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Retention of substituted coumarins using immobilized artificial membrane (IAM) chromatography: A comparative study with *n*-octanol partitioning and reversed-phase HPLC and TLC

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Abstract

The chromatographic indices log k_{wIAM} have been determined by HPLC on an immobilized artificial membrane column for a set of coumarin derivatives. The investigated compounds contain substituted amidoximes, or substituted heterocycles directly attached to the coumarinic skeleton. The log k_{wIAM} values were compared to previously reported data of octanol–water partition coefficients and extrapolated capacity factors determined by reversed phase HPLC and TLC. The log k_{wIAM} values of the investigated compounds were found to be comparable with the corresponding log *P* values, although they constitute a lower lipophilicity scale due to the reduced hydrophobic environment of the IAM stationary phase. These features were exemplified in their interrelationship with a slope close to unity and a large negative intercept. In contrast their comparison with the corresponding HPLC and RP-TLC capacity factors revealed differences in the retention mechanism reflected in slopes lower than unity, which were postulated to be due to secondary interactions under the reversed phase chromatographic conditions. However, conformational effects in the molecular structures of the coumarin derivatives were found to have a similar impact in their affinity for the IAM and octadecyl silane stationary phases, while they did not affect their octanol–water partitioning.

Keywords: Immobilized artificial membranes; Octanol–water partition coefficients; HPLC; RP-TLC; Substituted coumarins

1. Introduction

HPLC and reversed phase TLC are considered very popular techniques for the assessment of lipophilicity, the physicochemical property of primary interest for the evaluation of ADME characteristics in drug design [1,2]. During the last decades numerous publications report correlations between chromatographic capacity factors and octanol–water log *P*, the reference lipophilicity parameter [3,4]. Standardization of the chromatographic conditions in HPLC have been suggested in order to obtain calibration curves for log *P* prediction, the ultimate goal being the estimation of solute transport across the biological barriers [5]. The development of immobilized artificial membrane (IAM) chromatography unfolded new perspectives in the application of HPLC for the rapid evaluation of drug partitioning into cell membranes. IAMs are monolayers of phospholipid molecules covalently bonded to a solid matrix, the surface of silica particles. IAM capacity factors have been successfully used to correlate drug permeability data [6–8]. In a recent review, technical aspects relevant to the proper measurement of IAM capacity factors have been discussed, while attempts to explore the molecular factors governing IAM retention are also reported in references [9–12]. The functional groups of the bonded phospholipids are considered to play an important role in retention especially if charged molecules are analysed. For small neutral compounds the intermolecular forces resemble those underlying partitioning in octanol/water and retention in reversed

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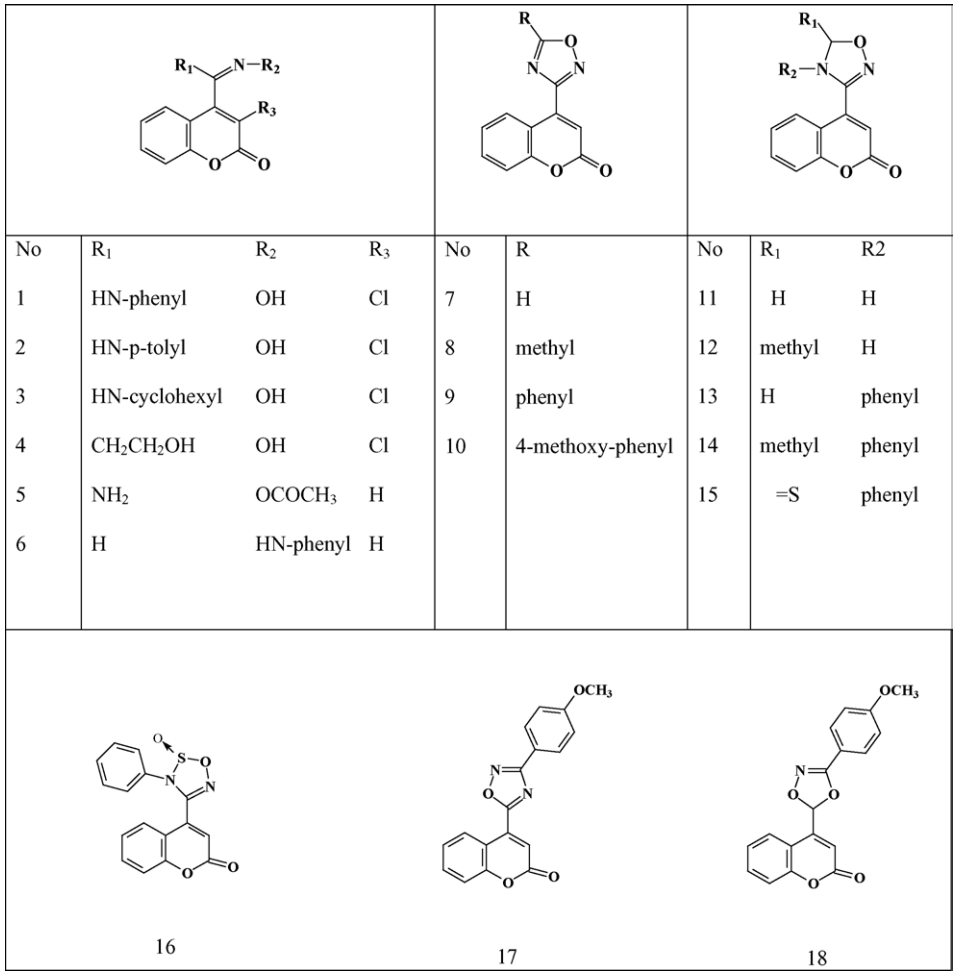

No	R_1	R_2	R_3
1	HN-phenyl	OH	Cl
2	HN-p-tolyl	OH	Cl
3	HN-cyclohexyl	OH	Cl
4	CH_2CH_2OH	OH	Cl
5	NH_2	$OCOCH_3$	H
6	H	HN-phenyl	H

No	R
7	H
8	methyl
9	phenyl
10	4-methoxy-phenyl

No	R_1	R2
11	H	H
12	methyl	H
13	H	phenyl
14	methyl	phenyl
15	=S	phenyl

16 17 18

Fig. 1. Chemical structures of the investigated coumarin derivatives.

phase HPLC. Thus besides the hydrophobic/solvophobic interactions, polar interactions mainly expressed as H-bond acceptor basicity are the predominant factors in IAM retention. According to the results of LSER analysis however the hydrophobic term seems to have a smaller positive contribution in IAM retention compared to its contribution in octanol/water partitioning [12]. Moreover, it is considered that the IAM surface provides a hydrophobic environment that resembles a RP-C3 HPLC column, whereas for lipophilicity assessment RP-C18 stationary phase are used [13]. Nevertheless, further investigations are needed to support the above-mentioned postulations since to date the reports found in literature cover only a few series of homologous compounds.

In the light of the above considerations the IAM retention of a series of neutral coumarin derivatives was assessed and compared to their partitioning in octanol–water, as well as to their behaviour in reversed phase HPLC and TLC, previously reported [14,15]. The coumarin derivatives have been synthesized as anti-inflammatory and antioxidant agents [16–18]. They contain a substituted amidoxime moiety or a substituted heterocyclic ring, directly attached at position 4 of the coumarinic skeleton, creating a conjugated double bond system in most cases (Fig. 1). This chemical environment is responsible for a considerable contribution of electronic effects to lipophilicity [14], while conformational differences were found to affect retention leading to discrepancies between octanol and water partition coefficients and reversed phase chromatographic indices in some cases [15].

2. Materials and methods

All compounds have been synthesized and purified in the Laboratories of Pharmaceutical Chemistry, Department of Pharmacy and of Organic Chemistry, Faculty of Chemistry, University of Thessaloniki. Methods and physical data are reported in references [16,17]. Coumarin was used as a reference compound and was purchased from Sigma–Aldrich Co. Acetonitrile was HPLC grade and purchased from Lab-Scan Analytical Sciences Ltd., Ireland. Water was de-ionised and

further purified by means of a Milli-Q Plus water purification system (Millipore Co., USA).

2.1. IAM chromatographic conditions

The HPLC isocratic pumping system consisted of a GBC Model 1126 pump and a Rheodyne Model 7725i injector with a 20 μl loop, which were coupled to a GBC Model LC1210 UV–vis detector operated at 254 nm. Data acquisition was performed using WinChrom chromatography software package Version 2.1.

The stationary phase consisted of an IAM.PC.DD2 column (Regis Technology, Morton Grove, IL, USA). The mobile phase consisted of pure water or of acetonitrile–water mixtures. Acetonitrile was added at concentrations ranging from 20 to 35%. The mobile phase was filtered through a 0.20 μm nylon membrane before use.

The flow rate was 1, 2 or 3 ml/min.

The compounds were dissolved in methanol at concentrations ~20 μg/ml. The chromatographic retention time t_r of each compound was measured and converted to log k according to the equation

$\log k_{IAM} = \log[(t_r - t_o)/t_o]$, to being the retention time of potassium bichromate. Each measurement was performed at least in dublicate when organic modifier was used and in triplicate for mobile phase consisting of pure water.

2.2. Octanol–water partition coefficients

Experimental octanol–water partition coefficients, log P, were taken from reference [14].

2.3. Reversed phase HPLC log k_{wBDS} and TLC R_{Mw}

Extrapolated capacity factors determined by HPLC using a BDS column and by RP-TLC on RP-18 plates were taken from reference [15].

3. Results and discussion

The derivatives **4**, **5**, **7**, **8**, **11–13** and coumarin could be analysed using pure water as mobile phase with retention times not exceeding 30 min. The capacity factors determined in this way correspond to the actual $\log_{wIAM}$ values and are presented in Table 1.The more lipophilic derivatives (**1–3**, **6**, **9**, **10**, **14–18**) were strongly retained using pure water, thus for their elution acetonitrile was added as organic modifier in the mobile phase. To keep the same conditions for all compounds acetonitrile was used at percentages 20, 25, 30 and 35% and log k_{wIAM} values at 100% water were derived by linear extrapolation plotting of the isocratic capacity factors, log k_{IAM}, versus the percentage (v/v) of organic modifier in the eluent, according to Eq. (1)

$$\log k_{IAM} = -S\varphi + \log k_{wIAM} \quad (1)$$

Table 1
Actual and extrapolated IAM capacity factors of the investigated coumarin derivatives

No.	log k_{wIAM} (actual)	log k_{wIAM} (extra)	S	r
1		1.64 ± 0.01	4.36 ± 0.12	0.999
2		2.05 ± 0.05	5.30 ± 0.44	0.993
3		1.78 ± 0.04	4.58 ± 0.38	0.993
4	0.05			
5	0.35			
6		3.01 ± 0.07	6.37 ± 0.61	0.991
7	1.10	0.95 ± 0.12	3.05 ± 0.44	0.980
8	1.26	1.12 ± 0.12	3.3 ± 0.42	0.984
9		3.14 ± 0.25	6.48 ± 0.90	0.981
10		2.94 ± 0.13	6.01 ± 0.48	0.994
11	1.09	0.94 ± 0.12	3.0 ± 0.44	0.978
12	1.12			
13	1.78	1.64 ± 0.12	3.75 ± 0.44	0.990
14		1.61 ± 0.09	4.29 ± 0.08	0.9997
15		1.89 ± 0.10	4.71 ± 0.37	0.994
16		1.50 ± 0.02	3.68 ± 0.18	0.998
17		3.24 ± 0.04	7.12 ± 0.38	0.997
18		2.14 ± 0.04	4.74 ± 0.12	0.9997
Coumarin	0.67			

φ being the percentage of acetonitrile added in the mobile phase in order to determine the isocratic log k_{IAM} values.

In order to examine the effect of acetonitrile in the extrapolated log k_{wIAM} values the same procedure was used also for the less retained compounds **7**, **8**, **11** and **13**. The extrapolated capacity factors along with the corresponding slopes S and the statistical data are included in Table 1. A very good linearity between log k_{IAM} and φ was observed in all cases. In addition log k_{wIAM} values correlated well with the corresponding slopes S, an indication of the uniformity in the retention mechanism within the investigated series of compounds. Eq. (2) describes the relationship between log k_{wIAM} and S.

$$\log k_{IAMw} = 0.599(\pm 0.028)S - 0.901(\pm 0.141)$$
$$n = 15 \quad r = 0.987 \quad s = 0.137 \quad F = 487.5 \quad (2)$$

The new slope of Eq. (2) is close to the value 0.5 reported by Barbato et al. [10] as indicative of a common property exhibited by the solutes for the hydrophobic expulsion process in the IAM/acetonitrile system in comparison with a value ca. 1 established for the ODS/methanol system [19]. It should be noted however that the negative intercept of Eq. (2) is smaller than the corresponding intercept reported by Barbato et al. (intercept value reported: 1.245). This differentiation may be related to the use of a different type of IAM column by these authors (IAM.PC.MG) [10].

Comparison of the extrapolated capacity factors of compounds **7**, **8, 11** and **13** with the actual log k_{wIAM} values showed that the latter were a little higher. Correlation between actual and extrapolated log k_{wIAM} led to Eq. (3) with excel-

Table 2
Corrected $\log k_{wIAM}$ values, octanol–water partition coefficients and reversed phase chromatographic indices

No.	$\log k_{wIAM}$[a]	$\log P$[b]	$\log k_{wBDS}$[c]	R_{MW}[c]
1	1.78	2.58	2.96	3.04
2	2.18	2.89	3.54	3.43
3	1.92	2.78	3.69	3.92
4	0.05	0.23	1.03	0.94
5	0.35	1.0	1.12	1.19
6	3.14	3.63	4.46	4.65
7	1.1	1.95	2.3	2.11
8	1.26	2.12	2.65	2.53
9	3.26	3.22	4.56	4.72
10	3.06	2.98	3.63	–[d]
11	1.09	1.98	2.23	2.83
12	1.12	1.93	2.07	2.3
13	1.78	2.45	2.87	3.01
14	1.75	2.70	2.98	3.407
15	2.02	3.03	3.4	3.68
16	1.64	2.16	3.26	3.11
17	3.36	3.07	4.81	5.058
18	2.54	3.04	3.97	4.135
Coumarin	0.67	1.39	1.69	2.03

[a] Corrected by means of Eq. (3).
[b] Taken from reference [14].
[c] Taken from reference [15].
[d] Not available.

lent statistics but with a slope slightly lower than 1 and a small but significant positive intercept.

$$\log k_{wIAM}(\text{actual}) = 0.987(\pm 0.008)\log k_{wIAM}(\text{extra}) + 0.161(\pm 0.009) \quad (3)$$
$$n = 4 \quad r = 0.999 \quad s = 0.005 \quad F = 15154.1$$

These results indicate a small systematic influence of acetonitrile in the retention of the investigated coumarin derivatives. Therefore, Eq. (3) was used as a calibration equation to correct the extrapolated $\log k_{wIAM}$ in order to use them together with the actual $\log k_{wIAM}$. The corrected $\log k_{wIAM}$ are presented in Table 2.

3.1. Comparison of IAM retention with octanol–water partitioning

The corrected $\log k_{wIAM}$ were correlated to octanol–water $\log P$ values (Table 2) and regression Eq. (4) was obtained.

$$\log k_{wIAM} = 1.066(\pm 0.107)\log P - 0.738(\pm 0.270) \quad (4)$$
$$n = 19 \quad r = 0.923 \quad s = 0.383 \quad F = 98.1$$

It should be noted that the correlation was slightly worse ($r = 0.916$) if the uncorrected extrapolated values were used together with the directly measured $\log k_{wIAM}$ values.

A visual inspection of the relationship between $\log k_{wIAM}$ and $\log P$ is provided by Fig. 2a. It can be seen that the $\log P$ values lie below the broken line, which represents the ideal 1:1 correlation. Exceptions in this tendency were observed

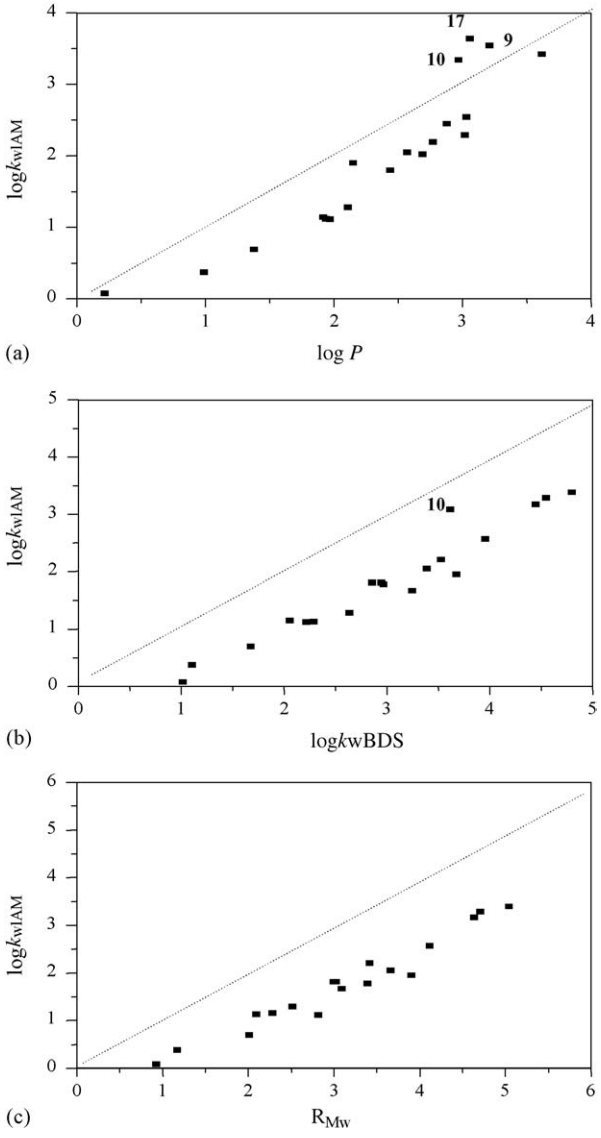

Fig. 2. Comparison between corrected $\log k_{wIAM}$ values and (a) $\log P$, (b) $\log k_{wBDS}$ values and (c): R_{Mw} values.

for the phenyl substituted oxadiazoline derivatives **9**, **10** and **17**, which possess $\log P$ values above the broken line. These compounds showed considerably enhanced retention leading to $\log k_{wIAM}$ higher than the corresponding $\log P$ values. Moreover the two isomers **10** and **17** showed differentiation in their retention behaviour, the latter being eluted considerably slower. The retention behaviour of compounds **9**, **10** and **17** is comparable to their behaviour in reversed phase chromatography and will be further discussed in Section 3.2. To the other end of the plot presented in Fig. 2a, in the region of polar compounds, a curvature appears in agreement with an analogous observation previously reported by Testa and coworkers for compounds with $\log P$ close or smaller than zero [12]. Exclusion of derivatives **9**, **10** and **17** as well as of the hydrophilic derivative 4 ($\log P = 0.23$) led to Eq. (5), which

does not differ from Eq. (4) but has improved statistics.

$$\log k_{wIAM} = 1.025(\pm 0.065)\log P - 0.812(\pm 0.161)$$
$$n = 15 \quad r = 0.974 \quad s = 0.167 \quad F = 244.6 \tag{5}$$

The slope ca. 1 of Eq. (5) denotes that the substituents attached on the coumarinic skeleton exhibit an analogous effect in IAM retention and in octanol–water partitioning and the two processes should be considered homoenergetic. Thus, the $\log k_{wIAM}$ values of the investigated compounds are fully comparable with the $\log P$ values, although they constitute a lower lipophilicity scale due to the reduced hydrophobic environment of the IAM stationary phase.

3.2. Comparison with reversed-phase chromatography

Extrapolated capacity factors $\log k_{wBDS}$ and R_{MW} obtained under reversed phase conditions are taken from reference [15] and are included in Table 2. They were determined using methanol as organic modifier on a BDS column and RP-18 plates, respectively. Their values are considerably larger than the corresponding IAM capacity factors. As illustrated in Fig. 2b and c, respectively, all $\log k_{wBDS}$ and R_{Mw} values lie below the broken line and no curvature is observed in the region of more polar compounds. Compounds **9** and **17** did not deviate from the scatter plots since they showed analogous enhanced retention in all three chromatographic systems. Compound **10** was strongly retained in HPLC but considerably less than its positional isomer **17** (R_{Mw} not available for compound **10**). The behaviour of compounds **9**, **10** and **17** was discussed in reference [15] and attributed to conformational effects. According to the results of molecular modelling compound **10** tends to occupy a rather extended conformation, while its positional isomer **17** and compound **9** are completely planar in differentiation from the folded conformation of the other coumarin derivatives [15]. It seems that these conformational effects have an analogous impact on IAM retention. However the reduced co-planarity of compound **10** did not affect its affinity for the IAM stationary phase. Thus, although it was less retained than compound **17**, it attained a larger than expected $\log k_{wIAM}$ value and consequently lies higher apart in the $\log k_{wIAM}/\log k_{wBDS}$ scatter plot (Fig. 2b).

Correlation between the $\log k_{wIAM}$ and $\log k_{wBDS}$ led to Eq. (6).

$$\log k_{wIAM} = 0.861(\pm 0.053)\log k_{wBDS} - 0.800(\pm 0.172)$$
$$n = 19 \quad r = 0.968 \quad s = 0.249 \quad F = 254.8 \tag{6}$$

Compound **10** was found to be an outlier. Its exclusion from the regression analysis led to Eq. (6a) with improved statistics.

$$\log k_{wIAM} = 0.838(\pm 0.038)\log k_{wBDS} - 0.773(\pm 0.119)$$
$$n = 18 \quad r = 0.984 \quad s = 0.173 \quad F = 493.6 \tag{6a}$$

Eq. (7) describes the relationship between $\log k_{wIAM}$ and R_{Mw} values.

$$\log k_{wIAM} = 0.801(\pm 0.045)R_{Mw} - 0.772(\pm 0.148)$$
$$n = 18 \quad r = 0.976 \quad s = 0.212 \quad F = 322.1 \tag{7}$$

The large negative intercepts of Eqs. (6a) and (7) are comparable to that of Eq. (5) and reflect the reduced hydrophobic environment in IAM stationary phase. However, in contrast to Eq. (5) the slopes of Eqs. (6a) and (7) are lower than 1, indicating that apart from the partition mechanism secondary forces (e.g. silanophilic interactions) may be involved in the reversed phase retention behaviour of the investigated coumarin derivatives. This issue was also discussed in reference [15].

4. Conclusions

The $\log k_{wIAM}$ values of the investigated coumarin derivatives were found to be fully comparable with the corresponding $\log P$ values, although they constitute a lower lipophilicity scale due to the reduced hydrophobic environment of the IAM stationary phase. In contrast, their comparison with the corresponding HPLC and RP-TLC capacity factors revealed differences in the retention mechanism, exemplified in interrelationships with slopes lower than unity. This behaviour may reflect secondary interactions under the reversed phase chromatographic conditions. However conformational effects in the molecular structures of the coumarin derivatives were found to have a similar impact in their affinity for the IAM and octadecyl silane stationary phases, while they did not affect their octanol–water partitioning.

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