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Genotoxicity testing of esterified propoxylated glycerol (EPG)

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ABSTRACT

Four versions of esterified propoxylated glycerols (EPGs) were evaluated for potential genotoxicity using a range of *in vitro* and *in vivo* assays. H-EPG-05 HR/SO 9:1, H-EPG-05 soyate, and H-EPG-14 soyate were non-mutagenic in reverse mutation assays (maximum concentration 1000 μg/plate) using *Salmonella typhimurium* and *Escherichia coli*. Heated and unheated H-EPG-05 HR/SO 9:1 and EPG-05 HR/ST 45:55 were likewise non-mutagenic in reverse mutation assays in *S. typhimurium* strains TA98 and TA100 (maximum concentration 5000 μg/plate). H-EPG-05 HR/SO 9:1, H-EPG-05 soyate, and H-EPG-14 soyate, were devoid of mutagenic activity in a mouse lymphoma assay in L5178Y tk +/− cells (maximum concentration 200 μg/plate for H-EPG-05 HR/SO 9:1; 100 μg/plate for H-EPG-05 soyate and H-EPG-14 soyate), and a chromosomal aberration test using human lymphocytes (maximum concentration 50 μg/plate for H-EPG-05 HR/SO 9:1 and H-EPG-05 soyate; 60 μg/plate for H-EPG-14 soyate). All assays were conducted with and without metabolic activation. Additionally, H-EPG-05 HR/SO 9:1, H-EPG-05 soyate, and H-EPG-14 soyate were non-genotoxic in unscheduled DNA synthesis tests in rats (maximum dose 2000 mg/kg). Based on the results of these assays it was concluded that these versions of EPG were not genotoxic under any of the conditions of the assays performed.

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1. Introduction

The potential genotoxicity of four versions of EPG[1] was evaluated in the following series of standardized studies: bacterial reverse mutation (Ames) assay; *in vitro* mouse lymphoma $TK^{+/-}$ gene mutation assay; *in vitro* mammalian chromosomal aberration test; and unscheduled DNA synthesis (UDS) test with mammalian liver cells *in vivo/ex vivo*. The bacterial reverse mutation assay alone (with *Salmonella typhimurium* strains TA98 and TA100) was used to screen heated versions of EPG that might be used for frying and baking.

2. Materials and methods

This series of studies was sponsored by ARCO Chemical Company, Newton Square, Pennsylvania, and conducted at Covance Laboratories, Inc. (formerly Corning Hazelton, Inc.) facilities in North Yorkshire, England, Madison, Wisconsin (USA), and Vienna, Virginia (USA) at various times between March, 1992 and January, 1993, except for studies of heated and unheated EPG, which were conducted at between December, 1994 and June, 1995. The studies were performed in compliance with the principles of Good Laboratory Practice (GLP) regulations of one or more of the following: United Kingdom; United States Food and Drug Administration (FDA); Organization for Economic Cooperation and Development (OECD); and Japanese Ministry of Agriculture, Forestry and Fisheries (MAFF).

2.1. EPG

The EPG versions tested were: H-EPG-05 HR/SO 9:1 (Lot # 753489, unless otherwise specified); H-EPG-05 soyate (Lot # 753568); H-EPG-14 soyate (Lot # 753569); and EPG-05 HR/ST 45:55. Each EPG version was provided by the sponsor.

2.2. Bacterial reverse mutation assay (S. typhimurium and Escherichia coli)

The bacterial mutagenicity (Ames) assay using standard plate incorporation and preincubation methods was used (Ames et al., 1975; Maron and Ames, 1983; Green and Muriel, 1976). *S.*

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[1] The nomenclature used to identify specific EPG version is based on the total number of propylene glycol units attached to the glycerol back bone, the source or identity of the fatty acids attached to the propylene glycol units, and the hydrogenation status of the final product. For example; H-EPG-05 HR/SO 9:1 is an EPG in which 05 represents the mean number of propylene glycol units per glycerol, HR/SO represents high-erucic acid rapeseed oil/soybean oil in a 9:1 ratio. The initial "H" indicates that the product is hydrogenated converting all fatty acids into their fully saturated counterpart. For example, erucic acid is converted to behenic acid.

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typhimurium strains (TA98, TA100, TA1535 and TA1537) were obtained from the National Collection of Type Cultures (London, UK). *E. coli* strains WP2 pKM101 and WP2 uvrA pKM101 were obtained from the Cancer Research Unit, University of York (York, UK) and Glaxo Group Research Ltd. (Greenford, UK), respectively.

The study consisted of three independent experiments, a toxicity range-finding test conducted in strain TA100 only, and two mutation assays. EPG (H-EPG-05 HR/SO 9:1; H-EPG-05 soyate; H-EPG-14 soyate; EPG-05 HR/ST 45:55) was tested in triplicate in the presence and absence of the postmitochondrial fraction of the liver homogenates (S9) from Sprague–Dawley rats pre-treated with Aroclor 1254 (Batch 0367, Molecular Toxicology, Inc., Annapolis, MD). The results of the range-finding test (data not shown) showed no toxic effect at concentrations of 8–5000 μg/plate, but precipitation of the test material was evident at 1000 and 5000 μg/plate. EPG was therefore tested at concentrations of 1.6, 8, 40, 200 and 1000 μg/plate in Experiment 1. Since these treatments were nontoxic, treatments in the presence of S9 in Experiment 2 included a preincubation step. Additionally, a narrower range of concentrations (62.5, 125, 250, 500 and 1000 μg/plate) was used to examine those most likely to elicit a mutagenic response. Negative or solvent (acetone) and positive controls were included in quintuplicate and triplicate, respectively, with and without S9 mix. Positive control chemicals consisted of: 2-nitrofluorene (2NF, Koch-Light, Haverhill, UK); sodium azide (NaN_3, Sigma Chemical Co., Poole, UK); 9-aminoacridine (AAC, Koch-Light, Haverhill, UK); 4-nitroquinoline 1-oxide (NQO, Fluka Chemicals Ltd., Glossop, UK); and 2-aminoanthracene (AAN, Sigma Chemical Co., Poole, UK).

Assay responses were considered positive if: (1) the assay was valid (the assay was considered valid if: (i) the mean negative control counts fell within the range of the laboratory's historical control data; (ii) positive control chemicals induced clear increases in revertant numbers in appropriate strains, and an active S9 preparation where appropriate; and (iii) no more than 5% of the plates were lost due to contamination or some other unforeseen event); (2) a significant response from Dunnett's test ($p \leqslant 0.01$) was observed and the data set showed a significant dose correlation ($p \leqslant 0.05$); (3) the positive responses described in (2) were reproducible.

2.3. Bacterial reverse mutation assay (S. typhimurium)

A modified (preincubation) Ames assay (Yahagi et al., 1975; Maron and Ames, 1983) was used to assess the mutagenic potential of: (1) H-EPG-05 HR/SO 9:1, heated (Lot # 850364) and unheated (Lot # 850324) *vs*. palm/rapeseed oil (60:40), heated (Lot # 850363) and unheated (Lot # 850362); and (2) EPG-05 HR/ST 45:55, heated (Lot # 900407) and unheated (Lot # 850308) *vs*. cottonseed oil, heated (Lot # 900406) and unheated (Lot # 900405).

S. typhimurium histidine strains TA98 and TA100 were incubated with the test materials at concentrations of 100, 250, 500, 1000, 2500, and 5000 μg/plate, in the presence and absence of S9 mix (Batch 0544, Molecular Toxicology, Inc., Annapolis, MD). Tester strains were received directly from Dr. Bruce Ames (Department of Biochemistry, University of California, Berkeley).

Vehicle (acetone) and positive controls were tested concurrently in the presence and absence of S9. Positive control chemicals consisted of: AAN (Sigma Chemical Co., Poole, UK); 2NF, Aldrich Chemical Co., Gillingham, UK); and NaN_3 (Sigma Chemical Co., Poole, UK).

EPG (all concentrations), vehicle control, and positive controls were plated in triplicate. Specifically, 0.5 mL of S9 mix (metabolic activation) or 0.5 mL of 0.1 M phosphate buffer (no metabolic activation) was added to a test tube. Bacterial tester strains (100 μL) and the appropriate volume of vehicle or test article was then added and incubated for 20 min at 37 °C. After incubation, 2.0 mL of top agar was added to the mixture and poured onto a minimal bottom agar plate. Positive control articles were plated using a 50 μL plating volume. The plates were incubated for 48 h at 37 °C, then evaluated for the number of revertant colonies.

The study consisted of two independent tests, the initial mutagenicity assay and a confirmatory assay (Experiments 1 and 2, respectively). A response was considered positive if the test material produced a concentration-related increase of at least twofold in the mean number of revertants per plate in at least one of the tester strains over the appropriate vehicle control value.

2.4. In vitro mouse lymphoma $TK^{+/-}$ gene mutation assay

The ability of EPG (H-EPG-05 HR/SO 9:1; H-EPG-05 soyate; H-EPG-14 soyate) to induce mutation at the thymidine kinase (tk) locus in L5178Y tk +/− mouse lymphoma cells (American Type Culture Collection) was examined, in accordance with Organization for Economic Co-operation and Development (OECD) guideline 476, using the procedures described by Cole et al. (1983, 1990). The study consisted of three independent tests: a cytotoxicity range-finding test and two mutation assays. Each assay was conducted in the absence and presence of S9 (Batch 0544, Molecular Toxicology, Inc., Annapolis, MD).

Immediately prior to the assay, EPG was dissolved in analytical-grade acetone (with warming at 37 °C) to give the required concentration. Further dilutions were made using analytical-grade acetone (Batch number MC 1370 in range-finding and Experiment 1; K 17184406 in Experiment 2). Test chemical solutions were protected from light and used within approximately 1.5 h of initial formulation. Positive controls, NQO (Fluka Chemicals Ltd., Glossop, UK) and benzo (a) pyrene (Aldrich Chemical Co., Gillingham, UK), were used for non-activation and activation assays, respectively. Negative controls were comprised of treatments with the solvent acetone diluted 100-fold in the treatment medium. The basic medium was RPMI 1640 supplemented with gentamycin (100 μg/mL), fungizone (2.5 μg/mL), and pluronic (0.5 mg/mL) (RPMI A). The growth medium (RPMI 10) was RPMI A supplemented with 10% (v/v) heat-inactivated horse serum. The cloning medium (RPMI 20) was RPMI 1640 supplemented with gentamycin (100 μg/mL), fungizone (2.5 μg/mL), and 20% (v/v) heat-inactivated horse serum. Cofactor solutions, with and without S9, were also added to the cultures (1–19 mL of cell culture).

The mouse lymphoma cells were suspended in 50-mL centrifuge tubes containing at least 10^7 cells in 18.8 mL of RPMI 5 (cells in RPMI 10 diluted with RPMI A to give a final concentration of 5% serum). In the range-finding assay (data not shown), cells were exposed to EPG at concentrations ranging from 3.125 to 100 μg/mL. Single cultures were prepared and positive controls were not included. Solvent or test material (0.2 mL), and S9 mix or 150 mM KCl were added to the cultures. After 3 h of shaking at 37 °C, tubes were centrifuged (200×*g* for 5 min), cells were washed with phosphate buffered saline and resuspended in 20 mL RPMI/tube. Cell concentrations were adjusted to 8 cells/mL and, for each dose, 0.2 mL was plated into 32 mL wells. The plates were incubated at 37 °C in a humidified incubator gassed with 5% (v/v) CO_2 in air for 12 days. Wells containing viable clones were identified and counted.

For Experiments 1 and 2, the assays generally followed the range-finding assay procedures. Duplicate cell cultures (single for positive control) were exposed to H-EPG-05 HR/SO 9:1 at concentrations ranging from 12.5 to 200 μg/mL; H-EPG-05 soyate and H-EPG-14 soyate were tested at 25–100 μg/mL. Following treatment, cell densities were determined using a Coulter counter and the concentrations adjusted to 2×10^5/mL. An aliquot of each cell

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