Lysinoalanine Content of Formulas for Enteral Nutrition

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ABSTRACT

Casein and caseinates are the main ingredients of formulas for enteral nutrition. Their manufacturing procedure and the thermal treatments necessary to assure microbiological stabilization and satisfactory shelf-life of the end-products are particularly favorable for the formation of lysinoalanine (LAL), a cross-linked amino acid that is considered a useful marker of the thermal damage and reduced digestibility of proteins.

The lysinoalanine content of 18 different kinds of formulas for enteral nutrition was determined by HPLC after derivatization. The liquid formulas have an average value of 528 $\mu\text{g/g}$ protein LAL, ranging from 160 to 800 $\mu\text{g/g}$ protein (average content of formulas for pediatric use 747 $\mu\text{g/g}$ protein). These values are rather high considering that the average value detected in UHT-treated drinkable milk is 117 $\mu\text{g/g}$ protein. In principle, the preparation of caseinates and the thermal stabilization of the end products are the two steps more favorable for the formation of LAL. The fact that the five samples stabilized by an UHT-treatment have an average value of 512 $\mu\text{g/g}$ protein suggests that the LAL content depends more on the quality of the starting ingredients than on the sterilization process. A better selection of the starting ingredients should improve the quality of formulas for enteral nutrition, which is very desirable when formulating foods for consumers with very high nutritional demands.

(Key words: lysinoalanine, thermal damage, cross-linked amino acids, enteral nutrition)

Abbreviation key: LAL = lysinoalanine; FMOC = 9-fluorenylmethylformate; SPE = solid phase extraction.

INTRODUCTION

Sodium caseinate and, more rarely, soybean protein isolates are the main ingredients for the preparation of formulas for enteral nutrition, an important sector of artificial nutrition. Unfortunately, the ingredient

manufacturing process and thermal treatments applied to end products for assuring microbiological stabilization and satisfactory shelf-life may induce antinutritional transformations, which are particularly relevant since these products may be the sole nutrient source for many patients for very long periods.

In particular, protein-bound amino acids may undergo modifications in their side chains, that may require the participation of sugars, as in the Maillard reaction, or not, as in the formation of cross-linked amino acids. The most investigated of these unusual amino acids is lysinoalanine [LAL, N^ϵ -(*R,S*-2-amino-2-carboxyethyl)-*S*-lysine], which has been used as a marker of thermal damage to foods because it has the analytical advantage of being stable in the acidic conditions of protein hydrolysis (Maga, 1984; Friedman, 1999).

The mechanism of the formation of LAL is relatively simple: the first step is the elimination of a leaving group from *O*-phosphorylserine, *O*-glycosylserine, or cystine to generate a dehydroalanine residue, which may undergo Michael addition by the nucleophilic side chain of another amino acid, such as the ϵ -amino group of lysine. This process is favored by aqueous alkali treatments, such as those applied in preparing some milk derivatives, in particular sodium caseinate, and soybean protein concentrates (Friedman, 1999).

Many studies have investigated the effects of the formation of LAL on protein functionality and nutritional value. Generally the concentration decrease of essential amino acids is not the most relevant consequence, but LAL, being like a bridge or a cross-linker between two different parts of the protein chain (Pellegrino et al., 1998), impairs the approach of enzymes with the consequence of decreasing protein digestibility. For this reason, many groups have studied the digestibility of proteins treated with alkali (Savoie et al., 1991; Anantharaman and Finot, 1993; Guo et al., 1999) and other nutritional and toxicological consequences of LAL formation in foods (Karajiannis et al., 1980; Maga, 1984; Friedman, 1999). Adverse effects on growth, protein digestibility, protein quality, and mineral bioavailability and utilization have been described (Sarwar et al., 1999).

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There are also indications about toxicity, as LAL was shown to provoke lesions in rat kidney cells causing nephrocitomegaly (Friedman et al., 1984; Friedman and Pearce, 1989). Although the effects seem very species-specific, these observations have promoted investigation on humans, in particular on preterm infants. The higher level of Maillard reaction products and LAL in infant formulas compared to breast milk had no influence on creatinine clearance or electrolyte excretion and there was no evidence of tubular damage as determined by the urinary excretion of four kidney-derived enzymes, however feeding with formulas resulted in a general increase in urinary microprotein levels (Langhendries et al., 1992).

The thermal damage of the formulas for parenteral nutrition has been investigated by several groups, especially regarding the formation of color and the degradation of essential amino acids (Fry and Stegink, 1982; Labuza and Massaro, 1990; Stegink et al., 1981). We verified, instead, that the thermal damage of formulas for enteral nutrition had never been studied before, although caseinates are particularly susceptible to the formation of LAL. This has prompted us to collect samples of commercial formulas and to quantify this cross-linked amino acid as a marker of their nutritional quality.

MATERIALS AND METHODS

Samples

The commercial formulas for enteral nutrition are produced by three companies and were provided by the pharmacies of three Italian hospitals. Samples **A**, **E**, **I**, and **L** are indicated for normocatabolic patients; samples **B**, **M**, **O**, and **Q** for patients with increased nutrient requirements, such as anorexic ones; samples **C** and **T** are for pediatric use; samples **D** and **F** for nephropathic patients; sample **P** for immunodeficiency; sample **R** for patients affected by pulmonary pathologies; sample **N** for diabetic patients; samples **G**, **H**, and **S** are enriched with fiber. The samples were liquid, with the exception of **Q** that was powdered. Details of the formula composition are reported in Table 1.

Materials

All chemicals for derivatization, solid phase extraction, and HPLC were of high purity. 9-Fluorenylmethylchloroformate (**FMOC-Cl**) was purchased from Fluka (Switzerland). Paper filters (589², 90 mm i.d.) were purchased from Schleicher & Schuell (Germany). The amino cartridges (Bakerbond, 500 mg/3 ml) were purchased from Baker (The Netherlands). The chromatographic column (Amino-Quant, 200 × 2.1 mm i.d.) and

Table 1. Composition of the commercial formulas for enteral nutrition.

Sample	Nutrient contents (g/100 ml) reported on the labels				
	Proteins			Carbohydrates	Lipids
	Total	Casein	Other proteins		
A ¹	4.0	4.0		12.3	3.9
B ¹	6.0	6.0		18.4	5.8
C ¹	2.8	2.8		12.2	4.5
D	1.2	1.2		12.3	5.1
E ¹	5.0	5.0		17.5	6.5
F ¹	4.0	4.0		23.6	10
G	4.0	4.0		12.3	4.0
H	4.1	4.1		14.4	3.54
I	4.1	2.9	1.2 ³	14.2	3.5
L	3.7	2.8	0.9 ³	13.2	3.9
M	5.4	5.4		20.5	6.0
N	3.4	3.4		12.5	3.2
O	6.6	5.3	1.3 ³	14.8	4.0
P	4.3	4.3		13.4	2.8
Q ²	34	34		53.2	0.8
R	6.2	6.2		10.6	9.2
S	4.2	4.2		13.4	3.4
T	3.0	2.4	0.6 ⁴	11.0	5.0

¹UHT-sterilized.

²Powdered formula (nutrient contents amounts expressed as percentage on dry weight).

³Soy protein.

⁴Lactalbumin.

the guard column cartridge (ODS-Hypersil C18, 5 μ m, 20 × 2.1 mm i.d.) were purchased from Hewlett Packard (Germany).

LAL Determination

The procedure for LAL determination included sample acid hydrolysis, FMOC derivatization of the amino compounds, selective solid phase extraction (**SPE**) to purify LAL derivatives, and subsequent HPLC analysis with fluorescence detection (Pellegrino et al., 1996). The HPLC elution solvents were prepared from stock solution I (0.5% tetrahydrofuran and 0.1% ethyl acetate in 30 mM potassium acetate; vol/vol/vol) and stock solution II (80% acetonitrile in 100 mM sodium acetate; vol/vol). The stock solutions were mixed (vol/vol) for preparing the elution solvent A (I:II=70:30) and the elution solvent B (I:II=25:75). The elution solvent C was 100% of stock solution II. An aliquot of 20 μ L of each sample purified by SPE was injected into the HPLC column protected by a guard column cartridge; the separation was carried out by a linear elution gradient. Fluorescence detection was performed with excitation at 266 nm and emission at 310 nm. Under the conditions described, the isomers SR- and SS-LAL were eluted as two different peaks with t_R 25 and 26 min respectively.

RESULTS

This study was based on LAL formation because it was considered a better marker than Maillard reaction products of the nutritional quality of caseins. Compounds such as furosine are not formed during casein manufacture because of the lack of reducing sugars (Pellegrino et al., 1996).

The analysis of LAL can be performed by different methods, for example by ion-exchange chromatography (Friedman et al., 1984; Sanderson et al., 1978) or by GC/MS after derivatization of both amino and carboxy groups (Büser and Erbersdobler, 1988; Hasegawa et al., 1987; Liardon et al., 1991). In recent years, however, HPLC has become more and more important. Warthesen and Wood-Rethwill (1984) have reported a method in reversed phase after derivatization of the three amino groups with dansylchloride, that has been improved in more recent times (Faist et al., 2000; Moret et al., 1994). Another HPLC method, based on derivatization with FMOC-Cl, solid phase extraction, reverse phase chromatography and fluorescence detection, has been proposed by Pellegrino et al. (1996) for the quantification of LAL at very low level in cheese.

The last method was selected for our study, because it is very sensitive, with a threshold limit around 1 $\mu\text{g/g}$ protein. As already indicated by Pellegrino et al. (1996), LAL appears in the chromatograms as a double peak due to the presence of the S,S- and S,R-diastereoisomers. The application of this methodology to products for enteral nutrition was very simple and gave good reproducibility (the standard deviation was 2–3%). In total 18 different kinds of commercial formulas were analyzed (for each of them at least two samples from different lots were obtained, the differences of LAL concentration in different lots may reach 9–10%). The samples were labeled with letters, the therapeutic indication is reported in Materials and Methods, whereas details of the composition, obtained from the labels, are reported in Table 1. Each sample was analyzed in triplicate: the results are shown in Figure 1.

Only the powdered formula **Q** did not contain LAL in detectable amounts, whereas this contaminant was detected in all the liquid formulas: its amount ranged from 150 to 800 $\mu\text{g/g}$ protein (13 samples contained more than 400 $\mu\text{g/g}$ protein, 8 samples contained more than 600 $\mu\text{g/g}$). Two of the most contaminated samples were for pediatric use (**C** and **T**, 677 ± 61 and 817 ± 75 $\mu\text{g/g}$ protein, respectively). Samples **I**, **L** and **O**, that contained about 25% of soybean proteins, had a LAL content (253 ± 22 , 679 ± 73 , and 537 ± 11 $\mu\text{g/g}$ respectively) comparable to products containing only casein or caseinates.

Figure 1. LAL contents of commercial formulas for enteral nutrition (see Table 1 for the composition of the products). Error bars refer to the standard deviation.

Very variable contents were detected in the 5 samples (**A**, **B**, **C**, **E**, **F**) that had been sterilized by an UHT treatment, as indicated in the label.

DISCUSSION

Animal studies have demonstrated that alkaline and/or heat treatments cause significant reduction in the digestibility of casein and also soybean proteins and a drastic negative effect on protein quality, as measured by rat growth methods (Sarwar et al., 1999). The mineral status of rats is compromised too and the kidney iron contents of rats fed with treated proteins are lower than that of rats fed with untreated proteins. Moreover liver copper levels of male and female rats fed with treated proteins are up to three folds higher than those found in rats fed with untreated proteins (Sarwar et al., 1999), even if some effects appear to be reversible, when the feeding with proteins characterized by high LAL contents is ceased (Struthers et al., 1977; Struthers et al., 1980). Although human studies (Langhendries et al., 1992) have shown only a general increase in urinary microprotein levels, whereas other parameters seem to be unaffected, the results on animals suggest that special attention should be paid at least to the preparation of foods for consumers with particular nutritional demands. Certainly patients requiring enteral nutrition belong to this category.

So far infant formulas are certainly the foods, which have been investigated in most detail from the point of view of LAL formation. Most of these studies were performed in the 80's (Bellomonte et al., 1987; De Koning and Van Rooijen, 1982; Friedman, 1999; Fritsch

and Klostermeyer, 1981; Resmini et al., 1985), when the level of LAL could reach 150 to 920 $\mu\text{g/g}$ protein in powdered formulas and 160 to 2100 $\mu\text{g/g}$ protein in liquid formulas. These results induced manufacturers to look for milder technologies for manufacturing the ingredients and for stabilizing the end-products, which resulted in a dramatic decrease of the LAL content in the following years. A recent investigation have shown that current commercial spray dried formulas, produced with very mild technologies, contain only traces of LAL, whereas commercial liquid formulas 131 $\mu\text{g/g}$ protein (Arnoldi, 2000; D'Agostina et al., 2002), comparable with UHT-treated drinkable milk (Faist et al., 2000). The consistent improvement of the quality of these products in respect to the past indicates that a careful choice of the manufacturing procedures permits to reduce considerably the thermal damage to proteins.

The liquid formulas for enteral nutrition investigated in this study have an average value of 528 $\mu\text{g/g}$ protein LAL, ranging from 160 to 820 $\mu\text{g/g}$ protein (only 3 samples out of 17 have less than 350 $\mu\text{g/g}$ protein). These values are rather high, for example much higher than in UHT-treated drinkable milk, average 117 $\mu\text{g/g}$ protein (Faist et al., 2000), and the values detected in the two samples for pediatric use (C and T, which contained 677 and 817 $\mu\text{g/g}$ protein of LAL, respectively) are particularly worrying.

The two steps more favorable for the formation of LAL are the preparation of caseinates and the thermal stabilization of the end-products. The indication of the stabilization method on these formulas is optional, however, the 5 samples stabilized by an UHT-treatment, as indicated in the label, have an average value of 512 $\mu\text{g/g}$ protein, suggesting that the LAL contents depends more on the quality of the starting ingredients than on the sterilization process of the end-products. Rennet casein has an average content of 114 $\mu\text{g/g}$ protein (range 21–178 $\mu\text{g/g}$), whereas sodium and calcium caseinates an average content of 977 $\mu\text{g/g}$ protein (Pellegrino et al., 1996), showing that the alkali process for producing caseinates is particularly favorable for the formation of LAL. However, the LAL content may vary from 128 to 2992 $\mu\text{g/g}$ protein (Pellegrino et al., 1996) depending on the manufacturing conditions, a fact that clearly shows the possibility of producing caseinates with satisfactory nutritional features. A better selection of the raw ingredients should improve the quality of formulas for enteral nutrition, a very desirable achievement while formulating products for consumers with very high nutritional demands, at least in the case of products for pediatric use.

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