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Determination of the lipophilicity of some peptides

Effect of surface pH of silica

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ABSTRACT

The lipophilicities of 22 peptides were determined by reversed-phase thin-layer chromatography using methanol as organic modifier in the concentration range 0–90 vol.-% on silica supports with surface pH values of 2.0, 4.5, 6.0, 7.5 and 9.0. Only one of the 22 peptides (Trp–Ala–Ile) followed the general rule, *i.e.*, its R_M value decreased linearly with increasing proportion of organic modifier over the whole concentration range. Most peptides exhibited typical silanophilic retention behaviour, the R_M value decreasing with increasing organic phase concentration in the lower concentration range and then increasing with further increase in the proportion of organic modifier. In some instances the lipophilicity increased linearly with increasing proportion of methanol. The silanophilic effect depended not only on the structure of the peptide, but also on the surface pH of the silica support. The retention behaviour of peptides can be well described by a polynomial function, the linear and quadratic forms of methanol concentration and surface pH being the independent variables. Principal component analysis showed that the presence of a ring structure in the peptides has the greatest impact on their retention behaviour, the overall polarity (basic, neutral or acidic) being of secondary importance. The number of amino acids in the peptide has a negligible effect on the chromatographic behaviour.

INTRODUCTION

Reversed-phase thin-layer chromatography (RPTLC) has been extensively applied to the determination of the lipophilicity of bioactive molecules [1,2]. To increase the accuracy of the lipophilicity determination, linear correlations have been calculated between the R_M values and the concentration of organic mobile phase in the eluent; the R_M value extrapolated to zero organic phase concentration (R_{M0}) was regarded as the most accurate estimate of liophilicity [3,4]. However, with peptides [5], quaternary amino steroids [6] and crown ether derivatives [7,8], no linear correlation was found between the R_M value and the concentration of the organic mobile phase. The R_M value decreased with increasing organic phase concentration in the lower concentration range, reached a minimum and then increased with further increase in the organic phase ratio. This phenomenon was tentatively explained in terms

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of silanophilic effects at higher organic phase concentrations, the solute molecules having an enhanced probability of access to the silanol groups uncovered by the impregnating agent. The interaction with the free silanol groups results in an increased retention and an increased apparent lipophilicity [5]. The adsorptive side-effect of free silanol groups can be eliminated or decreased by the addition of alkylamines [9] or salts [10] to the eluent.

Recent research indicates that in RPTLC the adsorptive character of the support has a considerable influence on retention, even after impregnation [11–13], because the adsorptive sites not covered with the impregnating agent also affect the binding of solutes. This finding indicates that the surface pH of the support may have some impact on the retention of polar compounds even after impregnation. It has recently been established that the surface pH of silica influences the RPTLC retention of dansylamino acids [14] and of free amino acids [15].

To our knowledge, the structural chracteristics of solutes accounting for the silanophilic effect and the influence of the surface pH of the silica support on it have never been studied in detail. We assumed that, owing to their amphipathic character, peptides are ideal test solutes for studing the effects outlined above. Reversed-phase chromatography has been extensively applied to separate peptides on both analytical [16] and preparative scales [17,18]. The retention dependend on the type [19] and on the density of the hydrophobic ligand [20]. Moreover, reversed-phase chromatography has been utilized as a physico-chemical tool for the study of peptide behaviour at hydrophobic liquid–solid interfaces which mimic biological lipid bilayers. It helped to identify and characterize both the hydrophobic interaction sites and the existence of conformational equilibria of peptides such as β-endomorphin [21,22], luteinizing hormone-releasing hormone [23], myosin kinase analogues [24] and human growth hormone related peptides [25,26].

The objectives of this work were to determine the contributions of the physico-chemical parameters of peptides and supports to the silanophilic effect in the RPTLC of some peptides.

EXPERIMENTAL

Kieselgel 60 H (Merck, Darmstadt, F.R.G.) was used as a support. Its pH was adjusted to 3.0, 4.5, 6.0, 7.5 and 9.0 is described in ref. 14. Layers of 0.25 mm thickness were prepared on 20 × 20 cm glass plates and impregnated by predevelopment in 5% paraffin oil [27] in *n*-hexane. The application of the less reproducible laboratory-made layers instead of ready-made layers was motivated by the fact that the pH modification of the silica surface cannot be carried out with acceptable accuracy under other experimental conditions. No leakage of the adsorbed paraffin layer was observed in similar RPTLC systems [28]. Untreated Kieselgel 60 H plates served as controls (pH = 6.55). The chemical structures of the peptides are given in Table I. The peptides were dissolved in water–1-propanol (2:1, v/v) at a concentration of 2 mg/ml, and 2 μl of each solution were spotted onto the plates. Methanol was applied as the organic phase in the concentration range 0–90% (v/v) at 10% intervals. After development, the peptides were detected with ninhydrin. For each experiment, five independent parallel determinations were carried out.

When for a given RPTLC system the peptide spot remained at the start, or was

TABLE I

CHEMICAL STRUCTURES OF PEPTIDES

All amino acids had the L-configurations. β-Abu = β-aminobutyric acid; γ-Abu = γ-aminobutyric acid; γ-Ape = γ-aminopentanoic acid; γ-Amh = γ-amino-δ-methylhexanoic acid; γ-Ava = γ-aminovaleric acid.

Compound No.	Chemical structure	Compound No.	Chemical structure
1	β-Abu–ala	12	Thr–Ile–Pro
2	Phe–Ala	13	Pro–Thr–Ile–Pro
3	β-Abu–β-Abu	14	Trp–Ser–Tyr–Gly
4	γ-Amh–γ-Amh	15	Trp–Ala–Ile
5	γ-Ava–γ-Abu	16	Arg–Thr–Asn–Thr–Gly
6	γ-Ava–γ-Ava	17	Lys–Ala
7	β-Ala–β-Abu	18	Ala–Lys–Pro–Lys
8	Ala–Thr	19	Reduced glutathione
9	Gly–Leu–Gly	20	Oxidized glutathione
10	Gly–β-Abu–Gly	21	Gly–Gly
11	γ-Glu–Cys–Gly	22	Ala–Ala

very near to the front (deformed spot shape), or the relative standard deviation of five parallel determinations was higher than 10%, the data were omitted from the calculations.

As our data indicated that the retention of peptides depended simultaneously and non-linearly on the methanol concentration in the eluent and on the surface pH of silica, stepwise regression analysis [29] was applied to select the chromatographic conditions that significantly influence the retention. Stepwise regression analysis is a special case of multi-linear regression analysis. It automatically eliminates from the selected equation the insignificant independent variables, thus increasing the information power of the calculation. Although stepwise regression analysis is a multi-linear technique, with pretransformation of the variables (*i.e.*, $x_2 = \log x_1$) it is suitable for calculating non-linear correlations.

The R_M value of peptides defined by eqn. 1 [2] was taken as the dependent variable.

$$R_M = \log (1/R_F - 1) \tag{1}$$

The linear and quadratic forms of the methanol concentration and the silica surface pH were taken as independent variables (total four independent variables). The number of accepted variables was not limited and their partial F value was set to $F = 2$. The F values characterize the level of significance of the individual independent variables. A higher F value means a higher significance level. The calculation was carried out separately for each peptide.

To elucidate the similarities and dissimilarities between the retention behaviours of peptides and the chromatographic parameters, principal component analysis (PCA) was applied [30]. PCA is the method of choice when the relationship between all parameters without one being the dependen variable is of paramount interest. The main advantages of PCA in chromatography are (a) clustering chromatographic systems or solutes according to their retention behaviour and (b) the possi-

bility of extracting one or more background variables having a concrete physico-chemical meaning for the retention.

The peptides were taken as observations and the parameters (the four slope values of the independent variables of the stepwise regression analysis) served as variables. When the stepwise regression analysis proved that the slope value of a given independent variable did not deviate significantly from zero, the zero value was included in the PCA. Peptides 21 and 22 were not included in the PCA. Two-dimensional non-linear mapping of the PC loadings and variables was also carried out [31]. The peptides showing similar retention behaviour form clusters on the two-dimensional map of PC variables whereas the peptides with different retention characteristics are situated far from each other on the map.

RESULTS AND DISCUSSION

Peptides can follow the general rule, their R_M value decreasing linearly with increasing proportion of organic modifier over the whole concentration range. However, this regular behaviour occurred only with Trp–Ala–Ile on the layers with a surface pH of 6.0. In the other instances two types of retention behaviour were observed (Fig. 1). Most peptides exhibited typical silanophilic retention behaviour, the R_M value decreasing with increasing organic phase concentration in the lower concentration range, and then increasing with further increase in the proportion of the organic modifier. Some peptides showed anomalous behaviour over the whole concentration range, their lipophilicity increasing with increasing methanol concentration. The silanophilic effect depended not only on the structure of the peptide, but also on the surface pH of the silica support (Figs. 2 and 3). The differences in the retention are generally small at acidic pH. This pH dependence suggests that the paraffin oil did not entirely cover the silica surface and the free silanol groups influence the retention. The strength of electrostatic interactions between the polar groups of

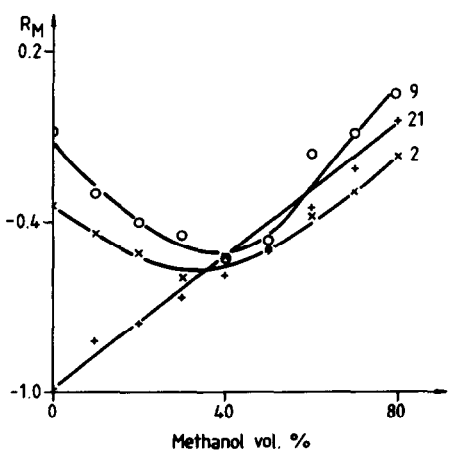

Fig. 1. Dependence of R_M values of some peptides on the methanol concentration in the eluent. Numbers indicate peptides in Table I. Surface pH of the silica support = 4.5.

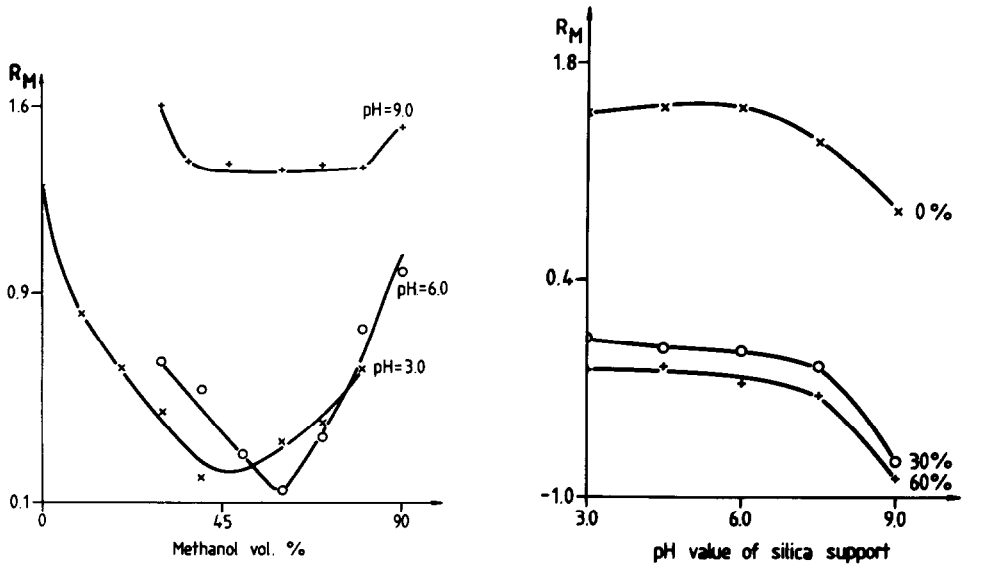

Fig. 2. Dependence of R_M value of peptide 18 on the methanol concentration in the eluent and on the surface pH of the silica support.

Fig. 3. Dependence of R_M value of peptide 13 on the surface pH of the silica support and on the methanol concentration in the eluent.

peptides and the active sites on the silica surface depends on the differences in their relative polarities resulting in modified retention. As the dissociation of the polar groups of peptides has to decrease at high organic phase contents, the influence of surface pH was expected to decrease at higher methanol concentrations. However, our data did not support this hypothesis, the effect of the surface pH of silica prevailing also at high organic phase concentrations (Fig. 3). This observation can be explained by the assumption that the polar groups of peptides are in dissociated or partially dissociated form even at higher methanol concentrations, which is sufficient to influence their interaction with the active sites on the silica surface.

The results of stepwise regression analysis are given in Table II. The F values show that the equations selected by the stepwise regression analysis fit the experimental data well, the significance level being over 99.9% except in one instance. The calculations entirely support our previous qualitative conclusions. The slope values indicate that the retention behaviour of peptides depended not only on the methanol concentration in the eluent, but also on the surface pH of silica, that is, the silanophilic effect is influenced markedly by the surface pH. However, the character of the correlation showed great variety. The significant differences between the slope values of the peptides demonstrate that their retention behaviours deviate significantly from one another, that is, the structural parameters considerably influence not only the methanol but also the pH dependence.

The goodness of fit of the equations showed that the retention behaviour of peptides can be modelled well with linear or quadratic functions even with the silanophilic effect. The results of PCA are given in Table III. The first and second princi-

TABLE II

DEPENDENCE OF R_M VALUE OF PEPTIDES ON THE pH OF THE SILICA SURFACE AND ON THE METHANOL CONCENTRATION (C, %, v/v) IN THE ELUENT

Results of stepwise regression analysis (n.s. = not significant).

$$R_M = a + b_1\text{pH} + b_2\text{C} + b_3\text{pH}^2 + b_4C^2$$

No. of	n	a	F	b_1	$b_2 \cdot 10^2$	$b_3 \cdot 10^2$	$b_4 \cdot 10^4$
1	58	−0.35	42.5	n.s.	−1.06	−0.17	1.6
2	46	−0.86	37.1	0.43	−2.23	−4.91	2.1
3	58	−0.87	37.1	2.44	−1.23	−2.23	0.2
4	48	2.84	16.8	−0.72	−1.44	5.82	n.s.
5	58	−0.36	39.8	0.09	−1.20	−0.86	1.7
6	54	−0.27	66.8	n.s.	−0.74	−0.21	0.2
7	53	−0.60	88.7	0.11	−0.85	−1.25	1.8
8	47	−1.28	33.5	0.45	−1.33	−5.12	0.3
9	48	−1.11	26.3	0.50	−2.22	−5.36	0.3
10	47	−1.08	40.4	0.30	−0.67	−3.41	1.7
11	51	−0.77	10.3	n.s.	n.s.	n.s.	0.8
12	46	−0.25	76.6	0.48	−4.09	−5.70	3.8
13	56	0.36	103.5	0.44	−5.29	−4.59	4.4
14	43	2.87	70.9	n.s.	−8.31	−1.19	5.9
15	39	2.68	158.4	n.s.	−3.55	−1.27	n.s.
16	57	0.58	35.0	n.s.	−2.37	n.s.	2.8
17	56	−0.48	9.8	0.40	−1.32	−3.41	1.7
18	49	1.12	41.9	n.s.	−3.31	1.44	3.0
19	18	−0.87	65.7	n.s.	n.s.	−9.31	1.7
20	12	−0.18	116.2	n.s.	−5.17	n.s.	7.9
21	46	−1.91	155.3	0.39	1.26	−4.42	n.s.
22	54	−0.87	186.1	−0.06	1.12	n.s.	n.s.

TABLE III

RESULTS OF PRINCIPAL COMPONENT ANALYSIS

No. of principal component	Eigenvalue	Sum of variance explained (%)
1	2.18	54.53
2	1.56	93.50
3	0.24	99.45

Principal component loadings

No. of variable	No. of principal component		
	1	2	3
1	0.85	0.51	0.04
2	−0.55	0.76	0.34
3	−0.86	−0.50	0.08
4	0.64	−0.69	0.34

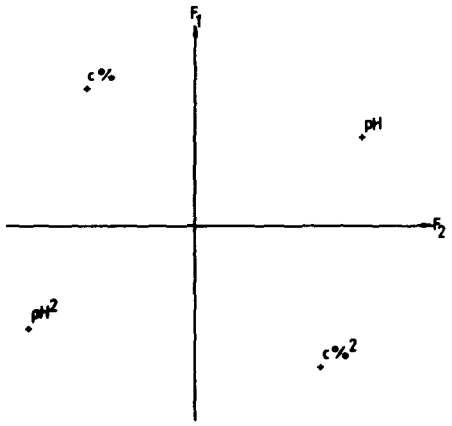

Fig. 4. Two-dimensional map of PC loadings. pH = surface pH of silica support; $c\%$ = methanol concentration in the eluent (vol.-%).

pal components explain most of the total variance, the former explaining 54% of it. The loadings of the two pH variables (variable 1 = pH, variable 3 = pH^2) are the highest in the first principal component, so this PC can be regarded as the effect of surface pH. The methanol concentration (variable 2) and the second power of the methanol concentration (variable 4) have the highest loadings in the second principal component, that is, this PC contains the effect of the organic modifier.

The two-dimensional map of PC loadings (Fig. 4) shows the clustering of chromatographic parameters taking into consideration simultaneously the retention beha-

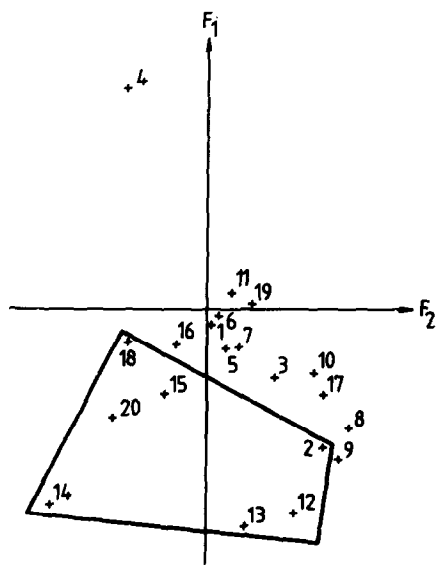

Fig. 5. Two-dimensional map of PC variables. Numbers indicate peptides in Table I.

viour of peptides 1–20. All the chromatographic parameters (linear and quadratic forms of methanol concentration and surface pH) are widely separated on the map. This finding that each of them separately influences the retention of peptides, that is, each variable is necessary to describe the retention behaviour of peptides.

The two-dimensional map of PC variables (Fig. 5) shows the clustering of peptides, taking into consideration simultaneously the effect of all four chromatographic parameters. Neither the alkaline (peptides 16, 16 and 18) nor the acidic (peptides 11, 19 and 20) side-chains account for the clustering of peptides, which means that the overall polarity of a peptide has a negligible effect on the retention characteristics. The peptides do not form clusters according to the number of amino acids, that is, the number of amino acids in the peptides does not influence the retention characteristics appreciably.

The peptides with a ring structure in the amino acid side-chain (Trp and Pro) form a cluster. This result suggests that the dimensions of the amino acid side-chain (bulkier ring structures) mainly influence the retention behaviour of peptides in RPTLC.

REFERENCES

1 C. B. C. Boyle and B. V. Milborrow, *Nature (London)*, 208 (1965) 537.
2 G. L. Biagi, A. M. Barbaro and M. C. Guerra, *J. Chromatogr.*, 41 (1969) 371.
3 J. Draffehn, B. Schonecker and K. Ponsold, *J. Chromatogr.*, 205 (1980) 113.
4 T. Cserháti, *Chromatographia*, 18 (1984) 18.
5 K. E. Bij, Cs. Horváth, W. R. Melander and A. Nahum, *J. Chromatogr.*, 203 (1981) 65.
6 É. János, T. Cserháti and E. Tyihák, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 5 (1982) 634.
7 T. Cserháti, M. Szögyi and L. Györfi, *Chromatographia*, 20 (1985) 253.
8 A. Nahum and Cs. Horváth, *J. Chromatogr.*, 203 (1981) 53.
9 S. G. Weber and W. G. Tramposh, *Anal. Chem.*, 55 (1983) 1771.
10 S. G. Weber and J. D. Orr, *J. Chromatogr.*, 322 (1985) 433.
11 M. C. Guerra, A. M. Barbaro, G. Cantelliforti, M. T. Foffani, G. L. Biagi, P. A. Borea and A. Fini, *J. Chromatogr.*, 216 (1981) 93.
12 W. F. Giesen and L. H. M. Janssen, *J. Chromatogr.*, 237 (1982) 199.
13 T. Cserháti, Y. M. Darwish and Gy. Matolcsy, *J. Chromatogr.*, 270 (1983) 97.
14 Z. Illés and T. Cserháti, *J. Planar Chromatogr.*, 1 (1988) 231.
15 Z. Illés and T. Cserháti, *J. Planar Chromatogr.*, 2 (1989) 92.
16 A. J. Albert, *J. Chromatogr.*, 444 (1988) 269.
17 L. R. Snyder, G. B. Cox and P. E. Antle, *J. Chromatogr.*, 444 (1988) 303.
18 G. B. Cox, P. E. Antle and L. R. Snyder, *J. Chromatogr.*, 444 (1988) 325.
19 G. Jilge, R. Janzen, H. Giesche, K. K. Unger, J. N. Kinkel and M. T. W. Hearn, *J. Chromatogr.*, 397 (1987) 71.
20 K. D. Lork, K. K. Unger, H., Brückner and M. T. W. Hearn, *J. Chromatogr.*, 476 (1989) 135.
21 M. I. Aguilar, A. N. Hodder and M. T. W. Hearn, *J. Chromatogr.*, 327 (1985) 115.
22 M. T. W. Hearn and M. I. Aguilar, *J. Chromatogr.*, 352 (1986) 35.
23 M. T. W. Hearn and M. I. Aguilar, *J. Chromatogr.*, 359 (1986) 31.
24 M. T. W. Hearn and M. I. Aguilar, *J. Chromatogr.*, 392 (1987) 33.
25 A. W. Purcell, M. I. Aguilar and M. T. W. Hearn, *J. Chromatogr.*, 476 (1989) 113.
26 A. W. Purcell, M. I. Aguilar and M. T. W. Hearn, *J. Chromatogr.*, 476 (1989) 125.
27 E. Schulek (Editor), *Pharmacopoea Hungarica*, Vol. II, Egészségügyi Kiado, Budapest, 1954, p. 411.
28 T. Cserháti, Gy. Ösapay and M. Szögyi, *J. Chromatogr. Sci.*, 27 (1989) 540.
29 H. Mager, *Moderne Regressionsanalyse*, Salle, Sauerlander, Frankfurt a.M., 1982, p. 135.
30 K. V. Mardia, J. T. Kent and J. M. Bibby, *Multivariate Analysis*, Academic Press, London and New York, 1969.
31 J. W. Sammon, Jr., *IEEE Trans. Comput.* C18 (1969) 401.