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Analytical Methods

Phenylketonuria: Protein content and amino acids profile of dishes for phenylketonuric patients. The relevance of phenylalanine

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ABSTRACT

Phenylketonuria is an inborn error of metabolism, involving, in most cases, a deficient activity of phenylalanine hydroxylase. Neonatal diagnosis and a prompt special diet (low phenylalanine and natural-protein restricted diets) are essential to the treatment. The lack of data concerning phenylalanine contents of processed foodstuffs is an additional limitation for an already very restrictive diet.

Our goals were to quantify protein (Kjeldahl method) and amino acid (18) content (HPLC/fluorescence) in 16 dishes specifically conceived for phenylketonuric patients, and compare the most relevant results with those of several international food composition databases.

As might be expected, all the meals contained low protein levels (0.67–3.15 g/100 g) with the highest ones occurring in boiled rice and potatoes. These foods also contained the highest amounts of phenylalanine (158.51 and 62.65 mg/100 g, respectively). In contrast to the other amino acids, it was possible to predict phenylalanine content based on protein alone. Slight deviations were observed when comparing results with the different food composition databases.

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1. Introduction

Phenylketonuria (PKU) was first described in 1934 by Asbörn Föling (Centerwall & Centerwall, 2000). It is an autosomal recessive inborn error of the metabolism that occurs due to mutations in the gene that codifies the phenylalanine hydroxylase (PAH) (Belanger-Quintana, Burlina, Harding, & Muntau, 2011; Scriver & Kaufman, 2001; Smith & Lee, 2000), which is responsible for converting dietary phenylalanine (Phe) into tyrosine (Tyr) in the liver. The location and type of mutation within the gene determine the severity of the phenotype (Mallolas et al., 1999). A decrease of PAH concentration and/or lower enzyme activity results in persistent elevated Phe blood and tissue concentrations, with potential toxic effects, particularly for the developmental brain (Blau, van Spronsen, & Levy, 2010; de Groot, Hoeksma, Blau, Reijngoud, & van Spronsen, 2010; van Spronsen & Enns, 2010). Early diagnosis in the neonatal period, with a screening program (Blau, Hennermann, Langenbeck, & Lichter-Konecki, 2011), and prompt dietary treatment are essential to prevent severe mental retardation and to achieve a good patient prognosis (Blau et al., 2010; de Groot et al., 2010). Indeed, patients with late diagnose and/or poor metabolic control tend to evidence signs of cognitive dysfunction, namely, developmental delay, progressive intellectual impairment, seizures, attention deficit/hyperactivity disorder, decreased autonomy, behavioral problems, such as aggression, anxiety or social isolation (Demirkol, Gizewska, Giovannini, & Walter, 2011; Rocha & Martel, 2009; Scriver & Kaufman, 2001). Untreated patients may also present other clinical signs such as a mousy odour, eczema, reduced pigmentation, reduced growth and microcephaly (Rocha & Martel, 2009).

Although some new therapeutic approaches have been studied in order to improve the quality of life of PKU patients (as neutral amino acids, glycomacropeptide, tetrahydrobiopterin, Phe ammonia lyase or gene therapy) (Blau et al., 2010; Feillet & Agostoni, 2010; Rocha & Martel, 2009; van Spronsen & Enns, 2010), dietary management remains the mainstay of treatment (MacDonald, Rocha, van Rijn, & Feillet, 2011; van Spronsen & Enns, 2010). Nutritional guidelines and statements vary between countries (Demirkol et al., 2011), but low protein and Phe-restricted diet

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are common (Feillet & Agostoni, 2010) as well as the need to continue the treatment throughout life. In order to limit the intake of Phe, dietary management of PKU patients restrict natural-protein food (Feillet & Agostoni, 2010). Therefore, based on nutritional recommendations and on PKU patients' individual tolerances, dietary treatment includes protein substitutes (Phe-free AA mixtures) and a wide range of low protein products (like bread, biscuits, cereals, pasta, flour, milk substitutes, cheese substitutes, egg substitutes, soups, candies, amongst others) to make up the energy needs of the PKU patients. In addition to these, the diet also incorporates strictly controlled amounts of natural foods, essentially fruits, vegetables and other natural foods or food products with low protein content (Bremer, Anninos, & Schulz, 1996; Weetch & MacDonald, 2006).

It is, therefore, of utmost importance to know the nutritional information of natural and processed food products included in these patients' diet, especially protein and Phe. It is often reported there is scarcity of information about Phe content particularly in food composition databases (FCD). In Europe, for example in UK's or Denmark's FCD, when available, Phe contents often concerns raw foods and, rarely, cooked composite meals. Some countries, as Portugal, do not have the complete aminogram of foods available in national FCD. In these cases, international data has to be consulted. Nonetheless, it is important to be aware that many factors (soil type, season, geography, type of processing.) may influence nutrient composition of foods and data cannot always be extrapolated from one country to another.

The aim of this work was to study the protein and amino acid contents of 16 low protein dishes selected from a list of recipes specifically planned for PKU patients (Almeida, 1995, 2001) and prepared with special ingredients acquired in Portugal, as well as to compare the most relevant results (protein and Phe) with the information described in several FCD (when available).

2. Materials and methods

2.1. Standards and reagents

Dansyl chloride and amino acid standards (L-alanine (Ala), L-arginine (Arg), L-asparagine (Asn), L-aspartic acid (Asp), L-glutamic acid (Glu), L-cysteine (Cys), L-glycine (Gly), L-glutamine (Gln), L-histidine (His), L-isoleucine (Ile), L-leucine (Leu), L-lysine (Lys), L-methionine (Met), L-phenylalanine (Phe), L-ornitine (Orn), L-proline (Pro), L-serine (Ser), L-threonine (Thr), L-tryptophane (Trp), L-tyrosine (Tyr) and L-valine (Val)), as well as the internal standard L-norleucine (Nor) were all obtained from Sigma (St. Louis, MO, USA).

Purified water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). HPLC-grade acetonitrile and N,N-dimethylformamide (DMF) were from Fluka (Madrid, Spain). All other chemicals used were of analytical grade.

2.2. Samples

Most of the 16 low protein dishes were selected from a list of recipes specifically planned for PKU patients (Almeida, 1995, 2001), which included soups, main courses, desserts and other daily basic foods, as described in Table 1. Regular ingredients were bought in local supermarkets. Dietetic low protein and low Phe products – egg substitute, low protein chocolate candies, low protein flour, low protein milk substitute, low protein pasta, low protein shortbread biscuits and low protein vermicelli – were kindly provided by Centro de Genética Médica Doutor Jacinto de Magalhães, INSA IP, Porto, Portugal.

2.3. Sample preparation

The dishes were prepared and cooked according to the instructions (Almeida, 1995, 2001) using domestic scale utensils and equipment. After being cooked, the food samples were mashed until completely homogenised (Moulinex Classical A320R1, Moulinex, France) and protein content determined immediately. Samples were kept frozen at −20 °C until analysis (total amino acids).

2.4. Protein determination

Protein content was determined using the Kjeldahl method (Büchi Labortechnik AG, 2007). Briefly, 1 g of each homogenised sample was digested with 96% sulfuric acid, using a catalytic mixture. Digestion was performed in a Büchi® apparatus (Automat K-438, Büchi Labortechnik AG, Swiss). The ammonia formed was distilled and collected in a solution of boric acid (Distillation Unit K-360, Büchi®, Büchi Labortechnik AG, Swiss), which was then titrated with sulfuric acid. In this way, the nitrogen content of the samples was determined. Protein content was calculated using the conversion factor 6.25 (Tontisirin, MacLean, & Warwick, 2003). Analyses were performed in duplicate.

2.5. Amino acids determination

2.5.1. Hydrolysis

The acidic hydrolysis used for the determination of the amino acids was performed as described elsewhere (Fountoulakis & Lahm, 1998; Paramas, Barez, Marcos, Garcia-Villanova, & Sanchez, 2006) with slight modifications. Briefly, 1 g of sample was weighted into a screw-cap tube to which 3 ml of 6 mol L^{-1} HCl were added. Oxygen was removed using a N_2 stream. The tubes were tightly capped and heated at 110 °C for 24 h. The extracts were neutralized with 12 mol L^{-1} KOH to pH 7.

The alkaline hydrolysis was performed to determine tryptophan content, as described by Yust et al. (Yust et al., 2004) with slight modifications. Briefly, 1 g of sample was weighted into a screw-cap tube to which 3 ml of 4 mol L^{-1} KOH were added. Oxygen was removed using a N_2 stream. The tubes were capped and heated at 110 °C for 4 h. The extracts were neutralized with 12 mol L^{-1} HCl to pH 7.

Both procedures were carried out in triplicate.

2.5.2. Derivatization

Dansyl chloride derivatives were prepared, in duplicate, according to Navarro, Aristoy, & Izquierdo (1984). Briefly, the hydrolysates obtained were centrifuged (Heraeus Sepatech Labofuge Ae, Haraeus Instruments, Germany) at 5000 rpm for 5 min. A supernatant aliquot (500 μl) was transferred to a screw-cap tube and 500 μl 2 mol L^{-1} Na_2CO_3 added followed by 200 μl dansyl chloride (2%), 60 μl norleucine (400 μg/ml) and 1 ml acetone (final pH between 9.5 and 10.5). The tubes were capped and heated to 110 °C for 10 min. After cooling to room temperature in the dark, and 1 ml of the derivatized extract was centrifuged (Heraeus Sepatech Biofuge Pico, Haraeus Instruments, Germany) at 13,000 rpm for 5 min. The supernatant was transferred into amber glass vials (Supelco, Bellefonte, PA, USA) and analysed by RP-HPLC/fluorescence.

2.5.3. RP-HPLC/fluorescence analysis

The chromatographic analysis was carried out in a HPLC integrated system equipped with an AS-950 automated injector, a PU-980 pump, and a FP-920 fluorescence detector (Jasco, Japan) programmed for excitation at 335 and emission at 514 nm.

The chromatographic separation was achieved with a RP-Tracer Excel ODS-A column (5 μm; 250 × 4 mm) from Teknokroma (Spain) operating at controlled temperature of 40 °C (Jasco CO-2060 Plus, Jasco, Japan).

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