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Physicochemical interaction of some benzodiazepine derivatives with amino acids and phospholipids

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Summary

The interaction of 18 benzodiazepine (BDZs) derivatives with amino acids and with the membrane phospholipid dipalmitoylphosphatidylcholine (DPPC) was studied using charge-transfer reversed-phase chromatography and differential scanning calorimetry, respectively. A weak hydrophobic interaction was observed between the BDZs and Trp. Midazolam and medazepam were strongly bound to dicarboxylic amino acids, the strength of interaction being higher in ionic environment. BDZs modified the phase transition parameters of DPPC, indicating direct interaction. The enthalpy of the main transition of DPPC showed a nonlinear dependence on the specific hydrophobic surface area of BDZs, thus demonstrating the hydrophobic nature of the interaction.

Introduction

During the last decade, benzodiazepine derivatives (BDZs) have found growing acceptance and application in therapeutic practice (Kelley et al., 1989). BDZs are of considerable importance, having hypnotic, tranquillizing and anticonvulsant properties. As the range of available BDZs has been extended rapidly over the same period of time, much work was aimed at the elucidation of their mode of action. BDZs interact with central receptors through the GABA : chloride ion channel macromolecular complex (Cecchi et al., 1989); the binding proteins contain four hydrophobic sequences about 20 amino acids in length (Haefely, 1989). The apparent molecular weights of the peripheral and central BDZ-binding sites are quite similar: 215 000 and 260 000–270 000, respectively (Saano et al., 1989). The proteins can bind BDZs reversibly and irreversibly (Schmitz et al., 1989), and many nonneuronal tissues contain pharmacologically distinct binding sites for BDZs (Hirsch et al., 1988a), human serum also binding BDZs (Menke et al., 1989). The data mentioned above neither exclude the possibility of direct interaction of BDZs with membrane phospholipids in the proximity of the chloride ion channel nor contain information about the exact binding site or sites of BDZs on the proteins. It was suggested that vari-

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ous binding sites may exist for different BDZs (Hirsch et al., 1988b). In order to improve separation, the application of modifying agents contained in the support was introduced in thin-layer chromatography (TLC) more than 20 years ago (Berg and Lam, 1964). The relative differences in R_f values determined in the cases of support with and without compounds suitable for charge-transfer interactions have been proposed as a measure of the strength of interaction (Harvey and Halonen, 1966). Early studies applied charge-transfer chromatography in adsorptive TLC using non-polar solvents (Dwivedy et al., 1967; Parihar et al., 1967), however, the interactive forces determined in organic eluents cannot readily be related to interactions taking place in an aqueous biological system. In recent research, charge-transfer chromatography has been applied to study biologically important molecular interactions (Slifkin et al., 1982; Slifkin and Singh, 1984; Gullner et al., 1986).

Differential scanning calorimetry (DSC) has been extensively employed to investigate the interactions of phospholipids with various bioactive compounds such as metalloporphyrins (Groves and Neumann, 1989), peptides (Naider et al., 1989) and herbicides (Szögyi et al., 1989). Greater knowledge of the relationships between the phase transition parameters of phospholipid-bioactive compound mixtures and the physicochemical characteristics of the bioactive molecular may help in the elucidation of the molecular basis of such interactions (Szögyi and Cserháti, 1987).

Our objectives concerned the elucidation of interactions between a number of BDZs, amino acids and synthetic phospholipids by charge-transfer chromatography and DSC, and the determination of the various physicochemical parameters for BDZ in the interaction.

Experimental

The chemical structure of each of the BDZs is compiled in Table 1. The compounds were purchased from Hoffmann-La Roche (Basel, Switzerland: compound nos 1, 2, 11 and 14), Gedeon Richter (Budapest, Hungary: nos 3, 4, 6, 10, 13 and 15), Wyeth Labs (Princetown, NJ: no. 5), VEB Arzneimittelwerk (Jena, G.D.R.: no. 7), POLFA Pharmaceutical Works (Yelenia Gora, Poland: no. 8), Up John Pharmaceutical Works (Kalamazoo, MI: no.9), H. Mack Chemische Pharmazeutische Fabrik (Illertissen, F.R.G.: no. 12), Gödecke AG (Augsburg, F.R.G.: no. 16), Hoechst AG, (Frankfurt, F.R.G.: no. 17) and Egis Pharmaceutical Works (Budapest: no. 18). Amino acids were obtained from REANAL (Hungary). Each amino acid was of analytical purity and of the L configuration. Dipalmitoylphosphatidylcholine (DPPC) was purchased from Serva (Heidelberg, F.R.G.) and used without further purification.

Silcoplat F_{254} plates (Labor MIM, Budapest) were impregnated with a 5% solution of paraffin oil in *n*-hexane as described (Cserháti et al., 1983). The BDZs were separately dissolved in methanol at a concentration of 1 mg/ml; 3 μl of each solution was spotted onto the plates. The eluent was water : methanol (3 : 2 v/v). The amino acids were separately dissolved in the eluent at a concentration of 25 mM. Amino acid free eluent served as a control. After developing, the plates were dried at 105 °C and the BDZs detected by means of their UV absorption spectra. As the mobility of the amino acids may deviate from that of eluent (Cserháti and Szögyi, 1988a), the amino acid fronts were detected with ninhydrin. All experiments were run in quadruplicate.

The R_M values of the BDZs, characterizing the molecular lipophilicity on reversed-phase thin-layer chromatography (RPTLC), were determined according to Eqn 1 (Biagi et al., 1969):

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

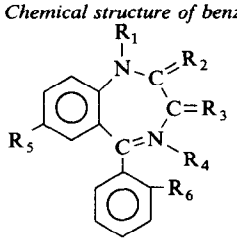
Since the mobilities of the amino acids differ from that of the eluent, the R_M values of BDZs were corrected for the different mobilities of the amino acids via the following equation:

$$R_{MC} = R_{MO} - (R_{MO} - R_M)/R_f \quad (2)$$

where R_{MC}, R_{MO} and R_M denotes the R_M values for a BDZ as corrected for the mobility of the amino acid, as determined in amino acid-free

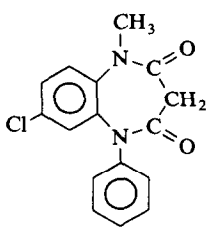
TABLE 1

Chemical structure of benzodiazepine derivatives

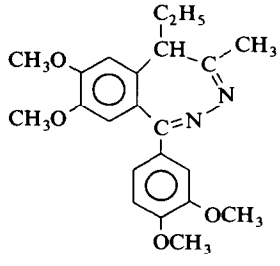


General structure

Compound no.	Trivial name	R_1	R_2	R_3	R_4	R_5	R_6
1	7-amino-nitrozepam	-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	chlordiaze-poxide	-H	$-NHCH_3$	-H	-O	-Cl	-H
9	alprazolam	$R_1-C(CH_3)=N-N=R_2$		-H	-H	-Cl	-H
10	desmethyl-diazepam	-H	=O	-H	-H	-Cl	-H
11	flunitra-zepam	$-CH_3$	=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	$R_1-C(CH_3)=N-CH_2-R_2$		-H	-H	-Cl	-F
15	medazepam	$-CH_3$	-H	-H	-H	-Cl	-H
16	prazepam	[b]	=O	-H	-H	-Cl	-H

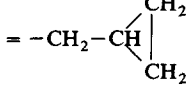


17 clobazam



18 tofisopam

[a] = 2-pyridinyl instead of phenyl group.

[b] = 

eluent and amino-acid-containing eluent, respectively, with R_f representing the R_f value of the amino acid front.

To extract the maximum amount of information from our data, principal component (PC) analysis (Mardia et al., 1979) was applied, the BDZs and amino acids being the variable and observed parameters, respectively. As Arg and Lys

showed very low mobility (R_f 0.10 and 0.05) in our chromatographic system these data were omitted from calculations. The percentage of variance explained was set to 99%. To avoid the loss of information due to normalization, calculation was carried out on the covariance matrix instead of the correlation matrix. A two-dimensional nonlinear map of PC loadings and variables was also calculated (Sammon, 1969). The amino acids form clusters on the map of PC variable when showing similar responses to the effect of BDZs, whereas they are widely separated in the case where their responses are markedly different. On the map of PC loadings, the BDZs interacting similarly with the amino acids form groups whereas those with differing effects are separated.

As the strongest interaction was observed between BDZs 14 and 15 and the dicarboxylic amino acids aspartic acid and glutamic acid, in these cases the change in lipophilicity of compounds 14 and 15 was determined at Asp and Glu concentrations of 5, 10, 15 and 20 mM. To assess the effect of a physiological concentration of ion on the strength of interaction, the same experiments were carried out at a final concentration of 0.16 M sodium chloride.

To determine the relative strength of interaction, linear correlations were calculated between the R_M values of compounds 14 and 15 and the Asp and Glu concentration (C mM) in the eluent for each BDZ-amino acid pair in ion-free and in ionic eluent.

$$R_M = a + bC \quad (3)$$

The value for the slope (b) of Eqn 3 is related to the strength of interaction (Cserháti and Szögyi, 1988b). To judge whether the differences between the effects of the dicarboxylic amino acids, the compounds and the presence of ions were significant or not, the corresponding b values were compared by the method of paired means (Weber, 1967). The melting properties of DPPC in the presence of some BDZs were determined via DSC. DPPC and the BDZs were dissolved in chloroform in a molar ratio of 10:1. The chloroform was removed by evaporation under a nitrogen atmosphere, then water was added to the BDZ-DPPC mixtures at a ratio of 4:1 (w/w). The samples were for 30 min vigorously mixed in a vortex mixer. Measurements were carried out in a Du Pont 990 Thermoanalyzer at a heating rate of 5 °C per min, within a sensitivity range of 0.1–0.2 mW/cm. The equipment was calibrated using indium.

The temperature of the pretransition (T_p), that of the main transition (T_m), and the half-width of the main transition ($\Delta T_{1/2}$) characterizing the cooperativity were determined. The enthalpy of the main transition (ΔH) was evaluated in a separate experiment. Each measurement was carried out in quadruplicate.

To establish the molecular parameters of BDZs that account for the interaction with DPPC, correlation was carried out between the phase transition parameters of DPPC-BDZ mixtures and the lipophilicity value and specific hydrophobic surface area (SHS) of the BDZs taken from the paper of Valko et al. (1989). As the exact type of correlation (linear or quadratic) had not previously been determined, both forms were included in calculations. To select the independent variables with a significant influence on the DPPC-BDZ interaction, stepwise regression analysis was performed (Mager, 1982). Calculations were made three times, the main transition temperature, peak half-width and enthalpy of the main transition representing the dependent parameters, the linear and quadratic forms of lipophilicity and SHS values being in each case the independent variables. The number of accepted variables was not limited; the significance level of acceptance was set to 95%.

Results and Discussion

The uncorrected mean R_M values of BDZs and the R_f values of amino acid fronts are compiled in Table 2. The coefficient of variation was below 8% in every case. With the exception of the interaction between dicarboxylic amino acids and compounds 14 and 15, the change in lipophilicity caused by the amino acids is fairly low. This suggests that the BDZs interact weakly with free amino acids, and thus that their strong binding to proteins generally cannot be attributed to binding

TABLE 2

100.R_M values of benzodiazepine derivatives

Compound no.	Control	Amino acid																	
		Ala	Asn	Asp	Cys	Gln	Glu	Gly	His	Ile	Leu	nLeu	Met	Phe	HPro	Pro	Ser	Thr	Trp	Val
1	−15	−6	7	0	−1	7	10	4	−23	−1	−24	−16	−26	−9	−15	−31	−13	−25	−13	−33
2	63	60	78	82	65	73	81	72	56	65	54	57	45	71	57	50	61	53	64	49
3	72	68	84	83	79	83	84	82	63	70	63	66	52	69	65	60	70	61	65	60
4	87	85	95	93	94	94	97	92	77	89	78	86	64	83	80	72	82	74	82	74
5	96	92	98	99	97	97	92	93	84	88	88	90	72	82	90	77	82	81	85	81
6	76	69	74	78	65	73	73	75	57	70	73	75	48	60	85	62	71	62	63	63
7	75	67	79	86	69	76	72	82	67	66	72	75	52	65	76	65	79	65	56	74
8	117	114	124	117	113	120	109	119	115	110	118	124	94	114	110	103	118	110	99	113
9	129	125	132	140	123	129	124	130	121	117	131	131	102	114	120	117	120	118	104	116
10	114	110	113	117	108	115	103	112	110	102	116	113	91	99	107	104	108	106	95	102
11	92	86	86	94	78	85	77	81	83	72	86	85	69	75	94	85	90	87	73	86
12	115	106	108	109	102	108	98	102	107	96	112	111	91	103	116	107	111	110	98	110
13	130	129	126	137	118	126	123	123	124	117	134	133	108	119	130	124	129	128	106	128
14	174	169	173	121	167	164	114	162	159	151	181	167	151	165	169	167	173	180	142	169
15	213	194	212	124	210	181	110	194	186	180	188	213	180	196	189	195	194	195	169	199
16	186	167	173	186	162	168	158	166	170	152	173	169	148	166	175	168	163	168	150	169
17	91	77	78	83	73	74	66	77	74	68	76	77	66	74	83	80	85	81	69	78
18	131	107	115	122	109	110	102	113	110	101	111	109	101	106	124	115	127	125	94	110

of a single amino acid. We assume that more than one amino acid participates in the binding of BDZ to a protein involving both hydrophobic and electrostatic interactions.

The first three PC components account for, 49.24, 30.71 and 12.20% of the total variance, i.e., the binding characteristics of all 18 BDZs can be ascribed to three hypothetical compounds. This does not signify that they exist, but merely indicates the mathematical possibility for such a case. The three proposed compounds give rise to more than 90% of the total variance, in other words, the characteristics of the BDZs can be expressed via three background variables.

A two-dimensional nonlinear map of PC loadings is shown in Fig. 1. The BDZs are distributed over the map according to their strength of interaction with all the amino acids. Apart from compounds 14 and 15, the BDZs form a fairly compact cluster, which is indicative of similar interactions with amino acids. The distinct difference of compounds 14 and 15 from the others is due to their specific interaction with dicarboxylic amino acids. As dicarboxylic amino acids are strongly polar (Skagerberg et al., 1987), we assume that their interaction with BDZs 14 and 15 is hydrophilic in nature. However, as regards BDZs 14 and 15, this suggests that they are more basic in character as compared to the other derivatives. This suggestion is only partly valid, since midazolam is the least acidic of the BDZs investigated (Allen et

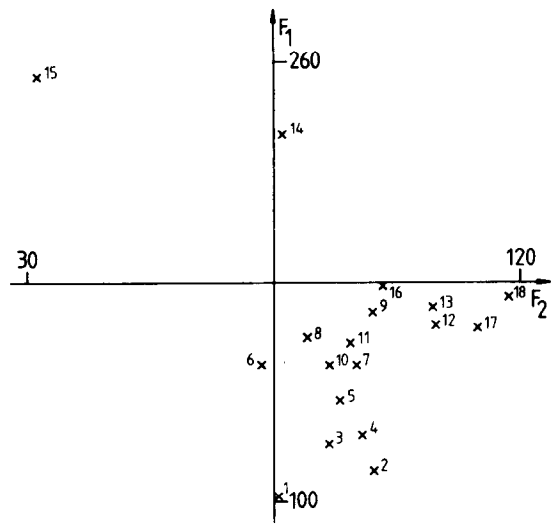


Fig. 1. Two-dimensional nonlinear map of PC loadings. Number of iterations, 269; maximum error, 1.51×10^{-2}. Numbers refer to benzodiazepine derivatives in Table 1.

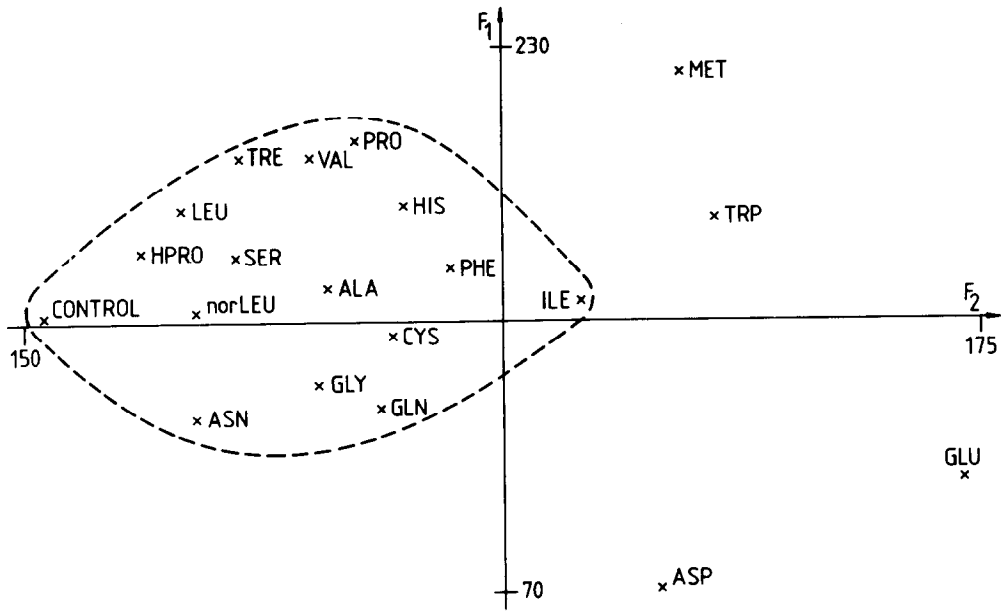


Fig. 2. Two-dimensional nonlinear map of PC variables. Number of iterations, 148; maximum error, 1.62×10^{-2}.

al., 1987) whereas medazepam is more acidic than chlordiazepoxide (Colburn and Jack, 1987) which does not undergo any interaction with dicarboxylic amino acids. The effect of Glu and Asp can be tentatively explained by the finding that their side chain differs from that of all other amino acids in the doublet acceptor energy levels inducing a repulsive effect (Sneddon et al., 1988) and hence this deviation may account for the phenomenon observed.

A two-dimensional nonlinear map of PC variables is shown in Fig. 2. The amino acids are distributed over the map in accordance with their interaction with the BDZs. Asp and Glu display the greatest deviation in behavior. This can be attributed to the specific interaction with compounds 14 and 15 discussed above. Trp and Met are also distinctly different compared to the majority of amino acids. In the case of Trp, this effect can be explained by the supposition that, as a consequence of being the most lipophilic amino acid (Asao et al., 1987), Trp binds to BDZs through hydrophobic forces. The deviation in binding behavior for methionine cannot be explained simply in terms of hydrophobic or hydrophilic attractive forces. The nature of the interaction remains unclear and further investigation is required in order to establish the details.

The parameter values for Eqn 3 are compiled in Table 3. Linear correlation yields a good fit to the experimental data, the significance level in each case being over 99%. The t probe for the paired means demonstrated that the difference between the binding capacity of the amino acids is not significant ($t = 1.80$) indicating that Asp and Glu interact in a similar manner with compounds 14 and 15. However, compound 15 binds more strongly to the amino acids in both ionic and ion-free eluent than does compound 14 ($t = 6.33$). The effect of ions on the strength of interaction is also significant ($t = 4.49$), interaction being stronger in an ionic environment. This indicates that binding of the compounds to proteins is favored by a physiological ion concentration.

TABLE 3

Parameters of linear correlation between the R_M value of compounds 14 and 15 in the presence of glutamic and aspartic acids and sodium chloride

	a	$-b$	S_b	r
Ion-free eluent				
Glu-comp. 14	191	1.68	0.07	0.9983
Glu-comp. 15	242	4.06	1.00	0.9439
Asp-comp. 14	178	2.38	2.36	0.9469
Asp-comp. 15	204	3.71	0.63	0.9591
Ionic eluent				
Glu-comp. 14	197	3.38	0.19	0.9970
Glu-comp. 15	236	5.74	0.83	0.9797
Asp-comp. 14	199	4.52	0.77	0.9724
Asp-comp. 15	246	7.60	1.58	0.9596

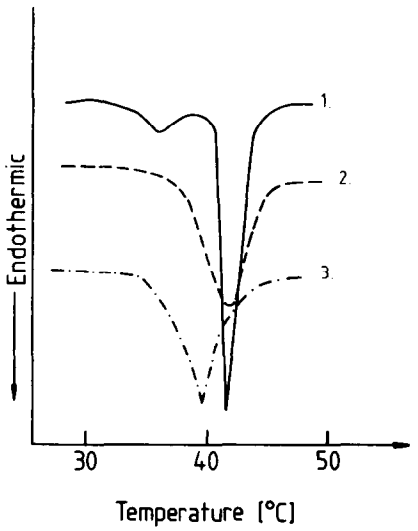


Fig. 3. DSC curves of DPPC (1), DPPC : medazepam (2) and DPPC : oxazepam (3) mixtures. Molar ratio 10 : 1.

A number of phase transition curves are shown in Fig. 4. The effects exerted by BDZs on the phase transition parameters of DPPC include a decrease in the main transition temperature, disappearance of the pretransition and an increase in the peak half-width. All these changes indicate that the BDZs interact directly with DPPC, thereby modifying the intermolecular organization of the membrane phospholipids, which may result in an altered functional capacity. The data in Table 4 fully support our previous conclusions. Each BDZ causes unambiguous changes in one or more of the phase transition parameters of DPPC, thus demonstrating the occurrence of direct interaction between the two molecular species. The fact

TABLE 4

Effect of benzodiazepine derivatives on the pre- (T_p) and main transition temperature (T_m), enthalpy (ΔH_m) and half-width ($\Delta T_{1/2}$) of the main transition of DPPC-water dispersion (SD, standard deviation)

Compound no.	T_p	T_m (°C)		$\Delta T_{1/2}$ (°C)		ΔH_m (mJ/mg)	
		Mean	SD	Mean	SD	Mean	SD
Control	present	41.5	0.1	1.5	0.1	56.0	2.3
4	absent	39.5	0.2	2.8	0.3	36.3	4.6
8	absent	41.0	0.3	2.7	0.3	39.2	5.1
9	present	41.0	0.2	1.5	0.2	37.4	1.1
13	absent	39.3	0.1	2.6	0.2	39.5	3.8
15	absent	41.7	0.2	4.1	0.4	41.2	1.6
16	present	40.8	0.1	1.4	0.1	38.4	2.9

that various BDZs influence the phase transition parameters of DPPC to different extents suggests that the nature of the interaction may vary according to the particular DPPC-BDZ pair. This hypothesis is supported by the results obtained on stepwise regression analysis (Table 5). Neither the main transition temperature nor the peak half-width showed a significant dependence on the lipophilicity or specific hydrophobic surface area (SHS) of the BDZs. However, a clear relationship was found to exist between the enthalpy of the main transition and the SHS values of the BDZs. The equation fits the experimental data well, the level of significance being above 99% (cf. F values), and accounts for 94% of the total variance (see r^2 values). The relationship is markedly non-linear, i.e., there exists an optimal value for the hydrophobic surface area. On the basis of the present data, we assume that the BDZs interact with the non-polar fatty acid chain region of DPPC, the interaction depending nonlinearly on the hydrophobic surface area of BDZs and possessing a hydrophobic character.

TABLE 5

Relationship between the enthalpy of the main transition temperature (ΔH_m) of DPPC and the specific hydrophobic surface (SHS) of benzodiazepine derivatives: results of stepwise regression analysis

	SHS	$(SHS)^2$
b	−4.91	0.34
S_b	1.11	0.12
β weight	−2.45	1.58

$\Delta H_m = a + b_1 SHS + b_2 (SHS)^2$; $n = 7$, $F_{calc.} = 31.58$, $F_{99\%} = 30.82$, $r^2 = 0.9404$, $a = 55.89$.

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