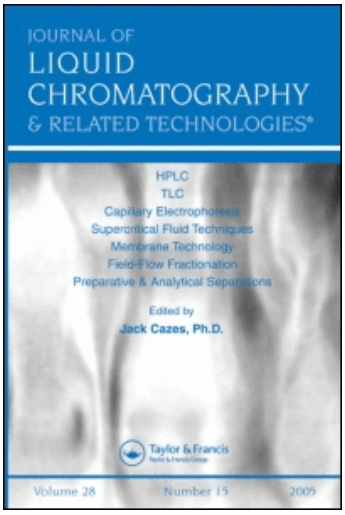

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ABSTRACT

The lipophilic character of a series of new isoxazolyl-naphthoquinones has been studied. Partition chromatography procedures (RP-HPLC and RP-TLC) were developed for determining the log $P_{o\text{-}w}$ (n-octanol/water partition coefficient) values. The experimental values from these methods were validated by comparison with those obtained from the conventional shake flask method, except with the more lipophilic **4**, **6** and **7** because they have poor water solubility. Also, the log $P_{o\text{-}w}$ was calculated using a CLOGP computer programme.

INTRODUCTION

The log $P_{o\text{-}w}$ (partition coefficient in n-octanol/water), which is closely related to the transport properties of drugs and their interaction with receptors, is a conventionally used hydrophobicity parameter in studies of quantitative structure-activity relationships.[1,2]

This parameter, a useful descriptor of global lipophilicity of therapeutic agents, has been traditionally obtained by the shake-flask method.[3] In addition, the partition chromatography is widely used in the evaluation of lipophilicity,[4] and its application for such a purpose follows from the relationship between suitable retention indices and partition coefficient, P_{o-w}, determined in the chromatographic system. Such relationships are based upon the observed similarities in the hydrophobic partitioning processes occurring in an n-octanol/water mixture and in a reverse-phase system with an aqueous mobile phase. A suitable index in the thin-layer chromatography (TLC) is R_m, which is related to the experimental R_f value and depends linearly on the log P_{o-w},[5] which is equivalent to the logarithm of the capacity factor, log k', obtained by high-performance liquid chromatography (HPLC).[6]

Calculation of log P_{o-w} from experimental values of R_m or log k' is based on the application of Collander's linear relationships for the chromatographic and the reference system n-octanol/water.[7]

The purpose of this paper was to examine the relationships between the log P_{o-w} values of some new isoxazolyl-naphthoquinone derivatives with an amino group in the 4-position of the isoxazole ring, measured by the conventional shake flask method and by reversed-phase chromatography (RP-HPLC and RP-TLC). Also, comparison with the log P_{o-w} calculated using CLOGP computer programme[8] is included.

Knowledge of these parameters for this type of compounds is supported on their important potential biological properties.[9]

EXPERIMENTAL

Materials

The isoxazolyl-naphthoquinone derivatives **1-7** (Figure 1) were prepared as previously described.[10,11] Compounds **1-4** have pK_a values of 8.97, 8.19, 7.29, and 7.39 respectively (unpublished results calculated by a spectrophotometric method). On the other hand, the absorption spectra in water of compounds **5-7** did not suggest that ionization occurs, so the aqueous phases used in the experiments were unbuffered. All other chemicals and solvents were of analytical reagent grade and were used without further purification. HPLC-grade methanol was purchased from Sintorgan®. Water reagent grade was generated by a Millipor Milli-Q Water Purification System. The n-octanol 99% was purchased from Sigma®.

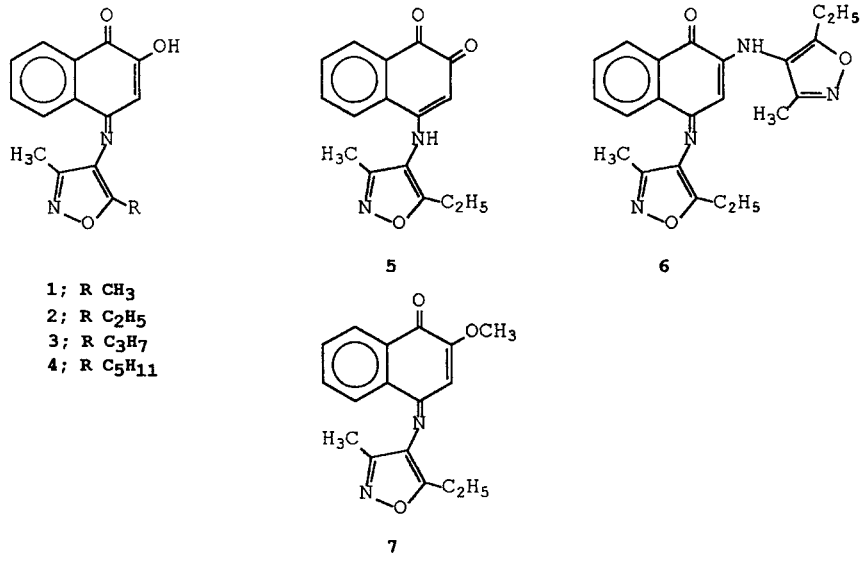

Figure 1. Structure of isoxazolylnaphthoquinones used in this work. **1** = 2-hydroxy-N-(3,5-dimethyl-4-isoxazolyl)-1,4-naphthoquinone-4-imine, **2** = 2-hydroxy-N-(3-methyl-5-ethyl-4-isoxazolyl)-1,4-naphthoquinone-4-imine, **3** = 2-hydroxy-(3-methyl-5-propyl-4-isoxazolyl)-1,4-naphthoquinone-4-imine, **4** = 2-hydroxy-(3-methyl-5-pentyl-4-isoxazolyl)-1,4-naphthoquinone-4-imine, **5** = N-(3-methyl-5-ethyl-4-isoxazolyl)-1,2-naphthoquinone-4-amino, **6** = 2-(3-methyl-5-ethyl-4-isoxazolylamino)-N-(3-methyl-5-ethyl-4-isoxazolyl)-1,4-naphthoquinone-4-imine, **7** = 2-methoxy-N-(3-methyl-5-ethyl-4-isoxazolyl)-1,4-naphthoquinone-4-imine.

Chromatographic Procedures

The HPLC system consisted of a KONIK 500G liquid chromatograph equipped with a loop injector (Rheodyne Model 7125), a UV-V-KNK-029-757 absorbance detector with the wavelength set at 241 nm, a Spectra Physics 4600 Data Jet integrator, and a 150.0 x 4.00 mm MicroPak MCH-5-n-cap LC 18 5-μm HPLC column (VARIAN). The mobile phase composition ranged from 50 to 80% (v/v) methanol with water. The flow rate was 1.0 mL/min. Analytes were dissolved in methanol and then they were injected separately from each other in triplicate and in a random order to obtain their retention characteristics. The chromatographic capacity factor, k', of each compound was calculated by the equation: $k' = (t - t_o)/t_o$, where **t** is the compound's retention time and $\mathbf{t_o}$ is the retention time of methanol. The log k' value at 0% MeOH, log k'_w, was calculated by linear extrapolation from the plot of log k' value against methanol concentration.

TLC was performed using precoated TLC plates RP-18 F_{254}, 20x20 cm in size, purchased from Merck. A 10 μL volume of a methanolic solution of the compounds was applied to the plates with aid of a HAMILTON syringe. The starting points of the compounds were positioned 20 mm from the bottom edge of the plate and at least 25 mm from the side of the plate with 10 mm between each other. Plates were developed in a chromatographic chamber containing 100 mL of mobile phase under conditions of equilibrium. Plates were run up to 20 mm below the upper end of the plate and then were dried in a stream air. Methanol-water mixtures containing 0.05 M KCl with proportions of methanol ranging from 60 to 80% were used as eluents. Spots were detected under UV light (254 nm) and iodine vapors. R_m values were calculated according to the equation: $R_m = \log [(1/R_f) - 1)]$.

Shake Flask Method

The method of Rauls and Baker was used.[12] Standard solutions of the compounds in 50% aqueous ethanol (2.5 mL) were added to a mixture of 3.0 mL of n-octanol-saturated water and 5.0 mL of water-saturated octanol. The mixture was placed in a constant-temperature bath (25º) and shaken several times on a vortex apparatus and the two phases were then allowed to equilibrate at 25º for 24 h. The phases were separated by centrifugation at 1000 rpm for 10 min and the concentration in aqueous phase was determined spectrophotometrically with a SHIMADZU UV-160A (UV/visible recording spectrophotometer). For each compound, four replicates were performed according to the above procedure, so the aqueous concentration used for computation was the mean value. The n-octanol/water distribution (P_{o-w}) of the compounds was taken as the ratio between the difference in concentration before and after shaking ($C_o - C_{aq}$) and the aqueous concentration (C_{aq}) according to the equation: $P_{oct} = (C_o - C_{aq})/C_{aq}$.

RESULTS AND DISCUSSION

Log $P_{RP\text{-}HPLC}$ Determination

The HPLC method used in this study involves the generation of log k' vs. percent of methanol in the mobile phase plots that are extrapolated to the y-axis (100% water mobile phase) to produce an estimated log k'_w value. Calibration of the system was carried out by injecting dilute solutions of nine accurately known log P_{o-w} "standards". These compounds, together with their corresponding log P_{o-w} values in parenthesis, are barbital (0.65), acetanilide (1.16), benzene (2.01), β-naphthylamine (2.26), α-naphthol (2.71), toluene

Table 1

Linear Regression Data for Mobile Phase Composition Versus RP-HPLC Capacity Factors (Log k')

Compound	Methanol Range in Mobile Phase (%)	Slope	Log k'w	r
Barbital	50 - 80	-0.0277	1.2566	0.985
Acetanilide	50 - 80	-0.0256	1.4063	0.989
Benzene	60 - 80	-0.0315	2.4434	0.986
β-Naphthylamine	60 - 80	-0.0347	2.4624	0.987
α-Naphthol	50 - 80	-0.0369	2.7013	0.989
Toluene	60 - 80	-0.0369	3.0661	0.984
Thymol	65 - 80	-0.0452	3.6138	0.965
Anthracene	65 - 80	-0.0521	4.7639	0.957
Phenanthrene	65 - 80	-0.0511	4.6540	0.975
1	50 - 80	-0.0289	1.8496	0.980
2	50 - 80	-0.0337	2.3152	0.986
3	50 - 80	-0.0381	2.8147	0.985
4	65 -75	-0.0428	3.5165	0.889
5	50 - 80	-0.0426	3.1970	0.992
6	60 -80	-0.0589	4.9139	0.983
7	60 - 80	-0.0493	4.0619	0.986

(2.74), thymol (3.30), anthracene (4.45), and phenanthrene (4.46). Table 1 shows the most important parameters of the log k' - % MeOH relationships: the "slope", the log k'_w and the correlation coefficients (r) values for each of the standard compounds.

A plot of log k'_w against log P_{o-w} for the set of standards (Figure 2) was linear, the least -squares linear regression of the calibration data produces the best fit equation:

$$P_{o-w} = 1.0421 (\pm 0.05) \log k'_w - 0.4153 (\pm 0.15) \quad (1)$$
$$n = 9, r = 0.985$$

where n is the number of data used, r the correlation coefficient and the 95% confidence limits on the regression coefficients are given in parenthesis.

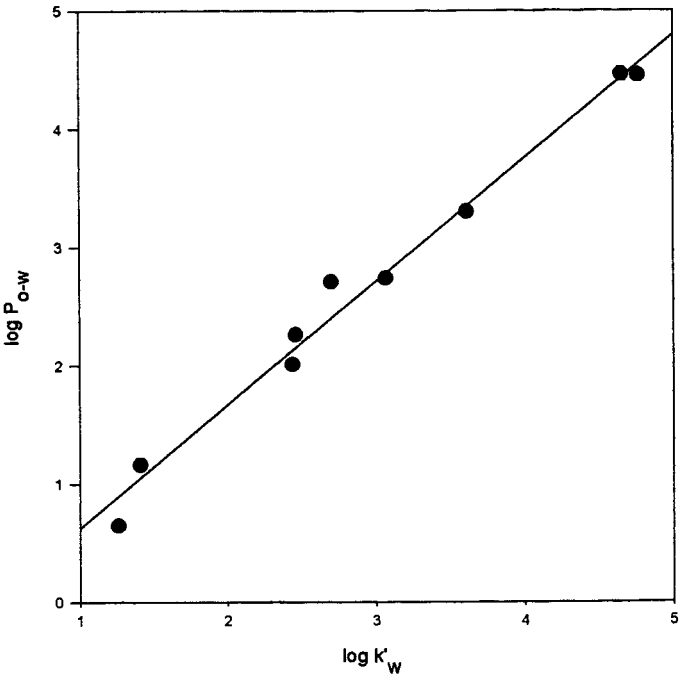

Figure 2. Calibration plot of n-octanol/water partition coefficient (P_{o-w}) and their theoretical capacity factor for a 100% aqueous mobile phase (log k'_w) corresponding to the standard compounds.

Capacity factors for compounds **1-7** were also determined. From these data, log k'_w values were determined for each isoxazolyl-naphthoquinone by linear extrapolation (Table 1), and log P_{o-w} values were calculated by substitution of these log k'_w values into the equation 1. Log P_{o-w} determined by this method (log $P_{RP-HPLC}$) are listed in Table 3.

Log P_{RP-TLC} Determination

R_{mw} values (Table 2) were obtained through linear extrapolation of R_m values determined at different methanol/water mixtures, to which a fixed concentration of KCl was added in order to suppress silanophilic interactions of eventually uncovered silanol groups.

Table 2

Linear Regression Data for Mobile Phase Composition Versus RP-TLC (R_m)

Compound	Methanol Range in Mobile Phase (%)	Slope	R_{mw}	r
Phenol	65 - 80	-0.0234	2.4291	0.948
Acetanilide	65 - 80	-0.0377	3.6091	0.983
2-Hydroxy-1,4-naphthoquinone	60 -75	-0.0976	8.0305	0.968
1,4-Naphthoquinone	65 - 80	-0.1099	9.9156	0.983
Thymol	65 - 80	-0.2622	22.6166	0.977
Anthracene	65 - 80	-1.5798	132.1670	0.985
1	65 - 80	-0.0469	4.3489	0.998
2	65 -80	-0.0687	6.1857	0.989
3	65 - 80	-0.1008	8.9478	0.988
4	65 - 80	-0.2909	24.684	0.974
5	65 - 80	-0.1781	15.3674	0.968
6	65 - 80	-0.6843	56.8412	0.967
7	65 - 80	-0.5252	44.5964	0.983

Partition coefficients for the following standard compounds were correlated with the extrapolated R_{mw} values to produce a calibration plot (Figure 3a): phenol (1.14); acetanilide (1.42); 2-hydroxy-1,4-naphthoquinone (1,55); 1,4-naphthoquinone (1.71), and thymol (3.30). Least- squares linear regression of the calibration data produced the best fit equation:

$$\log P_{o\text{-}w} = 0.1040\ (\pm\ 0.011)\ R_{mw} + 0.8545\ (\pm\ 0.13) \qquad (2)$$
$$n = 5,\ r = 0.967$$

To obtain the unknown log $P_{RP\text{-}TLC}$ of the very lipophilic solutes (**6** and **7**), the following compounds were used to produce the calibration plot: acetanilide (1.42), 2-hydroxy-1,4-naphthoquinone (1.55), 1,4-naphthoquinone (1.71), and anthracene (4.45) (Figure 3b). The linear regression from the calibration data rendered the following equation:

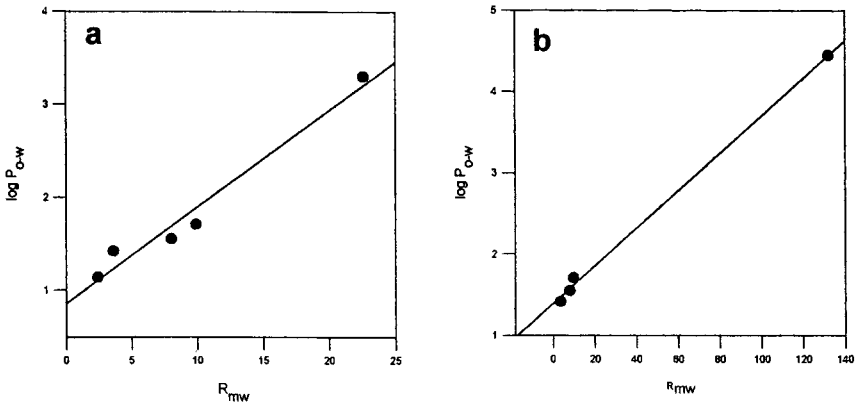

Figure 3. Calibration plots of n-octanol/water partition coefficients (P_{o-w}) and their R_{mw} values for the standard compounds.

Table 3

Prediction of Log P_{o-w} Values for the Isoxazolyl-Naphthoquinones

Cpd.	Log P_{o-w}	Log P RP-HPLC	Δ^a	Log P RP-TLC	Δ^b	CLOGP	Δ^c
1	1.04	1.51	-0.47	1.31	-0.27	1.78	-0.74
2	1.57	2.00	-0.43	1.50	0.07	2.31	-0.74
3	1.80	2.52	-0.72	1.79	0.01	2.84	-1.04
4	---	3.25	---	3.42	---	3.90	---
5	2.14	2.92	-0.78	2.45	-0.31	2.54	-0.40
6	---	4.71	---	2.71	---	3.79	---
7	---	3.82	---	2.43	---	2.57	---

$\Delta^a = \log P_{o-w} - \log P_{RP-HPLC,}$. $\Delta^b = \log P_{o-w} - \log P_{RP-TLC}$, $\Delta^c = \log P_{o-w}$ - CLOGP.

$$\log P_{o-w} = 0.02316\ (\pm 0.0007)\ R_{mw} + 1.3925\ (\pm 0.05) \qquad (3)$$
$$n = 4,\ r = 0.998$$

Log P_{RP-TLC} values of the isoxazolyl-naphthoquinones **1-5** and **6, 7** were calculated by substitution of the R_{mw} values into the equations 2 and 3, respectively (Table 3).

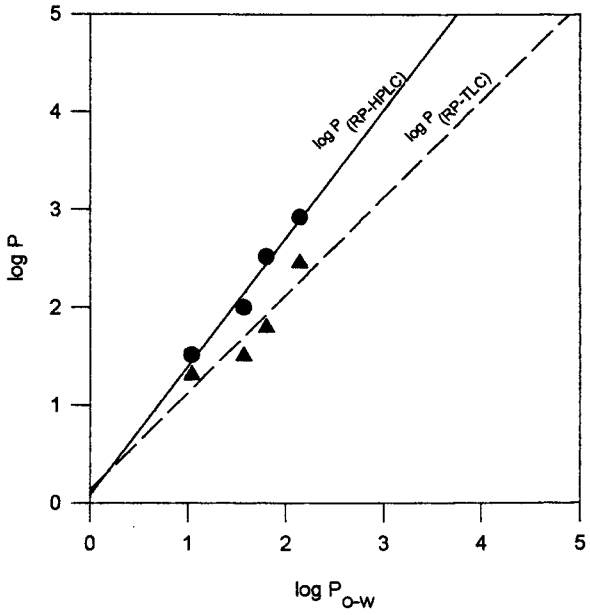

Figure 4. Correlation plots of log $P_{(RP\text{-}HPLC)}$ vs log $P_{o\text{-}w}$ and log $P_{(RP\text{-}TLC)}$ vs log $P_{o\text{-}w}$.

Shake Flask Log $P_{o\text{-}W}$ Determination

Partition coefficient values of isoxazolyl-naphthoquinones, determined in the n-octanol/water system, are listed in Table 3.

The great insolubility of **4**, **6** and **7** in water did not allow direct measurement of the partition coefficients by the conventional shaking flask method.

Correlation Between Different Techniques

Figure 4 shows two log P correlations. A plot of log $P_{RP\text{-}HPLC}$ vs log $P_{o\text{-}w}$ exhibits a slope of 1.3109 and an intercept of 0.0910 ($r = 0.973$); a plot of log $P_{RP\text{-}TLC}$ vs log $P_{o\text{-}w}$ exhibits a slope of 0.9962 and an intercept of 0.1313 ($r = 0.851$); and a plot, not shown, of CLOGP vs log $P_{o\text{-}w}$ exhibits a slope of 0.8094 and an intercept of 1.0422 ($r = 0.698$).

CONCLUSIONS

Although the shake flask method has become the standard technique for quantifying the hydrophobicity of organic compounds, it is particularly problematic when the compound is highly insoluble in either of the solvent phases. Derivatives **4**, **6** and **7** are slightly water-soluble compounds and their log $P_{o\text{-}w}$ could not be determined by this method.

Experimental log $P_{o\text{-}w}$ values were compared with the corresponding experimental values calculated from other methods (Table 3). The log $P_{RP\text{-}HPLC}$ values were generally slightly higher as shown for negative values of Δ^a = log $P_{o\text{-}w}$ - log $P_{RP\text{-}HPLC}$. The relationship between the log $P_{RP\text{-}HPLC}$ and the log $P_{o\text{-}w}$ gave the best correlation coefficient (r = 0.973).

The log $P_{RP\text{-}TLC}$ were in the same range as the corresponding log $P_{o\text{-}w}$ values, although the relationship between them is not as good as that obtained with the RP-HPLC technique (r = 0.851), but good enough if compared with those found in the literature.

The calculated partition coefficients by the CLOGP program are not in good agreement with the measured n-octanol/water partition coefficients because this program does not provide the values for some fragments entered via SMILES, resulting in values of zero.[13,14] These poor correlations, were also observed by other authors.[15,16]

Comparison between the log $P_{o\text{-}w}$ and CLOGP values indicate that the last method greatly over-estimated the hydrophobicity of all studied compounds, obtaining the worst correlation coefficient (r = 0.698).

The above results led us to conclude that the log P values determined by both chromatographic methods are in good agreement with those determined by the shake flask method. Thus, chromatographic techniques can be applied to determine the log $P_{o\text{-}w}$ of the studied isoxazolyl-naphthoquinones.

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