

## **Retention characteristics of CN, NH<sub>2</sub> and diol precoated high-performance thin-layer chromatographic plates in the adsorption and reversed-phase separation of some benzodiazepine derivatives**

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(First received December 11th, 1989; revised manuscript received March 28th, 1990)

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### ABSTRACT

The retentions of eighteen benzodiazepine (BZD) derivatives on CN, diol and NH<sub>2</sub> high-performance thin-layer chromatographic layers was determined using eight adsorption (carbon tetrachloride–ethyl acetate) and five reversed-phase (water–methanol) eluent systems. In most instances a linear correlation was found between the  $R_M$  value of BZD and the ethyl acetate or methanol concentration in the eluent, which allows the calculation of the optimum eluent composition. In aqueous eluents the lipophilicity of BZD determines the retention, that is, the plates behave as real reversed-phase stationary phases. The differences in the reversed-phase retentions of BZD were lower than in adsorption chromatography, advocating the application of adsorption chromatography for their separation. The low retention capacity and elongated spot shape make the NH<sub>2</sub> plate unsuitable for the reversed-phase separation of BZD without further modification of the composition of the mobile phase. As chlordiazepoxide and medazepam form distorted spots on CN and diol plates, for the best separation of these BZD, NH<sub>2</sub> plates in the adsorption separation mode are recommended.

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### INTRODUCTION

In recent years, high-performance thin-layer chromatography (HPTLC) has found growing acceptance and application among modern separation techniques<sup>1</sup>. Earlier results have already been reviewed<sup>2</sup>. Up to now mainly silica HPTLC plates have been applied for the separation of aflatoxins<sup>3–5</sup> and chlorthalidone and its impurities<sup>6</sup>. Amino phases have recently been applied to separate  $\beta$ -carotenes<sup>7</sup> and glucose and fructose<sup>8</sup>.

Benzodiazepine derivatives (BZD) have found growing application in modern therapeutic practice<sup>9</sup>. BZD are of considerable importance, having hypnotic, tranquillizing and anticonvulsant properties. As the range of BZD 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 TLC<sup>10,11</sup> or pH-gradient TLC<sup>12</sup>. Earlier high-performance liquid chromatographic (HPLC) methods have been reviewed<sup>13</sup>. Both adsorption<sup>14</sup> and reversed-phase methods<sup>15</sup> have been used in the HPLC separation of BZD, and gas chromatography (GC) has also been frequently applied<sup>16,17</sup> sometimes with mass spectrometry<sup>18</sup>. The performances of the various chromatographic methods (TLC, GC and HPLC) have been compared<sup>19</sup>.

The retention of a solute has been successfully predicted in binary eluent systems by various semi-empirical and theoretical approximations<sup>20-25</sup> using the various forms of eluent composition characteristics (preferably the concentration of the strong solvent in the eluent) as independent variables. The objectives of this work were to study and to compare the retention behaviours of some HPTLC layers in the adsorption and reversed-phase separation of some BZD.

#### EXPERIMENTAL

CN F<sub>254</sub>S, NH<sub>2</sub> F<sub>254</sub>S and DIOL F<sub>254</sub>S HPTLC precoated plates (Merck, Darmstadt, F.R.G.) were applied both for adsorption and reversed-phase chromatography without any pretreatment.

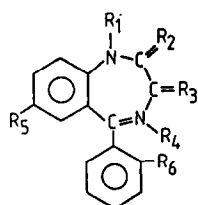
The structures of BZD are given in Table I. The compounds were purchased from Hoffmann-La Roche (Basle, Switzerland) (compounds 1, 2, 11 and 14), Gedeon Richter (Budapest, Hungary) (compounds 3, 4, 6, 10, 13 and 15), Wyeth Labs. (Princeton, NJ, U.S.A.) (compound 5), VEB Arzneimittelwerk (Jena, G.D.R.) (compound 7), POLFA Pharmaceutical Works (Jelenia Góra, Poland) (compound 8), Upjohn Pharmaceutical Works (Kalamazoo, MI, U.S.A.) (compound 9), H. Mack Chemische Pharmazeutische Fabrik (Illertissen, F.R.G.) (compound 12), Gödecke (Frankfurt, F.R.G.) (compound 16), Hoechst (Frankfurt, F.R.G.) (compound 17) and EGIS Pharmaceutical Works (Budapest, Hungary) (compound 18). The BZD were separately dissolved in methanol at a concentration of 1 mg/ml and 500 nl of each solution were spotted onto the plates with a Camag (Muttens, Switzerland) Micro Applicator. Both adsorption and reversed-phase developments were carried out in sandwich chambers of the same dimensions. Carbon tetrachloride-ethyl acetate mixtures served as eluents for adsorption chromatography in the concentration range 0-100% (v/v) ethyl acetate (eight eluent mixtures). Water-methanol mixtures were applied for the reversed-phase separation in the concentration range 0-80% (v/v) methanol (five eluent mixtures). After development the plates were dried at room temperature and the BZD spots were detected with a Shimadzu CS-930 dual-wave-length TLC scanner at 280 nm.

#### MATHEMATICAL METHODS

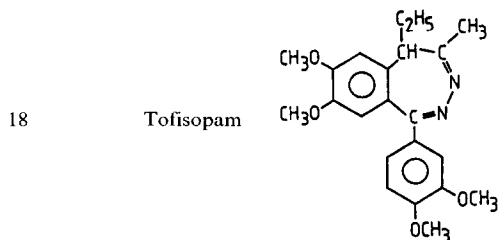
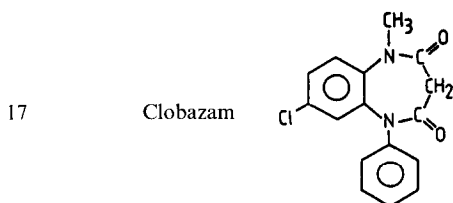
The  $R_M$  values were calculated separately for each BZD in each eluent:

$$R_M = \log (1/R_F - 1) \quad (1)$$

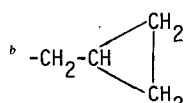
TABLE I  
STRUCTURES OF BENZODIAZEPINE DERIVATIVES



Compound No.	Common name	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>	R <sub>5</sub>	R <sub>6</sub>
1	7-Aminonitrozepam	H	=O	H	H	NH <sub>2</sub>	H
2	Bromazepam	H	=O	H	H	Br	<sup>a</sup>
3	Uxepam	CH <sub>3</sub>	=O	H	CONH <sub>2</sub>	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 <sub>2</sub>	H
7	Clonazepam	H	=O	H	H	NO <sub>2</sub>	Cl
8	Chlordiazepoxide	H	NHCH <sub>3</sub>	H	O	Cl	H
9	Alprazolam	R <sub>1</sub> C(CH <sub>3</sub> )=NN=R <sub>2</sub>		H	H	Cl	H
10	Desmethyldiazepam	H	=O	H	H	Cl	H
11	Flunitrazepam	CH <sub>3</sub>	=O	H	H	NO <sub>2</sub>	F
12	Clorazepat	H	(OH) <sub>2</sub>	COOH	H	Cl	H
13	Diazepam	CH <sub>3</sub>	=O	H	H	Cl	H
14	Midazolam	R <sub>1</sub> C(CH <sub>3</sub> )=NCH <sub>2</sub> R <sub>2</sub>		H	H	Cl	F
15	Medazepam	CH <sub>3</sub>	H	H	H	Cl	H
16	Prazepam	<sup>b</sup>	=O	H	H	Cl	H



<sup>a</sup> 2-Pyridinyl instead of phenyl group.



Linear correlations were calculated separately for each compound and for each plate type in both the adsorption and reversed-phase modes:

$$R_M = a + b \cdot C \quad (2)$$

where  $R_M$  = the actual  $R_M$  value of a compound determined at  $C\%$ (v/v) ethyl acetate or methanol concentration;  $a$  = the  $R_M$  value of a compound extrapolated to zero ethyl acetate or methanol concentration;  $b$  = decrease in the  $R_M$  value caused by a 1% increase of ethyl acetate or methanol concentration; and  $C$  = ethyl acetate or methanol concentration (%v/v).

On combination of eqns. 1 and 2, the  $R_F$  value, being the dependent variable, can be expressed as a function of the slope and intercept values of eqn. 2:

$$R_F = 1/(1 + 10^a \cdot 10^{bC}) \quad (3)$$

The optimum eluent composition for the separation of any pairs of BZD derivatives occurs when the  $R_F$  value expressed by eqn. 4 is a maximum:

$$R_F = 1/(1 + 10^{a_1} \cdot 10^{b_1C}) - 1/(1 + 10^{a_2} \cdot 10^{b_2C}) \quad (4)$$

where  $a_1$ ,  $b_1$ ,  $a_2$  and  $b_2$  are the intercept and the slope values of eqn. 2 for the BZD pair to be separated and  $C$  is the concentration (%v/v) of ethyl acetate or methanol in the eluent. Eqn. 4 has a maximum when its first derivative equals zero.

To assess the real reversed-phase character of the separation in water-methanol mixtures, the intercept and slope values of BZD determined on CN and diol plates were linearly correlated with the corresponding values determined in traditional (silica support impregnated with 5% paraffin oil in *n*-hexane) reversed-phase chromatography<sup>26</sup>.

The intercept ( $a$ ) and slope ( $b$ ) values of eqn. 2 characterize not only the retention behaviour of BZD but also the retention characteristics of the plates. To compare the BZD and the plate types, taking into consideration simultaneously their retention on each plate and in each eluent system, principal component analysis (PCA)<sup>27</sup> was applied. The parameters of eqn. 2 (intercept and slope values for CN, diol and NH<sub>2</sub> plates in the adsorption mode and intercept and slope values for CN and diol plates in the reversed-phase mode, total ten parameters) were taken as variables. The NH<sub>2</sub> plates in this instance were unsuitable for reversed-phase chromatography, so these values were excluded from the calculation. Individual BZD served for observations except compounds 14 and 15 (total 16 observations). The omission of compounds 14 and 15 from the calculations was motivated by the fact that eqn. 2 was not significant for compounds 14 and 15 on CN plates. To visualize the results, the two-dimensional non-linear maps of PC loadings and variables were also calculated<sup>28</sup>. The non-linear mapping technique calculates the distances in the multi-dimensional space between the variables (parameters of eqn. 2) and observations (individual BZD), then it projects the relative distances in a plane with minimum error. In this way the distributions of parameters and BZD on the two-dimensional map are related to the PC loadings and PC variables; they are derived from them but they are not identical with them. The axes  $F_1$  and  $F_2$  are on both maps coordinates without any concrete physical meaning. To

elucidate the impact of the physico-chemical parameters of BZD on their retention, the first three sets of PC variables containing the overwhelming majority of information was correlated with the lipophilicity, specific hydrophobic surface area<sup>26</sup> and  $pI$  value<sup>29,30</sup> of BZD. As the exact type of correlation between the dependent and independent variables had not previously been determined, we applied stepwise regression analysis<sup>31</sup> to overcome this difficulty. The PC variables were separately taken as dependent variables and the linear, logarithmic, quadratic and reciprocal forms of lipophilicity, specific hydrophobic surface area and  $pI$  values (total 12 variables) were taken as independent variables. The number of accepted variables was not limited; the acceptance limit for the individual independent variables was set to 95%.

## RESULTS AND DISCUSSION

In most instances the BZD spots were symmetrical without any distortion. However, on  $NH_2$  plates using the reversed-phase separation mode (eluent water), the BZD were fairly near to the front and had very elongated spots, that is, the  $NH_2$  plates showed negligible retention capacity and therefore they are not suitable for the reversed-phase separation of BZD with the mobile phase used here. In adsorption chromatography compounds 8 and 15 produced elongated spots on diol and CN plates (Fig. 1) but not on  $NH_2$  plates. This means that for the separation and determination of the BZD in mixtures containing these compounds the  $NH_2$  plates are strongly recommended (for the separation possibilities of other derivatives, see the conclusions below).

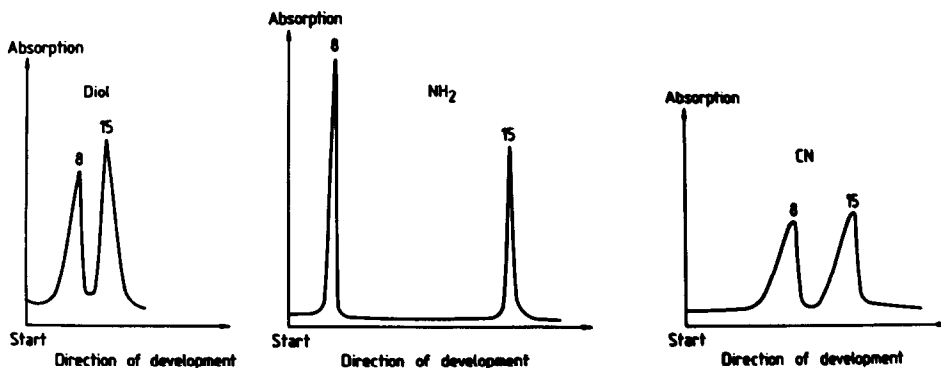


Fig. 1. Densitogram of the spots of compounds 8 and 15 (see Table I) on various layers. Eluent:  $CCl_4$ -ethyl acetate (1:1, v/v).

The dependence of the  $R_M$  values of some structurally different BZD on the eluent composition is shown in Figs. 2-4. The  $R_M$  values decreased in each instance with increasing concentration of ethyl acetate or methanol, that is, the BZD did not show any anomalous retention behaviour<sup>32,33</sup> which would make the application of eqn. 2 questionable. It also implies that the retention of BZD is governed in each instance by a single retention mechanism. The character of the dependence of the

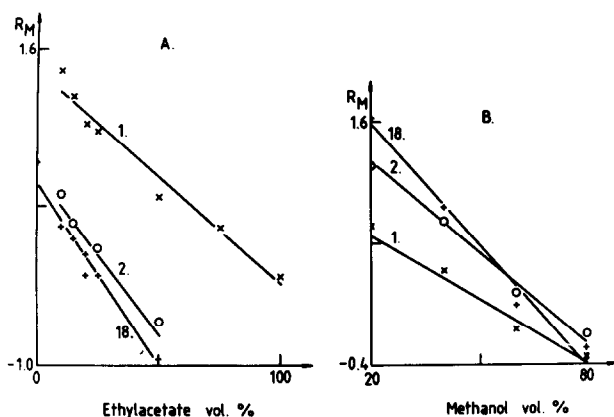


Fig. 2. Dependence of  $R_M$  value of some benzodiazepine derivatives on the ethyl acetate and methanol concentrations in  $\text{CCl}_4$  and water, respectively. CN plates. Numbers refer to compounds in Table I.

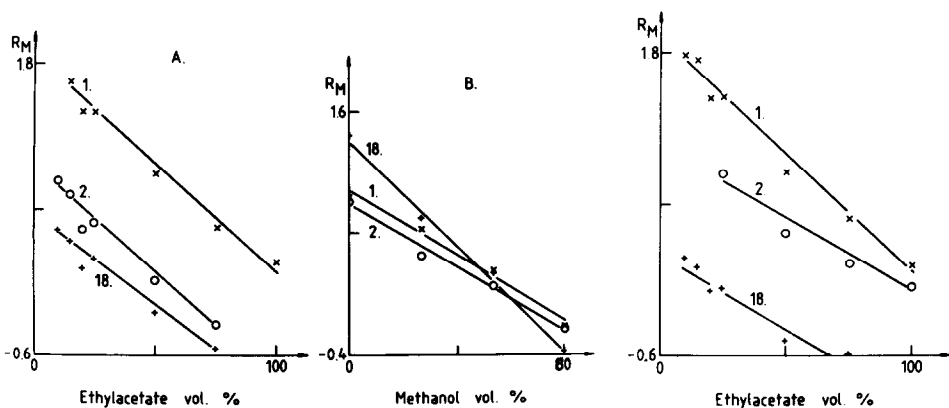


Fig. 3. Dependence of  $R_M$  values of some benzodiazepine derivatives on the ethyl acetate and methanol concentrations in  $\text{CCl}_4$  and water respectively. Diol plates. Numbers refer to compounds in Table I.

Fig. 4. Dependence of  $R_M$  values of some benzodiazepine derivatives on the ethyl acetate concentration in  $\text{CCl}_4$ .  $\text{NH}_2$  plates. Numbers refer to compounds in Table I.

retention on the eluent composition is different for the different plates, which means that the retention characteristics of the plates differ considerably. The parameters of the linear correlation between the  $R_M$  values of BZD and the eluent composition are given in Tables II–IV. In most instances the linear correlation fitted well to the experimental data, the significance level being over 95%. The high significance level of the correlation (low standard deviation of the slope and intercept values) makes it probable that the application, of eqn. 4 leads to a correct estimation of the optimum separation conditions. With CN plates the intercept and slope values show a higher diversity in adsorption than in the reversed-phase separation mode. This finding suggests that for the separation of BZD on CN plates adsorption chromatography is to be recommended. The same is valid, but to a lesser extent, to diol plates. We have to

TABLE II

PARAMETERS OF LINEAR CORRELATIONS BETWEEN THE  $R_M$  VALUES OF BENZODIAZEPINE DERIVATIVES AND THE ETHYL ACETATE ( $C_1$ ) AND METHANOL ( $C_2$ ) CONCENTRATIONS IN THE ELUENT WITH CN HPTLC PLATES

Compound No.	$R_M = a + b C_1$				$R_M = a + b C_2$			
	$a$	$-b$	$s_b$	$r$	$a$	$-b$	$s_b$	$r$
1	142.7	1.77	0.15	0.9834	115.5	1.87	0.17	0.9917
2	53.6	2.41	0.34	0.9715	170.5	2.36	0.16	0.9953
3	110.7	1.33	0.10	0.9856	168.5	2.50	0.20	0.9936
4	51.6	2.67	0.39	0.9696	167.5	2.46	0.20	0.9932
5	56.0	2.70	0.36	0.9747	208.5	2.93	0.21	0.9947
6	36.5	2.42	0.31	0.9769	194.5	2.57	0.36	0.9809
7	39.3	2.54	0.29	0.9807	210.0	2.84	0.22	0.9940
8	72.9	0.87	0.10	0.9666	202.0	2.83	0.40	0.9808
9	177.1	1.31	0.13	0.9771	195.5	2.65	0.20	0.9942
10	79.8	3.84	0.41	0.9776	195.5	2.74	0.21	0.9941
11	23.8	2.74	0.29	0.9785	215.0	2.84	0.28	0.9904
12	73.7	3.60	0.38	0.9783	196.0	2.76	0.22	0.9940
13	6.3	2.34	0.22	0.9829	214.5	2.89	0.22	0.9940
14		Not significant				Not significant		
15		Not significant			341.8	4.02	0.53	0.9913
16	13.3	5.22	0.65	0.9848	251.0	3.30	0.52	0.9878
17	66.1	2.90	0.32	0.9765	193.5	2.60	0.21	0.9933
18	51.6	3.07	0.34	0.9770	221.5	3.25	0.35	0.9888

TABLE III

PARAMETERS OF LINEAR CORRELATIONS BETWEEN THE  $R_M$  VALUES OF BENZODIAZEPINE DERIVATIVES AND THE ETHYL ACETATE ( $C_1$ ) AND METHANOL ( $C_2$ ) CONCENTRATIONS IN THE ELUENT WITH DIOL HPTLC PLATES

Compound No.	$R_M = a + b C_1$				$R_M = a + b C_2$			
	$a$	$-b$	$s_b$	$r$	$a$	$-b$	$s_b$	$r$
1	181.1	1.73	0.11	0.9917	94.5	1.77	0.15	0.9928
2	92.9	1.75	0.16	0.9831	82.2	1.69	0.14	0.9930
3	171.5	1.82	0.16	0.9815	68.5	1.52	0.12	0.9943
4	101.9	2.18	0.16	0.9893	78.4	1.63	0.13	0.9934
5	107.1	2.25	0.15	0.9909	88.8	1.83	0.14	0.9944
6	66.4	2.00	0.16	0.9878	71.4	1.48	0.13	0.9920
7	72.3	2.12	0.17	0.9870	87.9	1.75	0.16	0.9913
8	152.3	1.25	0.09	0.9877	119.8	1.78	0.25	0.9817
9	176.0	1.33	0.10	0.9857	104.1	2.09	0.12	0.9966
10	59.5	1.83	0.16	0.9846	103.4	2.03	0.16	0.9937
11	11.6	1.66	0.14	0.9868	75.7	1.64	0.12	0.9943
12	57.8	1.78	0.16	0.9847	102.2	2.01	0.15	0.9942
13	-8.8	1.20	0.12	0.9809	102.4	2.03	0.16	0.9937
14	170.8	1.07	0.12	0.9826	166.1	1.97	0.38	0.9651
15	61.0	0.41	0.03	0.9862	187.4	2.03	0.34	0.9734
16	-21.9	1.51	0.15	0.9865	123.3	2.33	0.24	0.9899
17	46.0	2.42	0.23	0.9869	69.8	1.61	0.09	0.9967
18	52.2	1.45	0.14	0.9824	138.4	2.90	0.17	0.9967

TABLE IV

PARAMETERS OF LINEAR CORRELATIONS BETWEEN THE  $R_M$  VALUE OF BENZODIAZEPINE DERIVATIVES AND THE ETHYL ACETATE ( $C_1$ ) CONCENTRATION IN THE ELUENT WITH  $NH_2$  HPTLC PLATES

Compound No.	$R_M = a + b C_1$			
	$a$	$-b$	$s_b$	$r$
1	191.0	1.88	0.11	0.9908
2	106.0	1.17	0.19	0.9735
3	199.3	1.85	0.11	0.9931
4	206.2	1.38	0.10	0.9894
5	227.0	1.58	0.25	0.9759
6	151.2	1.59	0.11	0.9883
7	144.8	1.54	0.09	0.9911
8	158.3	1.28	0.09	0.9874
9	150.6	1.59	0.17	0.9732
10	106.4	1.43	0.11	0.9861
11	37.3	2.20	0.19	0.9887
12	106.6	1.43	0.10	0.9879
13	5.5	1.84	0.17	0.9880
14	57.3	1.00	0.08	0.9843
15	Not significant			
16	-15.2	1.82	0.24	0.9747
17	44.8	1.38	0.09	0.9901
18	21.6	1.12	0.10	0.9819

stress that the conclusion outlined above are valid only for the compounds investigated and do not contain any information about the retention behaviour of the plates with other solutes. The significant correlations between the corresponding reversed-phase parameters (Table V) prove that both CN and diol plates behave as reversed phases in aqueous eluents, that is, the retention of solutes is governed mainly by their lipophilicity. Although the regression coefficients indicate significant linear correlations they are not high enough to allow the application of CN or diol plates for the determination of the lipophilicity of BZD.

TABLE V

PARAMETERS OF LINEAR CORRELATIONS BETWEEN THE INTERCEPT AND SLOPE VALUES OF BENZODIAZEPINES DETERMINED ON VARIOUS PLATES IN THE REVERSED-PHASE MODE

$y = a + bxn$				$n$	$a$	$b$	$s_b$	$r$
$y$		$x$						
Parameter	Plate type	Parameter	Plate type					
Intercept	Diol	Intercept	rp	18	1.89	31.95	7.09	0.7480
Slope	Diol	Slope	rp	18	0.64	0.16	0.05	0.6478
Intercept	CN	Intercept	rp	17	36.18	54.25	10.44	0.8017
Slope	CN	Slope	rp	17	0.72	0.26	0.07	0.6885

<sup>a</sup> rp = Traditional reversed-phase plate.



Principal component analysis proved that the ten variables can be substituted with three hypothetical ones that explain more than 80% of the total variance (Table VI). PC loadings show that each parameter of the CN plates and many parameters of the diol and NH<sub>2</sub> plates have a high loading in the first PC, that is, this PC can be tentatively identified with the retention characteristics of CN plates and with those retention characteristics of diol and NH<sub>2</sub> plates which are similar to those of CN plates. The second PC consists mainly of the high loadings of diol plates, so it can be considered as a background variable with diol-type retention characteristics.

TABLE VI  
RESULTS OF PRINCIPAL COMPONENTS ANALYSIS

<i>Principal component No.</i>	<i>Eigenvalue</i>	<i>Variance explained (%)</i>		
1	4.90	49.00		
2	2.19	21.94		
3	1.20	11.96		
4	0.69	6.86		

<i>Principal component loadings</i>				
<i>Variable No.</i>	<i>Principal component No.</i>			
	<i>1</i>	<i>2</i>	<i>3</i>	<i>4</i>
1	-0.66	0.58	-0.04	0.23
2	0.71	-0.36	0.24	0.47
3	0.88	-0.09	-0.01	-0.38
4	0.89	0.06	0.08	-0.35
5	-0.84	0.49	0.07	-0.12
6	-0.35	-0.68	0.55	0.08
7	0.62	0.73	0.08	0.07
8	0.71	0.60	0.14	0.23
9	-0.83	0.08	0.24	-0.24
10	-0.05	-0.33	-0.86	0.11

The results of stepwise regression analysis proved that the first PC variable correlated quadratically with the specific hydrophobic surface area of BZD ( $a = -5.75$ ;  $b = 0.09$ ;  $s_b = 0.02$ ;  $r = 0.7812$ ), that is, this physico-chemical parameter has the greatest influence for the first background variable. With the second and third PC variables no significant correlation was found, that is, these variables are influenced to physico-chemical parameters that were not included in the calculations.

On the two-dimensional non-linear map of PC loadings (Fig. 5) the intercept (points 1, 5 and 9) and slope (points 2, 6 and 10) values of eqn. 2 calculated from the adsorption chromatographic data for each plate type form distinct groups. We again draw attention to the fact that the coordinates of the two-dimensional maps ( $F_1$  and  $F_2$ ) were calculated from the PC loadings and PC variables, but  $F_1$  and  $F_2$  are not identical (or simply related) to the PC loadings in Table VI and PC variables (not

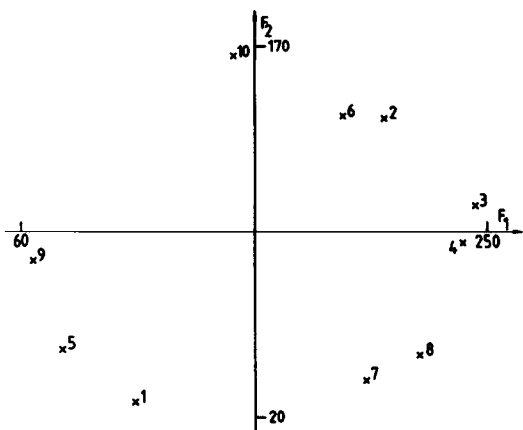


Fig. 5. Two-dimensional non-linear map of PC loadings. Number of iterations, 51; maximum error,  $2.82 \cdot 10^{-2}$ . Numbers refer to the parameters of eqn. 2. Intercept (1) and slope (2) values on CN plates, adsorption system; intercept (3) and slope (4) values on CN plates, reversed-phase system; intercept (5) and slope (6) values on diol plates, adsorption system; intercept (7) and slope (8) values on diol plates, reversed-phase system; intercept (9) and slope (10) values on  $\text{NH}_2$  plates, adsorption system.

given). The four reversed-phase parameters (points 3, 4, 7 and 8) also form a cluster. This finding proves again that the plates show different retention capacities and selectivities in adsorption and reversed-phase chromatography. The BZD do not form separate clusters according to either the number or the type of substituents on the two-dimensional non-linear map of PC variables (Fig. 6). This result indicates that each substituent has a similar impact on the retention. The map also contains information about the separation capacity of the CN, diol and  $\text{NH}_2$  plates for BZD. The greater the difference between two BZD the higher is the probability that they can be separated in one or more chromatographic systems. The very close positions of compounds 10 and 12 on the map indicate that they can hardly be separated with the chromatographic systems applied.

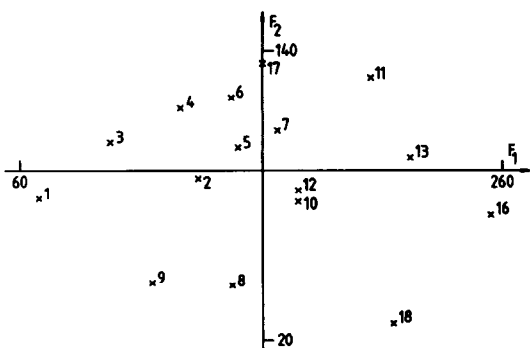


Fig. 6. Two-dimensional non-linear map of PC variables. Numbers refer to benzodiazepine derivatives in Table I. Number of iterations, 103; maximum error,  $2.82 \cdot 10^{-2}$ .

## CONCLUSIONS

CN, diol and NH<sub>2</sub> HPTLC plates seem to be suitable for the separation of most benzodiazepine derivatives. Except for the NH<sub>2</sub> plates, the separation can be carried out both with adsorption (carbon tetrachloride–ethyl-acetate) and reversed-phase (methanol–water) eluents, but the separation efficiency was higher in adsorption than in reversed-phase systems in all instances.

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