THE PAPER CHROMATOGRAPHY OF STEROID ESTERS

R. W. H. EDWARDS*

Kings College Hospital Medical School, London (Great Britain)

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Chromatographic procedures for the separation of steroids are now well established and have been the subject of reviews by BUSH[1], HEFTMANN[2], NEHER[3] AND EDWARDS[4]. Di-ketones, di-esters and keto-esters of the steroids generally move with the solvent front and, until recently, have only been adequately resolved by reversed phase partition techniques. KRITCHEVSKY AND CALVIN[5] treated the paper with Quilon (stearato-chromic chloride) and KRITCHEVSKY AND TISELIUS[6] treated it with silicone.

NEHER AND WETTSTEIN[7] impregnated the paper with phenyl cellosolve and eluted with hexane in a non-reversed phase procedure but this, like the reversed phase methods, leads to gross contamination of the steroids when recovering them unchanged after chromatography.

ZANDER AND SIMMER[8] modified the solvent mixture of BUSH[9] and obtained improved separations, illustrated by the R_F** change for progesterone from 91 to 72. This type of solvent mixture, petroleum or ligroin with aqueous methanol, is ideal when recovery of the steroid is required and the procedure involved is capable of completion in much shorter times than are the previously described methods. EDWARDS[4] showed that the resolution could be improved by employing partition between anhydrous methanol and petroleum at 4°, giving an R_F of 25 for progesterone and an effective separation of esters.

The resolution of these relatively "low-polar" steroid derivatives is further improved in the partition between formic acid and light petroleum recently reported in preliminary form[10] and now to be fully described.

EXPERIMENTAL

The general procedures of steroid chromatography are described elsewhere[4] and only special points will be elaborated.

Solvents

Formic acid, A.R.; light petroleum, b.p. 80–100°, A.R.; methanol and benzene were used as supplied.

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* Miles Ames Fellow, 1957 to 1959. Present address: The Institute of Child Health, The Hospital for Sick Children, Great Ormond Street, London, W.C. 1.

** R_F is expressed as the percentage fraction of the distance moved by the solvent front.

Apparatus

(1) The "Universal Chromatography Apparatus" described by SMITH[11] and supplied by Messrs. Aimer Products, 56 Rochester Place, London, N.W. 1, was used. The Datta frame carries five papers in a vertical position for ascending chromatography. For the equilibration it is suspended by cross wires and a rod (Fig. 1) and then lowered into the solvent in the tray for the chromatographic run.

(2) Cylindrical jar (30 cm high, 10 to 15 cm diam.) and lid with central hole (7 mm diam.).

Paper

Whatman grades numbered 1, 2, 4 and 3MM supplied in 25 cm squares with corner holes are assembled into the frame or sewn into cylinders by cotton thread and used in the jars.

Steroids

Reference steroids were commercial products or were kindly supplied by Dr. W. KLYNE from the M. R. C. Reference steroid collection. Formates were prepared on an

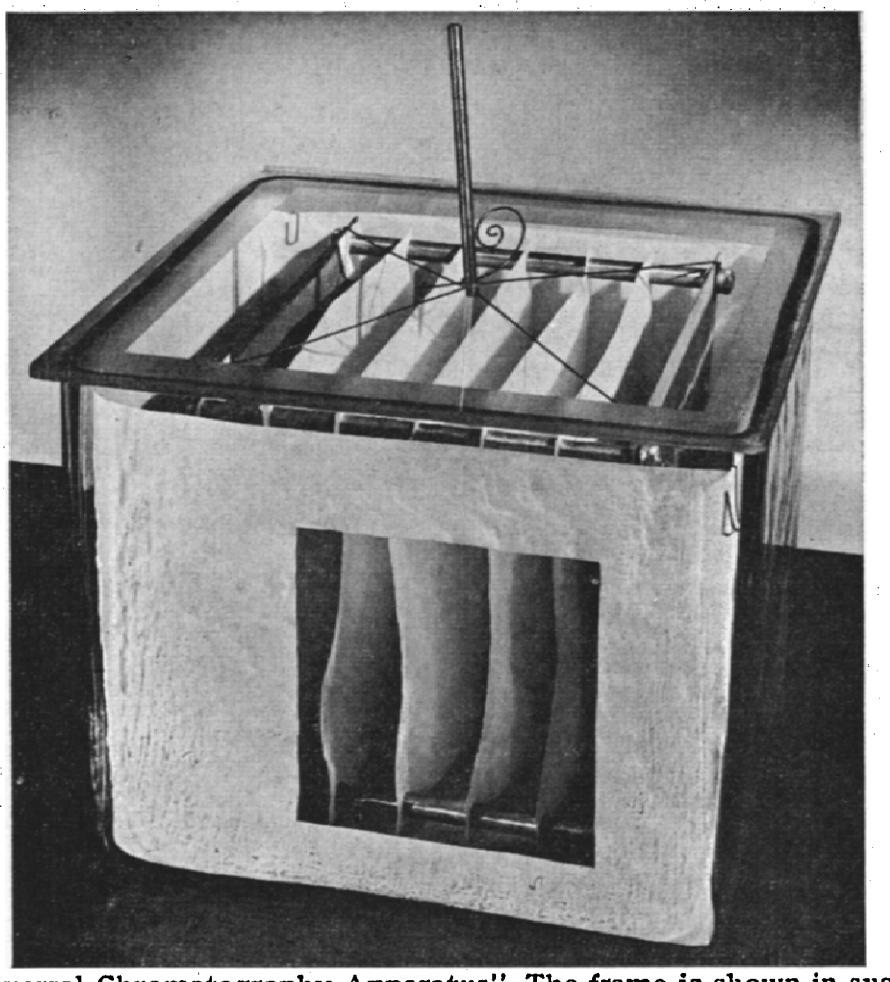

Fig. 1. The "Universal Chromatography Apparatus". The frame is shown in suspended position for equilibration. The curled pin is removed and the frame lowered into the mobile phase to start ascent of solvent.

approximate 100 μg scale by standing the steroids in formic acid solution overnight. The excess acid was evaporated in a stream of nitrogen at 40°; no further purification was employed. Acetates and propionates were prepared by treating 100 μg quantities of steroid with the respective anhydride (0.2 ml) and pyridine (0.2 ml). After standing overnight the solvents were removed in a stream of nitrogen at 40°, assisted when necessary by the additions of small volumes of toluene.

Location of steroids on chromatograms

Steroids were located[4] by use of characteristic physical and chemical properties, including the absorption of 254 mμ ultraviolet light, reaction with alkaline *m*-dinitrobenzene and reduction of phosphomolybdic acid.

Chromatography

The steroids to be chromatographed were placed on origins 2 cm apart along a line drawn 4 cm from one edge of the 25 cm square paper and elution was effected by the ascending solvent by one of two procedures.

(*1*) *The Universal Apparatus.* Up to five papers were mounted in the frame, separated from one another by dural collars. Between the outer collars and the side plates of the frame were placed small paper "washers" to absorb condensation from the metal. Two cross wires were fixed to the top four corner nuts and these wires passed through holes near one end of a brass rod (6 mm diam.) at the point where they crossed, and by this means the frame could be suspended freely by lifting the rod (Fig. 1).

The walls of the Universal Chromatography tank were lined with filter paper (3MM), except for a window cut to permit observation of the movement of the solvent front. This paper was soaked with stationary phase (200 ml) placed in the bottom of the tank and surrounding the dural trough charged with mobile phase (200 ml). The rod carrying the frame and papers was passed through the central hole of the lid of the tank and was pinned so that the frame hung, evenly balanced, from the lid when it was raised. On sealing the lid on the tank with silicone grease the chromatogram papers were suspended in position for equilibration. After 4 h at room temperature the chromatographic run was started by lowering the frame into the trough of mobile phase.

(*2*) *The jar chromatographic apparatus.* The jar was lined with paper (3MM) except for an observation window (5 × 5 cm). This paper was soaked with stationary phase (25 ml) placed in the bottom of the jar and surrounding a Petri dish. The chromatogram was sewn into a cylinder (with a 5 mm gap between the meeting edges) by knotted strands of cotton at three evenly spaced intervals along the length. The lid was sealed in position with silicone grease and after 4 h equilibration the mobile phase (10 ml) was added through a long stemmed funnel inserted temporarily through the central hole and which touched the Petri dish.

In each apparatus the ascent of the solvent front to within 1 cm of the top of the papers took about 45 min at room temperature. The papers were then removed and the solvent evaporated in a stream of air. After location of the steroids the R_F values were calculated and expressed as the percentage fraction of the distance moved by the solvent front.

Solvent mixtures

Formic acid and light petroleum were mixed in the proportions given in Table I with the inclusion of other solvents as required.

TABLE I

R_F VALUES OF STEROIDS AND STEROID ESTER IN FORMIC ACID–LIGHT PETROLEUM MIXTURES

The R_F values of non-esterified alcohols were observed after 0.5 h equilibration at approx. 18°, those of the other steroids after 4 h equilibration.

Designation		*F0*		*F1*				*F2*	
*Solvents**	*Formic acid*	*100*		*100*				*100*	
	Light petroleum (80–100°)	*100*		*90*				*50*	
	Benzene							*50*	
	Methanol			*10*					
*Steroid***		*Free*	*Ft*	*Free*	*Ft*	*Ac*	*Pr*	*Free*	*Ft*
Cholestenone		96		98					
Oestrone									22
Androstane derivatives									
5α-Androstan-17-one		95		97					
Androst-5-en-3β-ol			93		93				
5α-Androstane-3α,17β-diol			77		84				87
5β-Androstane-3α,17β-diol			78		88				89
Androst-5-ene-3β,17α-diol			76		86				89
Androst-5-ene-3β,17β-diol			78		85				89
17α-Methyl-5α-androstane-3β,17β-diol		11	48		66				82
17α-Methyl-androst-5-ene-3β,17β-diol			48		55				79
5α-Androstan-3β-ol-17-one			25		54	62	77		68
5α-Androstan-3α-ol-17-one		7.5	32	11	51	59	78	55	68
5β-Androstan-3α-ol-17-one		5.5	29	8	52	57	73	31	74
5β-Androstan-3β-ol-17-one		6	30					47	82
5β-Androst-9(11)-en-3α-ol-17-one		7.5	32	11	58	64	81	51	76
Androst-5-en-3β-ol-17-one		5	28	9	51	56	74	29	72
5β-Androstan-17β-ol-3-one		5	25	9	43				75
Androst-4-en-17β-ol-3-one			8	3	20	23	42	16	49
Androst-4-en-17α-ol-3-one			8	3	19	20	39	16	49
Androsta-[illegible],4-dien-17β-ol-3-one									29
17α-Methylandrost-4-en-17β-ol-3-one		0		6		32		25	
19-Nor-androst-4-en-17β-ol-3-one			8		16				48
5α-Androstane-3,17-dione		5		13				46	
5β-Androstane-3,17-dione		5		13				45	
Androst-4-ene-3,17-dione		3		3				20	
Androsta-1,4-diene-3,17-dione								10	
Androst-4-ene-3,11,17-trione				2				24	
Androst-4-en-11β-ol-3,17-dione				0				2	
5β-Androstane-3α,11β-diol-17-one					3				27
Androst-4-ene-11β,17β-diol-3-one					2				13
Pregnane derivatives									
5α-Pregnan-3β-ol			91		93				
5β-Pregnan-3β-ol			83		93				
5α-Pregnane-3α,20α-diol			86		88				93
5β-Pregnane-3α,20α-diol			86		88				94
Pregn-5-ene-3β,20α-diol			85		88				92
Pregn-5-ene-3β,20β-diol			84		89				93
5α-Pregnane-3α,17α,20α-triol			23		36				70

(continued on p. 216)

TABLE I (*continued*)

Designation		*Fo*		*F1*				*F2*	
*Solvents**	*Formic acid*	*100*		*100*				*100*	
	Light petroleum (80–100°)	*100*		*90*				*50*	
	Benzene							*50*	
	Methanol			*10*					
*Steroid***		*Free*	*Ft*	*Free*	*Ft*	*Ac*	*Pr*	*Free*	*Ft*
5β-Pregnane-3β,16β,20α-triol			22						73
5α-Pregnan-3α-ol-20-one			70	35	76				86
5β-Pregnan-3α-ol-20-one			65	28	77				89
Pregn-4-en-20β-ol-3-one			19		28	33	52		72
Pregn-5-en-3β-ol-20-one			61	23	76	80	86	60	86
Pregna-5,16-dien-3β-ol-20-one								58	86
5α-Pregnane-3,20-dione		19		36				68	
5β-Pregnane-3,20-dione		19		36				68	
Pregn-4-ene-3,20-dione		4		11				48	
Pregna-4,16-diene-3,20-dione								49	
16α-Methyl-pregn-4-ene-3,20-dione								62	
Pregn-4-ene-3,11,20-trione				0				8	

* Top phase is mobile phase.
** Ft = formate; Ac = acetate; Pr = propionate; mono-, di- or tri-ester as appropriate.

RESULTS

Many solvent mixtures were tried but the three given in Table I were extensively investigated because of the separation of the three more important groups of steroid derivatives.

Formylation of 3α, 3β, 11α, 17α- and 17β-androstane, 20α and 20β hydroxyl groups was observed during equilibration since, with longer equilibration, a substance of low mobility on the chromatogram was replaced by a substance of higher mobility which possessed the same R_F as the formate prepared independently. 11β- and 17α-pregnane hydroxyls were not esterified. When acetates or propionates were chromatographed there was no evidence of exchange of formoxy- for acetoxy- or propoxy-groups.

Reliability of the chromatographic procedure

R_F values of reference steroids in a given solvent mixture were constant to within 3 units and independent of, (1) position on the paper, (2) weight of the paper (*i.e.* Whatman grade 1, 2, 4 or 3MM), (3) position of paper in the frame, (4) type of apparatus employed (Universal apparatus or jar) and, (5) occasion of run.

Effect of variation of solvent constitution

Substitution of lower or higher boiling grades of light petroleum or ligroin for the 80–100° grade had little effect except that "tailing" occurred with the highest boiling fractions.

Load of steroid

When portions progesterone from 100 to 2000 μg were applied to origins of a single paper and run in the F2 system, little enlargement of the spots occurred up to 500 μg/cm². At 1000 μg/cm² spot diameter increased by 50% and at twice this oad the diameter was nearly trebled.

DISCUSSION

The procedures described facilitate the paper chromatography of those androstane and pregnane derivatives which possess two weakly polar substituents, such as ketones or esterified alcohols. The 4-h equilibration prior to chromatography is the time required for formylation of the unesterified hydroxyl groups. The 11β-hydroxyl and the 17α-hydroxy-pregnane are the anticipated exceptions since these are hindered groups of the type discussed by KLYNE[12]. This condition of esterification is mild and similar to that of formylation in formic acid solution at 100m temperature (pyridine catalyst is not required).

Whilst the use of the light petroleum and formic acid mixture was suitable for the chromatography of the esters of dihydroxy-steroids, addition of methanol or benzene was necessary for the esters of mono-ketonic alcohols and the diketones.

Pairs of isomeric steroids were separated to a lesser or greater degree. Thus, whilst epimeric 3, 5 (including -5-ene-), 17 and 20 substituents were not well resolved, if at all, larger scale isomers were resolved as shown by the separation of the groups of androstan-3-on-17-ols and androstan-17-on-3-ols.

The effect of substituents tended to be much greater, particularly that of the polar groups, such as the 11-ketone and 11-hydroxyl. A somewhat smaller but still powerful polar effect was associated with the addition of a -4:5- double bond in conjugation with a -3-ketone, as illustrated by the decrease of R_F by 23 units when testosterone was formed. No such change was associated with the -16:17- double bond in conjugation with a 20-ketone (from pregnenolone). The isolated -9:11- double bond when added to aetiocholanolone produced an R_F increase of 6 units. This decreased polarity must be explained by an association with benzene of the mobile phase, rather than with formic acid of the stationary phase.

The difference of property of pregnane derivatives in comparison to otherwise similar androstane derivatives can be considered as due to a substituent, that of the two carbon side chain. It is possible that other changes in the carbon skeleton would have similar marked effects but the following few examples are hardly enough for generalisation.

Addition of a methyl group has an effect, since 16α-methyl-progesterone is considerably less polar than progesterone and testosterone is less polar than its 19-nor-analogue. The effect of the 17α-methyl substituent in the androstane series is masked by the concomitant change of the 17β-hydroxyl to tertiary character, but 17β-acetoxy-methyl-testosterone is less polar than testosterone acetate.

Esterification had the most pronounced effect since the free steroids have very much lower R_F values than the esters. Each esterifying group had a different effect, polarity decreasing in the order formate, acetate, propionate. Such esterification had a much greater effect on R_F than a corresponding change to a ketone which results in an inversion of the order of separation of the major groups, diols, ketols and diones, when comparing the formic acid chromatography with procedures for free steroids, such as light petroleum–methanol partition[9].

Application of the light petroleum and formic acid mixtures to the chromatography of steroids of a higher degree of oxygenation and to that of crude mixtures of biological origin is in hand. Valuable results are expected, particularly since the steroids and their esters are highly soluble in both phases of the solvent mixture.

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SUMMARY

1. Two phase solvent mixtures are described in which di-substituted steroid ketones and esters have partition coefficients such that they may be readily separated by partition chromatography on paper.

2. 80 steroids and derivatives have been characterised in these solvent systems and their R_P values are presented. Notable features include the resolution of otherwise similar and ostane and pregnane derivatives, 3-hydroxy-17-ketone from 17-hydroxy-3-ketones in the androstane series, aetiocholanolone from its -9:11- unsaturated analogue and substantial inversion of general order of separation as compared with the order observed in procedures for unesterified steroids.

3. Up to 0.5 mg of steroid or derivative per cm^2 may be chromatographed from each origin.

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