Application for the Approval of (*R*)-3-hydroxybutyl (*R)*-3-hydroxybutyrate

Under

Regulation (EC) No 258/97 of the European Parliament and of the Council of 27th January 1997 Concerning Novel Foods and Novel Food Ingredients

NON-CONFIDENTIAL

July 24, 2013

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ADMINISTRATIVE DATA

Name and Address of Applicants/Manufacturers

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Name and Address of Company Responsible for Dossier

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SUMMARY

Approval is sought under Regulation (EC) No 258/97 of the European Parliament and of the Council of 27th January 1997 concerning novel foods and novel food ingredients, for the approval of (*R*)-3-hydroxybutyl (*R*)-3-hydroxybutyrate, referred to as D-β-hydroxybutyrate ester, as a food ingredient (European Parliament and the Council of the European Union, 1997).

D-β-Hydroxybutyrate ester has been developed as an oral source of ketones, which will be utilised as an energy source for athletes and persons undergoing strenuous exercise or conditions leading to rapid energy depletion. Ketone bodies have many beneficial metabolic functions within the body, and are the only fuel source that can completely sustain brain energy requirements when blood glucose levels fall. This is of specific relevance in physiologic conditions such as prolonged exercise, which may profoundly decrease blood glucose, and deplete brain glycogen stores. The combination of heavy sustained exercise, and persistent cognitive attention places great strain on the energy capacity of the brain and may compromise performance. Ketone bodies (when present in significant quantities) exert an inhibitory effect on glycogen breakdown (glycogenolysis) pathways by reducing the rate of glucose oxidation. Ketone bodies can be substituted for glucose, potentially sustaining energy requirements for longer.

D-β-Hydroxybutyrate ester is produced *via* an enzyme-catalysed transesterification reaction of ethyl-D-hydroxybutyrate and (*R*)-1,3-butanediol. The final product contains ≥97.5% D-β-hydroxybutyrate ester.

D-β-Hydroxybutyrate ester is intended for use as an ingredient in food supplements in liquid, powder (sachet), bar, and gel form that will be marketed to high-performance athletes only. Considering that D-β-hydroxybutyrate ester-containing products will be consumed only by high-performance athletes and persons undergoing extreme energy expenditures, the products will be consumed in a pattern consistent with supplement use and labelled in accordance with Directive 2002/46/EC (European Parliament and Council of the European Union, 2002). The ingredient will never be used in mainstream foods. It is therefore likely that D-β-hydroxybutyrate ester-containing products will be used intermittently and by a small section of the population. The addition of D-β-hydroxybutyrate ester to the proposed products will result in intakes that will not exceed 0.36 g/kg body weight/serving. It is envisaged that a maximum of 2 to 3 servings per day will be consumed, resulting in a maximum daily intake of 1.07 g/kg body weight. Based on an average 70 kg adult, this maximum will relate to 75 g D-β-hydroxybutyrate ester per day.

Following consumption, D-β-hydroxybutyrate ester undergoes complete hydrolysis to D-β-hydroxybutyrate and (*R*)-1,3-butanediol, with the latter being further metabolised to D-β-hydroxybutyrate and acetoacetate in the liver. This is confirmed by results of studies in which blood levels of D-β-hydroxybutyrate, acetoacetate, and (R)-1,3-butanediol were elevated in rats following dietary or gavage administration of D-β-hydroxybutyrate ester,

while the ester was absent or detected at very low levels. Additionally, in an *in vitro* study in which solutions of the ketone ester were incubated with fresh human plasma, hydrolysis was found to be rapid and complete. It also has been shown that the consumption of D-β-hydroxybutyrate ester in healthy adult volunteers resulted in elevated plasma levels of D-β-hydroxybutyrate and acetoacetate, but no detection of the ester.

The safety of D-β-hydroxybutyrate ester is supported by the results of a 28-day toxicity study conducted in rats in which the consumption of diets in which 30% of the calories came from D-β-hydroxybutyrate ester (12 and 15 g/kg body weight/day in male and female rats, respectively) did not cause adverse effects. Rats in the test group consumed less feed and gained less weight than control animals fed carbohydrate or fat-based diets; similar findings have been documented in studies of ketogenic diets. Between-group differences were noted in selected haematology, coagulation, and serum chemistry parameters; however, values were within normal physiological ranges and/or were not accompanied by other changes indicative of toxicity. There were no findings associated with the ketone ester upon gross and microscopic evaluation.

Results from a developmental toxicity study in rats also support the safety of D-β-hydroxybutyrate ester. Administration of 2 g D-β-hydroxybutyrate ester/kg body weight/day *via* gavage on days of gestation (DGs) 6 through 20 did not affect reproductive performance or litter parameters. The overall incidence of foetal alterations was higher in the test group; however, these were not considered to be attributable to the test substance.

In a physical endurance study, men consumed a vitamin water drink containing 1.23 g D-β-hydroxybutyrate ester/kg body weight or 1.44 g dextrose/kg body weight (divided into 3 drinks of equal volume) before and during a 2-hour cycling exercise session. D-β-hydroxybutyrate ester was well tolerated under conditions similar to the intended conditions of use, with no significant differences in symptom severity between the D-β-hydroxybutyrate ester and control arms of the study. In another human study, D-β-hydroxybutyrate ester was administered at doses of 140, 357, and 714 mg/kg body weight 3 times daily over a period of 5 days (equivalent to 420, 1,071, and 2,142 mg/kg body weight/day). No abnormal changes in haematology, clinical biochemistry, or urinalysis parameters were observed. Moreover, blood ketone levels and glucose levels did not deviate from ranges deemed to be safe. Vital signs also were stable throughout the course of the study, and no treatment-related abnormalities were reported upon physical examination. Some gastrointestinal effects related to D-β-hydroxybutyrate ester were reported at the highest dose tested, which likely resulted from the dosing matrix and the requirement to consume the beverage rapidly.

In addition to ingredient-specific data, the safety of D-β-hydroxybutyrate ester for its intended use is corroborated by studies conducted on its metabolites, D-β-hydroxybutyrate and (R)-1,3-butanediol. These include a 2-year study in which rats were administered diets comprising up to 10% (R)-1,3-butanediol (approximately 5 g/kg body weight/day) with no adverse effects. Similarly, no adverse effects resulted from the consumption of diets

containing (R)-1,3-butanediol at levels up to 3.0% (0.75 g/kg body weight/day) in dogs for 2 years.

In a reproductive and developmental toxicity study conducted on 1,3-butanediol (isomer not specified), it was shown that, in 5 successive breedings of rats, consumption of 5 to 24 g/kg body weight/day (approximately 2.5 to 12 g/kg body weight/day) was not associated with teratological effects. The control and test animals were comparable with respect to gestation, viability, and lactation indices. The results of *in vitro* studies (with isolated embryos) suggest that physiologically relevant levels of β-hydroxybutyrate, particularly, the L-isomer, may disrupt normal embryogenesis. Given that the majority of (R)-1,3-butanediol is metabolised to the ketones (R)-3-hydroxybutyrate and acetoacetate, while approximately one-third of (S)-1,3-butanediol is converted to ketone bodies, data from studies on 1,3-butanediol provide indirect evidence of the safety of β-hydroxybutyrate. As the administration of 1,3-butanediol in the aforementioned reproductive and developmental study did not produce teratogenic effects, nor did the developmental toxicity study conducted on D-β-hydroxybutyrate ester, the relevance of the results observed in the *in vitro* studies conducted with β-hydroxybutyrate in isolated embryos is questionable.

Data related to the safety of (R)-1,3-butanediol and D-β-hydroxybutyrate ester in humans are limited; however, sodium D,L-β-hydroxybutyrate has been administered orally to children with acyl-CoA dehydrogenase deficiency or with persistent hyperinsulinaemic hypoglycaemia at dose levels up to 1 g/kg body weight/day with no side effects.

In a study in which adult volunteers consumed 15 g 1,3-butanediol (enantiomer not specified) incorporated into bread for 7 days, no adverse effects on selected haematology or blood chemistry parameters. A significant decrease in blood glucose was observed, but it should be noted that such effects on glucose levels were not observed in the above-mentioned human study in which subjects consumed up to 2.1 g D-β-hydroxybutyrate ester/kg body weight/day for 5 days.

The safety of D-β-hydroxybutyrate ester is further supported by animal feeding trials of a similar ketone ester, namely (R,S)-1,3-butanediol mono- and diacetoacetate. A bolus intragastric dose of approximately 1.3 g/kg body weight administered to pigs did not alter standard clinical chemistry parameters nor have deleterious side effects. Likewise, the administration of repeated oral doses of (R,S)-1,3-butanediol diacetoacetate over a 300-minute period (equivalent to 1,054 to 1,144 mg/kg body weight) or a single bolus dose of 439 to 477 mg/kg body weight (R,S)-1,3-butanediol diacetoacetate to dogs did not alter clinical chemistry; moreover, no signs of distress in any of the animals during or following the experiment were observed.

D-β-Hydroxybutyrate ester is a synthetic compound and does not occur endogenously; however, the metabolites of D-β-hydroxybutyrate ester are ketones, which are normally produced in the body. Maximal ketone body production is approximately 185 g/day during times of limited glucose availability, indicating that the human body has the capacity to handle high concentrations of circulating ketones. Furthermore, there is a long history of use

of ketogenic diets, which, similar to D-β-hydroxybutyrate ester, result in elevated ketone levels.

Based on the available information on the safety of D-β-hydroxybutyrate ester, its metabolites (D-β-hydroxybutyrate and (R)-1,3-butanediol), and the related ketone ester, R,S)-1,3-butanediol mono- and diacetoacetate, the proposed use of D-β-hydroxybutyrate ester as an ingredient added to selected food supplement products targeted to specific population groups for consumption on a supplemental basis does not present a safety concern. The capacity of the human body to produce and utilise ketones, as well as the long history of use of ketogenic diets, also support the safety of D-β-hydroxybutyrate ester for its intended use.

GENERAL INTRODUCTION

TΔS Limited (TΔS) proposes to market (*R*)-3-hydroxybutyl (*R*)-3-hydroxybutyrate, referred to as D-β-hydroxybutyrate ester, for use as an ingredient to be added to specific food supplement products in Europe. D-β-Hydroxybutyrate ester has been developed as an oral source of ketones, which will be utilised as an energy source for athletes and persons undergoing strenuous exercise or conditions leading to rapid energy depletion. Ketone bodies have many beneficial metabolic functions within the body, and are the only fuel source that can completely sustain brain energy requirements when blood glucose levels fall. This is of specific relevance in physiologic conditions such as prolonged exercise, which may profoundly decrease blood glucose, and deplete brain glycogen stores. The combination of heavy sustained exercise, and persistent cognitive attention places great strain on the energy capacity of the brain and may compromise performance. Ketone bodies (when present in significant quantities) exert an inhibitory effect on glycogen breakdown (glycogenolysis) pathways by reducing the rate of glucose oxidation. Ketone bodies can be substituted for glucose, potentially sustaining energy requirements for longer.

Approval is sought under Regulation (EC) No 258/97 of the European Parliament and of the Council of 27th January 1997 concerning novel foods and novel food ingredients (hereafter referred to as EC 258/97), and accordingly, this submission has been prepared pursuant to the Commission Recommendation of 29 July 1997 concerning the scientific aspects and the presentation of information necessary to support applications for the placing on the market of novel foods and novel food ingredients (hereafter referred to as the Commission Recommendation of 1997) (European Parliament and the Council of the European Union, 1997).

Article 1(2.) of EC 258/97 states that the regulation "…shall apply to the placing on the market within the Community of foods and food ingredients which have not hitherto been used for human consumption to a significant degree within the Community and which fall under the following categories…(c) foods and food ingredients with a new or intentionally modified primary molecular structure;". D-β-hydroxybutyrate ester is thus considered a novel food/food ingredient (European Parliament and the Council of the European Union, 1997).

Section 4 of the Commission Recommendation of 1997 outlines recommendations made by the Scientific Committee on Food (SCF) pertaining to the "Scientific Classification of Novel Foods for the Assessment of Wholesomeness", which facilitates the safety and nutritional evaluation of a given novel food/food ingredient. Of the 6 classes identified, D-β-hydroxybutyrate ester would be classified in Class 1 as a pure chemical or simple mixture from non-GM sources, since it is a synthetic molecule and it is produced without the use of genetic modification. D-β-Hydroxybutyrate ester does not have a history of use in the community. Accordingly, D-β-hydroxybutyrate ester would be further allocated under Sub-Class 2.2: "the source of the novel food has no history of food use in the Community". The essential information requirements corresponding with this classification are outlined in a detailed list below, and are expanded upon in separate sections throughout the document,

forming the basis of the application (Recommendation 97/618/EC - Commission of the European Communities, 1997).

I	Specification of the Novel Food
II	Effect of the Production Process Applied to the Novel Food
III	History of the Organism Used as the Source of the Novel Food
IV-VIII	Not Applicable
IX	Anticipated Intake/Extent of Use of the Novel Food
X	Information on Previous Human Exposure to the Novel Food
XI	Nutritional Information on the Novel Food
XII	Microbiological Information on the Novel Food
XIII	Toxicological Information on the Novel Food

For each category (I through XIII), structured schemes have been developed by the SCF, which consist of a decision-tree-like set of questions designed to elicit sufficient data for a comprehensive safety and nutritional evaluation of the novel food. As outlined below in Sections I through XIII, the required questions are identified and subsequently addressed with the appropriate data. While category X (Information on Previous Human Exposure to the Novel Food) is not required for Class 1, Sub-Class 2.2 novel foods, information on human exposure to the metabolites of D-β-hydroxybutyrate ester has been included in Section X.

As detailed herein, the safety of D-β-hydroxybutyrate ester is supported by the purity of D-β-hydroxybutyrate ester (chemical purity ≥97.5%), safety data for the final D-β-hydroxybutyrate ester product, and safety data from additional published and unpublished toxicological and clinical data. Safety is corroborated by the capacity of the human body to produce and utilise ketones, as well as the long history of use of ketogenic diets, which, similar to D-β-hydroxybutyrate ester, elevate blood ketone levels.

I SPECIFICATIONS OF D-β-HYDROXYBUTYRATE ESTER

Based on the SCF guidelines, the following questions must be answered in the affirmative to ensure sufficient information pertaining to the specifications of the novel food:

- “Is appropriate analytical information available on potentially toxic inherent constituents, external contaminants and nutrients?”
- “Is the information representative of the novel food when produced on a commercial scale?”
- “Is there an appropriate specification (including species, taxon *etc.* for living organisms) to ensure that the novel food marketed is the same as that evaluated?”

These questions have been addressed collectively in Sections I.a through I.f.

I.a Common Name or Usual Name

D-β-hydroxybutyrate ester

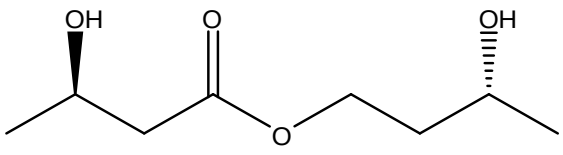
I.b Chemical Name

(*R*)-3-hydroxybutyl (*R*)-3-hydroxybutyrate

I.c Chemical and Physical Characteristics

Molecular formula:	$C_8H_{16}O_4$
Molecular weight:	176
Physical form:	Colourless oil
Taste:	Slight bitter, sharp taste; no odour
Enantiomeric Excess:	> 99% (*R*)-1,3 butanediol (Diacel) > 99% Ethyl (*R*) 3-hydroxybutyrate (Julich)
Density:	1.0731 g/mL at 22°C
Boiling point:	145°C at 1.8 Torr (Pope Scientific, July '07)
Significant impurities:	Ethyl-hydroxybutyrate < 0.5%; (*R*)-1,3-butanediol (< 2%) (starting materials)
Storage:	Room temperature
Optical activity product:	[α]23.4/D -46.2°, c = 1 in water [a]23.4/D -38.7°, c = 1 in ethanol

I.d Structural Formula



I.e Product Specifications and Analyses for D-β-Hydroxybutyrate Ester

The chemical specifications for D-β-hydroxybutyrate ester are presented in Table 1.e-1.

Table I.e-1 Chemical Specifications for D-β-Hydroxybutyrate Ester

Specification Parameter	Specification	Method [a]
D-β-hydroxybutyrate ester content	≥97.5%	GC/MS
Ethyl R 3-hydroxybutyrate content	<0.5%	GC/MS
R-1,3-butanediol content	<2.0%	GC/MS
Heavy Metals		
Mercury (Hg)	<0.008 mg/kg	UD030
Arsenic (As)	<0.1 mg/kg	UD031
Lead (Pb)	<0.005 mg/kg	UD032:ICPMS/005
Cadmium (Cd)	<0.005 mg/kg	UD033:ICPMS/005

GC/MS, gas chromatography/mass spectrometry; ICPMS, inductively coupled plasma mass spectrometry
[a] Certificates of analysis and analytical methods for specifications are provided in Appendix A

Example batch analysis results corresponding to the aforementioned specifications are provided in Table I.e-2.

Table I.e-2 Summary of the Chemical Product Analysis for 3 Consecutive Lots of D-β-Hydroxybutyrate Ester

Specification Parameter	Specification	Manufacturing Lot		
		Batch 7	Batch 8	Batch 9
D-β-Hydroxybutyrate ester[1]	≥ 97.5%	98.8%	97.7%	98.2%
Ethyl R 3-hydroxybutyrate	< 0.5%	0.1%	0.1%	0.1%
R 1,3-Butanediol	< 2.0%	0.7%	1.4%	0.8%
Total	100.0%	99.6%	99.2%	99.1%
Heavy Metals				
Mercury (Hg)	< 0.008 mg/kg	< 0.001 mg/kg	< 0.001 mg/kg	< 0.001 mg/kg
Arsenic (As)	< 0.1 mg/kg	0.012 mg/kg	0.013 mg/kg	0.011 mg/kg
Lead (Pb)	< 0.005 mg/kg	< 0.005 mg/kg	< 0.005 mg/kg	< 0.005 mg/kg
Cadmium (Cd)	< 0.005 mg/kg	< 0.001 mg/kg	< 0.001 mg/kg	< 0.001 mg/kg

[1] "D-β-Hydroxybutyrate ester content" represents (*R*)-3-hydroxybutyl (*R*)-3-hydroxybutyrate, 3-betahydroxybutryl 1,3-butanediol monoester, and β-hydroxybutyrate 1,3-butanediol diester. These esters were grouped together in the product specifications and analyses because they are handled in the same manner in the body.

As shown in Table I.e-3, over 99% of the material is accounted for by D-β-hydroxybutyrate ester and its starting materials. The remaining percentage (less than 1%) not accounted for in the batch analyses is due to the derivatising reagent used for analytical identification purposes. The derivatising reagent is not present in the neat compound, as shown by the presence of small peaks in the chromatographic analysis of the reagent alone (Appendix B). These very minor compounds can be subtracted from the chromatographic peak profiles, resulting in purity that approaches 100%. It also should be noted that there is variability in the gas chromatography/mass spectrometry measurements.

I.f Other Chemical Characterisation

Three consecutive lots of D-β-hydroxybutyrate ester have been analysed for the elements carbon, hydrogen, and nitrogen. Based on the formula of D-β-hydroxybutyrate ester ($C_8H_{16}O_4$), the samples were expected to contain 54.53% carbon, 9.15% hydrogen, and 0% nitrogen. The results of the analyses are presented in Table I.f-1.

Table I.f-1 Elemental Analysis of D-β-Hydroxybutyrate Ester

	%C	%H	%N
Lot 7			
Analysis 1	54.05	9.21	ND
Analysis 2	54.04	9.28	ND
Lot 8			
Analysis 1	54.14	9.26	ND
Analysis 2	54.07	9.27	ND
Lot 9			
Analysis 1	54.01	9.24	ND
Analysis 2	54.31	9.23	ND

C = carbon; H = hydrogen; N = nitrogen; ND = below limit of detection (0.3%)

During the synthesis of D-β-hydroxybutyrate ester, ethanol is produced as a by-product; however, ethanol is removed *via* vacuum. Three lots of D-β-hydroxybutyrate ester were analysed using mass spectrometry for residual ethanol; the results of these analyses are presented in Table 1.f-2 below. The levels of residual ethanol are negligible and likely would not have been detected using methods other than mass spectrometry. Moreover, according to the International Conference on Harmonisation (ICH) guidelines for residual solvents in pharmaceuticals for human use, ethanol is considered a solvent with low toxic potential and residual ethanol at levels of 0.5% are acceptable without justification (ICH, 2011).

Table I.f-2 Analysis of D-β-Hydroxybutyrate Ester for Residual Ethanol

Manufacturing Lot	Residual Ethanol (%)
Lot 7	0.03
Lot 8	0.06
Lot 9	0.08

II EFFECT OF THE PRODUCTION PROCESS APPLIED TO D-β-HYDROXYBUTYRATE ESTER

Based on the SCF guidelines, the following questions must be addressed to ensure sufficient information pertaining to the effect of the production process applied to the novel food:

- "Does the novel food undergo a production process?"
- "Is there a history of use of the production process for the food?" If no, "does the process result in a significant change in the composition or structure of the novel food compared to its traditional counterpart?"
- "Is information available to enable identification of the possible toxicological, nutritional and microbiological hazards arising from use of the process?"
- "Are the means identified for controlling the process to ensure that the novel food complies with its specification?"
- "Has the process the potential to alter the levels in the novel food of substances with an adverse effect on public health?"
- "After processing is the novel food likely to contain microorganisms of adverse public health significance?"

These questions have been addressed collectively in Sections II.a through II.c.

II.a Raw Materials Used in the Manufacturing Process

Table II.a-1 Specifications of Raw Materials	
Specification Parameter	**Specification**
(*R*)-1,3-Butanediol	>99.0%
Ethyl (*R*) 3-Hydroxybutyrate	>99.0%

II.b Manufacturing Process

The D-β-hydroxybutyrate ester is produced *via* an enzyme-catalysed transesterification reaction of ethyl-D-hydroxybutyrate and (R)-1,3-butanediol that is catalysed by a lipase enzyme (see Figure II.b-1).

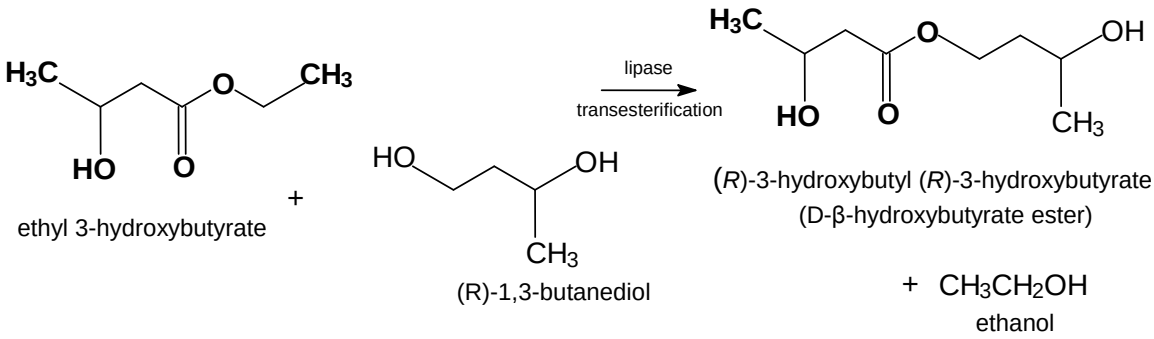


Figure II.b-1 Schematic Overview of the Manufacturing Process for D-β-Hydroxybutyrate Ester

II.c Stability of D-β-Hydroxybutyrate Ester

The stability of the D-β-hydroxybutyrate ester mixed with water (1:1) at different temperatures and different pH levels was assessed over a 330-day period. As shown in Figures II.c.1 to II.c.3, D-β-hydroxybutyrate ester generally remained stable when stored at temperatures ranging from 4 to 37°C and pH levels ranging from 3 to 10 throughout the storage period (shown in hours).

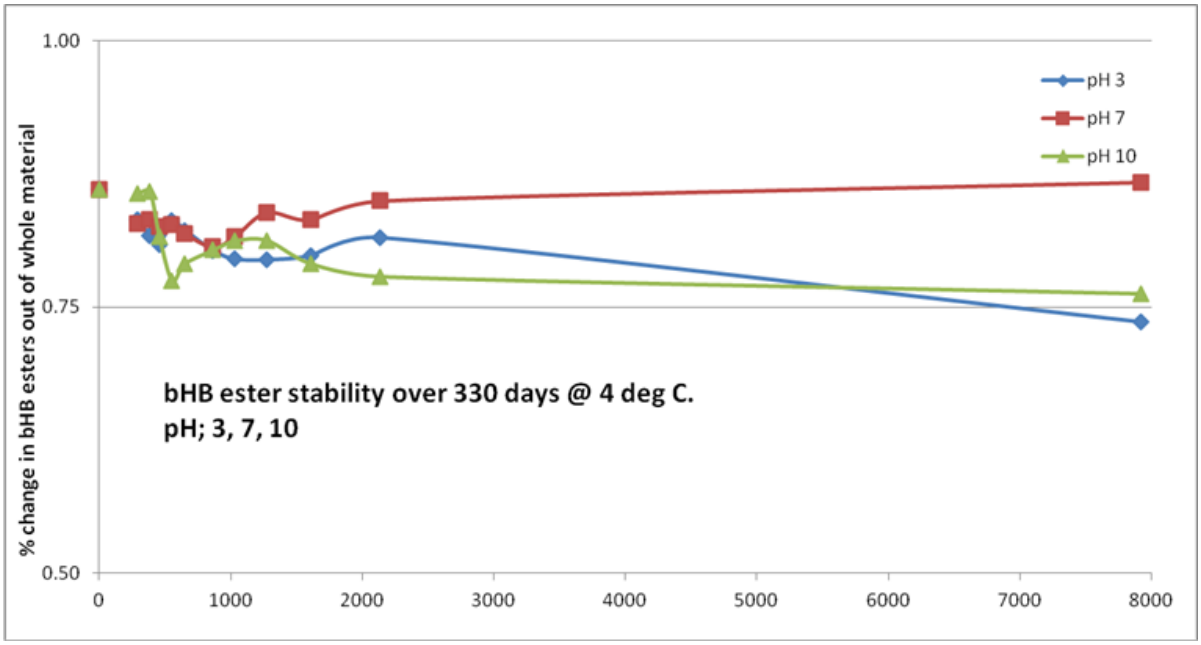


Figure II.c.1 Stability of D-β-Hydroxybutyrate at 4°C and pH of 3, 7, and 10

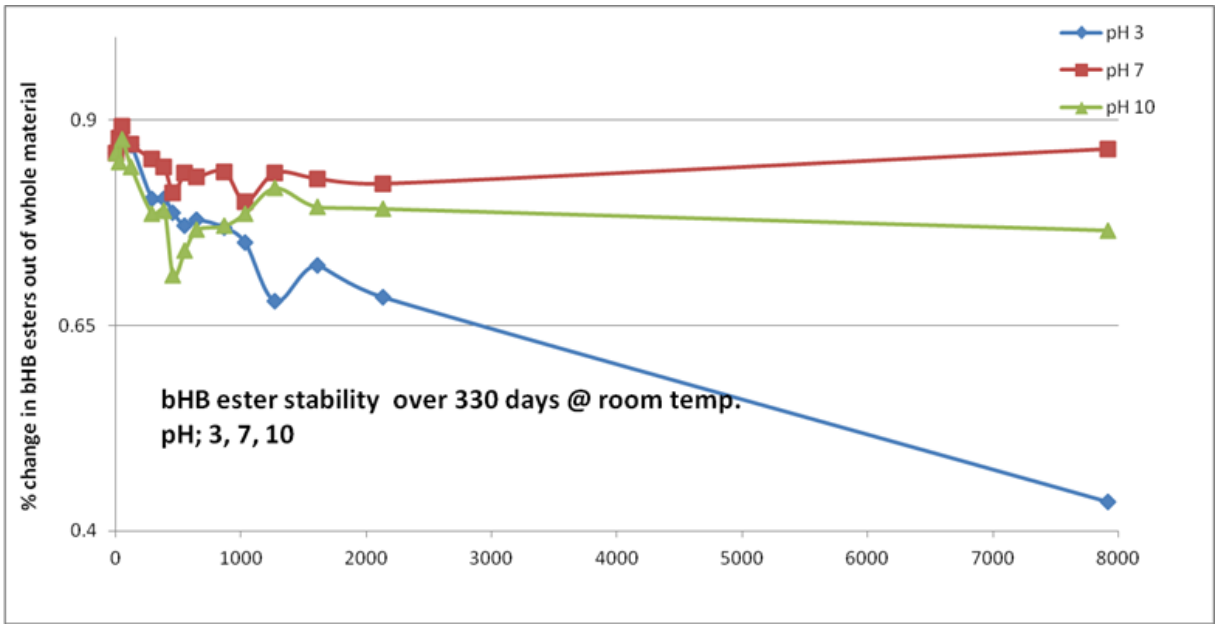


Figure II.c.2 Stability of D-β-Hydroxybutyrate at Room Temperature and pH of 3, 7, and 10

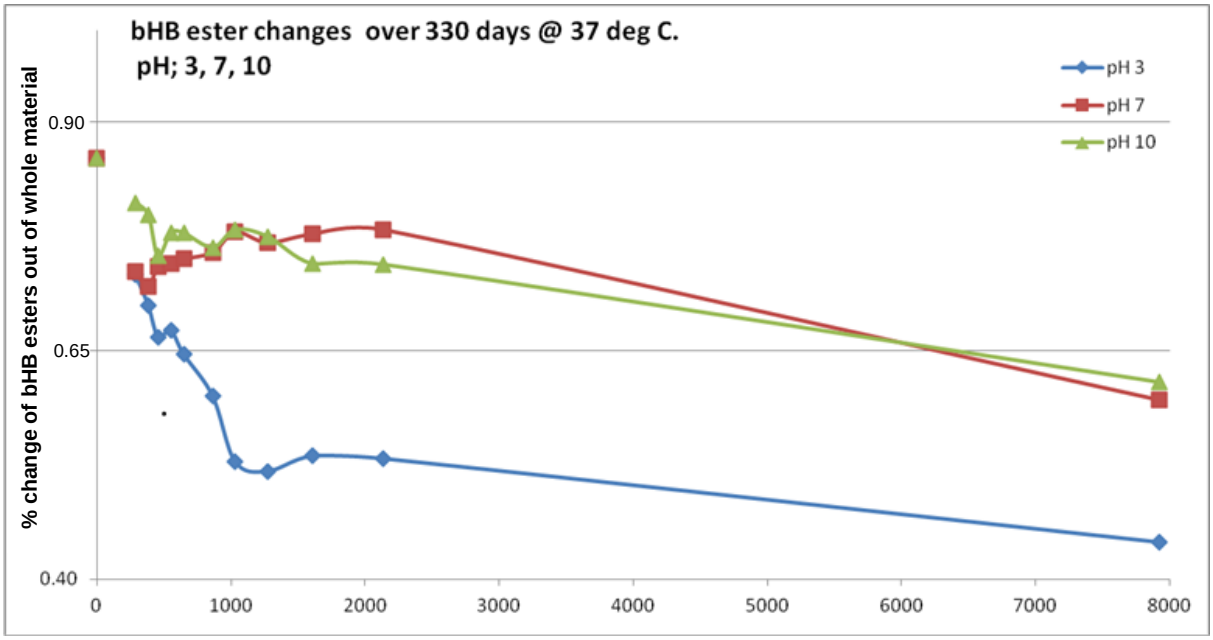


Figure II.c.3 Stability of D-β-Hydroxybutyrate at Elevated Temperatures (37°C) and pH of 3, 7, and 10

III HISTORY OF THE SOURCE ORGANISM OF D-β-HYDROXYBUTYRATE ESTER

Based on the SCF guidelines, the following questions must be addressed to ensure sufficient information pertaining to the history of the source organism:

- “Is the novel food obtained from a biological source, *i.e.*, a plant, animal or microorganism?”
- “Has the organism used as the source of the novel food been derived using GM?”
- “Is the source organism characterised?”
- “Is there information to show that the source organism and/or foods obtained from it are not detrimental to human health?”

D-β-hydroxybutyrate ester is produced *via* a synthetic process; therefore, the aforementioned questions are not applicable.

IV-VIII NOT APPLICABLE

IX INTAKE/EXTENT OF USE OF D-β-HYDROXYBUTYRATE ESTER

Based on the SCF guidelines, the following questions must be addressed to ensure sufficient information pertaining to the intake/extent of use of the novel food:

- “Is there information on the anticipated uses of the novel food based on its properties?”
- “Is there information to show anticipated intakes for groups predicted to be at risk?”
- “Will introduction of the novel food be restricted geographically?”
- “Will the novel food replace other foods in the diet?”

These questions have been addressed collectively in Sections IX.a through IX.b.

IX.a Estimated Consumption of D-β-Hydroxybutyrate Ester as Indicated for Food Supplement Use

IX.a.1 Introduction

TΔS proposes to market D-β-hydroxybutyrate ester in the EU in selected categories of food supplement products (liquids, powder (sachet), bars, and gels) designed exclusively for sports people and individuals undergoing extreme energy expenditure. The powder (sachet) products are to be diluted in water. D-β-Hydroxybutyrate ester will be added to these products to result in intakes that will not exceed 0.36 g/kg body weight/serving. It is envisaged that a maximum of 2 to 3 servings per day will be consumed, resulting in a maximum daily intake of 1.08 g/kg body weight. Based on an average 70 kg adult, this maximum will relate to 75 g D-β-hydroxybutyrate ester per day. These products will be marketed specifically to high-performance athletes.

As D-β-hydroxybutyrate ester-containing products will be used as specialised, food supplement products for consumption by high-performance athletes during exercise rather than as conventional foods for use by the general population, intakes of D-β-hydroxybutyrate ester were calculated on the basis of the proposed use-levels of the ingredient in each product category and the directions for use. A detailed intake assessment using European food consumption datasets was not conducted because the ingredient will not be consumed by the general population.

IX.a.2 Exposure Assessment

D-β-Hydroxybutyrate ester intakes were calculated based on the proposed use levels for bars, gels, powder (sachet), and liquids and possible combinations of these products following the directions for use of 2 (Table IX.a.2-1) to 3 (Table IX.a.2-2) servings per day. Estimated intakes on a per kilogram body weight basis were calculated using an average adult body weight of 70 kg.

With 2 servings per day, the highest estimated intake of D-β-hydroxybutyrate ester would be from 2 liquid or powder (sachet) supplements per day (50 g/day or 0.71 g/kg body weight/day), and the lowest estimated intake would be from 2 bars per day (22 g/day or 0.31 g/kg body weight/day).

With 3 servings per day, the highest estimated intake of D-β-hydroxybutyrate ester would be from 3 liquid or powder (sachet) supplements per day (75 g/day or 1.07 g/kg body weight/day), and the lowest estimated intake from 3 bars per day (33 g/day or 0.47 g/kg body weight/day).

Table IX.a.2-1 Total Daily Intake of D-β-Hydroxybutyrate Ester Based on 2 Servings per Day				
Bars (n servings) 11 g D-β-HB per serving	**Gels (n servings), 18 g D-β-HB per serving**	**Liquid or powder Supplements (n servings), 25 g D-β-HB per serving**	**Total D-β-hydroxybutyrate ester**	
			g/day	**g/kg body weight/d**
2	0	0	22 g	0.31
0	2	0	36 g	0.51
0	0	2	50 g	0.71
1	1	0	29 g	0.41
0	1	1	43 g	0.61
1	0	1	36 g	0.51

D-β-HB = D-β-hydroxybutyrate ester

Table IX.a.2-2 Total Daily Intake of D-β-Hydroxybutyrate Ester Based on 3 Servings per Day				
Bars (n servings) 11 g D-β-HB per serving	**Gels (n servings) 18 g D-β-HB per serving**	**Liquid or powder Supplements (n servings) 25 g D-β-HB per serving**	**Total D-β-hydroxybutyrate ester**	
			g/day	**g/kg body weight*/d**
3	0	0	33 g	0.47
0	3	0	54 g	0.77
0	0	3	75 g	1.07
2	1	0	40 g	0.57
2	0	1	47 g	0.67
1	0	2	61 g	0.87
1	2	0	47 g	0.67
1	1	1	54 g	0.77
0	2	1	61 g	0.87
0	1	2	68 g	0.97

D-β-HB = D-β-hydroxybutyrate ester
*Assuming a body weight of 70 kg.

IX.a.3 Summary and Conclusions

In summary, D-β-hydroxybutyrate ester intakes were calculated based on possible combinations of bars, gels, and liquid or powder (sachet) supplements following the directions for use of 2 to 3 servings per day. Based on 2 servings per day, the highest estimated intake of D-β-hydroxybutyrate ester was from an intake of 2 liquid or powder (sachet) supplements per day (50 g/day or 0.71 g/kg body weight/day), and the lowest estimated intake was from an intake of 2 bars per day (22 g/day or 0.31 g/kg body weight/day). Based on 3 servings per day, the highest estimated intake of D-β-hydroxybutyrate ester was from an intake of 3 liquid or powder (sachet) supplements per day (75 g/day or 1.07 g/kg body weight/day), and the lowest estimated intake was from an intake of 3 bars per day (33 g/day or 0.47 g/kg body weight/day). Based on the specialised use of D-β-hydroxybutyrate ester as an energy source during exercise in elite athletes rather than use in conventional foods by the general population, these values are considered to be

reasonable estimates of consumption. The high cost and sharp, bitter taste of products containing D-β-hydroxybutyrate ester will deter the general population from using these products for general energy purposes.

IX.b Labelling Instructions

D-β-Hydroxybutyrate ester is proposed for use in selected food supplement products. As per Directive 2002/46/EC (European Parliament and Council of the European Union, 2002), Article 1 "concerns food supplements marketed as food stuffs and presented as such. These products shall be delivered to the ultimate consumer only in a pre-packaged form" (European Parliament and the Council of the European Union, 2002). D-β-Hydroxybutyrate ester would be considered an "other substance with a nutritional or physiological effect" and products containing the ingredient must be labelled in accordance with the requirements outlined in Article 6(3) of Directive 2002/46/EC.

Without prejudice to Directive 2000/13/EC (European Parliament and the Council of the European Union, 2000), the labelling for products containing D-β-hydroxybutyrate ester shall bear the following particulars:

a) the names of the categories of nutrients or substances that characterise the product or an indication of the nature of those nutrients or substances;

b) the portion of the product recommended for daily consumption;

c) a warning not to exceed the stated recommended daily dose;

d) a statement to the effect that food supplements should not be used as a substitute for a varied diet; and,

e) a statement to the effect that the products should be stored out of reach of young children.

The consumption of food supplements containing D-β-hydroxybutyrate ester, therefore, will be controlled to the consumer and should not pose a safety concern.

X INFORMATION FROM PREVIOUS HUMAN EXPOSURE TO D-β-HYDROXYBUTYRATE ESTER

Based on the SCF guidelines, the following questions must be answered in the affirmative to ensure sufficient information pertaining to previous human exposure to the novel food:

- “Is there information from previous direct, indirect, intended or unintended human exposure to the novel food or its source which is relevant to the EU situation with respect to production, preparation, population, lifestyles and intakes?”

- “Is there information to demonstrate that exposure to the novel food is unlikely to give rise to mitochondrial, toxicological and/or allergenicity problems?”

These questions have been addressed collectively in Sections X.a and X.b.

X.a Natural Occurrence of D-β-Hydroxybutyrate Ester in the Diet

The D-β-hydroxybutyrate ester is a synthetic compound with no history of human consumption; however, its metabolites, namely (R)-1,3-butanediol and D-β-hydroxybutyrate, occur in nature. Although no quantitative data were available (levels not specified), the diol has been detected in parmesan cheese and Cupuacu, which is a commonly consumed fruit in Amazonia, Brazil, and Venezuela (Nijssen *et al*., 1996). In addition to its natural presence in the diet, 1,3-butanediol also is added to foods for use as a flavouring agent and solvent for flavouring agents (Burdock, 2009; FCC, 2010). Daily consumption of 1,3-butanediol as a result of its addition to foods is estimated to be approximately 0.008 mg/kg body weight/day (Burdock, 2009).

D-β-hydroxybutyrate has been detected in cow's milk, with levels ranging from 10 to 631 µM (Larsen and Nielsen, 2005). Since ketone bodies are of mitochondrial origin, other foods of animal origin are likely to contain small amounts of D-β-hydroxybutyrate, though quantitative data were not identified in the literature.

X.b Potential Allergenicity Concerns

Three batches of D-β-hydroxybutyrate ester (Batch #7, 8, and 9) have been analysed for protein (≥ 10 kDa) using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE). Protein was not detected in the analysed samples (Appendix D).

XI NUTRITIONAL INFORMATION ON D-β-HYDROXYBUTYRATE ESTER

Based on the SCF guidelines, the following question must be answered in the affirmative to ensure sufficient nutritional information pertaining to the novel food:

- “Is there information to show that the novel food is nutritionally equivalent to existing foods that it might replace in the diet?”

This question has been addressed in Section XI.a.

XI.a Nutritional Information on D-β-Hydroxybutyrate Ester

As mentioned, D-β-hydroxybutyrate ester is a synthetic compound that does not have a history of use in the diet; therefore, it will not be used to replace existing foods in the diet. The caloric value of D-β-hydroxybutyrate ester has been determined to be 4.7 kcal/g.

XII MICROBIOLOGICAL INFORMATION ON D-β-HYDROXYBUTYRATE ESTER

Based on the SCF guidelines, the following question must be addressed to ensure sufficient microbiological information on the novel food:

- “Is the presence of any microorganisms or their metabolites due to the novelty of the product/process?”

This question has been addressed in Section XII.a.

XII.a Microbiological Specifications and Analyses for D-β-Hydroxybutyrate Ester

The microbiological specifications for D-β-hydroxybutyrate ester are presented in Table XII.a-1.

Table XII.a-1 Microbiological Specifications for D-β-Hydroxybutyrate Ester

Specification Parameter	Specification (CFU/mg)	Method
Escherichia coli	< 5	HPA Standard Method F17, issue 2.4 May 2005
Moulds	< 10	BS 4285-3.6: 1986
Yeasts	< 10	BS 4285-3.6: 1986

BS = British Standards; CFU = colony forming unit; HPA = Health Protection Agency

Microbiological analyses for 5 batches of D-β-hydroxybutyrate ester are presented in Table XII.a-2.

Table XII.a-2 Summary of the Microbiological Product Analysis for 5 Lots of D-β-Hydroxybutyrate Ester

Specification Parameter	Specification (CFU/mg)	Manufacturing Lot				
		Batch 1	Batch 2	Batch 3	Batch 4	Batch 5
Escherichia coli (CFU/mg)	<5	<5	<1	<1	<5	<1
Moulds (CFU/mg)	<10	<10	<1	<1	<10	<1
Yeasts (CFU/mg)	<10	<10	<1	<1	<10	<1

CFU = colony forming unit

XIII TOXICOLOGICAL INFORMATION ON D-β-HYDROXYBUTYRATE ESTER

Based on the SCF guidelines, the following questions must be addressed to ensure sufficient toxicological information pertaining to the novel food:

- “Is there a traditional counterpart to the novel food that can be used as a baseline to facilitate the toxicological assessment?”
- “Compared to the traditional counterpart, does the novel food contain any new toxicants or changed levels of existing toxicants?”

or

- “Is there information from a range of toxicological studies appropriate to the novel food to show that the novel food is safe under anticipated conditions of preparation and use?”
- “Is there information which suggests that the novel food might pose an allergenic risk to humans?”

These questions have been addressed collectively in Section XIII.a.

XIII.a Introduction

The safety of D-β-hydroxybutyrate ester is supported by the results of studies conducted on the compound, specifically, a 28-day toxicity study in rats, a 66-day rat study that included selected safety parameters, a developmental toxicity study in rats, and 2 human studies in which subjects consumed D-β-hydroxybutyrate ester for 1 or 5 days. Safety is corroborated by animal feeding trials on the related compound 1,3-butanediol mono- and diacetoacetate.

Following oral administration, D-β-hydroxybutyrate ester is fully hydrolysed to D-β-hydroxybutyrate and (R)-1,3-butanediol in the gut, with the latter being further metabolised to D-β-hydroxybutyrate and acetoacetate in the liver, as discussed in Section XIII.b. Thus, data on each of the metabolites were used in support of the safety of D-β-hydroxybutyrate ester.

D-β-hydroxybutyrate ester is a synthetic compound that does not occur endogenously; however, the only metabolites of the ester are the ketones D-β-hydroxybutyrate and acetoacetate, which are normally produced in the body. As discussed in Section XIII.f, maximal ketone body production is approximately 185 g/day during times of limited glucose availability, indicating that the human body has the capacity to handle large amounts of ketones. Furthermore, there is a long history of safety of ketogenic diets, which, similar to D-β-hydroxybutyrate ester, elevate ketone levels.

XIII.b Metabolic Fate

JECFA conducted an evaluation of the metabolism of a group of aliphatic primary alcohols, aldehydes, carboxylic acids, acetals, and esters containing additional oxygenated functional groups (JECFA, 2000). It was noted by JECFA that the aliphatic esters, such as methyl or ethyl 3-hydroxyhexanoate, are expected to undergo hydrolysis to their corresponding alcohols and that the presence of a second oxygenated functional group has little if any effect on hydrolysis of the ester (JECFA, 2000). The hydrolysis of these esters is catalysed by carboxylesterases or esterases distributed throughout the intestinal tract, blood, liver, and other tissues (Heymann, 1980; Anders, 1989).

A study has been conducted to investigate the kinetics of orally administered D-β-hydroxybutyrate ester (Appendix E). Twenty-two male and female Wistar rats, weighing 350 to 500 g, were implanted with an atrial cannula 24 hours prior to the experiment, and were then gavaged with 0.5 (n=3 males), 1.0 (n=3 males and 3 females), or 5.0 (n=7 males and 6 females) g/kg body weight of D-β-hydroxybutyrate ester in a 50:50 mixture with fat-free sour cream. Metabolite levels [D-β-hydroxybutyrate, acetoacetate, (R)-1,3-butanediol, and D-β-hydroxybutyrate ester] were measured in whole blood at 0, 15, 30, 60, 120, 240, and 360 minutes by gas chromatography-mass spectrometry, following a 3-step derivatisation procedure to chemically modify the analytes for GC-MS analyses since the compounds contained a mixture of functional groups (carboxylic acid, hydroxyl groups, and ketones) that required alteration prior to GC-MS analysis. The hydroxyl groups were

derivatised to their trimethylsilyl ethers (TMS), the carboxylic functional groups were esterified to the pentafluorobenzyl esters (PFB) and the ketone (AcAc) was derivatised to the methyloxime derivative. The steps were carried out sequentially on 20 µL of the extract mentioned above. The derivatising reagents were prepared freshly for use and the dried samples were handled in the routine procedures for derivatising the functional groups. That is samples were brought up in 20 µL of each reagent and heated to 60°C for 5 to 12 minutes depending on the reaction step and then each reagent was evaporated under a stream of nitrogen. Samples were finally brought up in 50 µL of TMS and acetonitrile (1:1). One µL of the sample was injected onto a 30 metre capillary DB-1 column and the instrument was operated in the electron impact mode and scanned for the appropriate ions. Analytes eluted from the column between 5.5 to 15 minutes after injection and were quantified using 2 internal standards (1,4 butanediol and D4-BHB) that were added to the samples prior to derivatisation. Initially, known quantities of the D-β-hydroxybutyrate ester, butanediol, 13C-AcAC were added to samples in commensurate amounts and used as external references to each of the time point samples. Peak area counts of the analytes and the internal standards were used to compute quantities present in whole blood. Repeated extraction of whole blood samples and repeated derivatisation of aliquots of extracts were used to determine precision of the methods. Reproducibility was approximately 15%.

At a dosage of 0.5 g/kg body weight of the D-β-hydroxybutyrate ester, mean blood D-β-hydroxybutyrate and (R)-1,3-butanediol levels were 0.5 mM and 0.055 mM, respectively, returning to baseline levels by 6 hours. Blood acetoacetate and the D-β-hydroxybutyrate ester were not detectable when the animals were gavaged with 0.5 g/kg body weight of the D-β-hydroxybutyrate ester. At a dosage of 1.0 g/kg body weight, maximal blood D-β-hydroxybutyrate and acetoacetate levels were 1.1 and 1.5 mM, respectively, in males. In females, maximal blood D-β-hydroxybutyrate was 0.78 mM (acetoacetate was not detectable). Maximal blood levels of (R)-1,3-butanediol were 0.13 mM and 0.17 mM in males and females, respectively, while maximal blood levels of the D-β-hydroxybutyrate ester were not detected in males or females. All metabolites returned to baseline by 6 hours.

At a dosage of 5 g/kg body weight, blood D-β-hydroxybutyrate and acetoacetate were maximal at 30 minutes, reaching 12.1 mM and 5.9 mM, respectively, in males and 8.5 mM and 6 mM in females, respectively. Maximum total blood ketone levels were 18 mM and 14.5 mM in males and females, respectively, decreasing at 6 hours to 5.4 mM in both sexes. 1,3-Butanediol was maximal at 1 hour, reaching 1.3 mM and 2.9 mM in males and females, respectively; by 6 hours, blood (R)-1,3-butanediol decreased to approximately 0.2 mM in both sexes. D-β-Hydroxybutyrate ester was maximal at 15 minutes, reaching 0.05 and 0.11 mM in males and females, respectively. The rate constant for D-β-hydroxybutyrate ester disappearance from blood was 0.049 min^{-1} for males and 0.107 min^{-1} for females, with a half-life of 14 minutes for males and 6.5 minutes for females.

This study provides evidence that only limited amounts of the ketone ester are present intact systemically following oral administration of large amounts, while circulating levels of

ketones (*i.e.,* D-β-hydroxybutyrate and acetoacetate) are readily increased. Similar findings have been reported in other studies. In a 66-day rat study where the ketone ester was incorporated into the diet, described in detail in Section XIII.c1, the intact D-β-hydroxybutyrate ester was not detected in the blood (limit of detection at 1 µM) (Appendix F). In contrast, levels of D-β-hydroxybutyrate were increased by nearly 2-fold in animals fed diets supplemented with the ketone ester compared to controls. Moreover, the metabolic fate of the D-β-hydroxybutyrate ester has been examined in an ascending dose study conducted in healthy volunteers (6/group) (Clarke *et al.*, 2012a). Plasma levels of D-β-hydroxybutyrate and acetoacetate were readily elevated following administration of a single drink of the ketone ester (140, 357, and 714 mg/kg body weight), while the intact compound was not detected. Maximum plasma levels of ketones were achieved within 1.5 to 2.5 hours, reaching 3.30 mM and 1.19 mM for β-hydroxybutyrate and acetoacetate, respectively, at the highest amount of the ketone ester tested. In this study, the elimination half-life was found to range from 0.77 to 3.06 hours for β-hydroxybutyrate, and from 8 to 14 hours for acetoacetate. No gender differences in the pharmacokinetic parameters of D-β-hydroxybutyrate or acetoacetate were reported.

The kinetics of D-β-hydroxybutyrate ester hydrolysis in human plasma also have been examined *in vitro* (Appendix G). In this assay 1.5, 2, 2.25, 2.5, 2.75, 3.0, and 5 mmol/L solutions of D-β-hydroxybutyrate ester were incubated with 200 µL of fresh human plasma at 37°C for 75 minutes. The D-β-hydroxybutyrate content of the plasma was assayed every 15 minutes. At the lowest tested amount, the rate of D-β-hydroxybutyrate ester hydrolysis was 0.014 mM/minute, while at the highest amount tested the rate of hydrolysis was 0.041 mM/minute. The K_m for this reaction was calculated to be 23.8 mM and the maximum rate of metabolism (V_{max}) was calculated to be 0.24 mM/minute. The results of this assay indicate that the D-β-hydroxybutyrate ester would be rapidly hydrolysed into (R)-1,3-butanediol and D-β-hydroxybutyrate in plasma, in agreement with the findings described above.

XIII.c Studies on D-β-Hydroxybutyrate Ester

XIII.c.1 Repeated Dose Studies

The toxicity of D-β-hydroxybutyrate ester was evaluated in a 28-day study (Clarke *et al.*, 2012b). Sixty, 9-week-old Wistar rats (10 animals per sex per group) received a diet containing D-β-hydroxybutyrate ester[1] (providing 31% of calories from D-β-hydroxybutyrate ester) or 1 of 2 control diets [a fat-based diet, providing 34% of calories from fat, or a carbohydrate (CHO)-based diet providing 70% of calories from CHOs]. The ketone ester diet was formulated to contain 30% of the calories from the D-β-hydroxybutyrate ester, resulting in a ketone ester intake of 12.0 and 15.1 g/kg body weight/day over the 28-day study for male and female rats, respectively. Animals were monitored for clinical signs, body weight, and food consumption. At the end of the study period, haematology, coagulation,

[1] In the 28-day, 66-day, and developmental toxicity rat studies, the test material consisted of ethyl-(*R*)-3-hydroxybutyrate (~1%) and (*R*)-1,3-butanediol (~1%), which were the starting materials, and the ketone ester [~98%, representing (*R*)-3-hydroxybutyl (*R*)-3-hydroxybutyrate (~94%), 3-betahydroxybutryl 1,3-butanediol monoester (~1%), and di-β-hydroxybutyrate 1,3-butanediol diester (~3%)].

clinical chemistry, and urinalysis parameters were assessed, organs were weighed, and gross and microscopic examination was undertaken.

Rats in the ketone ester group consumed significantly less diet and gained significantly less weight compared with rats in the control groups. Decreased food consumption may have been caused by the palatability of the diet containing D-β-hydroxybutyrate ester. Additionally, the reduced food consumption and weight gain in this study are consistent with reports of decreased hunger, reduced energy intakes, and increased weight loss in subjects consuming low-carbohydrate ketogenic diets compared to low-fat diets or medium-carbohydrate non-ketogenic diets (McClernon *et al.,* 2007; Johnstone *et al.*, 2008).

All animals survived to the scheduled necropsy date. Daily cage side observations and detailed weekly physical examinations showed no treatment-related toxicity in animals of both sexes in all 3 diet groups. The exception was one male rat in the ketone ester-fed group which showed piloerection, decreased food consumption, and weight loss. Piloerection was not observed after the third week of dosing and although this animal did not fully regain initial body weight, it was gaining weight and otherwise appeared normal by the end of the study.

Urinalysis values did not significantly differ among groups. Between-group differences were noted in several haematology, coagulation, and clinical chemistry parameters; however, mean values were within normal physiological ranges. The only exception was noted in lactate dehydrogenase (LDH) levels, which were significantly higher in ketone ester-fed rats (both sexes compared to control animals). The increases were small in magnitude and were not associated with haemolysis or histological findings. LDH levels vary greatly in rats, as demonstrated by the historical control ranges for Sprague-Dawley rats in the testing facility of 100 to 7,201 U/L in males and 20 to 5,236 U/L in females. LDH levels in the ketone ester-fed rats were within this range and thus were not considered to be of toxicological relevance. The observed elevated levels may be related to the release of LDH during regular handling of rats, such as grasping, dosing, *etc.* (Yerroum *et al*., 1999); blood collection procedures (Friedel *et al*., 1974); or enzyme release from cellular elements during clotting (Friedel and Mattenheimer, 1970). Furthermore, LDH levels were unaffected by D-β-hydroxybutyrate ester consumption in the 66-day rat study described below. Similarly, no subjects had LDH levels above the normal range in the 5-day clinical study discussed in Section XIII.c.3.

There were no statistically significant differences in the absolute weight and in the body/brain weight ratios among groups for the following organs: spleen, liver, adrenals, testes, kidneys, prostate, lungs, heart, thymus, brain, pituitary, seminal vesicles and ovaries. The uterine weight of ketone ester-fed rats was smaller than the uterine weight of female rats fed the CHO and fat diets. No difference was noted in the relative uterus weight, indicating that the difference in absolute uterine weight was a result of the smaller body weight of female rats fed the ketone ester diet, and not an adverse effect of the ketone ester. It also should be noted that uterine weights of rats in all 3 groups were within the laboratory's historical range for uterine weight.

No gross or histopathological abnormalities of toxicological significance were observed in the heart, kidney, stomach, duodenum, ileum, colon, or brain. Two male rats and 4 female rats that received the fat diet, as well as 1 female rat given the ketone diet, had slight yellow discoloration of livers, presumed to be fat accumulation. Liver vacuolation was observed in all female rats in all 3 groups; likewise, necroinflammatory foci were observed in some animals (males and females) in all groups. Given that these findings were present in all groups and that liver function enzyme levels were within normal ranges, it is unlikely that they were related to consumption of the D-β-hydroxybutyrate ester. The diets were formulated by the separate addition of macronutrients (fat, CHO, ketone ester) to the rat chow, so the vitamin and mineral compositions of each diet were diluted, which may explain the observed findings.

With respect to muscle tissue, myocyte necrosis and repair, as well as focal histiocytosis, were noted in several animals in all 3 groups. Muscle changes were graded as minimal with the exception of 1 male and 1 female in the ketone ester group; effects in these animals were graded as mild. There were no animals in which muscle changes were graded as moderate, marked, or severe.

Based on the results of the study, the authors concluded that that consumption of D-β-hydroxybutyrate ester at 30% of the total calorie intake (12.0 g/kg body weight/day and 15.1 g/kg body weight/day in male and female rats, respectively) in the diet did not result in adverse effects.

A 66-day study was conducted to examine the effect of oral administration of the D-β-hydroxybutyrate ester on the physical performance and cognitive function of the rat, as well as any effect on the general health of the animals (Appendix F). A total of 50 young male Wistar rats were procured for the study. The rats were randomised to receive one of three 1.76 kcal/g diets: 1) a Western diet (n=20), 2) a high-CHO diet (n=10) or 3) a ketone ester diet (n=20) the compositions of which are detailed in Table XIII.c.1-1.

Table XIII.c.1-1 Composition of Test Diets

Diet	Fat (% kcal)	Protein (% kcal)	Carbohydrate (% kcal)	Ketone Ester (% kcal)
Western	34	27	39	0
Carbohydrate	4	26	70	0
Ketone	4	27	39	30

The physical performance of the rats was measured using a treadmill and cognitive performance was measured using an 8-arm radial maze. On the first day of the experimental protocol (Day 0), the rats consumed standard chow diets and began to become habituated to the maze. Each rat was placed in the maze twice daily for 14 days to allow them to locate the rewards placed in each arm of the maze. On Day 7 of the experimental protocol the rats began their treadmill habituation, during which belt speed and incline were gradually increased until all rats were able to complete a 5-minute session at a speed of 10 metres/minute on a 5° incline. The rear of the treadmill delivered a small electric stimulus

of <1.6 mAmps. On Days 15 through 17 of the protocol the rats were subjected to baseline assessments of physical ability and cognitive function. The physical assessment consisted of a 5 minute session on the treadmill at 10 metres/minute on a 5° incline, with the speed increasing by 1 metre/minute every minute thereafter until the rat fatigued. Fatigue was defined as either the point at which the rats could no longer keep pace with the treadmill or when the rat had received the longest permitted electric stimulus (not greater than 5 seconds). This assessment was repeated 3 times daily with a minimum of 1 hour of rest allowed between each assessment. After the third session on the treadmill the rats were placed in the maze for their cognitive assessment. In the maze, the movement of the rats was recorded until they located all 8 rewards or 5 minutes had elapsed.

On Day 18 the rats were rested and placed on the experimental diets. The rats were allowed 4 days to acclimate to the diets before physical and cognitive assessments were made, which continued through to Day 26. At baseline the physical performance of all rats was comparable. After consumption of the experimental diets a significant change in baseline performance was observed only in the rats consuming the ketone ester diet. These rats increased the distance run during the physical assessment by approximately 22% over baseline and ran approximately 32% further than rats consuming the other diets. Similarly, the time required to complete the maze was the same for all rats at baseline and only the ketone ester fed rats displayed a significant change in this marker of cognitive function. The ketone ester rats completed the maze 38% faster than rats on the Western or CHO diets. Additionally, the D-β-hydroxybutyrate ester fed rats made significantly more correct decisions before making an error compared to baseline trials. Following the physical and cognitive function test days, 10 rats consuming the Western diet and 10 rats consuming the ketone ester diet were euthanised and blood and tissue samples were collected. The remaining rats were left in their cages and pair fed the diets for an additional 7 weeks. At the end of 66 days of diet consumption the rats were sacrificed and blood and tissue samples were collected.

The average caloric intake during the run-in was 33 kcal/day and the average caloric intake during the 5-day test period was 39 kcal/day. Body weights remained constant during the 5-day test period and, as a result, the consumption of the ketone ester diet was calculated to provide approximately 11.7 g/kg body weight/day during this time. Similarly, no significant differences were observed in the body weight gain of the animals consuming the Western, the CHO, or the ketone ester diet over the course of the following 7 weeks, during which the rats consumed an average of 53 kcal/day, providing an average D-β-hydroxybutyrate ester intake of 13.7 g/kg body weight/day.

The body weights and heart weights of rats consuming the Western diet and the ketone ester diet did not differ significantly after 9 or 66 days of diet consumption. As expected there were no corresponding differences in the ratio of body weight to heart weight. After 9 and 66 days of diet consumption the epididymal fat weight and the ratio of epididymal fat weight to body weight was significantly lower in the rats consuming the ketone ester diet than in those consuming the Western diet. These parameters also were significantly lower

in the rats consuming the high-CHO diet for 66 days, compared to those consuming the Western diet. The plasma β-hydroxybutyrate levels of the rats fed the Western diet were consistently approximately 2-fold lower than those of the rats fed the ketone ester diet, 0.41 mM compared to 0.85 mM after 9 days of diet consumption and 0.48 mM compared to 0.91 mM after 66 days of diet consumption. No significant difference was observed between the levels of (R)-1,3-butanediol and acetone present in the plasma after consuming the Western and ketone ester diets for 9 days. At no time point was the D-β-hydroxybutyrate ester detected in the blood. After 9 days of consuming the experimental diets, the plasma cholesterol and triglyceride levels were significantly lower in the rats fed the ketone ester diet than those fed the Western diets. After 66 days these parameters were 52 and 40% lower, respectively, in the D-β-hydroxybutyrate ester fed rats. The plasma glucose of the rats on the Western diet did not differ significantly from that of the rats on the ketone ester diet after 9 days; however, after 66 days the plasma glucose was 33% lower in the rats of the D-β-hydroxybutyrate ester group compared to those in the Western group. Plasma free fatty acid levels and LDH activity were not significantly different in rats consuming either diet at any time point. Additionally, no significant differences were observed in the analysis of tissues collected from Western and ketone ester-fed rats after 9 days of diet consumption.

In both the 28-day and 66-day rat studies, rats consumed D-β-hydroxybutyrate ester at levels 11 to 13 times higher than the maximum estimated intake in humans based on the proposed uses. It would have been difficult to achieve a larger margin of safety in the aforementioned studies because the ingredient is a macronutrient and will be consumed at a maximum level of 1.07 g/kg body weight/day. To attain a margin of safety of 100, the test diets in these studies would have to provide the ingredient at a level of 107 g/kg body weight/day, which would not be possible due to displacement of nutrients and the palatability of the ingredient.

XIII.c.2 Reproductive and Developmental Toxicity

A developmental toxicity study was conducted wherein 25 pregnant Crl:WI(Han) rats per group were administered 2 g/kg body weight D-β-hydroxybutyrate ester or reverse osmosis deionised water (as a control substance) once daily by oral gavage on Days 6 through 20 of gestation (DGs 6 to 20), at a dosage volume of 2 mL/kg body weight (Clarke *et al.*, 2012b). On DG 21, rats were euthanised, Caesarean-sectioned, and examined for gross lesions. All foetuses were examined for external abnormalities. Approximately one-half of the foetuses in each litter were examined for visceral abnormalities, while the remaining foetuses were examined for skeletal abnormalities.

Decreased body weight gains and body weight corrected for gravid uterine weight were observed in dams administered D-β-hydroxybutyrate ester compared to controls; food consumption was similarly reduced. These findings are expected given that the test article provided caloric value whereas the control article (water) did not. Maternal ALT and ALP values were lower in the test group relative to controls; however, in the 28-day study, no significant effects on ALT were noted in female rats fed the ketone ester diet and ALP levels

were only lower in comparison to the fat control group but not the CHO control group. Moreover, no gross or histopathological effects suggestive of liver toxicity were noted.

Pregnancy occurred in 22 rats in the control group and 24 rats in test group. The litter averages for corpora lutea, implantations, the percentage of preimplantation loss, litter sizes, live and dead foetuses, early and late resorptions, the percentage of postimplantation loss, the percentage of resorbed conceptuses, and the percentage of live male foetuses were comparable among the groups.

Male foetal body weights in the test group were significantly lower compared to controls; however, combined foetal weights did not significantly differ, the percent difference from the control group was less than 5%, and the average value was within historical ranges at the test facility.

There were no significant between-group differences in the litter or foetal incidences of any gross external, soft tissue, or skeletal abnormalities (malformations or variations), nor were there differences in foetal ossification site averages. The number of foetuses with any alteration observed and percentage of foetuses within a litter with any alteration observed were significantly higher in the D-β-hydroxybutyrate ester group compared to controls. These findings were driven by skeletal variations; however, it should be noted that the incidences of skeletal variations did not significantly differ between groups. Therefore, the observed skeletal variations were not considered to be of toxicological concern.

The authors concluded that D-β-hydroxybutyrate ester did not adversely affect the development of rats exposed to the ingredient *in utero* at a level of 2 g/kg body weight/day.

XIII.c.3 Human Studies

The safety and tolerability of D-β-hydroxybutyrate ester have been investigated in human studies. Ingestion of the D-β-hydroxybutyrate ester in a meal replacement milkshake beverage was without adverse effects in participants administered a single dose at up to 714 mg/kg body weight in the pharmacokinetic study described in Section XIII.b. More recently, a clinical study was undertaken to assess the tolerability of D-β-hydroxybutyrate ester under the intended conditions of use (see Appendix H). In this randomised, blinded, placebo-controlled, cross-over study, citrus-flavoured sports water drinks were used as the matrix for the ingredient as they mask the flavour of D-β-hydroxybutyrate ester more effectively compared to milkshake. Forty-two adult men consumed a vitamin water drink to which was added 1.23 g β-hydroxybutyrate ester/kg body weight or 1.44 g dextrose/kg body weight (both drinks also contained 0.1 g fructose/mL). The subjects drank a total volume of 7.6 mL/kg body weight (approximately 578 mL total volume), divided into 3 drinks of equal volume, at 10 min before starting the exercise session, and at 65 min and 130 min of the cycling session (so 1.23 g β-hydroxybutyrate ester/kg body weight consumed within 140 min). There was a 72-hour washout period between each test protocol.

Subjects completed a Gastrointestinal Symptoms Questionnaire 7 times during the protocol, at 0, 40, 65, 105, 130, 170, and 195 min of the exercise session. In the questionnaire, subjects rated the severity of symptoms on a scale of 0 (none) to 8 (unbearable) for upper abdominal problems (heartburn, bloating, nausea, and vomiting), lower abdominal problems (intestinal cramps, abdominal pain, flatulence, and diarrhoea), and systemic problems (dizziness, headache, muscle cramp, and urge to urinate). The most severe score for each symptom was chosen as the final score for each subject. A score of 0 was classified as "none", scores of 1, 2, or 3, were classified as "mild", scores of 4 or 5 were classified as "moderate", scores of 6 or 7 were classified as "severe", and a score of 8 was classified as "unbearable". Blood samples were collected concurrently with the completion of the questionnaire for the measurement of D-β-hydroxybutyrate, glucose, lactate, glycerol, insulin, and free fatty acid levels.

Blood D-β-hydroxybutyrate levels increased to ~1 mM 10 min after the first drink of ketone monoester, rising with each successive drink to reach ~4.0 mM at the end of exercise. Levels of glucose, lactate, glycerol, insulin, and free fatty acids in the blood were not adversely impacted by the consumption of D-β-hydroxybutyrate ester.

As shown in Figure XIII.c.3-1, gastrointestinal symptoms occurred in few subjects, and there were no significant differences in symptom severity between the D-β-hydroxybutyrate ester and placebo arms of the study. No severe adverse events occurred. The findings of this study demonstrate that D-β-hydroxybutyrate ester is well-tolerated under the intended conditions of use (*i.e.*, provided at a dose consistent with the proposed uses or the ingredient, consumed before and during exercise, and consumed in reasonable volumes in a sports drink matrix).

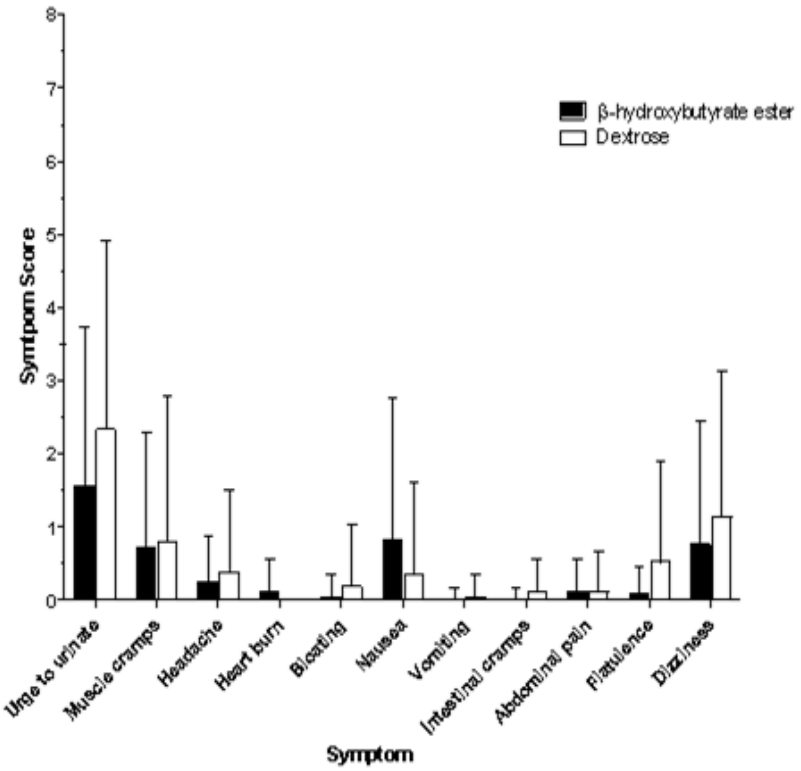


Figure XIII.c.3-1 Gastrointestinal Symptoms in Physical Endurance Study (Appendix H)

A study in which healthy volunteers drank only the D-β-hydroxybutyrate ester in a milkshake drink for 5 days also has been conducted (Clarke *et al.*, 2012a). Participants (6/sex/group) consumed a meal replacement milkshake beverage containing 140, 357, or 714 mg/kg body weight of the ketone ester 3 times daily, resulting in daily intakes of 420, 1,071, and 2,142 mg/kg body weight/day. Each 100 g serving of the meal replacement milkshake contained approximately 84 kcal and 5.2 g of the ketone ester. To standardise total daily caloric intake to 34 kcal/kg body weight, subjects in the low-dose (420 mg/kg body weight/day) and mid-dose (1,071 mg/kg body weight/day) groups received Ensure®, a water-based beverage, as a supplemental formula. Subjects receiving the lower doses of β-hydroxybutyrate ester consumed a lower total amount of liquid (ketone monoester-containing milkshake + Ensure®) than those who received the high dose of β-hydroxybutyrate ester (Table XIII.c.3-1). It should be noted that the highest daily dose administered in this study (2,142 mg/kg body weight) is beyond the maximum estimated daily intake of D-β-hydroxybutyrate ester based on the proposed uses (1,200 mg/kg body weight) and that the matrix in which the ingredient was delivered (milkshake) will not be utilized under the intended conditions of use. Nevertheless, the results of this study are included for the purposes of completeness.

Table XIII.c.3-1 Amount of D-β-Hydroxybutyrate Ester-Containing Milkshake and Ensure Consumed per Occasion in 5-Day Human Study (Clarke *et al.*, 2012a)

Dose of ketone monoester (mg/kg body weight)	Amount of ketone drink consumed (g/kg body weight)	Mean body weight (kg)	Amount of ketone drink consumed (g)	Amount of Ensure consumed (g/kg body weight)	Amount of Ensure consumed (g)	Total amount (ketone drink + Ensure) consumed (g)
140	2.69	76.1	205	8.72	664	868
357	6.87	73.8	507	5.35	395	902
714	13.73	77.7	1,066	0	0	1,066

Ketone and glucose levels were carefully monitored to ensure that β-hydroxybutyrate levels remained within the normal range (0 to 5.5 mM) and that hypoglycaemia or hyperglycaemia did not develop over the course of the study.

No abnormal changes in the levels of blood lipids, as well as haematology, clinical biochemistry, or urinalysis were observed following all doses in both the single and repeated dose study. Moreover, blood ketone levels and glucose levels did not deviate from ranges deemed to be safe, with blood D-β-hydroxybutyrate not exceeding 5.5 mM, and glucose levels remaining above 65 mg/dL (2.5 mM) and below 400 mg/dL (22.2 mM). Blood ketone levels increased in all subjects following milkshake consumption, indicating that the test substance was absorbed. Vital signs also were stable throughout the course of the study, and no ketone-related abnormalities were reported upon physical examination for all participants.

Adverse events that were deemed possibly to be treatment-related were observed in 4 out of 12 participants in the low-dose group and 1 out of 12 participants in the mid-dose group. The low and mid doses of β-hydroxybutyrate ester were generally well tolerated, and the few adverse events reported were all considered to be mild and only “possibly” related to β-hydroxybutyrate ester consumption. Mild gastrointestinal symptoms were observed in only 2 individuals receiving the low dose (420 mg/kg body weight/day; 1 incidence of nausea in 1 female and 1 incidence of flatulence in 1 male), and only 1 individual receiving the mid-dose (1,071 mg/kg body weight/day; 1 male with 1 incidence of diarrhoea and decreased appetite).

At the highest dose administered (2,142 mg/kg body weight/day), although adverse events were reported in 12 out of 12 subjects, in most instances the effects were considered to be mild and occurred only once in each subject during the 5-day dosing period. Most of the adverse events reported were gastrointestinal in nature (flatulence, nausea, diarrhoea, constipation, vomiting, and abdominal distension and pain), some of which were deemed to be “highly probable” in their relation to D-β-hydroxybutyrate ester consumption, being attributed to the consumption of a large amount of a thick milk-based beverage (1,066 g in the high-dose group) over a short duration. Subjects were initially asked to consume the drink (1.1 Litres) within 1 minute on each occasion, though this time period was extended to 30 minutes when some of the individuals reported vomiting. Additionally, the taste of D-β-

hydroxybutyrate ester, which is bitter and sharp, was likely also a contributing factor to the reported nausea and vomiting.

The reported gastrointestinal effects occurred at a lower frequency in individuals who consumed lower doses of D-β-hydroxybutyrate ester because they received a smaller volume of the test substance-containing milkshake as shown in Table XIII.c.3-1. Consumption of Ensure®, a water-based beverage, would not be expected to elicit the same gastrointestinal effects as an equal volume of milkshake.

As mentioned, most of the reported gastrointestinal effects were mild and only occurred once during the study. Repeated occurrences of a gastrointestinal effect in the same individual were reported only in 15 instances (*i.e.*, in 13 instances, the reported effect occurred twice in the same individual, and in 2 instances, the reported effect occurred 3 times in the same individual). The effects did not progress in severity, with the exception of vomiting, which progressed from moderate to severe in 1 individual, who was subsequently discontinued from the study because of the vomiting. Since vomiting occurred immediately after consumption of the milkshake, this effect may be attributed to the taste of D-β-hydroxybutyrate ester, as well as the large volume consumed within a short duration, rather than to a toxicological effect of the test substance. A second subject in the high-dose group was discontinued from the study due to reports of several adverse effects, of which the gastrointestinal effects and chest pain were considered to be "probable" or "highly probable" in their relation to the treatment. These effects may be attributed to the large volume of milkshake consumed over a short period of time

In addition to the gastrointestinal effects, headaches, dizziness, lethargy, and somnolence were reported in some participants, although these were considered to be mild in severity and were deemed to be "probable" in relation to D-β-hydroxybutyrate ester treatment. All other adverse events reported were considered mild in severity with "probable" or "possible" relation to ketone ester consumption. The adverse events reported at all doses of ketone ester resolved spontaneously by the end of the study, with the exception of the positive faecal occult test observed in one individual in the lowest dose group.

The reported adverse events in the 5-day human study are indicative of a physiological response to the food matrix (milkshake) and dosing regimen (consumption of ~1.1 L within 1 minute), rather than a toxicological effect of β-hydroxybutyrate ester *per se*. The generally mild and isolated nature of the reported adverse events helps to support this conclusion.

In summary, results of the physical endurance study (Appendix H) support the safety of D-β-hydroxybutyrate ester under the proposed conditions of use (provided at a total daily amount of 1.23 g/kg body weight, consumed before, during and/or following exercise, and consumed in reasonable volumes of less than 200 mL in a sports drink matrix). While gastrointestinal effects were noted in the high-dose group (2,142 mg/kg body weight/day) from the study reported by Clarke *et al.* (2012a), these effects may be attributed to the study design (*i.e.*, matrix, volume, and directions for use; see Table XIII.c.3-2 for a comparison of the 2 clinical studies). Additionally, only 2 mild adverse effects were reported in the mid-dose group

(1,071 mg/kg body weight/day), who consumed D-β-hydroxybutyrate ester at levels consistent with the intended uses of the ingredient. As such, findings of the Clarke *et al.* (2012a) study provides corroborative evidence of safety.

Table XIII.c.3-2 Comparison of Human Studies Conducted on β-Hydroxybutyrate Ester

Reference	Food Matrix	Instructions for Use	Dose of β-hydroxybutyrate ester per occasion (mg/kg body weight)	Daily dose of β-hydroxybutyrate ester (mg/kg body weight) 3 drinks/day	Volume per drink (mL)*	Daily Volume (mL)*
Unpublished study (Appendix H)	Vitamin water drink	Subjects asked to consume the drink within 5 to 10 minutes	410	1,230	193	578
Clarke *et al.*, 2012a	Milkshake	Subjects initially asked to consume the drink within 1 minute on each occasion; time period extended to 30 minutes	140 357 714	420 1,071 2,142	868 902 1,066	2,605 2,706 3,200

*Amount of drink consumed was reported in grams by Clarke *et al*. (2012a); for the purposes of comparing studies, 1 g was considered equivalent to 1 mL volume.

XIII.d Studies Pertaining to the Safety of the Metabolites of D-β-Hydroxybutyrate Ester

As discussed in Section XIII.a, D-β-hydroxybutyrate ester is metabolised to (R)-1,3-butanediol and D-β-hydroxybutyrate, which are the compounds identified in the blood following dietary administration of the ester. Thus, data considered pivotal in the demonstration of the safety of the ketone ester include toxicological data on each of the metabolites, as presented below.

XIII.d.1 1,3-Butanediol

1,3-Butanediol is reported to occur in nature and to be consumed in the diet through cheese and fruit and due to its use as a flavouring agent and solvent for flavouring agents (Burdock, 2009; FCC, 2010). 1,3-Butanediol occurs naturally in 2 enantiomers forms, R and S. In an *in vitro* study, differences between the metabolism of the R and S forms of 1,3-butanediol in perfused livers collected from fed and starved rats were investigated. The R and S forms of 1,3-butanediol were reported to be taken up by perfused liver at the same rate; however, the majority of (R)-1,3-butanediol was converted to ketone bodies (80 to 102% of the administered dose), (R)-3-hydroxybutyrate and acetoacetate, whereas only 29 to 38% of administered S-1,3 butanediol was converted to physiological ketone bodies with the balance converted to S-3-hydroxybutrayte, lipids, and carbon dioxide (Desrochers *et al*.,

1992). The exclusive administration of the R enantiomer of 1,3-butanediol is therefore likely to have a more pronounced effect on the ketone body content of the blood. These *in vitro* results have been mirrored in studies conducted in rats, pigs, and chicks, where the administration of oral doses of 1,3-butanediol (R,S not specified) resulted in the formation of D-β-hydroxybutyrate and acetoacetate (Romsos *et al*., 1975). Ingestion of 1,3-butanediol, provided as part of a high-fat basal diet at a concentration of 17 to 19%, was shown to significantly reduce free fatty acid synthesis in the liver of rats. As 1,3-butanediol is metabolised in the liver to D-β-hydroxybutyrate and acetoacetate *via* alcohol and aldehyde dehydrogenase (Veech and Mehlman, 1972), consumption of alcohol together with (R)-1,3-butanediol may potentiate the effects of alcohol.

XIII.d.1.1 Subchronic and Chronic Studies

The long-term toxicity of (R)-1,3-butanediol was evaluated in both rats and dogs. No adverse effects related to the administration of (R)-1,3-butanediol were observed in Sprague-Dawley rats provided the diol in the diet at concentrations of up to 10% (approximately 5 g/kg body weight/day) for a period of 2 years (Scala and Paynter, 1967). In this study, groups of 30 male and female weanling rats were administered diets comprising 1.0, 3.0, or 10% (R)-1,3-butanediol. Throughout the 2-year study, the clinical and physiological health of the rats was observed and blood and urine samples were collected and analysed at baseline, and after 4, 20, 52, and 104 weeks. After 1 year, 10 animals from each group were euthanised and gross and histological exams were conducted and this procedure was repeated at the conclusion of the experimental period for all surviving animals. No discernible adverse effects were observed by the authors at any of the dose levels. No significant differences were observed in the survival or health of the rats in any of the treatment groups.

Likewise, in a 2-year non-rodent study, no adverse effects were observed in pure-bred beagles administered (R)-1,3-butanediol in the diet at concentrations of up to 3% (approximately 750 mg/kg body weight/day) (Scala and Paynter, 1967). In this study, similar protocols were employed as the rodent study with 36 beagles administered diets comprising 0.5, 1.0, or 3.0% 1,3-butanediol. The dogs were examined at baseline and after 4, 20, and 104 weeks of consuming the diets. After 1 year, 2 dogs were sacrificed and autopsied, with all surviving dogs sacrificed at the end of the experimental period. No significant differences were observed in the food consumption, body weight, organ weight, blood chemistry, or urinalysis of the dogs in any group. Additionally no adverse effects were observed to result from the consumption of diets containing 1,3-butanediol. Based on the absence of any toxicity at the high-dose level in dogs, JECFA established an Acceptable Daily Intake (ADI) of 4 mg/kg body weight for 1,3-butanediol (JECFA, 1980). The Committee noted, however, that it would be desirable to conduct a multigenerational reproductive/teratology study with 1,3-butanediol.

XIII.d.1.2 Developmental and Reproductive Toxicity Studies

A study investigating the effect of 1,3-butanediol (enantiomer not specified, likely to be R,S) on neuronal and liver protein synthesis in developing offspring of Sprague-Dawley rats administered 1,3-butanediol in the drinking water at a concentration of 9% during gestation and lactation was identified (Khawaja *et al*., 1978). Protein synthesis in the liver of 8- and 18-day-old pups of 1,3-butanediol-treated dams was significantly reduced, whereas amino acid incorporation by free and membrane-bound ribosomes was increased. At 18 days, pups in the 1,3-butanediol-group were noted to exhibit a slightly higher neuronal RNA content in the cerebral cortex. Like in the liver, protein synthesis in neurons was inhibited in 18-day-old pups from mothers treated with 1,3-butanediol, whereas amino acid incorporation into polypeptides in the neuronal perikarya was increased in 8-day-old pups. The implications of these findings in terms of development were however not elucidated. With respect to reproductive parameters, the length of gestation was not affected by 1,3-butanediol.

A reproductive toxicity study was identified in which the effects of 1,3-butanediol (R, S not specified) were examined on 5 successive matings of Wistar rats (Hess *et al*., 1981). In this study, groups of 50 rats consumed diets comprising 0 (control), 5, 10, or 24% 1,3-butanediol [approximately 0, 2,500, 5,000, or 12,000 mg/kg body weight/day (U.S. FDA, 1993)] for a period of 4 weeks. After 4 weeks, blood and urine samples were collected from 10 rats of each sex, following which all rats of the F_0 generation were paired and mated. The female rats continued to consume the control and test diets throughout mating, gestation, and the lactation phases of the study. Following the birth of the F_{1A} generation, females from the F_0 generation were again mated to produce a second set of pups referred to as the F_{1B} generations. At 11 weeks of age the pups from the F_{1A} generation were paired and mated to produce the F_2 generation, which included 5 successive litters from the same F_{1A} parental animals, these being designated the F_{2A} through to the F_{2E} generations. This successive breeding was completed within 77 weeks, at about the time that the F_{1A} parental animals were approximately 88 weeks of age.

The only notable finding, which would not otherwise have been detected in a standard OECD-Guideline compliant 2-generation reproductive toxicity study, was a gradual decrease in pregnancy (fertility) rates across all dose levels with each successive mating of the F_{1A} parental animals. A further decrease in the fertility rate was noted in the fourth successive breeding of the F_{1A} parental animals (*i.e*., the F_{2D} group) fed 1,3-butanediol at 24% in the diet. In the fifth successive mating, no high-dose (the 24% dietary 1,3-butanediol group) parental animals produced an F_{2E} litter. Overall, in the fifth mating of the F_{1A} parental animals, decreased fertility relative to previous matings occurred in all groups, including controls, although the effect was more pronounced in rats treated with 1,3-butanediol, especially at the 24% dietary concentration. No significant differences in gestation, viability, or lactation indices were observed between the control and the test groups (this analysis excluded the last mating in the high dose group which did not produce any pups) in the F_{2A} through F_{2D} groups (*i.e*., the first 4 successive matings of the F_{1A} rats).

The significance of the decreased fertility rates reported in the fifth successive mating of the F_{1A} parental animals needs to be considered in light of the normal reproductive ageing process in female Wistar rats and in the context of results that would have been reported if the study followed a standard 2-generation protocol.

First, as already mentioned, if the Hess *et al*. (1981) study had followed a standard 2 generation reproductive study protocol, no indications of an adverse effect of 5, 10, or 24% 1,3-butanediol on fertility would have been detected. As it is, there were no adverse effects on gestation, pup viability, survival at 4 days post partum, or on live pup weight. Similarly, there were no effects of 1,3-butanediol on the incidence of soft tissue abnormalities. Incomplete ossification of sternebrae (mid- and high-dose groups) and missing sternebrae (high-dose group) were reported at an increased incidence, a finding indicative of slightly delayed foetal growth, not of a teratogenic effect or of overt reproductive toxicity.

Second, the finding of decreased fertility in the F_{2E} generation, with the high-dose group producing no litters, is not entirely surprising given that by this time the rats were about 88 weeks of age and in various stages of reproductive senescence. In the scientific literature there is considerable evidence showing that multiparous rats of this age have reduced fertility and fecundity rates compared to younger rats (Ingram *et al*., 1958; Miller *et al*., 1979; Lapolt *et al*., 1986; Matt *et al*., 1986, 1987a,b). Decreased fertility/fecundity appears to largely be the result of normal reproductive senescence processes involving luteinizing hormone secretion and circulating levels of oestrogen and progestin, all of which affect the normal, approximately 4-day, oestrus cycling patterns that occur in rats. In part, the loss of regular oestrus cycling and concomitant fertility in the rat is likely the result of one or more of: a) decreased luteinizing hormone response to the stimulatory effects of circulating oestrogen (Nass *et al*., 1984; Wise, 1984; Lu *et al*., 1985), b) altered neurotransmitter-mediated release of gonadotropic releasing hormone and subsequent effect on luteinizing hormone (LH) surge (Lapolt *et al*., 1986; Wise *et al*., 1989; Arias *et al*., 1996), and c) decreased progesterone secretion (Davis *et al*., 1977; Tsai *et al*., 2004), among other factors (Matt *et al*., 1987a,b; Wise *et al*., 1988). The age at which normal oestrus cycling is disrupted can show wide inter-animal variation and can be affected by parity (Nass *et al*., 1984; Lapolt *et al*., 1986), length of time of caging with fertile males (Nass *et al*., 1984), and caloric intake (McShane and Wise, 1996). In addition to effects specific to female reproductive function, reproductive ability, including fertility rates, in male rats decreases with advancing age (Hashizume *et al*., 1984; Hokao *et al*., 1993).

In a study similar in design to Hess *et al*. (1981), Matt *et al*. (1986) assessed the effect of breeding a group of female Long-Evans rats, once every 2 months beginning at 4 months of age and ending at 12 months of age (5 matings), on fertility and fecundity. In addition, Matt *et al*. (1986) assessed effects on oestrous cyclicity, foetal resorption rate, and on the patterns of progestin, androgen, and oestrogen secretion during these 5 successive generations. At 4 months of age, all rats showed regular oestrus cycling. By 8, 10, and 12 months of age, the incidence of regular cyclicity declined to 87, 62, and 42% respectively. The fertility rate in the 4-month old rats was reported to be 93%, with this rate declining to

79, 38, and 23% in the 8-, 10-, and 12-month-old rats, respectively. While nearly all of the rats with irregular or persistent oestrus cycles were infertile, the data indicated an additional gradual age-related increase in the percentage of rats maintaining normal oestrus cyclicity that failed to produce litters. Matt *et al*. (1986) reported an age-related increase in the number of foetal resorptions per number of implantation scars as assessed by laparotomy. There were no clear effects of successive breeding on blood levels of steroid hormones during Days 2 to 14 of gestation. However, on Days 16 and 19 of gestation, compared to 4-month-old rats, serum concentrations of estradiol, oestrone, testosterone, and androstenedione were lower in the 10- and 12-month-old rats. Based on their data, Matt *et al*. (1986) concluded that the age-related reduction in fertility is largely due to the loss of regular oestrous cycling and that middle-age pregnant female rats produce sufficient amounts of steroid hormones to maintain gestation. Decreased numbers of implantation scars were thought responsible for the decline in litter size over time and for the lower serum steroid concentrations during the end of the gestational period. The results of the Matt *et al*. (1986) study of untreated Long-Evans rats are highly consistent with those reported by Hess *et al*. (1981) during 5 successive breedings of their F_{1A} generation, including both controls and the 1,3-butanediol treated groups. It should be noted that the rats in the Hess *et al*. (1981) study were even older, up to 88 weeks of age, than those in the Matt *et al*. (1986) investigation (52 weeks).

Additional work by Matt *et al*. (1987a) provides further support for their hypothesis that cessation of regular oestrus cycling during ageing renders many females infertile, while other neuroendocrine-reproductive system changes or functional deficits are responsible for fertility loss in middle-aged rats that still maintain normal oestrus cycling patterns. This is consistent with the suggestion of Miller *et al*. (1979), who compared the effects of age on serial pregnancies initiated in Long-Evans rats at either 2 or 9 months of age, that loss of reproduction in aged regularly cycling rats is largely due to post-ovulatory factors. Similarly, Mattheij and Swarts (1991) have suggested that pregnancy loss or wastage in regularly cycling middle-aged rats is related to a reduction in the viability of ovulating eggs.

Given the preceding data, the decreased fertility seen in the fourth (high-dose 1,3-butanediol group) and the fifth successive (all groups, including controls) generations of F_2 rats relative to the first through third generations in Hess *et al*. (1981) follows the expected pattern of reproductive senescence known to occur in female rats.

In the fifth generation (F_{2E}), the loss of fertility in high-dose group (24% 1,3-butanediol in the diet) was greater than in the controls (0/25 pregnancies *versus* 10/25 in the controls). The apparent exacerbation of the loss of fertility in ageing female rats by high dietary concentrations (24%) of 1,3-butanediol may be of limited or no relevance to humans. First, the reproductive senescence patterns of the Wistar rat, the strain used in the Hess *et al*. (1981) study are unlike those of humans. Key differences are presented in Table XIII.d.1.2-1. Data for the rat are for the Sprague-Dawley strain, a strain derived from the Wistar rat.

Table XIII.d.1.2-1 Comparison of the Reproductive Senescence of Humans and Rats		
Parameter	**Human Female**	**Female Rat**
Time of onset (percent of lifetime)	Approximately 70%	30-40%
Site of action	Ovaries	Hypothalamus
Mechanism of occurrence	Follicle depletion	Impaired LH/FSH control
Overall LH surge	Maintained	Lost or greatly reduced
Cycle pattern	Anoestrus	Persistent oestrus
Oestrogen secretion	Decreased	Increased
Oestrogen: progesterone	No change	Increased
Prolactin secretion	Low	Continuous

FSH, follicle stimulating hormone; LH, luteinizing hormone.

Given the differing aspects of reproductive senescence in humans and rats, the findings in the Hess *et al*. (1981) study in ageing female rats subject to successive breeding are difficult to extrapolate to humans. In addition, the wide inter-animal variation in the time course over which reproductive senescence occurs in the rat (Huang and Mietes, 1975; Lu *et al*., 1979; Miller *et al*., 1979; Matt *et al*., 1986, 1987a,b; Wise *et al*., 1988) complicates the attribution of the reduction in fertility if the 4th and 5th breedings in the Hess *et al*. (1981) study as a treatment-related effect, since the effect, as expected, was found in the controls as well, just not to the same extent. In addition, it is plausible that the high-dose (24% 1,3-butanediol) exposure over the course of the 5 successive breedings produced nutritional or dietary stress on the animals (*i.e*., an induced ketogenic state), possibly accelerating the natural reproductive senescence pattern. In any case, human exposure to 1,3-butanediol arising from the metabolism of the D-β-hydroxybutyrate ester would not approach a dietary concentration of 24% nor would it occur over successive or multiple pregnancies. In the Hess *et al*. (1981) study, female rats were subjected to multiple pregnancies and were exposed to 24% 1,3-butanediol for upwards of 88 weeks, a significant proportion of their lifetime. Given the preceding analysis, a strong argument exists to consider the effects of fertility in the 4th and 5th breedings in the Hess *et al*. (1981) reproductive study of no relevance to humans.

XIII.d.1.3 Human Exposure

JECFA has established an ADI of 4 mg/kg body weight for 1,3-butanediol (R,S not specified). Short-term metabolic studies conducted in humans indicate that 1,3-butanediol can supply up to 10% of total dietary energy with no toxic effects, though there may be physiological, but not clinically significant, reductions in blood glucose levels (JECFA, 1980).

Kies *et al.* (1973) conducted a short-term 4-arm, crossover design clinical trial in which healthy volunteers consumed daily doses of 15 g 1,3-butanediol/day. The 1,3-butanediol was incorporated into bread that was consumed with or without supplemental urea by 12 healthy male and female volunteers. The urinary excretion of creatine and nitrogen were measured daily through 24-urine collection, and faecal nitrogen levels were analysed across samples collected as available over the 7-day period. The nutritional status of the volunteers

was measured by examining the nitrogen balance of each individual. Fasting blood samples were collected at the end of the experimental period and these were analysed for blood urea nitrogen, total serum proteins, haematocrit, haemoglobin, white blood cell counts, blood glucose levels, blood lactate levels, blood lipids, and plasma sodium, potassium, and chloride content. Administration of the 1,3-butanediol was reported by the authors to decrease the negative nitrogen balance observed in all study participants at baseline. Consumption of 1,3-butanediol did not significantly alter the serum protein, white blood cell counts, haematocrit, or haemoglobin levels. No significant difference in the blood lipid or plasma ion content was observed following 1,3-butanediol consumption compared to baseline values. A significant decrease in blood glucose was observed following consumption of the 1,3-butanediol containing bread. Overall, the authors concluded that the consumption of 15 g 1,3-butanediol/day for 7 days may represent a desirable energy source for humans.

As mentioned, JECFA indicated that the administration of 1,3-butanediol at levels providing up to 10% of energy intake resulted in hypoglycaemic effects in human subjects; however, these effects did not have an impact on the well-being of the subjects. The lack of safety concern associated with exposure to 1,3-butanediol from the proposed uses of D-β-hydroxybutyrate ester is supported by the results of a study in which subjects consumed up to 2.1 g D-β-hydroxybutyrate ester/kg body weight/day for 5 days, providing approximately 1 g 1,3-butanediol[2]. No adverse effects on blood glucose levels were observed, confirming the safety of this metabolite following its production from the metabolism of the ketone ester (Section XIII.c.3).

XIII.d.2 D-β-Hydroxybutyrate

D-β-Hydroxybutyrate [*(R)*-3-hydroxybutyrate] is an endogenously produced compound that has been identified at high levels in the blood of fasted individuals and in individuals consuming either very low-calorie diets or very high-fat, low-CHO, ketogenic diets. D-β-Hydroxybutyrate and acetoacetate undergo interconversion *via* the enzyme β-hydroxybutyrate dehydrogenase. Under normal physiological conditions, blood and urinary levels of ketones are <3 mg/100 mL and ≤125 mg/24-hour urine. During periods of limited glucose availability, humans produce approximately 150 g of ketone bodies per day (Reichard *et al*., 1974). Mild ketosis, characterised by ketone bodies in the blood at concentrations of 5 to 7 mM, is considered a normal physiological response to fasting in humans (Cahill, 1970). Under such conditions, D-β-hydroxybutyrate is detected in the blood at levels of 4 to 5 mM (approximately 42 to 52 mg/100 mL).

Increased ketone body production can be induced during the consumption of ketogenic diets. While the formulation of these diets may vary substantially, the “classic” ketogenic diet (used for over a century in the treatment of childhood refractory seizures) comprises, by weight, fat and protein+CHO in a ratio of 4:1, respectively. More recently, ketogenic diets

[2] 2.1 g D-β-hydroxybutyrate ester/kg body weight/day x [90.12 (molecular weight of 1,3-butanediol) / 176 (molecular weight of D-β-hydroxybutyrate ester)] = 1.1 g 1,3-butanediol/kg body weight/day

have proved popular among individuals seeking to achieve weight loss (*e.g*., "Atkins diet"); these diets differ markedly from the classic ketogenic diet in that protein consumption is generally not restricted. Given that, with excessive protein consumption, amino acids undergo conversion to glucose, diets such as the Atkins diet that do not restrict protein intakes are far less ketogenic than the traditional "classic" ketogenic diet.

XIII.d.2.1 Developmental and Reproductive Toxicity Studies

On the basis of data indicating that poorly controlled diabetes impaired fertility in women, Moley *et al*. (1994) undertook an *in vitro* analysis of the effects of compounds known to be elevated in the plasma of pregnant, diabetic, and hyperglycaemic or hyperketonaemia women in this condition (Ornoy and Zusman, 1991). For this purpose, the authors perfused isolated 2-cell mice embryos with elevated concentrations of metabolic intermediates such as glucose, ketone bodies, and insulin that represented exposure levels 50 to 100 times larger than those experienced by hyper- or hypoglycaemic women. For this study, female B6C3F1 mice were injected with gonadotropins to induce ovulation and then mated with male mice. Mating with male mice was confirmed by the presence of a vaginal plug and 48 hours later the mice were sacrificed by cervical dislocation and the embryos were harvested. All the embryos were at the 2-cell stage and these were placed in a culture dish with Ham's F-10 medium and incubated in a low oxygen environment for a period of 72 hours at 37°C. The development of the embryos was evaluated every 24 hours; at each time period, each embryo was scored as either degenerated, a 2, 3, 4, 6, or 8 cell blastomere, morula, blastocyst, or an expanded blastocyst. The control group of embryos was incubated in the media alone, while the test media also contained 7 mM amino acids, 5 mM sodium lactate, 160 µM glycerol, 250 mg/dl lipids, 10 mM acetoacetate, or 8, 16, or 32 mM DL-β-hydroxybutyrate.

No significant differences were observed in the development of the control embryos and those cultured with amino acids, lactate, glycerol, or lipids. A significant retardation in growth was observed in the embryos cultured with acetoacetate, with only 8% of the embryos reaching the 4-cell stage or above as compared to 97% of the control embryos. No significant difference in the rate of growth or the distribution of developmental stages was observed for the embryos cultures in 8 mM DL-β-hydroxybutyrate as compared to the control embryos. A significant retardation in growth was observed at all times point for the embryos cultured in higher doses of DL-β-hydroxybutyrate and the overall growth of the embryos was significantly delayed as compared to control results. In the control medium 75% of embryos reached the blastocyst stage or a higher stage after 72 hours of incubation as compared to 45 and 30% of embryos cultured in 16 and 32 mM DL-β-hydroxybutyrate, respectively. The authors interpreted these results as an indication that elevated blood levels of acetoacetate and DL-β-hydroxybutyrate may be responsible for the teratogenic effects associated with hyperglycaemia and hyperketonaemia.

A similar study was conducted with β-hydroxybutyrate (D,L not specified) by Sheehan *et al* (1985) using slightly older rat embryos. In this study, the embryos of Wistar rats were collected on Day 9.5 of gestation and placed in bottles containing balanced saline and

culture medium. β-Hydroxybutyric acid was added to the bottles at a level of 0 (control), 0.5, 1.25, 2.5, or 5.0 mg/mL corresponding to doses of 4, 10, 20, and 40 mM. The embryos were cultured in the bottles for 48 hours at 37°C in a low oxygen environment. After the 48 hours had elapsed the embryos were examined for abnormalities under a scanning electron microscope, and the yolk-sac diameter, crown-rump length, heart-beat, yolk-sac circulation and number of somites were scored for each embryo. No significant differences were observed between the control embryos and those cultured in a 4 mM solution of β-hydroxybutyrate. In the embryos exposed to 4 or 10 mM β-hydroxybutyrate, no significant differences were observed between the number of somites and the yolk-sac expansion of the treated and control embryos. Both of these parameters decreased significantly in the embryos cultured in higher doses of β-hydroxybutyrate. A significant decrease in crown rump length also was observed in the embryos cultured in 10 or 20 mM β-hydroxybutyrate and this measurement could not be completed for the embryos exposed to the highest dose of β-hydroxybutyrate as the embryos failed to rotate into the characteristic foetal position. As the concentration of β-hydroxybutyrate increased so did the number of abnormalities in embryo development observed and the classification of the abnormalities observed shifted from minor to major.

Similar results were observed by Zusman and Ornoy (1987), who examined the effects of incubation of rat embryos collected on Day 10.5 of gestation in serum containing 2, 5, or 8 mg/mL of β-hydroxybutyrate, equivalent to 16, 40, or 64 mM. It should be noted that while the authors of this study observed growth retardation and developmental abnormalities in the embryos exposed to β-hydroxybutyrate alone, the retardation and abnormalities were significantly more severe in embryos exposed to serum containing β-hydroxybutyrate, acetoacetate, and glucose, all 3 of which are elevated in the serum of diabetic individuals experiencing hyperglycaemia or hyperketonaemia.

Alterations in glucose utilization, ultimately leading to the disruption of normal embryogenesis during the period of neurulation, has been proposed as the mechanism of action whereby D-β-hydroxybutyrate, and more so the racemic mixture of β-hydroxybutyrate, induces malformations including neural tube defects, craniofacial hypoplasia, and cardiac abnormalities in rodent embryos (Hunter *et al*., 1987). Although the D-isomer induced malformations in the embryos, the incidence was lower than that observed with equivalent concentrations of the racemic mixture. Notably, however, the spectrum of defects observed in the rodent embryos *in vitro* was consistent with the defects observed in malformed infants of diabetic individuals. Since then, others also have suggested additional mechanisms of β-hydroxybutyrate-induced teratogenesis depending on the stage of embryonic development including a reduction in pyrimidine biosynthesis (Shum and Sandler, 1990).

The available literature indicates that β-hydroxybutyrate is embrytoxic *in vitro* at concentrations greater than 8 mM; however, the relevance of these data to *in vivo* exposure has not been established. No traditional *in vivo* reproductive or developmental toxicity studies have been conducted with β-hydroxybutyrate and therefore, for the purpose of this safety assessment, a developmental study conducted with 1,3-butanediol was considered.

As described in Section XIII.d.1, the majority of (R)-1,3-butanediol is converted to ketone bodies, (R)-3-hydroxybuturate and acetoacetate; in contrast, 29 to 38% of S-1,3 butanediol is converted to physiological ketone bodies with the balance converted to S-3-hydroxybutrayte, lipids, and carbon dioxide (Desrochers *et al*., 1992). On this basis, reproductive toxicity studies conducted with 1,3 butanediol provide indirect evidence of the safety of β-hydroxybutyrate. A reproductive toxicity study was conducted by Hess *et al.* (1981) using 1,3-butanediol provided by the Celanese corporation (CAS No. 107-88-0) (discussed in detail in Section XIII.d.1.2). No mention of any chirality (R or S form) or of any value for optical rotation for the 1,3-butandiol produced by Celanese was identified.

Assuming that the 1,3-butanediol administered in the study reported by Hess *et al.* (1981) consisted of a mixture of 50% (R)- and 50% (S)- enantiomers, at least 80% of the "R" form and approximately 30% of the "S" form would have been metabolised to β-hydroxybutyrate. On the basis of the metabolism of the "R" and "S" forms of 1,3-butanediol, the administration of diets containing 5, 10, or 24% 1,3-butanediol would be equivalent to dietary β-hydroxybutyrate levels of 3.25, 6.5, and 15.6%. Based on the molecular weights of 1,3-butanediol and β-hydroxybutyrate, consumption of these diets would provide daily β-hydroxybutyrate intakes of approximately 1,877, 3,755, and 9,013 mg/kg body weight/day (U.S. FDA, 1993). The administration of these β-hydroxybutyrate-equivalent levels was not associated with teratogenic effects. Moreover, results of a developmental toxicity study conducted on D-β-hydroxybutyrate ester indicate no adverse effects on the development of rats following *in utero* exposure to the ketone ester at a dose of 2 g/kg body weight/day on Days 6 through 20 of gestation (discussed in Section XIII.c.2). Therefore, the relevance of results observed in *in vitro* teratogenicity studies (conducted in isolated embryos) is questionable.

XII.d.2.2 Human Exposure

Ingestion of approximately 100 to 150 g of ketone bodies is expected to result in blood ketone body levels of 2 to 7 mM (Veech *et al*., 2001). In children with acyl-CoA dehydrogenase deficiency, oral administration of sodium *D*,*L*-β-hydroxybutyrate at dose levels of 80 to 900 mg/kg body weight/day resulted in peak blood levels of 0.19 to 0.36 mM of combined *D*,*L*-β-hydroxybutyrate and acetoacetate (Van Hove *et al*., 2003). Similarly, treatment of two, 6-month-old infants with persistent hyperinsulinaemic hypoglycaemia with oral *D*,*L*-sodium-β-hydroxybutyrate was reportedly tolerated with no side effects (Plecko *et al*., 2002). Administration of 0.9 to 1.0 g *D*,*L*-sodium-β-hydroxybutyrate/kg body weight/day resulted in blood β-hydroxybutyrate concentrations comparable to those observed following a 16- to 24-hour fast. β-Hydroxybutyrate levels also were observed to increase in the cerebrospinal fluid. The high ratio of β-hydroxybutyrate to acetoacetate supported that the increase in β-hydroxybutyrate levels was due to exogenous β-hydroxybutyrate administration.

XIII.e Studies Conducted with 1,3-Butanediol Diacetoacetate

Several pre-clinical trials were identified in which (R,S)-1,3-butanediol mono- and diacetoacetate esters were administered to various laboratory animals. (R,S)-1,3-butanediol mono- and diacetoacetate esters are similar in nature to D-β-hydroxybutyrate esters (*i.e.,* both are ketone esters), and most notably are metabolised to similar compounds, (R,S)-1,3-butanediol and acetoacetate, the latter which undergoes interconversion with D-β-hydroxybutyrate. Thus, safety data pertaining to (R,S)-1,3-butanediol mono- and diacetoacetate are presented in support of the safety of D-β-hydroxybutyrate ester.

The metabolism of (R,S)-1,3-butanediol acetoacetate esters following parenteral and intravenous administration has been examined in pigs (Desrochers *et al.*, 1995). Groups of 8 male pigs were administered infusions of 3 hours in duration that provided a dose of (R,S)-1,3-butanediol or mixtures containing 84:16 and 27:73 molar ratios of (R,S)-1,3-butanediol mono- and diacetoacetate, equivalent to 30% of the pigs' hourly caloric requirements. Control pigs were administered saline. Additionally, another group of pigs was administered the same compounds in bolus intragastric doses, with water employed as the control substance for these groups, at doses equivalent to 15% of the pigs' daily caloric needs. The daily caloric requirement for the pigs was reported by the authors to be 110 kcal/(kg body weight)$^{0.75}$, and the caloric value of the (R,S)-1,3-butanediol, the (R,S)-1,3-butanediol monoester, and the (R,S)-1,3-butanediol diester were reported to be 6.7, 5.9, and 5.6 kcal/g, respectively. Based on the reported starting weight of the pigs (20 to 25 kg), the required caloric intake, and the caloric value of the test compounds, the estimated doses of (R,S)-1,3-butanediol monoester and diester mixtures for pigs receiving the 3-hour infusions were approximately 107 mg/kg body weight/hour (between 6.8 and 8.0 g/pig) during the 84:16 mixture administration, and approximately 116 mg/kg body weight/hour (6.9 and 8.7 g/pig) during the 27:73 mixture administration. For pigs receiving the bolus intragastric dose, the dose of (R,S)-1,3-butanediol monoester was approximately 1,360 mg/kg body weight/day (between 27.2 and 34.1 g/pig) following the 84:16 mixture administration, and approximately 1,317 mg/kg body weight/day (27.8 and 32.9 g/pig) following the 27:73 mixture administration. Following the administration of all compounds, blood samples were collected from the pigs and blood ketone, 1,3-butanediol, and acetate levels were examined.

No significant increases in blood ketone, 1,3-butanediol, and acetate levels were observed in any of the control groups. A significant increase in all parameters was observed immediately after test compound administration, with the levels peaking approximately 240 minutes after administration. Following intravenous infusions, the largest increase in total ketone bodies and 1,3-butanediol was observed following the administration of (R,S)-1,3 butanediol. These increases were significantly larger than those observed following the administration of the monoester rich mixture and the diester rich mixture. Following the bolus intragastric administration of all 3 test compounds, the largest increase in blood ketone bodies was observed in animals administered the monoester and diester mixtures, with levels peaking approximately 30 minutes after administration and returning to baseline approximately 275 minutes after administration. The largest increase in 1,3-butanediol levels was again

observed in the animals administered (R,S)-1,3-butanediol alone. The authors reported that none of the pigs displayed any signs of discomfort during the trial and that no significant differences were observed in the standard clinical chemistry parameters examined at the beginning, middle, and end of the experimental period. The authors concluded that (R,S)-1,3-butanediol acetoacetate esters were well utilised as a nutrient by the pigs without producing deleterious side effects.

It has been reported that the consumption of ketogenic diets may decrease the frequency of seizures in patients with seizure disorders. Puchowicz *et al.* (2000) examined a dog model of therapeutic ketosis in which a ketotic state was induced by the administration of (R,S)-1,3-butanediol diacetoacetate. A total of 12 dogs were employed in the trial, and these were administered doses of (R,S)-1,3-butanediol diacetoacetate equivalent to 0 or 12% of their daily caloric requirements. The authors reported the daily caloric requirements of the dogs to be equivalent to 110 kcal/(kg body weight)$^{0.75}$ and the starting body weight of the dogs was reported to range between 18 and 25 kg. To provide 12% of the daily caloric requirement each dog would have to be administered between 20.6 and 26.3 g of (R,S)-1,3-butanediol diacetoacetate, which was reported by the authors to have a caloric value of 5.6 kcal/g. On a body weight basis these doses would be equivalent to between 1.0 and 1.1 mg/kg body weight. The (R,S)-1,3-butanediol diacetoacetate was administered in gelatine capsules administered over the course of 300 minutes. Additionally, to further elucidate the metabolism of (R,S)-1,3-butanediol diacetoacetate a single dog was administered a single bolus dose of (R,S)-1,3-butanediol diacetoacetate corresponding to 5% of the dog's daily caloric requirement, equivalent to between 8.6 and 11.0 g (R,S)-1,3-butanediol diacetoacetate, or 439 and 477 mg/kg body weight.

Following administration of a single bolus dose of (R,S)-1,3-butanediol diacetoacetate, the authors observed an increase in the acetoacetate, (R,S)-β-hydroxybutyrate, and butanediol concentrations in the blood with levels returning to baseline approximately 180 minutes after the administration. In the dogs receiving the repeated oral doses of R,S-butanediol diacetoacetate, no accumulation of acetone was observed in the blood over the course of the experimental period. No significant differences were observed in the plasma glucose concentrations of the control and treatment groups throughout the experimental period. All standard clinical chemistry analyses were reported to remain within normal ranges through the experiment period and the authors observed no signs of distress from any of the animals during or following the experimental period.

XIII.f Endogenous Production of Ketones

The rate of ketone formation is low when an adequate supply of carbohydrates is available; however, when blood glucose levels decrease, the rate of ketogenesis is increased, and ketones replace glucose as the primary source of energy. The normal 6- to 8-hour fasting blood ketone concentration of a healthy individual is reported to be approximately 0.5 mM, while after a 5- to 7-day fast, total blood ketone levels as high as 5 to 7 mM have been documented (Owen *et al*., 1967; Mensink *et al.,* 1992; VanItallie and Nufert, 2003). Typical

levels of β-hydroxybutyrate in the blood are reported to be approximately 0.2 mM, with levels increasing by up to 50 times during periods of limited calorie intake (Hall *et al*., 1984). The normal blood ketone level of pregnant women is slightly higher than that of other individuals, and pregnant women, because of their greater caloric requirements, achieve greater ketone levels during times of limited glucose availability (Paterson *et al.,* 1967; Gin *et al.*, 2006). Maximal ketone body production by a healthy adult liver and kidney is approximately 185 g/day, with ketones accounting for between 2 and 6% of an individual's energy needs after an overnight fast and up to 40% of energy needs after a 3-day fast (Reichard *et al.*, 1974; Laffel, 1999).

In most mammals including humans, the fatty acid oxidation product, acetyl-CoA, formed in the liver can enter the citric acid cycle or alternatively can be converted to ketone bodies, a metabolic process called ketogenesis (Nelson and Cox, 2000). Ketogenesis occurs mainly in the mitochondria of liver cells during times of limited glucose availability, when there is an increase in lipolysis and a concomitant reduction or saturation in acetyl-CoA oxidation in the citric acid cycle. The latter occurs as a result of the need for citric acid cycle intermediates to enter the gluconeogenic pathway to maintain blood glucose levels. Acetyl-CoA, the primary substrate in the citric acid cycle, is then diverted into the ketogenic pathway, resulting in the formation of the ketone bodies, acetoacetate and D-β-hydroxybutyrate. Acetoacetate and D-β-hydroxybutyrate are transported to extra-hepatic tissues, where they can be oxidised in the citric acid cycle and thus serve as an alternate source of energy. D-β-Hydroxybutyrate is oxidised to acetoacetate by D-β-hydroxybutyrate dehydrogenase, which is subsequently converted to acetoacetyl-CoA and finally to 2 acetyl-CoA molecules. While D-β-hydroxybutyrate dehydrogenase is found in the liver, 3-oxoacid-CoA transferase, which converts acetoacetate to acetoacetyl-CoA, does not occur in hepatic tissue. Thus, the liver cannot utilise ketone bodies as an alternate source of energy. In the fed state, the brain uses glucose as its primary source of energy; however, the brain also can use acetoacetate and D-β-hydroxybutyrate when these metabolites are available. Production of ketone bodies, and their transport to other tissues for conversion to acetyl-CoA, allows for continued oxidation of fatty acids, particularly when acetyl-CoA oxidation slows down. A reduction in acetyl-CoA oxidation may occur when intermediates of the citric acid cycle are being used in gluconeogenesis or during coenzyme A saturation.

The oral administration of the D-β-hydroxybutyrate ester can be considered a direct means of increasing systemic ketone levels, thereby providing additional acetyl-CoA substrates for the citric acid cycle.

XIII.g Ketogenic Diets

Ketogenic diets are high-fat, adequate protein, low-carbohydrate diets that induce and maintain ketosis in the body. The "classic" ketogenic diet, which consists of fats and carbohydrates in a 4:1 ratio, was developed in 1921 for use for the treatment of paediatric refractory epilepsy (Wilder, 1921), and continues to be utilised as an anticonvulsant dietary regimen.

In addition to their established role in the treatment of paediatric epilepsy, ketogenic diets have potential therapeutic applications in neurodegenerative diseases, including Alzheimer's (Reger *et al.*, 2004) and Parkinson's disease (Vanitallie *et al.*, 2005). Variations of the ketogenic diet also have been investigated for their therapeutic effects on obesity (Dashti *et al.*, 2003, 2006; Yancy *et al.*, 2004), diabetes (Westman *et al.*, 2008; Al-Khalifa*et al.*, 2009), cancer (Freedland *et al.*, 2008; Otto *et al.*, 2008; Seyfried *et al.*, 2008), and a number of other conditions.

Similar to ketogenic diets, D-β-hydroxybutyrate ester will be used to elevate blood ketone levels, given that it is metabolised to the ketones D-β-hydroxybutyrate and acetoacetate following ingestion. The long history of use of ketogenic diets provides corroborative evidence of D-β-hydroxybutyrate ester's safety for its intended use as an ingredient in food supplement products for high-performance athletes.

OVERALL CONCLUSIONS

D-β-Hydroxybutyrate ester has been developed as an oral source of ketones, which will be utilised as an energy source for athletes and persons undergoing strenuous exercise or conditions leading to rapid energy depletion. It is produced *via* an enzyme-catalysed transesterification reaction of ethyl-D-hydroxybutyrate and (R)-1,3-butanediol. The final product contains ≥97.5% D-β-hydroxybutyrate ester.

D-β-Hydroxybutyrate ester is intended for use by high-performance athletes as an energy source in food supplements in liquid, powder (sachet), bar, and gel form. Considering that D-β-hydroxybutyrate ester-containing products will be consumed only by athletes and persons undergoing extreme energy expenditures, the products will be consumed in a pattern consistent with supplement use and will be labelled in accordance with Directive 2002/46/EC (European Parliament and Council of the European Union, 2002). The ingredient will not be used in mainstream foods. It is therefore likely that D-β-hydroxybutyrate ester-containing products will be used intermittently and by a small section of the population. The addition of D-β-hydroxybutyrate ester to the proposed products will result in intakes that will not exceed 1.07 g/kg body weight/day. Based on an average 70 kg adult, this maximum will relate to 75 g D-β-hydroxybutyrate ester per day. It is envisaged that a maximum of 2 to 3 servings per day will be consumed. These products will be marketed to high-performance athletes only.

Like other aliphatic esters, D-β-hydroxybutyrate ester undergoes complete hydrolysis *via* carboxylesterases or esterases distributed throughout the intestinal tract, blood, liver, and other tissues (Heymann, 1980; Anders, 1989). The ketone ester is hydrolysed to D-β-hydroxybutyrate and (R)-1,3-butanediol, with the latter being further metabolised to D-β-hydroxybutyrate and acetoacetate in the liver (Tate *et al.*, 1971; Desrochers *et al.*, 1992). Pharmacokinetic studies in male and female Wistar rats demonstrate that, following the oral administration (gavage) of up to 5 g/kg body weight D-β-hydroxybutyrate ester, very low levels of the ester were detected in the plasma, and the small amount (<0.11 mM) detected was rapidly degraded. In a 66-day study in which rats were administered the ketone ester *via* the diet, the ketone ester was not detected in blood (Appendix F). This finding further supports complete hydrolysis of the ketone ester. Additionally, in an *in vitro* study in which solutions of 1.5 to 5.0 mM of the ketone ester were incubated with fresh human plasma, hydrolysis was found to be rapid and complete (Appendix G).

The metabolic fate of D-β-hydroxybutyrate ester also has been examined in humans (Clarke *et al.*, 2012a). Plasma levels of D-β-hydroxybutyrate and acetoacetate were readily elevated, reaching peak levels within 1.5 to 2.5 hours following administration of a single dose of the ketone ester (up to 714 mg/kg body weight), while the intact compound was not detected. The elimination half-life ranged from 0.77 to 3.06 hours for β-hydroxybutyrate, and from 8 to 14 hours for acetoacetate.

A 28-day toxicity study conducted in rats demonstrated that the consumption of diets containing 11.4% D-β-hydroxybutyrate ester (12 and 15 g/kg body weight/day in male and female rats, respectively) did not cause toxicological effects (Clarke *et al.*, 2012b). Rats in the ketone ester group consumed significantly less feed and gained significantly less weight than rats in the control groups. Decreased food consumption may have resulted from the palatability of the diet containing D-β-hydroxybutyrate ester. Additionally, these effects are consistent with reports of decreased hunger, reduced energy intakes, and increased weight loss in subjects consuming low-carbohydrate ketogenic diets compared to low-fat diets or medium-carbohydrate non-ketogenic diets (McClernon *et al.,* 2007; Johnstone *et al.*, 2008).

Clinical chemistry analysis revealed that LDH levels were significantly higher in ketone ester-fed rats (both sexes compared to control animals); however, the increases were small in magnitude and were not associated with changes in haemolysis parameters or histopathological effects. Microscopic findings in the liver were present in all groups and were not accompanied by effects on liver function parameters; therefore, they were not considered to be related to consumption of D-β-hydroxybutyrate ester.

Results from a developmental toxicity study in rats also support the safety of D-β-hydroxybutyrate ester. Administration of 2 g D-β-hydroxybutyrate ester/kg body weight/day *via* gavage on DGs 6 through 20 did not affect reproductive performance or litter parameters. Litter averages for corpora lutea, implantations, the percentage of pre-implantation loss, litter sizes, live and dead foetuses, early and late resorptions, the percentage of resorbed conceptuses, and the percentage of live male foetuses were comparable among groups. The overall incidence of foetal alterations was higher in the ketone ester group; however, there were no significant between-group differences in the litter or foetal incidences of any gross external, soft tissue, or skeletal abnormalities (malformations or variations).

In a physical endurance study, adult men consumed a vitamin water drink containing 1.23 g D-β-hydroxybutyrate ester/kg body weight or 1.44 g dextrose/kg body weight (divided into 3 drinks of equal volume) before and during a cycling exercise session. D-β-Hydroxybutyrate ester was well tolerated under conditions similar to the intended conditions of use, with no significant differences in symptom severity between the D-β-hydroxybutyrate ester and control arms of the study. In another human study, D-β-hydroxybutyrate ester was administered in a milkshake matrix at doses of 140, 357, and 714 mg/kg body weight 3 times daily over a period of 5 days (equivalent to 420, 1,071, and 2,142 mg/kg body weight/day). The ketone ester was generally well tolerated, although some gastrointestinal effects were reported at the highest dose tested. These effects were attributed to the food matrix (milkshake) and dosing regimen (consumption of ~1.1 L within 1 minute) rather than to a toxicological effect of the test substance. No abnormal changes in haematology, clinical biochemistry, or urinalysis parameters were observed. Moreover, levels of blood lipids, ketones, or glucose did not deviate from ranges that were deemed to be safe.

The safety of D-β-hydroxybutyrate ester is supported by the results of studies conducted on its metabolites, D-β-hydroxybutyrate and (R)-1,3-butanediol. Sodium D,L-β-hydroxybutyrate

has been administered orally to children with acyl-CoA dehydrogenase deficiency or with persistent hyperinsulinaemic hypoglycaemia at dose levels up to 1,000 mg/kg body weight/day with no side effects (Plecko *et al.,* 2002; Van Hove *et al.,* 2003). The results of *in vitro* studies (with isolated embryos) suggest that physiologically relevant levels of β-hydroxybutyrate, particularly the L-isomer, may disrupt normal embryogenesis (Sheehan *et al.,* 1985; Hunter *et al.,* 1987; Moley *et al.,* 1994); however, the results of an *in vivo* study with R,S-1,3-butanediol indicate that it is not teratogenic (Hess *et al.,* 1981). Because 100% and 30% of the R and S enantiomers, respectively, of 1,3-butanediol are metabolised to ketones (Desrochers *et al.*, 1992), studies on 1,3-butanediol indirectly provide information on the reproductive and developmental safety of β-hydroxybutyrate. Moreover, results of the rat developmental toxicity study on D-β-hydroxybutyrate ester indicated no adverse effects on development.

Almost all experimental studies on 1,3-butanediol identified administered both the (R)- and (S)- forms of butanediol, except for the chronic toxicity studies conducted by Scala and Paynter (1967). In this study, it was demonstrated that the 2-year oral administration of (R)-1,3-butanediol to Sprague-Dawley rats or to pure bred-beagles, at levels of 5 and 0.8 g/kg body weight/day, respectively, was not associated with toxicity. Hess *et al.* (1981) demonstrated, in 5 successive breedings of Wistar rats, that consumption of 5 to 24 g/kg body weight/day of 1,3-butanediol was not associated with teratological effects. The control and test groups were comparable with respect to gestation, viability, and lactation indices. The ADI set by JECFA for 1,3-butanediol is 4 mg/kg body weight/day (JECFA, 1980). Short-term human experimental studies indicate that 1,3-butanediol consumption may result in statistically, but not clinically significant reductions in blood glucose levels (Kies *et al.*, 1973; JECFA, 1980); however, it should be noted that no clinically significant effects on blood glucose levels were reported in the 5-day human study in which subjects consumed approximately up to 2 g D-β-hydroxybutyrate ester/kg body weight/day (Clarke *et al.*, 2012a).

The safety of D-β-hydroxybutyrate ester is further supported by animal feeding trials of a similar ketone ester, namely (R,S)-1,3-butanediol mono- and diacetoacetate. A bolus intragastric dose of approximately 1.3 g/kg body weight administered to pigs was not associated with alterations in standard clinical chemistry parameters or with deleterious side effects (Desrochers *et al.,* 1995). Likewise, the administration of repeated oral doses of (R,S)-1,3-butanediol diacetoacetate over a 300 minute period (equivalent to 1,054 to 1,144 mg/kg body weight) or a single bolus dose of 439 to 477 mg/kg body weight (R,S)-1,3-butanediol diacetoacetate to dogs was not associated with alterations in clinical chemistry analyses; moreover, the authors observed no signs of distress in any of the animals during or following the experiment (Puchowicz *et al.,* 2000).

D-β-Hydroxybutyrate ester is a synthetic compound and thus, does not occur endogenously; however, the metabolites of D-β-hydroxybutyrate ester are ketones, which are produced in the body. Maximal ketone body production is approximately 185 g/day during times of limited glucose availability, indicating that the human body has the capacity to handle large amounts of ketones. Furthermore, there is a long history of use of ketogenic diets, which

have been used for over a century in the treatment of paediatric refractory epilepsy, and more recently for other therapeutic applications. Ketogenic diets, similar to D-β-hydroxybutyrate ester, result in elevated ketone levels.

Based on the available information on the safety of D-β-hydroxybutyrate ester, its metabolites (D-β-hydroxybutyrate and (R)-1,3-butanediol), and the related ketone ester, R,S)-1,3-butanediol mono- and diacetoacetate, the proposed use of D-β-hydroxybutyrate ester as an ingredient added to selected products targeted to specific population groups for consumption on a supplemental basis does not present a safety concern. The capacity of the human body to produce and utilise ketones, as well as the long history of use of ketogenic diets, also support the safety of D-β-hydroxybutyrate ester for its intended use.

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