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The Importance of Quality Specifications in Safety Assessments of Amino Acids: The Cases of L-Tryptophan and L-Citrulline[1–3]

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Abstract

The increasing consumption of amino acids from a wide variety of sources, including dietary supplements, natural health products, medical foods, infant formulas, athletic and work-out products, herbal medicines, and other national and international categories of nutritional and functional food products, increases the exposure to amino acids to amounts far beyond those normally obtained from the diet, thereby necessitating appropriate and robust safety assessments of these ingredients. Safety assessments of amino acids, similar to all food constituents, largely rely on the establishment of an upper limit [Tolerable Upper Intake Level (UL)] considered to be a guide for avoiding high intake, above which adverse or toxic effects might occur. However, reliable ULs have been difficult or impossible to define for amino acids because of inadequate toxicity studies in animals and scarce or missing clinical data, as well as a paucity or absence of adverse event reporting data. This review examines 2 amino acids that have been associated with in-market adverse events to show how quality specifications might have helped prevent the adverse clinical outcomes. We further highlight the importance of various factors that should be incorporated into an overall safety assessment of these and other amino acids. In addition to the traditional reliance on the established UL, well-defined quality specifications, review of synthesis and production strategies, potential interactions with drugs, contraindications with certain disease states, and cautionary use within certain age groups should all be taken into consideration. *J Nutr* 2016;146(Suppl):2643S–51S.

Keywords: L-tryptophan, L-citrulline, quality specifications, upper limit safety assessments, eosinophilia myalgia syndrome

Introduction

In the United States, Western Europe, and Pacific Rim nations, there has been a strong and steady increase in the use of dietary supplements (DSs)[8] (1, 2) and other nutritional and functional food (FF) products. For the purposes of this article, the term "functional food" (FF) will apply to the broad and often ill-defined category of nutraceuticals, nutritional products, and athletic and work-out products, as well as better-defined categories of natural health products, medical foods, infant formulas, follow-up formulas, foods for special medical uses, foods for specified health uses, foods for special dietary uses, novel foods, and others not listed here. DSs and FF products contain a variety of ingredients, including botanical (herbal) ingredients, vitamins, minerals, and amino acids, and are usually formulated singly or in combination. Paradoxically, in combination with the increased use of DSs and FFs, there is growing consumer wariness with regard to the true health benefits of many of these

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[8] Abbreviations used: CM, citrulline-malate; DS, dietary supplement; EBT,1-1-ethylidenebis(tryptophan); EMS, eosinophilia-myalgia syndrome; FF, functional food; GFR, glomerular filtration rate; HOI, Highest Observed Intake; MAOI, monoamine oxidase inhibitor; OSL, Observed Safe Level; OTC, over-the-counter; ppm, parts per million; UL, Tolerable Upper Intake Level; USP, US Pharmacopeial Convention; *USP-NF*, US Pharmacopeia–National Formulary; 5-HT, 5-hydroxytryptamine; 5-HTP, 5-hydroxytryptophan.

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ingredients. In addition to suspicion about claims associated with many supplements, consumers rank the poor quality of the ingredients used in such products as a primary concern (3). Some authors have stated that the current government policies in the United States should be changed so as to require DSs to be regulated more stringently—for example, regulated similarly to over-the-counter (OTC) medications (4).

In the United States, more than half of the population uses DSs, many of which contain amino acids as the sole active ingredient or in combination with other active ingredients (1). Amino acids in DSs and FFs have become increasingly popular because they are believed to enhance performance in a number of ways, including increasing the secretion of anabolic hormones, modulating energy metabolism, preventing adverse effects of training, and increasing endurance. The functions of some of these amino acids do have the support of scientific evidence. For example, studies have established a cause-effect relation between creatine supplementation and an increase in short-term, high-intensity physical performance, although no such relation has been established between creatine, endurance capacity, and other measures of physical performance (5). In another example, an evidence-based review by the US Pharmacopeial Convention (USP) on β-alanine found that the evidence was insufficient to support the use of this amino acid to enhance athletic performance (6).

Most of the human exposure to amino acids occurs as part of a well-balanced diet. However, with the increasing consumption of amino acids from alternative sources such as DSs and FFs, it becomes increasingly important to ensure that appropriate and robust safety assessments are conducted on these ingredients. Safety assessments of amino acids have largely relied on the establishment of an upper limit [Tolerable Upper Intake Level (UL)] (7, 8). By definition, the UL is the intake above which adverse or toxic effects are likely to occur (7). Defining a UL involves various steps associated with risk assessment, including hazard identification (adverse events), dose response, exposure, and risk characterization. For many amino acids, the UL may be difficult or impossible to define because adequate toxicity studies in animals, safety studies in humans, and/or adverse event reporting data in humans may not exist (8). In fact, a review by the Institute of Medicine (9) on DRIs evaluated an extensive amount of clinical and animal data available at the time but was unable to determine a UL for any of the evaluated amino acids. Importantly, the report observed that, for all amino acids, risk emanated from their intake as DSs and FFs or when used as a food additive (e.g., glutamate as monosodium glutamate) rather than when ingested as part of the diet (9).

In some cases in which a UL cannot be defined, newer methods that use an Observed Safe Level (OSL) or Highest Observed Intake (HOI) have been incorporated into quantitative risk assessments for some amino acids (8). The OSL, HOI, and UL offer valuable information with regard to amino acid intakes that are expected to pose no risk of adverse health effects and therefore would be expected to be safe. However, a UL, an OSL, or an HOI alone is not sufficient to fully assess safety without addressing the quality of raw materials and other considerations such as potential interactions with other supplements and/or drugs, use in certain sensitive populations (i.e., children, elderly individuals, pregnant women, and others), and use in certain disease conditions.

In this review, we present 2 cases of amino acids used as DSs and in pharmaceutical products, L-tryptophan (L-Trp) and L-citrulline (L-Cit), in which serious in-market adverse events have occurred. We propose that if more stringent quality specifications, such as raw material identification and purity, would have been in place for these products, the adverse events could have been prevented. However, we note that the L-Trp example occurred in the 1980s, predating the enactment and implementation of rigorous DS current Good Manufacturing Practice requirements, which responsible manufacturers follow (10). Furthermore, since then, adverse event reporting requirements have been enacted and implemented to provide a postmarket safety net (11). Here we reiterate the importance of other considerations that should be incorporated into an overall safety assessment for ingredients such as amino acids. We posit that these principles can be applied across other DS and FF classes as well.

Quality Specifications for Amino Acids

The US Pharmacopeia–National Formulary (*USP-NF*) and other pharmacopeia, such as the British Pharmacopeia, European Pharmacopeia, and Japanese Pharmacopeia, as well as the Food Chemicals Codex and the Codex Alimentarius and Joint WHO/FAO Expert Committee on Food Additives all have quality specifications and standards for many amino acids of nutritional importance. The *USP-NF* also has quality monographs for other amino acids used as DSs or FFs; for example, 5-hydroxytryptophan (5-HTP) and L-Cit. *USP-NF* monographs typically have specifications that state the ingredient's strength and purity, methods for identification, limits for impurities, specific tests, packaging and storage, reference material(s), and standards. Monograph information for L-Trp and L-Cit is presented in **Tables 1** and **2**, respectively. In the following case studies, the absence of appropriate quality specifications may have contributed to in-market adverse events.

L-Trp—Case Study

Case study. L-Trp is a precursor of 5-HTP, which is the immediate precursor for neurotransmitter 5-hydroxytryptamine (5-HT; serotonin) (**Figure 1**). The serotonergic 5-HT system is known to modulate pain (13); thus, some athletes ingest L-Trp supplements (via DSs and/or FF products) to increase serotonin concentrations, with the intention of increasing their pain threshold during exercise (14). One study that examined the effects of L-Trp on endurance and sensation of effort reported that supplementation of athletes with 1.2 g L-Trp increased total exercise time by 49.4% when the subjects were running at 80% of maximal oxygen uptake (15). However, a subsequent study in trained athletes showed no differences between athletes who were supplemented with a total of 1.2 g L-Trp or 1.2 g placebo over a 24-h period before the test exercise (16). Other studies of the effect of L-Trp supplementation on aerobic endurance performance also reported no effects at 70–75% of maximal oxygen uptake. An evidence-based review thus concluded that L-Trp may not be an effective ergogenic substance (17). Because it is a precursor of serotonin, L-Trp has been used in many conditions believed to be mediated by serotonin (e.g., insomnia, mood alteration, and pain control) (18), despite the mixed evidence for its effectiveness for these conditions.

Upper limit and safety. At the Eighth Amino Acid Assessment Workshop that was held in 2011, participants discussed clinical data and animal data on L-Trp with a view to setting the UL (19). Participants pointed out that before the incidences involving L-Trp and eosinophilia-myalgia syndrome (EMS), L-Trp had been used as a supplement at intakes of ≤8 g/d for ≤8 wk

TABLE 1 Quality specifications for monographs of L-Trp ($C_{11}H_{12}N_2O_2$)[1]

	L-Trp monograph			
	US Pharmacopeia	British Pharmacopeia	European Pharmacopeia	Japanese Pharmacopeia
Purity, %	98.5–101.5	98.5–101.0	98.5–101.0	≥98.5
Identification				
Infrared spectroscopy	Spectrum compared with the reference standard spectrum	Not more intensely colored than reference solution	Not more intensely colored than reference solution	Spectrum compared with the reference standard spectrum
Optical rotation	−29.4° to −32.8°	−30.0° to −33.0°	−30.0° to −33.0°	−30.0° to −33.0°
Chromatography	Retention time same as reference standard in HPLC	Same as reference solution in TLC	Same as reference solution in TLC	Not more intense than the standard solution in TLC
Content assay				
Titrimetric test, %	98.5–101.5 (on a dried basis)	98.5–101.0 (on a dried basis)	98.5–101.0 (on a dried basis)	≥98.5 (on a dried basis)
Impurities				
Residue on ignition, %	≤0.1	N/A	N/A	≤0.1
Chlorides	≤0.05%	≤200 ppm	≤200 ppm	≤0.021%
Sulfates	≤0.03%	≤300 ppm	≤300 ppm	≤0.028%
Iron, ppm	≤30	≤20	≤20	N/A
Ammonium	N/A	≤100 ppm	≤0.02%	≤0.02%
Heavy metals, ppm	CG	≤10	≤10	≤20
Arsenic, ppm	CG	N/A	N/A	≤2
Sulfated ash, %	CG	≤0.1%	≤0.1%	N/A
Related compounds (individual)	Impurity 1: ≤0.01% (elutes before L-Trp); impurity 2: ≤0.03% (elute eluting after L-Trp)	Impurity A: ≤0.5 times the area of the principal peak in the chromatogram obtained with the reference solution; ≤10 ppm	Impurity A: ≤0.5 times the area of the principal peak in the chromatogram obtained with the reference solution; ≤10 ppm	N/A
Related compounds (total)	L-Trp–related compound A: ≤10 ppm (if present)	Sum of impurities with retention time less than that of L-Trp: ≤100 ppm Sum of impurities with retention time greater than that of L-Trp: ≤300 ppm Disregard limit: 0.02 times the area of the peak due to *N*-acetyltryptophan in chromatogram obtained with reference solution; disregard the peak due to *N*-acetyltryptophan	Sum of impurities with retention time less than that of L-Trp: ≤100 ppm Sum of impurities with retention time greater than that of L-Trp: ≤300 ppm Disregard limit: 0.02 times the area of the peak due to *N*-acetyltryptophan in chromatogram obtained with reference solution; disregard the peak due to *N*-acetyltryptophan	N/A
Specific tests				
Loss of weight on drying, %	≤0.3	≤0.5	≤0.5	≤0.30
pH	5.5–7.0	N/A	N/A	5.4–6.4
Packaging and storage	Preserve in well-closed containers	Store protected from light	Store protected from light	Containers: tight containers; storage: light resistant
Reference standards	Monograph for L-Trp (January 2015)	Monograph for L-Trp (January 2015) N/A	Monograph for L-Trp (January 2015) N/A	Monograph for L-Trp, 16th edition (2011) N/A

[1] CG, complies with US Pharmacopeia General Chapter Title 2232 Elemental contaminants in dietary supplements (12); N/A, not applicable; ppm, parts per million; TLC, Thin Layer Chromatography.

without serious adverse effects (19). Furthermore, it was noted that the rate of catabolism of L-Trp may be important in determining whether excess dietary intake could lead to adverse effects; therefore, the catabolism rate could be informative in determining the UL for L-Trp. Data from a study in rats showed that the urinary excretion ratio of anthranilic acid:kynurenic acid could be an indicator of intake amounts above which adverse effects are likely to occur, and therefore could be a marker for determining a UL for L-Trp (20). An earlier review proposed an approach for determining safe ULs for amino acids, which included the use of the maximum disposal rate as a metabolic marker. The oxidation response of an amino acid reaches a plateau when the metabolic limit to oxidize an amino acid is reached, and this inflection point could be considered the UL beyond which excess intake is likely to cause increased toxicity (21). Furthermore, protein degradation pathways are known to adapt with increased protein consumption, which probably also occurs with the ingestion of an excess of ≥1 amino acid (21). The current HOI and UL data for L-Trp and other amino acids are based on human observational data (9), but there have been no studies, to our knowledge, showing the relation between chronic consumption and the potential for adverse effects. Furthermore, the available data were insufficient to provide dose-response relations to establish a UL for L-Trp or any other amino acid. For L-Trp, relatively short-term ingestion (once or for 1 wk) in humans has shown that ingestion is linked to appetite suppression, nausea, and drowsiness. This information, coupled with the uncertainty regarding the possible role of

TABLE 2 Quality specifications for monographs of L-Cit ($C_6H_{13}N_3O_3$)

	US Pharmacopeia
Purity, %	98.0–102.0
Identification by infrared spectroscopy	Spectrum compared with reference standard
Optical rotation	24.6° to 26.5°
Chromatography	Retention time same as reference standard in HPLC
Content/assay	
HPLC, %	98–102 on dried basis
Impurities, %	
Residue on ignition	≤0.1
Chloride	≤0.02
Sulfate	≤0.02
Iron	None
Related compounds	
Individual	Impurity 1: ≤0.1
Total	≤2.0
Specific tests	
Loss on drying, %	≤0.2 of its weight
pH	None
Packaging and storage	Preserve in well-closed containers
Reference standards	L-Cit, acetyl-L-leucine

tryptophan in EMS, made it impossible to determine the UL for L-Trp (9). The UL should not be the only determinant of the safety of L-Trp or of other amino acids. Other factors should be taken into consideration, such as quality specifications of the material; review of manufacturing processes; potential for interaction with other foods, DSs, OTC medications, and drugs; age; disease; nutritional status of the target user; and adverse event reports.

Quality and safety. In the 1980s, an epidemic of EMS associated with supplemental L-Trp consumption occurred in the United States (22). EMS was reported in ~1500 individuals who were later found to have ingested L-Trp, which had been manufactured by a single company in Japan (Showa Denko KK) (23). The FDA banned the importation of L-Trp in March 1990, and with the imported amino acid no longer available in the marketplace the incidence of EMS rapidly declined. An analysis of case-associated L-Trp samples found 60 impurities, some of which were closely associated with the development of EMS. One impurity in particular, 1,1′-ethylidenebis(tryptophan) (EBT) (**Figure 2**), when administered to rodents was found to be associated with the development of some, but not all, features of EMS (24, 25). Upon further observation, it was noted that the manufacturer of L-Trp associated with causing EMS had introduced a new purification process into the production of L-Trp (26). Although the company had previously used a genetically engineered strain of *Bacillus amyloliquefaciens,* they had eliminated a purification step involving a carbon column. As a result, impurities that previously would not have reached the final product now appeared. The modified manufacturing procedure may have introduced microbiological impurities in the L-Trp supplements that were ultimately responsible for the outbreak of EMS (26). Studies with the use of samples from products that were associated with EMS compared with those not associated with EMS found that the EMS-associated L-Trp contained EBT, which may have been partly responsible for the occurrence of the EMS (27).

It has been noted that the L-Trp supplements that were involved in EMS met the pharmacopeia pharmaceutical standards at the time, which did contain specifications for quality that protected patients from many other safety hazards. This example, however, shows the need for new pharmacopeia standards to evolve as new information becomes available. New limits for impurities have since been incorporated into the USP quality monograph for L-Trp (Table 1) in response to the EMS outbreak. Manufacturers now have a stronger pharmacopeia monograph that contains stringent limits for impurities, which has allowed the reintroduction of safer L-Trp into the US market. USP monographs now include HPLC tests to determine whether a material meets the stringent criteria for organic impurities that elutes before the L-Trp peak (≤0.01%), after the L-Trp peak (≤0.03%), and for L-Trp–related compound A (EBT), the impurity associated with the EMS.

The L-Trp monograph provides an identity test that uses an infrared spectrophotometric method, specific rotation (−29.4° to −32.8°), and provides titration methods to determine content and strength (28). In addition, other specific tests include pH (5.5–7.0) and loss on drying (≤0.3%). Limits for inorganic impurities include tests for residue on ignition (≤0.1%), chloride (0.05%), sulfate (0.03%) and iron [≤30 parts per million (ppm)]. In addition, if L-Trp–related compound A is observed, further testing by HPLC methods is required to determine that it does not exceed 10 ppm, the lowest limit that can be measured on the basis of the current method's limit of detection. Finally, the USP is continuously revising the monograph to include the most appropriate and sensitive identification tests, an assay of content, and determination of the absence of contaminants. The tests for impurities with an L-Trp sample were included into the monograph as part of a continuous revision process and adaptability of compendial standards to include improved methods of testing and to update the monograph to address issues pertinent to safety.

A unique activity that supports the USP DS monograph development is the continuous surveillance of the peer-reviewed literature and government reporting portals for adverse events associated with DS articles. This activity is in keeping with the USP's "continuous revision" approach whereby USP expert committees continuously monitor safety information related to dietary ingredients. This continuous monitoring approach enables the early identification of potential safety concerns (29). It should be noted, however, that the quantity of adverse event reports in a spontaneous reporting system is not reflective of the frequency of adverse reports in the population of users, which is a result of

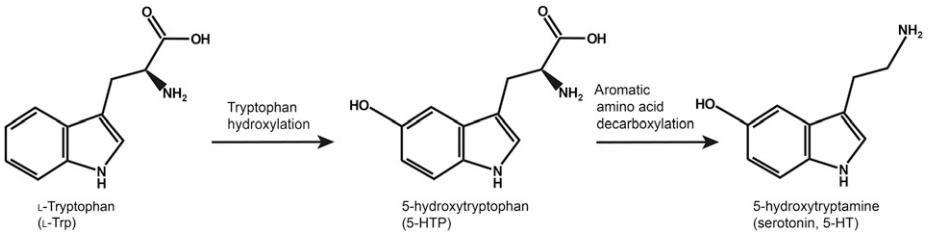

FIGURE 1 L-Tryptophan is a precursor for 5-hydroxytryptophan, which is the immediate precursor for the neurotransmitter 5-hydroxytryptamine, also known as serotonin.

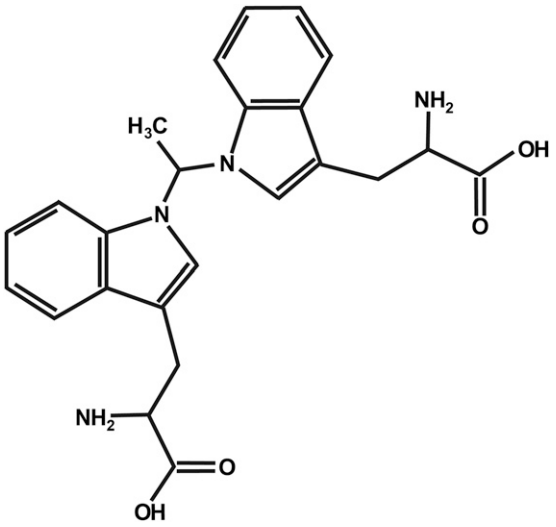

FIGURE 2 Structure of 1,1′-ethylidenebis (L-tryptophan), the contaminant implicated in the eosinophilia-myalgia syndrome associated with L-Trp from one particular manufacturer.

underreporting and the lack of information on volume sales of marketed products (30).

Interactions with other drugs and supplements. According to an annual survey conducted in 2014 on behalf of the Council for Responsible Nutrition, more than two-thirds (68%) of adults in the United States take DSs, more women than men use DSs, and usage increases with age, such that 74% of DS users are aged ≥55 y (1). Likewise, the use of multiple prescription and OTC drugs (polypharmacy) is also prevalent in the US population, and this trend follows the same pattern of usage as that of DSs (i.e., greater use by women and the elderly) (31). For example, in the United States, approximately one-third of the adult population aged 45–85 y take ≤5 prescription medications; of these adults, 52% concurrently ingest DSs, whereas 46% use other OTC medications, thereby increasing the risk of drug-drug and drug-supplement interactions (31).

The most common uses of L-Trp as a DS and/or an FF are for alleviation of insomnia and for mood improvement. Symptoms and side effects commonly associated with the use of oral L-Trp include drowsiness, sleepiness, dizziness, and occasionally muscle twitching or tremor (32). Although for individuals who use L-Trp to induce sleep, sleepiness and/or drowsiness would likely be the desired effects, for other uses somnolence may not be desirable and in some instances could be considered an adverse effect.

The concomitant use of L-Trp–containing DSs, OTCs, and drugs that induce sleep and drowsiness via 5-HT production could result in serotonin syndrome, a potentially life-threatening effect (33). Symptoms typically associated with serotonin syndrome include agitation, delirium, fever, hyperreflexia, tremor, hypertension, diarrhea, and myoclonus (34). The most common drug interactions associated with serotonin syndrome involve monoamine oxidase inhibitors (MAOIs) and serotonin selective reuptake inhibitors. For example, the concomitant use of MAOIs and tricyclic antidepressants greatly increases the risk of serotonin syndrome, with L-Trp and MAOI usage resulting in ≥1 death (34). In addition, when MAOIs and serotonin selective reuptake inhibitors are taken in combination with the OTC cough medication dextromethorphan, there is an increased risk of serotonin syndrome (35).

In animal studies, a pharmacodynamic interaction between L-Trp and various antitussives, including dihydrocodeine, noscapine, and dextromethorphan, was reported by Kamei et al. (36). In their study, rats were treated with various antitussive combinations and L-Trp (2.5 mg/g), and the L-Trp increased the antitussive effect of all 3 ingredients. The increased antitussive effect in this study was postulated to result from L-Trp–induced increases in brain 5-HT. On the basis of the results of the Kamei et al. study and other observations that increased serotonin concentrations in the brain have cough-suppressing effects (reviewed in reference 36) it is reasonable to suggest that L-Trp, when used in combination with dextromethorphan, one of the most commonly used OTC antitussives in North America, may enhance cough suppression.

Effect of age and nutritional status. It is currently unknown if L-Trp supplementation is teratogenic; thus, its use is cautioned during pregnancy. Bruni et al. (37) administered 5-HTP to children who were 3–10 y old in an open pharmacologic trial to determine its effect on modulating arousal and inducing long-term improvement in sleep terrors. The authors noted that 2 mg · kg^{-1} · d^{-1} administered at bedtime for 20 d produced no side effects. Others also noted that 5-HTP can be safely used in children with depression and sleep disorders (37).

The antidepressive effect of L-Trp was studied in a geriatric population (38). In that study, 20 adults (mean age: 81.9 y) were administered 6 g L-Trp/d for 6 wk. Apart from dry mouth, nausea, constipation, and drowsiness, no serious adverse effects were noted. In addition, L-Trp produced no observable differences in alleviating depression compared with placebo. Although the L-Trp study leg did show a positive trend, results were not substantially different compared with the placebo. In general, despite the absence of L-Trp–related adverse effects in this aged cohort, the use of DSs and FF products by the elderly should be given particular consideration because the potential for coadministration with prescription drugs is greater in this population.

Effects and health status. Caution is advised for L-Trp supplementation in individuals with liver cirrhosis and/or kidney disease (39, 40). Rössle et al. (39) studied the disposition of free and total L-Trp after an intravenous dose of 1.5 g L-Trp in patients with liver cirrhosis. Individuals with liver cirrhosis showed decreases in clearance of both free and total L-Trp (64% and 34%, respectively) as well as a 42% increase in the apparent volume of distribution of total L-Trp. The combined changes in clearance and apparent volume of distribution resulted in a 3-fold increase in the half-life of total L-Trp. These changes were not observed in patients with noncirrhotic liver disease. Elevated free L-Trp plasma concentrations were likely a result of impaired hepatic metabolism as a consequence of cirrhosis and not an alteration in L-Trp protein binding.

The influence of oral administration of L-Trp on glomerular filtration rate (GFR) in healthy subjects and in patients with glomerulonephritis has been studied (41). In patients with high proteinuria and impaired renal function, serum concentrations of L-Trp decreased, both before and after oral L-Trp loading. L-Trp oral loading increased GFR in healthy individuals and in patients with glomerulonephritis and proteinuria (<2 g/24 h), but in patients with renal dysfunction and high proteinuria (>2 g/24 h) L-Trp loading did not influence GFR. The aforementioned studies led to L-Trp supplementation being contraindicated in individuals with kidney and/or liver disorders (42).

L-Cit

Case study. Unlike L-Trp, the amino acid L-Cit is not an essential amino acid that must be obtained from the diet; furthermore, it is

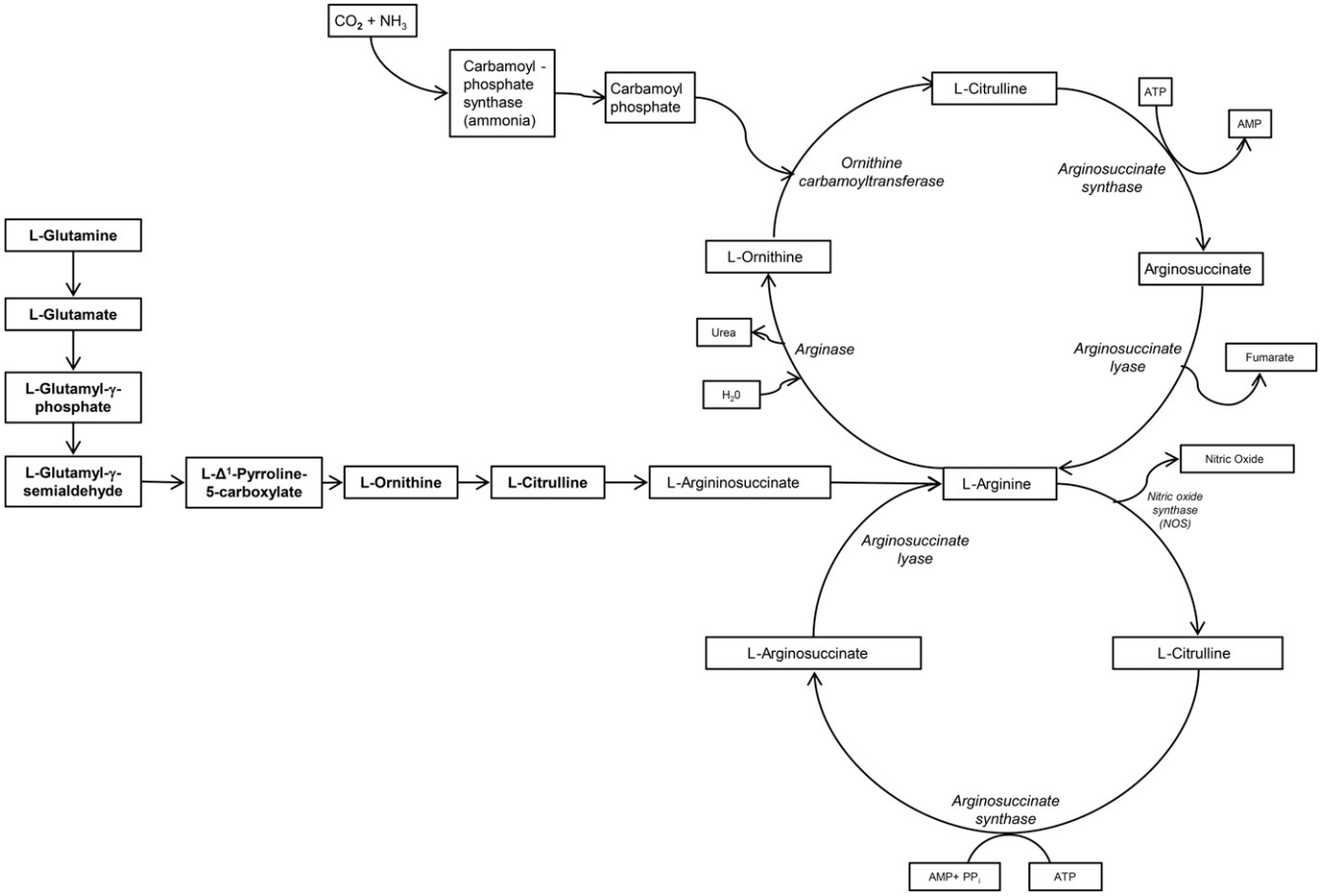

FIGURE 3 L-Citrulline is part of the urea cycle. It is involved in the de novo synthesis of L-Arg from L-Gln and the synthesis of NO. Supplementation with L-Cit can increase Orn and Arg plasma concentrations and subsequent NO.

not used in protein synthesis (i.e., nonprotein amino acid). However, L-Cit is synthesized in the intestinal mucosa and liver and can also be obtained from the diet. Common foods that contain L-Cit include vegetables such as melons, squash, cucumbers, and pumpkins (43). Two pathways exist for L-Cit biosynthesis: from recycled L-arginine (L-Arg) or L-glutamine (L-Gln). In the latter case, the enzyme ornithine transcarbamylase uses both ornithine and carbamoyl phosphate to produce L-Cit in enterocytes (43). It appears that the L-Arg pathway accounts for ~10% of circulating L-Cit, whereas the L-Gln pathway accounts for 90%. Thus, reductions in plasma L-Gln concentrations may also reduce plasma L-Cit.

L-Cit is involved in 3 important metabolic pathways (**Figure 3**): *1*) the de novo synthesis of L-Cit from L-Gln in the gut, *2*) the subsequent synthesis of L-Arg from L-Cit in the kidney along with the production of NO, and *3*) the intrahepatic transformation of ammonia to urea (44). As a result, supplementation with L-Cit can increase ornithine (L-Orn) and L-Arg plasma content, as well as promote ammonia recycling and NO synthesis. However, despite very high rates of arginine synthesis in the urea cycle, no net release of arginine by the liver has been reported.

L-Cit is used to treat rare, congenital urea cycle disorders characterized by hyperammonemia and various neurologic sequelae stemming from genetic deficiencies in enzymes that aid in clearing ammonia from the body. In many instances, L-Cit can be a life-saving medication for individuals with this disorder by helping them eliminate ammonia from the bloodstream and preventing it from accumulating to toxic concentrations. According to the National Urea Cycle Disorders Foundation, side effects of this disease can include metabolic instability and hyperammonemia. Elevated ammonia concentrations can lead to confusion, seizures, brain damage, coma, and even death (44).

In addition to its use to treat urea cycle disorders, L-Cit is used as a sports performance supplement to reduce fatigue and to improve endurance during prolonged exercise. The effects of citrulline-malate (CM) on skeletal muscle function in healthy rats were studied by using electrically stimulated gastrocnemius muscle to simulate exercise fatigue (45). The findings showed that the oral administration of CM (1 g/kg, 3 times/d for 48 h) had an ergogenic effect associated with the improvement of muscular contraction efficiency. The results for CM improvement on muscle function were attributed to a reduced ATP consumption and the known role of L-Cit in muscle byproduct elimination.

L-Cit supplementation was also shown to increase the expression of the main myofibrillar proteins and to induce a switch in muscle energy metabolism to a more aerobic state in malnourished aged rats (46). Two-year-old rats were diet-restricted for 12 wk followed by being fed a diet enriched with 5 g L-Cit $\cdot$ kg^{-1} $\cdot$ d^{-1} (extrapolated from doses classically used in humans) for 1 wk. After the dose administration, protein expression was analyzed from the isolated tibialis anterior muscle. Proteins analyzed included key enzymes involved in mitochondrial functioning, including respiratory chain, main myofibrillar constituents, enzymes involved in glycogenolysis, glycolysis, and

the Krebs cycle. The antioxidant properties of L-Cit represented one potential explanation for at least the effect on mitochondrial activity.

Upper limit and safety. L-Cit is Generally Recognized As Safe for oral use (47); however, this is primarily based on use in various food products, beverages, grains and pastas, and milk products. In humans, 15 g L-Cit taken acutely did not result in adverse effects, such as diarrhea or intestinal upset. Unlike L-Cit, even lower doses of L-Orn and L-Arg (e.g., 10 g as a bolus dose) were shown to cause osmotic diarrhea, largely as a result of their limited absorption and higher intraluminal concentration within the colon (48).

In the study by Moinard et al. (49), 8 young, healthy male volunteers were administered 4 oral doses (2, 5, 10, or 15 g L-Cit) in random order, with each dose separated by a washout period of 15 d. L-Cit was dissolved in 150 mL water and consumed rapidly. None of the volunteers suffered nausea or diarrhea or had any other notable side effect. The amino acid had no effect on hematologic or biochemical markers, nor did it have an effect on blood pressure. The effects of L-Cit loading on plasma insulin and growth hormone were also monitored, and these concentrations were not affected by L-Cit administration. L-Cit did accumulate in plasma; however, the increase in plasma L-Arg concentrations was less than expected (49).

Various clinical efficacy studies involving the repeated high-dose administration of L-Cit have been conducted. L-Cit intake at 5.6 g/d for 1 wk in otherwise healthy middle-aged men greatly reduced brachial-ankle pulse-wave velocity (an index of arterial stiffness). These findings were associated with increases in serum NO metabolites but had no impact on blood pressure (50). None of the study subjects experienced any adverse effects with high-dose L-Cit administration.

In a 6-wk study in which prehypertensive individuals consumed "watermelon extract" (1350 mg L-Cit with 650 mg L-Arg) 2 times/d, no adverse effects were reported (51). There was, however, a reduction in aortic and brachial pulse pressure (6 ± 2 and 8 ± 3 mm Hg, respectively), as well as a 7 ± 2–mm Hg reduction in aortic systolic blood pressure. Brachial blood pressure and aortic diastolic pressures were unaffected.

A 4-wk study examined whether L-Cit supplementation attenuated brachial blood pressure and/or modulated aortic hemodynamic responses at rest or during the cold pressor test. Participants (average age: 17 y) ingested 6 g L-Cit/d for 4 wk (52). Oral L-Cit supplementation did attenuate the brachial and aortic systolic blood pressure and aortic pulse pressure responses to the cold pressor test. No adverse effects were reported during this study.

Forty infants undergoing cardiopulmonary bypass and at risk of pulmonary hypertension were given 5 perioperative doses of oral L-Cit ($1.9\ g \cdot m^{-2} \cdot dose^{-1}$; equivalent to a 3.6-g adult male dose) to determine whether supplementation was safe and efficacious in increasing plasma L-Cit concentrations and decreasing the risk of postoperative pulmonary hypertension (53). Systemic blood pressure was monitored continuously during the 48-h study period. An adverse event was defined as a decrease in systemic mean blood pressure of >25% from baseline. The study found that blood pressure did not differ between the L-Cit and placebo groups, and there were no other serious adverse effects. Three patients died of postoperative complications within 30 d of surgery, but their deaths were considered unrelated to L-Cit administration. The authors concluded that oral supplementation with L-Cit may be effective in reducing postoperative pulmonary hypertension.

Quality and safety. In February 2014, the FDA alerted health care professionals, patients, and caregivers to serious adverse events (e.g., hyperammonemia) reported in patients who were administered L-Cit repackaged and distributed by Medisca, Inc. (54). The adverse events were associated with potentially subpotent L-Cit within 1 particular lot number (although subsequently expanded to cover other lots). Patients who had used this particular L-Cit product lot could have experienced high ammonia concentrations and their associated risks. After a thorough investigation by the FDA, it was determined that the suspected lot numbers did not contain L-Cit but instead contained *N*-acetyl-L-leucine.

A review of the Certificate of Analysis available at the company's website showed that the product met internal quality criteria. This indicated that the problematic batches of L-Cit had met the company's specification for content, recorded as 99.88% L-Cit by using a nonspecific titration analytical procedure (internal specification was 98.5–101.0% L-Cit). The Certificate of Analysis for the material showed results and specifications on other tests, which included loss on drying, solubility in water, residue on ignition, heavy metal limits ($\leq$1 ppm for any single heavy metal or $\leq$10 ppm total heavy metals), ammonium ($\leq$0.02%), chloride ($\leq$0.02%), sulfate ($\leq$0.2%), iron ($\leq$10 ppm), and pH (5.0–7.0).

This is another example in which appropriate quality testing standards would have helped avoid the life-threatening adverse events that occurred. USP, in response to this unfortunate event, is in the process of developing public quality standards for L-Cit and a quality monograph was published in the *Pharmacopeia Forum* on 30 October 2015 to seek public comments before the standards become official (55). Proposed specifications for the *USP-NF* L-Cit quality monograph are summarized in Table 2. For identity testing, the *USP-NF* monograph proposes identification by 3 complementary test methods, namely HPLC, Infrared (IR), and optical rotation, which together are a more specific testing modality. Content will also be determined by HPLC. The monograph also specifies limits for expected impurities and complies with the USP-NF general notices on determination of specific metals, generally by Inductively coupled plasma mass spectrometry (ICP-MS) (see chapter 232 in *Elemental Impurities—Limits*) (56), with limits set on the basis of daily intake amounts (see chapter 2232, *Elemental Contaminants in Dietary Supplements*) (12). USP quality standards are vetted by expert committee members who are knowledgeable about the article in question and have, unlike internal company methods, the benefit of this expert knowledge, which makes them more reliable.

Interactions with other drugs and supplements. L-Cit may interact with some prescription drugs, including medications used to treat hypertension, cardiovascular disease, and erectile dysfunction. However, to our knowledge, no clinical studies have been conducted to assess these types of interactions. Oral L-Cit supplementation has been shown to increase plasma L-Arg concentrations to an even greater extent than oral L-Arg supplementation. This is largely due to extensive presystemic hepatic metabolism of L-Arg (57). Therefore, the interaction potential of L-Arg with certain prescription medications should be considered. Caution is advised in the use of L-Arg supplements with antihypertensive agents, antidiabetic angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, drugs that increase coronary blood flow (e.g., nitrates), anticoagulants, and drugs for erectile dysfunction.

Effect of age and nutritional status. It is not currently known if there are negative effects of L-Cit supplementation on the unborn fetus; thus, caution is advised on its use during pregnancy. The short-term usage of L-Cit has been studied across a broad range of age groups at various doses and for limited periods of time with no reports of adverse events. However, any potential side effects that may be associated with long-term (chronic) supplementation remain to be determined.

Effects and health status. There are no known contraindications for the use of L-Cit in any disease populations. Nevertheless, it is unclear if L-Cit supplementation in certain populations should be avoided as a result of its ability to increase L-Arg plasma concentrations.

Conclusions and Recommendations

According to recent market research, retail sales of DSs (and FF products) in the United States have increased for the 11th consecutive year (2). Amino acid supplements are an especially popular category, particularly among athletes (14). However, with an increasingly aging population, amino acid supplementation is being considered as an alternative means of slowing muscle loss and enhancing physical activity in the elderly (58). Various amino acids are also added as food ingredients in energy bars as well as in diet and energy drinks. The increased use of these products supports the need for more robust safety assessments of individual ingredients.

In this review, we highlighted the need for an overall safety assessment of amino acids. In addition to the traditional reliance on the established UL, OSL, or HOI, other factors that need to be taken into account include well-defined quality specifications, review of synthesis and production strategies, potential interactions with drugs, contraindications within disease states, and cautionary use with certain age groups.

We presented 2 cases of amino acids used as DSs and FFs—L-Trp and L-Cit—in which serious in-market adverse events have occurred. In the example of L-Trp, the incidence of EMS associated with products from a particular manufacturer may have been the result of EBT contamination. The use of well-established specification limits for impurities as part of an overall quality profile could help prevent similar incidents from occurring in the future. Likewise, consideration of changes to synthesis and production strategies of raw materials for potential carryover impurities should routinely be included in overall safety assessments. Defined quality specifications, if followed by manufacturers, would likely have prevented the serious adverse events associated with the L-Cit example. Furthermore, current Good Manufacturing Practices and adverse event reporting requirements enacted in the United Sates postdated the adverse event that was reported for L-Trp. The use of public standards and specifications and adherence to appropriate test methods and acceptance criteria should be incorporated into a company's current Good Manufacturing Practices regimen.

Parallel to the increase in DSs and FF product use in the United States is the concomitant use of prescription medications. According to the most recent report from the CDC, 10.1% of the population uses ≥5 prescription medications (data for both males and females, age adjusted) (59). These data do not include the use of OTC drugs. The potential for DS-drug interactions is well documented and should be considered as part of an overall safety assessment for DS and FF ingredients (60). Interactions may result in pharmacodynamic and/or pharmacokinetic changes in the profile of a DS, FF, or drug. Documented adverse events exist for the combination of L-Trp and MAOIs resulting in the elicitation of the serotonin syndrome (34). We have highlighted several other potential interactions of L-Trp and L-Cit with various drug classes and/or specific medications.

Similarly, consideration should be given to patients with certain disease states who may be at increased risk of adverse effects when adding supplements to their diet. Individuals with compromised liver and/or kidney function in particular should exercise caution when using various DSs and FFs that include amino acids. Examples exist for potential adverse effects that can occur when L-Trp is used by individuals with various hepatic dysregulations (39).

In addition to the above-mentioned considerations, the importance of in-market safety surveillance and adverse event reporting is highlighted. The USP continuously monitors the literature and various government adverse event reporting portals as a means of staying up to date on any safety signals related to the articles with USP monographs. This information, together with other adverse events reported in clinical studies and case reports, informs the USP expert committee on whether the admission status of an article needs revision. Premarket identification of potential DS-drug interactions and/or sensitive populations may help define safety surveillance strategies. In summary, we propose that these principles could be applied when assessing the safety of other DS and FF ingredients as well.

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