Determination of Conformer-Specific Partition Coefficients in Octanol/Water Systems

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The first conformer-specific experimental partition coefficients are presented for octanol/water, the most widespread solvent system to predict lipophilicity of drugs. Rotamer populations in octanol and water were elucidated from ^{1}H NMR vicinal coupling constants and were combined with classical partition coefficients to obtain the conformer-specific ones. Feasibility of the determination of conformer-specific partition coefficients is exemplified on amphetamine and clenbuterol, two flexible drug molecules. Partition capacities of the amphetamine rotamers have been proven to be essentially equal. The conformers of clenbuterol, however, have been found to be greatly different in partition properties, which could be interpreted in terms of intramolecular interactions between the vicinal polar sites and the solvent-accessibility of the groups. The conformers could be put into order of their membrane-influx and -outflow propensities. Deviations between experimental and calculated log *P* values could also be interpreted in view of the species-specific partition coefficients.

Introduction

Membrane transition capacity is a key component of targeted drug delivery. Its physicochemical prediction is usually based upon the partition coefficient of the drug, expressed typically in terms of octanol/water log *P* values. The significance of partition in drug research has recently been recognized by international conferences and books devoted entirely to the field of lipophilicity and log *P* determinations.[1,2] Techniques for the measurement of partition coefficients include the classical shake-flask and stir-flask methods, dual-phase potentiometric titrations, reversed phase planar and liquid chromatographic procedures, cyclic voltammetry, centrifugal partition chromatography, counter-current distribution, rotating diffusion cells, etc.[2] By far the most widespread solvents for partition experiments are octanol and water, but other immiscible solvent pairs have also been applied. Several calculation-based predictive methods and their softwares also appeared to estimate partition coefficients.[3−6]

The idea of conformer-specific partition coefficients appeared in 1979.[7] Davies et al. defined the new species-specific parameter as the individual partition coefficient of distinct conformers. They hypothesized that membrane penetration of drugs is bound to particular conformers but they presented neither methodology nor experimental data. Thus, the thousands of experimental partition coefficients reported so far are of bulk (macroscopic) kind, i.e., the log *P* value refers to the molecule as a whole, disregarding the conformation states of the compound in question. The only exception occurs in our recent theoretical paper[8] in which independent literature NMR data and a bulk constant were combined to introduce the principle and feasibility of conformer-specific partition coefficient determination. In fact, no experimental conformer-specific octanol/water partition coefficient has been reported on any compound, although the number of species-specific physicochemical parameters is steadily increasing,[9−11] and several approaches appeared to find correlation between partition coefficients and stereochemical data. Pleiss et al. investigated a number of conformationally rigid phenethylamines[12] and extended the *f*-fragment calculations of Rekker[13] and Leo[14,15] with correction factors to take conformation into consideration. Hopfinger et al. used the solvent-dependent conformational analysis procedure (SCAP) to predict the octanol/water partition coefficient for 20 different compounds.[16] Most of these molecules, however, were either rigid, or the constants referring to flexible molecules were of bulk type, encompassing an undefined number and concentration of conformers. In subsequent studies on hydroxyureas, systematic differences have been found between experimental and calculated log P_{octanol} values, owing to differences in conformational tendencies between the two groups of the molecules studied.[17,18]An extensive study on diastereomers examined the influence of stereochemical factors on the log P_{octanol} values.[19] Differences in lipophilicity between diastereomers were interpreted in terms of macroscopic properties, such as van der Waals volume, surface area, dipole moment, ionization constant, and H-bonding capacity, and provided indirect experimental evidence for conformational effects on lipophilicity. To get further insight into conformer-specific partition, various computational approaches have also been elaborated. MLP, the calculated Molecular Lipophilicity Potential quantitated and validated on the basis of a large, experimental log *P* data set on nonionisable, rigid compounds, allows intra- and intermolecular interactions and offers a three-dimensional representation of lipophilicity.[20,21] MLP is a great computational achievement to describe the conforma-

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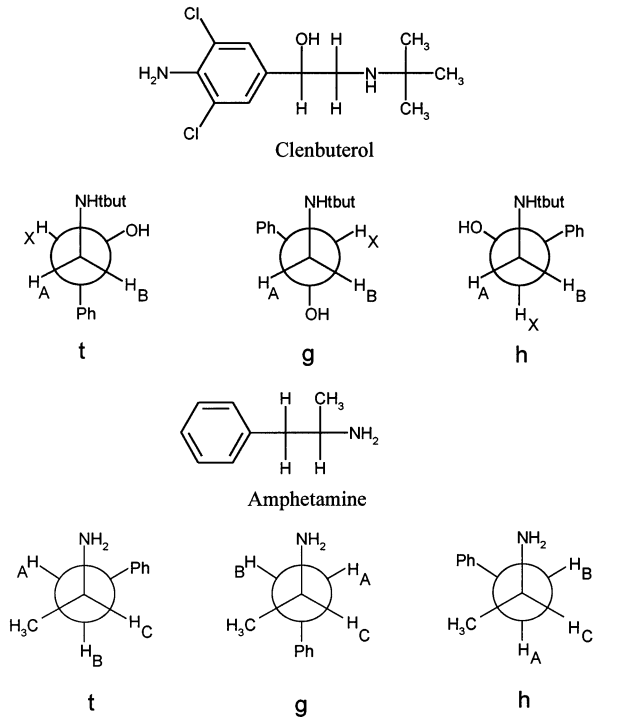

Figure 1. The structure and rotamers of clenbuterol and amphetamine.

tion-dependence of lipophilicity. Of the several adaptations of MLP,[22−24] the one on morphine glucuronides[22] could interpret the great differences between experimental and some calculated log *P* values, in terms of folded and extended conformations. This phenomenon is also called as "chameleonic behavior",[25] referring to medium conformity by hydrophobic collapse or hydrophilic folding.

The survey of the literature shows that conformer-specific partition coefficients are parameters of great importance, and sophisticated computational methods have been developed for their characterization. Nevertheless, no experimental partition coefficients of flexible molecules in octanol/water systems have been reported. The lack of such data is due to two facts: the necessary relationships and methodology have appeared very recently,[8] and NMR measurements in octanol are not routine tasks at all.

Here we report experimental conformer-specific partition coefficients in octanol/water system, for the first time. Conformers that preferably enter and exit the membrane are shown. The method is exemplified on amphetamine and clenbuterol, two small, flexible drug molecules with very different rotamer-specific partition behavior.

Results, Discussion

Figure 1 shows the constitutional formula and the staggered conformers of clenbuterol and amphetamine. Indices t, g, and h refer to rotamers where the vicinal, bulkiest groups are in trans, gauche, and hindered positions, respectively. By definition, the bulk (macroscopic) partition coefficient is the ratio of the total solute concentrations in the organic and aqueous phases:

$$P = \frac{c_o}{c_w} \tag{1}$$

The conformer-specific partition coefficients can be defined analogously:

$$p_g = \frac{[g]_o}{[g]_w} \tag{2}$$

$$p_t = \frac{[t]_o}{[t]_w} \tag{3}$$

$$p_h = \frac{[h]_o}{[h]_w} \tag{4}$$

We have earlier shown[8] that the conformer-specific and bulk partition coefficients are related by the α_{to}, ..., α_{hw} rotamer mole fractions, where indices refer to the rotamer and the phase, as follows:

$$p_t = \frac{\alpha_{t_o}}{\alpha_{t_w}} \cdot P \tag{5}$$

$$p_g = \frac{\alpha_{g_o}}{\alpha_{g_w}} \cdot P \tag{6}$$

$$p_h = \frac{\alpha_{h_o}}{\alpha_{h_w}} \cdot P \tag{7}$$

Thus, the rotamer-specific partition coefficients can be obtained in the knowledge of the bulk partition coefficients and the rotamer populations in the two solvents.

Rotamer populations in any solvents can be determined from vicinal $^1H-^1H$ NMR coupling constants for an ABC or ABX spin system.[26] Because of rapid interconversion among rotamers, the observed couplings are the weighted sums of the various gauche and trans coupling constants of individual rotamers, where weighting factors are the appropriate mole fractions. The following relationships encompass the parameters in clenbuterol.

$$^3J_{AX} = \alpha_t J_{Gt} + \alpha_g J_T + \alpha_h J_{Gh_{AX}} \tag{8}$$

$$^3J_{BX} = \alpha_t J_T + \alpha_g J_{Gg} + \alpha_h J_{Gh_{BX}} \tag{9}$$

$$\alpha_t + \alpha_g + \alpha_h = 1 \tag{10}$$

In eqs 8−10, $^3J_{AX}$ and $^3J_{BX}$ are the observed coupling constants between A−X and B−X protons, α_t, α_g, α_h are rotamer populations, J_T is the standard coupling constant belonging to 180° dihedral angle, J_{Gt}, J_{GhAX} and J_{Gg}, J_{GhBX} are standard coupling values belonging to 60° and 300° dihedral angles in rotamers t and h, and g and h, respectively.

By combining eqs 8−10 we obtain:

$$\alpha_t = \frac{(^3J_{BX} - J_{Gh_{BX}})(J_T - J_{Gh_{AX}}) - (^3J_{AX} - J_{Gh_{AX}})(J_{Gg} - J_{Gh_{BX}})}{(J_T - J_{Gh_{BX}})(J_T - J_{Gh_{AX}}) - (J_{Gt} - J_{Gh_{AX}})(J_{Gg} - J_{Gh_{BX}})} \tag{11}$$

$$\alpha_g = \frac{(^3J_{AX} - J_{Gh_{AX}})(J_T - J_{Gh_{BX}}) - (^3J_{BX} - J_{Gh_{BX}})(J_{Gt} - J_{Gh_{AX}})}{(J_T - J_{Gh_{BX}})(J_T - J_{Gh_{AX}}) - (J_{Gt} - J_{Gh_{AX}})(J_{Gg} - J_{Gh_{BX}})} \tag{12}$$

Table 1. Observed ^{1}H NMR Coupling Constants and Calculated Substituent Constants (λ) of Auxiliary Compounds

molecule	solvent	$^3J_{HH}$ (isopropyl protons)	λ
N-*tert*-butylisopropylamine	$CDCl_3$	6.34	1.11
	D_2O (basic pH)	6.41	1.03
2,6-dichloro-4-isopropylaniline	$CDCl_3$	6.92	0.48

Table 2. Observed and Standard Vicinal ^{1}H−^{1}H NMR Coupling Constants of Amphetamine and Clenbuterol in Octanol and D_2O

molecule	solvent	$^3J_{AX}$	$^3J_{BX}$	J_T	J_{Gt}	J_{Gg}	J_{GhAX}	J_{GhBX}
amphetamine	octanol	7.80	5.75	11.73	2.72	3.59	2.47	3.33
	D_2O	7.70	5.80	11.83	2.89	3.54	2.64	3.28
clenbuterol	octanol	3.30	9.75	11.11	2.59	4.33	2.88	1.14
	D_2O	5.35	8.05	11.29	2.73	4.24	2.97	1.47

Similar equations for amphetamine rotamer mole fractions can be derived analogously.[8] Calculation of the standard coupling constants is based on the relationship between vicinal ^{1}H−^{1}H coupling constants and the related torsional angles expressed in Karplus equations.[27] The observed vicinal coupling constants can now be measured with 0.02 Hz precision. Reliability of the rotamer populations therefore depends on the accuracy of the J_T, ... J_{GhBX} standard coupling constants. Of the several versions of Karplus-type equations, we used the reparametrized Haasnoot formula of Altona et al.,[28] the most specific such relationship today. This equation takes into consideration the dihedral angle, the relative position of the substituents and experimental group electronegativities, also named as substituent constants, specified for two groups of solvents. Altona et al. have reported empirical substituent constants of moieties such as OH, NH_2, phenyl, etc.,[28] for solvent classes of high and low dielectric constants. Differences between solvent dependences within a class (e.g., $CDCl_3$, and octanol in the solvent class of low dielectric constants) are negligible. Clenbuterol contains less common, composite groups such as *tert*-butylamino and 3,5-dichloro-4-aminophenyl groups. We have therefore performed independent experiments on related model compounds to determine the substituent constants from the observed ^{1}H−^{1}H NMR coupling values of the appropriate isopropyl derivatives as described before.[28] Results are shown in Table 1. The relative electronegativities of the *tert*-butylamino group are smaller than those of the amino group (1.19 in $CDCl_3$ and 1.10 in D_2O), which is accounted for the electron-donating effect of the *tert*-butyl moiety. The substituent constant of the 3,5-dichloro-4-aminophenyl group is nearly identical with that of the phenyl group (0.45), indicating that the phenyl substituents hardly influence the electronegativity effect of the phenyl ring, or the substituents in this arrangement provide an overall compensational effect. The measured and standard coupling constants of amphetamine and clenbuterol in D_2O and octanol, and the calculated rotamer mole fractions, are summarized in Tables 2 and 3.

Table 3 shows only insignificant solvent dependences for the three rotamer populations of amphetamine. Contrary to that, the rotamer populations of clenbuterol are greatly influenced by the solvent. Rotamer t, the most populated one in water becomes even more abundant in octanol, whereas rotamer g, the nearly "statistical" species in D_2O gets lower population in octanol. The sterically most hindered rotamer h remains the least populated one in both solvents.

Table 3. Calculated Rotamer Populations of Amphetamine and Clenbuterol in Octanol and D_2O

molecule	solvent	α_t	α_g	α_h
amphetamine	octanol	0.54 ± 0.07	0.33 ± 0.05	0.14 ± 0.05
	D_2O	0.52 ± 0.07	0.32 ± 0.05	0.16 ± 0.05
clenbuterol	octanol	0.84 ± 0.09	0.08 ± 0.04	0.08 ± 0.05
	D_2O	0.58 ± 0.07	0.30 ± 0.05	0.11 ± 0.05

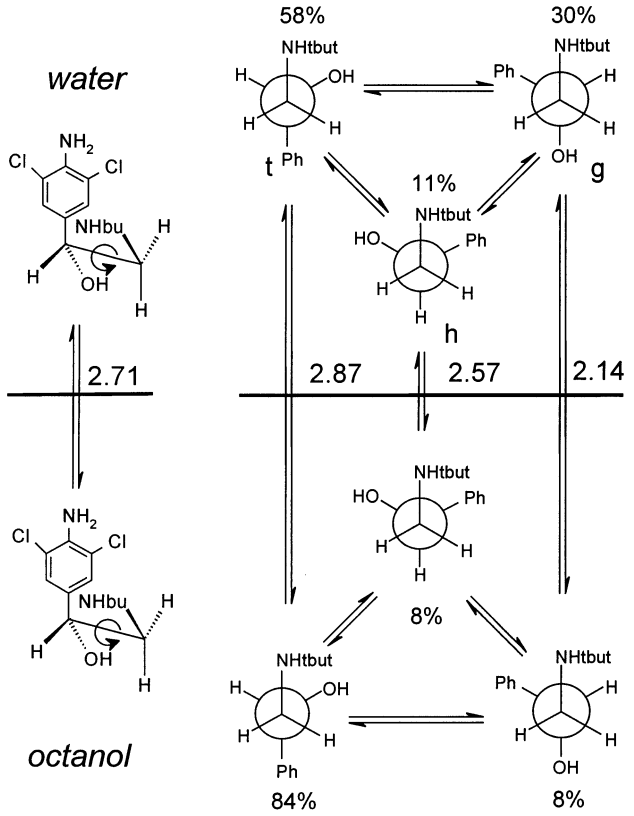

Figure 2. Scheme and data of clenbuterol partition in the octanol/water system at the macroscopic and conformer-specific levels. Partition coefficients are logarithmic values.

To obtain conformer-specific partition coefficients (eqs 5−7),[8] bulk partition coefficients and the rotamer populations in the two solvents have been determined. Surprisingly enough, no literature log *P* data can be found for clenbuterol. We have therefore carried out two types of determination: a predictive calculation by the atom/fragment contribution method of Meylan and Howard[29] (KOWWIN) and an experimental determination by the classical stir-flask method. The predicted value was log $P = 2.00$, whereas the experimental determination resulted in log $P = 2.71$. The deviation is significant and could only be interpreted in the knowledge of the conformer-specific log *p* values.

Since the rotamer populations of amphetamine in the partitioning solvents are virtually identical, their rotamer-specific partition coefficients do not differ from the bulk one.

The logarithmic value of the bulk- and conformer-specific partition coefficients of clenbuterol are shown in Figure 2. Uncertainties were calculated by the error proliferation method of Gauss and are summarized in Table 4. The value of log p_h bears high ambiguity

Table 4. Rotamer-specific Partition Coefficient Values and Confidence Interval Ranges of Clenbuterol

rotamer	p	log p
t	747 ± 132	2.87
g	138 ± 73	2.14
h	375 ± 291	2.57

because of the low populations in both solvents. Nevertheless, the order of rotamer populations is perfectly reasonable. The most lipophilic conformer of clenbuterol is t, with two adjacently situated polar groups that have the capacity to form hydrogen bond. This intramolecular, intermoiety interaction disfavors hydration and promotes apolar solvation. Concomitantly, the octanol solvation of the phenyl moiety is not hindered by the proximity of bulky, polar groups. The partition coefficient of rotamer h falls between that of rotamers t and g. In rotamer h the hydrogen bond formation between the *tert*-butylamino and hydroxyl groups is possible, but the octanol-solvation of the phenyl group becomes hampered by the gauche position of the bulky and polar *tert*-butylamino group. In rotamer g, neither factor that promotes octanol solvation occurs, but the most polar OH site gains best water-accessibility, making rotamer g the most hydrophilic species.

The rotamer-specific partition coefficient data shed light on diff-log $P = 0.71$, the great deviation between the experimental and calculated (predicted) bulk log P values. The predicted constant mainly resembles the partition coefficient of rotamer g. It indicates that this kind of prediction method is not able to take into account the intramolecular interactions in flexible molecules. Rather, it can approximate the lipophilicity of the conformer in which intramolecular interactions hardly exist. In general, if such a noninteraction species is a major one, the predicted "bulk" log P falls near the experimental bulk value. If, however, the "noninteraction" species is a minor one, and its conformer-specific log p value is different from that of the major ones, the predicted "bulk" constant is far from the experimental value. The conformer-specific partition coefficients of clenbuterol also indicate that rotamer t can well be assumed to be the leading species in membrane-influx processes, whereas rotamer g is predominant in membrane-outflow phenomena.

Experimental Section

Materials. D-Amphetamine sulfate, DL-clenbuterol hydrochloride, sodium deuterioxide, and deuterium chloride were obtained from Sigma, and deuterium oxide was from Merck. *N*-*tert*-Butylisopropylamine and 4-isopropylaniline were products of Aldrich, and *n*-octanol for UV spectroscopy was purchased from Fluka.

Determination of Vicinal ^{1}H−^{1}H NMR Coupling Constants. NMR spectra of amphetamine and clenbuterol were recorded in octanol and 0.1 N NaOD solutions after performing a partition experiment. The solvents were mutually saturated by stirring them overnight. The solutes were dissolved in the aqueous phase (0.1 N), and the solutions were stirred with an equal volume of water-saturated octanol for 4 h. Then the phases were separated by centrifugation (5000 rpm, 10 min) and were put into NMR tubes. The spectra were recorded at 25 °C on Bruker AM 400 and Bruker AM 200 spectrometers. Presaturation on the aqueous samples was used to suppress the HDO signal. Since nondeuterated octanol was used for the experiments, we put a coaxial insert filled with D_2O into the octanol samples to set the lock signal. To suppress the two largest octanol signals, the 1-$\bar{3}$-3-$\bar{1}$ pulse sequence was used.[30,31] All NMR spectra were recorded with a digital resolution of 0.02 Hz. Vicinal ^{1}H−^{1}H coupling constants were determined by simulation of the spin systems by the program SpinWorks 1.3.

Determination of log *P* Values. The octanol/water partition coefficients of amphetamine and clenbuterol were determined by the stir-flask method.[32,33] Octanol and 0.1 N NaOH solution were mutually saturated, and the phases were separated by centrifugation (5000 rpm, 10 min). A stock solution of amphetamine (4×10^{-3} M) and clenbuterol (saturated) were prepared using octanol-saturated NaOH solution. A part of these solutions were put aside for UV measurement. NaOH-saturated octanol was added at various volume ratios to the other part of the stock solution. Then the two-phase mixtures were intensively stirred for 4 h in thermostated double-walled glass cells at constant temperature (25.0 ± 0.1 °C). After separation of the phases by centrifugation, the absorbance of the aqueous solution and the octanol-saturated stock solution were measured by UV spectrophotometry with a Jasco V-550 UV/vis spectrophotometer against octanol-saturated 0.1 N NaOH solution at $\lambda_{max} = 258$ nm for amphetamine and $\lambda_{max} = 240$ nm for clenbuterol. The P value was calculated as follows:

$$P = \frac{A_0 - A_1}{A_1} \cdot \frac{V_w}{V_o} \tag{13}$$

where A_0 and A_1 represent the absorbance of the molecule in the aqueous phase before and after partitioning, V_w and V_o are the water and octanol volumes, respectively. Log P values are an average of six measurements.

Determination of Substituent Parameters. For the determination of relative group electronegativities of the *tert*-butylamino and 3,5-dichloro-4-aminophenyl groups, the ^{1}H−^{1}H NMR coupling constants of the aliphatic protons of N-*tert*-butylisopropylamine and 2,6-dichloro-4-isopropylaniline were determined in $CDCl_3$ and D_2O. 2,6-Dichloro-4-isopropylaniline was prepared by direct chlorination of 4-isopropylaniline under standard reaction conditions.[34,35] Excess chlorine was introduced into the solution of aniline in dry chloroform at room temperature. The reaction mixture was protected against light. The product was purified by silica gel column chromatography using benzene−methanol (4:1) solvent mixture as eluent.

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