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Separation of steroidal drugs on porous graphitized carbon column[1]

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Abstract

The retention time of 11 steroidal drugs was determined on a porous graphitized carbon (PGC) column using water–tetrahydrofuran mixtures as eluents. Linear correlations were calculated between the logarithm of the capacity factor (k') and the tetrahydrofuran concentration in the eluent. To find the molecular parameters significantly influencing the retention the intercept and slope values of the above relationship were correlated with the physicochemical characteristics of steroidal drugs using principal component analysis (PCA). Both the slope and intercept values show high differences proving that steroidal drugs can be successfully separated on a PGC column. Five principal components explained $>90\%$ of the total variance. Calculations indicated that both electrostatic interactions and sterical conditions can influence the retention of steroidal drugs on PGC column.

Keywords: Porous graphitized carbon column; Steroid derivatives; Mathematical data analysis

1. Introduction

Porous graphitic carbon support (PGC) has been developed in the last decade [1,2]. The development of this highly pH-stable type of column was motivated by the fact that the application of silica or silica based supports in high performance liquid chromatography (HPLC) is limited by low stability of silica at high pH values [3] and—in the case of reversed-phase silica based supports—by the undesirable electrostatic interactions between the polar substructures of solutes and the free silanol groups not covered by the hydrophobic ligand [4]. PGC column has been successfully used for the separation of diastereoisomers [5], geometric isomers [6], and various bioactive compounds such as taxol [7], neuroleptic agents [8], antihypoxia drugs [9], etc. The various practical applications of PGC column for the separation of a wide variety of compounds have recently been reviewed [10]. The effect of various physicochemical parameters of solutes on their retention behaviour on PGC columns has been studied in detail and the importance of steric and electronic

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interactions between solutes and stationary phase has been emphasized [11].

The most recent results in the study of the relationship between solute structure and retention as well as the retention mechanism of PGC have been compiled and critically reviewed [12]. The majority of studies involved the interaction between the surface of the PGC and the solutes. The number of papers studying the special interactions between the constituents of the mobile phase and solutes is surprisingly low. According to our knowledge only the effect of methanol, ethanol, 2-propanol, tetrahydrofuran, dioxane and 2-ethoxyethanol as organic modifiers was studied in more detail [13].

As the logarithm of the capacity factor generally depends linearly on the concentration of the stronger component in the eluent, this relationship has been frequently used for the determination of the retention capacity (intercept of the correlation) and for the calculation of the specific surface area of solutes in contact with the support (slope of the correlation [14]). It was assumed that the significant correlation between the intercept and slope values of a set of solutes indicates their structural homogeneity [15].

Many HPLC methods have been developed for the separation of bioactive steroids [16] using ion-pair HPLC [17], porous graphitic carbon column [18], cyclodextrin bonded phase [19] etc. However, the relationship between the retention parameters of steroids and the physicochemical parameters has not been elucidated in detail.

The objectives of the study were the determination of the retention behaviour of some steroidal drugs on PGC column using tetrahydrofuran as an organic modifier, to find the physicochemical parameters of solutes influencing the retention, to evaluate the retention data with principal component analysis followed nonlinear mapping, cluster analysis and varimax rotation and to compare the results of the various mathematical statistical methods.

The study was motivated by the assumption that the mixed retention mechanism of PGC makes possible the separation of steroidal drugs showing similar retention behaviour on traditional octadecylsilica column and the elucidation of the relationship between retention characteristics and physicochemical parameters may promote the rational design of an adequate HPLC system and its rapid optimization for the separation of any kind of steroidal drugs.

2. Experimental

The PGC column (Shandon Hypercarb 100 × 4.7 mm i.d. particle diameter 7 m) was purchased from Shandon Scientific (UK). The HPLC system consisted of a Liquopump model 312 (Labor MIM, Budapest, Hungary) pump, a Cecil CE-212 variable wavelength UV detector (Cecil Instruments, Cambridge, UK), a Valco injector (Valco, Houston, TX) with a 20 μl sample loop and a Waters 740 integrator (Water-Millipore, Milford, MA). Water–tetrahydrofuran mixtures were used as eluents, the tetrahydrofuran concentration varied between 30–75 vol.% in steps of 2.5 vol.%. The use of this very wide range of tetrahydrofuran concentration was motivated by the highly different hydrophobicity of the steroidal drugs. Flow-rate was 0.8 ml min^{-1} and the detection wavelength was set to 240 nm. The IUPAC names of steroidal drugs (Gift of Professor S. Görög, Gedeon Richter, Budapest, Hungary) are compiled in Table 1. The drugs were dissolved in the eluents at a concentration of 0.05 mg ml^{-1}. The column was not thermostated and each determination was run at room temperature. The retention time of each compound in each eluent was determined with three consecutive determinations. Linear correlations were calculated between the $\log k'$ value of drugs and the tetrahydrofuran concentration in the eluent:

$$\log k' = \log k'_0 + b \cdot C \quad (1)$$

where: $\log k'$, logarithm of capacity factor; $\log k'_0$, logarithm of capacity factor extrapolated to zero tetrahydrofuran concentration in the eluent (related to the retention capacity of the solute); b, change of $\log k'$ value caused by unit change (1 vol.%) of tetrahydrofuran concentration (related to the specific surface area of solutes in contact with the surface of PGC); and C, tetrahydrofuran concentration (vol.%).

In order to test the validity of the hypothesis that in the case of homologous series of solutes the slope and intercept values in Eq. (1) are strongly intercorrelated [20] linear correlation was calculated between the two chromatographic parameters.

Principal component analysis was used to find the physicochemical parameters of drugs influencing their retention behavior on PGC [21]. The parameters of Eq. (1) (slope $= \log k'_0$ and intercept $= b$ values) and various physicochemical characteristics of drugs (altogether 12 variables) were considered as variables and the steroidal drugs were the observations. The physicochemical parameters included in the calculation were: π, Hansch–Fujita's substituent constant characterizing hydrophobicity; H-Do, indicator variable for proton donor properties; M-RE, molar refractivity; F and R, Swain–Lupton's electronic parameters characterizing the inductive and resonance effect, respectively; σ, Hammett's constant characterizing the electron-withdrawing power of the substituent; Es, Taft's constant characterizing steric effects of the substituent; and B_1 and B_4, sterimol width parameters. The limit of the variance explained was set to 99.9%. To facilitate the evaluation of the results of PCA both two-dimensional nonlinear mapping [22] and cluster analysis was carried out on the PC loadings and variables. Varimax rotation around two axes [23] were carried out only on the PC loadings. To elucidate the influence of PCA on the data evaluation, cluster analysis was also applied to the original data matrix. Cluster analysis and nonlinear mapping technique are theoretically similar: both methods calculate and visualize the relative distances between the members of data matrix (in our cases: physicochemical and chromatographic parameters of drugs). To compare the information content of non-linear mapping and varimax rotation techniques linear correlations were calculated between their corresponding coordinates:

$$Y_{1-2} = a + b \cdot X_{1-2} \quad (2)$$

Y_{1-2} = first and second coordinates of varimax rotation; X_{1-2} = first and second coordinates of the two-dimensional non-linear map.

Table 1
IUPAC names of steroidal drugs

No of drug	IUPAC name
1	17-Hydroxy-19-norpregn-4-en-3,20-dione
2	11β,17,21-Trihydroxy-19-norpregn-4-en-3,20-dione
3	11β,16α,17,21-Tetrahydroxy-19-norpregn-1,4-dien-3,20-dione-16,17-acetonide
4	17-Hydroxy-19-norpregn-4-en-20-yn-3-one
5	17,21-Dihydroxy-19-norpregn-4-en-3,20-dione
6	7-(Acetylthio)-17-hydroxy-3-oxo-pregn-4-ene-21-carboxylic acid τ-lactone
7	17α-Pregna-2,4-dien-20-yno[2,3-d]isoxazol-17-ol
8	13-Ethyl-17-hydroxy-18,19-donirpregn-4-en-20-yn-3-one
9	Estra-1,3,5(10)-triene-3,17-diol
10	19-Nor-17α-pregna-1,3,5(10)-trien-20-yne-3,17-diacetate
11	19-Nor-17α-pregna-1,3,5(10)-trien-20-yne-3,17-diol

Table 2
Parameters of linear correlations between the logarithm of capacity factor and tetrahydrofuran concentration in the eluent

Compound no.	$\log k'_0$	$-b \cdot 10^2$	$s_b \cdot 10^3$	$r_{calc.}$
1	2.74	4.00	2.03	0.9962
2	1.29	2.31	4.77	0.8922
3	2.50	5.51	4.16	0.9806
4	2.22	4.47	2.46	0.9957
5	1.20	2.99	1.35	0.9919
6	2.27	3.83	2.52	0.9935
7	2.81	4.83	6.27	0.9680
8	2.41	4.49	3.24	0.9847
9	2.98	3.91	3.41	0.9888
10	2.17	3.87	2.16	0.9878
11	3.21	5.75	3.59	0.9885

$\log k' = \log k'_0 + b \cdot C$.
s_b, standard deviation of the slope b value.

3. Results and discussion

The parameters of Eq. (1) are compiled in Table 2. In each instance the relationship between the logarithm of capacity factor and the tetrahydrofuran concentration in the eluent was linear. The value of regression coefficient was high prov-

Table 3
Relationship between retention characteristics and physicochemical parameters for steroidal drugs

Eigen values	Total variance explained (%)				
3.59	29.89				
2.78	53.08				
2.40	73.08				
1.26	83.61				
0.84	90.59				
	Principal component loadings No of principal components				
Parameter	1	2	3	4	5
π	−0.45	−0.23	0.66	0.41	0.16
H-Ac	0.87	−0.02	0.06	−0.19	0.30
H-Do	0.61	−0.64	0.19	−0.07	0.40
M-RE	0.63	0.69	0.19	0.06	−0.09
F	−0.25	0.15	0.19	0.84	0.12
R	0.01	0.74	0.61	−0.17	−0.17
σ	−0.23	0.59	0.60	−0.26	−0.01
Es	−0.40	0.49	0.20	−0.09	0.64
B_1	0.88	0.42	−0.10	0.15	0.09
B_4	0.76	0.20	−0.11	0.45	−0.18
$\log k'_0$	−0.46	0.47	−0.67	−0.02	0.02
b	−0.16	0.52	−0.77	0.11	0.26

Results of principal component analysis

ing the applicability of Eq. (1). Both the slope and intercept values show high differences proving that this set of steroidal drugs can be successfully separated by PGC by using water–terahydrofuran eluent system.

Significant linear correlation was found between the chromatographic parameters calculated by Eq. (1):

$$\log k'_0 = 0.37 + (0.48 \pm 0.11) \cdot b$$

$$r_{\text{calc.}} = 0.8321 \quad r_{99\%} = 0.7348$$

This result indicates that the steroidal drugs behave as a homologous series of solutes even on PGC column. Although the correlation is significant it is not strong enough to substitute the two parameters with each other in any quantitative structure-retention relationship calculations.

The results of PCA are summarized in Table 3. Five principal components explain $>90\%$ of the total variance. This result indicates that the 12 physicochemical and chromatographic parameters can be substituted by five background variables with only 10% loss of information. Unfortunately, PCA does not prove the existence of such background variables as concrete physicochemical entities but only indicates its mathematical possibility. The chromatographic parameters together with the majority of physicochemical characteristics of drugs have high loadings in more than one principal components. The loading of hydrophobicity parameter (π) is the highest in the third principal component which explains only 20% of the total variance. This result indicates that the retention of solutes on PGC column is influenced to a lesser extent by the molecular hydrophobicity than on traditional reversed-phase columns, although the eluent systems are typical reversed-phase ones. The results of PCA suggest that other physicochemical characteristics than hydrophobicity can also influence the solute retention on PGC column. Thus, both sterical and polarity parameters have high loadings in the first three PCs. These results indicate mixed retention

mechanism: the planar ring structures of steroidal drugs can interact with the hexagonal carbon ring on the PGC surface while the hydrophobic substructures of steroidal drugs can form hydrogen bonds with the polar centers of the PGC.

Chromatographic parameters do not form distinct clusters with any of the physicochemical parameters included in the calculation on the two-dimensional nonlinear map of principal component loadings (Fig. 1). This finding suggests again that more than one physicochemical parameter is involved in the interaction of steroidal drugs with the surface of PGC support indicating mixed retention mechanism. Cluster analysis carried out on the original data matrix (Fig. 2A) and on the principal component loadings (Fig. 2B) show marked differences. This result may be due to the fact that PCA slightly modifies the original data matrix by extracting maximal information from it.

Solutes do not form separate cluster on the two-dimensional nonlinear map of principal component variables (Fig. 3) neither according to the degree of saturation of the ring structure nor according to the character and position of the substituents. This fact supports our previous assumption that electronic (degree of saturation of the ring structure) and hydrophobic interactive forces (character of substituents) and steric conditions (position of substituents) and are equally involved in the retention of steroidal drugs on PGC column. Clusters of solutes before and after PCA support our previous conclusions that PCA exerts a con-siderable impact on the clustering of solutes (data not shown).

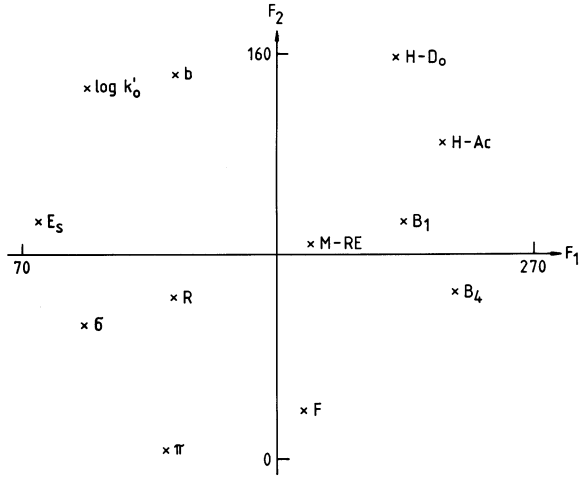

Fig. 1. Relationship between the various physicochemical parameters of steroidal drugs and their retention behaviour on porous graphitized carbon column. Two-dimensional nonlinear map of principal component loadings. Number of iterations: 124, maximum error: 6.09×10^{-2}. For symbols see Section 2.

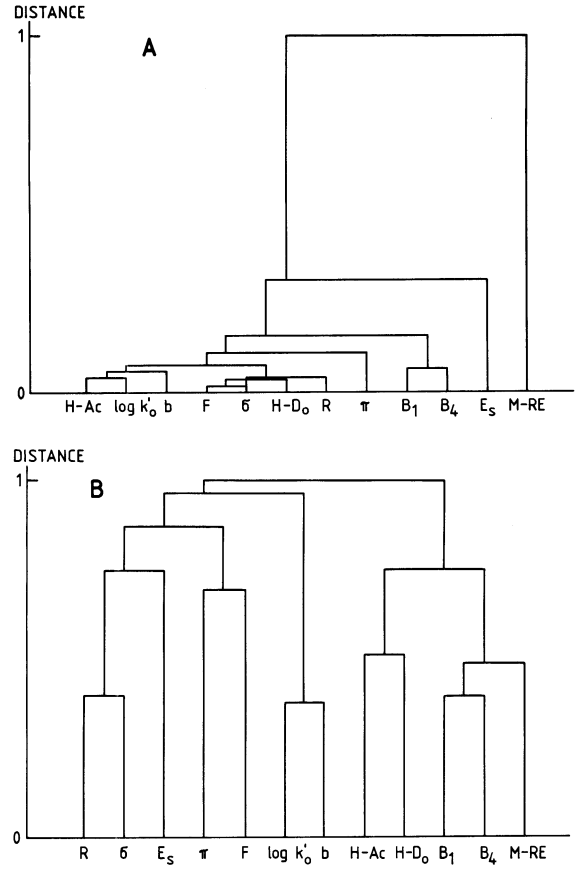

Fig. 2. Relationship between the various physicochemical parameters of steroidal drugs and their retention behaviour on porous graphitized carbon column. Cluster dendogram of the original data matrix (A) and principal component loadings (B). For symbols see Section 2.

Significant linear correlation was found between the first coordinates of nonlinear mapping and varimax rotation ($r_{calc.} = 0.7034$; $r_{98\%} = 0.6581$) but the second coordinates were not correlated. This result draws the attention to the fact that the in-formation content of the various multivariate mathematical-statistical methods may be

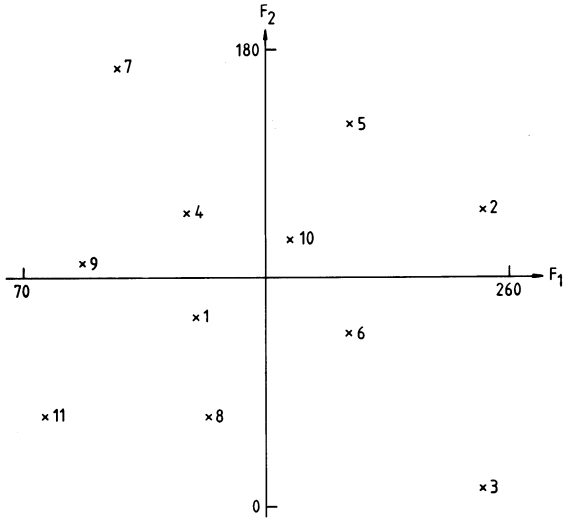

Fig. 3. Distribution of steroidal drugs according to their retention behaviour on porous graphitized carbon column. Two-dimensional non-linear map of principal component variables. Number of iterations: 122, maximum error: 4.67×10^{-2}. Numbers refer to steroidal drugs in Table 1.

different therefore the application of more than one method for the evaluation of the same data matrix is strongly advocated.

It can be concluded from the data that the mixed retention mechanism of PGC offers an interesting alternative for the separation of steroidal drugs which cannot be easily separated on traditional reversed-phase column.

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References

[1] M.T. Gilbert, J.H. Knox, B. Kaur, Chromatographia 16 (1982) 138.
[2] J.H. Knox, B. Kaur, G.R. Millward, J. Chromatogr. 352 (1986) 3.
[3] A. Berthod, J. Chromatogr. 549 (1991) 1.
[4] H. Tayar, H. Waterbend, B. Testa, J. Chromatogr. 305 (1985) 320.
[5] B. Kaur, LC-GC 3 (1990) 41.
[6] D. Berek, J. Novák, Chromatographia 30 (1990) 582.
[7] E. Forgács, Chromatographia 39 (1994) 740.
[8] G. Gu, C.K. Lim, J. Chromatogr. 515 (1990) 183.
[9] J.C. Berridge, J. Chromatogr. 449 (1988) 317.
[10] P. Ross, J.H. Knox, in: P.R. Brown, E. Grushka (Eds.), Advances in Chromatography, vol. 37, Marcel Dekker, New York, 1997, p. 121.
[11] E. Forgács, T. Cserháti, B. Bordás, Anal. Chim. Acta 279 (1993) 115.
[12] J.H. Knox, P. Ross, in: P.R. Brown, E. Grushka (Eds.), Advances in Chromatography, vol. 37, Marcel Dekker, New York, 1997, p. 73.
[13] M. Balcan, T. Cserháti, E. Forgács, Anal. Lett. 30 (1997) 883.
[14] C. Horváth, W. Melander, I. Molnár, J. Chromatogr. 125 (1976) 129.
[15] K. Valkó, J. Liq. Chromatogr. 7 (1984) 1405.
[16] A. Lagana, A. Marino, J. Chromatogr. 588 (1991) 89.
[17] V. Legrand-Defretin, C. Juste, R. Henry, T. Corting, Lipids 26 (1991) 578.
[18] B.J. Clark, in: S. Görög, E. Heftmann (Eds.), Adv. Steroid Anal. '90. Proceedings Symposium Anal. Steroids, 4th 1990, AkadKiadó, Budapest, 1991, p. 129.
[19] A.-H. Ahmed, S.M. El-Gizany, N.M. Omar, Anal. Lett. 24 (1991) 2207.
[20] T. Cserháti, Chromatographia 18 (1984) 318.
[21] K.V. Mardia, J.T. Kent, J.M. Bibby, Multivariate Analysis, Academic Press, New York, 1979, p. 90.
[22] J.W. Sammon Jr, IEEE Trans Comput. C18 (1969) 401.
[23] J.R. Llinas, J.M. Ruiz, in: G. Vernin (Ed.), Computer Aids to Chemistry, Horwood, Chichester, 1986, p. 47.