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Safety studies of L-alanyl-L-glutamine (L-AG)

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Abstract

The safety of L-alanyl-L-glutamine (L-AG) derived by fermentation using a recombinant *Escherichia coli* strain containing the L-amino acid α-ligase gene from *Bacillus subtilis*, was assessed in acute and subchronic toxicity studies in the rat. L-AG was tested *in vitro* in a bacterial reverse mutation assay and in a chromosome aberration assay. L-AG was not acutely toxic when administered to Sprague–Dawley rats by gavage at 2000 mg/kg bw. In a 14-day range-finding study, L-AG at up to 5% in the diet was without effect. In the 13-week dietary study, there were no toxicologically significant differences between the treated groups (1.0, 3.0 and 5.0% L-AG) and the controls (0% and 5% L-AG produced *via* a different method) with respect to body weight gain, feed consumption, feed efficiency, or the results of ophthalmological, haematological, clinical chemistry, and urinalysis evaluations. Three of 10 high-dose males had mild testicular changes, however, these were of exactly the same nature and severity as those that occur spontaneously, and were considered unlikely to be treatment-related. The NOAEL in both males and females was established as the highest dose tested at 3129 and 3601 mg/kg bw/day, respectively (5.0% in the diet). There was no evidence of genotoxicity of L-AG in the Ames assay or in the *in vitro* CHL cell chromosome aberration study.

Keywords: L-Alanyl-L-Glutamine; Alanine; Glutamine; Safety; Adverse; Acute; Toxicity; Clastogenicity; Mutagenicity; Subchronic; 13-Week study

1. Introduction

Glutamine is the most common amino acid in both blood and the intracellular fluid. In fact, one fifth or more of the amino acids in plasma are present as glutamine (Stein and Moore, 1954). In skeletal muscle, glutamine accounts for 50–60% of all amino acids (Bergstrom et al., 1974). Glutamine is associated with numerous physiological functions, including: protein synthesis, maintenance of gastrointestinal tract function, immune cell growth/maturation and function, and glutathione synthesis in the liver (Windmueller and Spaeth, 1980; Wilmore et al., 1988; Burke et al., 1989; Newshlome and Parry-Billings, 1990; Welbourne, 1995; Iwasa et al., 1996; Fürst et al., 1997; Huang et al., 2000; Tapiero et al., 2002; M'bemba et al., 2003).

In food matrices, and in the form of dietary supplements, glutamine is not stable under acid environments, and moreover, glutamine is poorly water soluble (Fürst et al., 1997; Fürst and Kuhn, 1998; Holeček et al., 2000). To overcome problems of stability and solubility, Kyowa Hakko Kogyo Co. of Japan has developed technology to produce a dipeptide of glutamine and alanine, namely L-alanyl-L-glutamine (L-AG), *via* fermentation using recombinant *Escherichia coli* K12, consisting of a host strain, constructed from strain JM101 (Stratagene Co., Ltd.), and a plasmid containing L-amino acid α-ligase gene from *Bacillus subtilis*. The L-AG produced is a white crystalline solid stable to both heat and acids, and is highly water soluble (568 g/L), thus increasing potential for absorption (Yagasaki et al., 2005). Under storage conditions, it was

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determined to be stable for 36 months at 25 ± 2 °C and 60 ± 5% relative humidity.

L-AG is currently used as part of total parenteral nutrition protocols in hospital settings (Burke et al., 1989; Stehle et al., 1989; Fürst et al., 1997; Morlion et al., 1998; Jiang et al., 1999; Goeters et al., 2002; Fuentes-Orozco et al., 2004). In these studies, infusion rates ranged from 0.3 to 0.5 g/kg body weight (bw)/day. There is no indication of adverse side-effects in patients treated with L-AG and/or other glutamine dipeptides. Similarly, no untoward side-effects have been reported in healthy individuals after either intravenous (i.v.) (Albers et al., 1989) or oral exposure (Klassen et al., 2000). These findings are not unexpected given that dipeptides such as L-AG are readily degraded into constituent amino acids during transport from the small intestine (Minami et al., 1992; Herzog et al., 1996; Klassen et al., 2000). Levels of alanine and glutamine in blood rise within 15–30 min of oral administration to both rats (Rogero et al., 2006) and humans (Klassen et al., 2000).

Given the potential utility of incorporating L-AG in food products to provide a readily available source of glutamine, and noting that classical preclinical toxicology studies have not been performed on L-AG, acute oral, 14-day subchronic and 90-day subchronic studies have been conducted. In addition, 2 *in vitro* assays of genetic toxicity, the Ames reverse mutation assay and the *in vitro* chromosome aberration test have been conducted. The results of these studies are reported forthwith.

2. Materials and methods

2.1. Test materials

The L-AG tested in all studies reported here was of 99.4–100.0% assayed purity. Based on the test material specifications, the pH, heavy metals content, loss on drying, and loss on ignition were 5.4–6.0, ⩽5 ppm, ⩽0.5%, and ⩽0.1%, respectively. L-AG was prepared by Kyowa Hakko Kogyo Co., Ltd. of Japan. The product was determined to be stable under the conditions employed and treatment durations in all of the studies performed. A comparative L-AG preparation was assayed to be of 100.1% purity. Both the test article and the comparative L-AG preparation were identical in terms of the specifications (*i.e.*, pH, ammonium, chlorine, sulphate, heavy metal, arsenic, loss on drying, and residue on ignition) for L-AG.

2.2. Acute toxicity study

In an acute toxicity study, using the acute toxic class method, L-AG (Lot No. F-050001) was administered by oral gavage to groups of 3 nulliparous, non-pregnant, female Sprague–Dawley CD (Crl: CD® (SD) IGS BR) rats supplied by Charles River (UK) Ltd., Margate, Kent, UK. The rats were approximately 8–12 weeks old and weighed from 200 to 227 g. Rats were housed in groups of 3 in suspended solid-floor polypropylene cages containing wood flakes for bedding material. The cages were kept in a room maintained within a temperature range of 19–25 °C and a humidity range of 30–70%. The room was provided with fresh air so as to allow at least 15 air changes per hour. The room was also maintained on a 12-h light and dark cycle. Food and water were provided *ad libitum*, except for the withdrawal of food the night before dosing and for 3–4 h post-dosing.

A group of 3 rats were administered the test material in aqueous solution by oral gavage (10 ml/kg bw) to provide a dose of 2000 mg/kg bw. Following confirmation of survival, a second group of 3 rats was similarly dosed at 2000 mg/kg bw. The 2000 mg/kg bw dose level was chosen as this represents a limit dose as detailed in the OECD Testing Guideline No. 423.

The rats were observed for clinical signs of toxicity at 0.5, 1, 2, and 4 h post-dosing. Thereafter, the rats were observed once daily. The body weights of each rat were recorded prior to test article administration and at 7 and 14 days post dosing. The rats were sacrificed by cervical dislocation 14 days post-dosing. Necropsy included an external examination and opening of the thoracic and abdominal cavities for observation of all major organs. No tissues were retained for histopathological analysis.

The study was conducted in compliance with OECD Test Guideline 423 (17 December 2001) and followed OECD Good Laboratory Practice (GLP) principles.

2.3. Subchronic toxicity study

A 90-day study was conducted to determine the effect of subchronic exposure to various doses of L-AG. Prior to beginning the study, a 14-day dose-range finding study was conducted.

2.3.1. Dose-range finding study

L-AG (Lot No. K850001) at dietary concentrations of 0, 1, 3, and 5% (w/w) was administered daily for 14 days to groups of 5 male and 5 female Sprague–Dawley CD SPF rats. The rats were supplied by Charles River Laboratories, Japan, Inc. The rats were housed individually in stainless steel cages kept in a room maintained within a temperature range of 21–23 °C and a relative humidity range of 43–58%. The room was provided with fresh air so as to provide 10–15 air changes per hour. The room was also maintained on a 12-h light and dark cycle. Food and water were provided *ad libitum*. Prior to study initiation, the rats were approximately 6 weeks of age. The males weighed between 184 and 205 g while females weighed between 137 and 161 g. All animals were allowed an 8 day quarantine/acclimation period.

The general condition of the rats was usually observed twice daily. Body weights and food consumption rates were recorded on dosing days 1, 4, 8, 11, and 15. From these data, feed efficiency and intake of L-AG were calculated. At study termination, blood was collected from all rats for determination of routine haematological and clinical chemistry parameters. Animals were killed by exsanguination from the abdominal aorta under ether anaesthesia following blood collection. Necropsy included an external examination and opening of the thoracic and abdominal cavities for observation of all major organs. The absolute and relative weights of the adrenals, spleen, heart, lung, liver, kidney, testis and ovary were recorded. Samples of these tissues were preserved for possible histopathological analysis. The study was conducted in compliance with animal welfare guidelines.

2.3.2. Subchronic study

Seventy-three, 5-week-old Sprague–Dawley SPF rats of each sex, supplied by Charles River Laboratories, Japan, Inc., were quarantined/acclimated for 7 days. Fifty of these animals of each sex were selected for inclusion in the treatment portion of the study. The males weighed between 187 and 217 g while females weighed between 143 and 170 g. The rats were housed individually in stainless steel cages kept in a room maintained within a temperature range of 19–25 °C and a relative humidity range of 32–71%. The room was provided with fresh air so as to provide 10–15 air changes per hour. The room was also maintained on a 12-h light and dark cycle. Food and water were provided *ad libitum*. Certificates of analysis of the feed and water were reviewed to prevent exposure to contaminants.

The 50 rats of each sex were randomly divided into 5 groups, each having 10 rats/sex to ensure heterogeneity of body weights. One group received basal diet (powdered CRF-1; Oriental Yeast Co., Ltd., Lot No. 050908) and served as the controls and 3 others received basal diet containing either 1, 3, or 5% (w/w) of L-AG (Lot No. 050001). A fifth group