Synthesis of triheptanoin and formulation as a solid diet for rodents

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Summary

Triheptanoin enriched diets have been successfully used in the experimental treatment of various metabolic disorders. Maximal therapeutic effect is achieved in the context of a ketogenic diet where triheptanoin oil provides 30-40% of daily caloric intake. However, studies using triheptanoin-rich diets in the pre-clinical setting are hindered by the difficulty to administer to lab animals as a solid foodstuff. In the present study, we have successfully synthesized triheptanoin to the highest standards of purity from glycerol and heptanoic acid, using sulfonated charcoal, previously studied in esterification reactions by Yurui and Prager (Aust. J. Chem. 1989, 42, 1003-1005), as a catalyst. Then, triheptanoin oil was formulated as a solid, stable and palatable diet using a ketogenic base and a combination of four commercially available formulation agents; hydrophilic fumed silica, hydrophobic fumed silica, microcrystalline cellulose and talc. Diet compliance and safety was tested on C57Bl/6 mice over a 15-week period, comparing overall status and body weight change.
Practical applications

This work provides a complete description of, (i) an effective and cost-effective synthesis of triheptanoin and, (ii) the formulation of a solid, stable and palatable triheptanoin-rich (39% of caloric intake) ketogenic diet for rodents. Rodent triheptanoin-rich diets have practical applications in pre-clinical screening of the therapeutic efficacy of triheptanoin in different rodent models of human diseases. On the other hand, using the same solidification procedure, other oils could be incorporated into rodent ketogenic diet to study its high dose/long term effect on mammal health and development. This approach could be extremely valuable as ketogenic diet is widely used clinically for epilepsy treatment.
Introduction

In mammals, glucose is the main metabolic substrate provided to peripheral tissues and brain. However, under certain circumstances such as long term fasting or ketogenic diet administration, a liver produces four carbon (C4) ketone bodies (acetoacetate and β-hydroxybutyrate), which are preferentially utilized by peripheral organs and brain, sparing glucose for glucose-dependent tissues, such as erythrocytes and retina. Since 1990s, ketogenic diet has been used clinically to treat refractory epilepsy [6], GLUT-1 deficiency syndrome [12] and pyruvate dehydrogenase deficiency [18].

Triheptanoin (also glycerol trienanthat; 1,2,3-trienanthoylglycerol; glycerol triheptanoate, trienantin) is a special non-natural triacylglycerol composed of 3 heptanoyl chains (C7:0). If added to ketogenic diet, it is metabolized in liver to non-natural ketone bodies such as β-keto-pentanoate and β-hydroxypentanoate. In peripheral organs and brain, those blood-born five carbon ketones (C5) can enter directly to the TCA (trigerboxylic acid, also Krebs) cycle via succinyl-CoA and, unlike the “classical” C4 ketone bodies, provide anaplerotic carbons. Anaplerosis is a process that replenishes TCA cycle intermediates, thus providing building-blocks for biosynthetic pathways. Triheptanoin-derived C5 could bypass various steps of intermediary metabolism and directly “energize” mitochondria [5]. This strategy has been successfully used in treatment of metabolic disorders such as mitochondrial fatty acid oxidation defects, pyruvate carboxylase deficiency and adult polyglucosan body disease [19-21]. Interestingly, according to a recent clinical trial, even patients suffering from Huntington’s chorea could benefit from triheptanoin diet [15]. In past years, basic research revealed that secondary derangement of mitochondrial metabolism accompany and worsen many complex disorders such as cancer, neurodegeneration and cardiovascular disease [6,8,10]. Intensive
screening for possible therapeutic effects of triheptanoin in different animal models of these diseases could open new horizons for medical dietary therapy of multiple aging-associated pathologies.

Although triheptanoin has been administered parentally in some studies [11], it has best anaplerotic potential (C5 ketone production) when administered with a ketogenic diet (high-protein and fat with up to one-third of dietary calories as triheptanoin). However, studies using triheptanoin/ketogenic diets in the pre-clinical setting are hindered by the difficulty to administer to lab animals as a solid foodstuff, due to the low viscosity of triheptanoin. In the present study, we have developed an efficient synthetic protocol to produce triheptanoin from simple precursors and elaborated a palatable, low spreading and easy to administered solid diet rich in triheptanoin.

The main goal of our synthetic protocol has been to develop an efficient, economic and environmentally friendly procedure that provides considerable quantities of triheptanoin with an outstanding level of purity, of crucial importance because triheptanoin is therapeutically used in long-term, high-dose protocols. Using sulfonated charcoal, previously studied in esterification reactions by Yurui and Prager [17], as a catalyst, instead of stoichiometric reagents and halogenated solvents, synthesis was cleaner, and more efficient. Next, we produced a formulation taking into consideration not only thickening or adsorption capacity of each additive, but also their possible interaction with ketogenesis (i.e., carbohydrate content) and median lethal dose in rodents. To achieve this goal, a combination of four commercially available formulation agents has been used in this work; hydrophilic fumed silica, hydrophobic fumed silica, microcrystalline cellulose and talc. Finally, compliance and safety was performed on C57Bl/6 mice comparing overall status and body weight change for 15 weeks.
2. Materials and Methods

2.1 Chemicals

Anhydrous glycerol (CAS: [56-81-5]) was purchased from Fluka, heptanoic acid (CAS: [111-14-8]) from Sigma-Aldrich, toluene, sodium hydroxide (NaOH), charcoal and sulphuric acid (H₂SO₄) from local supplier. Rheological additives (all pharmaceutical grade): hydrophilic fumed silica (AEROSIL® 200) and hydrophobic fumed silica (AEROSIL® R972) were purchased from Evonik Industries; microcrystalline cellulose (VIVAPUR® 101) and talc (Ph Eur. / USP quality) from Quimivita S.A.

High Protein Ketogenic Diet for rodents (Test Diet - 5TJR) was purchased from IPS Product Supplies Ltd. and normal rodent chow from Harlan.

2.2 Chemical synthesis of triheptanoin

Scheme 1. Esterification reaction.

2.2.1 General Methods

NMR spectra were recorded in CDCl₃ at 400 MHz (¹H) and 100.6 MHz (¹³C), and chemical shifts are reported in δ values downfield from TMS or relative to residual chloroform (δ = 7.26 ppm, 77.0 ppm) as an internal standard. Data are reported in the following manner: chemical shift, multiplicity, coupling constant, integrated intensity. Multiplicities are reported using the following abbreviations: t, triplet; dd, doublet of doublets; m, multiplet; tt, triplet of triplets. Evaporation of solvents was accomplished with a rotary evaporator. Thinlayer
chromatography was performed on SiO$_2$ (silica gel 60 F254), and the spots were located by 1% aqueous KMnO$_4$. Mass spectra were recorded with a LTQ spectrometer using electrospray ionization (ESI+) techniques.

2.2.2 Preparation of sulfonated charcoal catalyst [9]

A mixture of active charcoal (25 g) and sulphuric acid (75 mL, 96%) was heated at 260 °C and vigorously stirred overnight (18 h). Then the mixture was filtered off and washed with distilled water (1 L) until washings gave a negative barium chloride test. The filtrate was dried at 100 °C for 24 h.

2.2.3 Esterification Procedure

Sulfonated charcoal catalyst (4.20 g, w = 2.3%, based on glycerol) was added to a mixture of glycerol (176.85 g, 1.92 mol), heptanoic acid (1000 g, 7.68 mol, 4.0 equiv) and toluene (350 mL). The reaction mixture was heated under reflux (internal temperature: 140 °C) with water being removed in a Dean-Stark apparatus [Annotation 1].

Work-up; Method A: After 4 days the catalyst was removed by filtration (paper; Albet 400) with the aid of toluene (400 mL). Filtrate was washed with aqueous 15% NaOH (3 x 300 mL) [Annotation 2] and with distilled water (2 x 50 mL). Combined aqueous layers where extracted with toluene (1 x 100 mL) and organic layer was washed with distilled water (2 x 20 mL). Combined organic extracts were dried over sodium sulphate (Na$_2$SO$_4$), filtered and concentrated under reduced pressure to give transparent colourless liquid product. Product was
dried in vacuum drying oven (60 °C, 20-30 mbar, 48 h) to give transparent colourless oily liquid (802 g, 97.5%) with purity 99+% (GC/MS).

*Work-up; Method B:* 20 g scale experiment: The resulting filtrated was evaporated and the residue was fractionally distilled to give transparent colourless oil (20.14 g, 94%, 190°C, 1 mm Hg) with purity 99+% (GC/MS).

### 2.2.4 Analytical data

![Triheptanoin molecule](image)

**Figure 1:** Triheptanoin; atom numeration for NMR assignment

**$^1$H-NMR** (400 MHz, COSY, CDCl$_3$): $\delta$ 0.89 (t, $J = 6.8$ Hz, 9H, 3x $^2$CH$_3$), 1.25-1.31 (m, 18H, 3x $^4$CH$_2$, 5CH$_2$, 6CH$_2$), 1.61 (m, 6H, 3x $^3$CH$_2$), 2.31 (m, 6H, 3x $^2$CH$_2$), 4.15 (dd, $J = 11.9$, 6.0 Hz, 2H, 2xH$_A$), 4.30 (dd, $J = 11.9$, 4.3 Hz, 1H, 2xH$_A$), 5.27 (tt, $J = 6.0$, 4.3 Hz, 1H, H$_B$) ppm.

**$^{13}$C-NMR** (100.6 MHz, HSQC, CDCl$_3$): $\delta$ 13.9 (3xC-7, CH$_3$), 22.4 (3xC-6, CH$_3$), 24.7 (3xC-3, CH$_2$), 28.7 (3xC-4, CH$_2$), 31.3 (3xC-5, CH$_2$), 33.9 (2xC-2A, CH$_2$), 34.1 (C-2B, CH), 62.0 (2xC-A, CH$_2$), 68.8 (C-B, CH), 172.7 (C-1B, COOR), 173.1 (2xC-1A, COOR) ppm. **IR** (NaCl): 2930, 1745 (s, COOR), 1162 cm$^{-1}$. **GC-MS:** m/z 299 (21; C$_{17}$H$_{31}$O$_4^-$), 285 (10; C$_{16}$H$_{29}$O$_4^-$), 113 (100; C$_7$H$_{13}$O$^-$), 85 (16; C$_6$H$_{13}^+$). **Elemental Analysis:** Anal. Calcd for C$_{24}$H$_{44}$O$_6$: C, 67.26; H, 10.35. Found: C, 67.12; H, 10.64. [Annotation 3] **High Resolution Mass Spectrometry:** [C$_{22}$H$_{48}$N$_6$O$_6$ (M$^+$+NH$_4^+$)] 446.3474, calculated 446.3476. [Annotation 4] **UV-vis** (EtOH) = 211 nm (I$_{max}$).

**TLC:** $R_f$ = 0.26 (SiO$_2$, 2% MeOH in CH$_2$Cl).
Annotations to the synthesis:

1. 106 mL of water was collected, expected amount was 103.7 mL of water formed during the course of reaction and the excess is from the used solvent.

2. Error: C = 0.14%; H = 0.29% when 0.40% is permitted.

3. 0.42 ppm difference when 5 ppm is permitted.

4. When the reaction is finished, mixture contains 1.92 mol of unreacted heptanoic acid. For alkali refines 2 molar excess of base was used, i.e. 3.84 mol (153.6 g). Solution 15% (weight): 153.6 g of NaOH and 870 mL of water. Observation: Sodium salt of heptanoic acid can form temporal solid – soap, which is dissolved spontaneously with time.

2.3 Pharmaceutical formulation

2.3.1 Food mixture preparation and storage

The food mixtures were prepared under normalized conditions as follows: The standard ketogenic diet (KD) was placed into a warm water bath (30 °C) while the rheological additives were incorporated at room temperature to the oily phase (triheptanoin) under gentle stirring. Finally the thickened oily phase was added to the standard ketogenic diet and the mixture kneaded until homogeneity. Each sample was transferred in glass recipient hermetically closed. Once left standing at room temperature they were stored in refrigerator (4 ± 1 °C). The mixtures were examined 48 h after their elaboration. All measurements were replicated three times. Results are presented as mean value ± standard deviation.

2.3.2 Rheological characterization

2.3.2.1 Spreading capacity
The spreading capacity of ketogenic diet (KD), binary mixture (BM, *i.e.* 72-28 % KD and triheptanoin) and final triheptanoin-rich ketogenic diet (TKD) was assessed. The measurement principle consists of determining the area increase of a fixed volume of product (352 mm$^3$) squashed between two parallel planes, under the effect of the constant weight pressure (200 g) during a fixed period of time (1 min). For this determination an original apparatus developed in our laboratory was used [For detailed information see supplementary material].

2.3.2.2 Viscosity determination and viscoelastic behaviour

The rheological studies have been performed using the HAAKE RheoStress 1 rheometer: Haake PP60Ti plate sensor (6 cm diameter), connected to a temperature control Thermo Haake Phoenix II + Haake C25P.

Viscosity measurements at 25 °C were applied to triheptanoin and BM. They were carried out at three different shear rates (25, 50 and 100 s$^{-1}$) and recorded during 1 min after the corresponding three ramp–up periods (0 to 25 s$^{-1}$, 25 to 50 s$^{-1}$ and 50 to 100 s$^{-1}$) within 1 min.

Oscillatory tests were applied to the final TKD in order to determine the linear viscoelastic region. Oscillatory stress sweeps between 0.1 and 100 Pa were performed at 1 Hz. Four different gaps between plates were tested (1, 2, 3 or 4 mm). Frequency sweep tests were performed from 0.01 and 10 Hz at a constant shear stress within the linear viscoelastic region in order to determine the related variation of storage modulus ($G'$) and loss modulus ($G''$) at different temperatures (25, 30 y 35 °C). Both viscoelastic moduli are defined as follows: $G' = \tau_o/\gamma_o \cdot \cos \delta$ and $G'' = \tau_o/\gamma_o \cdot \sin \delta$ (where $\tau_o$ and $\gamma_o$ are the amplitudes of stress and strain and $\delta$ is the phase shift between them) [14,23,24].
2.4 Animals

Thirty-five female, three months old C57Bl/6 mice from our colony were housed under the standard conditions, with *ad libitum* access to water. Control group (n=12) received normal chow *ad libitum*. KD and TKD were stored at 4°C, according to the manufacturer’s recommendation. Fresh dose of KD and TKD were offered in amount 3g/day/animal twice a week, unconsumed rests were discarded. Animals were observed twice a week and weighted once a week. Study protocol was approved by the University of Barcelona Animal ethical committee.

3 Results and discussion

3.1 Triheptanoin synthesis from glycerol and heptanoic acid: high yield and purity.

To the best of our knowledge the first reported $^1$H RMN data of triheptanoin was published by Lie Ken Jie and co-workers [13]. Saturated triacylglycerols were prepared by esterification reaction of corresponding carboxylic acid with glycerol mediated by coupling reagent (dicyclohexylcarbodiimide - DCC) in dichloromethane media. Neither experimental details, nor information on yield are described. More recently Ataide and collaborators reported the chemical synthesis of tricaproin, triheptanoin and tricaprylin in the absence of both solvent and catalyst at high temperatures and under vacuum [2]. The process involved two stages under high temperature and vacuum and a 50% molar excess of heptanoic acid. Triheptanoin was isolated in 79% yield after purification by column chromatography (silica gel, hexane:chloroform 1:1).

There are many questions to be considered in the synthesis of acylglycerols, especially those of potential application in the food industry and as nutraceuticals. The methods explained
above can be excluded because coupling reagent (DCC) or excess of halogenated solvents in the purification step is used. Additional problems will be with scaling-up of the reaction under vacuum, which are potentially dangerous (especially with larger set-ups) and problematic for analytical sampling.

Therefore, we have developed an alternative procedure for the synthesis of triheptanoin that involves the esterification of heptanoic acid (33% molar excess) by glycerol in toluene under heterogeneous catalysis (sulfonated charcoal). The reaction was scaled up from 5 g to 800 g without loss of purity with yield 94-97%. In terms of “green chemistry metrics” [3] this process can be considered as environmentally friendly, because water is the only subproduct and the catalyst can be recycled. [9] The atom economy (AE) of this process is 88.8% and carbon efficiency (CE) is 75.5%. Mass intensity (MI), rigorous metrics - in which also non-binding reagents and solvents are included - is 1.85 g/g. Very good MI ratio confirms that our synthesis is highly efficient. This method to obtain highly pure triheptanoin is a clean, cost-effective, safe and easy-to-set-up.

All the analytical data (\(^1\)H-NMR, \(^{13}\)C-NMR, IR, etc.) are coincident with the previously reported data. Moreover, High Resolution Mass Spectrometry and Elemental Analysis were performed. Noteworthy, the previous assignation of the \(^{13}\)C RMN needs to be corrected due to the fact that bidimensional studies revealed that previously assigned peaks 31.32 (C-5) and 24.70 (C-3) are interchanged.

3.2 Combination of four pharmaceutical additives improves spreading capacity and viscoelastic parameters of the Triheptanoin-rich ketogenic diet (TKD).
In 2008, a research group from Federal University of Alagoas, Brasil, fed rats during 7 weeks with anaplerotic diet based on margarine, casein and triheptanoin with weight ratio of fat:(protein+carbohydrate) 3.5:1 but energy ratio close to 7:1. Unfortunately, no details on physical properties of this diet are available [4]. To the best of our knowledge, no formulation of solid triheptanoin-rich ketogenic diet for rodents has been described to date. Direct incorporation of requested amount of triheptanoin (28 weight%, corresponding to 39 calorie%) into a standard rodent ketogenic diet gives dense liquid consistency, difficult to administer to animals at room temperature in dose-controlled manner. Triheptanoin oil showed Newtonian behaviour and its viscosity at 25 °C was 14.15 mPa·s. In contrast, BM showed pseudoplastic rheological behavior and its viscosity decreases with shear rate: 5743±38 mPa·s (25 s⁻¹), 3407±13 mPa·s (50 s⁻¹) and 2105±8 mPa·s (100 s⁻¹). Spreading capacity of the KD was 255±7 mm² while spreading capacity of the BM was 562±11 mm². Four additives have been added; hydrophilic fumed silica (Aerosil® 200), hydrophobic fumed silica (Aerosil® R972), microcrystalline cellulose and talc (Table 1). The final TKD has a solid pasty texture with spreading capacity 349±24 mm² at room temperature.

Additive content already present in KD (Powdered cellulose) as well as mouse median lethal dose (LD50) were considered when determining the maximum amount of each additive beside their thickening or adsorption capacity [1,7,16,22].

Table 1: Content of triheptanoin-rich ketogenic diet (TKD).
According to the results of oscillatory stress sweeps, a plat gap of 2 mm and a constant shear stress of 2 Pa (20% of the critical value) were selected to perform the frequency sweep tests.

Oscillatory measurements applied to TKD sample showed prevalence of the elastic over the viscous behavior ($G' > G''$) at all studied temperatures (25ºC–35ºC) in the whole frequency range. No significant differences were detected within the temperature range (Figure 2).
3.3 Pilot testing in adult mice shows the diet is safe and palatable.

Final TKD was easy to weight and administer over the cage grid and was well tolerated by the animals. There was no significant difference in body weight variation between the three groups until week nine. After 12 weeks of diet administration, animals in TKD group showed continuously lower weight gain if compared to KD and control group. No abnormality in overall status of any animal was observed (Figure 3).

4 Conclusions

>From the chemical viewpoint, we have developed a cost-effective procedure of triheptanoin synthesis with evident advantages: i) just small excess of heptanoic acid is used, ii) the process is metal free, iii) only environmentally friendly solvents and catalysts are involved, iv) no column
chromatography or other purification of product is needed. Triheptanoin is isolated in 94-97% yield, with more than 99% purity (CG-MS) and no additional purification step is needed. Reaction was successfully scaled up from 5g up to 800g.

Combination of four pharmaceutical additives improved spreading capacity and viscoelastic properties of the pilot binary mixture and resulted in solid, palatable, non-toxic, stable and easy to handle formulation of rodent Triheptanoin-rich ketogenic diet. Finally, animal testing showed that this diet is well tolerated and could be used in wide variety of chronic experiments requiring anaplerotic, triheptanoin-rich ketogenic diet.

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Scheme 1. Esterification reaction.

Glycerol (1.0 equiv) + heptanoic acid (4.0 equiv) → Sulfonated Charcoal Catalyst (w = 2.3%) → Glycerol-triheptanoate (800 g SCALE, purity 97.5%, GC-MS = 98+%)
Figure 1.: Triheptanoin; atom numeration for NMR assignment
### Table 1

**Content of triheptanoin-rich ketogenic diet (TKD).**

<table>
<thead>
<tr>
<th>TKD Content</th>
<th>Content Parts</th>
<th>Mice LD&lt;sub&gt;50&lt;/sub&gt; [mg/g]</th>
<th>Content in daily food offer* [mg]</th>
<th>Maximal daily dose/body weight** [mg/g]</th>
<th>Maximal average [%] Mice LD&lt;sub&gt;50&lt;/sub&gt;</th>
<th>Caloric content [kcal/g]</th>
<th>Daily energy offer [kcal]</th>
<th>Energy [%]</th>
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<tr>
<td>Ketogenic diet (Test Diet - 5TJR) (in which powdered cellulose content 2.89%)</td>
<td>72</td>
<td>1993.0</td>
<td>99.7</td>
<td>5.2</td>
<td>10.4</td>
<td>61%</td>
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<tr>
<td>Triheptanoin oil</td>
<td>28</td>
<td>775.0</td>
<td>38.8</td>
<td>8.5</td>
<td>6.6</td>
<td>39%</td>
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<td>Aerosil® 200 (Hydrophilic fumed silica)</td>
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<td>3.2</td>
<td>41.5</td>
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</tr>
<tr>
<td>Aerosil® R972 (Hydrophobic fumed silica)</td>
<td>0.9</td>
<td>5</td>
<td>24.9</td>
<td>1.3</td>
<td>24.9%</td>
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<tr>
<td>Microcrystalline cellulose</td>
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<td>5</td>
<td>55.4</td>
<td>2.8</td>
<td>55.4%</td>
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<td>Talc</td>
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<td>110.7</td>
<td>5.5</td>
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<tr>
<td><strong>TOTAL</strong></td>
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<td>3000</td>
<td>150</td>
<td>17.0</td>
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</tbody>
</table>

* daily food offer 3 g (17.0 kcal)  
**minimal body weight 20 g  
n. d. not determined
Figure 2. Frequency dependence of the storage and loss moduli for Triheptanoin-rich diet.

Frequency sweeps at 25°C, 30°C and 35°C.
Figure 3. Animal body weight variation.


