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Short Communication

Use of high-performance liquid chromatography for quantitative structure–activity relationship studies of a series of potential contraceptive steroidal esters

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Keywords: *Contraceptive steroidal esters; reversed-phase high-performance liquid chromatography; quantitative structure–activity relationship.*

Introduction

The quantitative relationships between the lipophilic properties of drugs and their actions have been widely studied [1]. The lipophilic character of drugs has been commonly quantified using partition coefficients in *n*-octanol–water systems [2] and R_M values [3]. The determination of partition coefficients is a tedious process often complicated by lengthy analytical procedures, instability of the drug in solution and the tendency of the drug to dissociate or associate. Likewise, the measurement of R_M values using reversed-phase thin-layer chromatography has its disadvantages. Reproducibility of results is often difficult because of the problems associated with the standardization of the plates and the limits of visual detection.

To overcome these problems, many workers have used high-performance liquid chromatographic (HPLC) retention data as a measure of drug lipophilicity [4–7]. This has advantages of speed and reproducibility over conventional methods. In addition, the transfer equilibrium of solute molecules between the mobile and stationary phases takes place in a dynamic manner as in biomembranes.

The main objectives of this work were to investigate if a relationship exists between the biological activity and HPLC retention data of a series of synthesised norethisterone and levonorgestrel esters, to quantify any such relationship and to investigate the effects of mobile phase composition and substituents on the retention of these compounds.

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Experimental

Materials

The norethisterone and levonorgestrel esters were synthesized in this laboratory using thallous ethoxide [8] and trifluoroacetic anhydride [9]. Spectroscopic grade methanol (Uvasol, E. Merck Darmstadt) and deionised, distilled water (Milli-Q System, Waters Associates) were used for the preparation of the mobile phase.

Chromatographic conditions

Chromatography was carried out on a Perkin–Elmer LC-65T chromatograph equipped with a 2/2 pump module and fitted with a Rheodyne injector (model 7105) and a variable-wavelength UV detector linked to a Hewlett–Packard 3380S integrator. Separations were performed isocratically on 250 × 4.6 mm i.d. column containing Lichrosorb RP18 (E. Merck) with a 10 μm particle size. The mobile phases consisted of 90, 85, 80, 75 and 65% v/v methanol in water. The operating conditions of the chromatograph were as follows: column temperature of 37°C, flow rate of 0.8–5.0 ml/min depending on the mobile phase, and a detector wavelength of 244 nm.

Measurement of log $V_{R(w)}$

Samples for analysis were prepared from stock solutions of the esters in methanol diluted with the appropriate mobile phase. Acetone, which served as a non-retained compound to define the chromatographic hold-up volume, was added to each sample. A 10-μl injection was made in triplicate. Retention times, t_R, for each sample using the five mobile phase compositions, were recorded on the integrator and converted to adjusted retention volumes, V_R, using equation (1):

$$V_R = (t_R - t_o)\ (\text{flow rate}), \tag{1}$$

where t_o = retention time of acetone.
Log $V_{R(w)}$ values (log V_R at 100% aqueous mobile phase) were obtained from the y-intercepts of plots of log V_R versus percent methanol (v/v) in the mobile phase.

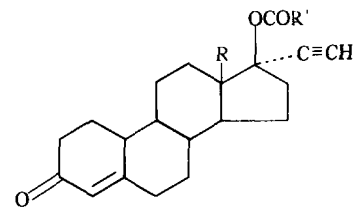
Results and Discussion

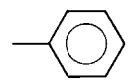
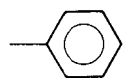
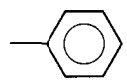
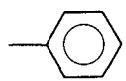
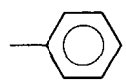
The steroids under investigation consisted of four homologous series of norethisterone and levonorgestrel esters. Their structures are shown in Table 1. The retention volumes were considered to be too dependent on the experimental conditions to be useful as indices of lipophilicity. Therefore extrapolation to 100% water as eluent (log $V_{R(w)}$) was carried out. Correction for solute ionization was not necessary since the esters are unionizable compounds.

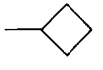
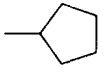
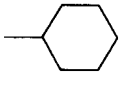
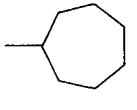
Effect of methanol on log V_R

Plots of the content of methanol in the mobile phase versus log V_R were linear (correlation coefficient >0.99; Table 1). Linearity has been observed also in other studies especially for hydrophobic compounds [6, 10]. Linearity is an indication of partition equilibrium of the esters between the two phases during elution and is an important criterion for the simulation of *in vivo* conditons [11]. Also, statistical analysis has shown that the change in log V_R with variation of the content of methanol,

Table 1
Structures of steroid esters and their log $V_{R(w)}$ values*



Ester	R	R′	log$V_{R(w)}$ (±2 s.d.)	m (±2 s.d.)	r†
I	$-CH_3$	$-CH_3$	4.09 (±0.78)	−0.0450 (±0.0098)	0.9905
II	$-CH_3$	$-CH_2CH_3$	4.41 (±0.31)	−0.0469 (±0.0040)	0.9985
III	$-CH_3$	$-(CH_2)_2CH_3$	4.84 (±0.32)	−0.0504 (±0.0040)	0.9985
IV	$-CH_3$	$-(CH_2)_3CH_3$	5.30 (±0.33)	−0.0542 (±0.0073)	0.9990
V	$-CH_3$	$-(CH_2)_4CH_3$	5.97 (±0.58)	−0.0604 (±0.0073)	0.9970
VI	$-CH_3$		5.37 (±0.50)	−0.0552 (±0.0063)	0.9975
VII	$-CH_3$	$-CH_2$ 	5.33 (±0.53)	−0.0560 (±0.0067)	0.9970
VIII	$-CH_3$	$-(CH_2)_2$ 	5.75 (±0.54)	−0.0589 (±0.0068)	0.9975
IX	$-CH_3$	$-(CH_2)_3$ 	6.17 (±0.56)	−0.0626 (±0.0071)	0.9975
X	$-CH_3$	$-(CH_2)_4$ 	6.55 (±0.59)	−0.0658 (±0.0074)	0.9975

XI	$-C_2H_5$	$-CH_3$	4.17 (±0.33)	−0.0449 (±0.0042)	0.9985
XII	$-C_2H_5$	$-CH_2CH_3$	4.60 (±0.37)	−0.0480 (±0.0047)	0.9980
XIII	$-C_2H_5$	$-(CH_2)_2CH_3$	5.00 (±0.39)	−0.0513 (±0.0050)	0.9980
XIV	$-C_2H_5$	$-(CH_2)_3CH_3$	5.44 (±0.41)	−0.0550 (±0.0052)	0.9985
XV	$-C_2H_5$	$-(CH_2)_4CH_3$	5.90 (±0.44)	−0.0587 (±0.0055)	0.9985
XVI	$-C_2H_5$		4.65 (±0.42)	−0.0486 (±0.0053)	0.9975
XVII	$-C_2H_5$		5.17 (±0.46)	−0.0525 (±0.0053)	0.9975
XVIII	$-C_2H_5$		5.57 (±0.49)	−0.0556 (±0.0062)	0.9975
XIX	$-C_2H_5$		5.96 (±0.49)	−0.0588 (±0.0062)	0.9980
XX	$-C_2H_5$		6.65 (±0.59)	−0.0650 (±0.0074)	0.9975)

*Calculated from the linear relationship between log V_R and percent methanol in the mobile phase.
†Correlation coefficient (n = 5).

represented by slope m in Fig. 1, increases with the chain length of the ester. Consequently it would be erroneous to compare log V_R values at a single methanol concentration since relative log V_R values for any two compounds depend on the methanol content in the mobile phase.

Effect of ester structure on log $V_{R(w)}$

Plots of log $V_{R(w)}$ versus ester chain length were linear for each set of esters (Fig. 2). Statistical analysis shows that all plots have a common slope, indicating that the change in log $V_{R(w)}$ per $-CH_2$ increment is a constant value independent of the manner in which the $-CH_2$ increments are arranged, i.e. in straight chains as in norethisterone and levonorgestrel aliphatic acid esters (I–V, XI–XV), in cyclic structures as in levonorgestrel cycloaliphatic acid esters (XVI–XX) or attached to an aromatic ring as in

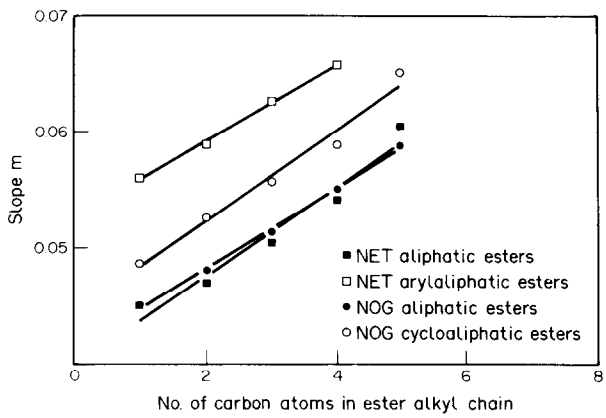


Figure 1
Relationship between slopes (m) and ester chain length.

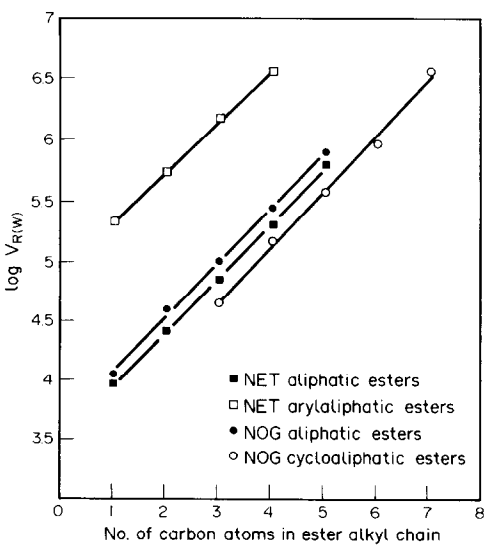


Figure 2
Linear relationship between log retention volume at 100% aqueous mobile phase ($V_{R(W)}$) and ester chain length.

norethisterone arylaliphatic acid esters (VI–X). The differences in *y*-intercepts (γ) of these plots are also useful indications of the contributions to log $V_{R(w)}$ from substituents on the molecules. These are summarised in Table 2. It is interesting to note that a phenyl group in the ester chain or an additional methylene group in the steroid ring increases lipophilicity whilst cyclic substituents in the ester chain decrease lipophilicity of the compounds. In addition, a $-CH_2$ group in the ester chain increases lipophilicity of the ester to a greater extent than a CH_2 group in the 13-carbon atom substituent.

Table 2
log $V_{R(w)}$ contributions due to phenyl groups, methylene groups and cyclic substituents

log $V_{R(w)}$ contribution				
Phenyl group	$\gamma_{NET\ arylaliphatic}$	−	$\gamma_{NET\ aliphatic}$	= 1.44
Methylene group in substituent on C-13	$\gamma_{NOG\ aliphatic}$	−	$\gamma_{NET\ aliphatic}$	= 0.15
Cyclic substituent	$\gamma_{NOG\ cycloaliphatic}$	−	$\gamma_{NOG\ aliphatic}$	= −0.36

NET = Norethisterone.
NOG = Levonorgestrel.
γ = y — intercept in plots shown in Fig. 2.

Correlation of log $V_{R(w)}$ *with biological activity*

Biological response data for fifteen of the synthesised esters (V, VI–XIX) were obtained from the WHO Project on long-acting steroidal contraceptive agents [12]. The duration of action of each ester, formulated as a microcrystalline suspension in an aqueous base, was determined in an oestrus–suppression assay which measures the length of time (days) in which cornification of vaginal epithelium is suppressed after injection of the suspension.

Log $V_{R(w)}$ values and biological response (BR) data were fitted into two quantitative structure–activity relationship (QSAR) models: the parabolic [13] [equation (2)] and bilinear [14] [equation (3)] models. These equations have been modified to equations (4) and (5), respectively for the purpose of this study.

$$\text{Log } 1/C = a\log (P)^2 + b\log P + c, \tag{2}$$

$$\text{Log } 1/C = a\log P - b\log (\beta P + 1) + c, \tag{3}$$

$$\text{Log } BR = a(\log V_{R(w)})^2 + b\log V_{R(w)} + c, \tag{4}$$

$$\text{Log } BR = a\log V_{R(w)} - b\log (\beta\log V_{R(w)} + 1) + c. \tag{5}$$

where c = molar concentration of a drug producing a standard response and
P = octanol–water partition coefficient.

Multiple regression analyses gave values for constants a, b and c. The non-linear term, β, in equation (5) was obtained by a stepwise iteration method outlined by Kubinyi and Kehrhahn [15]. With the exception of three compounds, norethisterone benzoate (VI), phenylacetate (VII) and levonorgestrel cyclopentanecarboxylate (XVIII), the relationship between log $V_{R(w)}$ and biological activity for the other esters was expressed by equations (6) and (7) (Fig. 3):

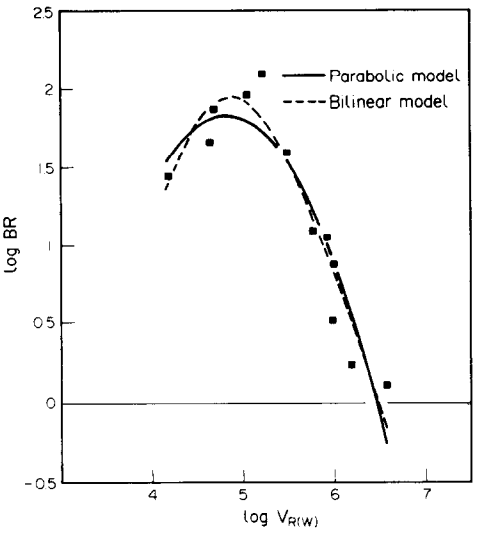


Figure 3
Log biological response (BR) of NET and NOG esters fitted with parabolic and bilinear model.

$$\text{Log } BR = 6.40\ (\pm 3.86)_2\log V_{R(w)} - 0.668\ (\pm 0.361)\ (\log V_{R(w)})^2 - 13.5\ (\pm 10.2) \quad (n = 12,\ r = 0.924,\ s = 0.257,\ F = 32.91), \tag{6}$$

$$\text{Log } BR = 2.46\ (\pm 1.3)\log V_{R(w)} - 4.23\ (\pm 1.70)\ (2.03 \times 10^{-5} \log V_{R(w)} + 1) - 8.35\ (\pm 5.56) \quad (n = 12,\ r = 0.951,\ s = 0.208,\ F = 52.71). \tag{7}$$

On comparing the statistical parameters r (correlation coefficient), s (standard deviation) and F (variance ratio) from both models, it was clear that the bilinear equation [equation (7)] was a better description of the activity–lipophilicity relationship.

The design of steroidal esters as long-acting agents has been based on the assumptions that regeneration of the parent steroid is necessary for activity and that the rate of *in vivo* hydrolysis of the ester determines the rate of release of the active parent steroid and hence its duration of activity. The results of the present study revealed that the change in hydrophobicity brought about by esterification plays a vital role in determining biological activity of the esters under investigation. Hydrophobicity determines the rate of release and dissolution of the esters from the formulation into tissue fluids as well as the rate of partitioning through biological membranes, and this indicates that these processes, and not the rate of *in vivo* hydrolysis, are the rate-determining steps in the production of the pharmacologically active compound.

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