Use of Principal Component Analysis for the Study of the Influence of Physicochemical Parameters on the Retention of Barbiturates on Porous Graphitized Carbon Column

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Porous graphitized carbon (PGC) has proved to be an excellent tool for the separation of positional- and stereoisomers of steroids, cephalosporin antibiotics and cresols.¹ The retention mechanism of PGC has been extensively discussed. Under non-polar conditions, PGC acted as an electron-pair acceptor of solutes.² Another study indicated the importance of dipole-induced dipole interactions in the retention mechanism.³ The influence of physicochemical parameters of some ring-substituted aniline derivatives on their retention on the PGC column has been studied in aqueous eluent systems. It has been established that the retention on the PGC column of ring-substituted aniline derivatives is mainly governed by the sterical parameters of the substituents.^{4,5}

Multivariate mathematical-statistical methods such as canonical correlation⁶ and cluster analysis (CA)⁷, step-wise regression analysis (SRA)⁸, and principal component analysis (PCA)⁹ have been frequently used to extract maximum information from retention data matrices of considerable dimensions.

The objective of our investigations was to study the retention behavior of some barbituric acid derivatives on a PGC column using ethanol-water mixtures as eluents, to assess the relationship between the physicochemical characteristics and retention parameters of barbituric acid derivatives by means of PCA combined with CA and SRA, and to elucidate the similarities and dissimilarities in the information content of the various calculation methods. The study was motivated by the fact that, to the best of our knowledge the combination of PCA, CA and SRA has not been used so far in the study of molecular structure-retention behavior relationships.

Experimental

The HPLC system was identical to that described in ref. 5. The flow rate was 1.0 ml min⁻¹ and the detection

wavelength was set to 240 nm. Mixtures of ethanol-water were used as eluents; ethanol concentration ranged from 70 to 90% v/v (in steps of 5% v/v). The eluents were not buffered. In agreement with the results of refs. 10 and 11, we found that the column temperature exerts a considerable effect on the retention. The column was thermostated at 22.5±0.5°C in order to reduce the influence of temperature oscillation. The chemical structure of barbituric acid derivatives is depicted in Table 1. They were dissolved in the eluents in 0.05 mg ml⁻¹ concentration. The retention time of each compound in each eluent was determined with three consecutive determinations. The logarithm of the capacity factor, k' was linearly correlated to the ethanol concentration, V (vol%):

$$\log k' = \log k'_0 + bC \quad (1)$$

where $\log k'_0$ and b are constants to be determined by the least square method.

For the determination of the molecular parameters significantly influencing the retention, the intercept and slope values of Eq.(1) were correlated with the physicochemical characteristics of barbituric acid derivatives, using PCA followed by CA and two-dimensional non-linear mapping. The physicochemical parameters included in the calculation were: π =Hansch-Fujita's substituent constant characterizing hydrophobicity;^{12,13} H-Ac and H-Do=indicator variables for proton acceptor and proton donor properties, respectively;¹⁴ M-RE=molar refractivity;¹⁵ F and R =electronic parameters characterizing the inductive and resonance effect, respectively;¹⁶ σ =Hammett's constant, characterizing the electron-withdrawing power of the substituent;¹⁷ Es=Taft's constant, characterizing steric effects of the substituent;¹⁸ B_1 and B_4 =Sterimol width parameters determined by the distance of substituents at their maximum point perpendicular to attachment.^{19,20} These physicochemical parameters were selected because it has been proven that they exert a considerable impact

Table 1 Chemical structure of barbituric acid derivatives

Compound No.	R ₁	R ₂	General structure R ₃	R ₄	X
1	H	H	H	H	O
2	methyl	methyl	H	H	O
3	3-pentyl	methyl	H	H	O
4	methyl	1-methylpentyl	H	H	O
5	ethyl	ethyl	H	H	O
6	ethyl	1-methylbutyl	H	H	O
7	ethyl	3-methylbutyl	H	H	O
8	ethyl	1-methylpropyl	H	H	O
9	ethyl	pentyl	H	H	O
10	butyl	1-methylpropyl	H	H	O
11	butyl	1-methylbutyl	H	H	O
12	butyl	3-methylbutyl	H	H	O
13	ethyl	octyl	H	H	O
14	ethyl	3-dimethyloctyl	H	H	O
15	allyl	<i>i</i> -propyl	H	H	O
16	allyl	<i>i</i> -butyl	H	H	O
17	allyl	1-methylbutyl	H	H	O
18	allyl	1-methylcyclohexenyl	H	H	O
19	allyl	2-cyclopentyl	H	H	O
20	ethyl	1-cyclohexenyl	H	H	O
21	ethyl	ethyl	H	H	S
22	ethyl	1-methylbutyl	H	H	S
23	allyl	1-methylbutyl	H	H	S
24	ethyl	1,3-dimethylbutyl	H	H	S
25	ethyl	phenyl	H	H	O
26	ethyl	ethyl	phenyl	H	O
27	ethyl	ethyl	benzoyl	H	O
28	ethyl	ethyl	benzoyl	benzoyl	O
29	ethyl	ethyl	<i>p</i> -Cl-benzoyl	H	O
30	ethyl	ethyl	<i>p</i> -NO ₂ -benzoyl	H	O
31	ethyl	ethyl	<i>p</i> -NO ₂ -benzoyl	<i>p</i> -NO ₂ -benzoyl	O
32	ethyl	phenyl	phenyl	H	O
33	ethyl	phenyl	benzoyl	methyl	O
34	ethyl	phenyl	<i>p</i> -NH ₂ -benzoyl	methyl	O
35	ethyl	phenyl	<i>o</i> -NO ₂ -benzoyl	methyl	O
36	ethyl	phenyl	<i>p</i> -NO ₂ -benzoyl	methyl	O
37	ethyl	phenyl	<i>m</i> -NO ₂ -benzoyl	methyl	O
38	ethyl	ethyl	<i>p</i> -NO ₂ -benzoyl	methyl	O
39	ethyl	ethyl	benzoyl	H	O
40	methyl	phenyl	benzoyl	methyl	O
41	ethyl	phenyl	benzoyl	H	O
42	ethyl	methyl	H	H	O
43	ethyl	ethyl	propyl	H	O
44	methyl	methyl	methyl	H	O

on chromatographic retention. The calculation of the physicochemical parameters of barbituric acid derivatives was carried out by using the additivity rule. We are well aware that the additivity rule does not take into consideration the position and deviation of the substituent from a theoretical plane of the main ring structure. As we could not find more precise structural characteristics in the literature, we had to use the additivity rule in spite of the fact that this represents a fairly rude approximation of the real situation. The calculated physicochemical parameters of barbituric acid derivatives are compiled in Table 2.

Results and Discussion

The retention of each barbituric acid derivative decreased with increasing concentration of ethanol in the eluent. The parameters of Eq.(1) are listed in Table 3. S_b and $s_{\log k'_0}$ values are the standard deviations of the intercept " $\log k'_0$ ", and slope " b " values. The " r " value is the correlation coefficient indicating the fitness of the equation to the experimental data. In each instance the relationship between $\log k'$ and organic phase concentration was linear and significant at the significance

Table 2 Calculated physicochemical parameters of barbituric acid derivatives

No. of barbituric acid derivatives	π	H-Ac	H-Do	M-RE	F	R	σ	Es	B1	B4
1	0	0	0	0	0	0	0	0	0	0
2	1.12	0	0	11.3	-0.08	-0.26	-0.14	-2.48	3.04	4.08
3	3.23	0	0	29.0	-0.10	-0.21	-0.14	-2.88	3.04	6.98
4	3.79	0	0	35.55	-0.14	-0.34	-0.15	-4.12	4.56	9.02
5	2.04	0	0	20.60	-0.10	-0.20	-0.14	-2.62	3.04	5.94
6	3.71	0	0	35.56	-0.15	-0.31	-0.22	-4.18	4.56	9.43
7	3.71	0	0	35.56	-0.15	-0.31	-0.22	-4.18	4.56	9.43
8	3.13	0	0	30.91	-0.15	-0.31	-0.21	-4.15	4.56	8.42
9	3.69	0	0	34.55	-0.11	-0.18	-0.15	-2.95	3.04	7.91
10	4.24	0	0	40.22	-0.16	-0.29	-0.22	-4.47	4.56	9.87
11	4.82	0	0	44.87	-0.16	-0.29	-0.23	-4.50	4.56	10.88
12	4.82	0	0	44.87	-0.16	-0.29	-0.27	-4.50	4.56	10.88
13	5.28	0	0	49.52	-0.17	-0.26	-0.23	-4.57	4.56	11.81
14	6.40	0	0	60.82	-0.25	-0.52	-0.39	-7.05	7.60	15.89
15	2.93	0	0	29.45	-0.04	-0.08	-0.13	-3.81	3.56	6.94
16	3.49	0	0	34.10	-0.05	-0.08	-0.20	-5.05	5.08	8.98
17	4.09	0	0	39.75	-0.09	-0.19	-0.27	-6.29	6.60	11.02
18	4.47	0	0	46.83	-0.20	-0.09	-0.18	-5.37	5.08	9.31
19	3.54	0	0	36.51	-0.16	0.04	-0.11	-3.85	3.56	7.76
20	3.53	0	0	36.99	-0.22	-0.08	-0.12	-3.34	3.56	6.46
21	3.10	-1	0	26.97	-0.11	0.33	-0.01	-3.14	3.39	5.37
22	4.77	0	1	41.93	-0.16	0.22	-0.09	-3.66	4.91	9.83
23	5.15	0	1	46.12	-0.10	0.34	-0.08	-8.43	6.81	11.42
24	5.33	0	1	47.58	-0.20	0.09	-0.16	-5.94	6.43	11.87
25	2.98	0	0	35.66	0.03	-0.18	-0.01	-5.13	3.22	6.08
26	4.00	0	0	45.96	-0.02	-0.28	-0.08	-6.44	4.74	9.05
27	3.09	1	0	50.93	0.20	-0.04	0.20	-7.22	5.40	11.92
28	4.14	2	0	81.26	0.50	0.12	0.54	-11.82	7.76	17.90
29	3.80	1	0	56.96	0.61	-0.19	0.57	-8.19	7.20	13.72
30	2.81	1	0	58.29	0.87	0.12	0.91	-8.81	7.10	14.36
31	3.58	2	0	95.98	1.84	0.44	1.96	-15.00	11.16	22.78
32	4.94	0	0	61.02	0.11	-0.26	0.05	-8.95	4.92	9.19
33	4.59	1	0	72.64	0.29	-0.15	0.26	-10.97	7.10	14.10
34	3.36	2	1	77.06	0.31	-0.53	0.13	-11.58	8.60	15.94
35	4.31	2	0	79.00	0.96	0.01	0.97	-12.56	8.80	16.54
36	4.31	2	0	79.00	0.96	0.01	0.97	-12.56	8.80	16.54
37	4.31	2	0	79.00	0.96	0.01	0.97	-12.56	8.80	16.54
38	3.37	2	0	63.94	0.84	-0.01	0.84	-9.98	8.62	16.40
39	3.65	1	0	56.58	0.17	-0.17	0.13	-8.39	6.92	13.96
40	4.13	1	0	66.99	0.30	-0.18	0.26	-10.90	7.10	13.17
41	4.03	1	0	65.99	0.33	-0.02	0.33	-9.73	5.58	12.06
42	1.58	0	0	15.95	-0.09	-0.23	-0.14	-2.55	3.04	5.01
43	3.59	0	0	35.56	-0.16	-0.28	-0.21	-4.22	4.56	9.35
44	1.68	0	0	8.65	-0.12	-0.39	-0.21	-3.72	4.56	6.12

Numbers refer to barbituric acid derivatives in Table 1. See text for abbreviations.

level of 95% confirming the applicability of Eq.(1). It should be emphasized that the calculation procedure represented by Eq. (1) is only one of the various extrapolation methods used in RP-HPLC, which often yield different $\log k'_0$ values for the same solute.²¹ The parameters of Eq. (1) are chemically meaningful in the case of homologous series of solutes when only a single

retention-governing solute property varies.²² The experimental data fit well to Eq. (1) the significance level in each case being over 99.9%. These results indicate the sound stability of the chromatographic system and the suitability of Eq. (1) to describe the relationship between the retention of barbiturates and the concentration of ethanol in the eluent.²³ The slope and

Table 3 Parameters of linear correlations between the logarithm of capacity factor and ethanol concentration in the eluent

Compound No.	$\log k'_0$	$s_{\log k'_0}$	$-b \times 10^2$	$s_b \times 10^3$	$r_{\text{calc.}}$
1	90.88	1.6	1.17	1.4	0.9988
2	95.22	2.8	1.08	1.3	0.9920
3	94.72	1.9	1.20	2.1	0.9896
4	110.20	2.1	4.68	1.3	0.9921
5	68.16	1.5	5.37	1.4	0.9978
6	114.17	2.5	6.40	0.2	0.9986
7	117.23	1.9	10.87	1.5	0.9945
8	121.62	2.7	12.85	1.6	0.9975
9	100.03	2.2	11.52	2.0	0.9917
10	98.76	1.7	1.80	0.9	0.9928
11	104.18	2.0	2.72	1.8	0.9893
12	94.17	1.5	2.15	1.4	0.9953
13	100.12	1.8	1.56	1.4	0.9909
14	100.26	1.7	1.85	1.5	0.9908
15	138.49	2.1	20.18	1.8	0.9934
16	118.82	2.2	12.12	2.2	0.9877
17	120.15	2.0	5.81	2.1	0.9897
18	129.26	1.6	15.23	1.9	0.9936
19	109.27	1.9	6.85	0.5	0.9957
20	127.22	1.8	15.27	1.5	0.9938
21	142.89	2.1	25.46	2.2	0.9903
22	106.25	2.2	5.33	1.5	0.9915
23	101.16	2.5	3.57	2.3	0.9894
24	39.51	1.0	13.21	1.4	0.9949
25	110.64	1.2	9.84	0.7	0.9987
26	108.45	2.5	6.86	1.1	0.9987
27	101.16	2.6	1.96	1.2	0.9930
28	103.54	2.2	2.11	1.4	0.9918
29	99.15	1.6	0.85	1.6	0.9946
30	118.56	0.9	1.16	1.8	0.9975
31	110.56	1.7	0.44	1.5	0.9941
32	99.17	1.5	0.64	1.3	0.9964
33	102.49	1.8	0.95	1.9	0.9885
34	108.41	1.4	0.81	1.2	0.9976
35	112.66	1.7	0.77	1.7	0.9928
36	110.63	2.1	0.80	1.6	0.9905
37	111.42	2.2	0.82	1.4	0.9962
38	113.07	1.8	0.56	1.9	0.9901
39	104.54	2.3	2.00	2.1	0.9896
40	101.25	2.5	1.24	2.3	0.9892
41	102.22	2.1	1.19	2.0	0.9901
42	95.47	1.6	1.37	0.3	0.9948
43	109.25	1.9	9.84	0.6	0.9976
44	130.04	1.7	18.98	1.4	0.9971

intercept values considerably differ, indicating that barbituric acid derivatives can be easily separated on the PGC column in an ethanol-water eluent system. The parameters in Table 3 allow the calculation of retention time differences for each pair of barbiturates at each eluent composition.

The results of PCA are compiled in Table 4. The

Table 4 Similarities and dissimilarities between the physicochemical parameters and retention characteristics of barbituric acid derivatives on porous graphitized carbon column (Results of principal component analysis)

Eigenvalue	Variance explained, %	Total variance explained, %		
6.55	54.62	54.62		
1.77	14.72	69.34		
1.31	10.95	80.29		
1.22	10.13	90.42		
Principal component loadings				
	No. of principal component			
Parameter	1	2	3	4
$\log k'_0$	-0.02	0.62	0.55	-0.41
b	-0.54	0.26	0.69	0.02
π	0.39	-0.67	0.42	-0.32
H-Ac	0.91	0.14	-0.21	0.00
H-Do	0.09	-0.61	0.29	0.57
M-RE	0.94	-0.17	0.11	-0.16
F	0.90	0.39	-0.12	0.13
R	0.37	0.27	0.39	0.70
σ	0.87	0.40	-0.08	0.20
Es	-0.96	0.02	-0.06	0.08
B ₁	0.94	-0.12	0.16	-0.11
B ₄	0.94	-0.17	0.10	-0.16

remaining four principal components explain only 10% of the total variance, so they are not included in Table 4. This does not mean that these data are totally ignored, but since their information content is relatively low, they do not require special discussion. They are included, however, in the calculation of both non-linear maps and cluster dendrograms. The overwhelming majority of the information contained in the original data matrix can be described by four background variables. In other words, four theoretical variables are sufficient to describe the relationship between the physicochemical parameters and retention characteristics of barbituric acid derivatives on a porous graphitized carbon column. Unfortunately, PCA does not define these variables as concrete physical or physicochemical entities, but only indicates their mathematical possibility. Steric parameters have high loadings in the first, PC indicating that PC1 represent the steric character of the data matrix. The slope value (b) of Eq. (1) has high loadings in the first and third PC, whereas the $\log k'_0$ value has high loadings in the second and third PC. The fact that both retention characteristics and various physicochemical parameters have high loadings in more than one principal component indicates that the relationship between chromatographic behavior and physicochemical parameters is probably complicated and more than one type of interactive forces are involved in the interaction of barbituric acid derivatives with the surface of PGC.

The two-dimensional non-linear map of principal

component loadings showed that chromatographic parameters form a loose cluster with the E_s value characterizing the steric effect of substituents; however, the E_s value is relatively far from the chromatographic parameters indicating the loose character of the relationship. The calculated hydrophobicity value π is not included in the cluster. These findings show that the retention of barbiturates on PGC column depends not only on molecular hydrophobicity, which has high values in the second and third PC, but also on other physicochemical parameters included in the calculation. These results further suggest that the retention characteristics of PGC differ from those of traditional reversed-phase columns. This observation is in good agreement with the finding of ref. 24. Similar conclusions can be drawn from the cluster dendrogram of principal component loadings, indicating that in this case the information content of both methods is identical and both can be used for the reduction of the dimensionality of the matrix of principal component loadings.

Barbituric acid derivatives with an additional ring structure form a cluster on the two-dimensional nonlinear map of principal component variables. Compound 26 is very near the cluster, only compound 17 with a branching, relatively long alkyl chain is situated between compound 26 and the cluster. Compound 25 is not included in the cluster. This is probably due to the fact that the unique phenyl group is less bulky than the substituents of barbituric acid derivatives in the cluster, resulting in different retention behavior on PGC, where the retention is considerably influenced by steric conditions of the solute molecules. The cluster formation suggests that these compounds may interact differently with the surface of PGC. It may be assumed that the additional ring structures of barbiturates also interact with the hexagonal graphitic substructures on the PGC surface by stacking interactions affected by this retention behavior of these derivatives. It must be stressed that this is only an assumption not supported by other physicochemical measurements.

The results of stepwise regression analysis indicating the impact of individual physicochemical parameters on the formation of PC variables entirely support our previous conclusion drawn from the two-dimensional nonlinear map of PC loadings. Steric parameters have the highest impact on the first PC, indicating again the marked importance of steric conditions on the retention of barbiturates on PGC. Hydrophobicity and electronic parameters greatly influence the other PCs, which explains the considerably lower ratio of variance. This result draws the attention to the fact that data other than steric parameters may only have a small impact on the retention.

Significant linear correlations were found between the $\log k'_0$ and b values of barbituric acid derivatives determined on a PGC column with ethanol organic modifier and those determined with methanol and acetonitrile organic modifiers:

$$\log k'_{0(\text{ethanol})} = 3.57 + (0.95 \pm 0.03) \cdot \log k'_{0(\text{acetonitrile})} \quad (3)$$

$$n=43 \quad r=0.9844$$

$$\log k'_{0(\text{ethanol})} = 14.13 + (0.84 \pm 0.05) \cdot \log k'_{0(\text{methanol})} \quad (4)$$

$$n=39 \quad r=0.9445$$

$$b_{0(\text{ethanol})} = -0.11 + (0.91 \pm 0.01) \cdot b_{0(\text{acetonitrile})} \quad (5)$$

$$n=43 \quad r=0.9960$$

$$b_{0(\text{ethanol})} = -0.10 + (0.86 \pm 0.02) \cdot b_{0(\text{methanol})} \quad (6)$$

$$n=39 \quad r=0.9924$$

The parameters of Eqs. (3) – (6) show that the nature of the organic modifier exerts negligible influence on the retention behavior of barbituric acid derivatives on the PGC column.

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