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Note

High-performance liquid chromatography in the evaluation of the lipophilicity of 17β-carboxamide steroid derivatives

P. MAES, P. FORMSTECHER*, P. LUSTENBERGER and M. DAUTREVAUX
Laboratoire de Biochimie Structurale, Faculté de Médecine, 1 Place de Verdun, 59045 Lille-Cédex (France)
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In recent years an increasing number of papers reporting the use of reversed-phase high-performance liquid chromatography (RP-HPLC) to evaluate the hydrophobicity of a wide variety of compounds have been published and reviewed[1]. A good correlation between the chromatographic capacity factor k' and the *n*-octanol–water partition coefficient P, the well known hydrophobic parameter widely used to find quantitative structure–activity relationships in pharmacology[2], has generally been found. In particular, an RP C_{18} stationary phase with a methanol–water or methanol–buffer mobile phase appeared to be the system of choice for the determination of RP-HPLC hydrophobic parameters, resulting in a strikingly good relationship between log k' and log P[1,3–6]. However the measurement by RP-HPLC of hydrophobic parameters for ionizable compounds is difficult and needs some corrections to account for ionization of the solute[6–11]. Obviously the capacity factor of ionogenic compounds in an RP-HPLC column is greatly affected by the eluent pH and, as the operating pH range of common reversed-phase silica columns is limited to 2–8, ionization cannot be avoided for organic bases with $pK_a > 8$.

In this study we tried to determine the relative lipophilicities of more than 30 dexamethasone 17β-carboxamide derivatives, by determining both their octanol–water partition coefficients using a conventional shake-flask procedure and their capacity factors k' on an RP C_{18} column equilibrated in a methanol–buffer mobile phase. The correlation between log k' and log P was then examined and the efficiency of a very simple correction for the ionization of ionogenic compounds was assessed. This work was required because the 17β-carboxamide steroid derivatives studied constitute an original class of bioactive compounds, displaying antiglucocorticoid activity in mammalian cells by acting at the glucocorticoid receptor level[12].

EXPERIMENTAL

Materials

Dexamethasone was purchased from Roussel Uclaf (Romainville, France) and *n*-octanol from Aldrich (Beerse, Belgium). 17β-Carboxamide derivatives were prepared as previously described[13]. Nucleosil C_{18} (10 μm) was obtained from Macherey, Nagel & Co. (Düren, F.R.G.) and was persilylated with hexamethyldisilazane (HMDS) and trimethylsilyl chloride (TMSCl) in hot pyridine to block potentially

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remaining silanol sites according to McCall[14]. All other chemicals were of analytical-reagent grade. HPLC was performed using a Waters 204/U/6/6/CM liquid chromatograph and a laboratory-packed persilylated Nucleosil C_{18} column (100 × 4.7 mm I.D.) with UV detection at 254 nm.

Partition coefficients

Shake-flask experiments. The conventional technique[2] was used with slight modifications. Each solute was dissolved in *n*-octanol and diluted to obtain a set of three concentrations ($4 \cdot 10^{-3}$, $2 \cdot 10^{-3}$ and $1 \cdot 10^{-3}$ *M*). Partition between *n*-octanol and 10 m*M* phosphate buffer (pH 7.4) was performed in 5-ml glass tubes equipped with PTFE screw-caps. The total liquid volume was 4 ml with an *n*-octanol to buffer volume ratio of 1:1. Tubes were inverted overnight on a rotating device at ambient temperature, then centrifuged at 1800 *g* for 30 min and samples of each phase were carefully withdrawn for steroid assay.

Chromatographic assay. The carboxamide concentration in each phase was determined by HPLC on a 100 × 4.7 mm I.D. Nucleosil C_{18} (10 μm) column with detection at 254 nm. The mobile phase was prepared from methanol and 10 m*M* phosphate buffer (pH 7.40). For each compound the proportion of methanol was adjusted to give a capacity factor $k' \approx 2$ and calibration was performed with a 10^{-4} *M* methanolic solution of the steroid to be assayed. Chromatography was performed at a flow-rate of 2 ml/min and a pressure of *ca.* 1500 p.s.i. Sample volumes ranged from 1 to 50 μl. For highly hydrophobic compounds the steroid concentration was very low in the aqueous phase and came close to the detection limit of the system. In this instance the aqueous phase was concentrated prior to injection on to the column.

Statistics

Correlation studies were performed using SAS statistics software on a VAX 11/780 computer.

RESULTS AND DISCUSSION

Thirty-eight 17β-carboxamide dexamethasone derivatives were synthesized and used for the determination of *n*-octanol–phosphate buffer partition coefficients and/or log *k'*.

Octanol–phosphate buffer partition coefficient

We measured the *n*-octanol-phosphate buffer partition coefficients, *P*, of 33 17β-carboxamide steroids (Table I) by direct determination using the shake-flask method followed by HPLC steroid assay.

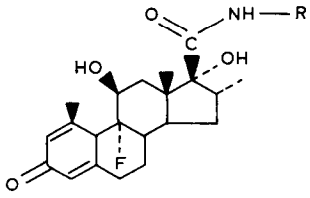
$$P = \frac{C_{\text{octanol}}}{C_{\text{phosphate}}}$$

where C_{octanol} = steroid concentration in the octanol phase and $C_{\text{phosphate}}$ = steroid concentration in the phosphate-buffer phase equilibrated at the physiological pH 7.4. For each compound the partition coefficient was determined in triplicate at three

different steroid concentrations, and P was taken as the mean value except for compound 8, which was so hydrophobic that its partition coefficient could not be measured ($C_{phosphate}$ below the detection limit). The calculated log P value was 5.78 for this compound and it has been pointed out that values of log $P_{oct} > 4$ obtained using the shake-flask method are unreliable if the usual centrifugation techniques are ap-

TABLE I

17β-CARBOXAMIDE DERIVATIVES OF DEXAMETHASONE: *n*-OCTANOL–WATER PARTITION COEFFICIENTS AND RP-HPLC RETENTION FACTORS



Compound No.	*R*	π_X*	*Log* P_m**	*Log* P_m^c***	*Log* P_c§	*Log* k'§§
1	H	−0.56	1.41		0.08	0.23
2	CH_3	0	1.54		1.54	0.27
3	CH_2CH_3	0.56	2.07		2.10	0.38
4	$(CH_2)_2CH_3$	1.02	2.59		2.56	0.49
5	$(CH_2)_3CH_3$	1.55	3.10		3.09	0.66
6	$(CH_2)_4CH_3$	2.13	3.64		3.68	0.86
7	$(CH_2)_5CH_3$	2.69	4.54		4.23	1.19
8	$(CH_2)_8CH_3$	4.24	n.d.		5.78	1.97
9	$CH(CH_3)_2$	1.12	2.43		2.66	0.55
10	$CH_2CH(CH_3)_2$	1.53	3.01		3.07	0.62
11	$C(CH_3)_3$	1.68	3.20		3.22	0.64
12	$(CH_2)_2CH(CH_3)_2$	2.09	3.74		3.63	0.83
13	$(CH_2)_3CH(CH_3)_2$	2.65	3.93		4.19	1.10
14	CH_2CCH	0.40	2.00		1.94	0.35
15	CH_2CH_2Cl	0.17	2.73		2.81	0.48
16	$CH_2C_6H_5$	1.96	3.28		3.50	0.79
17	$(CH_2)_2C_6H_5$	2.01	3.66		3.55	0.94
18	$CH(C_6H_5)CH_2OH$	0.93	2.89		2.47	0.56
19	$CH(CH_2C_6H_5)COOCH_3$	2.00	3.40		3.54	0.90
20	CH_2CH_2OH	−0.67	1.51		1.40	0.27
21	CH_2CN	−0.57	1.57		0.97	0.33
22	$CH(CH_2)_5$	1.96	3.54		3.50	0.79
23	$CH_2CH(OC_2H_5)_2$	1.32	2.65		2.86	0.73
24	CH_2COOCH_3	−0.01	1.69		1.53	0.34
25	CH_2COOH	−1.28	−1.44	1.20	0.26	−0.22
26	$(CH_2)_5COOH$	0.85	−0.31	2.29	2.39	0.69
27	$(CH_2)_2NH_2$	−1.04	−0.63	2.64	0.50	0.35
28	$(CH_2)_4NH_2$	−0.02	−0.72	2.30	1.54	0.44
29	$(CH_2)_6NH_2$	1.09	−0.63	2.61	2.63	0.56
30	$(CH_2)_8NH_2$	2.21	0.48	3.72	3.75	0.78
31	$(CH_2)_9NH_2$	2.77	0.89	4.13	4.31	0.97
32	$(CH_2)_2NH(CH_2)_2CH_3$	1.08	0.67	3.94	2.62	0.72

(Continued on p. 412)

TABLE I *(continued)*

Compound No.	*R*	π_X*	*Log* P_m**	*Log* P_m^c***	*Log* P_c§	*Log* k'§§
33	$CH_2CH(CH_2)_5$	2.51	n.d.§§§		4.05	0.99
34	$CH_2(C_6H_4)$-2-OCH_3	1.94	n.d.		3.48	0.70
35	$CH_2(C_6H_4)$-3-OCH_3	1.94	3.27		3.48	0.72
36	$CH_2(C_6H_4)$-4-OCH_3	1.94	n.d.		3.48	0.68
37	$CH_2(C_6H_4)$-4-NO_2	1.68	3.44		3.22	0.80
38	$CH_2(C_6H_4)$-4-Cl	2.67	n.d.		4.21	0.92

* Values obtained from ref. 16 and R = CH_2X for all compounds except 1, for which it was calculated from that for 2 by subtracting η_{CH_2}.

** P_m = *n*-octanol–phosphate partition coefficient measured by shake-flask method[2].

*** P_m^c = value of P_m corrected for ionization according to Hafkenscheid and Tomlinson[8] and using published pK_a values for free aliphatic amines and carboxylic functions[17].

§ Log *P*, calculated from log P_{RX} = log P_{RH} + π_X.

§§ Log k' = k' on RP C_{18} column using methanol–phosphate buffer as mobile phase.

§§§ n.d. = not determined.

plied[15]. When the variation of $C_{octanol}$ as a function of $C_{phosphate}$ deviated from linearity the values obtained from the lower steroid concentration were the only ones retained. From these experimental *P* values, log P_m (measured) was computed and

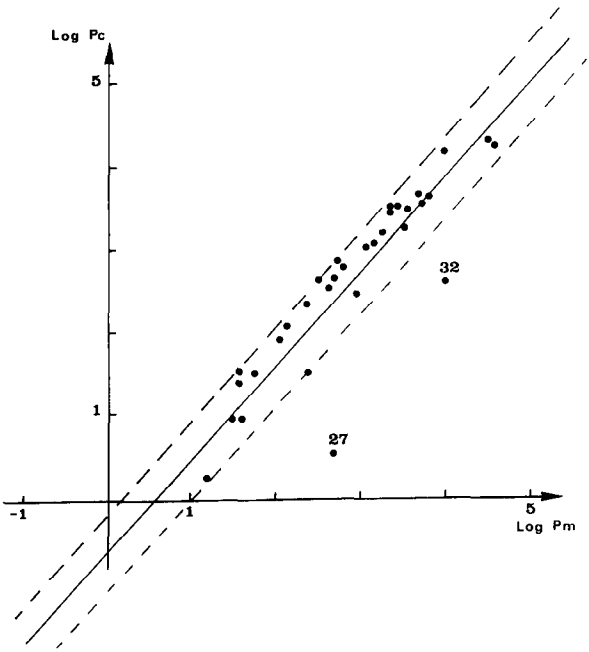


Fig. 1. Relationship between log P_m^c, *n*-octanol-phosphate buffer partition coefficient measured by shake-flask method and corrected for ionization, and calculated log P_c. Compounds are numbered as in Table I.

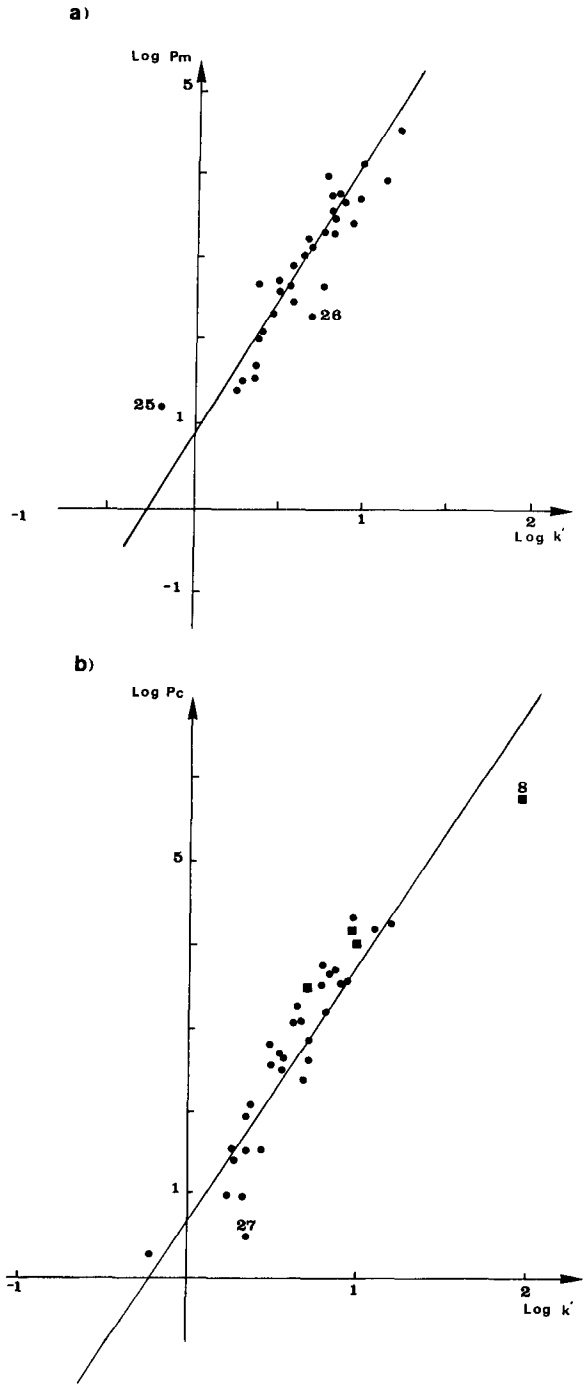


Fig. 2. (a) *n*-Octanol–phosphate buffer partition coefficient *versus* capacity factor k' measured by RP-HPLC on a Nucleosil C_{18} column with phosphate buffer–methanol (35:65) as mobile phase. (b) Relationship between log P calculated according to Hansch and Leo[16] and log k'. ■, Compounds for which log P_m was not determined (see Table I).

compared with log P_c (calculated) obtained from the additive approach using the parameter π defined according to Hansch[2] by

$$\log P_X = \log P + \pi_X$$

where P_X is the partition coefficient of a derivative, P that of the parent compound and π_X the hydrophobic constant of the substituent X. Compound 2 was the parent compound in our series and π_X was obtained from a published compilation[16]. Although we were dealing with an aliphatic series, we used the values given for the benzene solute system because this system is by far the most complete with π values reported for almost all substituents.

For the eight ionogenic compounds 25–32, the experimental measured partition coefficient P_m was corrected for ionization by resorting to the very simple equation proposed by Hafkenscheid and Tomlinson[8]:

$$P_m^c = P_m[1 + 10^{(pK_A - pH)_{aq}}]$$

with the subscript aq refers to the aqueous phase of the distribution system, P_m is the observed distribution coefficient at pH 7.4, *i.e.*, the ratio of the compound in the *n*-octanol phase (only non-ionized species) to the concentration of both ionized and non-ionized species in the aqueous phase, and P_m^c is the corrected value, *i.e.*, the "true" partition coefficient or ratio of non-ionized compounds in the two phases. To avoid the time- and compound-consuming pK_a determination for each ionogenic derivative, standard values determined for simple aliphatic amines and carboxylic acids were used[17]. When correlating log P_c with log P_m^c the following equation was found:

$$\log P_c = 1.096\ (\pm 0.010)\ \log P_m^c - 0.454\ (\pm 0.297) \qquad (1)$$

$$n = 33;\ r = 0.892\ (p < 0.0001);\ F = 120.32\ (31, 2)$$

The good fit observed (significant at the $p > 99.9$ level) and the fact that the measured and calculated log P values were similar supported our choice of π_X values obtained from the benzene solute system (Fig. 1). All the experimental data were in the ± 2 S.D. range around the expected values except for compounds 27 and 32 (-4 and -2.7 S.D., respectively). The poor fit for these two compounds, bearing an amino group at the same very short distance from the amide bond and the bulk of the steroid structure, suggested that these derivatives could be less ionized than expected, probably owing to some electronic and/or steric intramolecular interactions. However, for the other six ionogenic compounds the very simple correction used appeared to be satisfactory, even for compound 25, which bears an electronegative carboxylic group very close to the carboxamide nitrogen.

Correlation between HPLC retention factor k' and octanol–buffer partition coefficient

The capacity factor k' was determined for 38 17β-carboxamide derivatives using the same persilylated Nucleosil C_{18} column as for the determination of partition coefficientss. The mobile phase was 10 mM phosphate buffer (pH 7.4)–methanol

(35:65) for all compounds (Table I). The log k' values at a 65% methanol concentration were selected for this study because this methanol concentration in the mobile phase provided a log k' in the region of maximum accuracy for most of the 17β-carboxamide derivatives. Log k' values of the sample compounds were calculated from their retention times:

$$\log k' = \log(t_R - t_0)/t_0$$

where t_R is the retention time of the retained compound and t_0 that of unretained compounds (methanol peak).

When log P_m^c was plotted against log k' a significant correlation was found (Fig. 2a):

$$\log P_m^c = 2.816\ (\pm 0.218) \log k' + 1.122\ (\pm 0.146) \quad (2)$$
$$n = 33;\ r = 0.918\ (p < 0.0001);\ F = 167.24\ (31, 2)$$

The observed fit was better than that between log P_m^c and log P_c. Here again two compounds fall outside the ± 2 S.D. range. However, the deviations remained rather limited, of opposite sign and affected the two acidic derivatives 25 and 26 (-2.02 and $+2.25$ S.D., respectively), whereas an excellent fit was observed for all the basic derivatives tested, including compounds 27 and 32. Hence, in our original series the retentions on an RP C_{18} column equilibrated in an aqueous buffer–methanol mobile phase appeared to be well correlated with the measured *n*-octanol–water partition coefficients, an observation in good agreement with data previously published on various solutes such as *n*-alkylbenzenes[3], 5-nitroimidazoles[4], phenylcarbamates[5], *p*-benzoquinones[6] and others[1]. Therefore, resorting to k' values measured with 65% methanol, without the correction or extrapolation to 0% methanol sometimes recommended[10], and to very simply corrected P_m^c values could yield satisfactory structure–retention relationships.

Finally, the correlation between the calculated partition coefficient P_c and k' also appeared satisfactory (Fig. 2b):

$$\log P_c = 3.490\ (\pm 0.256) \log k' + 0.532\ (\pm 0.172) \quad (3)$$
$$n = 33;\ r = 0.926\ (p < 0.0001);\ F = 185.38\ (31, 2)$$

A strong deviation was observed only for compound 27 (-3.09 S.D.). Thus, surprisingly, the fits between log P_c and log k' and between log P_c^m and log k' appeared better than those between log P_c and log P_m^c. Moreover, when compounds for which no determination of P_m had been performed were added to the series, the equation became

$$\log P_c = 3.068\ (\pm 0.220) \log k' + 0.813\ (\pm 0.165) \quad (4)$$
$$n = 38;\ r = 0.919\ (p < 0.0001);\ F = 195.26\ (36, 2)$$

Compounds 27 and 8 fell outside the ± 2 S.D. range. Therefore, the correlation

appeared to be better respected by compounds that displaying medium polarity than by highly ionogenic compounds such as 27 or strongly hydrophobic compounds such as 8.

However, the overall correlation remained satisfactory. It follows from the results presented in this paper that for the expression of the hydrophobicity of 17β-carboxamide steroid derivatives it is possible to use the log k' values measured directly by RP-HPLC. These values were then used to establish quantitative structure–activity relationships between the chromatographic retention parameters of the steroids and the dissociation constant of the complexes formed with the glucocorticoid receptor[18].

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