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A COMPARISON OF CHROMATOGRAPHIC METHODS FOR THE ASSESSMENT OF THE DISTRIBUTION COEFFICIENTS OF ANDROGEN ESTERS

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SUMMARY

Gas–liquid and thin-layer chromatographic methods for the determination of androgen esters are described. Relative distribution coefficients for three homologous series are derived and compared with those obtained in two recent papers, by thin-layer[1] and paper[2] chromatography, respectively. Reasons are advanced for the differences between the three sets of results, and those for testosterone are correlated with biological observations.

INTRODUCTION

Two papers, describing the estimation of distribution coefficients of testosterone (17β-hydroxyandrost-4-en-3-one) esters by partition chromatography, have appeared in the literature recently. The first[1] used a modified BOYCE–MILBORROW[3] thin-layer chromatographic (TLC) technique, and yielded results which correlated with the haemolytic activities of the steroids. The second[2] used a Bush system on paper and gave results which were proportional to the ratios of the solubilities in water and cyclohexane, and which correlated with the durations of androgenic activity in the rat[4].

Both communications are based on eqns. 1 and 2

$$\log \alpha = R_M + k \quad (1)$$

$$\log \alpha_A = k' \log \alpha_B + k'' \quad (2)$$

where α signifies the distribution coefficient, with the suffixes A and B representing two different solvent systems; k, k', and k'' are constants, dependent on experimental conditions.

Eqn. 1 was developed theoretically by BATE-SMITH AND WESTALL[5] and eqn. 2 derived by COLLANDER[6] from experimental results. Combination of eqns. 1 and 2 yields eqn. 3, indicating that the R_M values from the one procedure should be proportional to those from the other

$$R_{M(A)} = k' R_{M(B)} + k''' \quad (3)$$

where k''' is a constant.

This is not the case with the measurements cited above, since eqn. 1 yields a linear relationship between R_M and the position in the homologous series whilst ref. 2 does not (Fig. 1A).

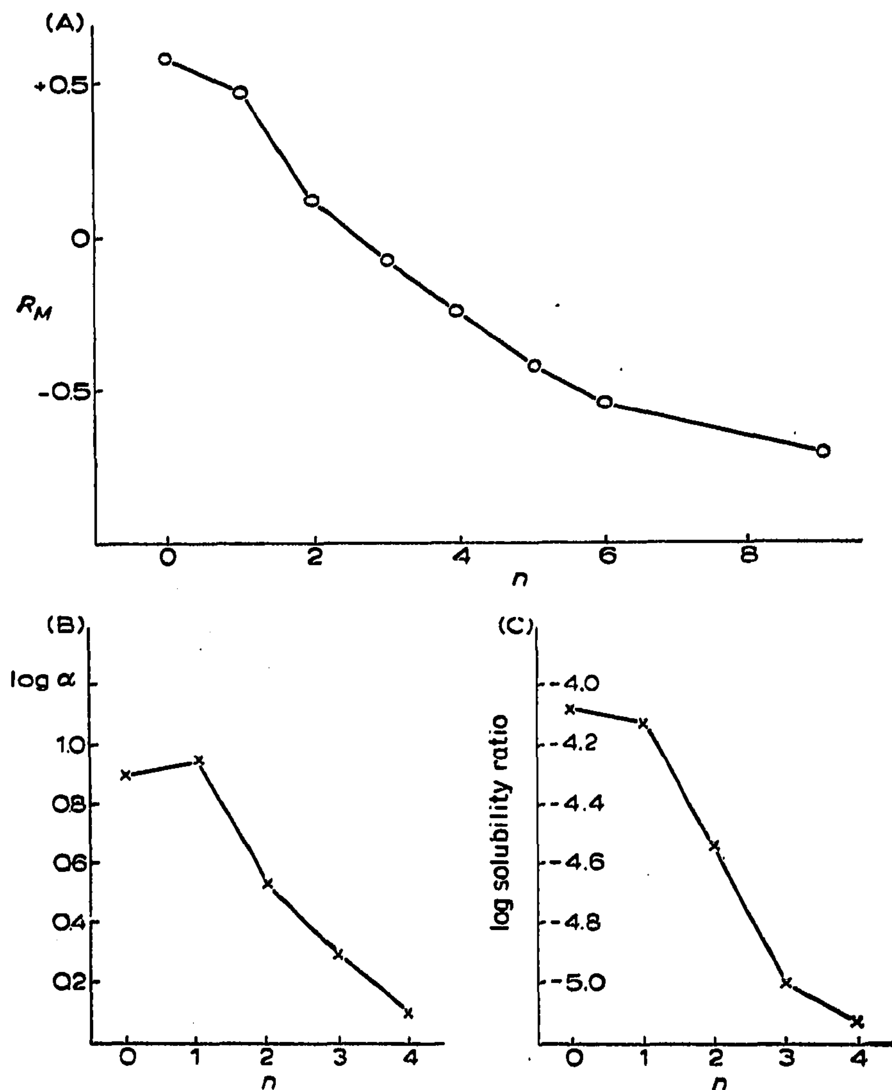

Fig. 1. Paper chromatographic and related data for the testosterone ester series $C_{19}H_{27}O_2CO-(CH_2)_nH$. (A) R_m *vs.* position in the homologous series (n). (B) Distribution coefficients (α) between the Bush system phases; plots of log α *vs.* n. (C) Solubility ratios between water and ethyl oleate.

A gas–liquid chromatographic (GLC) technique, described below, has been developed to determine steroid alcohols and their esters in mixtures with hydrolysing enzymes.

The three methods are compared and the differences between their results discussed.

EXPERIMENTAL

Materials

Formate esters were prepared by the method of RINGOLD *et al.*[7]. Nandrolone (17β-hydroxyoestr-4-ene-3-one) esters were prepared by adding a solution of the

appropriate acid chloride in dry acetone to a solution of nandrolone in a dry acetone–pyridine (1:1) mixture at 0°. The reaction mixture was poured into excess ice-water and extracted with benzene. The combined dry benzene extracts were evaporated to dryness and the residue purified by solution in benzene–*n*-hexane (1:1) and passage through neutral alumina. The desired portion of the eluate was carefully evaporated to a small bulk, when the ester crystallised out.

All the other esters were obtained by refluxing with the appropriate anhydrides in the presence of pyridine, and recrystallising from aqueous ethanol. Melting points agreed with those quoted in the literature.

Methods

Thin-layer chromatography. Silica gel plates (Plated R Polygram Sil-N-HR, Macherey-Nagel) were predried at 110° for 30 min and impregnated by immersing in a 5 % solution of liquid paraffin in *n*-hexane. The hexane was removed by evaporation at 40°. 5-μl samples of 1 % solutions in chloroform were applied to the plates in a random manner, thus eliminating plate variation. After equilibration, the plates were developed by the ascending method. Five different developing solvents were used, *viz.* 50, 55, 60, 70, and 80 % acetone in water, respectively. The location reagent was 2,4-dinitrophenylhydrazine solution (British Pharmacopoeia, appendix). Testosterone was used as marker on each plate.

BIAGI *et al.*[1] used Silica Gel G, impregnated with silicone DC 200, as stationary phase, and either aqueous acetone or aqueous methanol as mobile phase.

Gas–liquid chromatography. A Pye panchromatograph, equipped with a flame ionisation detector was operated isothermally at 240°. A glass column, 150 × 0.5 cm I.D., containing a stationary phase of 2.5 % w/w methyl silicone gum (SE-30) on AW Celite (100–120 mesh) was used. The carrier gas was nitrogen, with a flow-rate of 60 ml/min. Retention times were determined, with 5α-androstane as internal standard, using 1-μl samples of 1 % w/v solutions in chloroform.

Distribution coefficients. The two phases of the Bush system were prepared by shaking together the requisite volumes of formic acid, spectroscopic methanol, and spectroscopic *n*-hexane fraction, and leaving overnight to equilibrate. A weighed aliquot of ester was added to a suitable combination of the two phases and shaken to equilibrium; 0.1 ml of the upper layer was removed, adjusted to 10 ml with spectroscopic *n*-hexane fraction and assayed spectrophotometrically at 230 nm. The concentration in the lower layer was determined by difference. Each result, plotted in Fig. 1B, is the mean of five determinations.

RESULTS

Thin-layer chromatography

R_m values were calculated from R_F values, which were the mean of eight readings. These were plotted against the percentage acetone in the solvent system, and the best straight line through the points, calculated by least-squares analysis, was extrapolated to zero acetone concentration. Results are given in Table I, and the intercepts plotted against the number of carbon atoms in the ester chain in Fig. 2.

TABLE I

R_m VALUES[a] × 100 OF ANDROGEN ESTERS FOR THE TLC SYSTEM

	Testosterone						*Nandrolone*						*Androstanolone*					
% Acetone	80	70	60	55	50	0[b]	80	70	60	55	50	0[b]	80	70	60	55	50	0[b]
Formate	−82	−89	−48	−20	−3	187	−80	−92	−55	−37	−18	146	−48	−37	10	31	47	232
Acetate	−100	−72	−34	−12	7	210	−100	−72	−42	−27	−7	190	−64	−30	18	41	63	281
Propionate	−79	−46	−7	15	35	250	−92	−55	−25	5	19	244	−46	0	41	59	87	314
Butyrate	−68	−33	10	36	62	295	−76	−37	3	29	46	276	−43	2	62	83	110	384
Valerate	−59	−13	36	59	88	342							−27	21	86	110	131	410

[a] Calculated from R_F values relative to testosterone, which were the mean of eight readings.
[b] Determined by extrapolation.

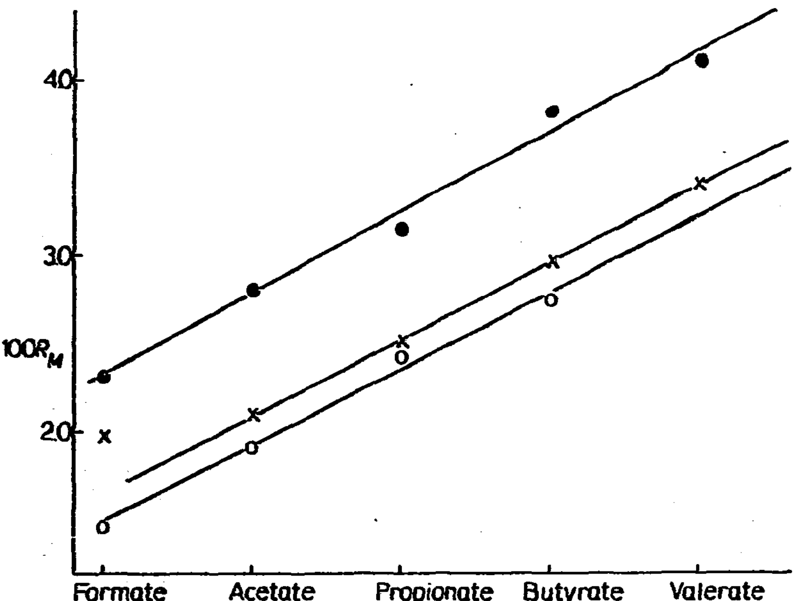

Fig. 2. Apparent R_m values of androgen esters between liquid paraffin and water. Plots of R_m *vs.* position in the homologous series (formate–valerate). ●, Androstanolone series; ○, nandrolone series; ×, testosterone series.

Gas–liquid chromatography

Retention times were recorded as the mean of three readings. These are shown in Table II.

TABLE II

RETENTION TIMES[a] OF ANDROGEN ESTERS

Ester	*Retention time (min)*		
	Testosterone	*Nandrolone*	*Androstanolone*
Formate	9.42	8.46	8.18
Acetate	10.02	9.06	8.94
Propionate	13.14	12.06	11.76
Butyrate	16.53	15.60	15.10
Valerate	21.78	—	20.10
5α-Androstane[b]	1.81	1.81	1.81

[a] Mean of 3 readings.
[b] Internal standard.

DISCUSSION

The retention time (t_R) of a component passing through a GLC column is given by eqn. 4 (ref. 8)

$$t_R = t_A(1 + K\alpha) \tag{4}$$

where t_A is the elution time for unabsorbed gas; α is the distribution coefficient; and K is a constant under constant conditions. Rearrangement of eqn. 4 gives eqn. 5

$$\log \alpha = \log \left(\frac{t_R}{t_A} - 1\right) + \log \frac{1}{K} \tag{5}$$

which indicates that the first term on the right-hand side is analogous to the R_M value of liquid–liquid partition chromatography. Like R_M and log α it is linearly related to free energy and should increase by a constant increment on ascending an homologous series. The function log $(t_R/t_A - 1)$ behaved in this way for androstanolone (17β-hydroxyandrost-3-one), nandrolone, and testosterone normal fatty acid ester series up to the valerate, when plotted against the number of carbon atoms in the ester chain. Three parallel straight lines were obtained, as shown in Fig. 3; the formates deviated from the plots, but this is common for first members of homologous series.

The TLC work by BIAGI *et al.*[1] was repeated to determine how the formate fitted into the scheme and to ensure that the linear relationship was not unique to the testosterone series. Conditions were modified slightly to improve reproducibility. Three parallel straight lines were again obtained on plotting R_M against the number of carbons in the ester chain. These are shown in Fig. 2. It thus appears that both techniques comply with the anticipated constant free-energy-increment for the CH_2 group.

In contrast, the R_M values for the testosterone esters in the Bush system

formic acid–methanol–petroleum ether are not linearly related to the number of carbon atoms in the ester chain. Instead, the ΔR_M value between formate and acetate is small, while the ΔR_M values for the subsequent CH_2 increments are larger, but become progressively smaller as the homologous series is ascended (Fig. 1A). This follows from the distribution coefficients between the two phases, since they vary in the same way as the R_M values (Fig. 1B).

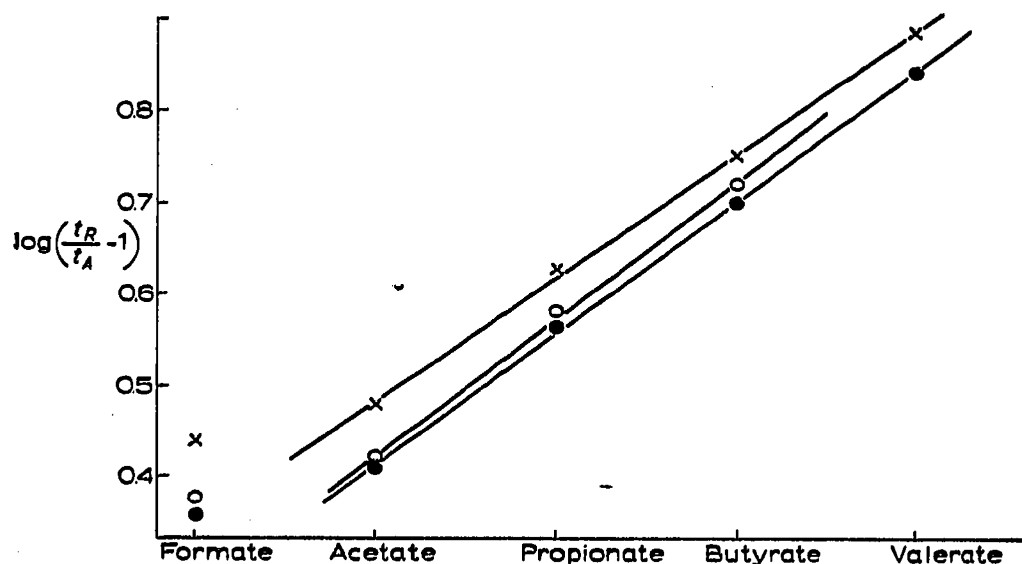

Fig. 3. GLC results for androgen esters. Plots *vs.* position in the homologous series (formate–valerate). ●, Androstanolone series; ○, nandrolone series; ×, testosterone series.

Distribution coefficients for the Bush system were comparatively easy to determine because the solubilities in the two phases were of a similar order. The differences between the solubilities in water and organic solvents are so great that the distribution coefficients corresponding to the TLC technique are almost impossible to determine directly. We can, however, speculate on the relative values of the distribution coefficients by considering the solubilities in water and ethyl oleate, which have been published by ROBERTS[9]. It is appreciated that the ratios of the solubilities in these two solvents cannot be directly equated to the distribution coefficients between water and liquid paraffin, but it can, as a first approximation, be assumed that the factors which cause the two terms to differ are reasonably constant from homologue to homologue.

The aqueous solubilities of the formate–valerate esters of testosterone decrease logarithmically as the series is ascended[10]. The solubilities of the same series in a range of organic solvents have been determined by JAMES AND ROBERTS[10]. All yielded a characteristic irregular profile, of which Fig. 4 is an example. The profile was confirmed theoretically and attributed to the irregular sequence in the melting points (Fig. 4). Subtraction of such an irregular profile from the logarithmic aqueous solubility plot gives a pattern similar to that obtained when the R_M values for the Bush system were plotted against the number of carbon atoms in the ester chain (Fig. 1A). Ethyl oleate gave a typical pattern and is reproduced in Fig. 1C. When one organic solvent is run against another, as in the TLC system, the irregular profiles, typified by Fig. 4, will cancel each other, giving a linear plot of the type shown in

Fig. 2. The solubilities of the lower testosterone esters in the stationary phase of the Bush system formic acid–methanol–petroleum ether have been determined, and shown to be logarithmically related to the number of carbon atoms in the ester chain, in a similar manner to that observed in water[11]. Since the mobile phase is typical of a non-polar organic solvent, this Bush system must be more representative of a distribution between water and a hydrophobic liquid than the TLC system.

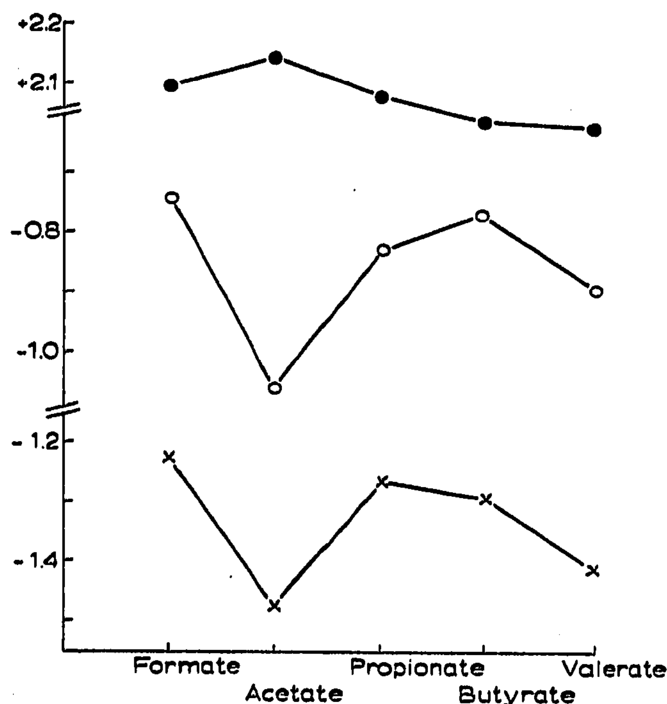

Fig. 4. Solubilities and related data for the testosterone ester series. ●, Log melting point (°C); ○, log ideal solubility, calculated as $\Delta H_f/2.303\,R \cdot ((T_m - T)/T_m T)$; ×, log mole fraction solubility in ethyl oleate.

There is considerable experimental evidence in favour of a constant $\Delta R_{M(CH_2)}$ increment in homologous series. The Bush system formic acid–methanol–petroleum ether does not conform here because it involves a low-polarity phase in combination with a phase having a polarity similar to that of water. It is probable that more exceptions to the constant $\Delta R_{M(CH_2)}$ have not come to light because in most systems in partition chromatography both phases are considerably less polar than water.

The mole fraction solubility (X_2) of a low-polarity solid in a low-polarity liquid is predicted by eqn. 6 (ref. 12)

$$-\log X_2 = \frac{\Delta H_f}{2.303\,R}\left(\frac{T_M - T}{T_M T}\right) + \log \gamma \qquad (6)$$

where T_M is the melting point, ΔH_f heat of fusion, T temperature, R the gas constant and γ the activity coefficient.

It has been shown[10] that, as a consequence of the manner in which the melting points change as the series is ascended for the testosterone esters, the first term on the right-hand side of eqn. 6 varies in a similar manner to the observed solubilities in organic solvents. The situation is illustrated in Fig. 4. For liquid solutes the first

term on the right-hand side disappears. If the profiles in Fig. 4 are a consequence of the variation in melting points, it would follow that the GLC results vary throughout the homologous series in a different way from the Bush-system R_M values because the operating temperature is in excess of the melting points of the esters.

The biological half-lives of the lower testosterone esters are logarithmically related to their distribution coefficients[4]. Since both R_M and log $(t_R/t_A - 1)$ are directly proportional to log α, they may be substituted for log α in such considerations. MIESCHER *et al.*[13] measured the androgenic responses in the rat after one injection of testosterone or its esters, and their results have been interpreted by DORFMAN AND SHIPLEY[14] as "times of maximum effect", the times at which the graphs of MIESCHER *et al.* reached a maximum. These increased as the series was ascended, except the result for the formate, which was the same as that for the acetate. The formate result will therefore deviate from a correlation with any property which changes by a constant increment as the homologous series is ascended. It was for this reason that it was considered important to ascertain the behaviour of this ester using the BOYCE–MILLBORROW technique. Fig. 2 shows that it does not follow the linear relationship between R_M and the position in the homologous series with the other esters. Least-squares analysis of R_M against log time of maximum effect (TM) produced a good correlation, not only for the formate–valerate esters, but also when testosterone was included. The relationship is expressed in eqn. 7

	n	r	s	
$\log TM = 0.173 + 0.310 R_M$	6	0.983	0.054	(7)

where n represents the number of compounds examined; r is the correlation coefficient; and s is the standard deviation.

The values for r and s indicate that the correlation is good, which is confirmed by the predicted result of 6.1 days for the formate by eqn. 7, compared with an observed result of 6.0 days. The GLC results gave an equally good correlation when only the five esters were considered, but failed when testosterone was included.

Thus, in all three methods the formate has failed to follow the same uniform progression in physical properties as the remainder of the series and as a consequence, is in line with the observed times of maximum androgenic effect in rats. The fact that this phenomenon is observed in the GLC results indicates that the deviation of the solubility of testosterone formate from the otherwise linear sequence is, at best, only partly due to its melting point. The major cause must occur at a molecular level and may be intermolecular hydrogen bonding between oxygen and hydrogen in neighbouring formate groups.

It must be emphasized that although good correlation is obtained with biological results for the compounds examined, it can only be expected to hold for a limited range of the homologous series, over which the plot of log TM against the position in the homologous series is approximately linear. When the results of BIAGI *et al.*[1] and BOWEN[11] *et al.* were compared over a longer range of homologues[15], BOWEN's Bush system fitted the biological results significantly better than the TLC results. This was particularly evident at the end of the range, where the TLC method predicted a TM value of 36 days for testosterone decanoate compared with an observed result of 21 days. The Bush procedure gave 22 days.

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