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Safety assessment of gamma-glutamylcysteine sodium salt ☆

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ABSTRACT

γ-Glutamylcysteine (GGC) is a relatively unexplored option for the treatment of chronic glutathione depletion related disorders that involve down regulation of GGC synthetase. High purity GGC (sodium salt) has only recently become available and, given its reactive capacity, required an investigation of its safety profile. In this report, GGC sodium salt was demonstrated to be safe according to Organisation for Economic Cooperation and Development (OECD) toxicology protocols for acute and repeated doses. No mortalities or adverse effects were observed in Wistar rats following the acute oral (gavage) administration of 2000 mg sodium GGC /kg body weight. No animal deaths occurred with daily administration (1000 mg/kg sodium GGC) over 90 days, with a post trial 28 day observation period. GGC had no significant effect on feed consumption, body weights, physical appearance, neurological behaviour and urine chemistry. No consistent significant differences between treatment groups were observed in haematological and clinical chemistry parameters. Similarly, no post-mortem necroscopically identified abnormalities could be attributed to GGC. Based on these observations, sodium GGC can be classed as not acutely toxic at 2000 mg/kg, with a no-observed-adverse-effect level (NOAEL) of at least 1000 mg/kg/day for systemic toxicology from repeated dose oral gavage administration.

1. Introduction

The thiol tri-peptide glutathione (L-γ-glutamyl-L-cysteinyl-glycine) is synthesised in the cytosol of all mammalian cells by a sequence of two ATP dependent enzyme catalysed reactions. In the first reaction, cysteine and glutamic acid are condensed by γ-glutamylcysteine synthetase (also known as γ-glutamate cysteine ligase) to form γ-glutamylcysteine (GGC). In the second reaction, GGC is condensed with glycine by glutathione synthase to produce glutathione (Anderson and Meister, 1983).

Glutathione levels have been reported to progressively decline in all tissues with age, and chronic glutathione depletion has been implicated in many health conditions including, Parkinson's and Alzheimer's diseases, AIDS, pulmonary diseases, diabetes, cystic fibrosis, haemolytic anaemia, myocardial infarction, schizophrenia, and chronic obstructive pulmonary disease (COPD) (Ballatori et al., 2009; Li et al., 2004).

Glutathione depletion can potentially arise from limitations in cysteine availability or from down regulation of expression or lowering of the specific activities of either of the two enzymes involved in its synthesis (Lu, 2009).

The levels of cysteine and methionine ingested in most normal diets are generally much higher than necessary for glutathione synthesis (Food and Nutrition Board, 2005; Jones et al., 2011). This would suggest that substrate limitation is not implicated as a causative mechanism for chronic glutathione depletion.

N-acetylcysteine (NAC) has proven efficacious in the treatment of acetaminophen overdose, where hepatic glutathione is rapidly and acutely depleted as it conjugates with an accumulating toxic metabolite, N-acetyl-p-benzoquinone imine (NAPQI). This leads to cysteine becoming a limiting substrate for glutathione synthesis and prevents cells from maintaining glutathione homeostasis, which can result in liver failure and death if untreated (Siegers et al., 1978; Dai and Cederbaum, 1995). Glutathione itself is not considered a superior option to NAC for treating acute glutathione depletion, as it is not taken up intact by most cells, and requires digestion by membrane bound gamma glutamyl transferase for cellular uptake. This involves cleavage of the bond between the

Abbreviations: GGC, γ-Glutamylcysteine; mg/kg or g/kg, mass per kilogram of body weight; OECD, Organisation of Economic Cooperation and Development; NOAEL, no-observed-adverse-effect level; GLP, Good Laboratory Practice.

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γ-glutamyl and cysteine residues and transfer of the γ-glutamyl moiety to another available amino acid. The two resulting products L-cysteinyl-glycine and the γ-glutamyl amino acid conjugate are then taken up by the cell, where they are hydrolysed by pepsidases (Anderson and Meister, 1983; DeLeve and Kaplowitz, 1991). This mode of uptake suggests that administration of exogenous glutathione may just simply provide a cysteine source for cells.

GGC synthetase is a heterodimer comprising a catalytic heavy subunit and a regulatory light subunit that modulates the catalytic subunit's activity via glutathione mediated non-allosteric feedback inhibition. The ratio of the two subunits varies between tissues and during ageing, with rodent studies showing that the regulatory subunit levels rather than the catalytic subunit levels decline with increasing age and that this corresponds to lowering of homeostatic glutathione levels, (Liu, 2002; Liu and Dickinson, 2003).

The glutathione synthetase enzyme, on the other hand, is a much simpler homodimer composed of two identical catalytic subunits and generally has a higher specific activity than GGC synthetase (Lu et al., 2009). Providing glutathione synthetase activity and glycine are both in excess, there should be negligible homeostatic cellular GGC content due to GGC's unregulated conversion to glutathione.

Animal and human cell line studies have shown that the expression of both synthetase enzymes can be induced when exposed to oxidative stress, which results in increased cellular glutathione synthesis capacity. Though induction of both enzymes is often coordinated, some compounds such as insulin, hydrocortisone and ethanol have been shown to only induce GGC synthetase and potentially lead to glutathione synthetase activity being the limitation in glutathione synthesis. (Lu et al., 2009).

The key issue in the etiology of many chronic glutathione depletion conditions that are related to a loss in GGC synthetase activity will be the supply of GGC rather than the supply of cysteine. That is, chronic glutathione depletion can be recast as an inability to synthesise sufficient GGC.

Comparatively little research has been conducted to explore GGC's potential as a therapeutic agent for glutathione replenishment. This may in large part have been due to lack of the commercially available quantities required to conduct the experimentation. Recently, new methods have been developed that are suitable for the industrial scale manufacture of GGC, (Bridge and Zarka, 2006). The few studies involving GGC administration have largely been conducted in rodents, and provide evidence that GGC does restore depleted cellular glutathione. Injected GGC increased glutathione levels in rat kidneys where glutathione was depleted by buthionine sulfoximine, (Anderson and Meister, 1983), and an intracerebroventricular injection of GGC was shown to increase glutathione levels in rat brains (Pileblad and Magnusson, 1992). In a larger recent study, GGC ameliorated oxidative injury in rat neurons and astrocytes *in vitro* and increased brain, liver and other major organ glutathione levels *in vivo*. It was concluded that GGC supplementation has potential for the treatment of clinical settings involving acute and chronic oxidant damage (Le et al., 2011).

GGC occurs in some foods such as green beans (5.7 μg/g wet weight (ww)) and spinach (1.0 μg/g ww), (Demirkol et al., 2004), and spices; mustard (11.5 μg/g dry weight, (dw)) and fenugreek (10.5 μg/g dw), (Manda et al., 2010). Higher levels occur in the whey protein fraction of bovine milk, where the majority of GGC is bound to serum albumin (at a 6:1 mole/mole ratio) and to β-lactoglobulin (1:1) (Bounous and Gold, 1991), which equates to a fresh milk GGC content in the order of 50 mg/l.

Whey protein supplementation has been shown in numerous animal model and human studies (at doses of 20–45 g/day, equating to approximately 150–375 mg GGC/day) to replenish glutathione levels. Associated observed physiological health improvements in immune function and exercise recovery during these studies have been attributed to the GGC content. (Birt et al., 1982; Bounous et al., 1989; Bounous and Gold, 1991; Lands et al., 1999; Micke et al., 2002; Zommara et al., 1998).

It is quite possible that much higher doses of GGC than could be reasonably ingested in the diet from food sources will be required to achieve a therapeutic benefit for the many glutathione depletion related health conditions. Though no evidence has been reported that ingestion of GGC containing foods causes any ill effects, it is essential before GGC can be considered as a dietary supplement, food ingredient, or therapeutic to determine whether it has any potential toxicity at high dosage. To date, no studies investigating the toxicology of GGC have been published.

The current studies were conducted to assess the acute and repeated dose safety profiles of the sodium salt of GGC (sodium GGC) in Wistar rats. All studies were conducted in accordance with OECD and GLP guidelines (OECD, 2001, 1998). For each trial, the limit doses were chosen on the basis that the absence of any observed toxicity at such high levels would support GGC's eligibility for the status of generally regarded as safe (GRAS). The limit doses were 2000 mg/kg for the acute dose and 1000 mg/kg per day for the chronic dosing study.

2. Material and methods

2.1. Test materials

GGC (CAS No. 636-58-8) was provided by Biospecialties International as a sodium salt. The purity of GGC was determined by HPLC on a Shimadzu system using an Alltima (Alltech) C18, 5 μm, and 250 × 3.6 mm column at 30 °C. The gradient elution was performed at a flow rate of 1 ml/min using a binary mobile phase consisting of 50 mM KH_2PO_4 adjusted to pH 2.5 with H_3PO_4 (A) and 50% (v/v) acetonitrile/water (B). Samples were injected (10 μl) at 100% mobile phase A, and mobile phase B was initiated at 2% and increased linearly to 15% B over 20 min. The analytes were detected by UV adsorption at 220 nm. The GGC content of the sample (Batch No. RX-013) used in this study was greater than 95.3% (90.3% reduced, 5.0% oxidised) with the minor contaminant being γ-glutamyl-GGC (4.3%). The sample contained traces (less than <0.4% in total) of cysteine, pyroglutamic acid, and γ-glutamylcystine; a mixed disulfide of GGC and cysteine. The identity of GGC was confirmed by Nuclear Magnetic Resonance (NMR) spectroscopy and by Thin Layer Chromatography against a Sigma Aldrich Standard (G0903).

2.2. Animals

All Wistar rats used in this study were bred in house by Bioneeds Laboratory Animals & Preclinical Services. The acute toxicity study used female non-pregnant, 10 week old rats of weight 154.0–160.0 g, and the 90 day study used 7–8 week old rats of both sexes, weighing between 145.2–165.0 g for males and 130.0–150.0 g for females.

2.3. Housing

The rats were housed under standard laboratory conditions in an air-conditioned environment with adequate fresh air supply (air changes 12–16 volumes per hour), at room temperature (19–25 °C), 51–62% relative humidity, with a 12 hours light and 12 hours dark cycle. For the acute study, the rats were housed singly, and for the repeated dose study, were housed in groups of two of the same sex.

Prior to commencing the experimental treatments, the rats were acclimatised for 6 days to the laboratory conditions. During this period they were monitored daily for any clinical indications of ill health. Veterinary examination of all animals was recorded