Food and Chemical Toxicology 55 (2013) 498–504

Contents lists available at SciVerse ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Toxicological evaluation of pure hydroxytyrosol

David Auñon-Calles, Lourdes Canut, Francesco Visioli *

Seprox Biotech, Madrid, Spain
Harlan Laboratories S.A., Barcelona, Spain
Laboratory of Functional Foods, Madrid Institute for Advanced Studies (IMDEA) – Food, Madrid, Spain

ARTICLE INFO

Article history:
Received 13 October 2012
Accepted 22 January 2013
Available online 1 February 2013

Keywords:
Hydroxytyrosol
Toxicity
NOAEL
Rats
Mediterranean diet
Functional foods

ABSTRACT

Of all the phenolic constituents of olives and extra virgin olive oil, hydroxytyrosol is currently being actively exploited as a potential supplement or preservative to be employed in the nutraceutical, cosmeceutical, and food industry. In terms of safety profile, hydroxytyrosol has only been investigated as the predominant part of raw olive mill waste water extracts, due to the previous unavailability of appropriate quantities of the pure compound. We report the toxicological evaluation of hydroxytyrosol and, based on the results, propose a No Observed Adverse Effects Level (NOAEL) of 500 mg/kg/d.

1. Introduction

In addition to its high proportion of oleic acid, extra virgin olive oil is rich in phenolic compounds, which other vegetable oils do not contain. Consequently, the purported healthful activities of extra virgin olive oil are being attributed to its phenolic components in addition to its fatty acid profile (Visioli, 2012). Indeed, it must be underlined that the field of olive phenols and health, as related to the cardiovascular system, is very advanced and more than 20 human trials describe the superiority of phenol-rich olive oil to other vegetable oils or sources of fat (Visioli and Bernardini, 2011). This notion has been reinforced by the recent European Food Safety Authority (EFSA) allegation (EFSA Panel on Dietetic Products, 2011).

Of all the phenolic constituents of olives and extra virgin olive oil, hydroxytyrosol is currently being actively exploited as a potential supplement or preservative to be employed in the nutraceutical, cosmeceutical, and food industry (Visioli and Bernardini, 2011). At the crossroad between pharma and nutrition (Visioli, in press), hydroxytyrosol has been proposed as cardioprotective (Visioli, 2012), neuroprotective (Schaffer et al., 2007), and chemopreventive (Visioli et al., 2004) agent. Of note, these activities are as yet to be fully demonstrated in humans.

In terms of safety profile, hydroxytyrosol has only been investigated as the predominant part of raw olive mill waste water (OMWW) extracts. No untoward effects have been demonstrated even at very high doses (D'Angelo et al., 2001; Christian et al., 2004; Soni et al., 2006) and two olive mill waste water nutraceuticals, i.e. Hidrox®, containing ~12% of polyphenols (~6% hydroxytyrosol) and Hytolive®, containing ~10–15% of hydroxytyrosol have been granted the Generally Recognized as Safe (GRAS) status. However, due to the previous unavailability of appropriate quantities, the toxicological profile of pure hydroxytyrosol has never been properly investigated.

We report the toxicological evaluation of hydroxytyrosol and, based on the results, propose a No Observed Adverse Effects Level (NOAEL).

2. Materials and methods

This study was performed in accordance with the procedures indicated by the following internationally accepted guidelines and recommendations: (1) Commission Directive 2003/63/EC of 25 June 2003 amending Directive 2001/83/EC of the European Parliament and of the Council on the Community code relating to medicinal products for human use. (2) Commission Directive 96/54/EEC of 30 July 1996. Annex IV A Part B. Method B.26. Sub-chronic oral toxicity test: 90-day repeated oral dose using rodent species. (3) Note for Guidance on Repeated Dose Toxicity. CPMP/SWP/1042/99. 27 July 2000. (4) ICH Topic S4A. Note for Guidance on Duration of Chronic Toxicity Testing in Animals (Rodent and Non Rodent Toxicity Testing; CPMP/ICH/300/95). (5) Guideline on the Evaluation of Control Samples in Non clinical Safety Studies: Checking for Contamination with the Test Substance. CPMP/SWP/1094/04 of 17 March 2005. (6) OECD Guideline for the Testing of Chemicals, Guideline 408 Repeated Dose 90-Day Oral Toxicity Study in Rodents, 21 September 1998.

* Corresponding author. Address: Laboratory of Functional Foods, IMDEA – Food, CEI UAM + CSIC, C/Faraday 7, 28049 Madrid, Spain. Tel.: +34 91 2796986; fax: +39 02700426106.
E-mail address: francesco.visioli@imdea.org (F. Visioli).

0278-6915/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fct.2013.01.030

Rats (Rat, Wistar Hannover RccHan™: WIST, from Harlan Laboratories, B.V., 50% males and 50% females; age at start of treatment 7–11 weeks; body weight range at start of treatment: males 222–285 g, females: 164–203 g) were acclimatized for 13 days between the arrival date and treatment start. During acclimatization, the animals were examined by a veterinary surgeon and only animals without any visible signs of illness were used for the treatment. Animals were provided with Pelleted standard Harlan Teklad 2014C rat/mouse maintenance diet and tap water *ad libitum*.

Hydroxytyrosol was administered orally (by gavage) to daily for a 13-week period at dose levels of 5, 50, and 500 mg/kg/day. The test item was prepared not earlier than seven days before dosing, by dissolution in distilled water (Aqua B. Braun, B. Braun Medical, Rubí, Barcelona, Spain). The prepared formulations were stored refrigerated and protected from light until the time of administration. Administration volumes were 10 mL/kg body weight. The animals from the Control group were treated similarly with the vehicle (distilled water).

Four groups (each comprising 10 animals per sex) that were sacrificed after 91–92 days of treatment were used for toxicity testing (allocation A). Five additional animals per sex in groups 1 and 4 were used for a four-week recovery period (allocation B) to assess the reversibility or progression of any test item-related changes. During weeks 1 and 8 of treatment, formulation samples from each group were analyzed to determine their hydroxytyrosol content.

Ophthalmoscopic examination results, clinical signs, food consumption and body weights were recorded periodically during acclimatization, treatment and recovery periods. Functional observational battery and measurements of locomotor activity and grip strength were performed during week 13 of treatment and at the end of recovery period. At the end of the dosing period and at the end of the recovery period, blood samples were drawn for hematology, clinical biochemistry and urinary analyses.

At the end of treatment, all allocation-A animals were sacrificed, necropsied and examined postmortem. Histological examinations were performed on organs and tissues of all Control and high-dose group animals and on any gross lesions occurring in allocation-A animals. All allocation-B animals were sacrificed, necropsied and examined post mortem after a 4-week treatment-free recovery period. The aim of the recovery period was to evaluate the reversibility of any alterations recorded during the treatment period.

2.1. Clinical signs

Cage-side clinical observations were carried out at least twice daily. During the administration period, the animals were observed before and after the treatment to detect signs of toxicity. Detailed clinical signs were recorded at least once daily. These observations were performed by removing the animal from the cage. The observations included, but were not be limited to, changes in the skin, fur, eyes and mucous membranes, in the respiratory, circulatory, central and autonomic nervous systems, in the somatomotor activity, behavior and reflexes.

2.2. Functional observational battery

During the last week of treatment, relevant parameters from a modified Irwin screen test were performed on all rats from Control and high-dose groups (Allocations A and B). These observations were based upon the procedures used for the detailed weekly observations. Any abnormal findings were recorded and graded in severity. The hind- and forelimb grip strength was performed using a push–pull strain gauge (Bioseb 100143). Locomotor activity was measured quantitatively with an ambulatory monitoring system (AMS) from Medical Instruments GmbH (Langgöns, Germany) (FMI) and DeMeTec GmbH (Langgöns, Germany). Activity of the animals was recorded in 10-min intervals over a 60-min period also performed in low and intermediate dose groups during treatment week 13. During the last recovery week, the functional parameters (grip strength and locomotor activity) of all Control and high-dose animals (Allocation B) were measured.

2.3. Body weight

The body weights of all animals were recorded during the week before beginning treatment, twice weekly during the treatment weeks 1–13 and weekly afterwards and before sacrifice. The rats undergoing the recovery period were weighed weekly during this period. The mean body weights per group and sex were calculated twice a week from the individual weights.

2.4. Food intake

Before beginning treatment and then once a week during the treatment period and during the recovery period, food intake per cage was recorded and the weekly mean intake per rat was calculated.

2.5. Ophthalmoscopy

Ophthalmoscopic examinations (Boretsky et al., 2011) included the cornea, crystalline, lens, conjunctivae, sclera, iris and fundus. During each examination, the pupils were dilated by instilling 1% cyclopentolate hydrochloride eye drops.

2.6. Clinical laboratory investigations

Blood samples were extracted from the retro-orbital plexus under light isoflurane anesthesia. The animals were fasted in metabolism cages for approximately 16–18 h before blood sampling but allowed access to water *ad libitum*. The samples were collected early in the working day to reduce biological variation caused by circadian rhythms. Urine was collected into a specimen vial during the 16–18 h fasting period.

Blood and urine were analyzed by routine laboratory methods. In particular, we assessed: red blood cells; hemoglobin; hematocrit; red blood cell distribution width; mean corpuscular volume; red cell distribution width; mean cell hemoglobin; mean corpuscular hemoglobin concentration; platelets; reticulocytes; reticulocytes with low fluorescence; reticulocytes with median fluorescence; reticulocytes with high fluorescence; white blood cells; neutrophils; lymphocytes; monocytes; eosinophils; basophils; large unstained cells; prothrombin time; activated partial thromboplastin time; glucose; urea; creatine; bilirubin test; cholesterol; triacylglycerols; aspartate aminotransferase; alanine aminotransferase; alkaline phosphatase; creatine kinase; gamma-glutamyl transferase; calcium; phosphates; sodium; potassium; chloride; protein; albumin; globulin; albumin globulin ratio; and total bile acids.

2.7. Pathology

2.7.1. Sacrifice and macroscopic examination

At the end of the treatment, all allocation-A animals were deprived of food, deeply anaesthetized with sodium pentobarbital administered intraperitoneally and then exsanguinated by excision of the axillary vessels and aorta. Allocation-B animals were sacrificed at the end of the recovery period. Full necropsies were performed on all animals, which included examination of the external surface of the body, all orifices, cranial, thoracic and abdominal cavities and the observation of the organs both in situ and after evisceration.

Samples of the following tissues and organs were collected from all animals at necropsy and fixed in neutral phosphate buffered 4% formaldehyde solution (10% formalin): adrenal glands; aorta; bone marrow (femur) (air-dried); brain – including section of medulla/pons, cerebral and cerebellar cortex; epididymis (Bouin's solution); esophagus; eyes with optic nerve (Davidson's fixative); femur (with articular surface); heart; intestine, large (cecum, colon, and rectum); intestine, small (duodenum, jejunum, andileum); kidneys; larynx; liver; lungs, with main bronchi and bronchioles; lymph nodes (mandibular and mesenteric); mammary gland area; nasal cavity; ovaries; pancreas; pituitary gland; prostate and seminal vesicles; salivary glands (mandibular, sublingual, and parotid); sciatic nerve; skeletal muscle (thigh); skin (abdominal); spinal cord (cervical, mid-thoracic, and lumbar); spleen; sternum (with bone marrow); stomach (glandular and non-glandular); testes (Bouin's solution); thymus; thyroid (including parathyroid gland, whenever possible); tongue; trachea; urinary bladder; uterus (cervix corpus and oviducts); and vagina.

All organ and tissue samples were weighted, embedded, and cut at an approximate thickness of 2–4 μm and stained with hematoxylin and eosin.

2.8. Statistical analyses

The following statistical methods were used to analyze body weight, grip strength, locomotor activity, food consumption, clinical laboratory data, organ weights and ratios, ophthalmoscopy, and macroscopic findings: (1) Dunnett's test (many-to-one t-test) based on a pooled variance estimate was applied if the variables could be assumed to follow a normal distribution for the comparison of the treated groups and the Control groups for each sex; (2) The Steel-test (many-one rank test) was applied instead of Dunnett's test when the data could not be assumed to follow a normal distribution; and (3) Fisher's exact-test was applied to the ophthalmoscopy and macroscopic findings.

3. Results

No mortality was recorded in any group. Because no dose-dependent effect was recorded (see below) we only report data on Allocation A animals.

3.1. Clinical signs

Salivation was recorded before and/or after the administration in all animals from group. This clinical sign was detected on day three until the end of treatment. Salivation after administration was observed sporadically in four males and two females from the 50 mg/kg group. Sporadic salivation after the administration was also observed in one 5 mg/kg group female. Other clinical signs such as scabs and desquamation in the sacral region, scabs at the base of the tail and hair loss on the head were observed in