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ORIGINAL RESEARCH

Retention–activity relationship studies of benzodiazepines by micellar liquid chromatography

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ABSTRACT: Solute partitioning into lipid bilayers of biological membranes is the basis for drug and metabolite uptake, passive transport across membranes and bioaccumulation. In order to emulate *in vitro* the partitioning process in the biomembranes, different approaches have been proposed. The use of micellar solutions as mobile phases in reversed-phase liquid chromatography (micellar liquid chromatography, MLC) has proven to be valid in the prediction of the biological activities of local anesthetics, catecholamines and barbiturates. In this paper we focus our attention on benzodiazepines. The retention of benzodiazepines using different concentrations of Brij35 as micellar mobile phase in modified C_{18} stationary phase was studied. Quantitative retention–activity relationships (QRAR) in MLC were investigated for these compounds. An adequate correlation between the capacity factors (log *k*) and the toxicity (log LD_{50}) and anxiolitic activity (log ED_{50}) of benzodiazepines was obtained. Copyright © 1999 John Wiley & Sons, Ltd.

INTRODUCTION

The biological activity of a drug could be viewed as a summation of the different interactions that a compound undergoes both during transport through the biological membranes and in reaction with the active site. These interactions are assumed to be governed by the chemical structure of the compound (Hansch, 1971; Goldberg, 1983). A change in the structure can result in a change in biological response. For a group of drugs with similar structural features that act in a specific receptor site, the biological activity is basically determined by the solute partitioning into lipid bilayers of biological membranes. As these membranes have a lipid nature, hydrophobic compounds tend to cross through these membranes easily. Electronic and steric properties of compounds are also of great importance.

In order to emulate *in vitro* the partitioning process in the biomembranes, different approaches have been proposed. Measurement of the partition coefficient in the biphasic octanol–water solvent system, log *P*, has been one of the most commonly used methods (Hansch and Fujita, 1964; Hansch and Leo, 1987). However, the organic solvent–aqueous partitioning systems are good models for solute–membrane partitioning only when polar group interactions between the solute and the phospholipid bilayer are minimal or absent (Rodgers and Wong, 1980). Another approach to studying solute–membrane interactions is to obtain liposome partition coefficients from liposome suspensions of phospholipids (Katz and Diamond, 1974). However, although liposomes can model both polar and non-polar solute–membrane interactions, the method is time consuming and experimentally laborious.

Chromatography is a powerful technique for the measurement of physicochemical parameters. The application of chromatographic parameters in structure–activity relationships gives rise to new field, quantitative retention–activity relationships (QRAR) (Kaliszan, 1987; Dorsey and Khaledi, 1993; Lambert, 1993). Firstly, the retention of compounds on reversed-phase liquid chromatography using octadecyl silica (ODS) stationary phases was used to correlate biological activity. However, with this stationary phase the interactions between solutes and the polar lipid head groups of biomembranes are not modeled (Ong *et al.*, 1995). In order to overcome this limitation, different reversed-phase stationary phases

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Abbreviations used: AL, alprazolan; BR, bromazepam; CB, clobazam; cmc, critical micellar concentration; CR, clorazepato; Dl, diazepam; FL, flunitrazepam; LA, lorazepam; LM, lormetazepam; MI, midazolam; MLC, micellar liquid chromatography; ODS, octadecyl silica; OX, oxacepam; QRAR, quantitative retention–activity relationships; QSAR, quantitative structure–activity relationships; RMSEC, root-mean-square error of calibration; RMSECV, root-mean-square error of cross-validation; TE, tetrazepam.

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NEURELIS, INC., EX2066, page 1
PADAGIS US LLC v. NEURELIS, INC.
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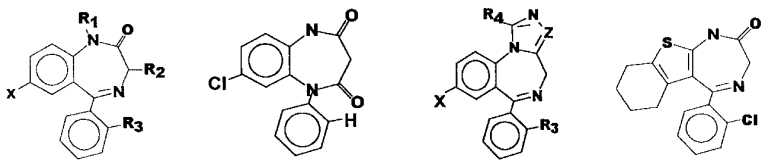
Table 1. Structure and log *P* values for non-ionic forms of the benzodiazepines studied



(A) (B) (C) (D)

Compound	General structure	R_1	R_2	R_3	R_4	X	Z	log P[a]
Bromazepam	A	-H	-H		—	-Br	—	1.83
Clorazepato	A	-H	-COOH	-H	—	-Cl	—	2.67
Diazepam	A	$-CH_3$	-H	-H	—	-Cl	—	3.18
Flunitrazepam	A	$-CH_3$	-H	-F	—	$-NO_2$	—	2.35
Lorazepam	A	-H	-OH	-Cl	—	-Cl	—	2.48
Oxazepam	A	-H	-OH	-H	—	-Cl	—	2.31
Lormetazepam	A	$-CH_3$	-OH	-Cl	—	-Cl	—	2.37
Tetrazepam	A	$-CH_3$	-H		—	-Cl	—	3.90
Clobazam	B	—	—	—	—	—	—	2.51
Alprazolam	C	—	—	-H	$-CH_3$	-Cl	N	2.06
Midazolam	C	—	—	-F	$-CH_3$	-Cl	C	2.7
Bentazepam	D	—	—	—	—	—	—	3.36

[a] Values were taken from Hansch (1990).

have been synthesized with the aim of incorporating polar groups in the chromatographic surfaces. Chromatographic surfaces have been synthesized by covalently immobilizing phospholipids onto silica propyl amide particles (Ong *et al.*, 1995 1996; Liu *et al.* 1995) or by immobilizing liposomes in capillary continuous beds with covalently linked C_4 or C_8 alkyl ligands (Zhang *et al.*, 1996).

A simpler and reproducible approach uses micellar liquid chromatography (MLC). MLC is a mode of reversed-phase liquid chromatography, which uses a surfactant solution above the critical micellar concentration (cmc) as mobile phase (Armstrong and Nome, 1981; Arunyanart and Cline-Love, 1984). The use of micellar solutions produces the adsorption of surfactant monomers to the stationary phase, providing it with both hydrophobic and electrostatic sites of interaction (Berthod *et al.*, 1986; Medina-Hernández and García Alvarez-Coque 1992). There are a number of similarities between the mobile phase–modified stationary phase in MLC and the membrane–water interface. The stationary phase modified by the adsorption of surfactant monomers structurally resembles the ordered array of the membranous hydrocarbon chains. In addition, the hydrophilic/hydrophobic character of surfactants adsorbed could be expected to resemble the polar membrane regions.

Successful applications of MLC to quantitative retention–activity relationships have been reported to describe substituted phenol bioactivity (Breyer *et al.*,1991), the anesthetic potency of local anesthetics (Escuder-Gilabert *et al.*, 1998a, b), the hypnotic activity of barbiturates (Cuenca-Benito *et al.*, 1998), and the α- and β-adrenergic activity of catecholamines (Sanchis-Mallols *et al.*, 1997). In this paper we have focused our attention on benzodiazepines.

Benzodiazepines are the most widely used anxiolytic drugs. They are also used as muscle relaxants, in the treatment of insomnia and some forms of depression, and in controlling severe epileptic seizures and schizophrenia. All benzodiazepines are absorbed orally. The equilibrium between plasma and brain is reached quickly, because all of them are sufficiently hydrophobic and they cross the blood–brain barrier easily (Litter, 1993). Studies have been performed on the structure–activity relationships of the 1,4-benzodiazepines (Clarck and Giudice, 1978). The results indicate that various substituents determine the ligand's pharmacological properties: its affinity to bind the receptor, and its intrinsic efficacy or capability in producing a functional change in the receptor.

In this paper, the usefulness of micellar liquid chromatography to establish the retention–activity relationships of benzodiazepines is studied. The advantages and limitations are discussed of using a single parameter (e.g. retention in MLC) for describing the activity of such a short data series of drugs (e.g. benzodiazepines).

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NEURELIS, INC., EX2066, page 2
PADAGIS US LLC v. NEURELIS, INC.
IPR2025-00465, U.S. PATENT 11,241,414

EXPERIMENTAL

Instruments and measurement. A Hewlett-Packard (HP) 1100 chromatograph with an isocratic pump, a UV–visible detector and an HP Vectra computer were used (Palo Alto, CA, USA). Data acquisition and processing were performed on an HP Vectra XM computer (Amsterdam, The Netherlands) equipped with HP-Chemstation software (A0402, 1996). The solutions were injected into the chromatograph through a Rheodyne valve (Cotati, CA, USA) with a 20 μL loop. A Kromasil C_{18} column (5 μm, 150 × 4 mm) and a guard column of similar characteristics (35 × 4 mm) (Scharlau, Barcelona, Spain) were used. The mobile phase flow rate was 1 mL/min. The detection was performed in UV at 240 nm. All the assays were carried out at room temperature.

Reagents and standards. Mobile phases were prepared using aqueous solutions of polyoxyethylene (23) lauryl ether (Brij35, Acros Chimica, Geel, Belgium). The pH of the micellar eluent was adjusted to 7.4 with 0.05 M phosphate buffer. It was prepared with disodium hydrogen phosphate and sodium di-hydrogen phosphate (analytical reagent, Panreac, Barcelona, Spain). In order to reproduce the osmotic pressure of biological fluids, NaCl (9.20 g/L; purissim, Panreac) was added to the micellar mobile phase. Benzodiazepines were obtained from differents Spanish pharmaceutical laboratories: alprazolam (AL; Trankimazin,® Upjohn farmoquímica, S.A., Madrid, Spain), lorazepam (LA) and lormetazepam (LM; Orfidal® and Loramet,® Wyeth-Orfi, S.A., Madrid, Spain), flunitrazepam (FL), bromazepam (BR), diazepam (DI) and midazolam (MI; Rohipnol,® Lexatin,® Valium® and Dormicum,® Productos Roche, S.A., Madrid, Spain), clorazepato (CR) and tetrazepam (TE; Tranxilium® and Myolastan,® Sanofi Winthrop, S.A., Barcelona, Spain), clobazam (CB; Noiafren,® Hoechst farma, S.A., Barcelona, Spain), and oxacepam (OX; Adumbran,® Boeringer ingelheim, S.A., Barcelona, Spain). Bentazepam was kindly donated by Knoll S.A., Madrid. Stock standard solutions of benzodiazepines were prepared by dissolving 10 mg of the compound in 10 mL of phosphate buffer. Working solutions were prepared by dilution of the stock standard solutions using phosphate buffer solution. The solutions were stored in the refrigerator at 4°C.

Barnstead E-pure, deionized water (Sybron, Boston, MA, USA) was used throughout. The mobile phase and the solutions injected into the chromatograph were vacuum-filtered through 0.45 and 0.22 μm Nylon membranes, respectively (Micron Separations, Westboro, MA, USA). All retention factors were obtained by triplicate injections of compound solutions.

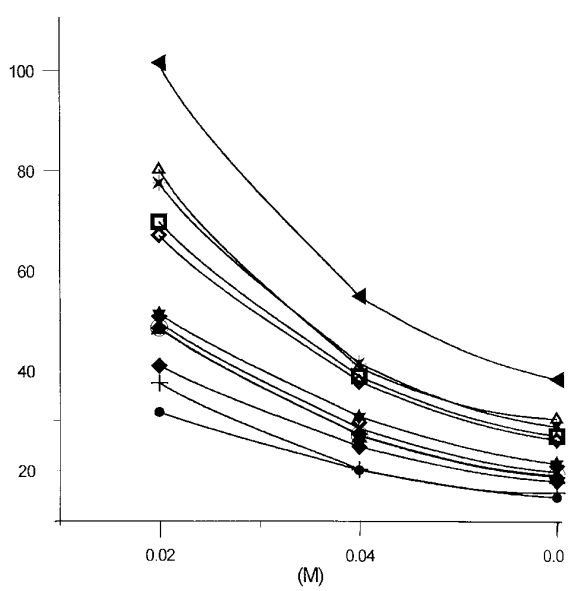

Figure 1. Effect of Brij35 concentration in mobile phase on the retention of benzodiazepines: (●) BR; (+) AL; (◆) CB; (○) OX; (▲) LM; (×) LA; (★) FL; (□) DI; (◊) CR; (*) BE; (△) MI; and (▲) TE.

RESULTS AND DISCUSSION

Structure–retention relationships

Table 1 shows the structure and log *P* values for the non-ionic forms of the studied benzodiazepines. The core of all benzodiazepines is a benzene ring joined to a seven-membered 1,4-diazepine ring. The retention of benzodiazepines was measured using Brij35 as micellar mobile phase. The mobile phase pH was adjusted to 7.4 to obtain experimental conditions as close as possible to physiological pH. At this pH value all benzodiazepines studied except clorazepato are neutral or slightly positively charged while clorazepato is negatively charged. Figure 1 shows the effect of the Brij35 mobile phase concentration (0.02, 0.04 and 0.06 M) on the retention of compounds. As can be observed, for most hydrophobic compounds (tetrazepam, bentazepam, diazepam, midazolam and clorazepato), large changes in the retention were obtained upon increasing the Brij35 concentration in the mobile

Table 2. Retention data (log *k* values at 0.02 M Brij35) and biological activities of benzodiazepines

Drug	log *k*	LD_{50}[a] (mg/kg)	ED_{50}[b] (mg/kg)
Bromazepam	1.50	3050	34.2
Alprazolam	1.57	2010	
Clobazam	1.61	2000	38
Lormetazepam	1.68	1148	28.5
Oxazepam	1.68	1370	28.5
Lorazepam	1.69	1160	
Flunitrazepam	1.71	870	23.6
Clorazepato	1.82	700	11.7
Diazepam	1.84	710	12
Midazolam	1.90	60	
Tetrazepam	2.00		

[a] LD_{50} indicates the dose of drug causing death within 72 h in 50% of the mice treated orally. Data from *Index Merck*, 9th edn.
[b] ED_{50} indicates the dose of drug causing activity in 50% of mice treated orally tested against maximum electroshock (evaluated using the antipentylenetetrazole test). Data from (1970).

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NEURELIS, INC., EX2066, page 3
PADAGIS US LLC v. NEURELIS, INC.
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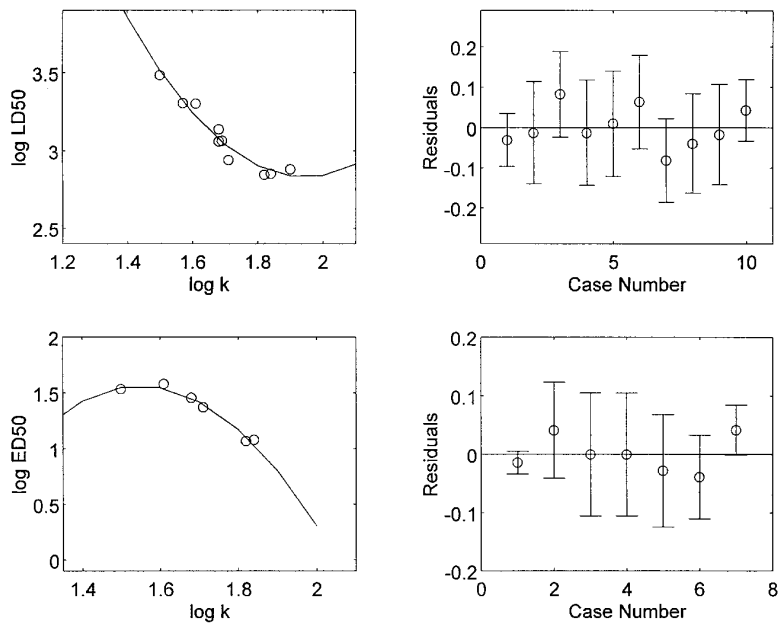

Figure 2. log k–log (LD_{50}) (upper left part) and log k–log (ED_{50}) (lower left part) relationships for different benzodiazepines at 0.02 M Brij35 concentration in mobile phase. Residuals with their confidence intervals at 95% probability level (right part) for both QRAR models are included.

phase, while for the less hydrophobic compounds (bromazepam, alprazolam, flunitrazepam, oxazepam, lorazepam, lormetazepam and clobazam) the retention was scarcely modified.

When the log k values of compounds obtained for a certain mobile phase were correlated with the corresponding log P values for the non-ionic forms of compounds, relatively low correlation coefficients were obtained: $r^2 = 0.89$, 0.87 and 0.91 for 0.02, 0.04 and 0.06 M Brij35 concentrations, respectively. This may suggest that benzodiazepine retention not only depends on the hydrophobic interactions but also on the compound's ionization degree and steric properties.

Retention–activity relationships

QRAR models for benzodiazepine derivatives. Interpretation of the benzodiazepine structure–activity relationships (Clarck and Giudice, 1978) indicates that molecular features determine the drug–receptor interaction and consequently the biological activity. Since chromatographic retention in MLC is expected to also be related to these molecular features, the activity prediction can be made directly from log k data (QRAR) without going via molecular property determination or estimation (QSAR). Table 2 shows the biological activities of benzodiazepines found in the literature.

Table 3. Statistical analysis and predictive features of the QRAR models, log (activity) = $a + b$ (log k) + c (log k)^2 corresponding to the retention data obtained using a 0.02 M Brij35 mobile phase[a]

Activity (n)	$a \pm$ ts (p-value)	$b \pm$ ts (p-value)	$C \pm$ ts (p-value)	R^2 (r-adj.^{2})	SE	F (p-value)	RMSEC	RMSECV	RMSECVi
log LD_{50} (10)	16 ± 8 (0.0028)	-13 ± 10 (0.0145)	4 ± 3 (0.0253)	0.95 (0.93)	0.057	63.28 (<0.0000)	0.048	0.078	0.061
log ED_{50} (7)	-13 ± 9 (0.0151)	19 ± 11 (0.0079)	-6 ± 3 (0.0059)	0.98 (0.97)	0.038	86.23 (0.0005)	0.029	0.105	0.042

[a] n, number of available activities; ts, 95% confidence interval for coefficient estimates; r-adj.^{2}, r^2 adjusted for degrees of freedom; SE, standard error of the estimate; F, F-ratio; RMSEC, root mean square error of calibration; RMSECV, root mean square error of cross-validation (leave-one-out); RMSECVi root mean square error of cross-validation (leave-one-out) for interpolated data.

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NEURELIS, INC., EX2066, page 4
PADAGIS US LLC v. NEURELIS, INC.
IPR2025-00465, U.S. PATENT 11,241,414

Other biological activities were not included because the number of available data was too low ($n < 5$). Figure 2 shows the relationships between two biological activities of benzodiazepines and their retention data together with the residual plots. The first (Fig. 2, upper part) is the toxicity of benzodiazepines expressed as log LD_{50}. The second parameter studied was the clinical activity of the benzodiazepines expressed as log ED_{50} (Fig. 2, lower part). As can be observed, the dependence between the biological and retention data seems to be nonlinear. In fact, when linear models were assayed, the residual values for extreme data were statistically non-zero (95% probability). This nonlineal dependence is usual in structure–activity relationship studies [QSAR models, eg $\log(1/C) = a \log P + b (\log P)^2 + c$] (Hansch, 1990).

The QRAR models obtained by adjusting the logarithm of biological activities and the retention of compounds obtained using 0.02, 0.04 and 0.06 M Brij35 concentration as micellar mobile phase were, respectively:

$$\log(LD_{50}) = 16(4) - 13(4)\log k + 3.3(1.2)(\log k)^2; \quad r^2 = 0.94 \tag{1}$$

$$\log(LD_{50}) = 12(5) - 10(7)\log k + 3(2)(\log k)^2; \quad r^2 = 0.91 \tag{2}$$

$$\log(LD_{50}) = 17(4) - 20(6)\log k + 7(2)(\log k)^2; \quad r^2 = 0.94 \tag{3}$$

$$\log(ED_{50}) = -13(3) + 19(4)\log k - 6.1(1.1)(\log k)^2; \quad r^2 = 0.98 \tag{4}$$

$$\log(ED_{50}) = -12(3) + 21(5)\log k - 7.7(1.6)(\log k)^2; \quad r^2 = 0.98 \tag{5}$$

$$\log(ED_{50}) = -10(4) + 20(6)\log k - 8(2)(\log k)^2; \quad r^2 = 0.97 \tag{6}$$

where the numbers in brackets represent the standard deviation of the estimates.

Table 3 shows the statistical analysis and the predictive features of the QRAR models obtained using 0.02 M Brij 35 mobile phase. As can be observed, since the *p*-values for QRAR models are less than 0.05, there are statistically significant relationships between log LD_{50} or log ED_{50} and log *k* at the 95% confidence level. In both cases, the coefficients are significant at 95% confidence level ($p < 0.05$). The standard error (SE) can be used to construct prediction limits for new observation. Based on these results, it is possible to estimate the activities of data not available in Table 2, so the estimated log LD_{50} value for tetrazepam is 2.8 ± 0.3 and the estimated log ED_{50}s for alprazolam, lorazepam, midazolam and tetrazepam are 1.56 ± 0.12, 1.43 ± 0.12, 0.8 ± 0.18 and 0.3 ± 0.3, respectively.

Some of the previous estimations are interpolations, but there are also extrapolated values. Therefore, it is interesting to compare the predictive ability of the models in terms of cross-validated data. We suggest the comparison between the fit error (e.g. the root-mean-square error of calibration, RMSEC) and the prediction error based on cross-validation (e.g. root-mean-square error of cross-validation, RMSECV). The RMSEC tells us about the average deviation of the model from the data:

$$\mathrm{RMSEC} = \sqrt{\frac{\sum_{i=1}^{n}(\hat{y}_i - y_i)^2}{n}} \tag{7}$$

where $\hat{y}_i$ is the predicted activity when all the *n* molecules are included in the model formation. In contrast, the RMSECV is a measure of the model ability to predict new activities. RMSECV is defined as in eqn 7 except that *y* are predictions for molecules not included in the model formulation (eg each one of the calibration molecules is used as test in turn for the model chosen on the remaining molecules, performing the procedure $n-1$ times, which is referred as 'leave-one-out cross-validation'). Since in the RMSECV parameter both interpolation and extrapolation information are mixed, we propose an additional parameter for measuring only the interpolation information (e.g. excluding the two extreme data, after ordering them by their log *k* values): 8

$$\mathrm{RMSECVi} = \sqrt{\frac{\sum_{i=2}^{n-1}(\hat{y}_i - y_i)^2}{n-2}} \tag{8}$$

It is expected that new molecules of the same family of compounds as those used for model calibration should not behave as outliers, so the predictive ability of the model must be well defined by RMSECV and RMSECVi. On the other hand, the more differences there are between RMSEC and RMSECV or RMSECVi values, the lower QRAR model robustness is, and then more caution must be taken in future predictions.

The RMSECV, RMSEC and RMSECVi values obtained for the QRAR models studied are shown in Table 3. As can be seen, when the log LD_{50} values were used as dependent variables, both RMSECV and RMSECVi values were relatively similar and similar with respect to the RMSEC value. This suggests that both interpolations and extrapolations of LD_{50} activities based on the current QRAR model should be reasonably adequate. In contrast, in the ED_{50} case, the RMSECV value is notoriously larger than RMSECVi or RMSEC values, which indicates that some caution must be taken with extrapolated ED_{50} data. In spite of this, the

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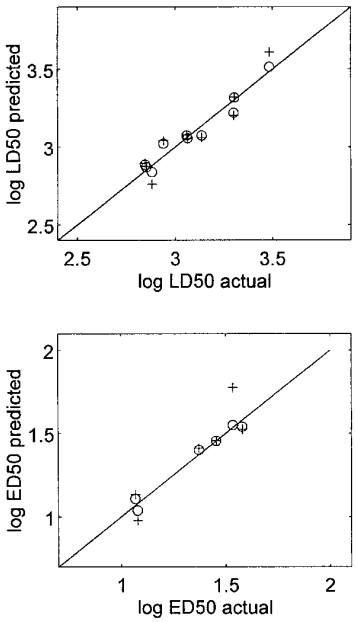

Figure 3. Validation plots for QRAR models: predicted log (LD_{50}) (upper part) and log (ED_{50}) (lower part) vs actual activities. Fitted (○) and cross-validated (+) results are shown.

information obtainable may be useful from a practical point of view (e.g. an almost null log ED_{50} activity for tetrazepam is expected according to the current QRAR model).

The predictive ability of the QRAR models can be also evaluated from the validation plots (Fig. 3), which show the predicted (fitted and cross-validated) vs actual activities for the available data (Table 2). As can be seen, the ability of log *k* values to describe LD_{50} and ED_{50} activities of benzodiazepines is adequate.

In addition, the log *k* parameter was clearly superior to the classical QSAR descriptor, log *P*. Worse results were obtained using polynomial ($r^2 < 0.73$) or linear ($r^2 < 0.55$) models using log *P* as independent variable, and in all cases the estimates were statistically non-significant at 95% probability level.

QSAR vs QRAR models for short data series. In computational quantitative structure–activity relationship (QSAR) studies, physicochemical characteristics of compounds such as hydrophobic, electronic and steric properties are used as parameters to correlate activity with structures using different chemometric approaches. In classical QSAR based on MLR analysis, initially the number of samples is required to be at least five times (preferably more than 10 times) the number of molecular descriptors in order to obtain both interpretative and predictive equations, which is the main purpose of QSAR studies. However, often it is almost impossible to process enough data for QSAR studies of a family of drugs, sometimes because there is a low number of available compounds and sometimes because their biological activity data have not been studied. In this nearly collinear data situation, latent variables instead of the original descriptors [as obtained by principal component regression, (PCR) or partial least squares (PLS) methods] must be used. In this case, the descriptive ability of the model may be devalued for the benefit of predictive ability.

In our opinion, due to the widespread clinical use of certain drugs, the advantages of obtaining a method for estimation of potential activity of a new molecule (eg a new synthesized compound from a generic molecular structure) justify the development of predictive models. The use of a single parameter, instead of molecular descriptors, may solve QSAR-MLR drawbacks for short data series. This single parameter should imply hydrophobic, electronic and steric contributions to the free energy change in the biological response.

We consider that the retention (log *k*) of drugs in MLC satisfies this requisite, since the chromatographic column reproduces the biological environment quite well. On the other hand, log *k* values are empirical variables easily measurable and reproducible. This approach involving quantitative retention–activity relationships (QRAR) may be an alternative to QSAR models in order to obtain estimation, or at least useful qualitative information about drug activity.

CONCLUSIONS

Chromatographic surfaces modified by absorption of surfactants (MLC) resemble the lipidic bilayers of biological barriers. In addition, the retention of compounds in MLC, which depends on hydrophobic, electrostatic and steric properties of compounds, is obtained in flow conditions in similar way to the phenomena of absorption, transport, metabolism and excretion of drugs in the body. Consequently, a single MLC retention parameter, log *k*, is capable of describing the biological activity of benzodiazepines (QRAR) better than a single conventional molecular descriptor such as log *P* (QSAR). On the other hand, when the number of available activity data of compounds is low, multivariate QSAR models using additional molecular descriptors are not statistically adequate.

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PADAGIS US LLC v. NEURELIS, INC.
IPR2025-00465, U.S. PATENT 11,241,414