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Mechanism of retention of benzodiazepines in affinity, reversed-phase and adsorption high-performance liquid chromatography in view of quantitative structure-retention relationships

Roman Kaliszan*, Anna Kaliszan and Terence A. G. Noctor

Pharmacokinetics Division, Department of Oncology, McGill University, 3655 Drummond, Montreal, PQ H3G 1Y6 (Canada)

William P. Purcell

Department of Medicinal Chemistry, College of Pharmacy, University of Tennessee, 26 South Dunlap Street, Memphis, TN 38163 (USA)

Irving W. Wainer

Pharmacokinetics Division, Department of Oncology, McGill University, 3655 Drummond, Montreal, PQ H3G 1Y6 (Canada)

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ABSTRACT

Quantitative structure–retention relationships (QSRRs) were compared for a set of 1,4-benzodiazepine (BDZ) test solutes analyzed by high-performance liquid affinity chromatography (HPLAC), reversed-phase (RP) and adsorption (normal-phase, NP) high-performance liquid chromatography (HPLC). The HPLAC data reflected the enantioselective retention on a human serum albumin-based chiral stationary phase (HSA-CSP); RP-HPLC data were determined on a specially deactivated hydrocarbon-bonded silica material; NP-HPLC was performed on a graphitized carbon stationary phase. Molecular descriptors reflecting additive-constitutive properties of the solutes as well as their geometry and electron charge distribution were generated using molecular modelling software. The QSRR equations derived for each chromatographic mode involved different sets of molecular descriptors. Analysis of the physical meaning of the individual descriptors allowed for interpretation of separation mechanisms at molecular (submolecular) level and rationalization of the observed separation patterns. For HPLAC the contributions by hydrophobic, steric and electrostatic factors were quantified and accounted for the differential retention of enantiomers. Retention in RP-HPLC was demonstrated to be a net effect of both non-specific (dispersive) and directional (electrostatic) intermolecular interactions between solute and molecules of both stationary and mobile phases. QSRR equations derived for NP-HPLC proved the predominance of specific dipolar and charge-transfer attractive interactions. The QSRR-based models obtained appear a reliable and convincing proof for involvement of distinctive mechanisms in different HPLC systems.

INTRODUCTION

One of the most extensively studied manifestations of linear free-energy relationships are the quantitative structure–chromatographic retention relationships (QSRRs). These are statistically derived relationships between the structure of a solute

Correspondence to: Professor Irving W. Wainer, Pharmacokinetics Division, Department of Oncology, McGill University, 3655 Drummond, Suite 701, Montreal, PQ H3G 1Y6 Canada.

* On leave from Department of Biopharmaceutics and Pharmacodynamics, Medical Academy, Gen. J. Hallera 107, 80-416 Gdansk, Poland.

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and its chromatographic retention. Using the tools of QSRRs, the chromatographic column becomes a "free-energy transducer", translating differences in chemical potentials of solutes, arising from differences in structure, to chromatographic retention. One of the ultimate goals of QSRR studies, *i.e.*, accurate prediction of retention parameters prior to an experiment, appears rather remote, except for particular solutes and separation systems [1]. Like in other structure–property relationships, this limitation is due to the shortage of relevant, unequivocally defined and quantitatively precise structural descriptors.

However, this is not the case when QSRRs are used to gain insight into the molecular mechanism of chromatographic retention. Using this approach, it is possible to identify the dominant factors which define the interactions of solute molecules with chemical entities forming the chromatographic system. This can be achieved if statistically significant QSRRs are derived and if these equations approximate the experimental retention data for a representative set of model solutes. Although QSRRs are extrathermodynamic in nature and, as such, lack the formal rigor of thermodynamics, there is no doubt that mechanistic conclusions based on QSRRs are much more reliable than those based on qualitative comparisons of structure and retention of a limited number of preselected solutes.

QSRRs have been employed to analyze the mechanism of reversed-phase high-performance liquid chromatographic (RP-HPLC) separations of simple aromatic test solutes on octadecylsilica [2,3] and polybutadiene-coated alumina [4] stationary phase materials. Retention in adsorption HPLC on metallic stationary phases has also been analyzed in terms of QSRRs [5,6]. Recently, we succeeded in deriving quantitative structure–enantioselective retention relationships (QSERs) for a series of 1,4-benzodiazepines subjected to enantioselective high-performance liquid affinity chromatography (HPLAC) on a human serum albumin-based chiral stationary phase (HSA-CSP) [7].

For each of the above HPLC modes (affinity, reversed-phase, adsorption) the QSRR derived revealed different predominant properties of solutes which determined retention. However, different sets of test solutes employed in individual studies [2–7] preclude direct comparison of respective QSRRs and, thus of the mechanisms of retention. With that in mind predesigned, comparative QSRR studies were undertaken and are reported here.

EXPERIMENTAL

Materials

The series of 1,4-benzodiazepine test solutes comprised 9 achiral compounds, 4 single enantiomers and 16 individual isomers of 8 racemates. The solutes alprazolam, 4-hydroxy-alprazolam and triazolam were kindly supplied by Upjohn Labs. (Kalamazoo, MI, USA). The compounds Ro 23-0983/001, Ro 11-3128/002, Ro 14-8935/000, Ro 11-5073/000, Ro 23-1117/000 and Ro 23-3880/000 were generously provided by Hoffman-La Roche (Nutley, NJ, USA). Racemic oxazepam hemisuccinate, and its individual enantiomers, were gifts from Dr. Carlo Bertucci (Pisa, Italy). The remaining benzodiazepines as well as deuteromethanol ($C^2H_3O^2H$) and deuterium oxide (^2H_2O) were purchased from Sigma (St. Louis, MO, USA).

Apparatus

The chromatographic system consisted of a Spectroflow 400 pump; 480 injector module equipped with 20-μl loop; 783 programmable absorbance detector (all from ABI Analytical, Ramsey, NJ, USA). A DataJet integrator (Spectra-Physics, San Jose, CA, USA) or a Shimadzu (Kyoto, Japan) C-R6A integrator were used. In the case of affinity chromatography the column was thermostated using a CH-30 temperature regulating jacket (FIATron Laboratory Systems, Oconomowoc, WI, USA).

The experiments were carried out using a flow-rate of 1 ml min^{-1}.

Chromatographic conditions

Affinity mode [7]. The column (15 cm × 4.6 mm I.D.), packed with HSA-CSP was prepared according to Domenici *et al.* [8] by Shandon Scientific (Runcorn, UK).

Chromatography was carried out isocratically using the mobile phase based on sodium dihydrogenphosphate–disodium hydrogenphosphate (100 m*M*, pH 6.90), modified with 5% (v/v) propan-1-ol. Column temperature was maintained at 28 ± 0.1°C. Capacity factors (k') were calculated taking the signal of water as the dead time marker.

Reversed-phase mode. A Suplex pKb-100 column (15 cm × 4.6 mm I.D.) was purchased from Supelco (Bellefonte, PA, U.S.A).

Chromatography was carried out polycratically using eluents of following proportions (v/v) of methanol to buffer: 80:20, 70:30, 60:40, 50:50, 40:60, 30:70 and 20:80. The buffer of pH 7.00 was prepared by adding 0.1 *M* NaOH to a solution of 0.02 *M* CH_3COOH, 0.02 *M* H_3PO_4 and 0.02 *M* H_3BO_3.

Capacity factors were calculated assuming constant dead volume of the column. Corresponding numerical data were obtained by measuring signals of deuteromethanol ($C^2H_3O^2H$) or deuterium oxide (^2H_2O) chromatographed with neat methanol (CH_3OH) or water (H_2O) eluents, respectively [9].

Logarithms of capacity factors (log k') for individual solutes were plotted against the volume fraction of methanol in the eluent. Excellent linearity of the relationships in the whole eluent composition range studied (correlation coefficient $r > 0.995$) allowed for extrapolation of log k' to 0% methanol (100% buffer). Only the retention parameters normalized to pure buffer (log k'_w) were subjected to further analysis.

Adsorption mode. The Knox graphitized carbon [10] column Shandon Hypercarb S (10 cm × 4.6 mm I.D.) was obtained from Shandon Scientific.

Measurable retention data for all solutes, excepting oxazepam hemisuccinate, were obtained employing hexane–propan-1-ol (97:3, v/v) mobile phase. Column dead volume was obtained injecting $C^2H_3O^2H$ when neat CH_3OH was the eluent. With the value obtained one solute of 21 appeared excluded ($k' < 0$). The value of log k' of oxazepam hemisuccinate, corresponding to the hexane–propan-1-ol (97:3, v/v) eluent, was estimated by extrapolation to this composition of the mobile phase using the log k' data determined at hexane–propan-1-ol proportions 40:60, 50:50 and 55:45 (v/v).

Determination of structural descriptors

Structural analysis was carried out by means of a molecular modeling program InsightII (Biosym Technologies, San Diego, CA, USA) executed on an IBM (Austin, TX, USA) RISC System 6000 computer. Molecular geometry was optimized and the distribution of electron charge within the molecule was calculated by appropriate molecular orbital package (MOPAC) procedures [11,12] within InsightII. Angles and distances of interest were displayed and measured conveniently due to InsightII molecular graphics facilities.

The InsightII-generated structural descriptors which were considered included total energy, orbital energies, electron excess charges on individual atoms, dipole moments, planar and torsion angles formed by common sets of atoms and distances between selected structural moieties in a molecule. The structural descriptors which were eventually employed in QSRR equations included: submolecular polarity parameter (P_{SM}), molecular width (W), electron excess charge on carbon atom C(3) of 1,4-diazepine system [$C(3)$], angle formed by atoms C(2)–C(3)–N(4) of diazepine ring (β_{CCN}), energy of the lowest unoccupied molecular orbital (E_{LUMO}) and total dipole moment (μ). To determine P_{SM}, at first the difference in electron excess charge between the hydrogen atom at position C(3) and the most negatively charged atom in other substituent at C(3) was calculated. Next, that charge difference was multiplied by the distance (in Å) between these two atoms [7]. The width, W, was measured from the extremity of the phenyl substituent.

In addition to the descriptors generated by molecular modeling, meaningful in QSRRs appeared fragmental hydrophobic constants of substituents at position 7 (f_Y) and 2′ (f_X) according to Taylor [13], their sum (f_{X+Y}) and molecular refractivity of a whole molecule (M_R) calculated after Vogel [14]. Theoretical values of logarithms of octan-1-ol–water partition coefficient for whole molecules (CLOGP) were calculated by the ProLog program (CompuDrug Chemistry, Budapest, Hungary).

Deriving of QSRR equations

Logarithms of capacity factors and structural descriptors were mutually related by means of multiparameter regression analysis using the CSS package (StatSoft, Tulsa, OK, USA) run on a personal computer. The equations were derived by a stepwise regression analysis and next, refined by a standard regression method taking into consideration significance of individual descriptors, intercorrelations among them, number of data points and variable data range and distribution. The relationships derived were tested according to the requirements of a meaningful correlation analysis [15].

RESULTS AND DISCUSSION

Structures of 1,4-benzodiazepine (BDZ) test solutes are given in Fig. 1. Respective chromatographic data are collected in Table I and structural parameters are listed in Tables II and III.

HPLAC

The QSRR equations 1 and 2 were previously derived [7] which described retention of the first (log k'_1) and the second (log k'_2) eluting enantiomers in HPLAC on HSA-CSP. For achiral solutes it was assumed that $k'_1 = k'_2$ and the actual capacity factor confirmed both QSRR equations for individual enantiomers.

In eqns. 1 and 2 the values in parentheses are standard deviations of regression coefficients, R is the correlation coefficient, F is the f-test value, t is the t-test value and p is the significance level of individual variables and of the whole equation; n is the number of data points used to derive regression. Of 21 BDZs studied we had 17 second-eluting enantiomers available. In the case of eqn. 2 the number of data points (n) is 16 because (*S*)-temazepam was excluded from regression as an outlier.

Eqns. 1 and 2 suggest high stereospecificity of BDZ–HSA retentive interactions. BDZ solutes are assumed to interact with two main types of stereospecific binding sites: one nonenantioselective and another at which there is significant binding by only one enantiomer. Both binding sites were postulated [16] to be composed of a hydrophobic anchoring pocket (positive input by f_Y), a size restrictive region (negative input by W) and a cationic area repulsive towards positively charged carbon C(3) of diazepine system [negative input by $C(3)$]. In the case of the enantioselective binding site an extended cationic area is assumed to attract negatively charged atoms in the substituent at C(3) (positive input by polarity parameter, P_{SM}) providing that the actual configuration of a chiral molecule allows for close contact of interacting moieties. Hydrophobicity of other parts of BDZ seems to be of little importance for retention on HSA-CSP. Some positive input is provided by hydrophobicity of substituents at position 2′ of the phenyl system, but this is at the lowest limits of statistical significance. An even less informative term including β_{CCN} completely loses significance in eqn. 2.

One of the referees indicated that the number of descriptors incorporated in eqns. 1 and 2 is relatively high compared to the number of observations and thus, there was a possibility of chance correlations. The statistically significant equations with reduced number of variables are given in eqns. 1′, 1″, 2′ and 2″.

$$\log k'_1 = -1.7497 + \underset{t = 5.19,\ p \leqslant 10^{-4}}{0.3895\ (\pm 0.0751)\ f_Y} - \underset{t = -3.66,\ p \leqslant 2 \cdot 10^{-3}}{1.8392\ (\pm 0.5020)\ C(3)} - \underset{t = -3.31,\ p \leqslant 5 \cdot 10^{-3}}{0.1609\ (\pm 0.0485)\ W} + \underset{t = 2.36,\ p \leqslant 3 \cdot 10^{-2}}{0.0354\ (\pm 0.0150)\ \beta_{CCN}} + \underset{t = 1.85,\ p \leqslant 8 \cdot 10^{-2}}{0.1736\ (\pm 0.0939)\ f_X} \quad (1)$$

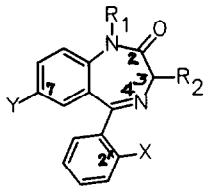
$$n = 21,\ R = 0.8814,\ F = 10.5,\ p < 2 \cdot 10^{-4}$$

$$\log k'_2 = 1.9922 + \underset{t = 7.78,\ p \leqslant 2 \cdot 10^{-5}}{0.8926\ (\pm 0.1147)\ P_{SM}} + \underset{t = 6.43,\ p \leqslant 8 \cdot 10^{-5}}{0.4830\ (\pm 0.0751)\ f_Y} - \underset{t = 5.63,\ p \leqslant 2 \cdot 10^{-4}}{4.1482\ (\pm 0.7367\ C(3)} - \underset{t = -2.20,\ p \leqslant 5 \cdot 10^{-2}}{0.1197\ (\pm 0.0544)\ W} + \underset{t = 1.63,\ p \leqslant 0.13}{0.1324\ (\pm 0.0814)\ f_X} \quad (2)$$

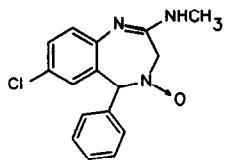
$$n = 16,\ R = 0.9702,\ F = 32.0,\ p < 8 \cdot 10^{-6}$$

$$\log k'_1 = -0.8218 + \underset{t = 4.54,\ p \leqslant 3.4 \cdot 10^{-4}}{0.3518\ (\pm 0.0775)\ f_Y} - \underset{t = -3.51,\ p \leqslant 2.9 \cdot 10^{-3}}{1.8866\ (\pm 0.5379)\ C(3)} - \underset{t = -3.77,\ p \leqslant 3.9 \cdot 10^{-3}}{0.1737\ (\pm 0.0516)\ W} + \underset{t = 1.86,\ p \leqslant 8.1 \cdot 10^{-2}}{0.0292\ (\pm 0.0157)\ \beta_{CCN}} \quad (1')$$

$$n = 21,\ R = 0.8521,\ F = 10.6,\ p < 2.1 \cdot 10^{-4}$$

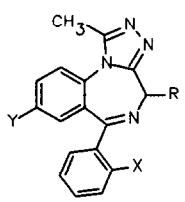


Compounds 2-15

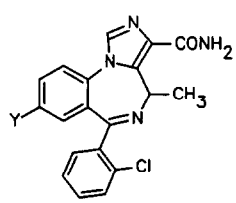


Chlordiazepoxide

	Compound	R_1	R_2	X	Y
1	Chlordiazepoxide	See above	-	-	-
2	(R,S)-Oxazepam H.	H	$OCO(CH_2)_2COO^-$	H	Cl
3	Nitrazepam	H	H	H	NO_2
4	Flunitrazepam	CH_3	H	F	NO_2
5	Clonazepam	H	H	Cl	NO_2
6	Delorazepam	H	H	Cl	Cl
7	Desmethyldiazepam	H	H	H	Cl
8	Diazepam	CH_3	H	H	Cl
9	(R,S)-Lormetazepam	CH_3	OH	Cl	Cl
10	(R,S)-Lorazepam	H	OH	Cl	Cl
11	(R,S)-Oxazepam	H	OH	H	Cl
12	(R,S)-Temazepam	CH_3	OH	H	Cl
13	(S)-Ro 14-8935/000	CH_3	CH_3	Cl	NH_2
14	(S)-Ro 23-0983/001	H	CH_3	Cl	F
15	(R,S)-Ro 11-3128/002	H	CH_3	Cl	NO_2



Compounds 16-19



Compounds 20 and 21

	Compound	R	X	Y
16	(R,S)-Alprazolam, 4-OH	OH	H	Cl
17	Alprazolam	H	H	Cl
18	Triazolam	H	Cl	Cl
19	(S)-Ro 11-5073/000	H	F	Cl
20	(S)-Ro 23-1117/000	-	-	F
21	(R,S)-Ro 23-3880/000	-	-	Cl

Fig. 1. Structures of 1,4-benzodiazepine test solutes.

TABLE I

HPLC retention parameters of a series of benzodiazepine solutes determined in affinity (HPLAC), reversed-phase (RP-HPLC) and normal-phase (NP-HPLC) modes.

No.[a]	HPLAC [7] $\log k'_1$[b]	HPLAC [7] $\log k'_2$[c]	RP-HPLC $\log k'_w$[d]	NP-HPLC $\log k'_{H\text{-}P}$[e]
1	0.8645	0.8645	2.9746	−0.5574
2	0.8512	1.8938	3.1550	1.5413[f]
3	0.6243	0.6243	2.6155	0.2791
4	0.4857	0.4857	2.7687	−0.0025
5	0.7679	0.7679	2.8696	0.4229
6	1.0614	1.0614	3.2752	−0.1663
7	1.0969	1.0969	3.2002	−0.4310
8	1.1216	1.1216	3.1588	−0.5452
9	0.7672	0.9745	3.1870	0.2142
10	0.8068	0.9360	3.0201	0.3082
11	0.6561	1.0261	2.8348	0.2764
12	0.5224	1.1793	2.8987	−0.0923
13	0.3892	–	2.4293	−0.7411
14	0.6628	–	3.1670	–[g]
15	0.7193	0.7193	2.9853	−0.1546
16	0.2648	0.4533	2.5959	0.5812
17	0.4200	0.4200	2.8580	0.0934
18	0.6243	0.6243	3.0971	0.4081
19	0.3838	–	3.0360	−0.2611
20	0.7404	–	3.1836	0.2447
21	1.0523	1.1156	3.4041	0.3374

[a] Solutes are numbered as in Fig. 1.
[b] First-eluting enantiomer.
[c] Second-eluting enantiomer.
[d] Extrapolated data corresponding to pure buffer.
[e] Determined at mobile phase composition hexane–propan-1-ol (97:3, v/v).
[f] Extrapolated from data determined at higher concentrations of propan-1-ol.
[g] Excluded solute; $k' = -0.0077$.

$$\log k'_1 = 2.6402 + \underset{t = 3.87,\ p \leqslant 1.2 \cdot 10^{-3}}{0.2998\ (\pm 0.0774)\ f_Y} - \underset{t = -2.89,\ p \leqslant 1.0 \cdot 10^{-2}}{1.5929\ (\pm 0.5504)\ C(3)} - \underset{t = -3.76,\ p \leqslant 1.6 \cdot 10^{-3}}{0.1996\ (\pm 0.0531)\ W}$$

$$n = 21,\ R = 0.8165,\ F = 11.3,\ p < 2.5 \cdot 10^{-4} \qquad (1'')$$

$$\log k'_2 = 2.2061 + \underset{t = 7.10,\ p \leqslant 2.0 \cdot 10^{-5}}{0.8458\ (\pm 0.1191)\ P_{SM}} + \underset{t = 5.91,\ p \leqslant 1.0 \cdot 10^{-4}}{0.4756\ (\pm 0.0804)\ f_Y} - \underset{t = -5.07,\ p \leqslant 3.6 \cdot 10^{-4}}{3.95\ (\pm 0.7792)\ C(3)}$$

$$- \underset{t = -2.40,\ p \leqslant 3.5 \cdot 10^{-2}}{0.1371\ (\pm 0.0572)\ W} \qquad (2')$$

$$n = 16,\ R = 0.9622,\ F = 34.28,\ p < 4.0 \cdot 10^{-6}$$

$$\log k'_2 = 1.0664 + \underset{t = 8.83,\ p \leqslant 1.0 \cdot 10^{-5}}{1.0116\ (\pm 0.1146)\ P_{SM}} + \underset{t = 4.82,\ p \leqslant 4.2 \cdot 10^{-4}}{0.4562\ (\pm 0.0946)\ f_Y} - \underset{t = -6.60,\ p \leqslant 3.0 \cdot 10^{-5}}{5.0103\ (\pm 0.7586)\ C(3)}$$

$$n = 16,\ R = 0.9418,\ F = 31.4,\ p < 6.0 \cdot 10^{-6} \qquad (2'')$$

TABLE II

MOLECULAR DESCRIPTORS DERIVED FROM THE GEOMETRY OPTIMIZED STRUCTURES OF BENZODIAZEPINE SOLUTES

No.[a]	P_{SM}[b]	C(3)[c]	W[d]	β_{CCN}[e]	E_{LUMO}[f]	μ^2[g]
1	0.0849	0.1035	9.30	108.01	−0.6967	8.8506
2	1.8635	0.2785	8.74	115.80	−0.9463	3.0976
3	0.0703	0.0960	8.54	112.61	−1.6124	3.1826
4	0.0609	0.0882	9.63	112.22	−1.3241	1.5475
5	0.0680	0.0966	8.67	112.25	−1.6027	0.9722
6	0.0635	0.0977	8.69	112.39	−0.8502	9.8596
7	0.0634	0.0979	8.59	112.45	−0.8534	6.7912
8	0.0578	0.0933	9.56	112.83	−0.8337	6.9907
9	0.6120	0.2388	9.76	112.66	−0.9072	13.1624
10	0.5953	0.2425	8.71	111.39	−0.9306	15.3664
11	0.7049	0.2451	8.60	109.46	−0.9260	10.7387
12	0.6113	0.2353	9.49	112.52	−0.8921	11.1156
13	0.0675	0.0549	10.02	114.06	−0.5324	29.2140
14	0.0600	0.0624	8.64	109.62	−0.8338	7.6286
15	0.0633	0.0651	8.70	109.48	−1.2830	0.9274
16	0.5862	0.3169	10.50	108.56	−1.3073	33.4662
17	0.0784	0.1722	10.29	109.84	−1.2428	27.4995
18	0.0730	0.1725	10.26	109.85	−1.2691	30.0414
19	0.0484	0.1379	10.24	108.51	−1.2497	27.9947
20	0.0320	0.1309	9.20	107.84	−1.1838	32.1489
21	0.0441	0.1184	9.22	107.45	−1.2276	33.8608

[a] Solutes are numbered as in Fig. 1.
[b] Submolecular polarity parameter.
[c] Electron excess charge on carbon at position 3.
[d] Molecular width in Å.
[e] Angle between atoms 2, 3 and 4.
[f] Energy of the lowest unoccupied molecular orbital (eV).
[g] Square of total dipole moment (D^2).

It seemed interesting to compare QSRRs derived for the same set of BDZs in the case of HPLAC with the QSRRs for RP-HPLC. RP-HPLC is widely used to determine hydrophobicity of solutes. The most recommended [17] chromatographic measure of hydrophobicity is log k'_w. This is the logarithm of capacity factor obtained by extrapolation of the linear part of the relationships between log k' and volume percent of organic modifier in binary aqueous eluent to the pure water eluent. The log k'_w data for BDZs are collected in Table I.

The standard reference parameter of hydrophobicity is logarithm of octan-1-ol–water partition coefficient, log P. Since log P is tedious to determine experimentally, computational methods for its evaluation have been developed [18]. Theoretical values of logarithms of octan-1-ol–water partition coefficient for the solutes used in this study, CLOGP, were calculated by the ProLog program and are presented in Table III. We were able to calculate apparently realistic log P values only for the first 15 structures in Fig. 1. Nonetheless, correlation between chromatographic measure of hydrophobicity, log k'_w, and the calculated hydrophobicity parameter was rather poor (R = 0.7500).

When undertaking QSRR studies on data derived by affinity HPLC (the starting hypothesis was that the first eluting enantiomer was retained solely due to non-stereo- and non-enantiospecific, hydrophobic interactions with HSA-CSP. We found that neither chromatographic hydrophobicity parameter, log k'_w, nor the calculated one, CLOGP, accounted for the first enantiomer retention (log k'_1). Correlation between log k'_1 and CLOGP was R = 0.5976 and that with log k'_w was R = 0.7410. In all subsequent QSRRs the log k'_w performed better

than CLOGP in contradiction to occasional suggestions [19,20] that the calculated hydrophobicity parameters can be more reliable than those derived by HPLC.

Eqns. 1 and 2, describing retention on HSA-CSP, comprise hydrophobicity terms. Attempts to replace these submolecular hydrophobicity parameters by log k'_w, a measure of the hydrophobicity of the whole molecule, yielded QSRR eqns. 3 and 4.

Eqns. 3 and 4 are highly significant statistically but their predictive value, as expressed by the correlation coefficient, is lower than that of the corresponding eqns. 1 and 2. Nevertheless, eqns. 3 and 4 which comprise a molecular width term, W, provide additional proof for the steric requirements of retentive interactions between BDZs and HSA-CSP.

The predictive value of QSRR regarding the second eluting enantiomer increases if, in addition to terms present in eqn. 4, the parameter f_X is introduced as in eqn. 5. The negative sign at the coefficient at f_X in eqn. 5 suggests that in this specific instance the parameter f_X reflects steric hindrance rather than solute's hydrophobicity.

The predictive potency of eqns. 3 and 5 is illustrated in Figs. 2 and 3, respectively. Intercorrelations among individual variables in eqns. 3–5 are highest for pairs log k'_1 *vs.* log k'_w (R = 0.7410), log k'_2 *vs.* P_{SM} (R = 0.6623) and log k'_2 *vs.* log k'_w (R = 0.6344); for other pairs of variables $R < 0.5$.

Reversed-phase HPLC

Retention in RP-HPLC, as quantified by log k'_w, is assumed to be due to hydrophobic intermolecular interactions [21]. From the QSRR point of view it seems interesting which structural features provide greatest input to what is called "hydrophobicity". The "best" regression equation describing log k'_w is shown in eqn. 6.

$$\log k'_1 = 0.2821 + \underset{t = 4.68,\ p \leqslant 1.9 \cdot 10^{-4}}{0.6246\ (\pm 0.1334)\ \log k'_w} - \underset{t = -3.17,\ p \leqslant 5.3 \cdot 10^{-3}}{0.1547\ (\pm 0.0487)\ W} \quad (3)$$

$$n = 21,\ R = 0.8431,\ F = 22.1,\ p < 4 \cdot 10^{-5}$$

$$\log k'_2 = -0.2740 + \underset{t = 6.36,\ p \leqslant 4 \cdot 10^{-4}}{0.8685\ (\pm 0.1366)\ \log k'_w} + \underset{t = 6.96,\ p \leqslant 2 \cdot 10^{-4}}{0.4534\ (\pm 0.0652)\ P_{SM}} - \underset{t = 3.70,\ p \leqslant 3 \cdot 10^{-3}}{0.1721\ (\pm 0.0465)\ W} \quad (4)$$

$$n = 16,\ R = 0.9548,\ F = 41.3,\ p < 10^{-6}$$

$$\log k'_2 = -0.6120 + \underset{t = 9.96,\ p \leqslant 10^{-5}}{1.0528\ (\pm 0.1057)\ \log k'_w} + \underset{t = 8.80,\ p \leqslant 10^{-5}}{0.4079\ (\pm 0.0464)\ P_{SM}} - \underset{t = -5.62,\ p \leqslant 1.6 \cdot 10^{-4}}{0.1798\ (\pm 0.0320)\ W}$$

$$- \underset{t = -3.79,\ p \leqslant 3 \cdot 10^{-3}}{0.2638\ (\pm 0.0695)\ f_X} \quad (5)$$

$$n = 16,\ R = 0.9807,\ F = 69.2,\ p < 10^{-6}$$

$$\log k'_w = 1.6541 + \underset{t = 12.04,\ p \leqslant 10^{-5}}{0.5081\ (\pm 0.0422)\ f_Y} + \underset{t = 5.25,\ p \leqslant 10^{-4}}{0.3084\ (\pm 0.0588)\ f_X} - \underset{t = -5.66,\ p \leqslant 5 \cdot 10^{-5}}{1.6641\ (\pm 0.2940)\ C(3)}$$

$$- \underset{t = -4.88,\ p \leqslant 2.4 \cdot 10^{-4}}{0.0103\ (\pm 0.0022)\ \mu^2} + \underset{t = 4.51,\ p \leqslant 4.1 \cdot 10^{-4}}{0.0155\ (\pm 0.0034)\ M_R} \quad (6)$$

$$n = 21,\ R = 0.9582,\ F = 33.7,\ p < 10^{-6}$$

TABLE III

MOLECULAR DESCRIPTORS OF BENZODIAZEPINE SOLUTES CALCULATED FROM ADDITIVE–CONSTITUTIVE STRUCTURAL CONSTANTS

No.[a]	f_Y[b]	f_X[c]	M_R[d]	CLOGP[e]
1	0.88	0.17	80.132	3.029
2	0.88	0.17	95.240	3.103
3	−0.11	0.17	74.994	2.048
4	−0.11	0.31	79.596	2.136
5	−0.11	0.88	79.828	2.788
6	0.88	0.88	78.338	3.788
7	0.88	0.17	73.504	3.048
8	0.88	0.17	78.342	2.919
9	0.88	0.88	84.620	2.853
10	0.88	0.88	79.782	2.982
11	0.88	0.17	74.948	2.242
12	0.88	0.17	79.786	2.113
13	−1.06	0.88	92.834	2.402
14	0.31	0.88	77.916	3.784
15	−0.11	0.88	84.476	3.307
16	0.88	0.17	87.272	–
17	0.88	0.17	85.828	–
18	0.88	0.88	90.662	–
19	0.88	0.31	91.916	–
20	0.31	0.88	95.288	–
21	0.88	0.88	100.358	–

[a] Solutes are numbered as in Fig. 1.
[b] Hydrophobic constant of substituent at position 7.
[c] Hydrophobic constant of substituent at position 2'.
[d] Molecular refractivity according to Vogel [14].
[e] Logarithm of octan-1-ol–water partition coefficient calculated theoretically.

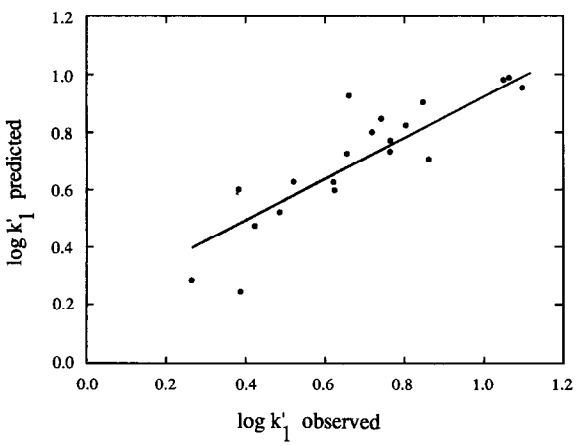


Fig. 2. Relationship between the retention parameters for the first-eluting enantiomer of 1,4-benzodiazepines (log k'_1) determined experimentally by HPLAC on HSA-CSP and calculated from log k'_w and W using eqn. 3.

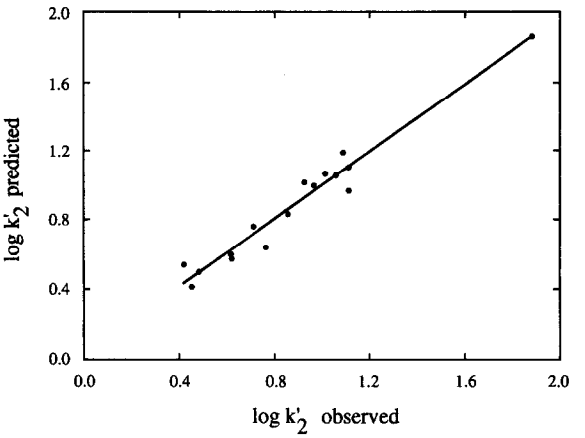


Fig. 3. Relationship between the retention parameters for the second-eluting enantiomer of 1,4-benzodiazepines (log k'_2) determined experimentally by HPLAC on HSA-CSP and calculated from log k'_w and descriptors using eqn. 5.

Intercorrelations among individual variables are low: the highest being $R = 0.6862$ for μ^2 *vs.* M_R and $R = 0.5850$ for log k'_w *vs.* f_Y. The predictive quality of eqn. 6 is illustrated in Fig. 4.

The variables f_Y and f_X in eqn. 6 are highly significant and the regression coefficients at these variables also differ significantly. This observation would suggest the different inputs to the reversed-

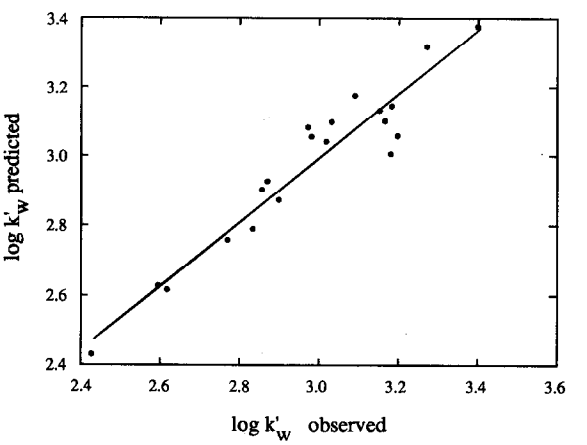


Fig. 4. Relationship between the retention parameters of 1,4-benzodiazepines corresponding to pure buffer eluent (log k'_w) determined experimentally by RP-HPLC on a hydrocarbon-bonded deactivated silica stationary phase and calculated from descriptors using eqn. 6.

phase retention of BDZs due to hydrophobicities of individual molecular regions. Additivity rules for fragmental hydrophobicity parameters usually require the application of some specific correction factors [13,18]. At first approximation, however, the variables f_Y and f_X can be replaced by their arithmetic sum, f_{X+Y}. The resulting eqn. 6′ is of high statistical quality.

Eqn. 6 shows that a molecular bulkiness related structural descriptor, M_R, provides positive input to hydrophobic retention, whereas the molecular polarity related descriptors, $C(3)$ and μ^2, account for the retention decreasing effects in RP-HPLC. This can be rationalized as follows:

(1) M_R reflects the ability of a solute to participate in dispersive (London type) intermolecular interactions. These nonspecific, molecular-size-dependent interactions are obviously stronger between a BDZ solute molecule and a bulky hydrocarbonaceous moiety of Suplex pKb-100 stationary phase than between the same solute molecule and small molecules forming a mobile phase. Thus, the larger M_R the stronger retention.

(2) The reverse holds true when considering parameters reflecting the ability of a solute to take part in more structurally specific, size-independent intermolecular interactions (so called polar interactions), like dipole–dipole, dipole–induced dipole, charge transfer and hydrogen bonding interactions. Such interactions, involving solute and polar molecules of eluent (water, methanol), will increase with the magnitude of polarity descriptors $C(3)$ and μ^2, being at the same time negligible in the case of the system involving the solute molecule and the hydrocarbon moiety of the stationary phase.

Eqn. 6 strongly resembles the QSRR equations describing retention on regular octadecylsilica [3] and polybutadiene-coated alumina [4] reversed-phase materials. In the later equations the net positive input to retention due to dispersive intermolecular interactions was quantified by such molecular-size-dependent descriptors as total energy, molecular-graph-derived indices, molecular refractivity or just molecular mass. The net negative input to retention was evaluated by means of a total dipole, a localized dipole or by a parameter reflecting maximum electron excess charge differences within the molecule.

If in QSRR analysis of the RP-HPLC retention data the submolecular polarity parameter, P_{SM}, is considered instead of the square of total dipole moment, μ^2, eqn. 7 results.

Replacing the variables f_X and f_Y in eqn. 7 by their sum, f_{X+Y}, results in eqn. 7′:

$$\log k'_w = 1.8226 + \underset{t = 9.73,\ p \leqslant 1.0 \cdot 10^{-5}}{0.4442\ (\pm 0.0456)\ f_{X+Y}} - \underset{t = -3.80,\ p \leqslant 1.6 \cdot 10^{-3}}{1.1866\ (\pm 0.3122)\ C(3)} - \underset{t = -3.59,\ p \leqslant 2.5 \cdot 10^{-3}}{0.0096\ (\pm 0.0026)\ \mu^2} +$$

$$+ \underset{t = 2.97,\ p \leqslant 9.0 \cdot 10^{-3}}{0.0120\ (\pm 0.0040)\ M_R} \qquad (6')$$

$$n = 21,\ R = 0.9302,\ F = 25.68,\ p < 1.0 \cdot 10^{-6}$$

$$\log k'_w = 2.8824 + \underset{t = 11.16,\ p \leqslant 10^{-5}}{0.5129\ (\pm 0.0460)\ f_Y} + \underset{t = -6.70,\ p \leqslant 10^{-5}}{0.3728\ (\pm 0.0597)\ f_X} - \underset{t = -6.70,\ p \leqslant 10^{-5}}{3.0641\ (\pm 0.4570)\ C(3)} +$$

$$+ \underset{t = 4.40,\ p \leqslant 4.5 \cdot 10^{-4}}{0.3164\ (\pm 0.0719)\ P_{SM}} \qquad (7)$$

$$n = 21,\ R = 0.9478,\ F = 35.4,\ p < 10^{-6}$$

$$\log k'_w = 2.8105 + \underset{t = 10.59,\ p \leqslant 1.0 \cdot 10^{-5}}{0.4658\ (\pm 0.0440)\ f_{X+Y}} - \underset{t = -5.81,\ p \leqslant 2.0 \cdot 10^{-5}}{2.7234\ (\pm 0.4684)\ C(3)} + \underset{t = 3.93,\ p \leqslant 1.0 \cdot 10^{-3}}{0.3097\ (\pm 0.0787)\ P_{SM}}$$

$$n = 21,\ R = 0.9329,\ F = 38.0,\ p < 1.0 \cdot 10^{-6} \qquad (7')$$

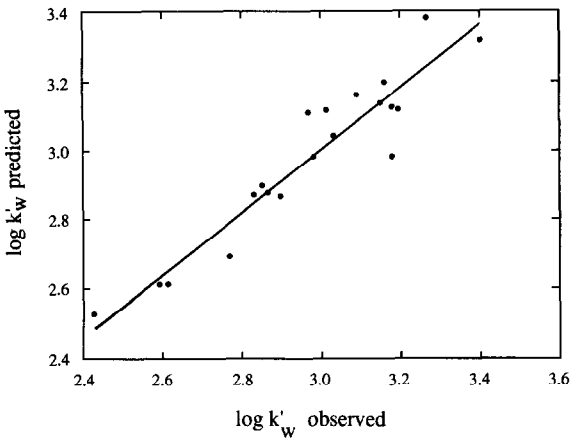


Fig. 5. Relationship between the retention parameters of 1,4-benzodiazepines corresponding to pure buffer eluent (log k'_w) determined experimentally by RP-HPLC on a hydrocarbon-bonded deactivated silica stationary phase and calculated from descriptors using eqn. 7.

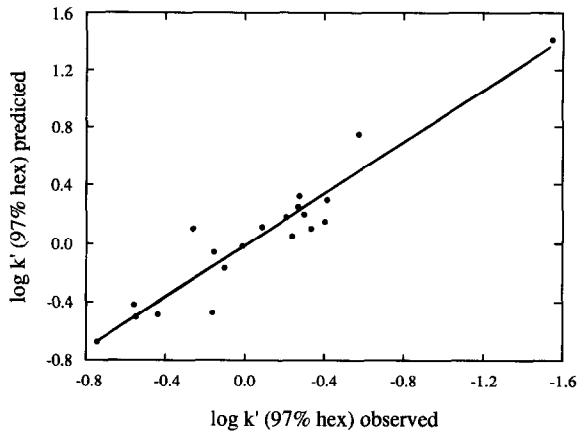


Fig. 6. Relationship between the retention parameters of 1,4-benzodiazepines [log k' (97% hex)] determined experimentally by NP-HPLC on a graphitized carbon stationary phase with hexane–propan-1-ol (97:3, v/v) eluent and calculated from descriptors using eqn. 8.

The statistical quality of eqn. 7 is similar to that of eqn. 6 as is its predictive value (Fig. 5). The highest intercorrelations among the variables of eqns. 7 and 7′ are between $C(3)$ and P_{SM} ($R = 0.7651$) and between log k'_w and f_{X+Y} ($R = 0.7781$). The parameter P_{SM} seems to be able to replace μ^2 and M_R in eqn. 6. This can, however, be fortuitous and discussion of P_{SM} as a net hybrid measure of dipolar and dispersive properties appears premature.

Adsorption HPLC

The QSRR equation derived for log $k'_{H\text{-}P}$ data (eqn. 8) has a different form from the relationships so far discussed. The relationship between the experimental and calculated log $k'_{H\text{-}P}$ is given in Fig. 6. There is an intercorrelation of $R = 0.7187$ between log $k'_{H\text{-}P}$ and P_{SM}. Other intercorrelations are $R < 0.5$. Similarity between eqn. 8 and eqns. 2 and 4, describing retention of second eluting enantiomer in HPLAC, is limited to the presence of submolecular polarity term, P_{SM}. Evidently, some kind of dipole–dipole and/or dipole–induced dipole interactions is operating at site II of HSA-CSP and in NP-HPLC on graphitized carbon. In the latter case there are no steric restrictions for effective interactions. Instead, the variables E_{LUMO} and (to a lesser extent) μ^2 account for retention. Equations comprising a measure of localized dipole similar to P_{SM} and the E_{LUMO} parameter as independent variables were previously derived to describe retention of a series of monofunctional benzene derivatives on metallic stationary phases [5,6]. The dominant retention mechanism postulated was an electron pair donor–electron pair acceptor interaction. This mechanism appears to operate also in the case of BDZs chromatographed on porous graphitic carbon under normal-phase conditions.

CONCLUSIONS

The results of this study demonstrate differences in mechanism of retention in affinity, reversed-phase and adsorption HPLC modes. At the same time, the conclusions which can be drawn from the

$$\log k'_{H\text{-}P} = -1.5582 + \underset{t = 10.26,\ p \leqslant 10^{-5}}{1.0250\ (\pm 0.0999)\ P_{SM}} - \underset{t = -7.17,\ p \leqslant 10^{-5}}{1.1220\ (\pm 0.1564)\ E_{LUMO}} + \underset{t = 2.19,\ p \leqslant 0.05}{0.0078\ (\pm 0.0036)\ \mu^2} \quad (8)$$

$$n = 20,\ R = 0.9428,\ F = 42.6,\ p < 10^{-6}$$

QSRRs are consistent with the commonly recognized qualitative models. The QSRRs identify retention affecting structural factors and provide quantitative estimation of their importance. Thus, a question arises whether QSRR analysis should not replace the speculations that a given property or functional group rather increases and another tends to decrease retention. Certainly, detection of informative and quantifiable structural descriptors still requires experience (and chemical intuition) but accessibility of modern molecular modelling facilities simplifies the task. QSRR analysis could be an important component in introduction of new separation modes and/or stationary phase materials.

QSRR analysis is especially challenging for highly stereospecific separations. We found a single report by Wolf *et al.* [22] on calculating the capacity factor of one of the enantiomers of a series of racemates chromatographed on cellulose triacetate. The authors used a negative electrostatic potential around the chiral centre of the solute, E_{00}, and the probability, Ω, of assuming a flat conformation by the molecule as variables in a regression equation. The enantiomer distinguishing structural parameter P_{SM} in our eqns. 2 and 4 is similar in nature to E_{00}, however, P_{SM} is simpler and more readily determinable. At the same time the retention on HSA-CSP appears to be a more complex process than that on cellulose triacetate. Eqns. 1 and 2 compensate for this complexity and were of help in establishing the topography of benzodiazepine binding sites on HSA [16,23].

QSRR regression equations are readily interpretable in mechanistic physical terms. There is another multivariable statistical approach to QSRR which is based on factorial methods of data analysis. The approach consists in condensing most of the information dispersed over a multitude of various molecular descriptors into a few abstract principal factors or components. It usually appears difficult to assign a definite physical meaning to individual principal components and the approach is rather aimed at prediction of retention data, *e.g.*, enantioselectivity [24]. Another approach attempts to associate substituents with contributions to the observed enantioselectivity [25].

Retention in RP-HPLC was found to be a net effect of non-specific, dispersive, bulkiness-dependent interactions and directional electrostatic interactions involving solute molecules and molecules of both mobile and stationary phases. Former interactions are stronger for a solute–stationary phase system than for a solute–mobile phase system. The reverse is true with polar interactions. QSRRs derived here support generally observed mechanism of RP-HPLC on hydrocarbonaceous stationary phase materials [2–4]. The material used here, Suplex pKb-100, is characterized by an extremely high level of deactivation. Suppression of silanophilic interactions has been confirmed by our observation of excellent linearity of dependence of log k' on organic modifier concentration in mobile phase at a wide eluent composition range. This property would facilitate determinations of reliable and reproducible chromatographic measures of the hydrophobicity of solutes.

A separate question is whether the chromatographic hydrophobicity scale parallels the reference log P scale determined by slow equilibration methods. This is probably not the case since the correlation between the normalized chromatographic hydrophobicity parameter, log k'_w, and the calculated log P (CLOGP) is low. Besides, hydrophobicity is a property which depends as much on the nature of the solutes considered as on the properties of the environment in which it is measured. Chromatographic and octan-1-ol–water experimental hydrophobicity parameters may differ more or less but this does not imply lower reliability of the HPLC approach. Chromatographic measurement of hydrophobicity is certainly more reliable than an *a priori* calculated log P.

The mechanism of retention in NP-HPLC on graphitized carbon revealed by eqn. 8 is as expected for adsorption chromatography. Retention is not affected significantly by molecular size of solutes. Non-specific, dispersive interactions are dominated by the more chemically specific directional electrostatic and charge transfer interactions. There are no geometric restrictions regarding the fitting of a solute to any definite binding (adsorption) site as it is the case with HPLAC.

As demonstrated here QSRR analysis provides rationale for different separation patterns observed in individual HPLC systems. Meaningful QSRRs can be of help in understanding fundamental processes at the basis of chromatographic separations and in a rationally guided search for optimum

separating systems. With further progress in molecular modelling and chemometrics also the analytical prediction of retention data will no longer be restricted to closely congeneric sets of solutes.

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