachloride[66], ferric chloride[1], antimony trichloride[86], iodine[28], and zinc chloride[77], have been described. Two comprehensive papers[4,57] tabulate data for the colours produced by oestrogens with numerous reagents. The detection of specific

groups in the oestrogen molecule, *e.g.* a phenolic group unsubstituted in the *ortho* position, or an *o*-dihydroxyphenol, has received special attention in a paper by AXELROD AND PULLIAM[5]. Tests to differentiate between hydroxyl groups at C-17 in the (α) and (β) configuration have been described by KÄGI AND MIESCHER[51]. DAVID[33] reported that oestriol-3,16α,17β alone gave a blue colour with a mixture of sulphuric and arsenious acids. DICZFALUSY, TILLINGER AND WESTMANN[35], however, found that oestriol-3,16β,17β and the 3-methyl ethers of oestriol-3,16α,17β and oestriol-3,16β,17β also formed a blue colour.

Derivatives of oestrogens in which the 3-hydroxy group is esterified will not react with reagents for the phenol group. This difficulty can be overcome[91] by hydrolysis of the ester *in situ*[102]. Phosphomolybdic acid[66] has been used to detect oestrogen esters whilst a mixture of *p*-phenolsulphuric and phosphoric acids has been used to detect oestrogen 3-methyl ethers[94]. These ethers have been detected also by examination, in ultra violet light, of chromatograms treated with sulphuric acid[81].

Detection of oestrogens or their derivatives by location of a radioactive atom present in the molecule is a most useful technique (*e.g.*[61]). BRODA[23] has reviewed methods for the detection of radioactive compounds on paper chromatograms whilst several other reports[42,64,87,90,97,101] have described the use of liquid scintillation counters for this purpose. The scope of this method is so wide that it will not be considered further in this review.

5. THE RELATIONSHIP BETWEEN THE MOBILITY AND CHEMICAL STRUCTURE OF SOME OESTROGENS

A study of the relationship between the sequence of mobility and chemical structure of a series of 17-oxo-steroids has been presented by SAVARD[89]. This author was able to show that the mobility of certain 17-oxo-steroids chromatographed on paper in ligroin–propylene glycol was inversely proportional to the polarity of the oxygen functions in the molecule.

Sufficient data from one particular solvent system are not available for a detailed analysis of this relationship for oestrogens. However, from data presented in the foregoing review the following observations can be made.

As expected from the rules proposed by SAVARD[89] the mobility sequence for the three "classical" oestrogens is oestrone > oestradiol-17β > oestriol-3,16α,17β. It is clear also that oestradiol-17α has a higher mobility than oestradiol-17β, indicating that the C-17α hydroxyl group is less hydrophilic than the C-17β hydroxyl group.

A study of the work of BREUER and his colleagues[17,70] indicates that the mobility sequence for the epimeric 3,16,17-oestriols is oestriol-3,16α,17α > oestriol-3,16β,17β > oestriol-3,16β,17α > oestriol-3,16α,17β. It is noteworthy that the *cis*-glycols (oestriol-3,16α,17α and oestriol-3,16β,17β) have a higher mobility than the corresponding *trans*-glycols (oestriol-3,16β,17α and oestriol-3,16α,17β).

A comparison of the effect on mobility of a hydroxyl group at C-6 and at C-16 can be made from data provided by BREUER, KNUPPEN AND PANGELS[18]. The mobilities of oestriol-3,6α,17β and oestriol-3,6β,17β, are lower than that of oestriol-3,16α,17β when chromatographed in chloroform–ethyl acetate–formamide. It is evident therefore, that, in this series of compounds, a hydroxyl group at C-6 is more hydrophilic

than one at C-16. An explanation of this effect is that the hydrophilic properties of the hydroxyl group at C-16 are reduced by hydrogen bonding to the adjacent hydroxyl group at C-17, whereas no such hydrogen bonding takes place at C-6.

A similar explanation can be made of the fact that the mobility of oestriol-2,3,17β is nearer to the mobility of oestriol-3,16α,17β than to that of oestriol-3,6α,17β. In this case the relative loss of the hydrophilic properties of the C-2 hydroxyl group is probably due to hydrogen bonding with the C-3 hydroxyl group. The mobility of oestriol-2,3,17β is slightly higher than that of oestriol-3,16α,17β but is lower than that of oestradiol-17β when chromatographed in methylene dichloride–aqueous acetic acid[31].

On the basis of the foregoing comparisons, it is evident that, for oestradiol-17β, the replacement of a hydrogen atom at C-6 in the (β) conformation by a hydroxyl group causes the greatest decrease in mobility, followed by substitution at C-6 (α), C-16 (α) and C-2. Sufficient data are not available to compare the mobilities of oestriol-2,3,17β and the four epimeric 3,16,17-oestriols.

(*Text continued on p. 13*)

TABLE I

R_F VALUES OR MOBILITIES IN cm/h (MARKED*) FOR OESTRONE

Solvent system	R_F	*Ref.*
Chloroform–formamide	0.80	52
Chloroform–formamide	0.93	83
Carbon tetrachloride–formamide	0.41	83
o-Dichlorobenzene–formamide	4.7*	1, 2
Ethylene dichloride–formamide	0.88	83
Toluene–formamide	0.78	83
Benzene–Skellysolv B (1:1)–formamide	2.7*	100
Carbon tetrachloride–ethylene glycol	0.50	96
Toluene–propylene glycol	0.20	71
Cyclohexane–triethylene glycol	0.03	95
Light petroleum–triethylene glycol	0.04	95
Carbon tetrachloride–triethylene glycol	0.16	95
Benzene–triethylene glycol	0.37	95
Light petroleum (100–120°)–methanol (1:1)	0.2	73, 74
Skellysolv C–methanol–water (5:4:1)	0.14	85
Benzene–Skellysolv C–methanol–water (33:67:80:20)	0.52	85
Benzene–Skellysolv C–methanol–water (33:67:80:20)	0.33	93
Benzene–Skellysolv C–methanol–water (40:60:70:30)	0.65	72
Benzene–methanol–water (10:8:2)	0.85	84
Benzene–methanol–water (10:7:3)	0.91	78
Benzene–methanol–water (100:55:45)	0.88	72
Toluene–methanol–water (100:75:25)	0.84	94
Toluene–ligroin–methanol–water (34:66:70:30)	0.61	94
Toluene–iso-octane–methanol–water (75:25:80:20)	0.78	72
Toluene–Skellysolv C–methanol–water (5:5:7:3)	0.74	85
Toluene–ethyl acetate–methanol–water (9:1:6:4)	0.86	94
Chloroform–benzene–*N* NH_4OH (1:9:1)	0.89	49
Benzene–hexane–methanol–2 *N* NH_4OH (65:35:15:15)	0.85	71
Benzene (alumina paper)	0.4	28
Benzene–iso-octane (2:1) (silicate paper)	0.69	45
Benzene (silicate paper)	0.88	45
Toluene–methanol (9:1)–*p*-toluenesulphonate	0.50	11
Toluene–*p*-toluenesulphonate	0.78	10
5% aq. K_3PO_4 saturated with *n*-butanol	0.35	11

TABLE II

R_F VALUES OR MOBILITIES IN cm/h (MARKED*) FOR OESTRADIOL-17β AND OESTRADIOL-17α

Oestradiol-17β		
Solvent system	R_F	*Ref.*
Chloroform–formamide	0.50	52
Chloroform–formamide	0.81	83
Carbon tetrachloride–formamide	0.11	83
o-Dichlorobenzene–formamide	1.8*	1, 2
Ethylene dichloride–formamide	0.75	83
Toluene–formamide	0.41	83
Benzene–Skellysolv B (1:1)–formamide	0.73*	100
Cyclohexene–formamide	0.73*	1
Carbon tetrachloride–ethylene glycol	0.12	96
Toluene–propylene glycol	0.03	71
Light petroleum (100–120°)–methanol (1:1)	0.07	73, 74
Light petroleum (80–100°)–methanol–water (10:8:2)	0.24*	78
Benzene–Skellysolv C–methanol–water (33:67:80:20)	0.21	85
Benzene–Skellysolv C–methanol–water (40:60:70:30)	0.28	72
Benzene–methanol–water (100:55:45)	0.79	72
Benzene–methanol–water (10:8:2)	0.70	84
Benzene–methanol–water (10:7:3)	0.77	78
Toluene–ligroin–methanol–water (34:66:70:30)	0.23	94
Toluene–iso-octane–methanol–water (75:25:80:20)	0.54	72
Toluene–Skellysolv C–methanol–water (5:5:7:3)	0.47	85
Toluene–methanol–water (100:75:25)	0.61	94
Toluene–ethyl acetate–methanol–water (9:1:6:4)	0.80	94
Chloroform–benzene–*N* NH_4OH (1:9:1)	0.78	49
Benzene–hexane–methanol–2 *N* NH_4OH (65:35:15:15)	0.65	71
Methylene dichloride–acetic acid–water (10:7:3)	0.78	31
Benzene (alumina paper)	0.1	28
Benzene–iso-octane (2:1) (silicate paper)	0.52	45
Benzene (silicate paper)	0.73	45
Toluene–methanol (9:1)–*p*-toluenesulphonate	0.21	11
Toluene–*p*-toluenesulphonate	0.27	10
5% aq. K_3PO_4 saturated with *n*-butanol	0.28	11

Oestradiol-17α		
Solvent system	R_F	*Ref.*
Chloroform–formamide	0.60	52
o-Dichlorobenzene–formamide	2.3*	1, 2
Benzene–Skellysolv B (1:1)–formamide	1.1*	100
Light petroleum (100–120°)–methanol–water (10:8:2)	0.35*	78
Benzene–methanol–water (10:7:3)	0.78	78
Benzene–iso-octane (2:1) (silicate paper)	0.38	45
Benzene (silicate paper)	0.57	45
Toluene–methanol (9:1)–*p*-toluenesulphonate	0.21	11
Toluene–*p*-toluenesulphonate	0.45	10
5% aq. K_3PO_4 saturated with *n*-butanol	0.30	11

TABLE III

R_F VALUES OR MOBILITIES IN cm/h (MARKED*) FOR THE EPIMERIC 3,16,17-OESTRIOLS

Oestriol-3,16α,17β		
Solvent system	R_F	*Ref.*
Methylene dichloride–formamide	0.6*	1, 2
Chloroform–formamide	0.04	17, 76
Chloroform–formamide	0.13	83
Chloroform–formamide	0.31*	20
o-Dichlorobenzene–formamide	0.2*	2
Carbon tetrachloride–formamide	0	83
Ethylene dichloride–formamide	0.07	83
Toluene–ethyl acetate–methanol–water (9:1:6:4)	0.33	94
Toluene–methanol–water (100:75:25)	0.08	94
Benzene–methanol–water (100:50:50)	0.13	73, 74
Benzene–methanol–water (100:50:50)	1.25*	58
Benzene–methanol–water (100:55:45)	0.09	72
Benzene–methanol–water (100:55:45)	0.37*	17, 22, 76
Benzene–methanol–water (100:55:45)	0.64*	20
Benzene–methanol–water (100:70:30)	0.10	78
Benzene–methanol–water (100:80:20)	0.16	84
Toluene–iso-octane–methanol–water (75:25:80:20)	0.03	72
Benzene–Skellysolv C–methanol–water (40:60:80:20)	0	72
Ethylene dichloride–acetic acid–water (10:7:3)	0.6	53, 54
Methylene dichloride–acetic acid–water (10:7:3)	0.54	31
5% aq. K_3PO_4 saturated with *n*-butanol	0.40	11
Toluene–methanol (9:1)–*p*-toluenesulphonate	0.02	11
Toluene–*p*-toluenesulphonate	0	10
Chloroform–benzene–*N* NH_4OH (1:9:1)	0.02	49
Benzene–iso-octane (2:1) (silicate paper)	0*	45

Oestriol-3,16β,17β		
Solvent system	R_F	*Ref.*
Chloroform–formamide	0.17	17, 20, 76
Chloroform–formamide	0.86*	63
Benzene–methanol–water (100:55:45)	0.48	20
Benzene–methanol–water (100:55:45)	0.44	72
Benzene–methanol–water (100:70:30)	0.39	78
Benzene–Skellysolv C–methanol–water (40:60:70:30)	0.02	72
Toluene–iso-octane–methanol–water (75:25:80:20)	0.16	72
Ethylene dichloride–acetic acid–water (10:7:3)	0.7	54

Oestriol-3,16α,17α		
Solvent system	R_F	*Ref.*
Chloroform–formamide	0.22	17, 76
Chloroform–formamide	1.8*	12

Oestriol-3,16β,17α		
Solvent system	R_F	*Ref.*
Chloroform–formamide	0.04	17
Benzene–methanol–water (100:55:45)	0.49*	17, 22, 76

TABLE IV

R_F VALUES OR MOBILITIES IN cm/h (MARKED*) FOR OESTROGENS WITH AN α-KETOL GROUP IN RING D AND 16-OXO-OESTRONE

Solvent system	R_F	*Ref.*
16-Oxo-oestradiol-17β		
Chloroform–formamide	0.33	17
Chloroform–formamide	1.2*	63
Chloroform–formamide	2.4*	62
Chloroform–formamide	2.0*	60
Benzene–methanol–water (100:55:45)	0.64	72
Benzene–methanol–water (100:50:50)	0.70	36
Toluene–iso-octane–methanol–water (75:25:80:20)	0.31	72
Benzene–Skellysolv C–methanol–water (40:60:70:30)	0.08	72
Benzene–light petroleum–methanol–water (33:67:80:20)	0.37*	17
5% aq. K_3PO_4 saturated with *n*-butanol	0.40	11
Toluene–methanol (9:1)-*p*-toluenesulphonate	0.11	11
16-Oxo-oestradiol-17α		
Chloroform–formamide	0.33	17
Benzene–light petroleum–methanol–water (33:67:80:20)	0.45*	17
16β-Hydroxyoestrone		
Chloroform–formamide	0.28	17
Chloroform–formamide	1.6*	60
Benzene–methanol–water (10:5:5)	0.58	36
16α-Hydroxyoestrone		
Chloroform–formamide	0.40	17
Chloroform–formamide	1.4*	63
Chloroform–formamide	2.2*	60
Benzene–methanol–water (10:5:5)	0.71	36
16-Oxo-oestrone		
Toluene–iso-octane–methanol–water (75:25:80:20)	0.49	72
Benzene–Skellysolv C–methanol–water (40:60:70:30)	0.17	72
Benzene–Skellysolv C–methanol–water (33:67:80:20)	0.14	93
Benzene–methanol–water (100:55:45)	0.84	93
Benzene–methanol–water (100:55:45)	0.75	72

TABLE V

R_F VALUES OR MOBILITIES IN cm/h (MARKED *) OF OESTROGENS WITH AN OXYGEN FUNCTION AT C-6

(Reference numbers are shown in brackets)

Oestrogens	*Solvent system*					
	Chloroform–formamide	*Monochlorobenzene–formamide*	*Chloroform–ethyl acetate (5:1)–formamide*	*Benzene–methanol–water (10:5:5)*	*Benzene–light petroleum–methanol–water (33:67:80:20)*	*Ethylene dichloride–acetic acid–water (10:7:3)*
6α-Hydroxyoestrone	0.59* (16)				2.8* (16)	
6β-Hydroxyoestrone	0.61* (16)					
6-Oxo-oestrone		0.23 (14)				
6α-Hydroxyoestradiol-17β			1.05* (18)			
6β-Hydroxyoestradiol-17β			0.91* (18)			
6-Hydroxyoestriol-3,16α,17β				0.01* (58)		0.1 (54)
6-Oxo-oestriol-3,16α,17β				0.27* (58)		0.4 (54)

TABLE VI

R_F VALUES OR MOBILITIES IN cm/h (MARKED *) OF OESTROGENS SUBSTITUTED AT C-2

(Reference numbers are shown in brackets)

Oestrogens	*Solvent systems*					
	Methylcyclohexane–propylene glycol	*Benzene–hexane–methanol–water (8:2:8:2)*	*Benzene–light petroleum–methanol–water (33:67:8:2)*	*Toluene–ligroin–methanol–water (67:33:70:30)*	*Methylene dichloride–acetic acid–water (10:7:3)*	*Ethylene dichloride–acetic acid–water (10:7:3)*
2-Methoxyoestrone	1.4* (6)			0.74 (37)		
2-Methoxyoestradiol-17β	0.2* (3)		0.81 (15)			
2-Hydroxyoestrone					0.73 (31)	
2-Hydroxyoestradiol-17β		0.2 (39)			0.59 (31)	
2-Hydroxyoestriol-3,16α,17β					0.23 (31)	0.2 (54)

Amongst oestrogens which have an α-ketol group in ring D certain comparisons may be made. All these compounds have lower mobilities than oestradiol-17β[17,72] which is in accordance with SAVARD's proposals[89]. 16-Oxo-oestradiol-17α has a higher mobility than 16-oxo-oestradiol-17β in a hydrocarbon–aqueous methanol system[17]. 16α-Hydroxyoestrone has a higher mobility than 16β-hydroxyoestrone in both benzene–aqueous methanol[30] and chloroform–formamide[60]. Both illustrations indicate that a hydroxyl group at either C-16 or C-17 in the (β) conformation is more hydrophilic than in the (α) conformation even in the presence of an adjacent ketone group.

TABLE VII

R_F VALUES OR MOBILITIES IN cm/h (MARKED *) FOR OESTROGEN DERIVATIVES OBTAINED BY REACTION WITH FAST BLACK SALT K

(Reference numbers are shown in brackets)

Derivative of	*Solvent system*		
	Toluene–light petroleum (35–60°)–ethanol–water (20:10:3:7)	*Toluene–light petroleum (35–60°)–ethanol–water (20:25:3:7)*	*Toluene–light petroleum (35–60°)–ethanol–water (10:20:1:9)*
Oestrone	0.95 (46)	0.91 (74)	0.29 (46)
Oestradiol-17β	0.81 (46)	0.63 (74)	0.09 (46)
Oestradiol-17α			0.32 (46)
Oestriol-3,16α,17β	0.12* (20); 0.07 (46, 13)		0 (46)
Oestriol-3,16β,17β	0.46 (13, 20)		
Oestriol-3,16α,17α	0.33* (12); 0.32 (13)		
Oestriol-3,16β,17α	0.70* (19)		
16α-Hydroxyoestrone	0.60 (13)		
16β-Hydroxyoestrone	0.56 (13)		
16-Oxo-oestradiol-17β	0.77 (13)		

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SUMMARY

The separations of steroid oestrogens achieved by paper chromatography have been reviewed with special reference to studies involving epimeric oestrogens and to oestrogens which have been isolated recently. Methods of detection of these compounds on paper chromatograms have also been discussed. The mobilities of thirty-four different oestrogens and their derivatives in many solvent systems have been tabulated.

It is hoped that this review will be found useful in providing a collection of data from which separations of mixtures of oestrogens can be planned.

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