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Differential Acute Toxicity of Tetrachlorobenzene Isomers to Oligochaetes in Soil and Water: Application of the Critical Body Residue Concept

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Abstract The acute, lethal potency of the 1,2,3,4-, 1,2,4,5- and 1,2,3,5-tetrachlorobenzene isomers was compared in the terrestrial and aquatic oligochaetes *Eisenia andrei* and *Tubifex tubifex*. 1,2,4,5-TeCB was neither lethal, nor produced any perceptible adverse effects, at lipid normalized concentrations predicted to be lethal according to the well-established critical body residue concept. If a narcotic is defined as a substance capable of inducing narcosis, rather than a substance displaying certain physical or chemical properties (e.g., log K_{ow}), then we do not believe these findings challenge the critical body residue because by the former definition, 1,2,4,5-tetrachlorobenzene is not a narcotic.

Keywords Differential toxicity · Tetrachlorobenzene · Narcotic · Critical body residue

The present study was prompted by the unexpected results of a baseline acute lethality test conducted in our laboratory that found 1,2,4,5-tetrachlorobenzene (TeCB) to be non-toxic to the earthworm *Eisenia andrei* at concentrations in which its isomeric counterparts, 1,2,3,4- and 1,2,3,5-TeCB, were toxic. A subsequent literature review of the acute toxicity of chlorinated benzenes revealed: (1) a general absence of toxicity data for the 1,2,4,5-TeCB isomer, (2) few publications reporting a differential toxicity between TeCB isomers (e.g., Chu et al. 1983), and (3) a general consistency for conducting bioassays with the 1,2,3,4-TeCB isomer (Carlson and Kosian 1987; Van Gestel et al. 1991; Belfroid et al. 1993; Leslie et al. 2004). Neither an explanation for this intentional, or incidental, isomer preference, nor a rationale for the absence of acute lethality data for the 1,2,4,5-TeCB isomer was found.

The significance of our initial results lies in their relationship and potential challenge to the well-established critical body residue (CBR) concept (McCarty and Mackay 1993). This concept predicts a specific response when the concentration of a chemical within an organism (body residue) reaches a threshold or critical concentration. In the context of lethal narcosis, this critical concentration is considered to be 2–8 mmol/kg tissue dry weight (McCarty and Mackay 1993). The present study compares the potency of these three TeCB isomers in terrestrial (*Eisenia andrei*) and aquatic (*Tubifex tubifex*) oligochaetes using lipid normalized body residues.

Materials and Methods

1,2,3,4- (98%; Acros Organics, Morris Plains, NJ), 1,2,4,5-, or 1,2,3,5-TeCB (98%; Sigma–Aldrich, St. Louis, MO) were dissolved in hexane (99%; Fisher Scientific, Itasca, IL) and amended into 600 g of Webster soil (Ames, IA; 2.4% organic carbon, 35.6% clay, pH 5.5, $\leq$2.0 mm particle size) or 300 g of silica sand according to Reid et al. (1998). The amended soil or sand was immediately transferred to an aluminum tray (soil) or beaker (sand) for 24 h to allow evaporation of the carrier solvent prior to placement and hydration with each test chamber. Spiked soil

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(200 g/replicate) was hydrated to 30% (w/w) with distilled water and spiked sand (100 g/replicate) was hydrated with 100 mL of dechlorinated water.

All static acute lethality tests involving soil were conducted with the earthworm *E. andrei* according to Environment Canada (2004). All static acute lethality tests involving sand were conducted with the aquatic worm *T. tubifex* according to a method adapted from Reynoldson et al. (1991). *E. andrei* were reared in-house within separated dairy solids (Ohio Agricultural Research and Development Center, Wooster, OH). Separated dairy solids served as both a substrate and source of food. *T. tubifex* were reared in-house within a mixture of dechlorinated water and sand and fed chopped Iceberg lettuce weekly. Neither organism was fed during testing.

To begin each soil test, ten adult, undepurated *E. andrei* were rinsed with tap water, weighed and randomly assigned to each test chamber. To begin each sand test, ten adult, undepurated *T. tubifex* were transferred directly from the culture stock into each test chamber. Soil test chambers (glass mason jars; 500 mL) were sealed with a screw collar and a metal lid with one, 2-mm perforation to allow atmosphere exchange. Sand test chambers (Pyrex beaker; 200 mL) were covered with a watch glass. All tests were conducted in a fume hood under continuous fluorescent lighting at 20 ± 2°C. Testing began 24 h after substrate hydration and involved six treatments, in triplicate, including a solvent control. Water loss during each test type was found negligible. Test chambers were monitored for test organism mortality every 24 h. All tests were terminated at 14 days or upon reaching an incipient lethal level. Mortality and exposure time was recorded. Test organisms were immediately extracted from each test chamber, rinsed with distilled water, transferred to a corresponding amber, glass vial (10 mL) and stored at −70 ± 2°C to retard any spontaneous chemical metabolism that may occur prior to chemical extraction. A valid test was considered one with a control treatment mortality of <10% (Environment Canada 2004).

The contents of each vial containing *E. andrei* were homogenized (20s) in hexane (4.0 mL) using an Omni International (Marietta, GA) tissue homogenizer. Following the completion of each homogenization, the homogenizer head was rinsed with hexane (1.0 mL) and the rinsate collected. Each vial was vortexed (10s), rotary extracted (30 rpm) for 1.0 h, and centrifuged at 1,500 x*g* (5.0 min; 20 ± 1°C). Vial contents were passed through a conditioned Bakerbond (Mallinckrodt Baker, Phillipsburg, NJ) cleanup column (10 mL; 1.5 g silica gel; 1.0 g sodium sulfate) under vacuum. Vial contents were extracted two more times using 3.0 mL hexane per extraction. Extract volumes were recorded and the extracts stored at 4 ± 2°C for chemical analysis.

The contents of each vial containing *T. tubifex* were immersed in 2.0 mL of methanol (HPLC-grade; Sigma–Aldrich, St. Louis, MO) and allowed to stand for 12 h at 4 ± 2°C before sonication (5.0 min). Following sonication, each vial received 1.0 mL of HPLC-grade water, was vortexed (30s), received an additional 3.0 mL of hexane, and was vortexed (30s) again. Each vial was centrifuged at 1,500 x*g* (5.0 min; 20 ± 1°C) and the supernatant decanted and passed through a cleanup column (10 mL; 1.5 g silica gel; 1.0 g sodium sulfate) under vacuum. Vial contents were extracted two more times using 3.0 mL hexane per extraction. Sample extract volumes were recorded and the extracts stored at 4 ± 2°C for chemical analysis.

Samples were analyzed using a Varian 3800 gas chromatograph coupled to a Varian 1079 split/splitless electron capture detector operating in splitless mode. Varian Star Chromatography workstation software (v 5.52) was used to perform data analysis. Method parameters were taken from Varian Application Note 788 (adapted from EPA Method 612). The injector (3.4 mm I.D.) and detector temperatures were 265°C and 300°C, respectively. The DB-5 narrow bore column (60 m; 0.1 μm film thickness; 0.25 mm I.D.) (Agilent Technologies, Santa Clara, CA) was held at 50°C for 2.0 min, ramped to 290°C at 5°C/min and held for 2.0 min for a total run time of 52 min. The carrier gas was helium and the make-up gas was nitrogen. Chlorinated benzene quantification limits ranged from 5.0 μg/L to 15 μg/L. All tissue samples were corrected for mass recovery which ranged from 90% to 95%. Accustandard, Inc. (New Haven, CT) standard number M-8121 (22 components; 1.0 mg/mL, hexane:acetone 97:3) was used for instrument calibration. The purity and identity of each of TeCB isomer was confirmed by an independent laboratory using mass spectrometry (Campus Chemical Instrument Center, Columbus, OH).

Body residue values were normalized to tissue dry weight as determined in the author's laboratory to be 15% of wet weight and 18% of wet weight for *E. andrei* and *T. tubifex*, respectively. LD_{50} and LC_{50} estimates were derived using the Trimmed Spearman-Karber method under automatic trim mode (Hamilton et al. 1977, 1978). Regression analyses were performed using SYSTAT versus 12 (Systat Software, Inc., Chicago, IL) and XLStat versus 2009.3 (Addinsoft, Inc., New York, New York).

Results and Discussion

1,2,4,5-TeCB was neither lethal, nor produced any discernable adverse effect, to either oligochaete. Body residues of 1,2,4,5-TeCB measured in *T. tubifex* were approximately 16 to 20 times higher (1,500 ± 725 mmol/kg lipid vs. 92 ± 22.5 mmol/kg lipid or 75 ± 15 mmol/kg

lipid) than the median lethal dose estimated for 1,2,3,4-TeCB and 1,2,3,5-TeCB, respectively (Table 1), and well above the body residue predicted by the CBR concept (66–266 mmol/kg lipid for *T. tubifex*) to be lethal (McCarty et al. 1992). Body residues of 1,2,4,5-TeCB measured in *E. andrei* were approximately 1.7–4.1 times lower (73 ± 23.3 mmol/kg lipid vs. 126 ± 0 13.3 mmol/kg lipid or 299 ± 15.9 mmol/kg) than the median lethal dose estimated for 1,2,3,4-TeCB and 1,2,3,5-TeCB, respectively. Although the body residue of 1,2,4,5-TeCB in *E. andrei* was lower than the other isomers, all three body residues fell within the concentration range (66–266 mmol/kg lipid) predicted by the CBR concept to be lethal (McCarty et al. 1992).

T. tubifex exposed to a nominal concentration of 1,2,4,5-TeCB (17,600 μmol/kg) 27 times higher than the nominal median lethal concentration estimated for 1,2,3,4-TeCB (642 ± 78 μmol/kg), and 20 times higher than the nominal median lethal concentration estimated for 1,2,3,5-TeCB (806 ± 60 μmol/kg), did not display any perceptible adverse effects (Table 1). Similarly, *E. andrei* exposed to a nominal concentration of 1,2,4,5-TeCB (4,651 μmol/kg) 13 times higher than the nominal median lethal concentration estimated for 1,2,3,4-TeCB (344 ± 23 μmol/kg), and 4 times higher than the nominal median lethal concentration estimated for 1,2,3,5-TeCB (1,037 ± 83 μmol/kg), did not display any perceptible adverse effects (Table 1).

Similar findings of a comparatively reduced 1,2,4,5-TeCB toxicity were reported by Chu et al. (1983) following a series of acute and sub-acute toxicity studies conducted with Sprague–Dawley rats. A markedly higher accumulation of 1,2,4,5-TeCB in fat and liver tissue relative to 1,2,3,4- and 1,2,3,5-TeCB was noted, with an hypothesis that the position of chlorine atoms around the benzene ring affected 1,2,4,5-TeCB isomer potency due to disproportionate partitioning into neutral lipid storage sites, an hypothesis that may explain the results of the present study.

The omission of a depuration phase as an explanation for observed TeCB concentrations was considered, and admittedly, the presence of gut contents may have influenced TeCB levels. While this possibility may have theoretical validity, it fundamentally ignores the following facts: 1) in two distinctly different organisms exposed in two distinctly different media (i.e., soil vs. water), two of the three TeCB isomers produced lethal narcosis, while one produced no perceptible adverse effects, 2) this comparison required the determination of TeCB concentrations in living *E. andrei* (1,2,4,5-TeCB) and dead *E. andrei* (1,2,3,4- and 1,2,3,5-TeCB) and thus it was impossible to depurate across all isomers given that deceased worms cannot be depurated, and 3) while it is entirely possible that 1,2,4,5-TeCB in the gut contents of live *E. andrei* contributed to the total body residue measured, this contribution was also present (theoretically) in the tests involving the 1,2,3,4- and 1,2,3,5-TeCB isomer and thus, at worst, would produce a unidirectional shift towards higher body residues, not a bi-directional shift as observed in the present study. When these facts are coupled with the findings of Rodriguez et al. (2001) that suggest *T. tubifex* preferentially feed on the silt and clay particle fraction of sediment, which was not present in the test medium, the importance of any gut content contribution to body residue is minimal.

The differential toxicity observed among TeCB isomers may have been the result of differential bioavailability and/or metabolism. However, the retarded bioavailability hypothesis was rejected following body residue analyses that indicated absorption, or adsorption, of the 1,2,4,5-TeCB isomer by both *T. tubifex* and *E. andrei*. In fact, body residues of 1,2,4,5-TeCB (*E. andrei*, 73 ± 23.3 mmol/kg lipid; *T. tubifex* 1,500 ± 725 mmol/kg lipid) were comparable to, or markedly greater than, the other two isomers from which a median lethal dose was estimated. Linear regression analyses indicated a direct relationship between nominal TeCB isomer exposure concentrations and body residue (Figs. 1, 2, 3). The accelerated metabolism hypothesis was rejected after confirming the absence of 1,2,4,5-TeCB metabolites on the chromatograms resulting from body residue analyses of those organisms exposed to 1,2,4,5-TeCB. This supports the expectation that significant TeCB metabolism should not occur, for even the metabolic rates of such compounds in organisms with well developed mixed function oxidase systems (e.g., mammals) under aerobic conditions and far longer exposure times, is poor

Table 1 Responses of *Tubifex tubifex* or *Eisenia andrei* to exposure to 1,2,3,4-, 1,2,3,5-, or 1,2,4,5-TeCB

Organism	LD_{50} (mmol/kg lipid)			LC_{50} (μmol/kg)		
	1,2,3,4-	1,2,3,5-	1,2,4,5-	1,2,3,4-	1,2,3,5-	1,2,4,5-
T. tubifex	92 ± 22.5	75 ± 15	1,500 ± 725	642 ± 78	806 ± 60	>17,600
E. andrei	126 ± 13.3	299 ± 15.9	73 ± 23.3	344 ± 23	1,037 ± 83	>4,651

T. tubifex LC_{50} values were derived from the nominal concentrations of the corresponding TeCB isomer in sediment (rather than water), given exposure to TeCB isomers at concentrations at, or below, their respective aqueous solubilities did not yield acute lethality (<14 days). Note that both LD_{50} and LC_{50} concentrations presented for the 1,2,4,5-TeCB isomer represent the highest concentration tested yet neither mortality, nor any perceptible adverse effects, were observed at those concentrations

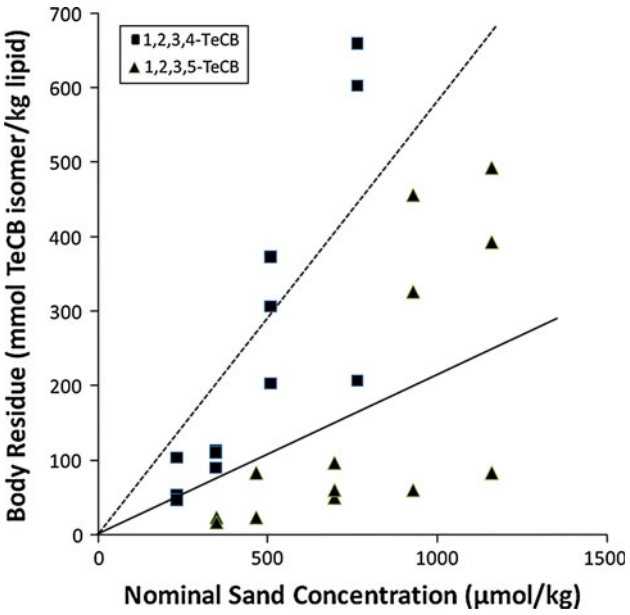

Fig. 1 Lipid normalized concentrations of 1,2,3,4- and 1,2,3,5-TeCB in *Eisenia andrei*. The *dashed line* represents a linear regression of 1,2,3,4-TeCB data resulting in a slope of 0.63 and a R^2 of 0.89 ($p = 0.001$; n = 12). The *solid line* represents a linear regression of 1,2,3,5-TeCB data resulting in a slope of 0.23 and a R^2 of 0.68 ($p = 0.004$; n = 14)

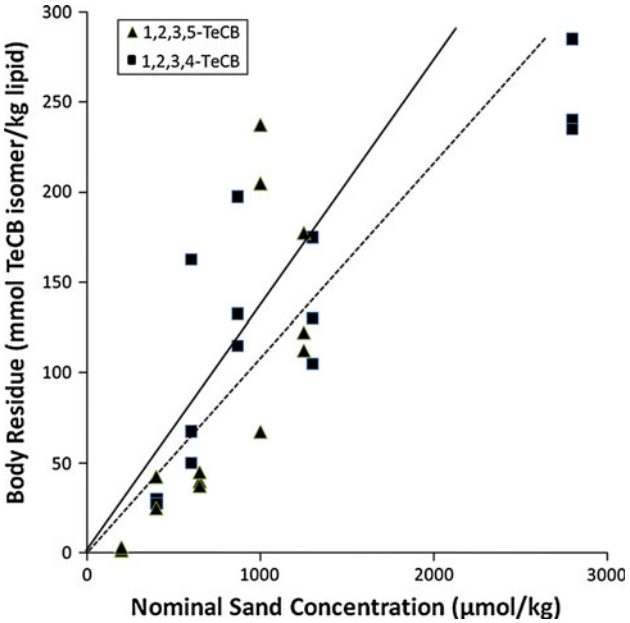

Fig. 2 Lipid normalized concentrations of 1,2,3,4- and 1,2,3,5-TeCB in *Tubifex tubifex*. The *solid line* represents a linear regression of 1,2,3,5-TeCB data resulting in a slope of 0.12 and a R^2 of 0.81 ($p = 0.001$; n = 14). The *dashed line* represents a linear regression of 1,2,3,4-TeCB data resulting in a slope of 0.10 and a R^2 of 0.91 ($p = 0.000$; n = 15)

(Johnston et al. 1997). Perhaps equally compelling is the fact that this lack of toxicity was observed in distinct test systems differing markedly in the physical and chemical

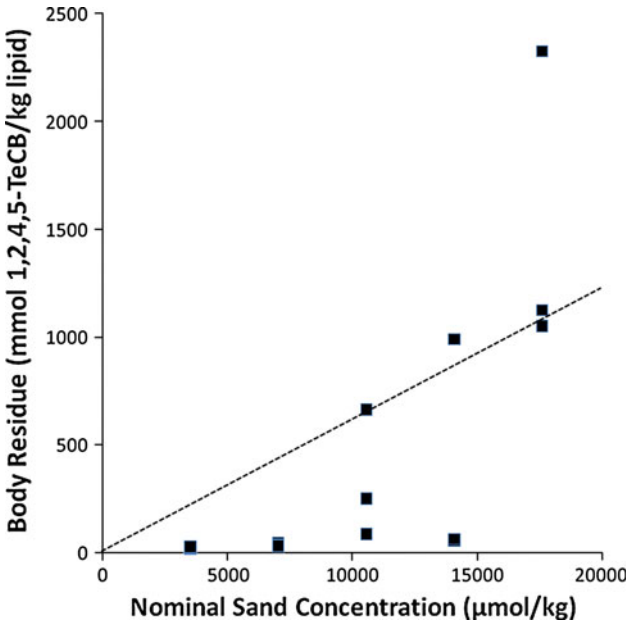

Fig. 3 Lipid normalized concentrations of 1,2,4,5-TeCB in *Tubifex tubifex*. The *dashed line* represents a linear regression of 1,2,4,5-TeCB data resulting in a slope of 0.05 and a R^2 of 0.60 ($p = 0.002$; n = 15)

characteristics of the test substrate, that is, an aquatic system (*T. tubifex*) featuring a mixed aqueous/solid phase and a terrestrial system dominated by a solid phase. Thus, if the 1,2,4,5-TeCB isomer is indeed entering the organism (which was not confirmed), and is poorly metabolized, then the observed differential toxicity may be better understood following a conceptual discussion of narcosis.

Another possibility for the observed lack of toxicity with 1,2,4,5-TeCB may have been the toxicity cutoff phenomenon. In assessing the toxicity and bioaccumulation of organic compounds, researchers have coined the term "toxicity cutoff" (Jonker and van der Heijden 2007) to describe a sharp decrease in toxicity in hydrophobic substances with a log K_{ow} greater than six due to significantly retarded uptake kinetics and/or steric hindrances. However, this hypothesis is not supported given that the log K_{ow} of the TeCB isomers tested in the present study ranged from 4.5 to 4.6, some 25 times less hydrophobic than the compounds with which this phenomenon has been observed.

Rand (1995) defines narcosis as "a general, nonspecific, reversible mode of toxic action that can be produced in most living organisms by the presence of sufficient amounts of many organic chemicals". He elaborates on this definition by stating "[narcotic] effects result from the general disruption of cellular activity" and that "hydrophobicity dominates the expression of toxicity in narcotic chemicals". Van Wezel and Opperhuizen (1995) provide further description of the candidate narcotic in that "nonspecific chemical action, such as narcosis, tends to be

Table 2 Select physical properties of tetrachlorobenzene isomers used in the present study as reported in the Concise International Chemical Assessment Document (CICAD) (2004)

Chemical	Abbreviation	CAS #	Molecular weight (g/mol)	Vapor pressure (25°C; Pa)	Aqueous solubility (25°C; mg/L)	Henry's law constant (25°C;kPa·m^3/mol)	Melting point (°C)	Log Octanol Water Partition Coefficient (K_{ow})
1,2,3,4-Tetrachlorobenzene	1,2,3,4-TeCB	634-66-2	215	5.2	12.1	0.2	47	4.5
1,2,3,5-Tetrachlorobenzene	1,2,3,5-TeCB	634-90-2	215	9.8	2.8	0.5	54	4.6
1,2,4,5-Tetrachlorobenzene	1,2,4,5-TeCB	95-94-3	215	0.7	2.1	0.2	139	4.5

elicited by non-polar substances, which are commonly, quantitatively identified via their octanol–water partition coefficient". Based on this collective informative, and prior to testing, the authors fully expected 1,2,4,5-TeCB to be a narcotic based upon physical and chemical properties similar to the other two TeCBs tested (Table 2), namely log K_{ow}, and an association with a class of compounds (chlorinated benzenes) that the literature has shown to act as narcotics. Yet from the results of this study, we can assemble these possible conclusions: (1) per Rand (1995), 1,2,4,5-TeCB is not a narcotic because it did not elicit narcosis, (2) 1,2,4,5-TeCB is a narcotic, for its log K_{ow} value (approximately 4.6) is typical of chemicals classified as narcotics, and therefore the results of this study challenge the CBR concept, (3) while the physical and chemical characteristics of 1,2,4,5-TeCB strongly suggest that 1,2,4,5-TeCB should elicit narcosis, it in fact does not (at least in the oligochaetes tested), indicating that there may be other metric(s) that can be applied, in concert with log $K_{ow,}$ to increase the probability that a given chemical will behave as a narcotic.

Supporting evidence for this third conclusion is found in Mayer and Holmstrup (2008), who observed an empirical, inverse relationship between chemical melting point and toxicity for hydrophobic organic compounds (HOCs), specifically, polycyclic aromatic hydrocarbons (PAHs) and springtails (*Collembola spp.*). Their work suggested that melting point may be a more important descriptor of chemical activity and toxicity than other chemical descriptors such as octanol–water partition coefficient or molecular size. Exposure to PAHs with low melting points (<110°C) resulted in complete mortality, while no mortality was observed in springtails exposed to PAHs with higher melting points (>180°C). The dissimilar melting points among TeCB isomers (139°C, 1,2,4,5-TeCB; 54°C, 1,2,3,5-TeCB; 47°C, 1,2,3,4-TeCB) and resulting toxicity are consistent with this hypothesis, suggesting that melting point should be considered in the criteria of physical and chemical properties used to describe putative narcotics. Mayer and Holmstrup (2008) further suggest that molecular symmetry may also be related to toxicity as well, because more symmetrical molecules tend to form more stable crystals and thus have higher melting points. The findings of the present study support this hypothesis as well, in that 1,2,4,5-TeCB displays the most symmetrical arrangement of chlorine atoms, the highest melting point, and a markedly lower toxicity than the 1,2,3,5- and 1,2,3,4-TeCB isomers.

In summary, the results of the present study, in conjunction with the implications of the research presented in Mayer and Holmstrup (2008), suggest that a definitive, properties-based classification (e.g., log K_{ow}) of a narcotic and any corresponding prediction of toxicity (e.g., CBR concept) based upon such a method of classification, may be not be absolute, and subject to exceptions such as 1,2,4,5-TeCB. Perhaps, as Mayer and Holmstrup (2008) imply, melting point, as an example, will emerge as a superior and/or concomitant metric to log K_{ow} in toxicity predictions employing a QSAR-like approach. Although the mechanism of the findings presented herein is open to debate, as is TeCB isomer absorption/adsorption, we conclude that if the definition of narcotic as a substance eliciting narcosis is accepted, then 1,2,4,5-TeCB does not challenge the CBR concept because by definition, 1,2,4,5-TeCB is not a narcotic.

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