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PREDICTION OF THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC RETENTION BEHAVIOUR OF SOME BENZODIAZEPINE DERIVATIVES BY THIN-LAYER CHROMATOGRAPHY

KLÁRA VALKÓ*
Central Research Institute for Chemistry, Hungarian Academy of Sciences, P.O. Box 17, H-1525 Budapest (Hungary)
SAROLTA OLAJOS
National Institute for Nervous and Mental Diseases, Laboratory of Pharmacokinetics, Budapest (Hungary)
and
TIBOR CSERHÁTI
Central Research Institute for Chemistry, Hungarian Academy of Sciences, P.O. Box 17, H-1525 Budapest (Hungary)

SUMMARY

The reversed-phase retention behaviour of eighteen benzodiazepine derivatives was studied using high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC). In each instance the retention decreased with increasing concentration of the organic mobile phase in the eluent, that is, no anomalous effect was observed. In reversed-phase TLC the R_M value of benzodiazepine derivatives depended linearly on the organic phase concentration and logarithmically on the buffer concentration in the eluent. A highly significant correlation was found between the HPLC log k_0 value and the reversed-phase TLC parameters, suggesting that TLC can be used for predicting the HPLC retention behaviour of benzodiazepine derivatives. As the TLC parameters explained about 75% of the total variance, the predictive power of TLC is limited.

INTRODUCTION

Benzodiazepine derivatives (BZDs) have found growing acceptance and application in the modern therapeutic practice[1]. BZDs are of considerable importance, having hypnotic, tranquillizing and anticonvulsant properties. As the range of BZDs available has expanded rapidly over the last 10 years, many chromatographic methods have been developed for their separation and identification. The early separations were based on adsorption thin-layer chromatography[2,3] (TLC) or pH-gradient TLC[4]. Earlier high-performance liquid chromatographic (HPLC) methods have been reviewed[5]. Both adsorption[6] and reversed-phase methods[7] have been used in the HPLC separation of BZDs, and gas chromatography (GC) has also been frequently applied[8,9]. The performances of the various chromatographic methods (TLC, GC and HPLC) have been compared[10].

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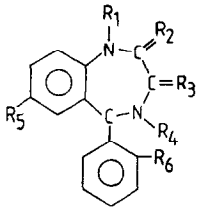
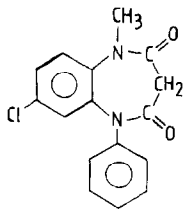
TABLE I

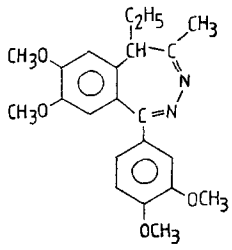
STRUCTURES OF BENZODIAZEPINE DERIVATIVES



Compound	*Common name*	R_1	R_2	R_3	R_4	R_5	R_6
1	7-Aminonitrazepam	H	=O	H	H	NH_2	H
2	Bromazepam	H	=O	H	H	Br	[a]
3	Uxepam	CH_3	=O	H	$CONH_2$	Cl	H
4	Oxazepam	H	=O	OH	H	H	H
5	Lorazepam	H	=O	H	H	Cl	Cl
6	Nitrazepam	H	=O	H	H	NO_2	H
7	Clonazepam	H	=O	H	H	NO_2	Cl
8	Chlordiazepoxide	H	$NHCH_3$	H	O	Cl	H
9	Alprazolam	$R_1C(CH_3)=$	$NN=R_2$	H	H	Cl	H
10	Desmethyldiazepam	H	=O	H	H	Cl	H
11	Flunitrazepam	CH_3	=O	H	H	NO_2	F
12	Clorazepat	H	$(OH)_2$	COOH	H	Cl	H
13	Diazepam	CH_3	=O	H	H	Cl	H
14	Midazolam	$R_1C(CH_3)=$	NCH_2R_2	H	H	Cl	F
15	Medazepam	CH_3	H	H	H	Cl	H
16	Prazepam	CH_2-cP[b]	=O	H	H	Cl	H



17 Clobazam



18 Tofisopam

[a] 2-Pyridinyl instead of phenyl group.

[b] cP = cyclopropyl.

The biological activity of a molecule is controlled by many factors, one of the most important being its lipophilicity, because penetration of the membranes of target organisms is governed by molecular lipophilicity[11,12]. Lipophilicity can be determined by the traditional method of partition between water and *n*-octanol[13], by HPLC[14,15] and by reversed-phase TLC (RP-TLC)[16]. The use of GC methods for determining lipophilicity has its limitations[17,18] and the results have sometimes been contradictory[19]. Chromatographic methods have some advantages: they are rapid and relatively simple, require only small amount of the compounds and the compounds need not to be very pure. When a compound contains one or more dissociable polar substituents, the pH of the eluent[20,21] and the ionic strength[22–24] modify the lipophilicity. As chromatographic retention data are used extensively in quantitative structure–activity relationship (QSAR) studies[25], comparison of the performances of various chromatographic techniques to determine lipophilicity is of great practical and theoretical importance.

The objectives of this work were to determine the lipophilicity of some benzodiazepine derivatives, to compare the lipophilicity values determined by HPLC and RP-TLC and to test the predictive power of RP-TLC for the HPLC retention behaviour of BZDs[26].

EXPERIMENTAL

The structures of the BZDs are given in Table I. The compounds were purchased from Hoffman-La Roche (Basle, Switzerland) (compounds 1, 2, 11 and 14), Gedeon Richter (Budapest, Hungary) (compounds 3, 4, 6, 10, 13 and 15), Wyeth Laboratories (Princetown, NJ, U.S.A.) (compound 5), VEB Arzneimittelwerk (Jena, G.D.R.) (compound 7), POLFA Pharmaceutical Works (Yelenia Gora, Poland) (compound 8), Upjohn Pharmaceutical Works (Kalamazoo, MI, U.S.A.) (compound 9), Mack Chemische Pharmazeutische Fabrik (Illertissen, F.R.G.) (compound 12), Gödecke (Augsburg, F.R.G.) (compound 16), Hoechst (Frankfurt, F.R.G.) (compound 17) and Egis Pharmaceutical Works, (Budapest, Hungary) (compound 18).

The HPLC system consisted of a Model 750 pump (Micromeritics, Norcross, GA, U.S.A.), Model OE-308 20-μl injector (Labor-MIM, Budapest, Hungary), a LiChrosorb RP-C18 column (250 × 4.6 mm I.D.) (Merck, Darmstadt, F.R.G.), a Model OE-308 variable-wavelength UV detector (Labor-MIM and a Type OH-850 recorder (Radelkis, Budapest, Hungary). The separations were carried out at room temperature, with detection at 230 nm. The compounds were dissolved in methanol to give a 1 mg/ml stock solution, which was then diluted with the eluent in a 1:19 (v/v) ratio. The dead volume was determined with 0.5 m*M* sodium nitrate solution. The retention times were determined with acetonitrile–0.06 *M* KH_2PO_4 (pH 4.8) eluent mixtures. The acetonitrile concentration was varied from 30 to 70 vol.-% in steps of 5%. The retention parameters of the BZDs were also determined in 0.15 *M* Sörensen buffer[27] (pH 7.4) at 40 and 50 vol.-% acetonitrile concentrations. Five independent determinations were performed with each eluent system.

The log k' values, measured with KH_2PO_4 buffer, were extrapolated to zero acetonitrile concentration separately for each BZD:

$$\log k' = \log k_0 + b \cdot C \quad (1)$$

where $\log k'$ is the actual $\log k'$ value of a BZD determined at C vol.-% acetonitrile concentration, $\log k_0$ is the $\log k'$ value of a compound extrapolated to zero acetonitrile concentration, b is the decrease in the $\log k'$ value caused by a 1% increase in the acetronitrile concentration and C (vol.-%) is the acetonitrile concentration.

For reversed-phase TLC at pH 4.8, Silcoplat F_{254} plates (Labor-MIM) were impregnated with paraffin oil, as previously described[28]. The stock solution for the HPLC experiments was applied; 3 μl of each solution were spotted on the plates. The eluent contained from 0 to 32.5 vol.-% of acetonitrile in steps of 2.5% and 6, 12, 30, 60 and 90 mM KH_2PO_4 solution. After development, the plates were dried at 105°C and the BZDs were detected by their UV absorption spectra. As it was previously established that the mobility of various buffers in RP-TLC may deviate from that of the eluent[29], the phosphate front was detected by use of the ammonium molybdate–tin(II)chloride reagent[30]. All experiments were performed in quadruplicate. The R_M values were calculated spearately for each individual spot and each eluent. It was assumed that the buffer and acetonitrile concentrations of the eluent may simultaneously influence the R_M value. Moreover, the exact type of correlation (linear or logarithmic) between independent (acetonitrile and buffer concentrations) and dependent (R_M value) variables was not previously established. We used stepwise regression analysis to select the independent variables influencing significantly the R_M value[31]. The R_M values were taken as dependent variables and the linear and logarithmic forms of acetonitrile and buffer concentrations (a total of four variables) as independent variables.

The significance level of accepted variables was set at 95%. This calculation allows the separation and determination of the relative effects of the acetonitrile and buffer concentrations on the retention behaviour of the BZDs, which in our case cannot be determined experimentally.

To study the effect of various organic modifiers on the RP-TLC retention of BZDs, their R_M values were determined at 40 and 50 vol.-% acetonitrile and methanol concentrations, the aqueous phase being Sörensen buffer (pH 7.4).

To assess the predictive power of RP-TLC for HPLC, two different methods were applied. The HPLC retention parameters of eqn. 1. were correlated with the results of the stepwise regression analysis described above. The $\log k_0$ and slope (b) values were separately taken as dependent variables. The R_{M0} value and the regression coefficients of the independent variables significantly influencing the RP-TLC retention of BZDs were taken as independent variables. Referring to the previous considerations, their linear, quadratic and logarithmic (if mathematically possible) forms were included in the calculations. The stepwise regression analysis was applied in this case under the same conditions as before.

The retention data obtained in Sörensen buffer (two HPLC and four RP-TLC retention parameters) were compared by principal component analysis (PCA)[32]. The data matrix consisted of the chromatographic parameters of the BZDs. The sum of variance explained was set at 99%. The non-linear map of PCA loadings and variables was also calculated[33].

RESULTS AND DISCUSSION

The dependence of the retention of some BZDs on the acetonitrile concentration in the eluent is shown in Fig. 1. In each instance the correlation follows the

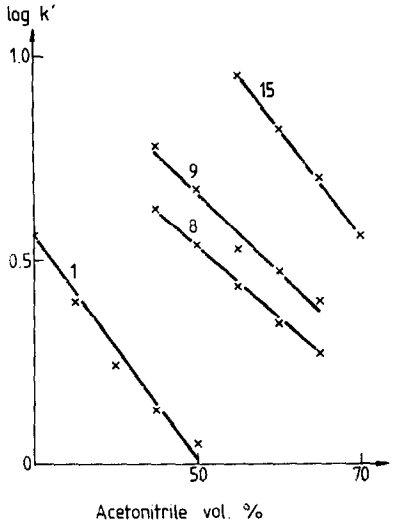


Fig. 1. Dependence of the HPLC retention of BZDs on the acetonitrile concentration in the eluent. Numbers refer BZDs in Table I.

general rule that the retention decreases logarithmically with increasing concentration of organic modifier. The parameters of eqn. 1 are given in Table II. The equation agrees well with the experimental data, the significance level in each instance being

TABLE II

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC RETENTION CHARACTERISTICS OF BENZODIAZEPINE DERIVATIVES: PARAMETERS OF EQN. 1

Numbers refer to BZDs in Table I.

Compound	*Log k_o*		*Slope · 10^3*	
	Mean	*Standard deviation*	*Mean*	*Standard deviation*
1	1.186	0.103	−23.07	2.41
2	1.231	0.038	−16.79	0.83
3	1.807	0.022	−29.10	0.47
4	1.802	0.090	−28.84	1.97
5	1.746	0.038	−27.00	0.76
6	1.479	0.020	−20.76	0.37
7	1.754	0.103	−25.47	1.95
8	1.448	0.042	−18.31	0.75
9	1.628	0.106	−19.26	1.91
10	1.699	0.032	−22.43	0.58
11	1.806	0.026	−23.97	0.43
12	1.618	0.054	−20.95	0.90
13	1.849	0.042	−22.01	0.70
14	1.514	0.056	−20.41	2.79
15	2.376	0.085	−25.86	1.30
16	2.328	0.094	−26.59	1.45
17	1.736	0.035	−22.64	0.59
18	1.799	0.059	−22.11	0.94

higher than 99.9% (the lowest regression coefficient was 0.9860). This result indicates that the linear approximation describes well the dependence of the log k' values of the BZDs on the acetonitrile concentration in the concentration range applied.

The coefficient of variation between the parallel determinations in RP-TLC never exceeded 6%. The mobility of the phosphate front in the RP-TLC experiments was near that of the eluent; the mean R_F value was 0.95. As the R_F value did not change systematically with either the acetonitrile or buffer concentration, we did not correct our retention data for the different mobilities of the phosphate front[34].

The dependence of the R_M value of some BZDs on the buffer concentration in the eluent is shown in Fig. 2. The data show that the dependence is markedly non-linear. The change is high at lower buffer concentration range and levels out at higher concentrations. This phenomenon can be explained by the assumption that the paraffin oil does not cover the active adsorption centers of the silica surface entirely. The free silanol groups also influence the retention; in our case they increase it. As the buffer is in a more or less dissociated form, its ions may be adsorbed on the silanol groups not covered by the impregnating agent. This adsorption results in a lower retention capacity.

The concentration dependence is of saturation character, because the number of active silanol groups is limited and decreases non-linearly with increasing concentration of free ions[35]. We are well aware that our data can also be explained by the salting-in effect[36]. However, taking into consideration the highly lipophilic character of BZDs this is not probable.

The parameters of the equations describing the dependence of the R_M values of BZDs on the acetonitrile and buffer concentrations are given in Table III. The blank entries in Table III are due to the fact that with compounds 1, 6, 7, 9, 11, 13 and 18

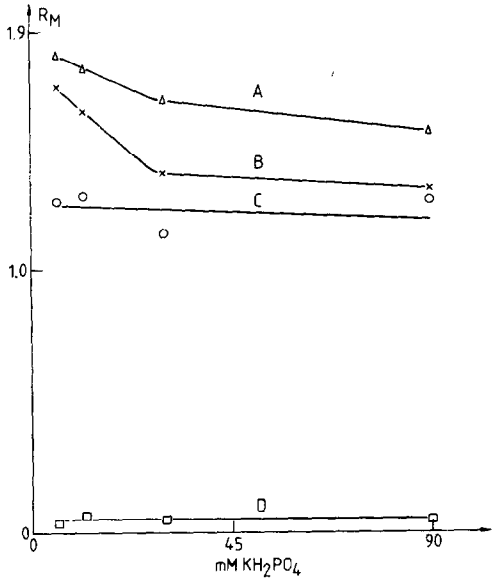


Fig. 2. Dependence of R_M values of some BZDs on the KH_2PO_4 concentration in the eluent at 25 vol.-% acetonitrile concentration. A = medazepam (compound 15); B = midasolam (compound 14); C = diazepam (compound 13); D = 7-aminonitrazepam (compound 1).

only the acetonitrile concentration influenced significantly the retention of BZDs. Therefore, the beta weights, b_2, and the standard deviation of the b_2 value are zero. In these instances, the beta weights of b_1 are equal to r^2. The equations fit the experimental data well; the significance level of the correlation in each instance was over 99.9% (see F values). The change in the independent variables accounts for about 91.75 and 98.40% of the change in the R_M value (see r^2 values). Except for compound 1, which is a metabolite, each compound has considerable lipophilicity (see R_{MO}

TABLE III

DEPENCE OF R_M VALUES OF BENZODIAZEPINE DERIVATIVES ON THE ACETONITRILE (C_1) AND BUFFER (C_2) CONCENTRATIONS IN THE ELUENT

Results of stepwise regression analysis. $R_M = R_{MO} + b_1C_1 + b_2\log C_2$. Numbers refer to BZDs in Table I.

Parameter	*Compound*					
	1	*2*	*3*	*4*	*5*	*6*
Sample number	23	20	18	20	18	19
R_{MO}	1.41	2.68	2.85	2.68	3.16	2.30
s	0.13	0.08	0.08	0.09	0.08	0.07
b_1	−5.30	−7.32	−7.37	−6.70	−7.92	−6.18
s	0.28	0.34	0.46	0.42	0.51	0.24
Beta weight		−1.08	−1.08	−1.09	−1.15	
b_2		−15.38	−12.94	−16.25	−23.38	
s		5.67	5.92	6.99	6.55	
Beta weight		−0.14	−0.15	−0.16	−0.26	
r^2	0.9451	0.9708	0.9666	0.9596	0.9599	0.9741
F		378.5	217.1	202.0	179.7	
	7	*8*	*9*	*10*	*11*	*12*
Sample number	19	18	17	18	18	18
R_{MO}	2.51	3.28	3.46	3.45	2.83	3.26
s	0.08	0.07	0.09	0.08	0.09	0.06
b_1	−6.67	−8.19	−8.89	−8.74	−7.53	−8.19
s	0.30	0.44	0.45	0.51	0.40	0.39
Beta weight		−1.11		−1.08		−1.08
b_2		−18.01		−15.10		−13.33
s		5.64		6.55		5.01
Beta weight		−0.19		−0.15		−0.14
r^2	0.9675	0.9740	0.9625	0.9709	0.9559	0.9806
F		281.0		250.2		379.1
	13	*14*	*15*	*16*	*17*	*18*
Sample number	17	15	16	14	19	17
R_{MO}	3.36	4.82	4.27	4.10	3.25	3.62
s	0.09	0.08	0-10	0.11	0.07	0.10
b_1	−8.44	−11.21	−8.67	−9.80	−8.19	−9.69
s	0.45	0.68	0.73	0.85	0.34	0.50
Beta weight		−1.26	−1.24		−1.08	
b_2		−38.94	−35.08		−13.82	
s		6.48	8.00		5.01	
Beta weight		−0.46	−0.46		−0.12	
r^2	0.9587	0.9656	0.9319	0.9175	0.9840	0.9613
F		168.6	88.9		490.9	

values), which makes it probable that they bind preferably to the lipophilic membrane substructures and/or to the hydrophobic core of proteins. The logarithmic form of the acetonitrile and the linear form of the buffer concentration do not influence the retention of BZDs significantly, *i.e.*, the lipophilicity depends linearly on the concentration of the organic modifier and, with some derivatives, logarithmically on the buffer concentration. The increase in both the acetonitrile and buffer concentration decreases the retention (see b_1 and b_2 values). The fact that the retention of some derivatives did not depend significantly on the buffer concentration in the eluent does not prove that the retention of these compounds is not influenced by the buffer concentration. This finding only indicates that in these instances the impact of the buffer concentration on the retention is probably low. Therefore, it is below the detection limit of our method. The relative importance of the two independent variables differs considerably (see beta weights). The impact of the acetonitrile concentration is 3–9 times higher than that of the buffer concentration. This result indicates that in the determination of the retention of BZDs the buffer concentration is of secondary importance.

The parameters of the equation describing the dependence of the log k_0 values on the RP-TLC characteristics of BZDs are given in Table IV. The changes in the independent variables selected by the stepwise regression analysis account for *ca.* 75% of the variance of the log k_0 value (see r^2 value). The calculated F value (see Table IV) is higher than the tabulated F value corresponding to the 99.9% significance level ($F = 9.34$), that is, the equation is highly significant.

Each RP-TLC parameter showed a significant correlation with the HPLC log k_0 value. However, their relative impacts were different. The beta weights show that the R_{M0} value has the highest and the buffer sensitivity the lowest impact on the log k_0 value. This is understandable because the R_{M0} and log k_0 values are theoretically similar parameters in RP-TLC and HPLC. As the correlation between the RP-TLC and HPLC retention data is highly significant, it can be assumed that the HPLC retention can be predicted on the basis of RP-TLC measurements. However, from a practical point of view, the fit of the equation (the RP-TLC parameters explain *ca.* 75% of the variance) is not sufficient to predict exactly the HPLC retention. We conclude that with BZDs the RP-TLC retention data are of limited value for predicting HPLC retention behaviour. This observation is supported by the finding that there was no significant correlation between the slope value of eqn. 1 and the RP-TLC parameters. This phenomenon can be explained by the assumption that the coverages

TABLE IV

DEPENDENCE OF THE LOG k_0 VALUES OF BENZODIAZEPINE DERIVATIVES ON THEIR RP-TLC PARAMETERS

Results of stepwise regression analysis. Log $k_0 = a + b_3R_{M0} + b_4b_1 + b_5b_2$, where R_{M0}, b_1 and b_2 are the parameters of the equation in Table III. $n = 18$; $F = 13.94$; $a = 2.00$; $r^2 = 0.7492$.

Parameter	R_{M0}	$b_1 \cdot 10$	$b_2 \cdot 10^2$
b	1.22	4.93	1.74
s	0.21	1.07	0.45
Beta weight	3.11	2.28	0.71

of the silica surface are different in RP-TLC and HPLC, resulting in different responses of the chromatographic system to changes in the organic modifier concentration.

The data matrix for the PCA is shown in Table V. The first PCA component contains most of the variance (eigenvalue 5.11; variance explained 85.24%). This finding indicates a strong relationship between the reversed-phase chromatographic systems studied. This result is in accordance with that of stepwise regression analysis, *i.e.*, the RP-TLC and HPLC retention mechanisms are similar but not identical. The second PCA component (eigenvalue 0.60; variance explained 9.92%) is of negligible importance.

The F_1 and F_2 axes of the two-dimensional non-linear maps do not have any concrete physical or chemical meanings. They only show the relative (projected in two dimensions) distances between the BZDs chromatographic systems in multi-dimensional space. The BZDs did not form separate groups on the two-dimensional non-linear map of PCA variables (Fig. 3). This finding indicates that the various substituents influence the retention to similar extents, *i.e.*, that there is no single substituent that governs retention. The six chromatographic systems on the two-dimensional non-linear map of PCA loadings form two distinct groups, one for the HPLC and the other for the RP-TLC systems (Fig. 4). This result supports our previous conclusions that the two methods may produce slightly different but correlated retention parameters.

TABLE V

RETENTION PARAMETERS OF BENZODIAZEPINE DERIVATIVES AT pH 7.4

Numbers refer to BZDs in Table I. I = log k' at 40 vol.-% acetonitrile concentration; II = log k' at 50 vol.-% acetonitrile concentration; III = R_M value at 40 vol.-% acetonitrile concentration; IV = R_M value at 50 vol.-% acetonitrile concentration; V = R_M value at 40 vol.-% methanol concentration; VI = R_M value at 50 vol.-% methanol concentration.

Compound	*No. of parameter*					
	I	*II*	*III*	*IV*	*V*	*VI*
1	0.222	0.035	−0.50	−0.58	−0.28	−0.40
2	0.581	0.301	−0.35	−0.47	−0.11	−0.19
3	0.653	0.216	−0.46	−0.56	−0.21	−0.41
4	0.669	0.390	−0.37	−0.53	−0.09	−0.31
5	0.734	0.368	−0.29	−0.41	−0.02	−0.23
6	0.734	0.368	−0.20	−0.41	0.15	−0.15
7	0.761	0.426	−0.11	−0.35	0.21	−0.11
8	0.778	0.442	−0.33	−0.45	−0.10	−0.27
9	0.928	0.558	−0.24	−0.37	−0.04	−0.24
10	0.932	0.561	−0.15	−0.37	0.18	−0.14
11	0.942	0.592	−0.12	−0.34	0.19	−0.10
12	0.885	0.515	−0.14	−0.35	0.16	−0.16
13	1.151	0.772	−0.09	−0.32	0.22	−0.08
14	1.431	1.014	−0.17	−0.37	0.05	−0.13
15	1.645	1.149	−0.10	−0.27	0.00	0.23
16	1.446	0.970	0.16	−0.16	0.59	0.09
17	0.934	0.584	−0.19	−0.40	−0.02	−0.25
18	1.073	0.661	−0.22	−0.40	−0.10	−0.29

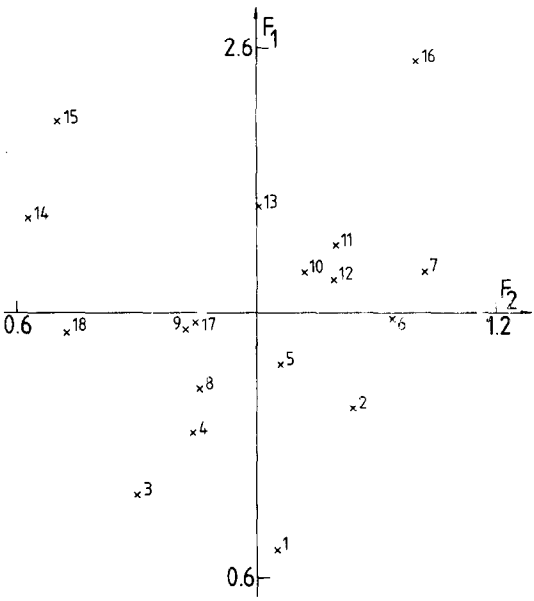


Fig. 3. Two-dimensional non-linear map of principal component variables. Number of iterations, 49; maximum error, $9.46 \cdot 10^{-6}$. Numbers refer to BZDs in Table I.

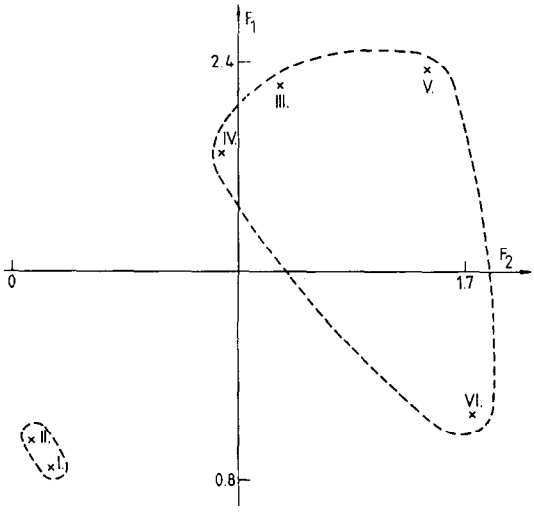


Fig. 4. Two-dimensional non-linear map of PCA loadings. Number of iterations, 142; maximum error, $3.73 \cdot 10^{-5}$. Numbers refer to chromatographic systems in Table IV.

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