Correlation of Contraceptive Activity of Norethisterone and Levonorgestrel Esters with $V_{R(w)}$ Values and Hydrolysis Rates

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The relationships between contraceptive activity, hydrophobic character and alkaline hydrolysis rates were studied in three homologous series of norethisterone and levonorgestrel esters. Hydrophobic character was expressed by the high-performance liquid chromatographic retention term, $V_{R(w)}$ or retention volume at 100% aqueous mobile phase. A parabolic and bilinear relationship was shown between $\log V_{R(w)}$ and $\log$ biological response. No correlation between second-order alkaline hydrolysis rates, k_2 , measured in 70% (v/v) aqueous dioxan and contraceptive activity was established.

Keywords — contraceptive steroid; quantitative structure–activity relationship; hydrophobicity; alkaline hydrolysis rate; hydrophobicity and contraceptive activity; hydrolysis rate and contraceptive activity

Introduction

In the search for potential long-acting contraceptive agents, the World Health Organization (WHO) has synthesized and studied the structure–activity relationship of more than 200 esters of norethisterone and levonorgestrel.¹⁾

The importance of a drug's physico-chemical properties in determining its biological activity has long been recognized. Of particular importance is hydrophobicity which is the major determinant of a drug's dissolution, distribution and availability in the body.²⁾

The usefulness of partition coefficients and R_M values in studying the correlation of chemical structure with biological activity have been demonstrated with phenols,^{3,4)} testosterone esters,^{4,5)} penicillins⁶⁾ and analgesics⁷⁾ among many others. More recently, high-performance liquid chromatographic (HPLC) retention data have also been successfully correlated with biological activity by Baker *et al.*⁸⁾ and Henry *et al.*⁹⁾

In the case of steroidal esters, another property which may be of importance to activity is the rate of hydrolysis. It has been postulated that steroidal esters act as prodrugs, thus requiring *in vitro* enzymatic or nonenzymatic hydrolysis in order to release the pharmacologically active

parent steroid. James *et al.*¹⁰⁾ have correlated enzymatic hydrolysis rates and androgenic activities of several testosterone esters in rats. Evidence for hydrolysis has also been indicated in a study whereby injections of norethisterone esters resulted in plasma levels of free norethisterone.¹¹⁾ On the contrary, no relationship between enzymatic hydrolysis rate and biological activity was found in a recent study¹²⁾ on 157 esters of norethisterone and levonorgestrel.

The purpose of the present work was to synthesize three series of norethisterone and levonorgestrel esters (I – XV) which have been screened under a special programme of WHO for duration of contraceptive activity¹³⁾ and subsequently, to study the relationships between biological activity, hydrophobicity and alkaline hydrolysis rates.

Synthesis was carried out initially with the conventional thallos ethoxide method of Herz *et al.*¹⁴⁾ in yields of 44–88%. A novel method which makes use of trifluoroacetic anhydride to generate the acylium ion of the esterifying acid, esterified the steroids in consistently higher yields (75–99%) and in shorter time.¹⁵⁾

Materials and Methods

Melting points were determined in a Gallen-

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Fig. 1

kamp melting point apparatus and are uncorrected. Specific optical rotations were recorded for 1% (w/v) solutions in $CHCl_3$ on a Leitz Polarimeter at 20 °C. Samples for analysis, hydrophobicity and kinetic measurements were dried over sodium hydroxide pellets under reduced pressure for 6 h.

Materials — Esters I, IV–VI were prepared using the thallous ethoxide method and II, III, VII–XV using the trifluoroacetic anhydride method. The purity and composition of the products were checked by melting point, optical rotations (Table I), elemental analyses, infrared (IR), ultraviolet (UV) and nuclear magnetic resonance (NMR) spectroscopy.¹⁵⁾ All compounds gave satisfactory analyses for C, H ($\pm 0.4\%$) and had appropriate IR and 1H -NMR data.

General Method for Preparation of Acid Chloride — In a dry system containing 5.0 g of the acid in 20 ml of dry benzene, an equimolar amount of freshly distilled thionyl chloride was slowly added. The mixture was heated under reflux for 3 to 7 h. The benzene and unreacted thionyl chloride were distilled at atmospheric pressure in a dry system. To remove residual thionyl chloride, another 20 ml of dry benzene was added and distilled. The desired acid chloride was then purified by distillation under reduced pressure.

Fig. 2

General Method for Esterification Using Thallous Ethoxide (Method A) — To a solution of 1.50 g (5.03 mmol) of steroid in 100 ml dry benzene, 0.43 ml (1.51 g, 6.03 mmol) of thallous ethoxide was added and the solution was heated under reflux in a nitrogen atmosphere with the exclusion of moisture. The solvent was slowly distilled and continuously replaced with dry benzene. After distilling a total of 100 ml solvent, the solution was cooled to 5–10 °C in an ice-bath. A solution of freshly distilled acid chloride (6.03 mmol) in dry benzene was added dropwise with stirring. The reaction was continued at room temperature, 28 °C, until thin-layer chromatography (TLC) of the reaction mixture indicated that the reaction had completed.

The reaction mixture was filtered to remove the thallous chloride formed. The benzene filtrate was washed with 10% aqueous sodium bicarbonate followed by distilled water until neutral, dried over anhydrous magnesium sulphate and evaporated to dryness under reduced pressure. The crude products were chromatographed on silica gel previously washed with *n*-hexane and eluted with mixtures of hexane–ether containing 12–18% (v/v) ethyl ether. Recrystalliza-

tion from methanol-chloroform yielded the pure esters.

General Method for Esterification Using Trifluoroacetic Anhydride (Method B) — In a dry system, a mixture of acid (6.40 mmol) and trifluoroacetic anhydride (10.62 mmol) in 40 ml dry benzene was heated under reflux for 0.5–2 h. On cooling to room temperature, 1.0 g (3.20 mmol) of steroid and 0.5 g of anhydrous sodium carbonate were added and heated under reflux until esterification was completed as indicated by TLC. Excess acid in the reaction mixture was removed by adding 10% aqueous sodium bicarbonate solution. The benzene layer was then washed with distilled water until neutral, dried over anhydrous magnesium sulphate and the benzene evaporated off under reduced pressure to yield the crude product which was purified by crystallizing in methanol-chloroform. In cases where the crude product was a gum, it was chromatographed on silica gel previously washed with *n*-hexane and eluted with a mixture of hexane-ether (4:1) to give the corresponding esters on crystallization.

$V_{R(w)}$ Measurements — Chromatography for hydrophobicity studies was performed on a Perkin-Elmer LC-65T chromatography module equipped with a 2/2 pump module and fitted with a variable-wavelength UV detector and a Rheodyne injector model 7105. The detector was linked to a Hewlett-Packard 3380s integrator.

The stationary phase was a 250 × 4.6 mm i.d. LiChrosorb RP18 column (E. Merck, Darmstadt) with a particle size of 10 μ m. The mobile phase, consisting of 90, 85, 80, 75 and 65% (v/v) spectroscopic grade methanol (Uvasol, E. Merck, Darmstadt) in deionized, distilled water (Milli-Q system, Waters Associates), was operated in the isocratic mode. The operating conditions of the chromatograph were: 37 °C, flow rate of 0.8 – 5.0 ml/min, depending on the alcohol content of the mobile phase, detector wavelength of 244 nm, absorbance range of 0.01 a.u.f.s. and sample volume of 10 μ l injected in triplicates.

Samples were prepared by diluting stock solutions of each ester in methanol with the appropriate mobile phase. Acetone, which served

as a non-retained compound to define the dead volume was added to each sample. Retention times, t_R , were recorded on the integrator for each sample at the 5 mobile phase compositions and converted to retention volumes, V_R , using Eq. 1. $\log V_{R(w)}$ values were obtained from the y -intercepts of linear regression plots of $\log V_R$ versus percent methanol in mobile phase.

$$V_R = (t_R - t_0) \times \text{flow rate (ml)} \quad (1)$$

where t_0 = retention time of acetone

Rate Measurements — The chromatographic system for quantitative analysis consisted of a Waters Associates model-45 pump fitted with a Rheodyne injector model 7161 and a series 440 fixed-wavelength UV detector. The detector was linked to a model 730 data module.

Separation was performed on a 30 cm × 3.9 mm i.d. 10 μ m Bondapak C18 column (Waters Associates). The mobile phase consisted of 78–88% (v/v) HPLC grade methanol (Unichrom), Ajax Chemical Co.) in deionized, distilled water (Milli-Q system, Waters Associates) operating in the isocratic mode. Operating conditions for the chromatograph were: ambient temperature (20 °C), absorbance range of 0.05 a.u.f.s. and sample volume of 10 μ l injected in triplicates. Flow rates (0.7–1.2 ml/min) and mobile phase composition were adjusted to give a total chromatographic retention time of 20 min.

Hydrolysis rate measurements were adapted from the methods of Vesely *et al.*¹⁶⁾ and Karunanithy *et al.*¹⁷⁾ Hydrolysis samples were prepared by mixing 4 ml of ester stock solution (0.0125 mmol/4 ml) with 6 ml of spectroscopic grade dioxan (E. Merck, Darmstadt)-aqueous sodium hydroxide 0.025 M (1:1) and maintained at a constant temperature of 37 ± 0.5 °C using a Grant Model SE-35 water-bath. Reactions were quenched at appropriate time intervals by adding 1 ml 0.1 M HCl solution. The reaction mixture was made up to 25 ml with the mobile phase. For analysis, 5 ml of the diluted solution was mixed with 1 ml of the internal standard solution (12 mg/25 ml) and made up to 10 ml with the mobile phase. The internal standards used

TABLE I. Structures, Physical Properties and Biological Response Data¹²⁾ of Synthesized Norethisterone and Levonorgestrel Esters

Ester	Empirical formula	Method of synthesis	Yield (%)	mp (°C)	$[\alpha]_D^{20}$ (deg)	$\log V_{R(w)}$ (± 2 S.D.)	$k_2 \times 10^{-3}$ 1 mmol ⁻¹ h ⁻¹	Biological response ^{a)}
I	C ₂₆ H ₃₆ O ₃	A	44.0	Oil	- 8.1	5.97 (± 0.58)	8.48	15.2 $\pm$ 3.1
II	C ₂₇ H ₃₀ O ₃	B	80.0	242–244	+34.9	5.37 (± 0.50)	3.52	2.4 $\pm$ 0.7
III	C ₂₈ H ₃₂ O ₃	B	74.5	193–195	-21	5.33 (± 0.53)	19.68	0.9 $\pm$ 0.4
IV	C ₂₉ H ₃₄ O ₃	A	63.2	148–150	- 6.7	5.75 (± 0.54)	14.70	24.9 $\pm$ 6.4
V	C ₃₀ H ₃₆ O ₃	A	87.9	147–149	- 4.6	6.17 (± 0.56)	8.51	3.5 $\pm$ 0.8
VI	C ₃₁ H ₃₈ O ₃	A	69.7	81– 82	- 3.4	6.55 (± 0.59)	8.08	2.6 $\pm$ 0.2
VII	C ₂₃ H ₃₀ O ₃	B	91.6	202–203	-38.5	4.17 (± 0.33)	18.58	33.82 ^{b)} ($r=0.95$)
VIII	C ₂₄ H ₃₂ O ₃	B	92.4	221–223	-34.9	4.60 (± 0.37)	10.58	58.11 ^{b)} ($r=0.98$)
IX	C ₂₅ H ₃₄ O ₃	B	98.4	211–214	-16.4	5.00 (± 0.39)	6.69	120.11 ^{b)} ($r=0.99$)
X	C ₂₆ H ₃₆ O ₃	B	98.4	168–170	-13.5	5.44 (± 0.41)	6.08	74.3 $\pm$ 15.6
XI	C ₂₇ H ₃₈ O ₃	B	96.2	89– 90	-10.8	5.90 (± 0.44)	6.30	22.6 $\pm$ 1.0
XII	C ₂₅ H ₃₂ O ₃	B	95.9	224–225	-42.2	4.65 (± 0.42)	3.97	92.62 ^{b)} ($r=0.99$)
XIII	C ₂₆ H ₃₄ O ₃	B	95.4	227–229	-14.7	5.17 (± 0.46)	6.14	140.49 ^{b)} ($r=0.95$)
XIV	C ₂₇ H ₃₆ O ₃	B	80.0	210–212	-11.3	5.57 (± 0.49)	5.15	3.9 $\pm$ 1.2
XV	C ₂₈ H ₃₈ O ₃	B	75.0	176–178	- 2.7	5.96 (± 0.49)	5.89	6.5 $\pm$ 2.6

a) Duration of estrus — supression at 16 mg dose per rat in days mean $\pm$ S.D. b) Extrapolated values at 16 mg dose per rat derived from log dose-response regression analysis. Regression coefficients are shown in parentheses.

were betamethasone (Glaxo), norethisterone acetate, propanoate, butanoate and pentanoate (synthesized).

Quantification was based on the peak area ratio of analyte to internal standard. The concentrations of the components in the hydrolysis mixture were obtained from the product of peak area ratio and conversion factor obtained from calibration curves found to be linear ($r > 0.998$) in the concentration ranges studied.

Results and Discussion

Hydrophobicity and Biological Activity

A summary of the experimental results is described in Table I. Initial studies have indicated that retention of the synthesized esters was not affected by pH and that mobile phase compositions of 65–90% (v/v) methanol in water yielded reproducible retention time, t_R , values.

Some studies have shown that HPLC retention data determined at a single mobile phase composition inadequately represents the hydrophobic character of a compound.^{18,19)} Others have observed that HPLC retention data at 100% water could be a good hydrophobicity

parameter because the effect of the hydrogen bonding factor can largely be eliminated by extrapolation of the retention values to 0% organic solvent.^{20–22)} Therefore, the procedure in this study was to employ $\log V_{R(w)}$ (Table I), extrapolated log retention volume, at 100% aqueous mobile phase, as the hydrophobicity parameter. Extrapolation was made possible because of the linear relationship between $\log V_R$ and percent methanol in mobile phase (Figs. 3–5, $r > 0.99$ at $p = 0.95$, $n = 5$). The effect of ester structure

Fig. 3. Relationship between $\log V_R$ and Methanol Concentration in Mobile Phase for Norethisterone Esters of Arylaliphatic Acids

■, III; □, IV; ●, V; ○, VI.

Fig. 4. Relationship between $\log V_R$ and Methanol Concentration in Mobile Phase for Levonorgestrel Esters of Aliphatic Acids

■, VII; □, VIII; ●, IX; ○, X; △, XI.

on $\log V_{R(w)}$ is revealed in Fig. 6 which shows a linear relationship between $\log V_{R(w)}$ and ester chain length is each of the homologous series, suggesting that $\log V_{R(w)}$, as in $\log P$, partition coefficient, is also an additive property of a molecule.

Biological response (BR) of the esters have been previously determined by the WHO¹³⁾ in an estrus-suppression assay which measured the length of time (d) in which cornification of vaginal epithelium was suppressed on injection of the test preparation. Using the data available which are included in Table I, quantitative structure-activity relationship (QSAR) studies were carried out using the parabolic model of Hansch *et al.*²³⁾ and bilinear model of Kubinyi²⁴⁾ which have been modified to Eqs. 2 and 3 respectively for the purpose of this study.

$$\log BR = a (\log V_{R(w)})^2 + b \log V_{R(w)} + c \quad (2)$$

Fig. 5. Relationship between $\log V_R$ and Methanol Concentration in Mobile Phase for Levonorgestrel Esters of Cycloaliphatic Acids

■, XII; □, XIII; ●, XIV; ○, XV.

Fig. 6. Linear Relationship between $\log V_{R(w)}$ and Ester Chain Length

■, norethisterone arylaliphatic esters; □, levonorgestrel aliphatic esters; ●, levonorgestrel cycloaliphatic esters.

$$\log BR = a \log V_{R(w)} - b \log(\beta V_{R(w)} + 1) + c \quad (3)$$

Multiple regression analyses gave values of constants a , b and c . The non-linear term, β , in Eq. 3 was obtained by a stepwise iteration proce-

Fig. 7. $\log BR$ of Norethisterone and Levonorgestrel Esters Fitted with the Parabolic (—) and Bilinear (---) Model

ture outlined by Kubinyi and Kehrhan.²⁵⁾ The relationship between the hydrophobic character of the esters and their biological activity is described by a parabolic and bilinear curve in Fig. 7 and can be quantitatively expressed by either of the following equations:

$$\begin{aligned} \log BR &= 6.40(\pm 3.86)\log V_{R(w)} \\ &\quad - 0.668(\pm 0.361)(\log V_{R(w)})^2 \\ &\quad - 13.5(\pm 10.2) \\ (n=12, r=0.924, s=0.257, \\ F=32.91) \end{aligned} \quad (4)$$

$$\begin{aligned} \log BR &= 2.46(\pm 1.30)\log V_{R(w)} \\ &\quad - 4.23(\pm 1.70)\log(\beta V_{R(w)} + 1) \\ &\quad - 8.35(\pm 5.56) \\ \beta &= 2.03 \times 10^{-5} \\ (n=12, r=0.951, s=0.208, \\ F=52.71) \end{aligned} \quad (5)$$

where r is the correlation coefficient, s the standard deviation, F the variance ratio and n is the number of esters tested. A comparison of the statistical parameters revealed a higher level of correlation with the bilinear model which is, however, not as significant as indicated in previous studies.

Alkaline Hydrolysis Rates and Biological Activity

Figure 8 shows the concentration-time

Fig. 8. Concentration-Time Profile of Levonorgestrel Acetate (■) and Its Parent Steroid, Levonorgestrel (□) under Alkaline Hydrolysis Conditions

course of ester VII and its parent steroid, monitored simultaneously using HPLC, during hydrolysis. At constant pH and temperature, the decomposition rates of the esters studied followed second-order kinetics. Rate constants were calculated from the slopes of kinetic plots (Eq. 6) which have been shown to be linear ($r > 0.96$ at $p = 0.95$, $n = 6-11$).

$$\ln \frac{(a-x)}{(b-x)} = \ln \frac{a}{b} + (a-b)k_2t \quad (6)$$

where x is the concentration of hydrolysed ester at time t , a and b are the initial concentrations of ester and sodium hydroxide, and k_2 is the second-order rate constant.

The data in Table I reveal the effect of ester structure on k_2 which could be attributed to a combination of steric and, to some extent, electronic influences on the carbonyl carbon of the molecule. The observed decrease in k_2 with increasing ester chain length is more likely to be caused by an increase in steric effect²⁶⁾ than by the weak electron-donating effect of the methylene group. The benzoate (II) exhibited an anomalously low hydrolysis rate because of stabilization of the ester by resonance.^{15,27)} In the

Fig. 9. Correlation of log BR with log k_2
●, norethisterone arylaliphatic esters; □, levonorgestrel aliphatic esters; ■, levonorgestrel cycloaliphatic esters.

cycloaliphatic series, the esters did not exhibit statistically different hydrolysis rates, suggesting that ring size neither influences electronic nor steric effects.

To determine the relationship between BR and k_2 , log BR was plotted against log k_2 . Figure 9 shows that there is no correlation between the two factors. There is however, indication of an optimal log k_2 which gives rise to maximum biological activity in the levonorgestrel aliphatic and norethisterone arylaliphatic series. The esters exhibiting maximal activity in these two series are IX and IV, respectively. Naderi and Fotherby¹²⁾ in a similar correlation study using rabbit liver preparations for enzymatic hydrolysis reported similar findings. No relationship between chemical structure and reactivity nor between biological activity and reactivity was established for the same esters studied.

Conclusion

The findings of this study indicate that the rate of hydrolysis is not the major factor determining the duration of biological activity of the esters. Instead, the results re-emphasize the vital role hydrophobicity plays in drug action. The hydrophobic character of the esters is important because it determines the rate of release and dissolution of the esters from the formulation into tissue fluids and the rate of partitioning through biological membranes.

The establishment of a relationship between biological activity and HPLC retention volume in this study also suggests that HPLC systems may be suitable models for *in vivo* partitioning. They may also be better than conventional octanol-water systems because such systems bear three important similarities to biological systems: (1) alkyl-bonded silica gel columns consist of alkyl side chains arranged in ordered arrays facing an aqueous mobile phase on one side and a polar (silica) surface on the other, (2) drug transfer is from a bulk aqueous phase to a monolayer system, and (3) transfer equilibrium of solute molecules between the mobile and stationary phases is dynamic in nature.

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