J. Pharm. Pharmacol. 1990, 42: 401–404
Received September 27, 1989

Normal Phase HPLC as a Model System of Specific Benzodiazepine-receptor Binding

GASTONE GILLI, MARIA CHIARA PIETROGRANDE* AND PIER ANDREA BOREA†

*Centro di Strutturistica Diffratometrica, Università di Ferrara, 44100 Ferrara, Italy, *Dipartimento di Chimica, Università di Ferrara, 44100 Ferrara, Italy and †Istituto di Farmacologia, Università di Ferrara, 44100 Ferrara, Italy*

Abstract—The hypothesis that benzodiazepines interact with their receptors in the CNS via hydrogen bonding mediated by the carbonyl and imine groups has been studied by correlating a physicochemical parameter affected by hydrogen bonds potentially accepted or donated by benzodiazepines with their binding constants to synaptosomal rat brain membranes. The reference physicochemical system chosen was normal phase high-performance liquid chromatography on μ-Bondapack NH_2 (propylamine groups chemically bonded to porous silica) eluted with mixtures of n-hexane and 2-propanol. A strong correlation has been found for binding of the implicated groups to the column and to the receptor.

Borea et al (1987) have suggested a general model for benzodiazepine-receptor interactions which accounts for both specific binding features of ligands of different chemical classes and for the nature of their intrinsic activity (agonistic, antagonistic or inverse agonistic). The model is based on a diffuse and substantially planar recognition site characterized by hydrogen bonds accepted by specific drug functional groups; the observed differences in pharmacological profile are accounted for by the different localization of different ligands inside this unique binding site. Our measurements agree with the hypothesis that the relevant hydrogen bonding acceptor groups are the carbonyl in position 2, the nitrogen in position 4, and sometimes a halogen atom in position 2′, whereas N_1- and 3-OH- or N_4-oxide groups are not involved in the drug-receptor binding process. The importance of the hydrogen bonding accepting groups in determining the standard enthalpy ΔH°_{bind} of the binding equilibrium is substantiated by the few binding measurements carried out at different temperatures (Kochman & Hirsch 1982) which show that ΔH°_{bind} values are of the order of -8 kcal mol^{-1} for benzodiazepine-2-one derivatives whereas it is $+5{\cdot}6$ kcal mol^{-1} for desmethylmedazepam, which lacks the carbonyl group.

We thought that the role of hydrogen bonding in the benzodiazepine-receptor interaction could be clarified by finding a physicochemical parameter characterised by hydrogen bonds potentially accepted (or donated) by the drug molecule and checking whether this quantity is functionally related to the value of the binding constant. The reference system chosen was normal phase high-performance liquid chromatography (HPLC) on a packing of μ-Bondapack NH_2, consisting of propylamine groups chemically bonded to porous silica, eluted with mixtures of n-hexane and 2-propanol; the polar solvent (propanol) would compete with benzodiazepines for hydrogen bonds donated or accepted by the NH_2 groups of the stationary phase, so the retention time (or retention volume) of any benzodiazepine could be considered a measure of its hydrogen bonding capability (Hammers et al 1979; Hurtubise et al 1981; Snyder 1983; Verzele & Van Damme 1987).

Correspondence to: P. A. Borea, Istituto di Farmacologia, Via Fossato di Mortara 64/b, 44100 Ferrara, Italy.

Materials and Methods

Benzodiazepines

Compounds studied are listed in Table 1.

Inhibitory binding constants K_i

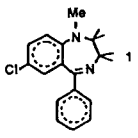
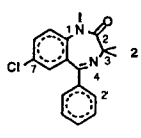
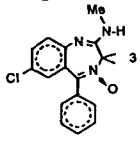
The inhibition of specific [^{3}H]diazepam binding to rat brain synaptosomal membranes was calculated as $K_i = IC50/(1+[C^*]/K_D)$ where [C*] is the concentration of the radioligand and K_D its affinity constant. The smallest K_i values are associated with compounds having the highest binding receptor affinities (Borea & Bonora 1983).

HPLC

Experiments were performed on a Spectra Physics Chromatograph (SP87100 pump and SP87500 organizer module) equipped with a Varian UV detector operated at 254 nm. A μ-Bondapack NH_2 column ($30 \times 0{\cdot}39$ cm) from Waters Associates (Milford, MA) was used and consisted of propylamine groups chemically bonded to Waters μ-Porasil (10μ porous silica). This packing has a surface area of about 325 m^2 g^{-1} and about 2 μmol m^{-2} of alkylamino groups (Majors 1980). Thus it can be calculated that the surface is covered by a packing of vertical chains having an area of about 17·9 Å^2/group and implanted on the surface at an average distance of 9·1 Å apart. Experiments were carried out with a mobile phase consisting of hexane in various mixtures with 2-propanol (HPLC-grade solvents). The flow rate was 1·5 mL min^{-1} and compounds were dissolved in 2-propanol (100–400 μg mL^{-1}).

Capacity factors were calculated as $k' = (t_r - t_o)/t_o$ where t_r is the measured elution time and t_o the elution time of a non-retained compound (toluene). Elution times were determined by injecting 10 μL in the system at $293{\cdot}15 \pm 0{\cdot}1$ K. For each benzodiazepine, 8–13 values of log k′ were measured and plotted against log x_s (mole fraction of the more polar

Table 1. Chemical formulae and chromatographic parameters (log k_1, b) for the compounds investigated. N_b is the number of groups hydrogen bonding with receptor (7-Cl excluded); N_{nb} is the additional number of groups interacting with the column but not with the receptor.

Compound		Substitution position								
no.	Formula	4	2	1	2′	3	log k_1	b	N_b	N_{nb}
										
1	1	—	—	Me	H	H	−1·14	−1·00	1	0
3	2	—	O	$CH_2cC_3H_5$	H	H	−0·82	−1·00	2	0
4	2	—	O	CH_2CF_3	H	H	−0·84	−1·08	2	0
7	2	—	O	Me	H	H	−0·62	−1·00	2	0
8	2	—	O	H	H	H	−0·56	−1·41	2	0
10	2	—	O	Me	Cl	H	−0·56	−1·09	3	0
14	2	—	O	Me	F	H	−0·54	−1·09	3	0
9	2	—	O	H	F	H	−0·53	−1·44	3	0
15	2	—	O	H	Cl	H	−0·49	−1·30	3	0
2	3	O	NHMe	—	H	H	−0·35	−1·19	1	1
5	2	—	O	H	H	OH	+0·10	−1·32	2	1
6	2	—	O	Me	H	OH	+0·11	−1·34	2	1
12	2	—	O	Me	Cl	OH	+0·57	−1·41	3	1
13	2	—	O	H	F	OH	+0·58	−1·41	3	1
11	2	—	O	H	Cl	OH	+0·62	−1·30	3	1

solvent); values of the slope b and the intercept log k_1 were determined according to equation 3 below by a least squares technique (average correlation coefficient = 0·997; e.s.d.'s on b and log k_1 in the ranges 0·01–0·33 and 0·005–0·04, respectively).

According to Snyder (1974) retention on silica, or functionalized columns eluted with binary mobile phases, can be accounted for by two main models. In the Soczewinski model (Soczewinski & Golkiewicz 1973) the solute (X) competes with the polar solvent (S) by forming discrete complexes $X-A_n^*$ or A^*-S with specific interaction points A^* of the substrate according to equation:

$$X + nA^* - S \rightleftarrows X\text{-}A_n^* + n\,S \qquad (1)$$

In this case the capacity factor k′ is calculated as:

$$\log k' = \log k_1 - n \log x_s \qquad (1a)$$

where k_1 is the capacity factor of the solute eluted with the polar solvent alone, x_s the mole fraction of the polar solvent and n the number of specific sites engaged by the solute molecule. In the Snyder model (Snyder 1968) of aspecific binding the substrate is covered by polar solvent molecules which are displaced by the solute according to equations:

$$X + n\,S_{ads} \rightleftarrows X + n\,S \qquad (2)$$

$$\log k' = \log k_1 - \frac{A_x}{A_s} \log x \qquad (2a)$$

where A_x/A_s is the ratio between the surface areas occupied by the solute and polar solvent, respectively, and other symbols have the usual meaning. In either model cancellation of solute-solvent interactions in solution and adsorbed phases is assumed and the functional form of log k′ is identical, that is:

$$\log k' = \log k_1 - b \log x_s \qquad (3)$$

and only the meaning of the slope b is different.

Results and Discussion

Table 1 shows the chemical formulae of the compounds investigated together with their measured values of log k_1 and b as defined in equation 3. Only log k_1 values show relevant variations, whereas the b values are confined in the restricted range of $1·2 \pm 0·2$. This result can be interpreted by assuming (according to equation 3) that the binding of the benzodiazepine molecule displaces a single molecule of the polar solvent, 2-propanol. The dependence of log k_1 on the number and nature of the hydrogen bond acceptors can be analysed in terms of a specific statistical technique, the Free-Wilson method (Free & Wilson 1964). This assumes that a specific molecular quantity (in the present case log k_1) can be expressed as the sum of a constant contribution μ and of as many individual group contributions α_{ij} as there are substituents j on the different positions i. The total α_i variation range, $\Delta\alpha_i$, is an indication of the relative importance of the substituent position in determining the physical property concerned. The data in Table 2 show that: (i) the relative importance of substituent position i (measured by $\Delta\alpha_i$) increases in the order $1 < 2 \approx 2' < 3$, (ii) large α_{ij} values are

Table 2. Individual group contributions α_{ij} obtained by the Free-Wilson analysis according to the model $\log k_1 = \mu + \sum_i \sum_j \alpha_i$ for HPLC measurements. R_1, R_2, R_3 and $R_{2'}$ are substituents in the indicated position of scheme 2; R is $CH_2cC_3H_5$ or CH_2CF_3 (compound 2 has been excluded from the analysis as it carries two substituents with unitary appearance frequency, so causing a singular regression matrix).

n = 13, r = 0·986, F = 35·88, $P < 0·01$, $\mu = -0·253$							
R_1	α_{1j}	R_2	α_{2j}	R_3	α_{3j}	R_2^1	α_{2j}^1
H	0·04	O	0·03	OH	0·59	Cl	0·14
CH_3	−0·02	H	−0·36	H	−0·37	F	0·09
R	−0·10					H	−0·14
$\Delta\alpha_i$	0·14		0·39		0·96		0·28

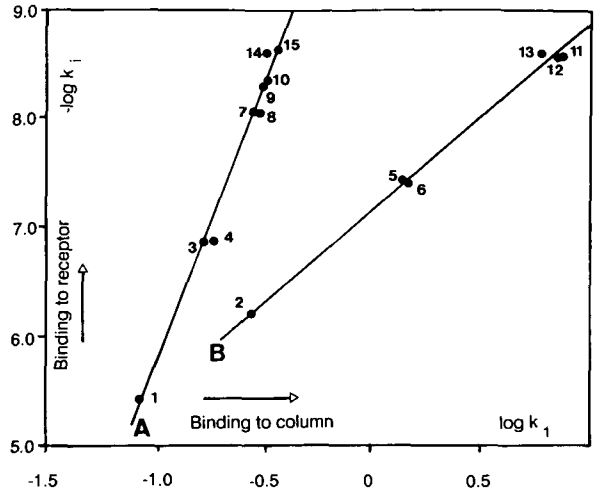


FIG. 1. Correlation between binding to benzodiazepine receptor (measured by $-\log K_i$, where K_i is the inhibitory constant) and to HPLC column (measured by $\log k_1$ as defined in eqns 1a, 2a) for all the 15 benzodiazepines considered. The regression lines $-\log K_i = a + b \log k_1$ have the following statistic parameters:

A: 11·08 (±0·17), b = 5·01 (±0·25) (n = 9, r = 0·992, F = 411·88, $P < 0·001$).
B: 7·12 (±0·03), b = 2·50 (±0·08) (n = 6, r = 0·998, F = 1069·07, $P < 0·001$).

systematically caused by the addition to the benzodiazepine skeleton of groups which are both hydrogen bonds donors and acceptors (OH in 3) or simply acceptors (halogen atoms in 2′, carbonyl in 2), the former being the most effective in increasing the binding to the column, and (iii) the highly significant regression parameters indicate that the contribution to binding of the different substituents is substantially additive. Therefore, it may be concluded that the chromatographic process in which benzodiazepines and polar solvent compete for the $-NH_2$ sites of the column can be interpreted in terms of a simple model. This model can be quantified as far as the $\log k_1$ values are concerned with the exception of chlordiazepoxide (3; compound 2) which could not be included in the regression analysis as it carries two unique substituents (2-NHMe and 4-O).

The correlation between receptor and chromatographic binding of benzodiazepine is shown in Fig. 1 in the form of a $\log K_i$ vs $\log k_1$ plot. There is a correlation only if compounds are divided into subsets, $S_1 = \{1, 3, 4, 7–10, 14, 15\}$ and $S_2 = \{2, 5, 6, 11–13\}$ (see last two columns of Table 1). This division can be justified by assuming that in subset S_1 all the hydrogen bonds acceptors (7-Cl, C_2=O, N_4, 2′-halogen) able to bind to the column are also involved in receptor binding, whereas in subset S_2 there is at least one substituent able to bind to the column but not to the receptor (see last two columns of Table 1). Detailed analysis of both receptor binding affinity data (Squires & Braestrup 1977; Mohler & Okada 1977; Borea 1983) and several proposed models of benzodiazepine-receptor interactions (Borea & Gilli 1984; Loew et al 1984; Borea et al 1987) indicates that such a division into subsets is justified by biochemical and pharmacological data so far collected and that the chemical group unable to bind to the receptor is certainly the hydroxyl group in position 3 for compounds 5, 6, 11, 12, 13 and, most probably, the *N*-oxide group in position 4 for chlordiazepoxide (compound 2).

There is a decrease of the slope of $\log K_i$ vs $\log k_1$ plot in compounds of subset S_2 when compared with those of subset S_1 (Fig. 1); all compounds binding more strongly to the column carry a 3-OH group which is both a hydrogen bond donor and acceptor and is the group making the largest individual contribution to $\log k_1$ (see Table 2).

The correlation parameters of the regression lines A and B of Fig. 1 (corresponding to subsets S_1 and S_2, respectively) are remarkably good though it is impossible to know, at present, if this is due to the particular choice of compounds in the sample. On each regression line a part of the variance is accounted for by the different number of hydrogen bonds formed: the compounds are clearly arranged in increasing order of $\log k_1$ according to the increasing number of hydrogen bonding groups. On line A compound 1 has the lowest number of such groups (N_4 and 7-Cl), compounds 3, 4, 7, 8 have one more (C_2=O) and the remaining compounds carry an additional 2′-halogen group. Likewise on line B compound 2 carries three groups ($N_4 \rightarrow O$, 2-NHMe and 7-Cl), compounds 5 and 6, four (N_4, C_2=O, 7-Cl and 3-OH) and compounds 11, 12, 13, five (an additional halogen in position 2′). This shows that the role played by the different groups in the binding to the column (and to receptor, with the exception of the 3-OH group) is substantially additive, though other specific effects are also operating (see for instance the different behaviour of compounds 3, 4 and 7, 8).

In conclusion, the idea of using normal phase chromatography on NH_2-functionalized columns as a tool for evaluating the ability of benzodiazepines to interact via hydrogen bonding has proved to be reasonably successful. Although the high correlation coefficient values of the $\log K_i$ vs $\log k_1$ regressions may be, in part, due to a fortuitous choice of compounds, there is little doubt about the strong correlation between the abilities to bind to the receptor and to the column for all the groups implicated in the drug-receptor interaction. The HPLC method seems to be able to have at least two interesting applications in the field of molecular pharmacology. The first is that it can indicate whether a specific hydrogen bond donor or acceptor group is primarily involved in the drug-receptor interaction provided pairs of values (K_i, k_1) are known for a sufficiently large number of compounds. The second is in the specific field of drug-receptor interaction thermodynamics; measurements of log P (Hansch 1969, P = partition coefficient) or R_M (Biagi et al 1969) in reversed phase chromatography are known to be indirect measures of drug-receptor interaction entropies and, likewise, the normal phase method could be used for estimating the corresponding interaction enthalpies.

Acknowledgement

The work was financially supported by C.N.R. (Rome); Progetto Finalizzato Chimica Fine II.

References

Biagi, G. L., Barbaro, A. M., Gamba, M. F., Guerra, M. C. (1969) Partition data of penicillins determined by means of reversed-phase thin layer chromatography. J. Chromatogr. 41: 371–378

Borea, P. A. (1983) De novo analysis of receptor binding affinity data of benzodiazepines. Arzneim. Forsch. 33: 1086–1090

Borea, P. A., Bonora, A. (1983) Brain receptor binding and lipophilic character of benzodiazepines. Biochem Pharmacol. 32: 603–607

Borea, P. A., Gilli, G. (1984) The nature of 1,4-benzodiazepines-receptor interactions. Arzneim. Forsch. 34: 649–653

Borea, P. A., Gilli, G., Bertolasi, V., Ferretti, V. (1987) Stereochemical features controlling binding and intrinsic activity properties of benzodiazepine-receptor ligands. Mol. Pharmacol. 31: 334–345

Free, S. M., Wilson, J. W. (1964) A mathematical contribution to structure-activity studies. J. Med. Chem. 7: 395–398

Hammers, W. E., Spanjer, M. C., De Ligny, C. L. (1979) Selectivity of nucleosil NH_2 as an adsorbent in high-performance liquid chromatography. J. Chromatog. 174: 291–305

Hansch, C. (1969) A quantitative approach to biochemical structure-activity relationships. Acc. Chem. Res. 2: 232–236

Hurtubise, R. J., Ussain, A., Silver, H. F. (1981) Effects of solvent composition in the normal phase liquid chromatography of alkylphenols and napthols. Anal. Chem. 53: 1993–1997

Kochman, L., Hirsch, J. D. (1982) Thermodynamic changes associated with benzodiazepine and alkyl β-carbolines-3-carboxylate binding to rat brain homogenates. Mol. Pharmacol. 22: 325–331

Loew, G. H., Nienow, J. R., Poulsen, M. (1984) Theoretical structure-activity studies of benzodiazepine analysis. Requirements for receptor affinity and activity. Ibid. 26: 19–34

Majors, R. E. (1980) Recent advances in HPLC packings and columns. J. Chromatog. Sci. 18: 488–492

Mohler, H., Okada, T. (1977) Benzodiazepine receptors: demonstration in the central nervous system. Science 198: 849 851

Snyder, L. R. (1968) Principles of Adsorption Chromatography. Marcel Dekker, New York, pp. 17–31

Snyder, L. R. (1974) Role of the solvent in liquid-solid chromatography. A review. Anal. Chem. 46: 1384–1389

Snyder, L. R. (1983) Mobile phase effects in liquid-solid chromatography. Importance of adsorption-site geometry, adsorbate delocalization and hydrogen bonding. J. Chromatog. 255: 3–27

Soczewinski, E., Golkiewicz, W. (1973) A simple molecular model for adsorption chromatography. Relationship between the R_M value and composition of phenols in systems of the type cyclohexane + polar solvent/silica. Chromatographia 6: 269–273

Squires, R. F., Braestrup, C. (1977) Benzodiazepine receptors in rat brain. Nature 266: 732–734

Verzele, M., Van Damme, F. (1987) Polarity and selectivity of polyol- and polyphenol-derivatized silica gels for normal-phase high-performance liquid chromatography. J. Chromatog. 393: 25–37