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Acute and repeated dose (28 days) oral safety studies of phosphatidyl-hydroxytyrosol

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ABSTRACT

Phosphatidyl-hydroxytyrosol, a carrier of hydroxytyrosol under the form of phospholipid with high antioxidant capacity, is being actively studied as a potential ingredient of functional foods and supplements. To support the safety, phosphatidyl-hydroxytyrosol has been examined in an acute and in a 28-day repeated dose oral toxicity studies in rats. Phosphatidyl-hydroxytyrosol administered in a single oral gavage dose of 2000 mg/kg of body weight (bw) resulted in no adverse events or mortality. In addition, phosphatidyl-hydroxytyrosol administered as a daily dose of 2000 mg/kg bw for 28 days by gavage resulted in no adverse events or mortality. No evidence or treatment related toxicity was detected during both studies. Data analysis of body weight gain, food consumption, clinical observations, blood biochemical, haematology, organ weight ratios and histopathological findings did not show significant differences between control and treated groups. It is concluded that phosphatidyl-hydroxytirosol orally administered to rats was safe and that no treatment-related toxicity was detected even at the high doses investigated in both acute (2000 mg/kg bw) and repeated dose (28-day) oral (2000 mg/kg bw) toxicity studies.

1. Introduction

The popularity of phenolic compounds in general as bioactive natural antioxidants is currently well-known. Within polyphenolic compounds, hydroxytyrosol, the major phenolic compound present in virgin olive oil, has been an attractive molecule in the last decades that has shown a great bioactivity and antioxidant power, which has been related to antiatherogenic, antiplatelet aggregation, anti-inflammatory, antimicrobial, and antitumor effects, or aging regulation (Cornwell and Ma, 2008; Granados-Principal et al., 2010; Killeen et al., 2011). Although hydroxytyrosol is well absorbed at the gastrointestinal tract, it is rapidly metabolized in enterocyte and liver and it is only found at minor levels in plasma and tissues (D'Angelo et al., 2001; De la Torre, 2008; Rubió et al., 2014). The production of "phenolipids", namely, lipophilized phenolics resulting from the union of a lipid to the phenolic moiety (Laguerre et al., 2010), has been explored by diverse authors to obtain carriers of hydroxytyrosol. Concerning novel derivatives of phenolic compounds, Casado et al. (2013, 2014) have recently developed a solid to solid reaction system for transphosphatidylation of phosphatidylcholine with two different phenylalkanols, namely tyrosol and hydroxytyrosol, obtaining a new phospholipid, phosphatidyl-hydroxytyrosol (PHT). Remarkable antioxidant activity of this new molecule for edible oils has been described. It was shown a significant antioxidant activity of PHT in diverse edible oils, which was comparable or even superior to hydroxytyrosol (Martin et al., 2014a). This new phospholipid (phosphatidyl-hydroxytyrosol, PHT) was proposed as a potential vehicle of hydroxytyrosol considering also the additional relevance of the phospholipid backbone. Phospholipids are well-known essential molecules for the maintenance of living cells as major constituents of cell membranes. Additionally, a positive impact of dietary phospholipids on human health, such as relevant implications in hypercholesterolemia, atherosclerosis, cardiovascular disease, inflammation and immunity, liver disorders, brain development, as well other chronic diseases has been pointed out (Espinosa-Salinas et al., 2011).

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On the other hand, due to their amphiphilic nature and surface-active properties, phospholipids are well-known as emulsifier ingredients in food, pharmaceutical, or cosmetic industry. The emulsifying properties are also related to the role of phospholipids to enhance the digestion and absorption of other molecules at the intestinal level, by contributing to the emulsification of lipid drops in the aqueous media, improving the formation of mixed micelles, and serving as vehicles of lipid products to the enterocytes (Borgström, 1993). This is particularly important as all current strategies for enriching food products. Furthermore, a high bioaccessibility has been described for PHT (Martin et al., 2014b), which means that after hydrolysis their lipid products are easily solubilized within the micellar phase for intestinal absorption (Fernandez-Garcia et al., 2009).

To date there have been no conventional safety *in vivo* studies published for high levels of polyphenolic compounds and there is need for further research on the possible adverse effects that might be exerted by the news phospholipids, such as PHT, to be used as functional ingredients in food. Because toxicology *in vivo* studies are necessary to assess the safety of a functional food previous commercialization, the aim of this study was to evaluate the acute and repeated dose (28 days) oral toxicity of PHT in rodent model to serve as scientifically defensible safe data.

2. Material and methods

2.1. Chemicals and reagents

Hydrogenated phosphatidylcholine, 90% purity (PC) (Phospholipon 90H) was purchased from Lipoid AG (Cham, Switzerland). Powdered preparation of Actinamadure Sp phospholipase D (EC 3.1.4.4) was acquired from Meito Sangyo CO (Nikko-Cho, Fuchu, Tokyo, Japan). According to the vendor specifications PLD contained 1500 U/mg (1 PLD Unit is defined as the amount of enzyme producing 1 μmol/h of choline from L-a-phosphatidylcholine when the enzyme solution is reacted at pH 8 at 30 °C). Hydroxytyrosol was acquired from SEPROX (Madrid, Spain). Anhydrous calcium chloride (PRS Grade), formic acid (98%), ethyl butyrate (99.5%), squalene (97%) triethylamine (99.5%), phosphatidic acid (1, 2-dioleyl-sn-glycero-3-phosphoric acid monosodium salt, 99%), and sodium acetate trihydrate (99%) were acquired from Sigma–Aldrich (St. Louis, MO, USA). Chloroform, 2-propanol, hexane, acetic acid, and methanol (HPLC grade) were purchased from Lab-Scan (Dublin, Ireland).

2.2. Phosphatidylcholine transphosphatidylation

The test substance, phosphatidyl-hydroxytyrosol (PHT) (86% PHT, 8% hydroxytyrosol, and 6% fully PC) was synthesized by enzymatic transphosphatidylation of hydroxytyrosol and PC (90% purity esterified with 85% stearic acid and 15% palmitic acid). The detailed procedure of the synthesis of PHT was scaled-up based on the procedure previously described (Casado et al., 2014). 245 g of Phospholipon 90H and 51.5 g of hydroxytyrosol were added to a mixture of ethyl butyrate and sodium acetate buffer in a 5-L stainless steel reactor coupled to an anchor stirrer at 200 rpm (Kiloclave Buchi Glass Uster, Switzerland). The total volume of the reaction mixtures was 2 L. The aqueous phase was comprised of 0.2 M sodium acetate buffer (pH 5.6). Then, phospholipase D (1% w/w) was added to the mixture. The reaction mixture was stopped after 20 h and washed first with ethyl butyrate and three times with distilled water. Finally the product mixture was freeze dried and stored at 4 °C. This procedure shows an adequate tool to prepare enough amounts of this new phospholipid (PHT) to test its potential as technological antioxidant and also to further investigate its potential biological activities and safety studies.

2.3. Purification of PHT by semi-preparative HPLC coupled to a fraction collector

The methodology utilized to purify PHT to be used as a standard for HPLC analyses was previously reported (Casado et al., 2014). A reaction mixture comprised of 24 mL was prepared to purify PHT. The reaction mixture was stopped after 20 h and extracted with an appropriate volume of chloroform/methanol/water/phosphoric acid (8/4/2/2, v/v/v/v). Centrifugation separated the mixture into two phases, an upper aqueous phase and a lower phase. The lower phase was recovered and purified via semi-preparative HPLC. A fraction collector coupled to the HPLC was also utilized. The flow rate was 5 mL/min. The column Kromasil Sil column (5 μm, 250 mm, 10 mm), acquired from Analisis Vinicos (Tomelloso, Spain), was maintained in a temperature-controlled column compartment at 55 °C. The mobile phase utilized consisted of a binary gradient of A: hexane and B: hexane/2-propanol/acetic acid/triethylamine (815/170/15/0.8, v/v/v/v). The method started at 1% B increasing to 13% in 10 min, and then increasing to 50% in 1 min. This percentage of B was maintained 16 min and reduced to 1% in 1 min, and the initial conditions were maintained 10 min. Several consecutive injections of 200 μL were effected to obtain enough PHT to be used as analytical standard.

2.4. HPLC analysis

1 μL of the final transparent solutions were analyzed on a Luna 5 μm HILIC diol column (250 mm, 4.60 mm, Phenomenex, Torrance, CA, USA) coupled to an Agilent (Santa Clara, CA, USA) 1200 Series HPLC containing a temperature-controlled column compartment, quaternary pump, autosampler, vacuum degasser, and a dual detection system comprised of an evaporative light scattering and a diode array detector. The flow rate was 1.5 mL/min. A splitter valve was used after the temperature-controlled column compartment and only 30% of the mobile phase was directed through the ELSD detector (3.5 bar and 41 °C). The column temperature was maintained at 55 °C. The mobile phase for the analysis of the product mixtures from PC transphosphatilydation with hydroxytyrosol consisted of a ternary gradient of (A) hexane, (B) hexane/2-propanol/acetic acid/triethylamine (815/170/15/0.8, v/v/v/v), (C) 2-propanol/water/acetic acid/triethylamine, (837/140/15/0.8, v/v/v/v). The method starts at 50% of A and 50% of B for 5 min increasing up to 100% of B in 5.1 min. Then up to 12% of C is added from 5 min to 7 min increasing up to 40% of C at 20 min. Then 100% of B is restored at 35.5 min and initial conditions at 36 min. These initial conditions are maintained for 9 min. Identification and quantification was carried out by using standards for each lipid class (i.e. PC, hydroxytyrosol and PHT) involved in the transphosphatilydation reaction. Calibration curves from each analytical standard were used over the calibration range of 1–50 μg. The lower calibration standard was used as the limit of quantification (LOQ).

2.5. Acute and repeated dose (28-day) oral toxicity studies

Wistar male and female rats (Charles River Inc., Marget, Kent, UK) were acclimated for 7 days prior to study initiation with an evaluation of health status. The rats were individually housed in polycarbonate cages with sawdust bedding and maintained in environmentally controlled rooms (22 ± 2 °C and 50% ± 10% relative humidity) with a 12 h light-dark cycle (light from 08.00 to 20.00 h). Food (A03 rodent diet, Scientific Animal Food and Engenieering, Villemoisson-sur-Orge, France) and water were available *ad libitum*. The rats were 56-days old at initiation of treatment. Acute oral toxicity – fixed dose procedure and repeated dose (28 days) toxicity oral studies were conducted in accordance with European Union guidelines (Council Regulation (EC) No. 440, 2008a,b). Both studies were undertaken in accordance with the ethics requirements and authorized by the Official Ethical Committee of the Complutense University.

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