Food and Chemical Toxicology 186 (2024) 114518

Contents lists available at ScienceDirect

Food and Chemical Toxicology

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

Toxicological evaluation of a fish oil concentrate containing Very Long Chain Fatty Acids

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ARTICLE INFO

Handling Editor: Dr. Bryan Delaney

Keywords:
Toxicity
Omega-3
Very-long-chain-fatty-acids
VLCFA
PUFA
Fish oil
EPAX EVOLVE

ABSTRACT

Very long chain fatty acids (VLCFA) have a chain length ≥24 carbons. Fish contain low levels of these fatty acids. A commercial oil called EPAX® Evolve 05 with an up-concentration of VLCFAs of approximately 10 times, has been developed as a dietary supplement by Epax Norway AS. A series of toxicological studies were performed using mice and rats to determine the safety and toxicity of repeat dosing with a gavage administered VLCFA formulation. The results suggest transient lipid accumulation in kidneys and liver. Lipid accumulation was seen with the test item and with the soya control but was not dose related. Liver and kidney lipid accumulation, whilst present in 14- day repeat dose study, was absent in a 90-day rat study. No treatment-effect was seen in urine analysis in any of the studies. No treatment-related effects were seen with a functional observation battery, ophthalmological examination, haematology, urine analysis, oestrus cycle, thyroid hormones, organ weight, or histopathology. In the 90-day study the liver enzymes ALP, AST and ALT were statistically significantly increased with test item but within control values. There were no associated histological findings in the liver suggesting there was no toxic effect and the normalisation of values for all liver enzymes in the recovery groups suggests an adaptive response rather than a prevailing toxic response. The no-observed-adverse-effect level (NOAEL) was determined as 1200 mg VLCFA/kg b.w./day.

1. Introduction

Traditionally, fish and marine diets have been considered part of a healthy lifestyle. The intake of sufficient amounts of omega-3 fatty acids became a general health consideration after the ground-breaking work by Bang and Dyerberg in the late 1970s associating marine lipids with cardiovascular health (Bang et al., 1971; Dyerberg et al., 1978). Since then, dietary levels of omega-3 fatty acids have been associated with a series of health benefits including cardiovascular, brain and eye health, child brain development, depression, systemic and local inflammation and lipid metabolism (Calder, 2017; Swanson et al., 2012). The production of highly concentrated omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), has become a world-wide commercial practice and manufacturing methods and quality assurance are well regulated.

Recently, the role of fatty acids with longer chain lengths, the Very-Long-Chain Fatty Acids (VLCFA) with C ≥ 24 has been investigated. These fatty acids are derived in vivo from the action of the enzyme family Elongation of Very Long Chain Fatty Acids 1–7 (ELOVL 1–7) (Agbaga et al., 2008; Gyening et al., 2023). Knock-out studies show that ELOVL4 and VLCFAs are important in visual acuity (Harkewicz et al., 2012), and skin function, with knock-out animals dying shortly after birth due to dehydration (Vasireddy et al., 2007). These findings are mirrored in humans harbouring a homozygous ELOVL4 mutation with individuals presenting with ichthyosis, severe neurological problems, small testes, and hypertonia (Aldahmesh et al., 2011; Diociaiuti et al., 2021; Xiao et al., 2019). Furthermore, a human autosomal dominant heterozygous mutation of ELOVL4 has been identified as the primary cause of Stargardt 3 macular dystrophy, a progressive, juvenile form of blindness (Agbaga et al., 2008). Oral supplementation with synthetic VLCFA can rescue visual acuity in an animal model of Stargardt 3 (Gorusupudi et al., 2021) showing that VLCFAs given orally can be utilised by a target tissue and likely undergo processing to the particular

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https://doi.org/10.1016/j.fct.2024.114518
Received 16 May 2023; Received in revised form 13 February 2024; Accepted 14 February 2024
Available online 20 February 2024

Abbreviations

A/G	Albumin/globulin
ADI	Acceptable dietary intake
ALP	Alkaline phosphatase
ALT	Alanine transaminase
APTT	Activated partial thromboplastin time
AST	Aspartate aminotransferase
b.w.	body weight
DHA	Docosahexaenoic acid
EDI	Estimated dietary intake
ELOVL	Elongation of Very Long Chain Fatty Acids
EPA	Eicosapentaenoic acid
HDL	High-density lipoprotein
LDL	Low-density lipoprotein
MTD	Maximum tolerated dose
NOAEL	No observed adverse effect level
PT	Prothrombin time
RDW	Red cell distribution width
TG	Triglyceride
VLCFA	Very long chain fatty acids
VLCPUFA	Very long chain poly-unsaturated fatty acids

active form required by the target tissue (for example a phospholipid form in the retina). Oils containing VLCFAs may be suitable as supplements for maintaining the structure and function of relevant tissues and organs.

Using standard commercial manufacturing processes, a fish oil with concentrated VLCFA has been developed (EPAX® Evolve 05). This oil has VLCFAs at a typical level of 150 mg/g with maximum levels up to 200 mg/g. Although VLCFAs exist naturally in fish and fish products, they have traditionally received little attention due to their low concentration and difficulty in detection. More recently, there has been increasing attention as studies show VLCFAs are involved in essential biological functions, as described above. Despite this interest, the lack of a product containing defined amounts of VLCFA has hindered interventional studies.

EPAX Evolve 05 is a concentrated VLCFA oil derived from natural fish oil and is therefore a composite of different fatty acids. The predominant fatty acid is DHA (approximately 50 % by weight of lipid), with VLC mono-unsaturated fatty acids (approximately 10–12% by weight of oil) and VLC polyunsaturated fatty acids (approximately 5–8%). Compositional analysis is provided in Supplementary Table S1. The composition of the soya oil control used in the 14- and 90-day studies is provided in Supplementary Table S2.

Here we report the safety and toxicological profile of EPAX Evolve 05 as investigated in a series of repeat-dose studies in mice and rats to define a Maximum Tolerated Dose/No Observed Adverse Effect Level.

2. Materials and methods

2.1. Test item

The test item (EPAX Evolve 05) is a marine fatty acid concentrate containing very long chain fatty acids, defined as fatty acids with a carbon chain ≥24 carbons. The manufacture of the oil follows a quality standard required for commercial production of a fish oil concentrate for human consumption. Fatty acids in natural fish oils are mostly present as triglycerides. In order to concentrate the oils, they are converted into ethyl esters, distilled and re-converted to triglycerides. This is a common industrial process for high concentrate omega-3 fatty acids used as supplements.

EPAX Evolve 05 was tested for elemental impurities and toxins according to industry standards (See Supplemental Table S3 for contaminant specifications).

Different oils have been tested in the described studies, as presented in Table 1. A 28-day repeat dose study in mice was the initial study and used a test item with VLCFA content higher than that of EPAX Evolve 05. This study was designed to give an initial indication of potential toxicity.

Test items for the 14- and 90-day study were tested for homogeneity and shown to have near identical concentrations throughout the oil. Testing of the gavage oil, up to two years after preparation showed stability (within 5% of expected values of VLCPUFA).

The rat 14-day and 90-day study doses, 40 mg VLCFA/kg b.w., 100 mg VLCFA/kg b.w., 300 mg VLCFA/kg b.w., and 600 mg VLCFA/kg b.w. VLCFA, used EPAX Evolve 05 oil as the test item. In order to gavage animals with higher doses (900 mg/kg b.w. and 1200 mg/kg b.w. VLCFA/day) without exceeding the limit of 4 ml/kg b.w., per day, a high concentrate VLCFA oil was made. The high concentrate oil had a VLCFA content approximately 2 times greater than EPAX Evolve 05. The VLCPUFA: VLCMUFA ratio in EVOVLE 05 was approximately 1.9 whereas the high concentrate was 1.3 meaning there was a greater amount of VLCPUFA content in the overall VLCFA. There is a possibility that toxic effects specific to VLCMUFA or VLCPUFA may be reduced when the ratio is changed. Most reports of LC-MUFA and LC-PUFA suggest health benefits rather than toxicity, however there is a possibility that LC-PUFA can result in toxicity from oxidation of the oils (Turpeinen et al., 1998) in this respect the concentrate VLCFA in this study with a high ratio of VLCPUFA will reveal PUFA toxicity. Unless otherwise stated, all doses presented in this report represent total VLC fatty acid content.

Where appropriate, oils were diluted with soya oil to provide the desired VLCFA concentration. These diluted oils are fully homogenous without any separation and were prepared under nitrogen. For the 14- and 90-day studies, single use bottles were prepared for each dosing group such that no bottle was opened more than once, and oil was not exposed to air. The bottles were stored in dry conditions below 25 °C. All oils were analysed at production for oxidation values which must meet strict criteria allowing for 2 years stability. The oils used for doses 600 and 1200 mg VLCFA/kg b.w did not undergo dilution.

The EPAX Evolve 05 dose corresponding to VCLFA dose used in the 14-day study and 90-day study are described in Table 2.

Test item composition for EPAX Evolve 05 used in the 14- and 90-day studies are provided in Supplementary Table S1. Composition of the soya oil control (also used as vehicle) is provided in Supplementary Table S2.

Gavage doses were evaluated for stability by GC-FID analysis of VLCFA content. The tested samples (ready for gavage use) were stable up to approximately 2 years (maximum time tested). Homogeneity was ensured by inversion of tubes. The fatty acids are fully soluble and there is no separation of fatty acids. To ensure this, a representative sample was tested with oil extracted from the bottom, middle and top of the bottle. Samples gave the same result showing no separation of oils.

2.2. Oral toxicity studies

2.2.1. 28-day repeat dose study in mice

An initial non-GLP, pilot study was performed by the Research Institute of Sweden (RISE). The study was performed with 8 C57/bl6 male mice per group, with a soya oil control (Group1), non-VLCFA -containing commercial fish oil (EPAX 3000 containing 160 mg/g EPA and 100 mg/g DHA; Group2), non-VLCFA-containing commercial fish oil control (EPAX 0460 containing 40 mg/g EPA and 600 mg/g DHA; Group3); Low dose VLCFA (81 mg/kg; Group 4), high concentrate VLCFA (162 mg/kg; Group5). Animals were housed 8 per cage. In this preliminary study, a recovery group was only performed for the highest dose group.

Test items were prepared at the GMP laboratory of the fish oil manufacturer. Test items were administered by oral gavage. Soya oil

Table 1
Content of VLCFA, EPA and DHA in test oils prior to dilution and final VLCFA dose in the studies.

	Fatty acid content of tested oil prior to dilution				Study dose	Study
Oil	Total VLCFA content (mg/g)	EPA (mg/g)	DHA (mg/g)	DPA (mg/g)	Total VLCFA (mg/kg b.w.)	
VLCFA concentrate	244	3	500	101	81 162	28-day repeat dose study in mice
EPAX Evolve 05	158	28	429	96	40 100 300 600	14-day repeat dose study in rats
EPAX Evolve 05	158	28	429	96	300 600	90-day repeat dose study in rats for doses 300 and 600 mg/kg/day
VLCFA high concentrate	312	2	321	77	900 1200	90-day repeat dose study in rats for doses 900 and 1200 mg/kg/day

Dose selection of the studies follows a dose escalation with the highest dose of 1200 mg/kg b.w.

Table 2
EPAX EVOLVE 05 dosing equivalent for test item doses.

VLCFA doses in studies	Equivalent EPAX EVOLVE dose
40 mg/kg b.w. VLCFA	0.2 g EPAX EVOLVE 05/kg b.w.
100 mg/kg b.w. VLCFA	0.6 g EPAX EVOLVE 05/kg b.w.
300 mg/kg b.w. VLCFA	1.9 g EPAX EVOLVE 05/kg b.w.
600 mg/kg b.w. VLCFA	3.8 g EPAX EVOLVE 05/kg b.w.
900 mg/kg b.w. VLCFA	5.7 g EPAX EVOLVE 05/kg b.w.
1200 mg/kg b.w. VLCFA	7.6 g EPAX EVOLVE 05/kg b.w.

was used as a vehicle oil. Male mice were chosen as there was no indication of gender bias in fish oil metabolism or safety (Blum et al., 2007; Kagan et al., 2014; Lewis et al., 2016) and future studies were planned for both genders. Soya oil was chosen as the vehicle control. Typical soya oil composition is presented in Supplementary Table S2 and contained no EPA or DHA.

Animals were assessed for clinical observations including observation of the eyes, body weight at study initiation, weekly and on termination, food and water consumption during the first and last week of dosing and during recovery. Clinical pathology was performed on all mice on the day of termination by heart puncture under isoflurane anaesthesia. The clinical chemistry parameters measured were: ALT, AST, cholesterol, triglycerides, bilirubin and creatinine. Haematology parameters measured were: erythrocytes, haemoglobin, haematocrit, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, mean red cell volume, red cell distribution width, reticulocytes, platelets, leucocytes, neutrophils, lymphocytes, monocytes, basophils, eosinophils, and large unstained cells. Histopathology of organs was performed on heart, liver, kidneys, spleen, thymus and brain. Group 1–5 animals were sacrificed on day 29. The study protocol was approved by the animal ethics committee of the Gothenburg region and the study adhered to EU regulations for animal research.

2.2.2. Maximum tolerated dose oral toxicity study (14-day repeat dose study) in rats

The objective of this study was to determine the maximum tolerated dose (MTD), and to assist in determining the No-observed-adverse-effect-level (NOAEL) of EPAX Evolve 05, following daily oral gavage administration to Sprague Dawley rats for 14 days. This study was conducted at a GLP laboratory by Vipragen Biosciences, India, selected and quality controlled by Vedic Life Sciences, India, with a protocol based on the OECD guideline for testing of chemicals, Section 4: Health Effects: No. 407, Repeated Dose 28-day Oral Toxicity Study in Rodents; Adopted: 3rd October 2008 with the following exceptions: the duration of the treatment period (14-day treatment period instead of 28 days), histopathology was performed on kidneys and liver only and total cholesterol was omitted. The study protocol was approved by the animal ethics committee, India, who determined the reduction in animal numbers based on the study endpoint of mortality. The study site was accredited by AAALAC, USA.

Oil for gavage feeding was prepared at the GMP laboratory of Epax Norway AS as single dose preparations for each day for each dosing group to prevent oxidation of the oil (i.e., one flask was used per day per dose). The VLCMUFA and VLCPUFA composition of the gavage oil is presented in Supplementary Table S31. Oil in each flask was bottled under a nitrogen blanket. Each oil was analysed for composition and oxidation parameters using Epax standard tests for commercial oils and a certificate of analysis issued. The oils were then diluted with soya oil to the relevant concentration. Homogeneity was ensured by instruction at the animal testing site to invert the tubes 3–5 times prior to use. Dosing was performed by daily gavage feeding with a water control, soya oil control, or an EPAX Evolve 05-based oil with an administered VLCFA dose of 40 mg/kg b.w., 100 mg/kg, 300 mg/kg and 600 mg/kg, this dose range represents approximately 1–15 times the equivalent human dose considered for supplemental use. The VLCFA supplemental dose in humans is 6,7 mg/kg b.w. and the conversion factor for a human equivalent dose was 6 (i.e., rat dose/6 = human equivalent dose; (Nair and Jacob, 2016)). A constant feed volume was maintained at 4 mL/kg b.w. per day for all doses by dilution in suitable quantities of soya oil. Each dosing group consisted of 3 male and 3 female Sprague Dawley rats (Charles River Labs, USA). Animals were housed according to sex and dosing group, with 3 animals per cage. All animals were acclimatised for 6 days prior to study start and were approximately 7 weeks old at study start. Animals were fed ad libitum with 5L79 (Charles River) feed with a crude fat content of >5%. All animals were assessed for viability (mortality/morbidity) twice daily, before and after gavage feeding. In addition, clinical examinations were performed during acclimatization. Clinical examination was performed with daily cage side examinations during the dosing period, weekly detailed examinations and twice daily recording of morbidity and mortality. Before treatment of the animals, ophthalmological examination was carried out by using a 3.5v Coaxial Ophthalmoscope (Welch Allyn., USA). The eyes of all the animals were observed at the termination of treatment by a veterinarian with an ophthalmoscope. Body weight was recorded for all the surviving animals individually on the day of acclimatization, grouping, and randomization, before dosing (day 1), day 8, and day 14. In addition, body weight was recorded on the day of fasting and the day just before euthanasia. Feed consumption was measured at least once weekly during the dosing periods, except when fasted for clinical pathology sampling.

Feed consumption of individual animals was calculated on a weekly basis using the weights of feed offered and feed left. At the end of the experimental period, following overnight fasting, blood samples were collected from all animals from the retro-orbital plexus while under isoflurane anaesthesia. Samples were collected for haematology and clinical chemistry analysis.

Blood samples were collected in tubes containing 10% tri-potassium ethylene di-amine tetra-acetic acid (K3-EDTA) and sodium citrate, for

haematology and coagulation parameters respectively. In the case of clinical chemistry, blood samples were collected in a plain tube, and serum was separated after centrifugation. The haematological evaluation was carried out for all the animal groups at the end of the treatment using a Horiba Medical: ABX SCIL Vet ABC haematology analyzer, USA. The following parameters were analysed: total leukocyte count, erythrocyte count, haemoglobin, hematocrit, platelet count, mean corp uscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, differential leukocyte count, and reticulocyte count.

Blood samples collected at the end of the experimental period for all the groups were subjected to coagulation analysis for Prothrombin Time (PT), Activated Partial Thromboplastin Time (APTT) and fibrinogen analysis.

The following serum clinical chemistry parameters were determined for all treated groups using Randox Daytona Plus, London; total protein, albumin, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, globulin, glucose, creatinine, serum urea, blood urea nitrogen, sodium, potassium, phosphorus, chloride, and A/G ratio.

In the last week of treatment, all rats were housed in metabolic cages overnight (reverse osmosis water available ad libitum without feed) and urine samples were collected early in the morning before blood collection at the end of the experiment period for all the surviving group animals. Urine samples from animals were collected in metabolic cages. Prior to necropsy, animals were transferred from conventional cages to metabolic cages and kept overnight for urine collection.

Volume and appearance were assessed as indications of kidney function, further tests were not performed since there was no suspicion that kidney function was affected.

At the end of the study animals were euthanized by CO_2 asphyxiation followed by exsanguination. All the animals were subjected to a detailed pathological examination at the end of the study period. All animals in the study were subjected to a full, detailed gross necropsy which included careful examination of the external surface of the body, all orifices, the site of administration, the cranial, thoracic, abdominal cavities, and their contents. The following organs were weighed and then preserved in 10 % neutral buffered formalin for fixation; liver, kidneys, adrenals, spleen, heart, thymus, brain, testes/ovaries, epididymites/uterus, prostate, thyroid, and pituitary gland.

The tissues were processed for routine paraffin embedding and 4- 5-μm sections were made and stained with Mayer's Hematoxylin Eosin stain. Oil Red O staining was performed on the liver and kidney sections of all the animals as a standard-practice stain for lipid analysis, due to H&E findings from the 28-day study.

2.2.3. 90-day oral toxicity study in rats

The study was performed in accordance with the OECD Guideline for the Testing of Chemicals; Health effects; Test Guideline No. 408, "Repeated dose 90-day oral toxicity study in rodents" Adopted on 25th June 2018.

This study was performed at VBNCRS (Vivo Bio Non Clinical Research Services, C/o. Vivo Bio Tech Ltd., India). The study was approved by CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) with registration number 1117/PO/RcBiBt/S/07/CPCSEA and AAALAC International. The study was approved by IAEC (Institutional Animal Ethics Committee) of the test facility under the project titled "90 Day Repeated Oral Toxicity study of Fish Oil containing Very Long Chain Fatty Acids (VLCFA) in Sprague Dawley Rats with 28 Day Recovery period", IAEC protocol No. VB/IAEC/03/2022/965/Rat/SD approved on 25/03/2022. All the procedures were conducted in compliance with the guidelines of CPCSEA, India. The study site was selected and quality assured by Vedic Lifesciences, India.

Oil for gavage feeding was prepared at the GMP laboratory of Epax Norway AS. The VLCMUFA and VLCPUFA composition of the gavage oil is presented in Supplementary Table S32. Each dosing group consisted of 10 male and 10 female Sprague Dawley rats aged 6–7 weeks at the time of study start, except for the recovery groups which comprised 5 male and 5 female rats per group. Animals were held in groups with a maximum of 3 per cage and had access to food and water ad lib. Animals were acclimatised for at least 5 days prior to study start. Animals were weighed on the day of receipt, before randomization, prior to initiation of dosing on Day 1 and weekly thereafter. Body weight was measured weekly. Feed consumption was calculated on a weekly basis by weighing food per cage. The feed used throughout the study was RM6 from Special Dietary Services, UK. This feed was chosen due to its low-fat content of approximately 2.4%. Animals were randomly assigned to one of 8 groups. All groups underwent gavage feeding daily with 4 mL/kg b.w. daily of test item composed of EPAX Evolve 05 in soya oil.

Animals were randomised to the following groupings: Group 1 received water, Group 2 soya oil control (4 mL/kg b.w.), Group 3 received 300 mg/kg b.w. daily of VLCFA, Group 4 received 600 mg/kg b.w. daily of VLCFA, Group 5 received 900 mg/kg b.w. daily of VLCFA, Group 6 received 1200 mg/kg b.w. daily of VLCFA, Group 7 soya oil control with an additional 28-day recovery period, Group 8 received 1200 mg/kg b.w. daily of VLCFA with an additional 28 days recovery period. The maximum rat dose of 1200 mg VLCFA/kg rat b.w. represents a human equivalent dose (HED) of 12 mg VLCFA/kg human b.w. when taking into account an FDA safety factor of 100 (FDA, 2016). With a human body weight of 60 kg, the HED is equal to 720 mg VLCFA/person. Since EPAX Evolve 05 contains a maximum of 200 mg VLCFA/g oil, the maximum daily human dose corresponds to 3,6 g of EPAX Evolve 05.

Oil for gavage feeding was prepared by Epax Norway AS as single dose preparations for each day for each dosing group to prevent oxidation of the oil (i.e., one flask was used per day per dose). Oil in each flask was bottled under a nitrogen blanket. Each oil was analysed for composition and oxidation parameters using Epax standard tests for commercial oils and a certificate of analysis issued. The oils were then diluted with soya oil to the relevant dose. Homogeneity was ensured by instruction at the animal testing site to invert the tubes 3–5 times prior to use.

A functional observation battery was performed during the last week of treatment in all surviving animals belonging to main groups and the last week of recovery for recovery groups. The functional observation battery included home cage observations, handling observations, open field observations, measurement of sensory reactivity, grip strength and foot splay.

All animals were observed for cage side clinical signs at least once daily during the study. A detailed clinical signs examination was performed outside of the cage for all animals on the day of randomization, and once weekly thereafter.

Ophthalmological examination, using a Welch Allyn direct ophthalmoscope was performed before the administration of the test item, i.e., after completion of randomization in all animals and the last week of treatment in all surviving animals belonging to the very high dose (G6) and control (G1), (G2) group of main groups. If signs were observed in high dose group animals, the ophthalmologic examination was extended to lower dose and recovery groups. Blood samples for clinical pathology were taken on day 91 from the main study groups and day 119 from the recovery groups. Blood samples were withdrawn through retro-orbital plexus under isoflurane anaesthesia for haematology and clinical chemistry analyses.

The haematology parameters analysed are presented in Table 3, Table 4, and Table 5.

Clinical chemistry values were assessed with a Siemens Dimension RxL 200 Clinical chemistry analyzer/Siemens Dimension RxL Max Clinical chemistry analyser and Easy Lyte plus Na+/K+/Cl-analyzer. In addition, the same parameters as described for the 14-day study were assessed with the following additional tests: calcium, total cholesterol, total bilirubin, triglyceride, LDL-cholesterol, and HDL-cholesterol. Blood samples (approximately 1.0 mL) were collected from all surviving animals (on day 91 for main groups and day 119 for recovery

Table 3
Haematology parameters measured in the 90-day repeat dose study.

Parameters	Units
White Blood Cells	($x10^3$ cells/μL)
Red Blood Cells	($x10^6$ cells/μL)
Haemoglobin concentration	g/dL
Haematocrit	%
Mean Corpuscular Volume	fL
Mean Corpuscular Haemoglobin	pg
Mean Corpuscular Haemoglobin Concentration	g/dL
Red Cell Distribution Width	%
Platelet Count	($x10^3$ cells/μL)
Mean Platelet Volume	fL
Prothrombin Time	Seconds
Activated Partial Thromboplastin Time	Seconds

Table 4
Differential leukocyte count measured in the 90-day repeat dose study.

Parameters	Differential Leukocyte Count	
	Relative	Absolute
	Units	Units
Neutrophils	%	$x10^3$ cells/μL
Lymphocytes	%	$x10^3$ cells/μL
Monocytes	%	$x10^3$ cells/μL
Eosinophils	%	$x10^3$ cells/μL
Basophils	%	$x10^3$ cells/μL

Table 5
Reticulocyte count measured in the 90-day repeat dose study.

Parameter	Reticulocyte Count	
	Relative	Absolute
	Units	Units
Reticulocyte count	%	$x10^9$ cells/L

groups) in pre-labelled clot activator vials for T3, T4 and TSH estimation. T3, T4 and TSH were determined using an ELISA method.

Urine analysis was performed for all animals on the day before sacrifice using Siemens Cliniteck status Urine analyser with the Multistix® 10 SG urine analysis strips. All animals were kept in metabolic cages for urine collection overnight. Measurements were made for: urine volume, colour, clarity, pH, specific gravity, blood, bilirubin, urobilinogen, ketones, protein, nitrite, glucose, and leucocytes.

The oestrus cycle of all surviving females was determined by taking vaginal smears on the day of necropsy. Main group animals were necropsied within 15 min to 4 h of vaginal smear collection. Recovery groups animals were necropsied within 35 min to 1.5 h of vaginal smear collection.

All animals of the main groups on day 91 and recovery groups on day 119 were humanely euthanized using carbon dioxide asphyxiation. The organs and tissues collected are listed in supplemental information in Table S11. All the organs (except eyes, testes and epididymides) were preserved in a 10% neutral buffered formalin solution. Eyes, testes and epididymides were collected and preserved in modified Davidson's fluid.

Liver, kidneys, adrenals, testes, epididymides, uterus, ovaries, thymus, spleen, lung, brain, pituitary, prostate and seminal vesicles with coagulation glands and heart were weighed as soon as possible after collection.

Complete histopathology on all preserved organs mentioned in Table S8 was carried out on surviving animals of controls and highest dose group, including any gross lesions seen in any dosing group. Fixed tissues were processed routinely for Hematoxylin and Eosin (H&E) staining. If treatment-related effects were seen, then additional analysis of the involved tissues from animals in the lower dose levels and recovery groups were examined. Additionally, all the tissues (as listed under organ collection) from animals found dead (if not autolyzed) or moribund were subjected to microscopic examination.

Assessment of lipid accumulation was performed using Oil Red O staining of the liver and kidney tissues from both control groups and the highest dose group as well as the recovery groups. Any findings led to the analysis of additional groups.

2.3. Statistical analyses

2.3.1. 28-day repeat dose study in mice

Histopathology results were presented using descriptive statistics. For haematology and clinical chemistry results, mean and standard deviation were presented and a *t*-test performed to compare the difference between the control and the treated groups.

2.3.2. Maximum tolerated dose oral toxicity study

Statistical analyses were performed using the Stat Plus program v6. All the data were checked for normality with normality test (Kolmogorov-Smirnov (Lilliefors), Shapiro-Wilk W, D'Agostino-Pearson Skewness, Kurtosis and Omnibus K2 tests). The data for each group of animals were subjected to analysis of variance (ANOVA). Values were given as mean ± standard deviation (SD). A *t*-test was done to compare the difference between the control and the treated groups. The statistical significance of differences was calculated with a one-way analysis of variance. All analyses and comparisons were evaluated at the 5% ($p \leq 0.05$) level.

2.3.3. 90-day repeat dose toxicity study

All continuous data such as body weight, body weight gain, feed consumption, haematology, clinical chemistry, urine analysis, thyroid estimation, functional observation battery, absolute organ weights and relative organ weights etc. were subjected to various statistical analyses using the software Systat Version No. 13". Data were evaluated for basic statistics, normality check by Shapiro Wilk's Test, homogeneity of variance by 'Bartlett's Test, ANOVA, ' 'Dunnett's two-sided test for equal variance and Dunnett T3 for unequal variance. In addition, data were evaluated by *t*-test in applicable cases such as recovery groups comparison. The parameters were analysed at a 5% ($p < 0.05$) level of significance.

3. Results

3.1. 28-day repeat dose study in mice

Two mice, one in Group 1 and one in Group 6, were euthanized prior to the date of termination. In both cases the causes were judged by a pathologist to be likely due to the extended gavage period.

No findings were seen for haematology (see Supplementary Fig. S1) or blood chemistry values (see Supplementary Fig. S2). From histopathology, one animal in Group 5 (high dose VLCFA) had minimal focal accumulation of mononuclear inflammatory cells in the myocardium of the left ventricle. This finding was isolated to 1 animal and not considered treatment related. Accumulation of fat in the liver was of special interest. Vacuolation, from H&E-stained tissue sections, was seen in all animals at various severity grades as shown in Table 6. Histopathologic grades were assigned as level 1 (minimal), 2 (slight), 3 (moderate), 4 (marked), or 5 (severe) based on an increasing extent and/or complexity of change, unless otherwise specified (Schafer et al., 2018).

In the kidney, renal vacuoles were seen in all animals as assessed by H&E staining. The severity appears to be related to VLCFA dose, see Table 7. The low dose group had 1 of 8 animals showing minimal severity. The high dose group had 3 of 8 animals showing minimal to slight severity. The high dose recovery group had 6 of 8 animals with

Table 6
Hepatic steatosis measured in the 28-day repeat dose study.

	Dose group	
	1 (Soy control)	5 (high dose, 162 mg VLCFA/kg b.w)
Number animals analysed	7	8
Number animals with hepatic vacuoles	7	8
Number animals with hepatic vacuoles	7	8
Minimal	2	2
Slight	1	4
Moderate	4	1
Marked	0	1

Table 7
Vacuolation in the kidney measured in the 28-day repeat dose study.

	Dose group				
	1 Soy control	2 Fish oil control	3 Fish oil control	4 Low dose VLCFA	5 High dose VLCFA
Number animals analysed	7	8	8	8	8
Number of animals with renal vacuoles	7	8	8	8	8
Number of animals with tubular vacuoles	1	2	2	1	3
Minimal	1	2	2	1	2
Slight	–	–	–	–	1
Moderate	–	–	–	–	–

minimal to moderate severity as determined following a 4-week recovery period, this suggests there may be a concern with the highest dose in the kidney.

One possible explanation for the occurrence of vacuoles in the kidneys of recovery animals may be that it is known that there is a high turnover of lipids in the kidney as they are routed to the mitochondria for energy production especially during periods of stress and/or nutrient restriction (Jarc and Petan, 2019). Future studies of toxicity should include staining to determine the lipid content of the vacuoles.

3.2. Maximum tolerated dose oral toxicity study (14-day repeat dose study) in rats

No mortality was observed at any dose level throughout the study. There were no observed changes in the mean body weights and net body weight gain in any of the groups treated with test item in either sex when compared to their respective vehicle control (Supplementary Figs. S3 and S4, net change and % change in body weight are presented in Supplementary Tables S4 and S5). There were no effects on clinical signs or eye abnormalities noted for any treatment groups or control groups.

Haematology parameters for either sex are presented in Supplementary Tables S6 and S7. No treatment related changes were observed.

No statistically significant changes were observed in the coagulation or clinical chemistry parameters in either male or female animals except for a statistically significant increase in glucose in male Group 5 (Supplementary Tables 8 and 9). A statistically significant decrease in MCH was noted for Group 2 (soya oil control) males compared to water (Group 1), and a significant increase in MCHC for Group 2 (soya oil control) compared to water (Group 1). These values were considered incidental.

No test-item related changes or statistical changes in urine volume were noted for urinalysis parameters in any of the groups (Supplementary Table S10).

In males, the spleen absolute organ weight in Group 4 showed a statistically significant increase compared to the water control group, but not when compared to Group 2 soya oil control. In females, the absolute organ weight of the liver and kidney in Group 6 showed a statistically significant increase compared to the water control group, but not when compared to Group 2 soya oil control. Increased organ weight may likely be due to lipid accumulation in vacuoles relative to Group 1 animals, although the more appropriate comparator is the Group 2 soya control. Liver findings from H&E staining of histopathology sections from male and female rats is presented in Table 8.

Liver histopathology showed that in male rats, 2 animals from Group 2, and 3 animals each from Groups 4, 5 and 6 had mild, minimal or moderate cytoplasmic vacuolation in the liver. In female rats, 2 animals from Group 4 and 1 animal from Group 5 had cytoplasmic vacuolation.

Kidney findings from H&E staining of histopathology sections from male and female rats is presented in Table 9.

In male rats, the kidney showed mild to moderate tubular degeneration in the following: 3 animals from Group 2 (soya control), 1 animal from Group 3, 2 animals from Groups 4, and 5 and 1 animal from Group 6.

In the female rats, the kidney showed minimal to moderate tubular degeneration in 1 animal each from Groups 2 and 5, and 3 animals from Group 6.

Changes described above appear to be a function of lipid intake since the soya group had an equal frequency of events as test-item groups.

Observations of microvesicular lipid accumulation are summarised in Table 10 and Table 11.

In male rats, lipid accumulation assessed by Oil-Red-O staining was seen in soya oil controls in both male and female animals and in Group 5. No dose relationship was seen.

In female rats, lipid accumulation was noted in the soya control and in Groups 5 and 6. In the liver, 1 animal showed accumulation in Groups 4, 5 and 6.

The observation of lipid accumulation was made with the soya control as well as groups with VLCFA. There was no apparent dose-response relationship, and no corresponding observations were made in clinical chemistry parameters. However, the study is limited by low animal numbers and the indication that lipid accumulation may occur led to inclusion of Oil-Red-O staining of liver and kidney sections in the proposed 90-day repeat dose study.

3.3. 90-day repeat dose oral toxicity study in rats

No mortality or clinical signs were observed in any dose group throughout the study. No dose related changes in feed consumption were noted and no significant differences were seen in weight gains between dosing groups (see Supplementary Figs. S5–6 and Tables S12–26). No significant changes were seen with a functional observation battery except for grip strength in Group 6 (highest dose of test item) males. The values were considered minor and values for all male Group 6 animals were within 2xSD of the control soya Group 2. This finding in grip strength was not seen in females and did not persist through the recovery period.

No ophthalmological abnormality was seen in Group 1 (water control), Group 2 (soya oil control) or Group 6 (highest test item dose). No statistically significant alterations were observed in absolute and relative organ weights in groups receiving the test item compared to controls in both male and female animals. No statistical differences in haematological parameters between the soya control group and test item were seen for any dose in female rats up to 90 days. A statistically significant increase in Red Cell Distribution Width (RDW) was seen in males for the highest dose group compared to the soya control group. The change, from 12.6 ± 0.31 % (Group 1 water control) to 13.2 ± 0.5 % (Group 6 1200 mg/kg), was only seen in male rats, was marginal to 2xSD of the soya oil group, was comparable to the water control group (Group 1)

Table 8
Liver histopathology findings all animals in the 14-day study.

Liver	Dose group					
	G1 Water Control	G2 Oil Control Soya	G3 40 mg VLCFA/kg b.w.	G4 100 mg VLCFA/kg b.w.	G5 300 mg VLCFA/kg b.w.	G6 600 mg VLCFA/kg b.w.
Mild cytoplasmic vacuolation male	0	1	0	1	1	1
Minimal cytoplasmic vacuolation male	0	1	0	2	2	1
Moderate cytoplasmic vacuolation male	0	0	0	0	0	1
Mild cytoplasmic vacuolation female	0	0	0	1	1	0
Minimal cytoplasmic vacuolation female	0	0	0	1	0	0
Moderate cytoplasmic vacuolation female	0	0	0	0	0	0

Table 9
Kidney histopathology findings all animals in the 14-day study.

Kidney	Dose group					
	G1 Water Control	G2 Oil Control Soya	G3 40 mg VLCFA/kg b.w.	G4 100 mg VLCFA/kg b.w.	G5 300 mg VLCFA/kg b.w.	G6 600 mg VLCFA/kg b.w.
Mild tubular degeneration male	0	1	1	1	1	0
Minimal tubular degeneration male	0	1	0	1	1	1
Moderate tubular degeneration male	0	1	0	0	0	0
Mild tubular degeneration female	0	0	0	0	0	0
Minimal tubular degeneration female	0	1	0	0	1	3
Moderate tubular degeneration female	0	0	0	0	0	0

Table 10
Number of male animals with microvesicular lipid observations after Oil Red O staining in a 14-day feed study.

Findings	Male groups (Dose (mg/kg rat)) (N = 3 for each group)					
	G1 Water Control	G2 Oil Control Soya	G3 40 mg VLCFA/kg b.w.	G4 100 mg VLCFA/kg b.w.	G5 300 mg VLCFA/kg b.w.	G6 600 mg VLCFA/kg b.w.
Kidneys - within normal limit	3	2	3	3	2	3
Microvesicular fatty change	–	1	–	–	1	–
Liver- within normal limit	3	1	3	1	2	3
Microvesicular fatty change	–	2	–	2	1	–

Table 11
Number of female animals with microvesicular lipid observations after Oil Red O staining in a 14-day feed study.

	Female groups (Dose (mg/kg rat)) (N = 3 for each group)					
Findings	**G1 Water Control**	**G2 Oil Control Soya**	**G3 40 mg VLCFA/kg b.w.**	**G4 100 mg VLCFA/kg b.w.**	**G5 300 mg VLCFA/kg b.w.**	**G6 600 mg VLCFA/kg b.w.**
Kidneys - within normal limit	3	2	3	3	2	2
Microvesicular fatty change	–	1	–	–	1	1
Liver- within normal limit	3	3	3	2	2	2
Microvesicular fatty change	–	–	–	1	1	1

and did not persist in the recovery groups and was therefore considered unrelated to the test item (see Supplementary Tables S27–28). The RDW data were also compared to historical control values for Sprague-Dawley rats. For male rats 8–16 weeks of age, the mean RDW was 12.7 ± 1.1 %.

Levels of T3, T4 and TSH were measured in all animals (see Supplemental Tables S29–30). For T3, significant increases were seen in Group 5 and Group 6 for males and no findings in females. T4 results were similar with statistical increases seen in males Groups 5 and 6, and no changes seen in females. For TSH, significant reductions were seen in male Group 3 and Group 4, and in female Group 6. All statistically

significant changes were within 2xSD of the Group 2 control except for T3 values in male rats. All statistically significant values were within 1 SD of historical controls.

Clinical chemistry measurements for male animals are presented in Table 12.

Clinical chemistry measurements for female animals are presented in Table 13.

Most noteworthy were statistically significant increases ($p < 0.05$) in liver enzymes for ALP (Groups 3–6), AST (Group 6) and ALT (Groups 5 and 6) compared to soya control (Group 2) for male rats only. AST and ALT values were within 2x the control mean (Group 1), and ALP values were outside of 2x control mean (Group 1 or Group 2). All ALP values were within the historical control range, as were values for AST. For ALT, the increase seen in Group 6 was outside of 2xSD of historical controls and within the 2xSD of control group 1. No correlative histopathological observations were seen, no changes were noted in female rats and recovery groups showed normal values of ALT, ALP and AST.

On closer inspection of individual animal data, it was found that for ALP one animal had a value of >2x (326 U/L) the mean value of the other 9 animals, i.e., the Group 6 mean was 173.1 ± 58.9 U/L including this animal, but 156.1 ± 25.7 U/L without this animal. The recovery group, Group 8 1200 mg/kg, did not show statistically elevated ALP relative to the recovery Group 7 soya control (Fig. 2). The cause for the apparent increase in ALP is currently unknown although likely to be an adaptive change in the liver as discussed elsewhere (Hall et al., 2012). Group results for AST, ALP and ALT are presented in Figs. 1–3 respectively.

In male rats dosed with VLCFA, statistically significant decreases were seen in total cholesterol (Groups 3–6 compared to soya control), LDL cholesterol (LDL) (Group 2 vs Group 1, and Groups 3–5 vs Group 2), HDL levels (HDL) (Groups 3–6 vs Group 2), globulin (Group 4 vs Group 2), phosphorus (Group 4 vs Group 2) and chloride (Group 5 vs Group 2), and a statistically significant increase in triacylglycerol (TG) was seen compared to soya control. In female rats, there was a decrease in total cholesterol (Group 4 and Group 6 vs Group 2), HDL (Groups 3, 5, 6 vs

Table 12
Clinical chemistry parameters for male rats at end of study from 90-day repeat dose study (N = 10).

Parameters	Treatment group - MALES					
	Group 1 Water control	Group 2 Soya control	Group 3 300 mg/kg	Group 4 600 mg/kg	Group 5 900 mg/kg	Group 6 1200 mg/kg
ALB g/dL	3.97 ± 0.17	3.90 ± 0.39	4.24 ± 0.30	4.10 ± 0.34	3.97 ± 0.19	4.14 ± 0.22
ALP U/L	79.60 ± 23.42	78.00 ± 7.26	120.0 ± 27.49[b]	143.4 ± 41.34[b]	143.2 ± 30.25[b]	173.1 ± 58.93[b]
ALT U/L	46.80 ± 29.71	30.40 ± 2.72	42.60 ± 16.77	62.20 ± 45.97	48.40 ± 15.20[b]	75.20 ± 21.46[b]
AST U/L	84.70 ± 24.83	82.80 ± 14.30	83.30 ± 18.96	103.80 ± 44.44	85.20 ± 16.88	104.2 ± 10.75[b]
BUN mg/dL	17.00 ± 2.54	14.90 ± 2.28	15.00 ± 2.67	16.10 ± 2.56	15.30 ± 2.26	16.40 ± 1.78
CA mg/dL	10.83 ± 0.93	10.76 ± 0.31	10.54 ± 0.87	10.18 ± 0.49	10.37 ± 0.33	10.54 ± 0.54
TB mg/dL	0.06 ± 0.03	0.07 ± 0.02	0.06 ± 0.04	0.06 ± 0.03	0.07 ± 0.03	0.07 ± 0.03
TG mg/dL	109.7 ± 30.51	73.90 ± 18.39[a]	111.7 ± 33.82	95.20 ± 15.93	112.1 ± 26.76[b]	95.00 ± 47.12
Chol mg/dL	86.20 ± 14.67	88.00 ± 10.68	56.80 ± 9.35[b]	50.10 ± 7.80[b]	61.20 ± 11.32[b]	61.40 ± 5.06[b]
LDL mg/dL	11.70 ± 1.34	15.50 ± 2.95[a]	10.50 ± 2.64[b]	11.40 ± 2.41[b]	12.40 ± 2.41[b]	12.70 ± 2.21
HDL mg/dL	69.60 ± 11.69	68.60 ± 8.19	44.00 ± 7.59[b]	36.20 ± 5.51[b]	43.20 ± 5.22[b]	41.20 ± 4.05[b]
Glu mg/dL	84.70 ± 18.71	87.50 ± 9.51	83.80 ± 18.12	75.10 ± 28.14	67.80 ± 18.82	86.70 ± 22.75
TP g/dL	6.75 ± 0.46	6.68 ± 0.57	6.82 ± 0.65	6.39 ± 0.52	6.62 ± 0.35	6.62 ± 0.27
Glob g/dL	2.78 ± 0.37	2.78 ± 0.38	2.58 ± 0.36	2.30 ± 0.28[b]	2.65 ± 0.31	2.49 ± 0.18
A/G ratio	1.45 ± 0.16	1.42 ± 0.20	1.66 ± 0.15[b]	1.80 ± 0.20[b]	1.52 ± 0.22	1.67 ± 0.15[b]
CRE mg/dL	0.50 ± 0.09	0,48 ± 0.07	0.46 ± 0.06	0.47 ± 0.03	0.49 ± 0.06	0.49 ± 0.09
Phos mg/dL	5.74 ± 0.68	5.89 ± 0.33	5.72 ± 0.63	5.16 ± 0.64[b]	5.48 ± 0.40	5.78 ± 0.67
Na^+ mmol/L	143.3 ± 1.06	143.0 ± 0.65	142.9 ± 1.01	142.8 ± 1.04	142.3 ± 1.01	142.8 ± 1.37
K^+ mmol/L	4.16 ± 0.34	4.12 ± 0.28	4.35 ± 0.23	4.30 ± 0.30	4.38 ± 0.19	4.44 ± 0.30
Cl^- mmol/L	104.0 ± 1.40	104.4 ± 0.89	103.4 ± 1.42	102.9 ± 1.38	101.8 ± 1.42[b]	102.6 ± 1.21
Urea mg/dL	36.38 ± 5.43	31.89 ± 4.89	32.10 ± 5.71	34.45 ± 5.47	32.74 ± 4.84	35.10 ± 3.80

[a] Statistically significant difference versus Group 1 Water control.
[b] Statistically significant difference versus Group 2 Soya control.

Table 13
Clinical chemistry parameters for female rats at end of study from 90-day repeat dose study (N = 10).

Parameters	Treatment group - FEMALES					
	Group 1 Water control	Group 2 Soya control	Group 3 300 mg/kg	Group 4 600 mg/kg	Group 5 900 mg/kg	Group 6 1200 mg/kg
ALB g/dL	4.24 ± 0.21	4.39 ± 0.36	4.48 ± 0.25	4.34 ± 0.19	4.44 ± 0.16	4.51 ± 0.14
ALP U/L	54.20 ± 8.79	60.80 ± 5.33	54.90 ± 14.84	65.50 ± 9.40	57.50 ± 7.86	69.70 ± 16.92
ALT UL	27.60 ± 4.88	26.50 ± 5.40	27.40 ± 5.78	30.70 ± 12.40	22.40 ± 4.33	28.50 ± 5.66
AST U/L	86.60 ± 10.49	89.40 ± 12.49	93.70 ± 17.40	90.10 ± 9.71	81.50 ± 13.14	101.0 ± 18.83
BUN mg/dL	19.10 ± 5.07	19.20 ± 1.69	18.70 ± 3.20	19.80 ± 2.04	18.80 ± 1.40	21.00 ± 3.43
CA mg/dL	10.77 ± 0.23	11.01 ± 0.39	11.15 ± 0.35	11.20 ± 0.32	11.10 ± 0.36	11.33 ± 0.39
TB mg/dL	0.13 ± 0.08	0.07 ± 0.05	0.05 ± 0.03	0.08 ± 0.02	0.07 ± 0.04	0.07 ± 0.02
TG mg/dL	48.20 ± 13.82	50.40 ± 13.82	48.10 ± 7.64	54.20 ± 10.71	43.90 ± 3.84	49.30 ± 7.10
Chol mg/dL	98.10 ± 24.75	91.70 ± 12.96	77.20 ± 14.75	74.50 ± 8.53[b]	77.20 ± 15.27	70.90 ± 13.83[b]
LDL mg/dL	10.50 ± 2.27	11.30 ± 2.16	9.30 ± 2.06	9.20 ± 1.69	9.00 ± 1.70	11.10 ± 2.85
HDL mg/dL	74.50 ± 17.09	70.90 ± 11.45	56.80 ± 10.98[b]	60.60 ± 8.02	59.50 ± 7.71[b]	54.00 ± 6.00[b]
Glu mg/dL	87.30 ± 10.06	96.80 ± 12.39	98.50 ± 28.24	88.90 ± 20.81	101.4 ± 16.13	91.70 ± 26.45
TP g/dL	6.77 ± 0.29	7.05 ± 0.39	7.23 ± 0.39	7.15 ± 0.28	7.24 ± 0.38	7.41 ± 0.29
Glob g/dL	2.53 ± 0.18	2.66 ± 0.48	2.75 ± 0.26	2.81 ± 0.16	2.80 ± 0.25	2.90 ± 0.21
A/G ratio	1.68 ± 0.13	1.75 ± 0.66	1.64 ± 0.16	1.55 ± 0.10	1.60 ± 0.11	1.56 ± 0.12
CRE mg/dL	0.64 ± 0.07	0.68 ± 0.06	0.63 ± 0.07	0.67 ± 0.05	0.58 ± 0.06[b]	0.63 ± 0.07
Phos mg/dL	5.16 ± 1.07	5.05 ± 0.72	5.49 ± 0.84	5.42 ± 0.66	5.27 ± 0.59	5.67 ± 0.54
Na^+ mmol/L	143.1 ± 1.56	141.6 ± 1.28[a]	141.6 ± 1.25	141.7 ± 1.35	142.3 ± 1.14	142.2 ± 1.83
K^+ mmol/L	3.66 ± 0.53	3.83 ± 0.27	3.76 ± 0.40	3.87 ± 0.48	3.69 ± 0.28	3.88 ± 0.49
Cl^- mmol/L	106.3 ± 1.17	105.1 ± 1.79	104.8 ± 1.06	104.7 ± 1.27	105.1 ± 0.92	104.4 ± 2.21
Urea mg/dL	40.87 ± 10.84	41.09 ± 3.61	40.02 ± 6.85	42.37 ± 4.37	40.23 ± 2.99	44.94 ± 7.34

[a] Statistically significant difference versus Group 1 Water control.
[b] Statistically significant difference versus Group 2 Soya control.

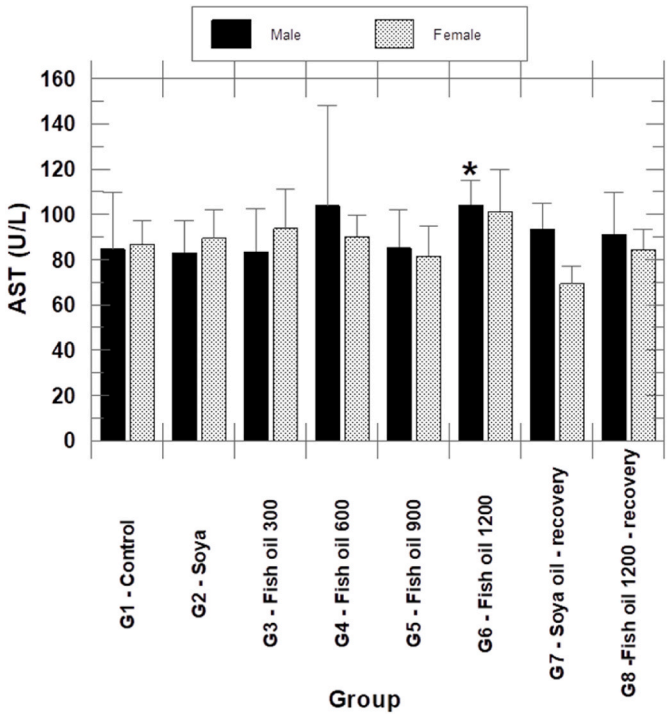

Fig. 1. AST levels in rats at 90 days * Indicates statistically significant changes at $p < 0.05$ when compared with Group 2.

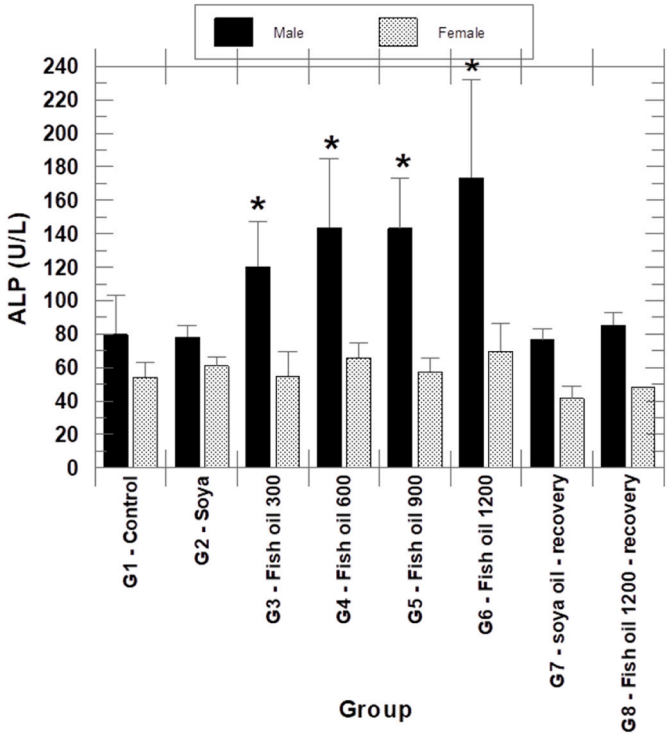

Fig. 2. ALP levels in rats at 90 days * Indicates statistically significant changes at $p < 0.05$ when compared with Group 2.

Group 2) and creatinine (Group 5 vs Group 2). No dose or treatment related relationships were seen. Therefore, the findings were considered incidental.

No test item related gross or histopathological findings were observed in any male and female animals in any treatment group on day

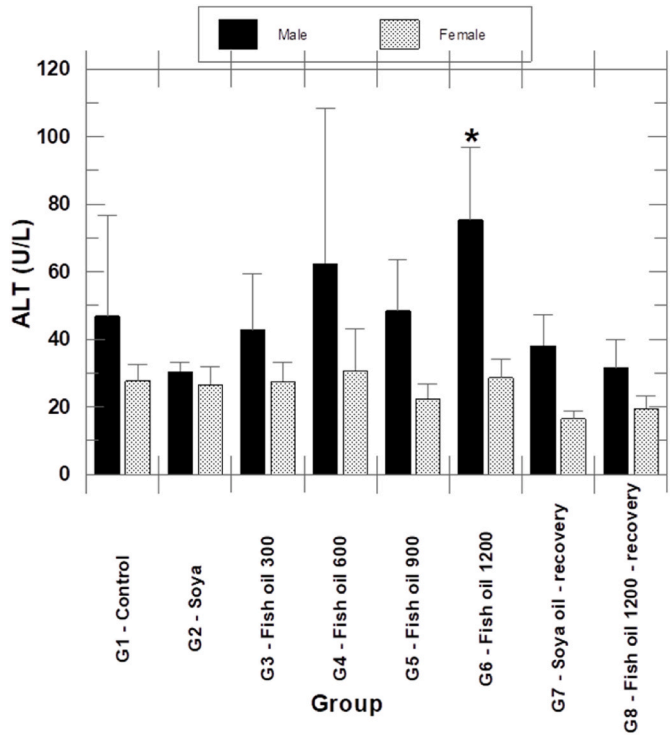

Fig. 3. ALT levels in rats at 90 days * Indicates statistically significant changes at $p < 0.05$ when compared with Group 2.

91 or recovery groups on day 119. Findings reported more than once with test item in male rats include: Histiocytosis (0/10, 4/10 and 10/10 for Groups 1, 2 and 6 respectively) and haemorrhages (1/10, 2/10, 4/10 for Groups 1, 2 and 6) in the lungs.

Basophilic tubules were noted in kidneys of 4/10, 5/10 and 3/10 male animals of G1, G2 and G6 groups, respectively. Basophilic tubules were noted in the kidneys of 1/10 female animals in each of the G2 and G6 groups, respectively; mononuclear cell infiltration was noted in the kidneys of 2/10 and 1/10 female animals of G2 and G6 groups, respectively. Findings were not dose related and were equally noted in the soya control Group 2 and Group 6. These findings were therefore not considered specific to the test item.

In male animals, hyaline casts were observed in the kidneys of 2/10 and 3/10 animals of G2 (soya control) and G6 (high dose) groups, respectively. In female animals, hyaline casts were observed in kidneys of 2/10 and 1/10 female animals of G2 and G6 groups respectively; mineralization was observed in kidneys of 1/10 female animal each of G1 and G6 groups and tubular dilation was observed in kidneys of 1/10 female animal of the G2 group (A; Berridge et al., 2016; Frazier et al., 2012; Hoenerhoff; RR).

Lipid accumulation was assessed by staining with Oil Red O staining. No accumulation of lipids, nor vacuolation was noted from the histopathological evaluation of the kidney or liver for control (Groups 1, 2) or highest dose group (Group 6) or follow-up groups (Groups 7, 8) therefore no further groups were assessed.

4. Discussion

EPAX Evolve 05 is a fish oil concentrate containing a maximum of 200 mg of VLCFA per gram fish oil. The lipid species in the oil have been consumed by humans whenever fish products are eaten, but to lower concentrations. Therefore, there were no suspected toxicological outcomes prior to initiating these studies. The initial 28-day study was therefore designed to provide indications of toxicological concern and produced the finding that lipid accumulation may occur on the liver and

kidneys and led to particular focus and commentary on liver and kidney steatosis and function.

In the 28-day study, steatosis was seen in the kidneys and liver for the soya control and VLCFA groups, without a dose relationship. Due to the findings of microvesicular vacuoles in the liver and kidneys in the 28-day mouse study, lipid staining of these organs was performed in all groups of the 14-day repeat study and the control groups, highest dose group, and recovery groups of the 90-day study.

Collectively, the results from the 28-day repeat dose study and 14-day repeat dose study showed no treatment related, biologically relevant findings other than lipid accumulation in the kidneys and liver. No accumulation was noted in the 90-day repeat dose study for any dose used.

In the 14-day study, groups receiving the test-item showed some microvesicular lipid accumulation, there was no dose relationship and accumulation was also seen in the soya control group. Lipid droplet accumulation may be due to causes such as obesity, lipodystrophy, non-alcohol fatty liver disease, infection, and cancer (Zadoorian et al., 2023). However, mice (28-day study) and rats (14-day study) did not show signs of obesity as determined by body weight. Lipodystrophy can be ruled out since this condition is often related to severe obesity-associated metabolic complications and there were no clinical signs of infection or cancer observed. Therefore, we propose that steatosis is likely due to an increase in the lipid load of the animals as seen in other studies with Sprague Dawley rats when fed a high fat diet (Ji et al., 2011). There were no clinically significant differences in organ weight. As described below, lipid droplets were not observed in kidney or liver samples in rats from the 90-day study suggesting an adaptive response.

In the 14-day study, renal tubular degeneration was noted in 4 animals in the highest dose group and 4 animals (3 male and 1 female) in the soya control suggesting there was no specific degeneration due to VLCFA content but rather this was related to total lipid load, although the low number of animals may be a limiting factor for determining specificity of events. No statistically significant differences in kidney organ weights were seen with male or female groups, nor any changes in urinalysis indicating no physiological impact of the test item. We therefore conclude that tubular degeneration was not specific to the test item and there was no evidence of specific toxicity from VLCFAs.

In the 14 -day repeat-dose study there were no clinically significant dose-related findings for clinical examinations, ophthalmology examinations, body weight, or assessments of haematology, blood clotting, clinical chemistry, or urine. The maximum tolerated dose from the 14-day repeat dose study was 600 mg VLCFA/kg/day.

An additional 90-day study was therefore planned with an additional test for lipid accumulation in liver and kidney. Doses were designed to overlap and extend the dosage used in the 14-day repeat dose study.

In contrast to the 14-day results, no lipid accumulation was seen in the kidneys or liver of rats in the 90-day study despite there being overlapping doses (300 and 600 mg VLCFA/kg b.w/day) and despite the total gavage volume of lipid administered per day being the same (4 mL/kg b.w) across studies. Interestingly, both 14- and 90-day studies had similar soya control groups, but steatosis was only noted in the 14-day study. A newly reported study assessing a marine derived oil with VLCFA at high doses (3500 mg/kg b.w. in mice) showed that after 8 weeks intervention there were no histopathological findings in heart, brain, liver, kidney or testes. The study also assessed Oil-Red-O staining of liver sections without evidence of lipid accumulation (Yang et al., 2023).

The lack of steatosis in the kidney and liver after 90-day exposure to the test item or control soya oil group may indicate a period of metabolic adaptation to the increased lipid. This phenomenon has previously been seen with erucic acid (C22:1 n-9) where rats fed erucic acid show myocardial lipidosis, hepatic steatosis and increased triglyceride levels in a dose dependent manner (Kramer et al., 1988). In the liver, the presence of erucic acid induces peroxisomal β-oxidation (Lazarow, 1994) leading to a gradual decline of hepatic lipid load (Bremer and Norum, 1982; Kramer et al., 1992; Sauer and J.K.G., 1983). In a 28-day repeat dose study of erucic acid in Charles River rats where groups were killed at 3, 7, 14 and 28 days, erucic acid accumulation in the liver reached a peak at 14-days and was not significantly different from controls at 28 days (Beare-Rogers and E.A Heggtveit, 1971).

The long chain fatty acids EPA and DHA also induce adaptive changes via receptor mediated down-regulation of hepatic genes associated with *de novo* lipogenesis and lipid peroxidation (Green et al., 2020). EPAX Evolve 05 contains approximately 50% DHA and although this is diluted in the test item with soya oil, there may well be an adaptive effect resulting from the activity of DHA. There are numerous studies describing the lipid modifying effects of EPA and DHA at a molecular and clinical level in the liver (Lytle et al., 2017; Musazadeh et al., 2023; Parker et al., 2012; Scorletti and Byrne, 2018; Watanabe and Tatsuno, 2020). One of the key regulators of hepatic lipid levels is the nuclear receptor PPAR-alpha. Activation leads to reduced *de novo* lipogenesis and increased peroxisomal and mitochondrial beta oxidation (Jump et al., 2013). Interestingly, EPA seems more potent than DHA in eliciting PPAR activity, and feeding studies with a VLCFA rich oil suggests there is significant retro-conversion of VLCFAs to EPA (Torrissen et al., 2021).

Lung histiocytosis was seen in 0/10, 4/10 and 10/10 for groups 1, 2 and 6 respectively. The performing laboratory considered this a result of gavage feeding since histiocytosis is a common event seen with prolonged gavage feeding. However, lung histiocytosis was only observed in animals receiving either soya oil or VLCFA indicating the possible presence of some residual oil entering the lung. The presence of alveolar histiocytosis may likely be caused by regurgitation of oil (both soya and VLCFA) following gavage administration (Sells et al., 2007). In addition, nonspecific macrophage responses to poorly soluble drugs are known to occur in the lungs. With respect to the accumulation of alveolar macrophages in the lungs of rats, the presence of alveolar macrophages in the lungs of rats administered soya oil or VLCFA does not appear to be associated with concomitant presence of other histopathological findings. Systemic measurements of white blood cells, haemoglobin and differential leukocyte count showed no effect of VLCFAs nor were coagulation parameters affected (see Supplementary Tables S27–S28) or associated clinical chemistry parameters (e.g., total protein, albumin). These biomarkers are recommended as additional tests for all types if histiocytosis for diagnostic purposes (Girschikofsky et al., 2013). Lung haemorrhages are also often associated with prolonged gavage feeding and a mild association with dose was noted. Taking this data collectively, there is an increase in histiocytosis in the highest VLCFA dose in male animals, but this is not supported by additional measurements and may therefore be due to prolonged gavage feeding.

PPAR activation may also occur with VLCFA due to the promiscuous ligand binding activity of PPAR (Varga et al., 2011), and this may therefore represent a pathway for an adaptive response. Indeed, a recent study by Yang et al. where mice were fed 3.3g/kg b.w. VLCPUFA for 8 weeks, gene expression of metabolic proteins were increased, and feed-induced hepatic steatosis was reduced. The study also reported no adverse events (In Press, Yang et al., NIH). Therefore, mechanisms for an adaptive response to increased levels of fatty acids have previously been described and could explain adaptive responses that may have taken place in the 90-day study (Marton et al., 2021).

Serum levels of hepatic marker enzymes such as AST, ALT, and especially ALP in the 90-day study show dose-response changes which were not apparent in the 14-day study. Increases in liver enzyme levels occurred in the absence of accompanying degenerative histopathological findings such as morphological changes in hepatocytes and there were no changes in liver weight. Changes in ALP and AST reported during 90-day oral administration of VLCFA are unlikely to have toxicological implications since there were no associated clinical pathology parameters and values were within historical control limits. The ALT level was significantly increased in Group 6 male rats and outside of historical controls, although within the standard deviation of the control

Group 1. AST levels were also raised in Group 3 with values ranges beyond those of Group 6. ALT was normalized in the recovery groups. These data, together with the normalisation in the recovery groups indicate that VLCFA likely induces a non-toxic, adaptive response with respect to these liver enzymes.

An examination of the 90-day results from individual animals, presented in Fig. 4, shows that the increase in ALP for Group 6 1200 mg/kg animals may be caused by one extreme outlier animal. The Group 6 mean value for ALP was 173.1 ± 58.9 U/L including this animal but 156.1 ± 25.7 U/L without this animal. An interquartile range was determined based on the group data with the upper fence value calculated as 211 U/L. The value for the animal in question was 326 U/L, well above the upper fence value and establishing this animal as a true outlier.

Irrespective of the outlier animal, there is an increase in ALP related to increasing dose of VLCFA. The increase in ALP was not associated with any morphological or histological changes in liver or kidneys. No changes in total bilirubin were seen, and no clinically significant changes were seen with other liver enzymes indicating a lack of overall toxicity. Of importance is that a 4-week recovery period reduced the increase in ALP to normal values resulting in values not significantly different from animals receiving soya oil vehicle.

It should also be noted that the feed used for the 90-day study contained less fat than that used in the 14-day study which may be a limitation in the study. The dietary fat intake for a 250g rat in the 14-day study from feed and test item was approximately 1.75g/day whereas for the 90-day study it was 1.38g/day (calculated from feed consumption data and % fat composition of feed).

Across the studies performed with VLCFAs, no gross lipid accumulation was seen around the heart, heart weight remained the same as controls, and was unaffected by the test item.

In conclusion, EPAX Evolve 05 is a fish-oil with a total VLCFA content of no more than 200 mg/g. Raised liver enzymes and histiocytosis in male rats were the most notable events in the 90-day repeat dose study. The presence of histiocytosis may be explained by gavage feeding since there were no associated changes in associated biomarkers and the reporting laboratory reported an association with gavage feeding.

Liver enzyme levels were raised but within control values. There were no associated histological findings in the liver suggesting there was no toxic effect and the normalisation of values for all liver enzymes in the recovery groups suggests an adaptive response rather than a prevailing toxic response.

The No Observed-Adverse-Effect-Level is therefore selected as 1200 mg/kg/day VLCFA in Sprague Dawley rats.

CRediT authorship contribution statement

Derek Tobin: Writing – review & editing, Writing – original draft, Project administration, Conceptualization. **Harald Svensen:** Writing – original draft, Methodology, Formal analysis. **Devanand Shanmugasundaram:** Writing – review & editing, Project administration, Methodology, Investigation, Formal analysis. **Bente Ruyter:** Writing – review & editing. **Iren Stoknes:** Writing – review & editing, Project administration, Conceptualization. **Michael Dornish:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Derek Tobin reports financial support was provided by Research Council of Norway. Derek Tobin reports a relationship with Epax Norway AS that includes: employment. Co-authors Iren Stoknes and Harald Svensen are employees of Epax Norway AS. Devanand Shanmugasundaram is an employee of Vedic Lifesciences. Michael Dornish is a toxicology consultant to Epax Norway AS.

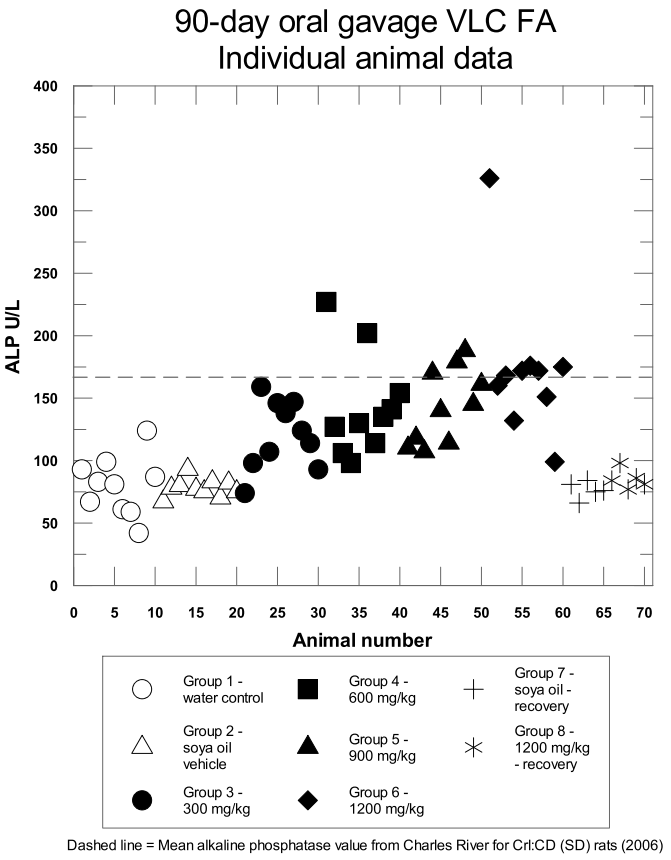

Fig. 4. ALP values from individual animals in the 90-day repeat dose study in rats.

Data availability

Data will be made available on request.

Acknowledgements

This work was funded from a research grant (321447) from the Research Council of Norway.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2024.114518.

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