Re-analysis of narcotic critical body residue data using the equilibrium distribution concept and refined partition coefficients

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14   Electronic supplementary information (ESI) available.

Abstract

Narcosis occurs as a result of accumulation of chemicals in phospholipid membrane. The toxic threshold concentration in membrane is thought to be relatively constant across different chemicals and species. Hence, estimating chemical concentrations in membrane is expected to reduce the variability of narcotic critical body residue (CBR) data. In this study, a high quality CBR data set for three aquatic species reported recently in the literature was evaluated with the internal equilibrium distribution concept. The raw wet-weight-based CBR values were converted to membrane-weight-based CBR by assuming that the chemical is distributed in storage lipid, membrane, protein, and water according to the respective equilibrium partition coefficients. Several sets of partition coefficients were compared for this analysis. The results were consistent with the notion that the use of a structural protein instead of serum albumin as surrogate for the body protein fraction could reduce the variability of CBR. Partition coefficients predicted by polyparameter linear free energy relationships (PP-LFERs) reduced the variability of CBR as much as or even more than experimental partition coefficients did. It is suggested that CBR data for chemicals with larger structural diversity and biological species with more distinct compositions are needed to evaluate further the equilibrium distribution concept and the constant membrane threshold hypothesis.

1. Introduction

The critical body residue (CBR) refers to the chemical concentration in an organism that causes a toxic effect.¹ For narcosis (also termed baseline toxicity or nonspecific toxicity), many experimentally determined CBR values fall within a narrow range, 2–8 mmol/kg-wet, over various chemicals and species.² However, data do exhibit a considerable variation, up to three orders of magnitude.² Inconsistent experimental settings may be a large contributing factor to the observed variation, while inadequacy of the wet-based whole body concentration as a metric for narcosis can also be a cause of the variability. Narcosis is thought to occur as a result of the accumulation of chemicals in membrane to the level where functioning or structure of the membrane is disrupted.³ This mechanism of toxicity is hypothesized to occur in a chemical- and species-independent manner; that is, there is a relatively constant threshold membrane concentration that causes narcosis. Since the target site is membrane, it is logical to consider the concentration in the membrane (i.e., in mmol/kg-membrane) as a more relevant surrogate metric for narcosis than the concentration in the wet body. The membrane concentration, however, cannot be directly measured and thus have to be estimated, and the value depends on the estimation method used.⁴

Recently, van der Heijden et al.⁵ reported a detailed study on narcotic toxicity of several neutral organic chemicals for three aquatic species. They performed experiments with a number of quality control measures, being aware of potential causes of experimental artifacts in CBR such as a variation in the aqueous exposure concentration and a depletion of oxygen. They converted their high quality data of wet-weight based CBR (CBR_{wet} in mmol/kg-wet) to membrane-weight-based CBR ($\text{CBR}_{\text{membrane}}$ in mmol/kg-membrane), assuming the equilibrium partitioning of the chemicals within the body. Then, van der Heijden et al.⁵ concluded that “Accounting for the chemicals’ internal distribution to different partitioning domains and relating effects to estimated concentrations in the target

compartiment (i.e., membrane) was expected to but did not decrease the overall variability, likely because the surrogate partition coefficients for membrane, storage lipid, protein, and carbohydrate that were used as input parameters did not sufficiently represent the actual partitioning processes.”

In the last several years, a series of detailed studies have been reported on the partition properties of relevant lipids and proteins to further our understanding in internal distributions of chemicals.⁶⁻⁹ In the current contribution, the CBR data from van der Heijden et al.⁵ are re-analyzed by calculating CBR_{membrane} using different sets of partition coefficients including both experimental and predicted values. The purpose is to evaluate whether refined sets of partition coefficients can reduce the variability of CBR data for nonspecific acute toxicity. Particularly, the role of the protein fraction in the equilibrium distribution of chemicals in test organisms is discussed in detail.

2. Methodology

2.1. CBR data

The CBR data from ref 5 were used for the analysis of this work. The data were measured for three aquatic species, *Lumbriculus variegatus* (California blackworm), *Hyalella azteca* (scud), and *Poecilia reticulata* (guppy), at 20°C. Bromophos-methyl was removed from consideration, as it appears to exert specific toxicity.⁵ This leaves six chemicals for the three species. For each of the 18 combinations, there are 4 to 28 measured values, which amount to 339 individual CBR values. It should be noted that, whereas this data set was consistently and carefully measured, replicates of CBR for a given combination of chemical and species do show a variability of 0.10-0.35 log unit standard deviations (with the mean of 0.17 for the 18 combinations). Thus, any data normalization with regard to the sorption capacity is not expected to improve the data coherence more than this range.

2.2. Equilibrium distribution model

The approach used in this work to estimate the membrane burden is based on the equilibrium distribution concept within the organism. It assumes that the concentrations of the chemical in all body compartments are in equilibrium with each other. The equilibrium partition coefficient between the whole organism and water [$L_{\text{water/kgorganism}}$] can be expressed as,¹⁰

$$K_{\text{organism/w}} = w_{\text{storage lipid}} K_{\text{storage lipid/w}} + w_{\text{membrane}} K_{\text{membrane/w}} + w_{\text{protein}} K_{\text{protein/w}} + w_{\text{carbohydrate}} K_{\text{carbohydrate/w}} + w_{\text{water}}/\rho_{\text{water}} \quad (1)$$

where w is the mass fraction of the component denoted with its subscript, K is the partition coefficient of the chemical from water to the respective component [$L_{\text{water/kgcomponent}}$], and ρ_{water} is the density of water (assumed to be 1 kg/L). In eqn (1), storage lipid, membrane, protein, carbohydrate, and water are included as significant partition phases in the body, as done by van der Heijden et al.⁵

To estimate $K_{\text{organism/w}}$ with eqn (1), all w and K values need to be known. The w values can be measured¹¹ or reference values are available for well-studied species such as rat and human.¹² K values need additional assumptions, because an actual organism is made up of diverse types of lipids, proteins, and other components. In a generic modeling approach, storage lipids are represented by triacylglyceride solvent such as olive oil, as the sorption properties do not significantly depend on the type of storage lipids.⁸ The membrane fraction is represented by phosphatidylcholine liposomes, which are considered a reasonable surrogate for phospholipid membrane in organisms.⁶ Currently, there is no widely accepted model protein to be used for $K_{\text{protein/w}}$. Serum albumin, a globular blood protein that has hydrophobic binding cavities with relatively low specificity, is often considered as a generic model. However, as reported previously,⁹ sorption of neutral organic chemicals to serum

albumin is generally stronger than that to structural proteins, which are the major proteins in terms of body mass and do not have specific binding pockets. So far, muscle protein is the only well-characterized structural protein in terms of the sorption property and thus is used as a model here. Carbohydrate is included in eqn (1), but it generally has relatively low sorption capacities^{13, 14} and its contribution to the total sorption capacity of animals is usually negligibly small.⁵

Each term of eqn (1) indicates the contribution of the respective biological component to the total sorption capacity of the body. The relative sorption capacity of component i, F_i , can thus be described by eqn (2).

$$F_i = \frac{w_i K_{i/w}}{K_{\text{organism/w}}} \quad (2)$$

The conversion of CBR_{wet} to $\text{CBR}_{\text{membrane}}$ can be accomplished using $K_{\text{membrane/w}}$ and $K_{\text{organism/w}}$.

$$\text{CBR}_{\text{membrane}} = \text{CBR}_{\text{wet}} \frac{K_{\text{membrane/w}}}{K_{\text{organism/w}}} \quad (3)$$

2.3. Mass fractions and partition coefficients used for modeling

For the mass fractions w in eqns (1) and (2), the values measured by van der Heijden et al.⁵ were used: thus, $w_{\text{storage lipid}} = 0.356$, $w_{\text{membrane}} = 0.644$, $w_{\text{protein}} = 0.622$, and $w_{\text{water}} = 0.794$ for *Lumbriculus*; $w_{\text{storage lipid}} = 0.603$, $w_{\text{membrane}} = 0.397$, $w_{\text{protein}} = 0.500$, and $w_{\text{water}} = 0.723$ for *Hyalella*; $w_{\text{storage lipid}} = 0.648$, $w_{\text{membrane}} = 0.352$, $w_{\text{protein}} = 0.604$, and $w_{\text{water}} = 0.777$ for *Poecillia*. For the partition coefficients between each body component and water, the following four sets of partition coefficients were used and compared.

2.3.1. Set 1: Experimental partition coefficients with BSA as surrogate protein

The first set of partition coefficients are those used by van der Heijden et al.,⁵ who considered triolein, palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) liposomes and bovine serum albumin (BSA) as surrogate phases for storage lipids, membrane, and proteins, respectively. These partition coefficients are shown in Table 1. The use of triolein and POPC liposomes as model lipids is in line with the current state of art, as explained in section 2.2. The consequence of using BSA to represent the sorption property of proteins will be discussed below.

Table 1. Partition coefficients from water to lipids and proteins.^a

	log K_{storage} lipid/w	log K_{storage} lipid/w	log $K_{\text{membrane/w}}$	log $K_{\text{membrane/w}}$	log $K_{\text{BSA/w}}$	log K_{muscle} protein/w	log K_{muscle} protein/w	log K_{ow}
	exp, 20°C	pred, 37°C	exp, 20°C	pred, 37°C	exp, 20°C	exp, 37°C	pred, 37°C	exp, 25°C
	ref 5		ref 5		ref 5	ref 9		ref 15
124-TCB	4.17	4.23	3.89	4.01	3.96	na	2.56	4.02
1234-TeCB	4.81	4.75	4.52	4.48	4.86	2.94	2.97	4.60
QBenz	4.99	5.04	4.69	4.50	4.12	2.71	3.08	4.90
234-TCA	3.58	3.17	4.12	3.74	3.54	na	2.44	3.33
2356-TeCA	4.59	3.88	4.79	4.55	4.14	na	3.16	4.10
4-C-3-MP	2.07	1.70	3.23	2.90	2.62	na	1.77	3.10
Pyr	5.71	5.44	5.75	5.35	5.29	3.67	3.71	4.88

^aFor prediction of partition coefficients, polyparameter linear free energy relationships (PP-LFERs) were used. na, not available; exp, experimental data; pred, predicted values. 124-TCB, 1,2,4-trichlorobenzene; 1234-TeCB, 1,2,3,4-tetrachlorobenzene; QBenz, n-pentachlorobenzene; 234-TCA, 2,3,4-trichloroaniline; 2356-TeCA, 2,3,5,6-tetrachloroaniline; 4-C-3-MP, 4-chloro-3-methylphenol; Pyr, pyrene.

2.3.2. Set 2: Experimental partition coefficients with muscle protein as surrogate protein

The second set includes the same experimental partition coefficients for storage and membrane lipids as Set 1 but considers chicken muscle protein instead of BSA as surrogate for all proteins. The experimental partition coefficients between muscle protein and water ($K_{\text{muscle protein/w}}$) are from ref 9 (Table 1). Missing experimental values were amended with the

146 predictions by polyparameter linear free energy relationships (PP-LFERs) (see below). As
147 Table 1 shows, $K_{\text{BSA/w}}$ is >1 log unit higher than $K_{\text{muscle protein/w}}$ and is as high as $K_{\text{membrane/w}}$.

149 2.3.3. Set 3: Experimental partition coefficients without protein

150 For comparison to Set 1 and Set 2, calculations were performed without considering
151 the protein fraction as a sorbing phase. This means that we assume $K_{\text{protein/w}} = 0$ in eqn (1) for
152 the calculation. The same experimental partition coefficients for storage lipid and membrane
153 as included in Sets 1 and 2 were used for Set 3.

155 2.3.4. Set 4: PP-LFER-predicted partition coefficients

156 It is frequently the case that experimental data for partition coefficients are
157 unavailable for the test chemicals. Thus, predictions of partition coefficients by a model
158 would be useful for future applications of the equilibrium partitioning concept as described
159 with eqns (1)–(3). Therefore, for Set 4, $K_{\text{storage lipid/w}}$, $K_{\text{membrane/w}}$, and $K_{\text{muscle protein/w}}$ were
160 predicted by PP-LFERs. PP-LFERs used here appear,

$$161 \quad \text{Log } K = c + eE + sS + aA + bB + vV \quad (4)$$

162 The symbols used are as follows: E , excess molar refraction; S , polarizability/dipolarity
163 parameter; A , solute H-bond donor property; B , solute H-bond acceptor property; and V ,
164 McGowan's molar volume. The lowercase letters are regression coefficients, which are
165 available for $\log K_{\text{storage lipid/w}}$, $\log K_{\text{membrane/w}}$, and $\log K_{\text{muscle protein/w}}$ in the literature.¹⁰ The
166 descriptors and parameters were retrieved from the literature using the UFZ-LSER database
167 (<https://www.ufz.de/index.php?en=31698>). All descriptors used are tabulated in Table S1 in
168 the ESI. The calculation of the leverage (see ref 16) suggests that predictions of $K_{\text{storage lipid/w}}$
169 for 2,3,5,6-tetrachloroaniline and 4-chloro-3-methylphenol might be considered

extrapolations, as the leverage exceeds three times the mean leverage of training chemicals. The other predictions are all within the domain of applicability.

	Set 1	Set 2	Set 3	Set 4
$K_{\text{storage lipid/w}}$	Experimental (triolein)			PP-LFER predicted (general storage lipid)
$K_{\text{membrane/w}}$	Experimental (liposome)			PP-LFER predicted (liposome)
$K_{\text{protein/w}}$	Experimental (BSA)	Experimental (muscle protein)	$K_{\text{protein/w}} = 0$ (no protein)	PP-LFER predicted (muscle protein)

Fig. 1 Four sets of partition coefficients used to estimate $\text{CBR}_{\text{membrane}}$ via eqns (1)-(3). The material that is considered representative of each fraction is indicated in the parentheses.

2.4. Evaluation of data variability

Variability of CBR data was evaluated by calculating the variance of the data. Levene's test was performed to evaluate whether there is a statistically significant difference in variance between two data sets. Statistical software R (3.1.2) with the "car" package was used for this calculation. The p value < 0.05 was considered significant.

3. Results and discussion

3.1. Relative contributions of storage lipid, membrane, protein and water to the total sorption capacity of organisms

In Fig. 2A, relative sorption capacities of individual biological components in *Lumbriculus variegatus* (California blackworm) estimated with the four sets of partition coefficients are presented. The plots for the other two species are similar (see Fig. S1 in the ESI). When BSA is used as the protein surrogate for obtaining partition coefficients (Set 1),

the protein fraction is estimated to contribute to the majority (53–97%) of sorption capacity of *Lumbriculus* for all compounds (Fig. 1A). The results are similar for *Hyalella* (44–97%) and *Poecilia* (30–94%). These high contributions of proteins are calculated as a result of the consistently high protein contents (50–62% of dry weight) in the organisms and the relatively high values of $K_{BSA/w}$, which are comparable to $K_{storage\ lipid/w}$ and $K_{membrane/w}$ (Table 1). The estimated predominance of protein, however, contradicts a body of previous research that indicates the major role of lipids in bioaccumulation, particularly for nonpolar compounds such as chlorobenzenes and aromatic hydrocarbons, which are also included in the used data set.

Fig. 2 Relative sorption capacities of individual biological components in *Lumbriculus variegatus* (California blackworm). The partition coefficients used for this calculation are summarized in Fig. 1. See Table 1 for the abbreviations of chemicals.

The plot with the Set 2 partition coefficients (Fig. 2B) is considerably different from that with Set 1. With muscle protein instead of BSA as surrogate protein, the protein fraction is estimated to contribute only 4–29% to the total sorption capacity for *Lumbriculus*, 3–33% for *Hyalella*, and 2–23% for *Poecilia*. Certainly, the true partition coefficient for the protein fraction of the test species may not be the same as for muscle protein. Also, the temperature

difference (20°C vs 37°C) may have a minor influence on the partition coefficient. Despite these uncertainties, it is worth noting that the use of a structural protein led to much smaller estimated contributions of the protein fraction to the total sorption capacity in comparison to serum albumin.

With the Set 2 partition coefficients, the protein contribution is estimated to be generally small. For example, a >20% contribution of the protein fraction to the total sorption capacity was estimated only for 1,2,4-trichlorobenzene and 4-chloro-3-methylphenol in *Lumbriculus*. As a result, the Set 3 partition coefficients (no protein sorption is assumed) exhibited a largely similar plot as compared to the Set 2 partition coefficients (Fig. 2C). PP-LFER predicted partition coefficients (Set 4, Fig. 2D) generated a plot that closely resembles the plot with the corresponding experimental partition coefficients (i.e., Set 2), reflecting the high agreement between experimental and predicted partition coefficients.

The relative sorption capacities of storage lipid and membrane depend on the chemicals and the species. Notable may be the generally low relative sorption capacity of storage lipid for 2,3,4-trichloroaniline, 2,3,5,6-tetrachloroaniline, and 4-chloro-3-methylphenol, predicted with the Sets 2 to 4 partition coefficients. This trend can be explained by the relatively strong H-bond donor property of these chemicals.¹⁰ For the two chlorobenzenes and pentylbenzene, roughly equal relative sorption capacities of storage lipid and membrane were estimated. For these chemicals, storage lipid is an alternative fate to membrane and decreases the fraction of chemicals in membrane.

3.2. Variability of CBR_{wet} and $CBR_{membrane}$

Using 339 raw CBR_{wet} data reported by van der Heijden et al.,⁵ $CBR_{membrane}$ was calculated using Set 1 to Set 4 partition coefficients. The results are plotted in Fig. S2 in the ESI and are summarized in Table 2. Fig. 3 compares the variance of CBR_{wet} and $CBR_{membrane}$.

When all 339 data are considered, variance of CBR_{membrane} calculated with the Set 1 partition coefficients is higher than that of CBR_{wet} . As already pointed out by van der Heijden et al.,⁵ the conversion of CBR_{wet} to CBR_{membrane} rather increases the data variability if BSA is used as protein surrogate. The same is true when the data specifically for *Lumbriculus* or for *Hyaella* are analyzed. This observation can be explained by the too high $K_{\text{protein/w}}$ specifically for serum albumin, as discussed above. That is, BSA does not represent the actual sorption property of body protein. In contrast, the Sets 2, 3, and 4 partition coefficients resulted in CBR_{membrane} with variations that are statistically not different from that of CBR_{wet} , or in the case of *Hyaella* and *Poecilia*, the variations become even smaller. Thus, with muscle protein used as model protein, calculating CBR_{membrane} does not increase, but rather, it could decrease the variability. Note that Sets 2 and 3 do not show a significant difference in any case. This means that the protein contribution appears to be relatively small for the data sets used and that considering the protein contribution does not reduce the overall variability more than just ignoring it. Thus, the current analysis does not prove that muscle protein is a preferred model structural protein to use. For further evaluation, CBR data with leaner organisms, for which the protein fraction should play a more important role, are needed. Significantly smaller variances of CBR_{membrane} for *Hyaella* and *Poecilia* using Set 3 (no protein) as compared to CBR_{wet} suggests that considering two types of lipids is sufficient for significantly reducing the variance from CBR_{wet} .

Table 2. Comparison of wet-weight-based (CBR_{wet}) and membrane-based CBR (CBR_{membrane}) (mean $\pm$ SD). The original experimental CBR_{wet} data are from ref 5.

	partition coefficients used	All data $n = 339$	<i>Lumbriculus</i> $n = 94$	<i>Hyaella</i> $n = 107$	<i>Poecilia</i> $n = 138$
log CBR_{wet}		0.75 ± 0.28	0.67 ± 0.21	0.79 ± 0.25	0.77 ± 0.32
log CBR_{membrane}	Set 1	1.72 ± 0.34	1.71 ± 0.37	1.79 ± 0.34	1.69 ± 0.31
log CBR_{membrane}	Set 2	2.15 ± 0.25	2.21 ± 0.23	2.25 ± 0.21	2.03 ± 0.25
log CBR_{membrane}	Set 3	2.22 ± 0.26	2.30 ± 0.22	2.32 ± 0.23	2.08 ± 0.25

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Fig. 3 Variance of CBR data. “Wet” refers to CBR_{wet} . “Set 1” to “Set 4” indicates the set of partition coefficients that was used to derive $CBR_{membrane}$. Symbols indicate significant difference evaluated with Levene’s test ($p < 0.05$).

It is interesting that PP-LFER-predicted partition coefficients (Set 4) exhibited the same or even smaller variability of $CBR_{membrane}$ as compared to experimental partition coefficients (Set 2). The SD of 0.21 for all data is close to the mean SD for a single species and a single chemical (0.17). As the descriptors needed for PP-LFER predictions are widely available in the literature and easily accessible via the web-based UFZ-LSER database, PP-LFERs could vastly expand the range of chemicals for which the equilibrium distribution concept can be applied and $CBR_{membrane}$ calculated. To evaluate whether PP-LFER predicted partition coefficients generally lead to a smaller variance than experimental partition coefficients, there needs to be more CBR data for chemicals with larger structural diversity and for organisms with more distinctly different compositions.

Note that the interspecies variability of CBR_{wet} reported in ref 5 is not substantial. A reason may be that the biological compositions of the three test species are not extremely different. For example, the content of membrane lipid in the three test species is 1.2–1.8 mass% on the wet-weight basis, that is, 1.5 times different at most. Also, for each chemical, the relative sorption capacity of membrane is only 1.1–2.2 times different between the three species (estimated with the Set 2 partition coefficients). As a result, conversion from CBR_{wet} to $CBR_{membrane}$ does not substantially change the relative CBR between the three test species.

If a realistic set of partition coefficients (i.e., Set 2) is used, the value of the $CBR_{membrane}$ is estimated to be 80–250 mmol/kg-membrane with the geometric mean of 140 mmol/kg-membrane, which is similar to but slightly higher than the previously proposed range of lipid-based CBR, 40–160 mmol/kg-lipid¹⁷ or 91–120 mmol/kg-lipid.² The cited

studies did not calculate specifically CBR_{membrane} but calculated total lipid-based CBR with assumptions that are different from those in this work, which could be a potential reason for the small difference from this work.

4. Conclusions

This study demonstrated that calculating the membrane burden could reduce the CBR variability if appropriate partition coefficients are used for the calculation. This result is in line with the claim that there is a relatively constant narcotic membrane concentration that is independent of the types of chemicals and biological species. It is strongly suggested that serum albumin not be used as a surrogate phase for the body protein fraction, as the use of serum albumin leads to unrealistically high partition coefficients and overestimate the sorptive contribution of the protein fraction to the total sorption, which, in the end, hampers the interpretation of toxicity data.

To evaluate further the validity of the equilibrium distribution concept for narcotic toxicity assessments, CBR data for more chemicals and species are needed, accompanied with data for partition coefficients and biological compositions. Particularly, CBR data for long aliphatic chemicals (which generally favor storage lipid over membrane) and strong H-bond donor compounds (for which the protein fraction has a relatively high sorptive contribution) will help evaluate whether the equilibrium distribution concept can explain chemical-to-chemical variability of CBR. Highly polar chemicals may more likely cause excess toxicity than chemicals with low polarity, which might pose a difficulty in obtaining narcotic CBR data for the former. What makes data interpretation more complicated is the fact that the identification of excess toxicity needs a model for estimating baseline toxicity. Refining the model for narcosis has an implication for the definition of excess toxicity as well. Moreover, additional data for species with various compositions such as those with a very

high fat content or with little lipid would help understand the interspecies variability. Such data will be most useful if they are as accurate, consistent, and transparent as those from van der Heijden et al.⁵

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