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Journal of Chromatography A, 667 (1994) 29–35

JOURNAL OF CHROMATOGRAPHY A

Determination of distribution coefficients for some 5-HT_3 receptor antagonists by reversed-phase high-performance liquid chromatography

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(First received July 27th, 1993; revised manuscript received December 20th, 1993)

Abstract

It is of major interest to determine the physico-chemical properties, dissociation constants and lipophilicity of potential drugs owing to their implication in absorption mechanisms. The HPLC capacity factors appear to be attractive and readily accessible lipophilic parameters. An isocratic HPLC method was designed using a C_8 column to determine the relative lipophilicity for two sets of 5-HT_3 receptor antagonists, including tropine and quinolizine analogues. Dimethyloctylamine was added to the eluent to mask silanophilic interactions and lead to a predominant partitioning mechanism. The capacity factors determined by a monocratic procedure showed good correlations with the distribution coefficients obtained by the shake-flask method, showing the validity of capacity factors as lipophilicity descriptors for close analogues.

1. Introduction

The importance of lipophilic properties and electrostatic effects in drug–receptor interactions has long been recognized [1,2]. The dissociation constants (pK_a) of compounds influence directly their lipophilicity and therefore their absorption mechanism and their interaction with specific receptors. Lipophilicity, which can be represented by the logarithm of the partition coefficient (log P) of a compound between octanol and an aqueous buffer, is one of the properties often modified to improve the biological activity or the transport properties of a molecule.

The use of reversed-phase chromatography has been proposed as an alternative to the traditional shake-flask procedure for the lipophilicity determination of compounds. Highly significant correlations have been reported between the logarithm of the octanol–water partition coefficients, log P, and the logarithm of the reversed-phase capacity factors, log k' [3,4].

The use of different types of HPLC systems (octanol-like, reversed-phase, polymer-based or deactivated columns) has been reviewed [5] and illustrates the difficulties in finding a common methodology for quantitative structure–activity relationship (QSAR) studies using chromatographic parameters. As the development of pharmaceutical drugs involves the synthesis of large series of analogues, the situation is favoured because correlations are generally ob-

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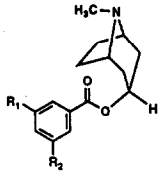
0021-9673/94/$07.00
SSDI 0021-9673(93)E0066-4

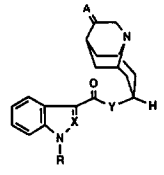


Compounds	Axial/Equat	R_1	R_2
1 (MDL 72222)	Ax	Cl	Cl
2	Eq	Cl	Cl
3	Ax	Me	Me
4	Eq	Me	Me
5	Ax	H	Me
6	Ax	F	F

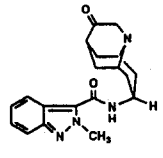
Fig. 1. Structures of tropine analogues.

tained between log k' and log P for close analogues [6].

In this paper, we report the determination of the capacity factors of a series of 5-HT_3 receptor antagonists with tropine- and quinolizine-like structures [7,8], and their comparison with the corresponding shake-flask distribution coefficients. The HPLC method was first optimized to ensure the measurement of the chromatographic



Compounds	Axial or Equat	X	Y	R	A
7 (MDL 73147)	Ax	CH	O	H	O
8	Eq	CH	O	H	O
9	Ax	CH	O	H	(H)(OH)
10	Eq	CH	O	H	(H)(OH)
11	Ax	CH	O	H	(H)(H)
12	Eq	CH	O	H	(H)(H)
13	Ax	N	NH	Me	O
14	Eq	N	NH	Me	O
15	Ax	N	NH	Me	(H)(OH)



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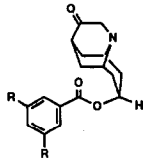

17 R = Me
18 R = Cl

Fig. 2. Structures of quinolizine analogues.

parameters with a predominant partitioning mechanism. The dissociation constants are also presented in order to highlight the importance of ionization. The structures of the compounds studied are given in Figs. 1 and 2.

2. Experimental

2.1. Reagents and materials

The 5-HT_3 receptor antagonists studied were synthesized at the Marion Merrell Dow Research Institute using the general scheme described previously [8] and were characterized by ^1H NMR spectrometry and elemental analysis (C,H,N). Their purities were checked by HPLC with UV detection. 1-Octanol and sulphuric acid were obtained from Merck (Darmstadt, Germany), dimethyloctylamine (DMOA) from ICN Pharmaceuticals (Plainview, NY, USA) and triethylamine (TEA) from Pierce (Rockford, IL, USA). The buffers were prepared with $NaH_2PO_4 \cdot H_2O$ and $Na_2HPO_4 \cdot 7H_2O$ (Merck) dissolved in water purified with a Milli-Q water system (Millipore) and the pH values were adjusted with dilute H_3PO_4 and NaOH (Merck). Acetonitrile (RS per HPLC grade) was obtained from Carlo Erba (Milan, Italy).

2.2. Determination of distribution coefficients (D) [9,10]

1-Octanol (500 ml) was washed three times with 50 ml of 2 *M* H_2SO_4, twice with deionized water, three times with 50 ml of 2 *M* NaOH and ten times with 50 ml of deionized water until the pH of the aqueous phase was neutral. Finally, the octanol was saturated with the buffer used.

The phosphate buffers were prepared by mixing the appropriate volumes of 0.1 *M* H_3PO_4, 0.1 *M* NaH_2PO_4, 0.1 *M* Na_2HPO_4 and 0.1 *M* NaOH to obtain the desired pH [11]. Volumes of 1–5 ml of approximately 10^{-3} *M* solutions of the compounds in the buffer or octanol were prepared and mixed with an equal volume of octanol or buffer, respectively, in 15-ml screw-capped vials. The mixtures were then shaken on

a reciprocal shaker at 24°C for 1 h. Afer centrifugation at 3900 rpm (24 600 *g*) for 10 min, the two phases were carefully separated and the concentrations of the compounds in the two phases were determined by HPLC. Usually partition experiments were run in duplicate and two HPLC analyses were carried out per sample. The distribution coefficients were calculated using the expression

$$D = \frac{\text{peak area in octanol phase}}{\text{peak area in buffer phase}}$$

2.3. Determination of capacity factors (k')

The k' values were determined on a Merck LiChroCART Superspher 60 RP-8e column (250 × 4 mm I.D.; 4 μm) with 600 ml of 0.01 *M* NaH_2PO_4, 400 ml of CH_3CN and 20 ml of DMOA (pH 7.4) at 40°C and a flow-rate of 1.2 ml/min using a Vista 5500 liquid chromatograph (Varian, Palo Alto, CA, USA) with a WISP 710 autosampler (Waters, Milford, MA, USA) and a UV 200 spectrophotometer (Varian) operated at 240 nm.

The k' value of each compound was calculated as $(t - t_0)/t_0$, where t is the retention time of the compound and t_0 the retention time of an unretained compound which was determined by injection of methanol.

2.4. Determination of protonation constants

Some of the compounds, *e.g.*, **7** (MDL 73147), were readily soluble at acidic pH in the 0.1 *M* KNO_3 medium used, but precipitated at pH values where deprotonation was more or less complete. It was therefore necessary to work with methanol–water (70:30) with no added inert salt.

For determination in water, 5×10^{-3} *M* solutions of the different compounds were obtained by dissolving a weighed amount of ligand (5–15 mg) in 5 or 10 ml of 0.1 *M* KNO_3 with an added excess of 1 *M* HCl (1.1–1.5 equiv.).

Titrations were performed using a semi-automatic Mettler DL20 Compact Titrator with a 1-ml burette (Mettler DV 401) and a combined microelectrode. Two types of electrodes were used: Metrohm EA 125 or Ross 8103 for the titration of small volumes, *i.e.*, 2 ml and Metrohm EA 120 for 5-ml volumes. The electrodes and pH meter were calibrated to read the pH values with six aqueous buffer solutions (pH 2.00, 4.00, 6.98, 9.94, 10.90 and 12.45). For the measurements in methanol–water (70:30), the glass electrode was conditioned overnight in the mixed solvent. Volumes of 2 or 5 ml of the 5×10^{-3} *M* solutions were back-titrated under nitrogen at 25°C with 0.1 *M* KOH. The Mettler titrator was linked to an IBM PC and the titration points were stored on floppy disk. Several programs were developed on the PC to calculate the equivalence points and plot the experimental titration curves. A simulation program was also written on the PC which allowed the calculation of the pK_a value by "best fit" between the experimental and simulated titration points.

3. Results and discussion

3.1. Determination of dissociation constants

It is well known that the partition coefficients (log P) and the capacity factors (log k') vary with pH in a sigmoidal manner for ionizable molecules [12–14]. Dissociation constants are key parameters if one wants to know the degree of ionization of a given compound. Tables 1 and 2 present pK_a values determined for some tropine and quinolizine analogues, respectively.

The values obtained for tropine and pseudotropine in methanol–water (70:30) mixture are 0.6–0.7 pH unit lower than in water. This decrease is in agreement with the variations observed for other amines [15].

The results for the quinolizine derivatives clearly show that the basicity of the tertiary amine of the quinolizine increases from the ketone **7** (MDL 73147) to the alcohol **9** and to the CH_2 analogues **11**; these increases are similar to those expected from the variations observed with various amines [2].

A wide range of acid dissociation constants

Table 1
Dissociation constants of the tropine analogues

Compound	pK_a [a]
Tropine	10.40[b], 9.85
Pseudotropine	10.05[b], 9.35
1 (MDL 72222)	8.70
2	8.45
3	8.90
4	8.60

[a] pK_a values obtained in methanol–water (70:30) at 25°C unless stated otherwise.
[b] pK_a values obtained in 0.1 *M* KNO_3 at 25°C.

were obtained from 6.35 for 7 to 9.2 for **11**, which leads to large differences in the ionization percentages at a given pH between 5 and 10. Therefore, it is essential to correlate log *D* and log *k'* determined at the same pH, as emphasized previously [16]. In this case the comparison of all the compounds as neutral species would require working at a pH above 10, which is not practical with silica-based reversed-phase packings. Polymer-based columns would be recommended for use at high pH but they appear to be less rugged [5] and have a poor separation efficiency owing to the low plate number. Nevertheless, the pH must not be considered as a limitation for chromatographic assessment of lipophilicity, as most of the time the pH of interest for pharmacological testing is the physiological pH 7.4, which is within the pH limitations for most reversed-phase packings.

Table 2
Dissociation constants of the quinolizine analogues

Compound	pK_a [a]
7 (MDL 73147)	6.35
9	8.10
11	9.17
13	6.35
15	8.60
18	5.95

[a] pK_a values obtained in methanol–water (70:30) at 25°C.

3.2. Effect of pH on retention times

The effect of pH on retention times is presented to demonstrate that log *k'* varies non-linearly with pH and therefore must be measured at the same pH as used for log *D* determinations.

As expected for these basic compounds, the retention times increase with increase in pH (Fig. 3). The pH variation experiments normally give sigmoidal curves. For the compounds studied the final plateau is not reached owing to the pH limitations of the column as discussed above except for **7** (MDL 73147), which has a lower pK_a of *ca.* 6.3. In addition, the pH effect appears much more important with the tropine analogues than with the quinolizine analogues: the presence of 40% of acetonitrile in the eluent gives shorter retention times for the quinolizine derivatives and hence the capacity factors are less affected by the pH changes than those of the tropine analogues, for which slight variations are easily noticeable owing to their much later elution.

3.3. Effect of amine modifier

The addition of dimethyloctylamine to the eluent decreases the retention times, as shown in

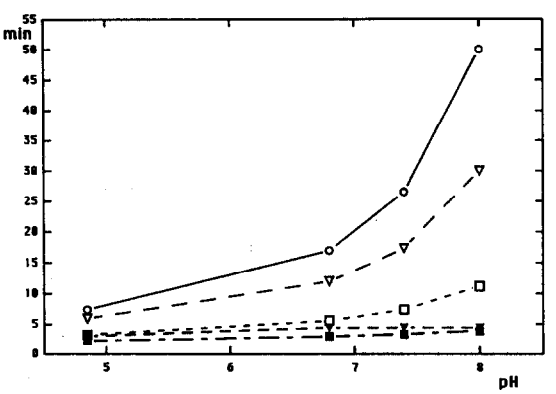

Fig. 3. Plot of retention times *versus* pH for various MDL compounds. Column, Merck 60 RP-8e; mobile phase, 0.01 *M* NaH_2PO_4–CH_3CN–DMOA (60:40:2, v/v/v) with adjusted pH; flow-rate, 1.2 ml/min; temperature, 40°C; UV detection at 240 nm. ○ = **1** (MDL 72222); ▽ = **3**; ▼ = **7** (MDL 73147); ■ = **9**; □ = **11**.

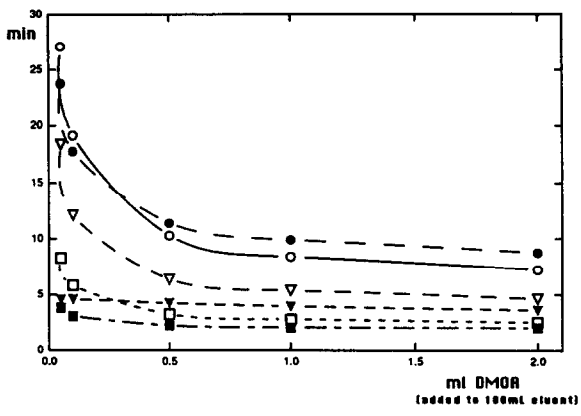

Fig. 4. Plot of retention times *versus* amount of DMOA for selected MDL compounds. Column, Merck 60 RP-8e; mobile phase, 0.01 *M* NaH_2PO_4–CH_3CN–DMOA (60:40:*x*, v/v/v) (pH 7.4); flow-rate, 1.2 ml/min; temperature, 40°C; UV detection at 240 nm. ○ = **1** (MDL 72222); ● = **2**; ▽ = **3**; ▼ = **7** (MDL 73147); ■ = **9**; □ = **11**.

Fig. 4. The effect is noticeable up to 0.5% DMOA and levels off between 0.5% and 2% DMOA. The tropine analogues appear to be more sensitive than the quinolizine analogues to the DMOA addition, except for **11**, which has a higher pK_a. Owing to their higher pK_a values, the tropine analogues show a greater percentage of protonated form at a given pH and therefore may interact more strongly with the residual silanols of the reversed-phase packing which are not fully masked by low percentages of DMOA. Similar variations were observed on adding triethylamine in the eluent. The addition of 1–2% of tertiary amine ensures the determination of the capacity factors resulting mainly from the partitioning mechanism, by minimizing the hydrogen bonding effects. The amine is dynamically coated on the stationary phase and interacts preferentially with the residual silanols, rendering them less accessible to hydrogen bonding with the eluted compounds.

Highly deactivated commercial columns may be used, as suggested by Kaliszan *et al.* [17], without a requirement for amine modifiers to obtain capacity factors as lipophilicity descriptors, but were not available at the beginning of this study. A deactivated column has subsequently been tested, but the peak shapes were not satisfactory and the addition of DMOA was still necessary [18].

3.4. Correlation between log D and log k'

The log k' values were obtained with an HPLC eluent containing 40% of acetonitrile rather than determining the log k'_w (capacity factor in a totally aqueous eluent). Although log k'_w is very attractive for constructing a lipophilicity scale [3,4,17], it is extrapolated from capacity factors measured at different percentages of organic modifier. A simplified and quicker method of lipophilicity determination in which a single measurement is made was found to be more suitable for our needs. In addition, correlations between log k' measured in aqueous organic mixtures and log P have been obtained for well defined classes of compounds [4,6].

Our approach after having optimized the chromatographic system by varying some fundamental parameters (percentage of organic modifier, buffering capacity) was to use these conditions for the measurement of capacity factors.

The capacity factors for the tropine analogues determined on a C_8 column are given in Table 3, along with the corresponding octanol–water distribution coefficients at the same pH (7.4). A significant correlation is obtained between log $D_{7.4}$ and log k'; the equation obtained by linear regression (Eq. 1) is given below and Fig. 5 shows the resulting plot.

$$\log D_{7.4} = 2.291\ (\pm 0.226) \log k' + 0.685\ (\pm 0.081) \quad (1)$$

$n = 6$; $r = 0.981$; S.D. = 0.114

The satisfactory linear correlation with a slope different from unity indicates similar physicochemical properties for the two partitioning processes, but with different intrinsic thermodynamic behaviours, which are termed homeoenergetic [19].

Similarly, a correlation was obtained for the quinolizine analogues (Eq. 2) between the capacity factors and log $D_{7.4}$ given in Table 4, as shown in Fig. 6.

Table 3
Capacity factors obtained with the C_8 column and distribution coefficients for the tropine analogues

Compound	k' [a]	Log k'	Log $D_{7.4}$ [a] (octanol–pH 7.4 buffer)
1 (MDL 72222)	3.19	0.50	1.78
2	4.21	0.62	2.03
3	1.66	0.22	1.34
4	2.01	0.30	1.51
5	0.96	−0.02	0.59
6	1.07	0.03	0.64

Chromatographic conditions for log k' determination: column, Merck LiChroCART 60 RP-8e (250 × 4 mm I.D.; 4 μm); mobile phase, 0.01 *M* NaH_2PO_4–CH_3CN–DMOA (60:40:2, v/v/v) (pH 7.4); flow-rate, 1.2 ml/min; temperature, 40°C; UV detection at 240 nm. Distribution coefficients, log $D_{7.4}$, between 1-octanol and 0.1 *M* phosphate buffer (pH 7.4); temperature, 24°C.
[a] Values are given ±0.05.

$$\log D_{7.4} = 1.616\,(\pm 0.152)\log k' + 1.719\,(\pm 0.077) \quad (2)$$
$$n = 12;\ r = 0.958;\ \text{S.D.} = 0.252$$

Again, in view of the slope value, the free energies involved in both processes are similar but not identical.

These correlations can be used to predict the partition coefficients of new compounds from their capacity factors, which are easily measured by chromatography. In both instances (tropine and quinolizine), the dynamic equilibrium descriptor of lipophilicity, log k', was found to correlate with the slow equilibrium descriptor, log D.

The poor correlation factor ($r = 0.82$) obtained on analysing both series of compounds together (Eq. 3) highlights that this approach must be used only for structurally related compounds.

Table 4
Log $D_{7.4}$ and log k' values obtained with the C_8 column for quinolizine analogues

Compound	k'	Log k'	Log $D_{7.4}$ (octanol–pH 7.4 buffer)
7 (MDL 73147)	1.39	0.143	2.33
8	1.41	0.149	2.25
9	0.27	−0.569	0.87
10	0.43	−0.371	1.36
11	0.42	−0.377	1.05
12	0.42	−0.377	1.35
13	0.97	−0.013	1.39
14	0.70	−0.157	1.53
15	0.16	−0.796	0.12
16	0.46	−0.337	0.85
17	6.01	0.779	2.85
18	8.97	0.953	3.10

Log k' and log $D_{7.4}$ determination: see comments in Table 3.

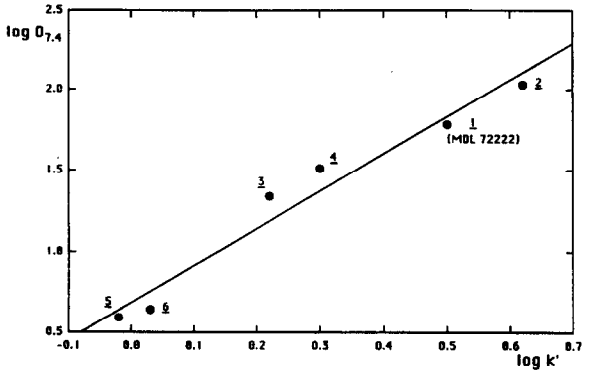

Fig. 5. Plot of octanol–buffer distribution coefficients (log $D_{7.4}$) *versus* capacity factors (log k'), obtained by reversed-phase HPLC, for six tropine analogues. Column, Merck 60 RP-8e; mobile phase, 0.01 *M* NaH_2PO_4–CH_3CN–DMOA (60:40:2, v/v/v) (pH 7.4); flow-rate, 1.2 ml/min; temperature, 40°C; UV detection at 240 nm.

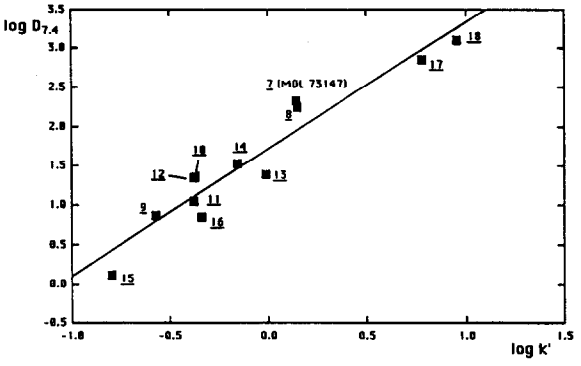

Fig. 6. Plot of octanol–buffer distribution coefficients (log $D_{7.4}$) *versus* capacity factors (log k'), obtained by reversed-phase HPLC, for twelve quinolizine analogues. Column, Merck 60 RP-8e; mobile phase, 0.01 *M* NaH_2PO_4–CH_3CN–DMOA (60:40:2, v/v/v) (pH 7.4); flow-rate, 1.2 ml/min; temperature, 40°C; UV detection at 240 nm.

$$\log D_{7.4} = 1.357\,(\pm 0.240)\log k' + 1.446\,(\pm 0.111) \quad (3)$$

$n = 18$; $r = 0.816$; S.D. = 0.455

Similar studies were conducted using the same set of quinolizine analogues with a C_{18} phase, but gave a poor correlation coefficient of 0.72 [18]. Other workers have reported better correlations with C_8 than with C_{18} stationary phases [20]. The residual silanols appear to be more easily accessible to the amine modifier (DMOA or TEA) for octyl than octadecyl phases, leading to a more efficient lowering of silanophilic interactions and therefore a partitioning system that better mimics the octanol–water system.

4. Conclusions

The measurement of HPLC retention data as lipophilicity descriptors presents many advantages over the determination of traditional shake-flask partition coefficients: it appears simple, rapid and readily automatable. In addition, it allows an estimation of hydrophobicity for highly lipophilic compounds or compounds available only in small amounts. The final interest for pharmaceutical molecules is the tentative correlation of the lipophilicity parameters with biological data, leading to QSAR studies. Obviously, the hydrophobic parameters are not the only ones that influence the biological activity, and electronic or steric parameters may play a significant role, but lipophilicity appears to be the main factor for membrane penetration and therefore bioavailability. A more detailed quantitative structure–retention relationship (QSRR) study with multivariate correlation would be necessary to determine the influence of the different parameters (hydrophobic, steric and electronic) on the retention of the tropine and quinolizine analogues in HPLC. Nevertheless, considering the range of variations generally observed with biological data and the good level of correlation between log k' and log $D_{7.4}$ for some ionizable 5-HT_3 receptor antagonists, the capacity factors obtained on a C_8 column in the presence of DMOA are considered to be good descriptors of relative lipophilicities.

5. Acknowledgement

We thank Dr. M.W. Gittos for his contribution to this work through his constant interest in the subject and the synthesis of the compounds.

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