Eur. J. Med. Chem. 33 (1998) 215–223

Short communication

IAM retention and blood brain barrier penetration

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(Received 10 July 1997; accepted 12 November 1997)

Abstract – A series of 1,3,5-triazines possessing bronchospasmolytic, antiinflammatory and central nervous system activities have been chromatographically characterized using commercially available immobilized artificial membrane (IAM) columns. The obtained capacity factors correlated well ($r = 0.97$) with the shake-flask octanol–buffer (log P) partition coefficient but less well ($r = 0.72$) with capacity factors obtained using RP-HPLC columns (log k'_w). Moreover it was shown that the potentiation of oxotremorine-induced tremors, observed for this class of compounds correlated well with both log P and log k'_{IAM} parameters. On the contrary such a correlation did not exist when using $\Delta\log P$ ($\log P_{octanol-water} - \log P_{cyclohexane-water}$). Thus the determination of log k'_{IAM} is a fast, reliable and efficient method for assessing lipophilic behaviour of 1,3,5-triazines in connection with CNS-related biological activities. © Elsevier, Paris

1,3,5-triazines / high-performance liquid chromatography (HPLC) / immobilized artificial membranes (IAM) / lipophilicity / blood brain barrier (BBB)

1. Introduction

The efficacity of a drug design process is critically dependent on the possibility of assessing, as early as possible, such properties as penetration of various biological membranes. Indeed these characteristics have a direct impact on the biological profile of the compound under investigation e.g. its main effect, side effect, bioavailability, etc. In medicinal chemistry laboratories partition properties have long been used as a means of evaluating the membrane penetration capabilities of molecules.

In addition to the classical octanol–water partition coefficients (log P), distribution coefficients (log $\Delta_{7.4}$) or $\Delta\log P$ ($\log P_{octanol} - \log P_{cyclohexane}$), chromatographic capacity parameters (log k', log k'_w) obtained by RP-HPLC on octadecylsilane (ODS) columns have been increasingly used as descriptors in SAR and QSAR relationships [1–3]. In an effort to mimick, as closely as possible, the 'in vivo' situation biological models of membranes, such as Caco-2 cells [4] mimicking the intestinal barrier or cocultures of astrocytes and endothelial cells [5] known to reproduce the characteristics of the blood brain barrier (BBB), have also been proposed.

The IAM (Immobilized Artificial Membrane) chromatographic columns first introduced in 1986 for separation purposes [6] have now been improved and shown to be a very efficient way of assessing lipophilic behaviour of molecules [7–10]. These columns, containing a monolayer of phosphatidylcholine immobilized on a silica support, are thought to mimick very closely a membrane bilayer [9] and are used in an HPLC system with a physiological buffer as eluent; the compounds under analysis are thus characterized by a capacity factor log k'_{IAM}.

Excellent correlations of this capacity factor have been observed not only with the octanol–water partition coefficient (log P) but also with a partition coefficient in an 'in vitro' model using DMPC liposomes, and with permeability of drugs through Caco-2 cells. This last point is especially important since oral bioavailability in man correlates well with Caco-2 cell permeability [11, 12]. In several cases it was shown that the IAM capacity factor is superior to log P as a descriptor of lipophilicity [13].

In this study a series of amino-1,3,5 -triazines possessing bronchospasmolytic, antiinflammatory and CNS-mediated activities [14, 15] have been characterized with respect to their lipophilic behaviour using

*Correspondence and reprints

IAM columns. The obtained capacity factors (k'_{IAM}) were compared with available log $\Delta_{7.4}$ (shake flask) data as well as with log k' (RP-HPLC on ODS column) data. However, our main goal was to seek a correlation between an 'in vivo' behavioural paradigm and retention on IAM columns. A salient feature of the before-mentioned triazines is their potential to enhance the tremorogenic action of oxotremorine (a cholinergic agent) while being themselves devoid of any cholinergic activity. The magnitude of this effect can represent therefore a direct measure of the ease with which these compounds cross the blood brain barrier. Such a biological profile might be of interest in pathological situations where there is a decrease in the cholinergic function (e.g. the Alzheimer disease). Indeed some of these compounds have been shown to possess potent activity in cognition tests in rats or mice (e.g. scopolamine induced amnesia).

We show that potentiation of oxotremorine-induced tremors observed for this class of compounds [15] correlated best with log $\Delta_{7.4}$ and log k'_{IAM} values.

2. Results and discussion

The pK_as of the above-mentioned triazines are between 5 and 6 and therefore these molecules are largely unprotonated at physiological pH (e.g. log P = log $\Delta_{7.4}$). Our goal was twofold:

a) to compare the log P with capacity factors obtained using IAM and RP (reversed phase) columns;

to study the correlation between these parameters and the potential to cross the blood brain barrier (BBB) as measured by a Central Nervous System (CNS)-mediated pharmacological property, potentiation of oxotremorine-induced tremor in mice.

In order to achieve these goals the triazines were divided in two groups:

- Group A (*table I*) included a series of 18 cyclopropylamino-1,3,5-triazines for which log P octanol–water coefficients have been previously measured using an in-house developed micro-shake flask procedure (see experimental part);
- Group B (*table II*) included a series of 15 alkylamino and acylamino-1,3,5-triazines for which data concerning potentiation of oxotremorine induced tremor (log PCTR; see experimental part) was available.

For the 18 triazines indicated in *table I* capacity factors (k'_w) have been determined using an RP8 column and different proportions of methanol in the mobile phase followed by extrapolation to 100% buffer (see experimental part). Moreover, for the same compounds capacity factors (k'_{IAM}) on two phospholipidic columns (IAM.PC.MG and IAM.PC.C_{10}/C_3) have also been determined. Plotting log $P_{octanol-water}$ measured for the above triazines against log k'_w showed appreciable, statistically significant deviation from a straight line with mediocre statistics (*figure 1*).

An unexpected difference was observed between structurally very closely related triazines. Thus triazines in entries 16 and 18 (*table I*) differ structurally only by a methylene group in the R2 substituent but a large deviation from the line is observed for compound 18.

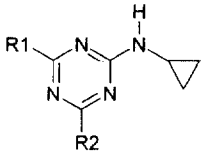
Since experimental determination of k'_w capacity factors involves a number of operations (e.g. the use of different methanol–buffer mixtures followed by extrapolation to 100% buffer), this in itself might account for the observed behaviour. Contrary to the above, the same plot but using results from an IAM.PC.MG column showed a much better correlation as seen by the R values, s values and cross validation factors (*figure 2*).

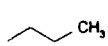
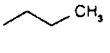
In this case only one operation involving the direct use of buffer eluent is needed for the k'_{IAM} determination. The same kind of correlation might be found when using a different type of IAM column (e.g. PC.C_{10}/C_3) as shown by the parallel lines depicted in *figure 3*. An increase in the capacity factors for all 18 triazines is observed when comparing the PC.C_{10}/C_3 column with the PC.MG column (*figure 4*).

In these cases only one operation involving the direct use of buffer eluent is needed for k'_{IAM} determination. Moreover it has to be pointed out that the analysis time using IAM columns is reduced by a factor of 8 and 2.5 compared to the micro-shake flask technique (log P) and measurement of log k'_w (RP 8 columns) respectively. The 'calibration line' established in *figure 1* was used to estimate the log $P_{octanol-water}$ partition coefficient for a series of 15 alkylamino and acylamino triazines (*table II*). These values (*table II*, column 4) are in quite good agreement with the ones measured by our micro-shake flask method (*table II*, column 6), sometimes even when extrapolation has been performed (see for example entries 20 and 26).

The difference in partition coefficients obtained while using octanol and cyclohexane as the organic phase (Δlog P) has been sometimes indicated in the literature as a good measure of hydrogen bond formation which has been linked with the ability of crossing the blood brain barrier [2, 16]. Therefore this parameter has also been included in our comparative study. Barrier penetration is an integrated and complex phenomenon which however might be approximated if some kind of relation (correlation) could be found between a partition parameter and a biological activity of interest. As stated previously we were interested to investigate whether such a correlation might be expected for the triazines under study (*table II*). The area under the dose effect curves (see experimental part) has been used in order to measure the percentage of tremor enhancement produced by

Table I. Cyclopropylamino-1,3,5-triazines used to establish calibration lines.

Group A triazines: 

Entry	R_1	R_2	log P_{Oct}	log k'_w	log k'_{IAM}
1			1.56	2.129	0.337
2			1.61	1.715	0.429
3			1.71	1.926	0.531
4			1.76	2.637	0.698
5			1.76	2.020	0.422
6			1.82	1.982	0.598
7		CH3	1.84	2.153	0.793
8			2.11	2.375	0.859
9			2.31	2.785	1.183
10			2.33	2.914	1.161
11			2.37	2.631	1.036
12			2.40	2.529	0.991
13			2.80	2.819	1.255
14			3.02	3.301	1.529
15			3.07	3.316	1.449
16			3.14	3.271	1.548
17			3.34	3.467	1.643
18			3.60	2.390	1.748

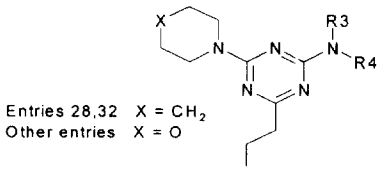
each triazine (log PCTR – oxotremorine induced tremor).

A plot against log k'_{IAM} leads to a quadratic (parabolic) curve indicating that about 80% of the variation in the observed effect is explained by the variation in k'_{IAM} capacity factors (*figure 5*).

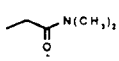
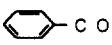
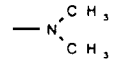
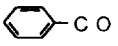
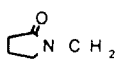
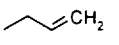
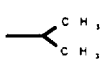
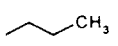
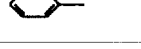
Even so the fit to a parabola may not look perfect but from a practical point of view it does give some

Table II. Alkyl and acylamino-1,3,5-triazines potentiating oxotremorine-induced tremor.

Group B triazines:



Entries 28,32 X = CH_2
Other entries X = O

Entry	R_3	R_4	log P^a	log k'_{IAM}	log P_{Oct} (M.S.F.)	log P_{Cyc} (M.S.F.)	Δ log P^b	log PCTR
19	H		0.51[c]	− 0.330	0.67	− 3.02	3.69	1.150
20	H	H	0.70[c]	− 0.194	1.55	− 2.01	3.56	2.083
21	H		1.61	0.471	2.33	0.87	1.46	1.845
22	H		1.94	0.703	1.64	0.18	1.46	2.110
23	CH_3		1.99	0.746	2.01	0.52	1.49	2.286
24	H	CH_3	2.02	0.762	2.15	1.00	1.15	2.124
25	H	CH_3CH_2	2.11	0.827	2.23	1.62	0.61	1.732
26	H		2.32	0.983	1.62	− 0.58	2.20	1.750
27	H	$-CH_2-C\equiv CH$	2.42	1.051	2.30	0.45	1.85	2.361
28	H	H	2.43	1.063	2.55	0.02	2.53	1.591
29	H		2.47	1.093	2.37	− 0.66	3.03	1.898
30	H		2.65	1.218	2.26	1.26	1.00	2.114
31	H		2.78	1.312	2.70	2.38	0.32	1.643
32	H	CH_3CH_2	3.12	1.561	3.34	2.78	0.56	1.230
33	H		3.61[c]	1.917	3.59	− 0.32	3.91	0.000

[a]log P estimated using the calibration established with UCB triazines of group A; [b]Δlog P via log P measured by micro-shake flask; [c]extrapolated value.

indication about the crossing of the blood brain barrier since the observed pharmacological effect is CNS-mediated. The maximal tremor enhancing effect is obtained for log k'_{IAM} around 0.5 and indeed other CNS-dependent properties are greatest for those triazines having the same k'_{IAM} capacity factors (not shown). A similar parabolic pattern is obtained when measured log $P_{octanol-water}$ is used for the correlation, the statistics being slightly better in this case (*figure 6*).

The above curve indicates that maximal biological effect (tremor enhancement) is obtained at a log P value of about 2 which is indeed in the range of optimal values (2–2.5) reported in the literature for efficient crossing of the blood brain barrier [17].

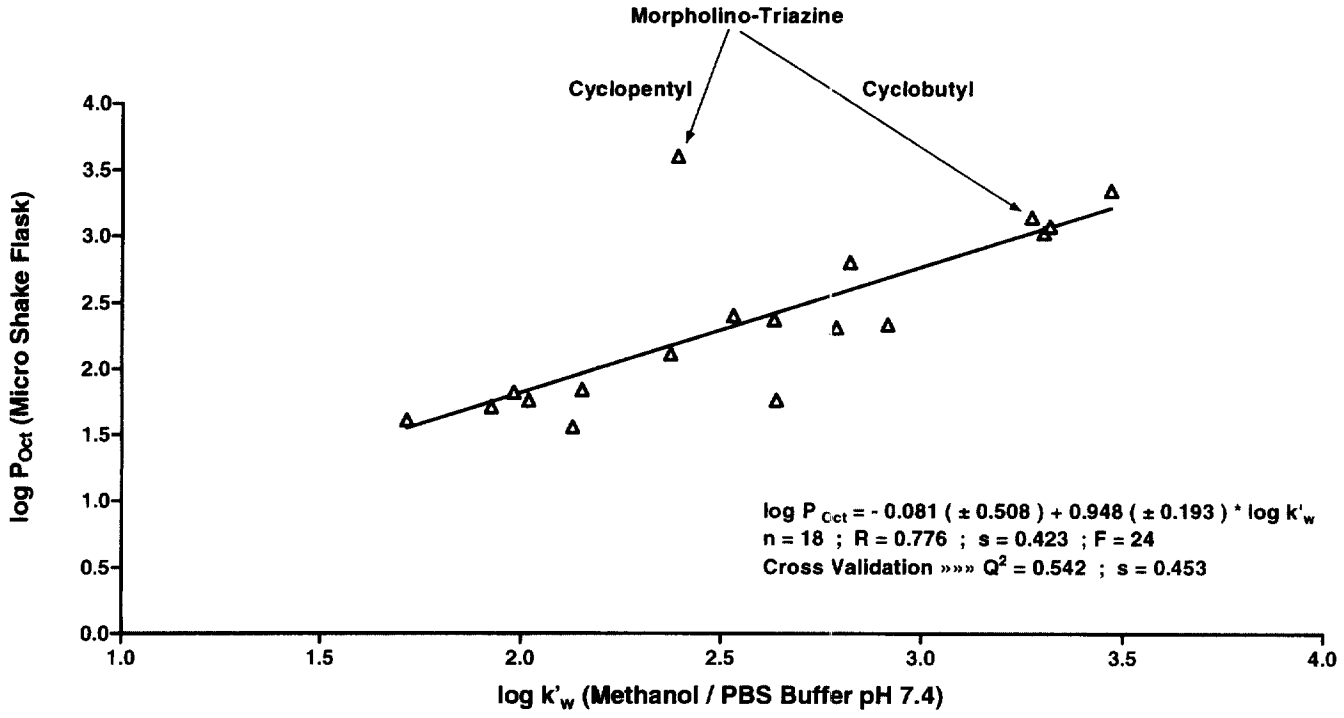


Figure 1. Group A triazines: log P_{Oct} vs. log k'_w.

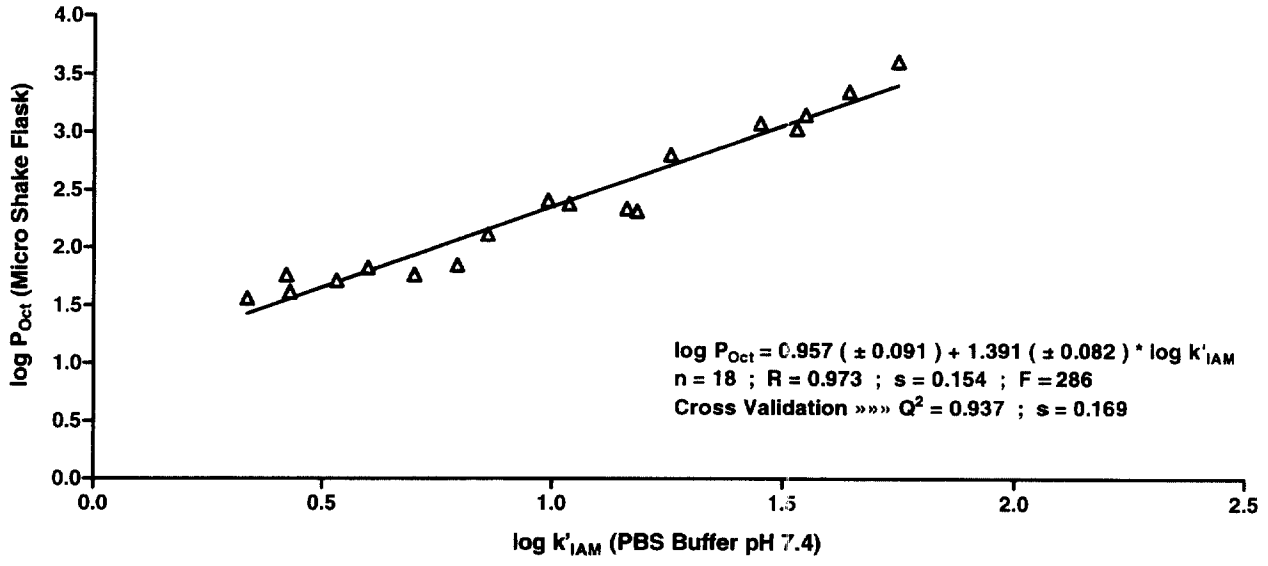


Figure 2. Group A triazines: log P_{Oct} vs. log k'_{IAM}.

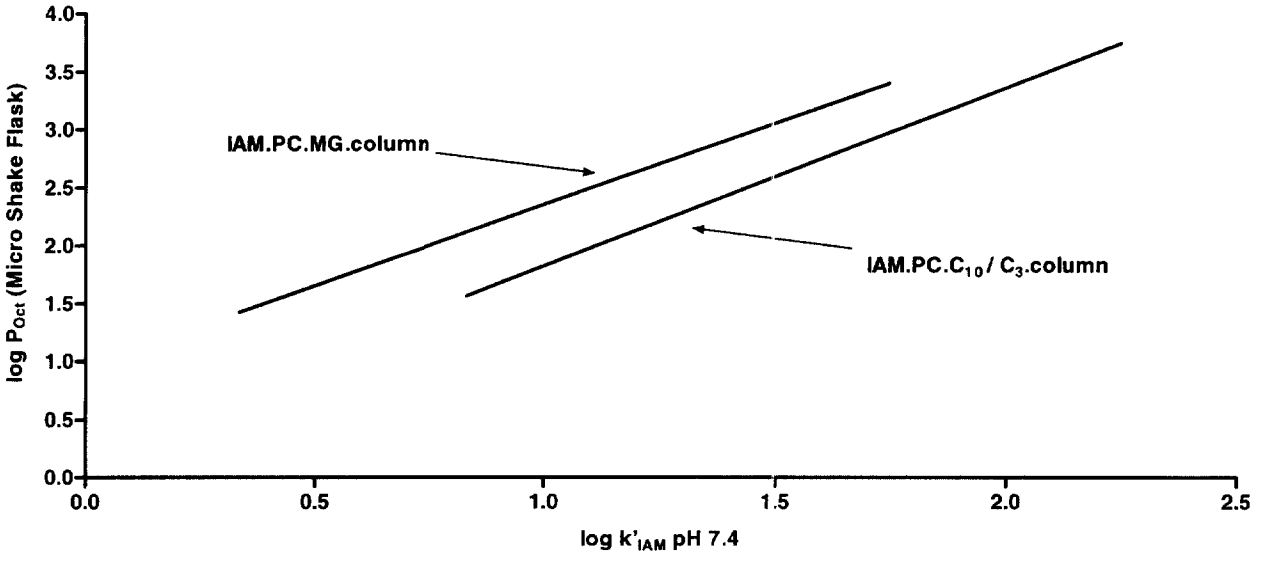


Figure 3. log P_{Oct} vs. log k'_{IAM}, pH 7.4.

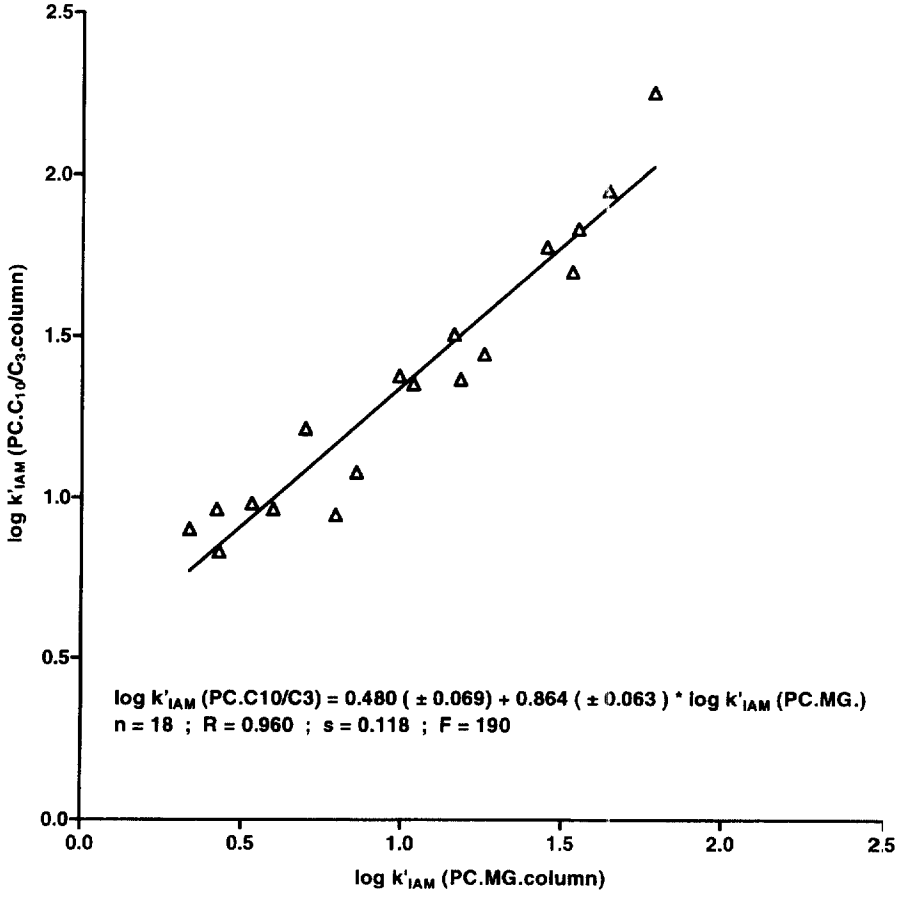


Figure 4. log k'_{IAM} (PC.C10/C3. column) vs. log k'_{IAM} (PC.MG. column).

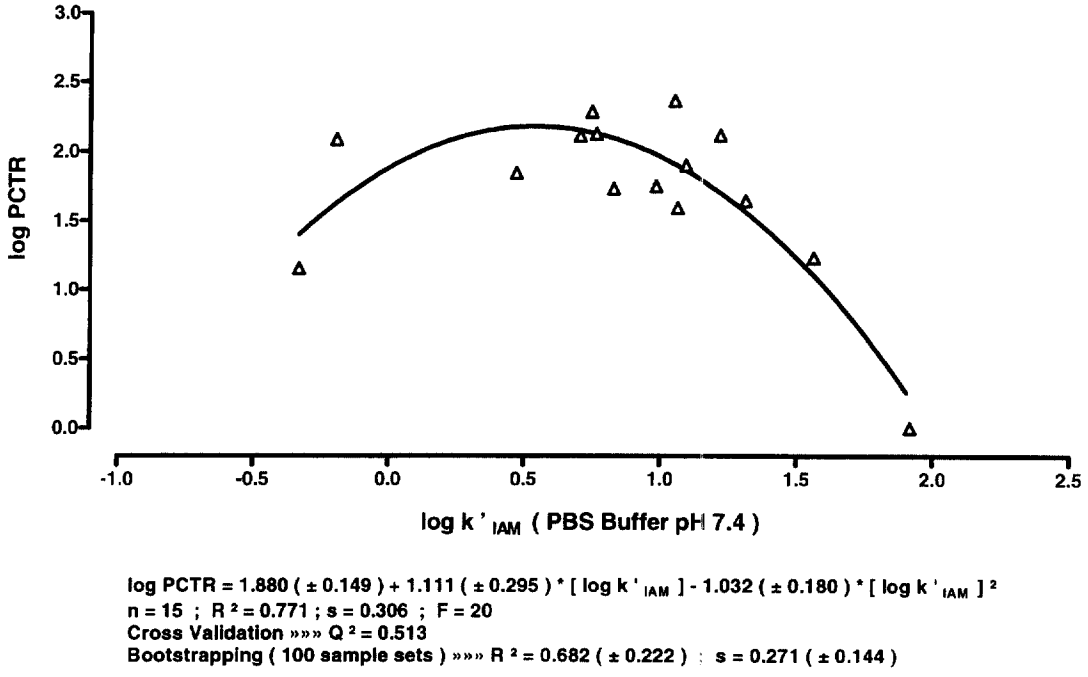


Figure 5. Group B triazines: log PCTR vs. log k'_{IAM} (PBS buffer pH 7.4).

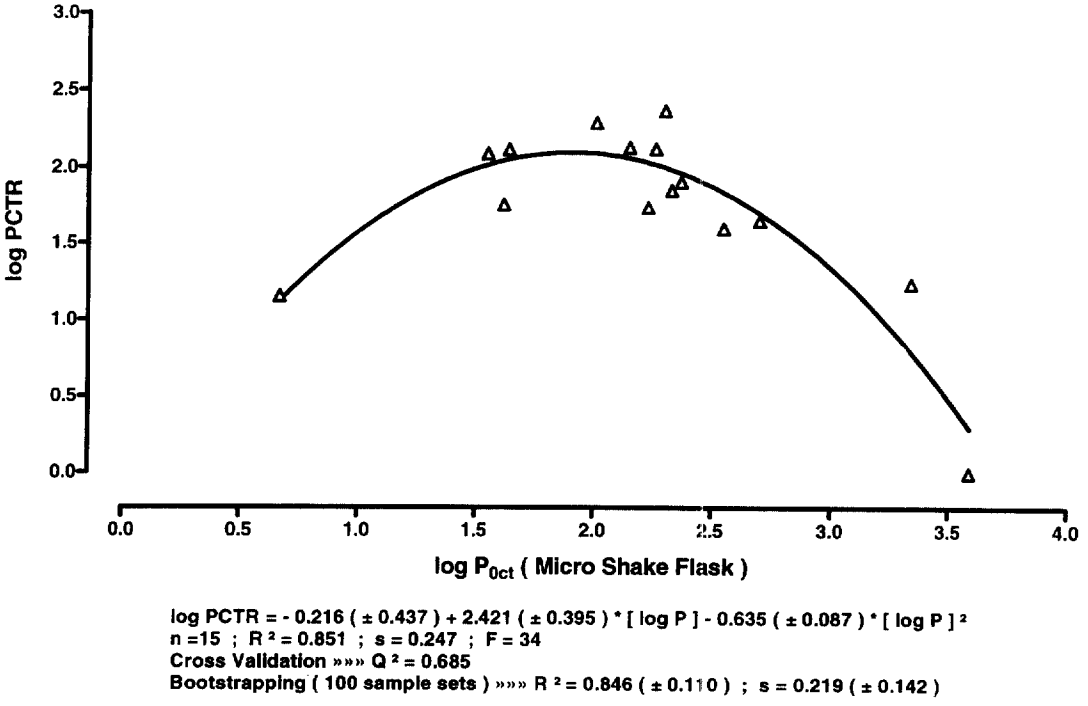


Figure 6. Group B triazines: log PCTR vs. log P_{Oct} (micro-shake flask).

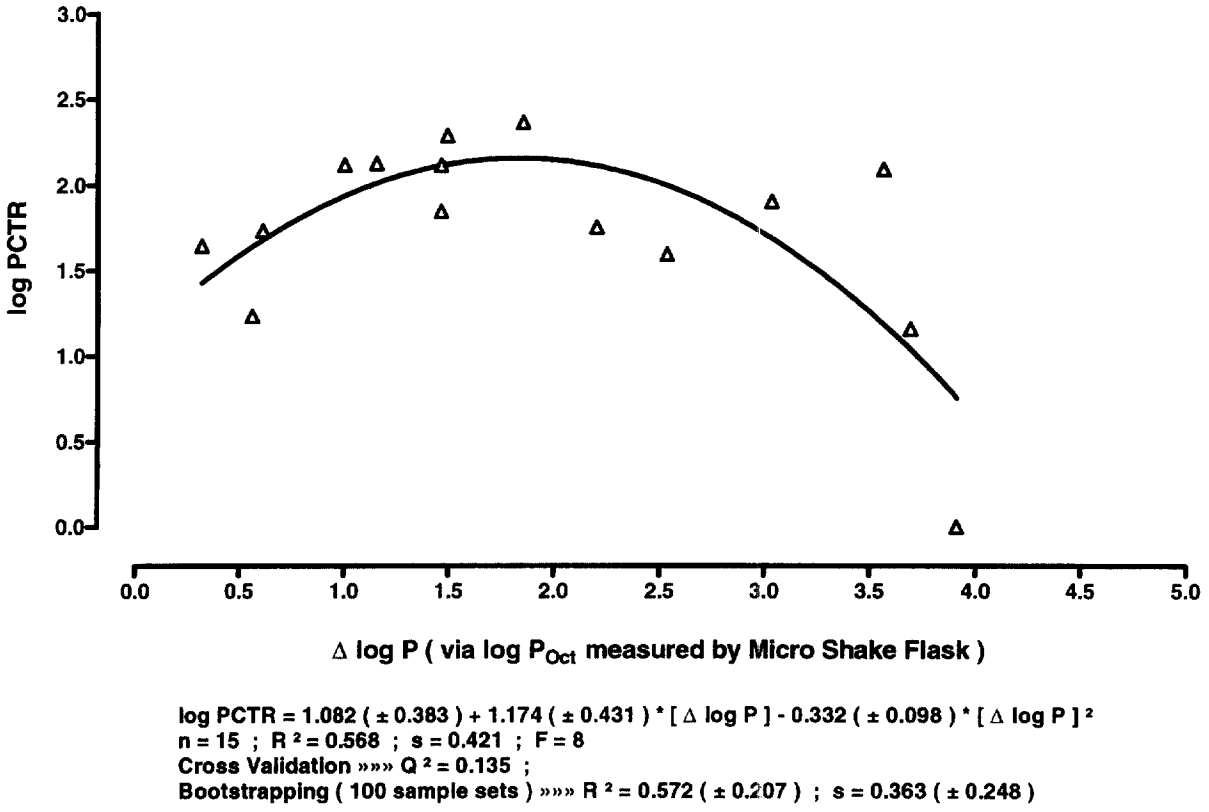


Figure 7. Group B triazines: log PCTR vs. Δlog *P*.

On the contrary the correlation of Δlog *P* (log $P_{\text{octanol-water}}$ – log $P_{\text{cyclohexane-water}}$) with tremor enhancement is almost non-existent (*figure 7*) indicating that in the present case this parameter is a poor descriptor of potential blood brain barrier crossing.

In conclusion we would like to emphasize the following points:

- determination of log k'_{IAM} (capacity factor using phosphatidyl choline columns) is an efficient and reliable way for assessing lipophilic behaviour of 1,3,5-triazines;
- for the series of molecules that was studied this capacity factor correlated well with the more traditionally used octanol–water partition coefficient but less well with capacity factors (log k'_w) obtained using a reversed phase column;
- about 80% of the variance in a CNS-mediated pharmacological activity (enhancement of oxotremorine-induced tremor) is explained by the variance in log k'_{AM} or log *P*. These results are by far superior to those obtained using Δlog *P*, a parameter sometimes thought to best describe a compound potential for crossing of the blood brain barrier.

Further work is ongoing in our laboratories in order to fully characterize the applications and limitations of these newer columns in solving medicinal chemistry problems.

3. Experimental protocols

3.1. Chemistry

The detailed synthetic procedures used for preparing triazines in *table I* and *table II* are described in [14, 15].

3.2. Pharmacology

Enhancement of oxotremorine-induced tremors has been assessed in male NMRI mice according to [16]. The detailed procedure utilized for the above-mentioned triazines is given in [15] (log PCTR in *table II* is obtained by comparing (%) the area under mean dose–effect curves for treated and control animals).

3.3. Partition coefficients (log P)

An in-house developed micro shake flask procedure was utilised. The immiscible biphasic system consisted of 1-octanol

(purris, grade, No. 74850 Fluka, Buchs, Switzerland) or cyclohexane (HPLC grade, No. C2508, Lab Scan, Dublin, Ireland) and physiological buffer (PBS at pH 7.4, 100-3, Sigma Diagnostics, St Louis, MO, USA) mutually saturated with each other.

The compound under investigation was dissolved in the one or the other phase over a ten-fold concentration range. Between 500–600 µL of the obtained solutions were sonicated (K72438 vibracell, USA and Bioblock Scientific, France) during 5 s with an equal volume of the other phase.

Phases were separated by centrifugation under 1000 *g* during 1 h (benchtop refrigerated centrifuge, Eppendorf, model 5403, Germany). Quantification was performed by HPLC analysis of both phases using preestablished calibration lines ($r \geq 0.99$).

For the cyclohexane–buffer system the organic solvent was evaporated under a nitrogen stream at room temperature and the residue was dissolved again in buffer or methanol (calibration lines are prepared in the same solvent). The final balance (the sum of concentrations found for the aqueous and organic phase) always matched the initial concentrations within 5 %.

The HPLC apparatus (Waters, Milford, MA, USA) included a model 616 pump equipped with a model 600S controller, a model 717 PLUS autosampler, a tunable UV model 486 detector (or model 996 photodiode array detector) and a chromatography manager Millennium version 2.10.

A Waters symmetry RP8 (5 µm; 100 Å; 50 x 3.6 mm) column was used; the mobile phase consisted of methanol–water mixtures 1.5 mM in phosphoric acid which were adjusted for each compound so as to obtain a retention time of about 7 min (flow 1 mL/min, UV detection at 210 nm, $T = 37$ °C).

3.4. Partition chromatography

Capacity factors on IAM columns (k'_{IAM}) were established using solutions (± 500 µm) of the various triazines in PBS buffer at pH 7.4. The previously described HPLC set-up was used. Two types of IAM columns were employed:
(a) PC.MG type pilot, 30 x 4.6 mm (Regis Tech Inc., Morton Grove, IL, USA) containing lecithin-COOH bonded to silica-propylamine with the unreacted propylamine moieties end-capped with methyl glycolate;
(b) PC.C_{10}/C_3, type pilot 30 x 4.6 mm (Regis Tech Inc., Morton Grove, IL, USA) containing the same phospholipid as above but with unreacted propylamine moieties end-capped with propanoic and decanoic acids.

The mobile phase consisted of the same phosphate buffered saline (pH 7.4) used to make up the solutions.

Citric acid monohydrate (No. 1204, Merck, Darmstadt, Germany) was employed as internal standard in order to establish the void volume of the columns. The following reference compounds were supplied with IAM columns (Regis Tech Inc., Morton Grove, IL, USA) and were used to correct the capacity factors during the lifetime of the column: benzoic acid, *p*-toluidine, *m*-nitroaniline and *p*-nitroaniline.

Acknowledgements

We gratefully acknowledge the skillful contribution and unshaken perseverance of Mrs L. Ellens for her careful physicochemical measurements of partition coefficients and capacity factors on IAM columns. We also acknowledge Mr E. Vande Water and Miss V. Galez for their assistance in preparing the manuscript.

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