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Dependence of the silanophilic effect on the concentration of preadsorbed salts and on the chemical structure of peptides in reversed-phase thin-layer chromatography

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ABSTRACT

The retentions of eighteen peptides in reversed-phase thin-layer chromatography were determined with methanol as the organic mobile phase. Before impregnation inorganic salts (LiCl, NaCl, KCl, $CaCl_2$ and $MgCl_2$) were preadsorbed on the silica support in the concentration range of 0.5–2 ml of 1 *M* salt solution per gram of silica. The majority of peptides showed a silanophilic effect: their R_M values first decreased to a minimum and then increased with increasing organic phase concentration. The retention of peptides depended quadratically on the methanol concentration and linearly on the salt concentration, on the hydrated ion radii and on the charge of the cation, that is, salts could not mask the silanophilic effect of free silanol groups even at higher concentrations. Principal component analysis (PCA) proved that the linear and quadratic forms of methanol concentrations have a similar effect on the retention of peptides; however, the salt concentration, the hydrated ion radii and charge of cations modify the retention differently. The PC variables did not correlate with the lipophilicity of peptides, proving the lipophilicity modifying effect of salts. No correlation was found between the parameters of quadratic functions fitted for salt-free and salt-containing systems, proving again the lipophilicity-modifying effect of salts. On the two-dimensional non-linear map of PC variables the peptides did not form cluster according to their structural characteristics, proving the participation of each peptide substructure in the peptide–salt interaction. However, the overall polarity of peptides influenced their salt sensitivity.

INTRODUCTION

In drug design, computer-assisted multivariate mathematical methods have gained growing importance and acceptance [1]. The lipophilicity of bioactive molecules is one of the physico-chemical parameters most frequently used in quantitative structure–activity relationship (QSAR) studies [2]. Reversed-phase thin-layer chromatographic (RP-TLC) methods have been extensively applied to determine the lipophilicity of many bioactive compounds [3]. To increase the accuracy of the lipophilicity determination, linear correlations have been calculated between the R_M values (characterizing the lipophilicity in RP-TLC) 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 the lipophilicity [4,5]. However, with peptides [6], quaternary amino steroids [7] and crown ether

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derivatives [8,9], 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 of the organic phase ratio. This phenomenon was tentatively explained in terms of silanophilic effect: at higher organic phase concentrations, the solute molecules have an enhanced probability of access to the silanol groups uncovered by the impregnating agent. The interaction with the free silanol groups results in increased retention and an increased apparent lipophilicity [6]. It was assumed and in some instances proved experimentally that the adsorptive side-effect of free silanol groups can be eliminated or reduced by the addition of alkylamines [10] or salts [11] to the eluent. To our knowledge, the concentration of salts needed to eliminate the silanophilic effect and the impact of the various physico-chemical parameters of salts (ion charge and hydrated ion radii) on the elimination process have never been studied in detail. Reversed-phase chromatography has been extensively applied to separate peptides on both the analytical [12] and preparative [13,14] scale. The retention depended on the type [15] and density of the hydrophobic ligand [16]. Moreover, reversed-phase chromatography has been applied to the study of peptide behaviour at hydrophobic liquid–solid interface 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 [17,18], luteinizing hormone-releasing hormone [19], myosin kinase analogues [20] and human growth hormone-related peptides [21,22].

The objectives of this work were to determine the effect of salts preadsorbed on a silica surface on the silanophilic effect and to elucidate the role of the salt concentration and that of ion charge and hydrated ion radii in the decrease in the silanophilic effect. We assumed that, owing to their amphipathic character, peptides are ideal test solutes to study the above effects.

EXPERIMENTAL

Kieselgel 60 H (Merck, Darmstadt, Germany) was used as a support. The salt solutions (0.5, 1 and 2 ml of 1 *M* NaCl, 1 ml of 1 *M* LiCl, KCl, $MgCl_2$ and $CaCl_2$ per gram of silica) were added to the silica before preparing the plates. Layers 0.25 mm thick were prepared on 20 × 20 cm glass plates and were dried overnight at room temperature and impregnated by predevelopment in 5% paraffin oil in *n*-hexane [23]. Untreated Kieselgel 60 H plates served as a control. We are well aware that the application of hand-made plates increases the inherent low reproducibility of TLC. However, the application of this method was motivated by the fact that the addition of salts cannot be carried out with acceptable accuracy under other experimental conditions.

The 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 on the plates. Methanol in water was applied as the organic mobile phase in the concentration range of 0–90 vol.% at 10% intervals. After development, the peptides were detected with ninhydrin. For each experiment, five parallel determinations were carried out.

For a given RP-TLC system, whenever the peptide remained at the start or was

TABLE I

STRUCTURES OF PEPTIDES

All amino acids had the L configuration. β-Abu = β-Aminobutyric acid; γ-Abu = γ-aminobutyric acid; γ-Ape = γ-amino-δ-methylhexanoic acid.

Compound No.	Structure	Compound No.	Structure
1	β-Abu–Ala	11	Glu–Cys–Gly
2	Gly–Gly	12	Thr–Ile–Pro
3	Phe–Ala	13	Pro–Thr–Ile–Pro
4	Ala–Ala	14	Trp–Ser–Tyr–Gly
5	γ-Ape–γ-Abu	15	Trp–Ala–Ile
6	γ-Abu–γ-Abu	16	Ala–Lys–Pro–Lys
7	Ala–β-Abu	17	γ-Glu–Cys–Gly
8	Ala–Thr	18	γ-Glu γ-Glu | | Cys–S–S–Cys | | Gly Gly
9	Gly–Leu–Gly		
10	Gly–β-Abu–Gly		

very near to the front (deformed spot shape), or the relative standard deviation of the five parallel determinations was higher than 10%, the data were omitted from the calculations. As our data indicated that the retention of peptides simultaneously depended on the methanol concentration in the eluent and on the concentration and on the type of the preadsorbed salt, stepwise regression analysis [24] was applied to select the variables that significantly influenced the retention.

The R_M value [25] of peptides defined by the following equation was taken as the dependent variable:

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

The linear and quadratic forms of the methanol concentration, the amount of preadsorbed salt, the charge and the hydrated ion radii of the cation were taken as independent variables (total five independent variables). The acceptance limit for the selected independent variables was set at 95% significance. The inclusion of the quadratic form of the methanol concentration was motivated by the finding that the irregular retention behaviour of peptides can be well described by a quadratic correlation [26]. Stepwise regression analysis was carried out separately for each peptide. To evaluate the retention-modifying effect of salts, linear correlations were calculated between the slope values of the independent variables concerning the effect of methanol (linear and quadratic form of methanol concentrations) determined in salt-free and salt-containing systems.

To elucidate the similarities and dissimilarities between the retention behaviour of peptides and the chromatographic parameters, principal component analysis (PCA) was applied [27]. The application of various multilinear regression methods requires the presence or selection of a dependent variable. However, in many instances one is not interested in the dependence of one parameter (dependent variable) on the other parameters (independent variables), the aim rather being to find the relationship

between all parameters without one being dependent variable. PCA complies with these requirements. The advantages of its application are the clustering of the variables according to their relationship, the possibility of extracting one or more background variables which may have a concrete physico-chemical meaning and the decrease in the number of variables.

The peptides were taken as observations, and the chromatographic parameters (the five slope values of the independent variables of the stepwise regression analysis) served as variables. The application of each slope value was motivated by the fact that these values are indicators of the retention behaviour of peptides. To avoid the information loss caused by the normalization, PCA was carried out on the covariance matrix instead of the correlation matrix. 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. The two-dimensional non-linear map of PC loadings and variables was also calculated [28]. The peptides (or chromatographic parameters) showing similar retention behaviour form clusters on the two-dimensional map. In most instances the coordinates of the map (F_1 and F_2) do not have any concrete physical or physico-chemical meaning. They only show the distribution of the variables in a plane which was reduced from the multi-dimensional PCA space.

To assess the role of the lipophilicity of peptides in the retention, linear correlations were calculated between the first three principal component variables and the lipophilicity of peptides. The lipophilicity values of peptides were taken from ref. 29 or calculated accordingly.

RESULTS AND DISCUSSION

Peptides exhibited typical silanophilic retention behaviour, the R_M value decreasing with increasing methanol concentration in the lower concentration range, and then increasing with further increase in the methanol ratio (Fig. 1). Apart from the silanophilic effect, this biphasic retention behaviour may be due to the adsorption of methanol at higher concentrations on the modified silica, which then results in a normal phase-like surface. The effect of salts depended on their concentration; however, the salt could not eliminate the silanophilic effect even at fairly high concentration (Fig. 2), which indicates that parameters other than the presence of free (uncovered) silanol groups may have some impact on the irregular retention behaviour of peptides. The cation type (charge and hydrated ion radii) also influenced the silanophilic effect (Fig. 3), as will be explained later.

The results of stepwise regression analysis are given in Table II. The F values show that the equations selected fit the experimental data well, the significance level being over 99.9% in each instance. The calculations entirely support our previous qualitative conclusions. The methanol concentration (linearly and/or quadratically) significantly influenced the retention of each peptide, proving again the irregular retention behaviour of peptides and the existence of the silanophilic effect. However, the character of the correlation showed high variance. The significant differences between the slope values of peptides indicates that their retentions deviate significantly from each other, that is, the structural parameters of a peptide considerably influence the nature of the silanophilic effect. The concentration of preadsorbed salts, the ion

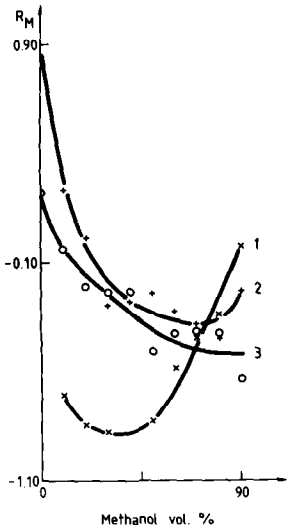
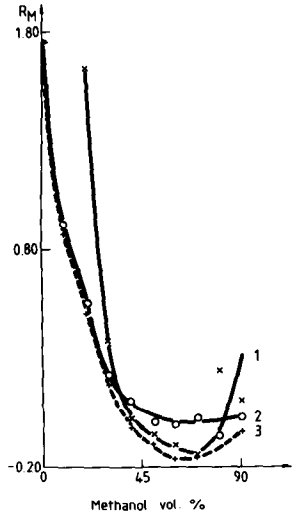


Fig. 1. Dependence of R_M value of some peptides on the methanol concentration in the eluent (1 ml of 1 *M* KCl per gram of silica). 1 = Peptide 2; 2 = peptide 12; 3 = peptide 3. For structures, see Table I.



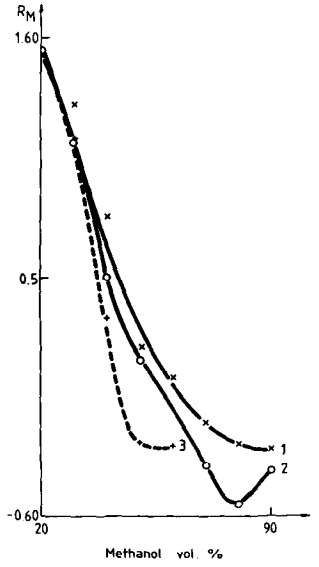


Fig. 2. Dependence of R_M value of peptide 14 on the methanol concentration in the eluent and on the amount of preabsorbed salt. Volume of 1 *M* NaCl per gram of silica: 1 = 2 ml; 2 = 1 ml; 3 = 0.5 ml.

Fig. 3. Dependence of R_M value of peptide 15 on the methanol concentration in the eluent and on the type of preadsorbed salt (1 ml of 1 *M* salt solution per gram of silica). 1 = $CaCl_2$; 2 = KCl; 3 = LiCl.

TABLE II

DEPENDENCE OF THE R_M VALUES OF PEPTIDES ON THE METHANOL CONCENTRATION (C) IN THE ELUENT AND ON THE PHYSICO-CHEMICAL CHARACTERISTICS OF SALTS

Results of stepwise regression analysis. $R_M = a + b_1C + b_2SC + b_3IR + b_4IC + b_5C^2$. SC = salt concentration; IR = hydrated cation radii; IC = cation charge.

Parameter[a]	No. of peptide[b]					
	1	2	3	4	5	6
n	42	47	47	52	67	65
a	−0.67	−1.13	−0.12	−0.78	0.15	0.03
$b_1 \times 10^{-3}$	n.s.[c]	n.s.	−24.2	5.30	−8.81	−7.14
$s_{b1} \times 10^{-3}$			2.78	6.85	1.67	1.79
p.c.			49.67	41.52	40.26	32.07
b_2	n.s.	0.13	n.s.	0.11	n.s.	n.s.
s_{b2}		0.06		0.05		
p.c.		12.20		12.25		
b_3	n.s.	n.s.	n.s.	−0.31	−0.24	−0.29
s_{b3}				0.10	0.08	0.08
p.c.				15.50	6.58	7.84
b_4	0.27	0.32	0.24	0.27	$8.26 \cdot 10^{-2}$	0.12
s_{b4}	0.07	0.06	0.05	0.05	0.03	0.03
p.c.	59.37	31.35	7.48	30.73	5.91	8.34
$b_5 \times 10^{-5}$	3.13	7.36	22.6	n.s.	11.0	12.4
$s_{b5} \times 10^{-5}$	1.21	0.78	3.00		1.78	1.93
p.c.	40.63	56.45	42.85		47.24	51.71
F	13.34	50.77	37.37	33.93	16.71	33.53
r^2	0.7388	0.7759	0.7181	0.7388	0.5148	0.6874
	7	8	9	10	11	12
n	63	52	50	50	27	47
a	−0.18	−0.44	−0.18	−0.67	−1.07	0.37
$b_1 \times 10^{-3}$	−4.74	−8.33	−17.9	−9.71	−11.4	−39.0
$s_{b1} \times 10^{-3}$	1.66	2.57	2.15	1.89	5.26	3.29
p.c.	26.94	12.59	47.58	34.33	28.46	52.29
b_2	n.s.	n.s.	n.s.	n.s.	0.26	n.s.
s_{b2}					0.10	
p.c.					9.73	
b_3	−0.22	n.s.	n.s.	n.s.	n.s.	n.s.
s_{b3}	0.07					
p.c.	7.39					
b_4	0.17	0.17	0.22	0.41	0.44	0.21
s_{b4}	0.03	0.05	0.04	0.04	0.09	0.06
p.c.	14.32	12.59	9.06	20.69	19.34	4.24
$b_5 \times 10^{-5}$	9.51	11.1	17.6	13.9	16.9	35.1
$s_{b5} \times 10^{-5}$	1.74	2.76	2.32	2.04	5.22	3.57
p.c.	51.34	48.30	43.37	44.97	42.47	43.47
F	41.29	12.91	30.18	68.60	16.82	62.04
r^2	0.7368	0.4414	0.6583	0.8141	0.7453	0.8088

TABLE II (*continued*)

Parameter	No. of peptide[b]					
	13	14	15	16	17	18
n	38	66	38	63	25	15
a	1.95	1.20	3.22	2.43	−1.19	−0.78
$b_1 \times 10^{-3}$	−25.7	−49.2	−111	−25.5	−47.4	n.s.
$s_{b1} \times 10^{-3}$	2.56	2.98	7.80	5.07	9.40	
p.c.	60.43	55.07	53.92	50.37	47.37	
b_2	−0.50	0.13	0.23	−0.29	1.39	n.s.
s_{b2}	0.13	0.06	0.07	0.10	0.65	
p.c.	22.55	2.07	2.15	7.87	6.14	
b_3	n.s.	n.s.	n.s.	−0.80	n.s.	n.s.
s_{b3}				0.22		
p.c.				9.75		
b_4	0.36	0.10	0.22	n.s.	0.69	n.s.
s_{b4}	0.13	0.05	0.06		0.14	
p.c.	17.02	1.84	2.20		15.62	
$b_5 \times 10^{-5}$	n.s.	38.7	74.3	17.1	35.9	14.2
$s_{b5} \times 10^{-5}$		3.13	6.69	5.32	10.78	
p.c.		41.01	41.73	32.03	30.87	
F	36.13	114.66	132.31	23.51	33.07	63.84
r^2	0.7559	0.8809	0.9396	0.6145	0.8630	0.8205

[a] Symbols: n = Number of independent observations (sample number); a = intercept (equal to the value of the dependent variable at zero concentration of the independent variables); b_{1-5} = slope values (change in dependent variable caused by a unit change in the independent variable); s_{b1-5} = standard deviations of the corresponding slope values; p.c. = path coefficient (%) = normalized slope values indicating the relative impact of the individual independent variables on the dependent variable independently of their dimension; F = calculated value of the F-test; r^2 = coefficient of determination indicating the ratio of variance explained by the independent variables.

[b] See Table I.

[c] Not significant.

charge and the hydrated ion radii of the cation sometimes, but not always, significantly influenced the retention. However, the path coefficient values (indicators of the impact of the given variable on the dependent variable independently of its dimensions) clearly showed that in most instances the effect of methanol concentration is much higher than that of salts.

The ions (we assume that the salts are more or less dissociated in water–methanol mixtures) occupying the free silanol groups on the silica surface modify the adsorptive capacity of the silica support. This effect prevails even after impregnation which proves indirectly that the paraffin oil does not cover all adsorption sites on the silica surface. We assume that the retention-modifying effect of salts is the result of at least two phenomena. The ions adsorb on the free silanol groups resulting in decreased retention. After saturating the available silanol groups, the non-adsorbed ions interact with the peptides, modifying their distribution between the stationary and the mobile phase (salting-out or salting-in effect). We assume that the ions with higher hydrated ion radii have a lower access to the active sites of the silica surface (smaller retention-decreasing effect), whereas a higher ion charge may result in a better adsorption capacity (enhanced retention-decreasing effect).

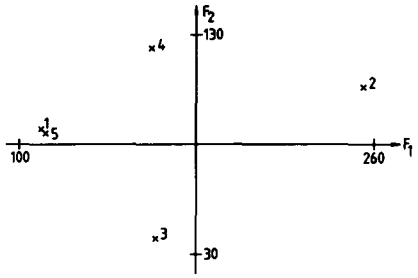
The observed retention behaviour is an interplay of the various interactions outlined above the exact molecular base of which is not well known and needs more investigation. These considerations explain why no linear correlation was found between the retention-modifying capacity of methanol in salt-free and salt-containing systems.

The first and second principal component explained 69.7 and 19.4% of the total variance, respectively, in other words there is a background variable (the first PC) which accounts for 69.7% of the effect of the five variables. Of course, the existence of a principal component does not necessarily mean the existence of a corresponding physico-chemical parameter but only indicates the mathematical possibility of such a parameter.

The two-dimensional non-linear map of PC loadings shows the similarities and dissibilarities of the five variables taking into consideration simultaneously the retention behaviour of all peptides (Fig. 4). The linear and quadratic forms of methanol concentration (points 1 and 5) are very close to each other on the map, indicating the similarity of their action on the retention.

The salt concentration, the ion charge and hydrated ion radii are widely separated on the map, suggesting that each of them separately influences the retention of peptides, that is, each variable is suitable for selectively modifying the retention.

The two-dimensional non-linear map of PC variables shows the clustering of peptides, taking into consideration simultaneously the effect of all chromatographic parameters (Fig. 5). The peptides did not form clusters according to the length of peptide chain or the presence of a bulky side-chain structure. This finding indicates that these molecular characteristics do not influence the salt sensitivity of peptides. The more polar peptides (alkaline peptide 16 and acidic peptide 17) are widely separated from each other and from the bulk of other peptides; however, the acidic peptide 18 does not show any deviating retention behaviour. This finding indicates that the salts



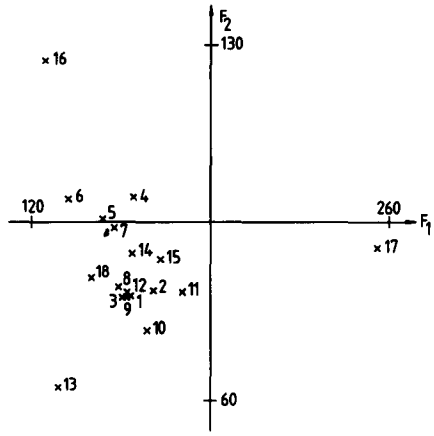


Fig. 4. Two-dimensional non-linear map of PC loadings. No. of iterations, 314; maximum error, $8.76 \cdot 10^{-3}$. 1 = Methanol concentration; 2 = salt concentration; 3 = hydrated cation radii; 4 = cation charge; 5 = square of methanol concentration.

Fig. 5. Two-dimensional non-linear map of PC variables. No. of iterations, 126; maximum error, $1.39 \cdot 10^{-2}$. Numbers refer to peptides in Table I.

have a greater effect on the retention of more polar peptides. This conclusion was supported by the fact that no significant linear correlation was found between the principal components and the lipophilicity of peptides.

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