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Short communication

Use of reversed-phase liquid chromatography for determining the lipophilicity of α-aryl-*N*-cyclopropylnitrones

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Abstract

The relationship between a reversed-phase high-performance liquid chromatography (RP-HPLC) retention parameter and various calculated log *P*-values of our previously synthesized α-aryl-*N*-cyclopropyl-nitrone derivatives was investigated. The RP-HPLC experiments were carried out with acetonitrile–water and methanol–water mixtures as mobile phases and with two kinds of stationary phases of different polarity. The retention parameter, $\log k_w$ was obtained by linear extrapolation of the $\log k$ retention to pure water as the mobile phase. The calculated log *P*-values were *C* log *P*, ACD/log *P*, *R* log *P*, *A* log *P*, LogKow, *X* log *P* and *M* log *P*. Statistically, highly significant correlations were found between $\log k_w$ and the calculated log *P*-values with squared correlation coefficients ranging from 0.771 (with *A* log *P*) to 0.956 (with *C* log *P*).

In addition, the comparative molecular similarity indices analysis (CoMSIA) method was also applied to correlate the $\log k_w$ retention parameter of the compounds with their molecular fields. Statistically significant CoMSIA models were obtained between $\log k_w$ and the hydrophobic and steric molecular fields of our compounds. The CoMSIA models describe how the structure of the nitrone derivatives influences (through hydrophobic and steric interactions with the stationary phase) the chromatographic retention of the compounds.

Keywords: Cyclopropylnitrones; Lipophilicity; HPLC; Calculated log *P*; CoMSIA

1. Introduction

In the central nervous system, stroke, multiple sclerosis and neurotrauma have been proposed to initiate a sequence of oxidative events, which ultimately lead to neuronal cell death [1–3]. Therefore, antioxidant therapy plays an important role in the treatment of free radical induced neurodegeneration. It has been well-established that organic molecules incorporating a nitrone moiety can act as free radical trapping agents and are capable of opposing oxidative challenges [4,5]. α-Phenyl-*tert*-butyl nitrone (PBN), one of the most commonly used nitrone-based free radical traps, has been shown to protect in several experimental models of neurodegenerative diseases [6,7]. The free radical scavenger capacity and the above pharmacological properties of nitrones as well as their brain penetration are influenced by the substitution pattern of these molecules. For example, PBN has a grater tendency to cross the blood–brain barrier as compared with pyridine *N*-oxide *tert*-butyl nitrone, an oxidated pyridine analogue, in accordance with their lipophilicity difference [8].

Distribution of a compound in vivo may be viewed as a series of partitioning steps, in conjunction with diffusion through several regions. The process involves partitioning between aqueous media and biological membranes (e.g. blood–brain barrier). The affinity of a compound for biological membranes may be represented by its lipophilicity [9]. Usually, the lipophilicity of a compound can be quantitatively characterized by log *P*, the logarithm of its

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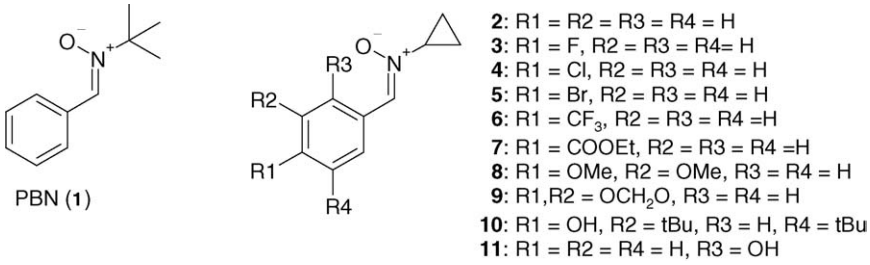

Fig. 1. The α-aryl-*N*-cyclopropylnitrones included in this study.

n-octanol/water partition coefficient [10]. The traditional experimental method for determining log *P* is the shake flask method. Nowadays, reversed-phase liquid chromatography (RP-HPLC) is widely used as an alternative technique to the tedious, time-consuming and poorly reproducible shake flask method, since the determined retention parameters (e.g. log k_w) can be correlated with log *P* and other lipophilicity parameters of compounds [11,12].

In the HPLC technique, the phase preference of a single solute can be expressed by the capacity factor *k*:

$$k = \frac{N_{stat}}{N_{mob}} \quad (1)$$

where N_{stat} and N_{mob} are the number of moles of the solute in the stationary and mobile phases, respectively. The capacity factor *k* can be calculated by:

$$k = \frac{t_R - t_0}{t_0} \quad (2)$$

where t_R and t_0 are the retention times of the substance under investigation and a nonretained compound, respectively. The sample *k* values are related to the volume fraction, φ, of the organic solvent in the mobile phase as:

$$\log k = \log k_w - S\varphi \quad (3)$$

The intercept log k_w corresponds to the retention in pure water as a mobile phase and represents the commonly employed chromatographic hydrophobicity parameter. *S* is a solute-dependent solvent strength parameter specific to the organic modifier on the stationary phase under consideration [13,14].

Another retention-related parameter has been introduced recently, the isocratic chromatographic hydrophobicity index, φ_0 [15,16]. The φ_0 value represents the volume fraction of the organic solvent in the mobile phase for which the amount of solute in the mobile phase is equal to that in the stationary phase, i.e. the capacity factor is 1 (log $k = 0$):

$$\varphi_0 = \frac{\log k_w}{S} \quad (4)$$

where φ_0 is equal to the ratio of the intercept and slope of Eq. (3).

In addition to determining the log *P*-values experimentally, they can also be predicted by using a number of log *P* calculation methods. The estimation of log *P* for complex structures may have a restricted importance because, so far, none of the available methods can take all the effects of molecular conformation, proximity and hydrogen bonds into consideration in the calculation procedure. On the contrary, these effects are simplified in many different computer-assisted methods. The methods can be arranged into three major groups: (i) methods based on fragmental constants; (ii) those based on atomic contributions; (iii) those based on conformation dependent properties [17].

In spite of the above-mentioned advantageous biological effects of nitrones and the fact that these biological effects are influenced by the lipophilicity of the compounds [18], relatively few experimental and calculated lipophilicity data are available for nitrones. Therefore, in the present paper, the lipophilicity of our previously synthesized α-aryl-*N*-cyclopropylnitrones [19] (Fig. 1) was investigated in two ways:

(1) the relationships between the chromatographic retention parameters of our compounds determined using RP-HPLC and their log *P* data calculated using various methods were analyzed and discussed;
(2) the above retention parameters of our compounds were investigated as a function of their hydrophobic and steric molecular fields using the comparative molecular similarity indices analysis (CoMSIA) method [20] in order to show another aspect of the relationship between the retention behaviour and the structure of our compounds.

2. Experimental

2.1. Materials

Two binary solvent systems, methanol–water and acetonitrile–water were used as a mobile phase. In the mobile phase, the content of organic component was varied between 40 and 70% with an increment of 5%. The methanol and the acetonitrile were of HPLC grade (Merck) and the water derived from a Milli-Q (Millipore, USA) water purification system (resistivity 18.2 MΩ cm). The eluents were pre-filtered through a 0.5 μm Fluoropore membrane filter (Millipore, USA) and degassed in an ultrasonic bath before use. The flow rate of the mobile phase was 0.5 ml/min

at room temperature; the amount of sample solution was 20 μl/injection.

Preparation of α-aryl-*N*-cyclopropylnitrones was carried out using an established method for the synthesis of nitrones, by direct condensation of the appropriate aldehyde with *N*-cyclopropyl-hydroxylamine [19].

Amounts of 5 mg/ml of each compound were dissolved in the eluent and diluted with the eluent to the concentration of 0.2 mg/ml. The solutions for the HPLC investigations were pre-filtered through a 0.45 μm Millex filter (Millipore, USA). Retention values of the compounds were calculated as the averages of three measurements for each solute–solvent combination.

2.2. High-performance liquid chromatography

The HPLC measurements were carried out using a Pharmacia LKB (Uppsala, Sweden) liquid chromatographic system consisting of an LKB 2249 gradient pump and an LKB 2141 variable wavelength detector set at 300 nm. Samples were injected using a Rheodyne 7161 valve (Cotati, CA, USA) fitted with a 20 μl loop. The columns used were Nucleosil-5 C-8, 250 mm × 4 mm i.d.; particle size 5 μm and Nucleosil-120 5 C-18, 250 mm × 4 mm i.d.; particle size 5 μm (Bio Separation Technologies, Budapest).

2.3. log P calculation

The log *P*-values of α-aryl-*N*-cyclopropylnitrones were calculated using the programs summarized in Table 1 [21–32].

As the electronic structure of the nitrone group implies zwitterionic, biradical and even hypervalent canonic forms (Fig. 2) [33], it should be mentioned how the log *P* calculation programs in Table 1 could handle this problem. ACD/log *P* was the only method recognizing both the ionic and the hypervalent forms of nitrones. On the other hand, the *C* log *P*, *A* log *P* and LogKow methods recognized only the hypervalent form, whereas the *X* log *P*, *M* log *P* and *R* log *P* methods could not recognize the nitrone structure at all. That means the latter methods handle the C, N and O atoms of the nitrone group as parts of other fragments.

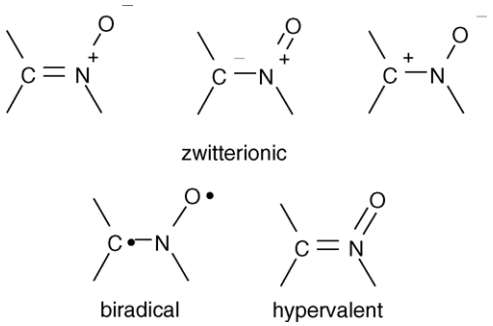

Fig. 2. Electronic structure of the nitrone group.

The calculated log *P*-values of our nitrone compounds are summarized in Table 2.

The intercorrelations of the calculated log *P*-values were also determined. Based on these intercorrelations, the calculation methods can be classified as follows. The first group includes *C* log *P*, *R* log *P*, LogKow and *X* log *P*, their correlation coefficients with each other falling in the range of 0.96 to 0.99. The second group includes ACD/log *P* and *A* log *P* with a correlation coefficient of 0.95. The correlation coefficients between groups 1 and 2 members range from 0.87 to 0.94. Finally, *M* log *P*'s correlation coefficients with the other calculation methods range from 0.77 to 0.92.

2.4. CoMSIA calculation

The calculations were carried out on a Silicon Graphics Octane workstation and on a Pentium PC. The structures were subjected to a full geometry optimization by the Spartan '04 program package [34] using the HF method with a split valence basis set 3–21*G*(*). A reliably low energy conformation was chosen for each molecule such that a proper alignment of the molecules was achieved. The CoMSIA analysis [20] was carried out using the SYBYL 6.8 program package [31]. The molecules were aligned according to their largest common substructure: Ph–C=N(–C)–O. The molecular fields were calculated using an sp^3 C as the probe atom with a charge of +1 and a grid spacing of 2 Å. Then the relationship between the retention parameter log k_w and the CoMSIA molecular fields was investigated using the partial least squares (PLS) method with the leave-one-out cross-validation technique and a filtering σ_{min} of 1.0.

Table 1
The log *P* calculation methods used in this study

Name	Software	Method
C log *P* [21,22]	SYBYL 6.8 [31]	Fragment-based
ACD/log *P* [23]	ACD/Labs package 8.0 [32]	Fragment-based
R log *P* [24]	LOGP, Rekker revised script, SYBYL 6.4 [31]	Fragment-based
A log *P* [25,26]	ALOGPS 2.1, Virtual Computation Chemistry Laboratory, http://www.vcclab.org/lab/alog*P*s	*E*-State indices (neural network)
LogKow [27]	LogKow (KowWin 1.6)	Fragment-based
X log *P* [28]	XlogP 2.0, Virtual Computation Chemistry Laboratory, http://www.vcclab.org/lab/alog*P*s	Atom contributions with correction factors
M log *P* [29,30]	MlogP 1.2	Counting atoms, bonds, fragments or functional groups

Table 2
Calculated log *P*-values for our nitrone compounds

	C log *P*	ACD/log *P*	*R* log *P*	*A* log *P*	LogKow	*X* log *P*	*M* log *P*
1[a]	1.23	1.25	4.06	1.40	1.21	3.14	2.59
2	0.35	0.38	3.35	0.65	0.65	2.39	1.91
3	0.71	0.56	3.59	0.71	0.85	2.55	2.32
4	1.28	1.54	4.08	1.00	1.29	3.01	2.47
5	1.43	1.14	4.28	1.16	1.54	3.19	2.61
6	1.62	1.36	4.37	1.22	1.61	3.31	2.89
7	1.25	1.32	4.39	1.37	0.97	2.75	2.11
8	0.42	0.41	3.49	0.68	0.29	1.95	1.37
9	0.73	-0.21	3.44	0.18	0.71	2.14	1.50
10	3.77	3.12	6.95	3.07	3.14	5.19	3.43
11	0.31	0.44	2.79	0.95	0.17	1.98	1.34

[a] PBN.

3. Results and discussion

Table 3 shows the log k_w and S parameters of Eq. (3) obtained for the acetonitrile–water eluent system. It can be seen that, as expected, the relationships between the log k_w retention values and the φ acetonitrile concentrations were found to be linear and statistically highly significant for each nitrone compound with both the Nucleosil-5 C-8 and the Nucleosil-120 5 C-18 columns.

The results obtained for the methanol–water eluent system are not reported here since they were nearly identical with the results obtained for the acetonitrile–water eluent system.

As seen from Table 3, the log k_w and S values of nitrones are significantly higher on the Nucleosil-120 5 C-18 stationary phase than on the Nucleosil-5 C-8 stationary phase. This means that the compounds enter into stronger interactions with the more nonpolar C-18 stationary phase [35]. Also, from the larger variation in log k_w and S on the C-18 stationary phase, it can be concluded that the structure of the nitrone compounds as well as the acetonitrile concentration in the mobile phase have a larger influence on the interactions of the compounds with the C-18 stationary phase than with the C-8 stationary phase.

Table 3
Parameters of Eq. (3) obtained for the acetonitrile–water eluent system

	C_8			C_{18}		
	log k_w	S	r^2	log k_w	S	r^2
1[a]	2.011	2.17	0.992	2.810	3.49	0.999
2	1.775	1.97	0.995	2.474	3.20	0.995
3	1.902	2.08	0.999	2.744	3.44	0.997
4	2.479	2.56	0.999	3.912	4.45	0.999
5	2.649	2.70	0.997	4.267	4.76	0.998
6	2.600	2.66	0.997	4.225	4.73	0.999
7	2.336	2.44	0.996	3.489	4.09	0.991
8	1.616	1.84	0.998	1.890	2.69	0.998
9	1.650	1.87	0.997	2.214	2.98	0.999
10	4.143	3.94	0.997	6.544	6.74	0.995
11	1.500	1.75	0.999	2.336	3.08	0.996

The r^2 is the squared correlation coefficient of the linear regression performed on 7 log $k - \varphi$ data points.
[a] PBN.

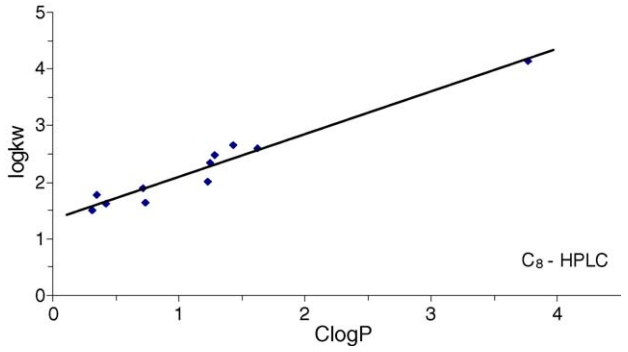

Fig. 3. Plot of log k_w determined on the Nucleosil-5 C-8 column vs. log *P* calculated using the *C* log *P* method.

The log k_w retention parameter values and the S slope values are also influenced by the character of the para substituent in the cyclopropylnitrone molecule and for the given two HPLC systems increase in the following order: –H < –F < –COOEt < –Cl < –CF_3 < –Br.

As it was suggested earlier, the log k_w values can be used as potential alternatives to the classical log *P* to express the lipophilic character of a compound. Table 4 summarizes the results of correlations between the log k_w retention parameter and the calculated log *P*-values of our nitrone compounds.

It can be seen that all the correlations in Table 4 are statistically highly significant and that the *C* log *P*, *R* log *P*, LogKow and *X* log *P* methods give much better correlations with log k_w than the ACD/log *P*, *A* log *P* and *M* log *P* methods, except one instance. Namely, ACD/log *P* and *R* log *P* give almost the same moderate correlation with log k_w on the C-18 column. Fig. 3 shows the best correlation in Table 4, the plot of the log k_w values obtained on the Nucleosil-5 C-8 column versus the log *P*-values calculated by the *C* log *P* fragment-based method.

Table 4 also shows that the correlation coefficients obtained on the C-8 column are somewhat higher than those obtained on the C-18 column. However, the correlations between log k_w and the calculated log *P*-values are similar with the two stationary phases. The reason is that the two retention parameters, log k_w on the C-8 column and log k_w on the C-18 column are highly correlated ($r = 0.99$).

Table 4
Linear relationships $\log k_w = a_1 + a_2 \log P$ between $\log k_w$ data and calculated $\log P$-values

	C_8					C_{18}				
	a_1	a_2	r^2	n	P	a_1	a_2	r^2	n	P
$C\log P$	1.340	0.757	0.956	11	0.0001	1.783	1.320	0.911	11	0.0010
ACD/log P	1.417	0.803	0.888	11	0.0024	1.899	1.416	0.868	11	0.0046
$R\log P$	−0.515	0.677	0.945	11	0.0002	−1.360	1.158	0.867	11	0.0047
$A\log P$	1.210	0.916	0.811	11	0.0193	1.559	1.594	0.771	11	0.0439
LogKow	1.221	0.903	0.954	11	0.0001	1.558	1.590	0.927	11	0.0005
$X\log P$	−0.066	0.803	0.946	11	0.0002	−0.698	1.411	0.915	11	0.0009
$M\log P$	0.017	0.997	0.773	11	0.0417	−0.651	1.796	0.787	11	0.0322

The a_1 and a_2 are regression coefficients, r^2 the squared correlation coefficient of the linear regression, n the number of nitrone compounds included in the regression and P is the statistical significance of the regression (in %).

The isocratic hydrophobicity index φ_0 was also determined for each compound with both stationary phases. However, due to the highly significant correlation of $\log k_w$ and φ_0 ($r = 0.96$ with both the C-8 and the C-18 columns) almost the same results were obtained with φ_0 as with $\log k_w$, therefore, these results are not reported here.

We also carried out a CoMSIA investigation to correlate the $\log k_w$ values of the compounds with their molecular fields. In accordance with the physical–chemical processes involved in the chromatographic separation and influencing the retention, the hydrophobic and steric CoMSIA fields were chosen as descriptors. The calculation resulted in a reliable CoMSIA model for both stationary phases. The statistical parameters were as follows:

- for Nucleosil-5 C-8: $q^2 = 0.494$, $c = 3$, $r^2 = 0.987$, $F_{3,7} = 178.1$, $s = 0.102$;
- for Nucleosil-120 5 C-18: $q^2 = 0.440$, $c = 3$, $r^2 = 0.990$, $F_{3,7} = 225.4$, $s = 0.162$.

where q^2 is the cross-validated squared correlation coefficient, c the number of PLS components, r^2 the nonvalidated squared correlation coefficient, $F_{3,7}$ the nonvalidated F-statistic with 3 and 7 degrees of freedom and s is the nonvalidated standard error of estimate.

The CoMSIA analysis identified regions of space around the molecules where an increased or decreased hydrophobic and steric interaction with the stationary phase increases the value of $\log k_w$, i.e. the retention. Thus, the CoMSIA models show that an increased hydrophobic and steric interaction at the R_1 substituent site as well as a decreased hydrophobic and steric interaction at the R_2 and R_4 substituent sites increase the retention of the compounds on both the C-8 and the C-18 columns.

4. Conclusion

In the present study, the relationship between the RP-HPLC retention parameter $\log k_w$ and various calculated $\log P$-values of our previously synthesized α-aryl-*N*-cyclopropylnitrone derivatives was investigated. In addition, the CoMSIA method was also applied to correlate the $\log k_w$ retention parameter of the compounds with their molecular fields.

Statistically, highly significant correlations were found between $\log k_w$ and the calculated $\log P$-values with both the Nucleosil-5 C-8 and Nucleosil-120 5 C-18 stationary phases. The best correlation was found with the $C\log P$ method on the C-8 column ($r^2 = 0.956$) and the LogKow method on the C-18 column ($r^2 = 0.927$), while the worst correlation was found with the $M\log P$ method on the C-8 column ($r^2 = 0.773$) and the $A\log P$ method on the C-18 column ($r^2 = 0.771$).

Also, we obtained statistically significant CoMSIA models between $\log k_w$ and the hydrophobic and steric molecular fields of our compounds with both stationary phases. The CoMSIA models describe how the structure of the nitrone derivatives influences (through hydrophobic and steric interactions with the stationary phase) the chromatographic retention of the compounds.

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