Acute toxicity classification for ethylene glycol mono-n-butyl ether under the Globally Harmonized System

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A B S T R A C T

Acute oral, dermal and inhalation toxicity classifications of chemicals under the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (GHS) should typically be based on data from rats and rabbits, with the tacit assumption that such characterizations are valid for human risk. However this assumption is not appropriate in all cases. A case in point is the acute toxicity classification of ethylene glycol mono-n-butyl ether (EGBE, 2-butoxyethanol, CAS 111-76-2), where acute toxicity data from rats or rabbits leads to an overly conservative assessment of toxicity. Hemolysis is the primary response elicited in sensitive species following EGBE administration and the proximate toxicant in this response is 2-butoxyacetic acid (BAA), the major metabolite of EGBE. The sensitivity of erythrocytes to this effect varies between species; rats and rabbits are sensitive to BAA-mediated hemolysis, whereas humans and guinea pigs are not. In this publication, a weight of evidence approach for the acute hazard classification of EGBE under GHS is presented. The approach uses acute toxicity data from guinea pigs with supporting mechanistic and pharmacokinetic data in conjunction with human experience and shows that adopting the standard method results in over-classification.

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1. Introduction

Ethylene glycol mono-n-butyl ether (EGBE, CAS No. 111-76-2) is a high production volume glycol ether solvent and is a component of a variety of products including hydraulic brake fluids, water-based coatings, and hard-surface cleaners (Boatman and Knaak, 2001). The hazards and risks associated with this solvent have been extensively reviewed (Boatman and Knaak, 2001; ECETOC, 2004; ECHA, 2010; EPA, 2010; IARC, 1998). Currently the harmonized hazardous substance classification for acute toxicity in the European Union for EGBE under the Dangerous Substance Directive (67/548/EEC) is Xn: R20/21/22 (harmful by inhalation, in contact with skin and if swallowed). This corresponds with the translated harmonized hazardous substance classification for acute toxicity of EGBE under Regulation (EC) No. 1272/2008 (Annex VI) which is Acute Toxicity Category 4 (‘harmful’) for oral, dermal and inhalation exposures. The Globally Harmonized System (GHS) classifications taken from the Dangerous Substances Directive represent the equivalent categories and do not take into account the different classification thresholds.

In early studies in rodents and rabbits, it was recognized that EGBE produces a hemolytic response characterized by the appearance of hemoglobinuria and by changes in a variety of blood parameters (Werner et al., 1943; Carpenter et al., 1956). Although first recognized following inhalation exposures, such effects are also present following oral and dermal administrations (Carpenter et al., 1956; Grant et al., 1985; Bartnik et al., 1987). Hemolysis is observed following both single and repeated exposures to EGBE, with apparent tolerance to EGBE-induced hemolysis developing in sub-acute or sub-chronic studies (Krasavage, 1986; Grant et al., 1985). Ghanayem et al. (1990) have shown that this tolerance to hemolysis is a consequence of the replacement of older and more susceptible erythrocytes with less susceptible, younger cells. Other reports have subsequently confirmed these findings (Sivarao and Mehendale, 1995).

The acute toxicity of EGBE varies among test species, but would generally be classified as low to moderate. Acute signs of toxicity in sensitive experimental animals include lethargy, labored breathing and ataxia, generally accompanied by clear evidence of hemolysis (Boatman and Knaak, 2001; ECETOC, 2004; ECHA, 2010). Pathological effects often noted in these acute studies and that are considered to be secondary to hemolysis may include hemorrhagic lungs, mottled livers, congested kidneys and spleens, red-stained fluid in
Table 1
Acute toxicity hazard categories and acute toxicity estimated cut-off values for oral, dermal and inhalation exposures according to the Globally Harmonized System of Classification and Labeling of Chemicals (GHS).

| Exposure route | Category 1 | Category 2 | Category 3 | Category 4 | Category 5 |
|---------------|------------|------------|------------|------------|------------|
| Oral (mg/kg bw)\(^{a}\) | 5          | 50         | 300        | 2000       | 5000       |
| Dermal (mg/kg bw)\(^{a}\) | 50         | 200        | 1000       | 2000       | 5000       |
| Vapors (mg/l)\(^{a}\) | 0.5        | 2.0        | 10         | 20         | Note 1     |

Note 1: No specific cut-off value is assigned for acute vapor inhalation for Category 5. A dose equivalent to a 2000–5000 mg/kg bw exposure from an oral or dermal exposure is specified.

\(^{a}\) Acute toxicity estimates are derived from LD\(_{50}\) values from animal studies where available.

urinary bladders, and hemoglobinuria (Boatman and Knaak, 2001). Hematuria was often observed in animals that died or were seriously affected. In reliable studies\(^3\) reviewed under the European Union REACH registration process (ECHA, 2010), LD\(_{50}\) values from acute oral toxicity studies in experimental animals ranged from 615 mg/kg bw to values in excess of 2000 mg/kg bw; LD\(_{50}\) values from acute dermal toxicity studies ranged from 435 mg/kg bw to values in excess of 2000 mg/kg bw; and LC\(_{50}\) values from inhalation studies ranged from ca. 400 ppm (2.0 mg/L, 7 h exposure) to values in excess of 3.9 mg/L (theoretical maximum achievable concentration).

The acute toxicity database for EGBE indicates the guinea pig to be relatively insensitive to the hemolytic effects that are observed in other sensitive species (Gingell et al., 1998; Boatman and Knaak, 2001; ECHA, 2010). This is reflected in generally low levels of acute toxicity in this species. In reliable studies conducted using the guinea pig, a LD\(_{50}\) value from an acute oral toxicity study of 1414 mg/kg bw has been reported; LD\(_{50}\) values from acute dermal toxicity studies in excess of 2000 mg/kg bw have been reported; and LC\(_{50}\) values from inhalation studies of approximately 400 ppm (2.0 mg/L) for a 7 h exposure, as well as values in excess of the maximum achievable vapor concentration of 633 ppm (3.1 mg/L) or 691 ppm (3.4 mg/l) for 1-h exposures have been reported. As in the case of the guinea pig, humans are generally insensitive to the hemolytic toxicity of EGBE. Under conditions of controlled human exposures, no overt toxicity or signs of hemolytic effects have been observed (Carpenter et al., 1956; Johanson, 1986; Johanson and Boman, 1991). In well-documented cases of intentional ingestions of large amounts of EGBE-containing products, coma with respiratory and other complications has been reported, but with little conclusive evidence of hemolysis (reviewed in Udden, 1996; Gualtieri et al., 2003; Hung et al., 2010). Metabolic acidosis is the critical effect observed in these poisonings with survival of all individuals following appropriate supportive care.

Under the guidelines for classification of acute toxicity according to GHS, the rat is the preferred species for evaluation of acute oral and inhalation toxicity and the rabbit the preferred species for acute dermal toxicity. Strictly applying the classification thresholds of this guideline may result in possible classifications for acute dermal toxicity Category 2, with the required values for classification under GHS. Expert judgment must be applied for weight of evidence from experimental animals, as well as additional considerations for human exposure.

For the purposes of classification, acute toxicity is defined as an adverse effect occurring following a single oral or dermal dose of a chemical. An adverse effect resulting from multiple doses administered within 24 h would also be considered an acute effect. An acute inhalation effect is defined as an adverse response following a 4-h exposure. The allocation of substances to one of five toxicity categories under GHS is shown in Table 1. The values shown represent numeric cut-off values for each category.

The general guidance provided for classifications under the GHS classification system as well as the related classifications under Regulation (EC) No. 1272/2008 (Classification, Labelling and Packaging (CLP) of substances and mixtures) recognize that animal data from acute toxicity studies will generally provide the acute toxicity estimates (ATE) as indicated by the cut-off values in Table 1. Although presented as estimates, in fact and in common practice, these default values become ipso facto the required values for classification. In other words, if reliable animal test data is available, and in the absence of reliable or contradictory human data, the values in Table 1 represent the default values for classification. As stated in the guidance for GHS classification system (UN, 2011), the rat is the preferred test species for acute oral and inhalation exposures. Thus, acute oral LD\(_{50}\) values from reliable studies in rats have precedence over other acute toxicity data from experimental animals. Similarly, acute inhalation 4-h LC\(_{50}\) values from studies conducted in rats are the preferred value for setting the acute inhalation classification. Acute toxicity data from either the rat or rabbit is preferred for evaluation of dermal toxicity. However, the GHS text (and the CLP legal text) also state that when experimental data for acute toxicity are available in several animal species, scientific judgement should be used in selecting the most appropriate LD\(_{50}\) value from among valid, well-performed tests. Therefore, when determining the acute toxicity of a substance, it is acceptable to deviate from the standard species (rats and rabbits) where there is a sound scientific basis for doing so.

2.2. Use of human data for hazard classification under GHS

It is clearly stated in the GHS guidance that the protection of human health and the environment is a primary goal of the harmonized hazard classification scheme. For the purpose of acute toxicity classification, reliable epidemiological data as well as experience gained from occupational exposures, medical surveillance, case reports or reports from national poison centers are all recognized sources of information. A weight of evidence approach is to be used for classification, with both human and animal data considered. In cases of a conflict, the quality and reliability of the results evaluated must be assessed to determine the final classification. Expert judgment must be applied for weight of evidence determinations both to assess quality and reliability as well as to assess confounding factors.

3. Summary of acute toxicity data for EGBE

Summarized in Tables 2–4 are acute toxicity data for EGBE from experimental animals following oral, dermal or inhalation exposures. The studies listed have been reviewed for reliability under the requirements of the EU REACH registration program and most
received acceptable ratings of 1 or 2 using the method of Klimisch et al. (1997).

3.1. Acute oral toxicity of EGBE in experimental animals

Table 2 lists acute toxicity data from experimental animals exposed by the oral route to EGBE. Acute oral toxicity has been most commonly measured in young adult rats, with reported LD₅₀ values varying significantly over a range of 615 mg/kg bw (BASF AG, 1968) to 2100 mg/kg bw (Carpenter et al., 1956). Hemolysis was observed in the majority of these studies, sometimes accompanied by evidence of hemolysis or any effects noted on erythrocytes. Gastrointestinal effects often noted included bloody urine and/or blood in the stomach and intestines and diffuse necrosis and hemorrhage of the gastric mucosa, indicative of a gastric irritant.

In a single reliable acute oral toxicity study conducted in mice, both fasted and fed male mice were treated by gavage with undiluted EGBE (Krasavage and Terhaar, 1981a). Fasted mice appeared somewhat more sensitive to EGBE than fed mice with LD₅₀ values of 1519 mg/kg bw (fasted) versus 2005 mg/kg bw (fed). In two older and less reliable acute oral toxicity studies by Carpenter et al. (1956), rabbits were reported to be the most sensitive of the species tested that included rats, mice, and guinea pigs. The LD₅₀ values reported in these studies were 320–370 mg/kg bw. These values seem low compared with those of other species but are consistent with more recent data for this species indicating complete mortality in both male and female rabbits at an administered dose of 695 mg/kg bw (BASF AG, 1967).

Two reviewed studies are available in guinea-pigs, one of which is a more recent GLP guideline study. The LD₅₀ values reported in these studies, 1414 and 1200 mg/kg bw, were comparable (Shepard, 1994a (as reviewed by Gingell et al., 1998); Carpenter et al., 1956). The same or similar clinical signs and pathology as displayed in other species were seen in these studies. However, in the more recent study of Shepard (1994a), there was no evidence of hemolysis or any effects noted on erythrocytes. Gastrointestinal irritation, as evidenced by salivation and changes in the gastric mucosa, may have contributed to the toxicity in this study.

3.2. Acute dermal toxicity of EGBE in experimental animals

Listed in Table 3 are acute dermal toxicity data for EGBE in experimental animals. In most cases, experimental conditions included occlusive exposures of 24-h duration. Data is presented for three species: the rat, rabbit and guinea pig.

In male and female rats, both occlusive and semi-occlusive exposures to undiluted EGBE are available and, using a weight of evidence approach, the LD₅₀ value in the rat can be considered to be >2000 mg/kg bw both under occluded and non-occluded conditions (Table 3). In rabbits, the results shown in Table 3 are generally consistent and indicate an increased acute dermal toxicity of EGBE in this species. In guinea-pigs, variations in LD₅₀ values were seen among the studies with a range from 230 mg/kg bw to

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Table 2

| Route/species/strain/sex/age | Dose | Exposure regime | Results (mg/kg bw)/evidence of hemolysis | References |
|----------------------------|------|----------------|------------------------------------------|------------|
| Rat/COBs CD BR/male         | 2.6–168 mM/kg bw (5 dose levels) | Gavage (no vehicle) | LD₅₀ = 1746 (fasted and fed animals)/hematoma; blood in stomach and intestines | Krasavage and Terhaar (1981a) |
| Rat/male–female             | 200, 400, 500, 640, 800, 1000, 1250 and 1600 mg/kg bw | Gavage (water) | LD₅₀ = 880 (male) LD₅₀ = 615 (female)/hemolytic urine; anemic musculature; hemoglobinuria | BASF AG (1968) |
| Rat/Wistar/male             | No data on doses administered | Gavage (water) | LD₅₀ = 1480/effects in Kidney and liver | Smyth et al. (1941) |
| Rat/Sherman and Carworth/male–female/5–6 weeks of age | No data on doses administered | Gavage (undiluted or in water) 5% or 10% dilutions | LD₅₀ (range-8 studies) >560 to <3000 (male) LD₅₀ = 2100 (male) LD₅₀ (range-6 studies) >530 to <2800 (female) LD₅₀ = 1850 (female)/hemoglobinuria; hemorrhaged lungs; mottled livers; congested kidneys | Carpenter et al. (1956), Dow (1952) |
| Rat/(CDF (F344 derived)/male | 130, 250, 500, 1000 and 2000 mg/kg bw | Gavage (no vehicle) | LD₅₀ >1000 to <2000 (est. ~1900)/staining of perianal region; necrosis of tails | Dow (1981) |
| Rat/Wistar/male             | 1128, 2257, 4515 and 9030 mg/kg bw | Gavage (no data on vehicle) | LD₅₀ = 2420/bloody salivation; dark livers; distended stomachs filled with liquid and gas; red colored kidneys and adrenals; blood in intestines | DOW (1980a) |
| Mouse/CD-1/male             | 2.6–168 mM/kg bw (5 dose levels) | GAVAGE (no vehicle) | LD₅₀ = 1519 (fasted) LD₅₀ = 2005 (fed)/hematoma; blood in stomach and intestines | Krasavage and Terhaar (1981a) |
| Mouse/male                  | No data on doses administered | Gavage (water) | LD₅₀ = 1230 | Carpenter et al. (1956) |
| Rabbit/male                 | No data on doses administered | Gavage (water) | LD₅₀ = 320 and 370 mg/kg bw (2 studies) | Carpenter et al. (1956) |
| Rabbit/male–female          | Approx. 695 and 1400 mg/kg bw | Gavage (water) | LD₅₀ = 695/hemoglobinuric nephrosis; bloody-red coloration in anterior chamber of eyes; decreased hematocrits; degenerative blood changes; lung edema | BASF AG (1967) |
| Dog/beagle/male–female      | Approx. 695 mg/kg bw | Gavage (water) | LD₅₀ = 695/no abnormalities noted | BASF AG (1966) |
| Guinea pig/Hartley/male–female/5–7 weeks of age | 500, 1000 and 2000 mg/kg bw | Gavage (distilled water) dose volume 1.5–2.0 ml | LD₅₀ = 1414/no evidence of hemolysis | Shepard (1994a), Gingell et al. (1998) |
| Guinea pig/male–female      | No data on doses administered | Gavage (water) | LD₅₀ = 1200/no evidence of hemolysis | Smyth et al. (1941), Carpenter et al. (1956) |

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* Unless otherwise noted, studies summarized were judged to have reliabilities of 1 or 2 using the scale and scoring system of Klimisch et al. (1997).
* A reliability rating of Klimisch 4 was assigned to this study. However, the results have been included as supporting information.
* Although judged of low reliability, this study has been included as supporting information.
3.3. Acute inhalation toxicity of EGBE in experimental animals

Listed in Table 4 are acute inhalation toxicity data for EGBE in experimental animals. Exposure durations reported in these studies ranged from 1 to 8 h thus complicating a direct comparison of the study results. No attempt has been made to correct for exposure durations. When interpreting the findings from these inhalation studies, it is also important to note that the calculated saturated vapor concentration of EGBE under ambient conditions is 791 ppm (3.9 mg/l) (DOW, 1974). Thus values in excess of this may represent nominal values. Maximum measured achievable vapor concentrations below this have been reported (DOW, 1994).

The acute inhalation toxicity of EGBE has been most commonly measured in the rat. Acute 4-h inhalation LC50 values in male and female rats were 2.4 and 2.2 mg/l, respectively (DOW, 1980b). LC50 values at or near the theoretical maximum vapor concentration have been reported for exposure durations from 1 to 8 h (MacDonald, 1982; Carpenter et al., 1956). There is some limited evidence to suggest that female rats are more sensitive than males (DOW, 1980b; MacDonald, 1982). Clinical signs and pathology indicative of hemolytic toxicity are seen.

In six, 7-h exposure studies in male rabbits at approximate vapor concentrations of 400 ppm (2.0 mg/l), an LC50 of 400 ppm (2.0 mg/l) was reported representing a mortality rate of 10/24 animals as an aggregate of the six studies (DOW, 1974).

In a more recent and well-documented acute inhalation toxicity study in male and female guinea pigs, test animals were exposed to maximum achievable vapor concentrations of EGBE for 1 h (DOW, 1994; Gingell et al., 1998). There was neither mortality nor adverse clinical effects noted in this study. There were also no significant adverse pathological effects reported. The LC50 values for guinea pigs in this latter study were 3.1 mg/l (females) and 3.4 mg/l (males), representing the maximum measured vapor concentrations achieved in the study. It is worth noting that at a theoretical maximum saturated vapor concentration of 3.9 mg/l, and given a standard respiratory minute volume in the guinea pig of 0.66 l/min/kg bw (Snipes, 1988), a 4 h inhalation exposure in a guinea pig would result in an internal dose (assuming 100% absorption) of 618 mg/kg bw, which is approximately half of the acute oral LD50 value in this species. Thus at experimentally-achievable vapor concentrations, a LC50 value from a 4-h exposure cannot be obtained in this species.

4. Human exposure data for EGBE relevant to an acute hazard classification

4.1. Controlled exposures of volunteers to EGBE

No evidence for hemolysis has been reported in any controlled laboratory exposures of human volunteers to EGBE by inhalation. In early studies by Carpenter et al. (1956), there was neither increased osmotic fragility of erythrocytes nor other signs of hemo-
Table 4
Acute inhalation toxicity values for EGBE.

| Route/species/strain/sex/age | Dose | Exposure regime | Results (mg/l)/evidence of hemolysis | References² |
|------------------------------|------|-----------------|-------------------------------------|-------------|
| Rat/Fischer 344/male–female/6–7 weeks of age | 867, 523 and 202 ppm (measured) | 4-h exposure/whole body | LC₅₀ = 2.4 (males) LC₅₀ = 2.2 (females)/red fluid discharge around urogenital region; enlarged and discolored kidneys; red-stained urine in bladders | DOW (1980b) |
| Rat/Wistar/male–female/8 weeks of age | 900–910 ppm | >1 to <7 h/3 studies | LC₅₀ > 4.4 (females) LC₅₀ > 4.4 (males)/blood in urine; necrosis of tails; pale eyes and feet | MacDonald (1982) |
| Rat/male–female | 1.44 mg/l (3 h) 4.25 mg/l (8 h) | 3 or 8 h exposures/whole body | LC₃₀ = 1.44 (3 h, no deaths) LC₃₀ = 4.25 (8 h, complete mortality)/hematruia; slight anemia; liver anemia; blood clotting in bladders | BASF AG (1968) |
| Rat/Sprague–Dawley/male–female | 2.25 mg/l (3 h), 4.26 mg/l (7 h) | 3 or 7 h exposures/whole body | LC₃₀ > 4.26/hemorrhagic urine; anemic ears; discolored liver; bloody ulcerations of the glandular stomach | BASF AG (1979) |
| Rat/male–female | 4.9 mg/l (measured) | >1 to <3 h | LC₅₀ > 4.9 mg/l/L (3 h) | Klimisch et al. (1988) |
| Rat/female (also, older animals studied) | 500 and 800 ppm | ≥4 to ≤8 h | LC₅₀ = 3.9 (4 h) | Carpenter et al. (1956) |
| Rabbit/male | 403 ppm (average) | 7 h/whole body | LC₃₀ > 2.0 mg/l/no signs of hemolysis | DOW (1974) |
| Dog/Beagle/male | 403 ppm (average) | 7 h/whole body | LC₃₀ > 2.0 mg/l/no signs of hemolysis | DOW (1974) |
| Guinea pig/Dunkin–Hartley/male–females/36 days of age | 633 ppm (measured, males) 691 ppm (measured, females) | 1 h/whole body | LC₃₀ = 3.1 (females) | DOW (1994), DOW (1998) |
| Guinea pig/male | 403 ppm (average) | 7 h/whole body | LC₃₀ > 2.0/no adverse clinical findings | DOW (1974) |

¹ Values of ppm EGBE were corrected to mg/l based on 4.914 mg/m³ = 1 ppm.
² Unless otherwise noted, studies summarized were judged to have reliabilities of 1 or 2 using the scale and scoring system of Klimisch et al. (1997).

Injury in 3 individuals exposed by inhalation at 195, 113 or 98 ppm of EGBE for up to 8 h. Seven male volunteers were exposed to 20 ppm of EGBE for 2 h while performing light work (Johnson, 1986). None of the exposed subjects in this latter study showed any of the adverse effects related to EGBE exposures.

4.2. Case studies involving accidental and intentional ingestions of EGBE

Reported cases of acute human poisonings with EGBE are rare and generally involve either accidental ingestion in pediatric cases or intentional ingestions in adults (Udden, 1996; Gualtieri et al., 2003; Hung et al., 2010). Dean and Krenzelok (1991) reviewed 24 pediatric poisoning cases reported to the Pittsburgh Poison Center (PPC) during a five month period from December of 1990 to April of 1991. These all involved glass or window cleaners containing EGBE at concentrations ranging from 0.5% to 9.9%. The ages of the children involved ranged from 7 months to 9 years. All incidents were reported to the PPC within 5 min of the actual exposures. The estimated quantities ingested ranged from 5 to 300 ml and all children were reported to be asymptomatic immediately following the ingestions. In the single greatest exposure among this group, a 2-year-old child was reported to have swallowed 300 ml of an 8% EGBE-containing glass cleaner representing approximately 24 ml of EGBE. In this latter case, the child underwent gastric lavage and was hospitalized for 24 h. Evidence of the toxicity of EGBE, as expressed in animals, and including hemolysis, central nervous system depression, metabolic acidosis and renal compromise were completely absent in these pediatric cases. In 22 of the reported 24 cases from this study, the patients were treated at home with simple dilution. All cases remained asymptomatic throughout an additional 48 h of telephone follow-up.

Summarized in Table 5 are a number of reported cases of intentional ingestion by adults of large amounts of EGBE-containing products. Reviews of these cases have been previously published (Udden, 1996; Gualtieri et al., 2003; Hung et al., 2010). Severe metabolic acidosis and coma are consistently reported in these poisoning cases. All patients required aggressive support with administration of fluids and mechanical ventilation. However, it is important to note that in all reported cases, patients recovered fully without subsequent symptomology. In those reported cases for which blood levels of BAA were measured, concentrations typically peaked at 2 days or after with little still present by 3 days. Maximum concentrations of BAA ranged as high as 3.64 mM (Table 5) and were generally in excess of levels that cause hemolysis in blood from sensitive species (Ghanayem and Sullivan, 1993), but well below a concentration of 10 mM, a level reported to show only the most minimal hemolytic effects in human blood (Udden, 2002).

In all but two of the cases listed in Table 5 (Gijzenbergh et al., 1989; Bauer et al., 1992), hemodialysis was employed to remove un-metabolized EGBE. In these reports there was no clear evidence for hemolysis as seen in sensitive laboratory species. In reports by Bauer et al. (1992) and Hung et al. (2010), non-hemolytic anemia was attributed to hemodilution as a result of hemodialysis. Other reported effects included renal insufficiency, thrombocytopenia and disseminated intravascular coagulation. As discussed by Udden (1996), these other effects reported were most likely not directly related to hemolysis. However, the exact etiology of these latter effects in certain human poisoning cases cannot be explained based on the limited data available. It can be concluded from these adult poisoning cases that EGBE is generally of a low order of acute toxicity in humans. High doses of EGBE in humans do not cause the characteristic hemolytic effects which have been shown to be critical for the acute toxicity expressed in rats, mice and rabbits.

5. Acute toxicity mode of action for EGBE

5.1. In vivo determinants of the hemolytic response to EGBE

Intravascular hemolysis is the major effect reported in acute toxicity studies in rats, mice and rabbits following EGBE administration. Both a dose- and concentration-dependent hemolytic anemia develops in rats following the administration of a single dose
of EGBE (Carpenter et al., 1956; Ghanayem et al., 1987a). The major urinary metabolite of EGBE, 2-butoxyacetate (BA), was originally confirmed to be the proximate hemolytic agent by Carpenter et al. (1956). In human poisoning cases as discussed above, metabolic acidosis is a commonly reported effect (Udden, 1996). Lactic acidosis is also observed in most of these cases of EGBE ingestion and it has been suggested that this may be a consequence of EGBE metabolism in humans (Hung et al., 2010). Thus, a combination of lactate production and BAA lead to the metabolic acidosis observed in human poisonings (Gijzenbergh et al., 1989). Other factors such as dialysis and hypotension may also contribute to the observed consequences of human ingestions (Udden, 1996; Hung et al., 2010). There are no comparable reports or studies of EGBE-induced acidosis in laboratory animals that can be used as a direct comparison to the effects seen in humans. At least in the case of the rat, the most extensively studied laboratory species, there is evidence that metabolic acidosis may be of much less consequence. In particular, this species more rapidly metabolizes and eliminates EGBE and its metabolites than do humans (Corley, 1996). Also, the tolerance induced in rats following either repeated or single sub-lethal doses of EGBE strongly argues for hemolysis as the primary toxicological response in this species, with other factors of only secondary importance (Ghanayem et al., 1992; Sivarao and Mehendale, 1995).

A number of detailed hematological investigations of EGBE toxicity have been conducted in the Fischer strain of rat. In a sub-acute oral toxicity study reported by Grant et al. (1985), four to five week old male F344 rats received 500 or 100 mg/kg EGBE for 4 days. These rats displayed decreased erythrocyte counts; increased relative weights of spleen, liver and kidneys; thymic atrophy; and lymphocytopenia. Microscopic examination of blood in this study was consistent with intravascular hemolysis and revealed increased numbers of circulating nucleated erythrocytes (normoblasts); pronounced anisocytosis, polychromasia and the presence of Howell Jolly bodies. All of these effects resolved within a 22-day recovery period, with the exception of relative weights of liver and spleen, which remained slightly raised. In studies by Ghanayem et al. (1987b), groups of adult (9–13 week) or young (4–5 week) male F344 rats were dosed EGBE by single gavage treatment at 32, 63, 125, 250 or 500 mg/kg bw. Significant decreases in circulating erythrocytes, hemoglobin concentrations, and hematocrit were seen in adult rats at doses of 125 mg/kg bw and above but in young rats only at 250 mg/kg bw or higher. The greatest changes occurred within 4–24 h. The onset of hemoglobinuria followed the decline in plasma hemoglobin levels and was again more pronounced for adult rats. Hematological changes were mostly resolved by 48 h. Histopathological changes in the liver including focal disseminated coagulative necrosis of hepatocytes and evidence of hemoglobin phagocytosis by Kupfer cells and hepatocytes were present at the two highest dose levels in adult rats but were absent in young rats. In concurrent metabolism studies, young rats excreted a greater proportion of the dose as carbon dioxide or urinary metabolites (Ghanayem et al., 1987b). It was proposed that the greater susceptibility of the older rats to the hemolytic toxicity of EGBE may be due, at least in part, to a greater proportion of BAA formed and to a depressed urinary excretion.

In a comparison of the in vivo hemolytic toxicity between rats (sensitive) and guinea pigs (insensitive), both species were given a sub-lethal gavage dose of EGBE at 250 mg/kg bw and blood parameters measured for up to 25 h (Ghanayem and Sullivan, 1993). As expected, rats showed significant declines in MCV, HCT, HGB and erythrocyte counts, associated with hemolysis. In guinea pigs, no changes in any of these parameters were recorded.

Thus, sensitive laboratory species such as rats, when exposed to acutely toxic doses of EGBE, display a number of responses secondary to hemolysis and including enlarged kidneys, blood in the bladder, bloody urine, and splenic lesions. In contrast, guinea pigs display less sensitivity to the acute toxicity of EGBE than either rats, mice or rabbits and do not display the adverse pathological effects associated with hemolysis as seen in the other sensitive species.

Carpenter et al. (1956) first identified BAA as the metabolite responsible for the hemolytic toxicity of EGBE by incubating the acid with blood from a variety of animal species and humans. In these studies, blood from rats, mice, and rabbits was more rapidly hemolysed than blood from monkeys, dogs, humans or guinea pigs when incubated at 37 °C in a 0.1% saline solution of sodium butyrate (BA). Also, inhalation exposures of rats, mice and rabbits led to increased osmotic fragility of erythrocytes while no similar effects were reported in monkeys, dogs, humans or guinea pigs, thus confirming the relevance of the in vitro responses (Carpenter et al., 1956).

Ghanayem and Sullivan (1993) have assessed the in vitro hemolytic response of BAA in blood from a variety of species including rats, mice, hamsters, rabbits, guinea pigs, dogs, cats, pigs, baboons and humans. In these studies, blood collected from 7.5% EDTA as anticoagulant was treated with 1.0 or 2.0 mM BAA concentrations. These concentrations were selected because previous in vivo studies indicated these blood levels were found to cause intermediate levels of toxicity (Ghanayem, 1989). Blood was incubated at 37 °C and samples collected at 1, 2 and 4 h and spun hematocrits obtained (HCT). Complete blood counts were obtained using an automated hematology analyzer and included the following: white blood cell counts, platelet counts, red blood cell (RBC) counts, mean cell volume (MCV), mean corpuscular hemoglobin (MCH),

### Table 5

Case reports of human adult EGBE ingestion.

| Reports                  | Age (years) | Sex   | Amount ingested (g) | Blood BAA (mM) | Hemoglobin level (g/dL) | Additional observations                                      |
|--------------------------|-------------|-------|---------------------|----------------|-------------------------|-------------------------------------------------------------|
| Rambourg-Schepens et al. (1988) | 50          | F     | 50–60               | 2.2* (Day 2)   | 9.7                     | Hemoglobinuria                                               |
| Gijzenbergh et al. (1989) | 23          | F     | 25–30               | 0.4* (Day 2)   | 8–9                     | Non-hemolytic anemia; Thrombocytopenia; adult respiratory distress syndrome (ARDS); renal insufficiency |
| Bauer et al. (1992)       | 45          | M     | 45                  | N/A            | 9.1                     | Disseminated intravascular coagulation                       |
| Litovitz et al. (1991)    | 87          | F     | Unknown             | N/A            | Unknown                | Profound metabolic acidosis; CNS depression; no evidence of hemolytic anemia |
| Gualtieri et al. (1995, 2003) | 18          | M     | 80–100              | 3.64 and 2.07* (Day 2) | Unknown                | Metabolic acidosis; no red cell dysmorphology or evidence of hemolysis |
| Hung et al. (2010)        | 53          | M     | 150–250 ml          | 13.1–10.7 (3 h post-admission) | N/A                    |                                                              |

* Estimate taken from a graphical representation of the results and corrected to mM concentration units based on an average serum concentration of 0.7 mg/dl creatinine for females.

b Intentional ingestions on 2 separate occasions.
and mean corpuscular hemoglobin concentration (MCHC). Blood from rodents, as represented by rats, mice and hamsters, displayed a time and concentration dependent increase in HCT and MCV when incubated with BAA, with more than a 45% increase above the corresponding control in MCV at 2 h and 6% decrease in RBC counts in rats and mice. At the higher concentration, RBC counts decreased 30% below control levels by 4 h. In hamsters, MCV increased greater than 15% and 35% above control levels at 1 or 2 mM BAA for 4 h, respectively. Although significant swelling occurred, no hemolysis of hamster blood was observed. Blood from rabbits incubated at 2 mM displayed greater than 20% increases in MCV above control levels at 2 h with a further increase to 39% by 4 h. Despite this extensive swelling of the RBCs, there was no significant change in RBC counts or hemoglobin concentrations suggesting hemolysis had not occurred under these in vitro conditions. The time- and concentration-dependent swelling of the erythrocytes of sensitive species leading to increased HCT and MCV is the effect most relevant to in vivo hemolysis. Such changes lead to decreased deformability of the erythrocytes, decreased ability to pass through small capillaries and subsequent removal of these damaged cells from circulation by the spleen.

Blood samples from a number of species tested by Ghanayem and Sullivan (1993) were insensitive to the hemolytic effects of BAA. Thus, blood from the guinea pig (Cavia porcellus) was essentially unaffected by incubations with 1 or 2 mM BAA for up to 4 h. Blood from two primate species were also assayed in these studies. Incubations of blood collected from healthy white adult humans with 2 mM BAA for up to 4 h caused only slight and statistically insignificant changes in MCV and HCT. In the case of blood from the yellow baboon, MCV increased in a time- and concentration-dependent manner to nearly 35% above control values after 4 h with 2 mM BAA. HCT in this species increased in a parallel fashion with MCV and hemolysis was significant at either the 1 or 2 mM BAA concentration by 4 h. The contrasting results from baboons and humans, both of the order primates, suggest the hemolytic sensitivity to BAA cannot be predicted solely based on order or class of mammal.

Udden (1996) has studied the effects of BAA on human erythrocytes from young (aged 31–56 years) versus old (aged 64–79 years) adults as well as blood from patients with hemolytic disorders. The increased toxicity of EGBE in older rats has been attributed to older erythrocytes (Ghanayem et al., 1987b). Incubation of erythrocytes from young versus old humans with 2.0 mM BAA produced no significant hemolysis. Similarly, erythrocytes from patients with sickle cell disease and those with hereditary spherocytosis were unaffected. It is notable that erythrocyte swelling with loss of the discocyte morphology is characteristic of BAA-induced changes in rats and this change is also a characteristic of hereditary spherocytosis.

Although when incubated at concentrations of 2.0 mM and below no significant effects on human erythrocytes have been observed, sub-hemolytic effects have been reported when human erythrocytes are incubated at high BAA concentrations (Udden, 2002). Incubations of human erythrocytes at concentrations 7.5 mM and 10 mM for 1–4 h resulted in decreased deformability, as measured by filtration, and slight but significant increases in MCV at the 10 mM concentration. Similar effects were observed when rat erythrocytes were incubated at 0.1 mM for 4 h. Thus, at 10 mM BAA, a 40% increase in filtration pressure was seen for human erythrocytes compared with a 64% increase in filtration pressure for rat erythrocytes incubated at a 100-fold lower concentration of 0.1 mM. Thus, a minimum 100-fold lower sensitivity of human versus rat erythrocytes was seen in this study.

Starek et al. (2008) have reported similar but less pronounced differences in the hemolytic effects of BAA between rat and human erythrocytes. Washed erythrocytes from healthy human donors or male Wistar rats were incubated in 10 mM Tris buffer for up to 3 h with BAA concentrations ranging from 6.0 to 18.0 mM (humans) or 1.0–5.5 (rats). EC50 values for changes in red blood cell counts (RBC), packed cell volumes (PCV) and mean corpuscular volumes (MCV) were then obtained. It is important to note that the EC50 value for PCV changes with BAA was 13.1 mM, but a similar EC50 value could not be determined for MCV changes since these were not large enough to allow calculation of this value. There are several reasons why this work does not lend itself to direct comparison with the greater body of work reported on the comparative effects of hemolysis by EGBE in different species. In particular, no discussion is given by these authors to the possible effects of the very high concentrations employed in these studies. A possible explanation for the effects reported is that cell damage and hemolysis, resulting in lowered RBC values, occurred due to uncontrolled and lowered pH; with these effects seen without the attendant cell swelling and the normally observed changes in PCV and MCV. These results also contradict the findings of other workers including Udden (2002) and Ghanayem (1989), who report only the most minimal and non-specific effects on human erythrocytes at 8.0 or 10 mM in vitro concentrations, and the work of Bartnik et al. (1987), who reported no hemolysis of human red blood cells incubated for 3 h with 15 mM BAA. Such high and physiologically irrelevant concentrations are considered of little or no use in the present human hazard assessment of EGBE.

6. Utilizing a weight of evidence approach to classify for acute toxicity

The application of expert judgment using a weight of evidence approach is a key feature of the hazard classification of chemicals under GHS (Morita and Morikawa, 2011; Hamilton et al., 2006). However, weight of evidence is a somewhat nebulous concept that does not have a set of well-defined tools and procedures for its implementation (Morita and Morikawa, 2011). In the ideal situation, all reliable scientific data relevant to the endpoint of concern undergoes expert review and subsequently is weighted to allow a final hazard classification. However, GHS is a Globally Harmonized System that is applied among many countries and regions of the world and with differences in the procedure used for its implementation. This often leads to discrepancies in the hazard classification of substances (Morita and Morikawa, 2011; OECD, 2010). This review is an attempt to perform a definitive assessment based on the hazard to humans that can be used as a basis to derive an acute toxicity classification for EGBE where ever GHS is implemented.

The categorization of acute hazard under GHS requires acute toxicity estimation (ATE) for placement into one of five hazard categories (Table 1). These categories are defined by various sharp cut-off values and the information used for the final category assignments is most often derived from inherently variable acute toxicity data derived from animal studies. The problems associated with this method are clearly illustrated in a recent publication by Hoffmann et al. (2010) in which a large dataset of acute oral toxicity data from experimental animals was compared for a total of 73 reference chemicals. Reliable studies, as defined by a Klimisch score of 1, “reliable”, or 2, “reliable with restrictions”, were scarce with data often available from studies over several decades old. Statistical analyses revealed that only ~50% of the substances would be unequivocally assigned to a single classification category (under GHS or EU categories); ~40% would unequivocally be classified within two adjacent categories; and ~10% would have LD50 ranges sufficiently large to span three or more classification categories.

In the current report, data from acute toxicity studies in experimental animals along with information from mode of action stud-
ies and human experience has been used to categorize the acute toxicity of EGBE. In sensitive rodent species, hemolysis of erythrocytes caused by the major metabolite of EGBE, BAA, has been shown to be responsible for the acute effects and mortality observed with this solvent (Carpenter et al., 1956). The central role of BAA as the proximate hemolytic agent in sensitive species has been further confirmed through in vivo studies employing metabolic inhibitors such as pyrazole and cyanamide, to block metabolism to the acid through alcohol and aldehyde dehydrogenases, respectively (Ghanayem et al., 1987a). Incubation of erythrocytes isolated from a variety of animal species with BAA indicates a large variation in the sensitivity to this hemolytic agent, with erythrocyte swelling and hemolysis occurring most readily and at low concentrations in blood from rats, mice and rabbits but absent in blood from humans and guinea pigs (Carpenter et al., 1956; Ghanayem and Sullivan, 1993). Similar incubations of erythrocytes isolated from healthy young or old humans or from patients suffering from congenital hemolytic disorders produced no significant hemolysis or morphological changes as seen in erythrocytes from the rat (Carpenter et al., 1956; Udden and Patton, 1994; Udden, 1996, 2000, 2002). In fact, more than 100-fold higher concentrations of BAA are required to produce even a minimal pre-hemolytic in human versus rat erythrocytes (Udden, 2002).

In more recent guideline studies conducted in guinea pigs, EGBE displayed a low acute toxicity following oral, dermal or inhalation exposures (Gingell et al., 1998). An acute oral LD$_{50}$ value of 1414 mg/kg bw (males and females) was reported in these studies and animals dying prior to study termination demonstrated pathology suggesting EGBE was a gastrointestinal irritant (Shepard, 1994a). There was no evidence of hemolytic toxicity in this acute oral study. Dermal administration under occlusive wrap at a limit dose of 2000 mg/kg bw produced no mortality or other signs of toxicity in the guinea pig (Shepard, 1994b). Inhalation exposures (1 h) at maximum achievable vapor concentrations of 633 (males) and 691 ppm (females) produced no mortalities or clinical signs of toxicity (DOW, 1994).

Perhaps the most convincing evidence of the low acute toxicity of EGBE in humans comes from accidental ingestions reported in pediatric cases or intentional ingestions in adults (Dean and Krenzelok, 1991; Udden, 1996; Gualtieri et al., 2003; Hung et al., 2010). In 24 pediatric poisoning cases reviewed, only two required hospitalizations with the remainder treated at home with palliative care (Dean and Krenzelok, 1991). In a number of adult poisoning cases severe metabolic acidosis was consistently observed. However, in all reported poisoning cases the patients survived and recovered without subsequent symptomology. In two exceptional cases, estimated quantities consumed of EGBE were 80–100 g (Gualtieri et al., 2003) or 150–250 ml (Hung et al., 2010). In one case, blood concentrations of BAA as high as 3.64 mM were reported but with no evidence of hemolytic anemia (Gualtieri et al., 2003).

7. Conclusions

Acute oral, dermal and inhalation toxicity values derived from rats and rabbits often serve as the basis for acute toxicity classifications. Both of these species show increased sensitivities to the intravascular hemolysis caused by BAA, the hemolytic metabolite of EGBE. This increased sensitivity in turn leads to an overly conservative acute hazard classification for this chemical. An extensive body of work over many decades and from a number of different investigators has clearly shown that the effects on the erythrocytes of sensitive species such as the rat and rabbit are not representative of the human response to this chemical and that the guinea pig is a more appropriate surrogate species for the acute hazard classification of EGBE.

7.1. Weight of evidence approach to assignment of an acute oral toxicity hazard classification for EGBE under GHS

Although varying considerably within and among species, the majority of the LD$_{50}$ values presented in Table 2 are consistent with a Category 4 acute oral toxicity classification under GHS. Specifically, all values are above the upper cut-off value of 300 mg/kg bw for the Category 3 classification, while some few of the reported LD$_{50}$ values fall near or slightly above the cut-off for the Category 4 classification (Table 1), i.e. into Category 5 (or no classification under the EU CLP regulation).

7.2. Weight of evidence approach to assignment of an acute dermal toxicity hazard classification for EGBE under GHS

In both the rat and rabbit, signs of the acute systemic toxicity of EGBE are evident and are consistent with exposures by other routes. As in the case of acute oral exposures, there is compelling evidence to suggest the rabbit is the most sensitive of the experimental animals when tested dermally. In the rat, clinical and pathological signs of hemolysis are evident generally at or above acute dermal toxicity LD$_{50}$ values of 2000 mg/kg bw and are more pronounced following occlusive exposures. Similar effects are observed in the rabbit but at acute dermal toxicity LD$_{50}$ values ranging from 435 to 1060 mg/kg bw (occlusive). Given the increased sensitivity of both the rat and rabbit to the hemolytic effects of EGBE, an effect that is not representative of the situation in humans, the guinea pig is considered the most representative species for assigning an acute dermal toxicity hazard for human exposures (LD$_{50}$ > 2000 mg/kg bw). Based on reliable and more recent studies of the acute dermal toxicity of EGBE in the guinea pig, as well as supportive mechanistic and well-documented human exposure data, a Category 5 acute dermal toxicity classification under GHS (or no classification under the EU CLP regulation) is the most appropriate outcome for EGBE.

7.3. Weight of evidence approach to assignment of an acute inhalation toxicity hazard classification for EGBE under GHS

In the rat and rabbit, two species sensitive to the hemolytic toxicity of EGBE, reported acute inhalation toxicity LC$_{50}$ values ranged from approximately 2 mg/l to values exceeding the theoretical calculated maximum achievable vapor concentration of EGBE (3.9 mg/l). Reported clinical and pathological effects from these studies confirm an acute hemolytic response in these species. In contrast, dogs exposed for up to 7 h at 2.0 mg/l were unaffected. Similarly, guinea pigs exposed at either 2.0 mg/l for 7 h or at maximum achievable vapor concentrations for 1 h were unaffected. Given the increased sensitivity of both the rat and rabbit to the hemolytic effects of EGBE, the guinea pig is considered the most representative species for assigning an acute inhalation toxicity hazard for human exposures. Based on reliable studies of the acute inhalation toxicity of EGBE in the guinea pig, as well as supportive mechanistic and well-documented human exposure data, no classification of EGBE for acute inhalation toxicity under GHS is warranted.

Conflict of interest

The authors claim no conflicts of interest in the preparation of this publication.
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