CHAPTER 4

Is 2-Hydroxypropyl-β-cyclodextrin a suitable alternative carrier to Human serum albumin for delivering FFA tracers by infusion in metabolic studies?

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Is 2-Hydroxypropyl-β-cyclodextrin a suitable alternative carrier to Human serum albumin for delivering FFA tracers by infusion in metabolic studies?

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Running head: Cyclodextrin as a carrier for FFA tracers

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Abstract

We investigated the feasibility of replacing human serum albumin (HSA) as the carrier for the intravenous delivery of free fatty acid (FFA) tracers with a chemically modified oligosaccharide, 2-hydroxypropyl-β-cyclodextrin (HP-β-CD). This study was initiated due to the stigma attached to blood products and physiological limitations related to administering exogenous HSA due to its potential to increase colloid oncotic pressure. HP-β-CD is promoted as an agent to aid in the delivery of hydrophobic "drugs" by intravenous administration. Thus we conducted a series of solubility tests and established that a molecular ratio of 1:3 and concentration ratio of 3.5:10.5 (mM) of FFA: HP-β-CD proved most successful, under optimal mixing conditions (using an incubation temperature of 65^{o}C and polypropylene plastic containers, which have a good conductivity). Five subjects participated in intravenous infusion trials with K^{+}[1-^{13}C]palmitate-HP-β-CD infused at 0.12 μmol.kg^{-1}.min^{-1} for 60 min during submaximal exercise. Three out of 7 infusion attempts proceeded without ill-effects, but 4 attempts resulted in irritation at the site of infusion; possibly due to unapparent incomplete solubility of the infusate. Comparison of the bioavailability of the FFA tracer when delivered with HP-β-CD or HSA based on expired carbon dioxide enrichment analysis (7.4±2.0‰ and 8.6±2.1‰ from HP-β-CD and HSA delivered FFA tracer, respectively) showed that HP-β-CD does not alter the capacity of the FFA tracer to be catabolised by tissues (P=0.4). Thus after refining the technique to perfect the infusate mixing protocol, HP-β-CD should make a suitable alternative FFA tracer carrier.

Key words: free fatty acid carbon isotopes; cyclodextrin; metabolic bio tracers; fat metabolism, exercise

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Introduction

The hydrophobic nature of free fatty acid (FFA) tracers, used in metabolic turnover studies, necessitates the use of a “carrier” to hold the tracer in solution for delivery by intravenous infusion. The carrier routinely used is human serum albumin (HSA); the primary natural carrier of FFA released into circulation following lipolysis [1]. HSA is used medically for the purpose of increasing plasma volume (due to the colloid osmotic properties of albumin) in patients with shock resulting from trauma, haemorrhage, operation or infection. In fact, plasma albumin is responsible for 80% of the colloid oncotic pressure. Therefore, when administering HSA to healthy individuals the amount administered must be carefully controlled and kept low to prevent changes to body fluid compartment volumes and possibly massive blood volume expansion. This is complicated by the necessity to maintain a low concentration of FFA tracer (~2.2mM) in the albumin solution to ensure solubility of the tracer. Furthermore, the use of stable tracers necessitates a higher infusion rate than with radioactive tracers to increase the enrichment above the naturally present background levels of the tracer. Therefore, in order to administer the appropriate infusion rate of a stable FFA tracer, a high flow rate is required and this increases the amount of albumin delivered. Consequently, the infusion period is limited, particularly during exercise when a higher infusion rate is required than at rest.

Moreover, since HSA is a blood product, this may dissuade volunteers from participating in research studies involving the infusion of HSA-bound FFA tracers. HSA, however, is extracted from blood that has undergone testing for infectious diseases and has been sterilized further by pasteurization for 10 hours at 60^{o}C, ensuring non-transmission of the type B Hepatitus virus and the Human Immunodeficiency Virus to recipients (South African Blood Transfusion Service).

For the above reasons, we considered the feasibility of using an alternative carrier for FFA tracers. Specifically, we wished to determine the feasibility of binding our FFA tracer to a complex carbohydrate polymer, 2-Hydroxypropyl-β-cyclodextrin (HP-β-CD). HP-β-CD is used for solubilising hydrophobic drugs or substances intended for intravenous administration, such as steroids [2,-4], prostaglandins [5] and thyropropin-releasing

hormone analogues [6]. It is also used to decrease the irritation of some drugs at the site of administration and to stabilize drugs that are unstable in aqueous environments [7].

Cyclodextrins are cyclic oligosaccharides composed of dextrose units that have a hydrophilic exterior surface, which promotes solubility and a hydrophobic core where the drug is bound by non covalent hydrogen bonds, Van der Waals and hydrophobic forces [8]. Alternatively, the drug is enclosed in a channel formed by several molecules of cyclodextrins [9]. Cyclodextrins are naturally produced from starch by the enzyme cyclodextrin glycosyltransferase by several organisms, such as Bacillus macerons [8]. HP-β-CD is a cyclodextrin that has been chemically modified to improve parenteral safety by increasing aqueous solubility [8]. The cyclodextrin-drug complex is dissociated on dilution of the infusate by plasma and extracellular fluid (ECF), and by cholesterols and other plasma lipids that have a high affinity for cyclodextrin and will displace the drug from the complex. HP-β-CD has limited tissue distribution and stays in the ECF due to its hydrophillic nature [7]. It has undergone numerous clinical trials on animals and humans to assess its safety for intravenous delivery [7]. One particular case study has confirmed long term safety following an acute intravenous administration of HP-β-CD at a dose of 7.5 g/day for 4 days in a young boy [10]. Recent studies have been using HP-β-CD for the intravenous delivery of dialysis drugs [11, 12], where doses of the order of 8 g of HP-β-CD is infused in patients over 1 hour [11].

In the current study, we wished to firstly, determine whether it would be possible by using specific mixing protocols utilising HP-β-CD to attain an infusate solution that holds a FFA tracer in solution. Secondly, after attaining solubility, the HP-β-CD-FFA tracer solution would be used in infusion experiments during exercise to compare the bio-availability of the tracer for cellular metabolism when using HP-β-CD as carrier versus HSA. The main intention of this initial investigative study was to elucidate the feasibility of applying this technique for specifically an exercise protocol which typically last 60 minutes.

Methods

Solubility experiments

The feasibility of HP-β-CD (*Encapsin*^{TM} HPB parenteral, Janssen Biotech N.V) as a means of solubilising the K^+[1-^{13}C]palmitate tracer (98% enriched, Isotec Inc., Miamisburg, OH) was tested by using the same general mixing protocol as in the preparation of the HSA-K^+[1-^{13}C]palmitate infusate, described previously [13]. The HP-β-CD used in this study, provided by Jannssen Biotech, is no longer commercially available following the closure of Janssen Pharmaceutica's biotechnology department. However, a replacement product called Hydroxyproply-β-cyclodextrin, Endotoxin controlled is available through RDI, division of Fitzgerald Industries International [14].

Various molar concentrations of palimitate and HP-β-CD were used to determine the highest possible concentration of the fat tracer that could be solubilsed relative to a safe dose of HP-β-CD. The molar ratio of FFA: HP-β-CD tested ranged from 1:1 to 1:10. In general, the FFA tracer and HP-β-CD was weighed to 5 decimal places in sterile weighing boats, transferred to sterile containers and heated at 60-65^{o}C in a water bath and then reconstituted in warm sterile water as separate aliquots as follows: the FFA tracer was reconstituted into 40% of the total volume and HP-β-CD into 60% of the total volume. The HP-β-CD aliquot was then filtered through a 0.2μm Millipore syringe filter into a new sterile container to ensure sterility. Both solutions were then re-incubated at 60-65^{o}C before combining as follows: the FFA tracer aliquot was filtered through a 0.2 μm Millipore syringe filter into the HP-β-CD solution. The filtering of the FFA solution had to be done rapidly in order to prevent cooling and precipitation of the FFA tracer. The combined mixture was well mixed, by manually swirling, and allowed to cool at room temperature. Solubility of the final solution was assessed by visual inspection for the presence of FFA tracer precipitates on the day following mixing.

Infusion trials

Ten young healthy volunteers participated in this study (Table 1). Five were included in the HP-β-CD-FFA trials (n=1 male and n=4 females) and 5 where included in the HSA-FFA trials (n=5 females). All subjects provided written consent to participate in the study after the procedures and risks had been explained. Subjects were all non-smokers. This study was granted ethical clearance by the Committee for Research on Human Subjects, University of the Witwatersrand (M980812).

Table 1. Characteristics of subjects who participated in either the free fatty acid tracer bound-hydroxypropyl-β-cyclodextrin (FFA-HP-β-CD) or free fatty acid tracer bound-human serum albumin (FFA-HSA) infusion trials

	FFA-HP-β-CD	FFA-HSA
Sample size	5	5
Age	25.8±5.5	22.4±2.5
Body mass (kg)	59.2±11.9	55.7±5.9
Height (cm)	166.6±14.9	163.4±6.3
VO_2max ($ml.kg^{-1}.min^{-1}$)	44.2±9.7	28.5±2.2
Submax exercise intenstity (%VO_2max)	59.2±3.8	61.4±3.5
Submax work load (Watts)	102±40.9	46.6±12

A discontinousVO_2max test was performed on a stationary bicycle ergometer (Excalibur 911 900, Lode, Groningen, Netherlands). A discontinuous protocol was chosen as it is well tolerated by sedentary subjects and most of the participants in this study did not exercise regularly. After a 10 min warm up subjects performed 3 min exercise bouts starting at 100 Watts and increasing in increments of 20 Watts interspersed by recovery periods. During the recovery period subjects were allowed to rest for as long as they felt necessary (10-20 min) or until their heart rate returned to 100 $beats.min^{-1}$. During the 3 min exercise bout subjects breathed through a one-way valve mouthpiece that allowed them to inhale

atmospheric air and all expired air was directed through a 3 L mixing chamber into a metabolic cart system (Oxycon-4, Mijnhardt, Bunnik, Netherlands). The oxygen and carbon dioxide analysers were zeroed using 100% nitrogen gas and calibrated using commercially available gas mixture of known O_2 and CO_2 content and room air. The system calculated and produced readings of VO_2 and VCO_2 from conventional equations every 30 seconds. VO_2max was considered to have been attained when VO_2 differed by less than 1.5 $ml.kg^{-1}.min^{-1}$ between successive workloads.

Subjects kept a record of all meals for 48 hours before the trial and 2 hours before arrival at the laboratory they ate a pre-packed meal (2062 kJ) consisting of a 175 ml yoghurt, 175 ml orange juice and 30 g granola bar, providing in total 74 g of carbohydrate, 14 g of fat and 12 g of protein. Subjects who performed the trial on a second occasion were required to follow their dietary records from the previous trial for 48 hours before the second trial.

At the start of each infusion trial, body mass was recorded and then a 20 G cannula was inserted into a vein on the subjects' right forearm for infusing. After 20 min of rest, subjects commenced exercise on a bicycle ergometer at 60%VO_2max for 90 min. After 30 min of exercise subjects received a 5.5 $mmol.kg^{-1}$ $NaH^{13}CO_3^-$ (98% enriched, Isotec Inc., Miamisburg, OH) priming dose in 0.1N sodium hydroxide and then a continuous infusion of either HP-β-CD-K^+[1-^{13}C]palmitate (n=5) or K^+[1-^{13}C]palmitate in 5% human serum albumin (HSA) (n=5) was started at an infusion rate of 0.12 $\mu mol.kg^{-1}.min^{-1}$ for the remaining duration of the exercise session (i.e. 60 min). (While resting FFA kinetic studies may require prolonged infusion periods, during moderate intensity exercise, a 60 min infusion period, run at a rate similar to the order of magnitude used in this current study, is more than sufficient for attaining a steady-state condition [15]. In fact, Sidossis et al. have previously shown steady-state for FFA tracers to be attained after only 15 min of infusing during moderate exercise [15]). As the concentration of potassium [1-^{13}C]palmitate in the infusate solution was fixed (2.2 mM for the HSA trials and varied during protocol development when using HP-β-CD (see table 2)), the flow rate was altered for each subject depending on their body mass to ensure an infusion rate of 0.12 $\mu mol.kg^{-1}.min^{-1}$. All solutions were freshly prepared under a laminar flow hood one to two days before the experimental trial. The 5% HSA solution was prepared from a 20 % HSA stock solution

(SA blood transfusion bank, Johannesburg, RSA) by diluting the required volume of stock with saline and incubating it in the water bath before use. All solutions passed a standard test (LAL, Gel clot test, sensitivity 0.06EU) to ensure that they were endotoxin-free before use (GX99010, Coatest Gel-LAL, Chromogenix, Mőlndal, Sweden). Indirect calorimetry measurements and expired air samples were collected in 2L Douglas bag after flushing the bags twice with the subjects expired air at rest, before onset of infusion and at 15 min intervals thereafter. Carbon dioxide was extracted from expired air by cryodistillation and carbon-13 CO_2 enrichment was determined by isotope ratio mass spectrometry (IRMS) (MS20, Kratos, UK).

Enrichment in expired carbon dioxide is the end result of carbon tracer oxidation and therefore cellular availability and utilisation of the tracer. Therefore, enrichment of the tracer in the expired air samples was used as an indication of bioavailability of the tracer during the HP-β-CD-K^+[1-^{13}C]palmitate infusion trials and the HSA-K^+[1-^{13}C]palmitate infusion trials.

Statistical analysis

Intra- and inter-individual coefficient of variation for expired air enrichment was determined as the standard deviation divided by the mean multiplied by 100. An unpaired t test was used to compare carbon-13 enrichment in expired air when FFA tracer was bound to HP-β-CD and HSA, $p<0.05$ was regarded as significant.

Results

Solubility tests

Solubility tests revealed that HP-β-CD is capable of holding the long chain FFA tracer in solution in a ratio of tracer to HP-β-CD of no less than 1:3 (Table 2).Thus three cyclodextrin molecules form an intermolecular hydrophobic channel in which the tracer is held. However, a lower molar concentration is preferable. That is, concentrations of the order of 6.9 mM FFA: 20.7 mM HP-β-CD resulted in a higher precipitation rate than 3.5 mM FFA: 10.5. mM HP-β-CD (Table2).

Table 2. Record of solubility tests and pilot infusion trials

	FFA:CD ratio		Incubation temp (°C)	Sample vol (ml)	Solubility	Comments or infusion trial outcome
	Molecular ratio	mM conc.				
Establish working molecular ratio to be at least 1:3	1:2	6.9:13.8	58	103.5	ppt	
	Sub-volume samples*					
	1:2	6.9:13.8	60	20	clear	
	1:3	6.9:20.7				
	1:4	6.9:27.6				
	Full volumes					
	1:2	2.3:4.6	60	137	ppt	multiple container transfer
	1:3	2.3:6.9				
	1:3	6.9:20.7		107.4	ppt	
á incubation temp to 65 °C. â Molar conc. ratio to 3.5: 10.5	Increase incubation temp.					
	1:3	6.9:20.7	65	107.4	clear	Infused into subject S1(a); vein closure after 75 min
	Decrease CD concentration					
	1:3	5:15	65	171	clear	Infused into S2(a); arm vein closed after 40 min
	Decrease CD concentration further					
	1:3	3.5:10.5	65	244	ppt	
Reconsidered molecular ratio, and discovered that container-type must be considered	Increase ratio of CD:FFA (sub-volume samples)					
	1:4	3.5:14	65	20	few ppt	
	1:5	3.5:17.5				
	1:6	3.5:21				
	1:8	3.5:28		30	clear	used 50 ml PP plastic centrifuge tubes
	1:10	3.5:35			ppt	used pyrex glass bottles
Experimented with different types of plastic conatiners. Polypropylene is possibly best. Small is better than large volumes	Type of container (only PP plastic) sub-volume sample					
	1:1	3.5:3.5	65	30	ppt	
	1:2	3.5:7			clear	
	1:3	3.5:10			clear	
	PP plastic 50 ml centrifuge tubes (full volume)					
	1:3	3.5:10.5	65	164 (4x41 ml)	clear	Infused S2(b); 60 min; no ill effects
	Larger 150 ml PS plastic containers					
	1:3	3.5:10.5	65	134	clear	Infused S3(a); 60 min; no ill effects
	Repeat attempt in S3(b)					
	1:3	3.5:10.5	65	134	clear	Infused veins irritated after 15 min
	New subject S4(a)					
	1:3	3.5:10.5	65	97	clear	Infused for 60 min; 1st vein closed 20 min, infuse into opposite arm vein 40 min
	New subject S5(a)					
	1:3	3.5:10.5	60	103.7	clear	vein close at 45 min

*Sub-volume samples where used in pilot solubility experiments whereas the full volume sample solubility tests where prepared as if for an infusion trial. Subjects are numbered S1-S5 and the first and second infusion trial is identified as a and b, respectively. FFA: CD – free fatty acid to cyclodextrin ratio; ppt- precipitate; PP- polypropylene; PS- polystyrene

Furthermore, incubation temperatures of greater than 60°C were shown to be preferable during mixing and prevention of cooling during the filtering of the FFA tracer solution into the HP-β-CD solution is critical to prevent precipitation of the FFA tracer before it successfully complexes with the HP-β-CD. In this regard the conductivity of the containers used proved to be important (Table 3). For reasons that remain unknown, pyrex glass was not suitable (Table 2). Plastics made of polystyrene had a substantially lower conductivity than ones made of polypropylene (Table 3). Furthermore, containers holding a smaller volume had a higher success rate for maintaining the solubility of the FFA tracer (Table 2); possibly due to a smaller volume of FFA tracer being filtered into the cyclodextrin solution. Thus the smaller volumes allowed for faster mixing and so limited the cooling down of the solutions, thereby preventing precipitation. However, the disadvantage of using containers that hold small volumes is that multiple samples must be individually prepared for a single total infusion dose.

Table 3. Conductivity of sample containers

T° in primary incubating container (°C)	T° in secondary incubating container (°C)		T° in sample container (°C)	
Water bath	Glass beaker	Plastic beaker	PP plastic (50 ml)	PS plastic (150 ml)
62	-	-	-	58.5
62	58	-	57	-
62	-	54	53.5	-
66	-	-	-	62
66	63	-	61	-
66	-	57	56	-

T° – temperature; PP – polypropylene; PS - polystyrene

Infusion trials

Successful infusion of FFA tracer complexed to HP-β-CD was achieved when administered in a molar ratio of 1:3 (Table 2). The infusion proceded for 60 min during submaximal exercise with no ill effects for 3 of the 7 trials (i.e. S1, S2b, S3a; Table 2). When the infusion was continued for longer than 60 min in one of these 3 successful trials (subject S1), the infusing vein closed at 75 min. However, this trial carried out in subject S1 was the

first infusion trial and a higher molar concentration of tracer and cyclodextrin was employed (i.e. 6.9 mM FFA: 20.7 mM HP-β-CD).

The 4 remaining infusion trials were terminated early or required catheterisation of a new vein in order to complete the 60 min infusion period (i.e. S2a, S3b, S4, S5; see Table 2) due to irritation of the infusing vein which resulted in eventual closure of that vein.

Breath enrichment: HP-β-CD versus HSA

From the infusion trials with the FFA tracer-cyclodextrin complex, three complete sets of breath enrichment data was available (S2b, S3a, S4, Table 2) for comparison with FFA-HSA infusion trials (Figure 1a).

FFA tracer infusion trials using the standard HSA carrier were run in five different subjects. Unfortunately expired air samples from 2 of these subjects were lost and thus reduced the sample size for the HSA-FFA group to n=3. However, each subject participating in the HSA-FFA infusion trials completed two replicate trials with one trial taking place in the subject's early follicular (EF) menstrual phase (serum oestrogen: 199.7 ±77 pmol.l^{-1} and serum progesterone: 1.4±0.82 nmol.l^{-1}) and a second trial in either the subject's late follicular (LF) (serum oestrogen: 873 pmol.l^{-1} and serum progesterone: 3.5 nmol.l^{-1}), mid-luteal (ML) (serum oestrogen: 342 pmol.l^{-1} and serum progesterone: 47.2 nmol.l^{-1}) or late luteal (LL) (serum oestrogen: 222 pmol.l^{-1} and serum progesterone: 9.5 nmol.l^{-1}) phase. The FFA-HSA trials were run twice in each subject during different menstrual phases to determine the full range of variability in plasma FFA utilisation and hence breathe enrichment that may occur within female subjects. The intra-subject coefficient of variability for breath enrichment (comparing the average over the final 30 min) from FFA-HSA infusion and exercise was 22.8%. The inter-trial coefficient of variability including all FFA-HSA infusion trials (n=6 trials) was 20.6%. The inter-trial coefficient of variability for breath enrichment averaged over the final 30 min of the FFA-HP-β-CD infusion trials was 26.9%. Thus the variability for breath enrichment during the HP-β-CD and HSA trials were essentially similar.

The mean delta enrichment over the last 30 min of exercise and infusion when corrected for background enrichment was 7.4±2.0‰ (range, 5.9-9.7) for the FFA-(HP-β-CD) and 8.8±1.8‰ (range, 6.6-11.1) for the FFA-HSA trials (Figure 1b). No significant difference was detected between breath enrichment when using a HSA or the cyclodextrin carrier (P=0.4).

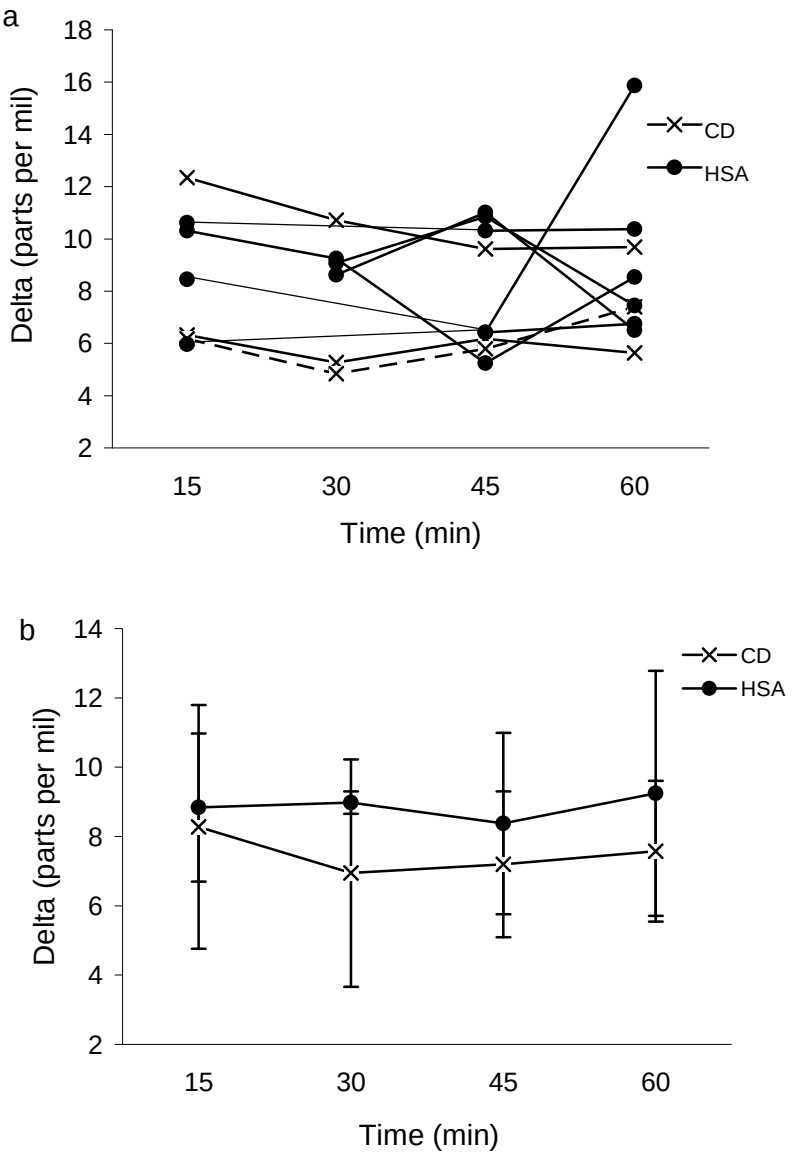

Figure 1. Carbon-13 enrichment in expired carbon dioxide during continuous infusion of either K^+[1-^{13}C]palmitate bound to HP-β-CD (solid circle) or K^+[1-^{13}C]palmitate bound to human serum albumin (x symbol) during submaximal exercise presented as (a) individual subject data, where the data set connected by a dashed (---) line identifies a male subject with marginally greater training status than the other subjects who were all sedentary females and (b) group mean and standard deviation.

Discussion

The most important finding of this study is that delivery of FFA tracers using HP-β-CD carrier did not affect bioavailability of the tracer when compared to delivery of the tracer using HSA carrier, based on breath enrichment data. The range for carbon-13 enrichment in expired air corrected for background levels when using the HP-β-CD carrier fell within the range measured when using HSA carrier, with no significant difference between the sample groups (Figure 1). This indicates that HP-β-CD may be used as an alternative carrier for intravenous delivery of FFA tracers.

The use of two different subject groups may be viewed as a potential limitation to our study design. However, due to the somewhat aversive nature of the protocol, we chose to include two separate subject groups in order to promote subject compliance. Great care was taken, however, to ensure that the groups were equally matched for age, and that they participated following a similar diet plan. Based on the VO_2max data presented in Table 1 it appears apparent that the subjects in the FFA-HP-β-CD group were more trained than the subjects in the FFA-HSA group. This may be a concern when comparing the CO_2 enrichment from a FFA tracer infusion between these groups, as training is known to increase FFA kinetics and oxidation even at equal relative exercise intensities [16]. Furthermore, gender is known to influence lipid metabolism, where men use less fat and more carbohydrate than women [17], and while the FFA-HSA group comprised only females, the HP-β-CD group included one male subject. However, of the three subjects from the FFA-HP-β-CD group whose CO_2 enrichment data was included in the comparison, two were women and had VO_2max values (on average 34.7 $ml.kg^{-1}.min^{-1}$) similar to the subjects in the FFA-HSA group and exercised at a similar work load (on average 65 Watts). The third subject from the FFA-HP-β-CD group was a male who had a higher VO_2max and submaximal work load (54 $ml.kg^{-1}.min^{-1}$ and 150 Watts). However, the CO_2 enrichment of this subject (Figure 1a) was within the range of the other subjects, possibly as a result of the positive effects of endurance training to promote fat oxidation that may have offset the normally lower levels of plasma fat oxidation experienced by males compared to females at equal relative intensities [18].

The multiple incidences of irritability of the infusion site when infusing the FFA-(HP-β-CD) complex is a concern. A possible cause for the irritation appears to be either incomplete solubility of the tracer in the infusate solution which is not readily apparent, or rapid dissociation of the tracer from the HP-β-CD carrier on entry into the plasma pool as a result of rapid dilution of the infusate solution when entering the circulation. The latter is known to be a factor when the ratio of drug to cyclodextrin is greater than 1:1 [4]. Various blood borne molecules are also known to have a high affinity for cyclodextrin, namely cholesterol or other blood lipids and will displace the FFA tracer from the cyclodextrin complex. It could be assumed that this rapid dissociation from the cyclodextrin complex on entry into the blood pool, if not removed by the circulation or taken up by endogenous albumin carrier, could cause local irritation at the delivery site. However, as not all FFA-HP-β-CD infusion trials in this current study resulted in irritation and closure of the infusion vein, the latter is most likely not the cause of irritability and vein closure. Furthermore, any rapid dissociation of FFA tracer from the HP-β-CD carrier on entry into the plasma pool can be expected to be easily picked up by circulating albumin, as the rate of infusion of the tracer is far below level that albumin normally copes with from endogenous FFA release from the adipose tissue, i.e. 0.12 μmol/kg.min was the infusion rate versus 8-12 μmol/kg.min which is the natural rate of appearance or release of FFA into circulation during moderate exercise [17, 19]. Lastly, the few incidences of irritability at the infusion site can also not be attributed to individual susceptibility as two of the subjects who repeated the infusion trial experienced one trouble-free trial and one trial with irritations. Therefore, it is most likely that the cause of the irritation in some of the trials but not others was due to unapparent incomplete solubility of the FFA tracer by the HP-β-CD in the prepared infusate solution and is possibly due to mixing conditions not being 100% ideal.

Conductivity of the plastic container may be the key factor determining success of complexing the FFA tracer with the HP-β-CD carrier, where the polypropylene 50 ml centrifuge tubes produced the most favourable temperature conditions for mixing and resulted in a trouble free infusion trial (subject S2b, see Table 2). The polystyrene plastic containers held larger volumes rendering them more convenient as they could hold the full

infusate sample, but only resulted in one successful infusion trial (subject S3a) and three with complications (subjects S3b, S4a, S5a). The polystyrene containers had a poorer temperature conductivity (4 ^{o}C lower than the incubating water bath) while polyproplyene containers only lost 1-2 ^{o}C relative to immediate incubating solvent (see Table 3). Future studies should experiment with using larger polypropylene containers.

The main difference between the HP-β-CD used in the current study (Encapsin^{TM}) and the one that is now commercially available (Hydroxypropyl-β-cyclodextrin, Endotoxin controlled) is what is termed the degree of substitution, with the new or replacement product having a slightly higher average degree of substitution (i.e. 4.3 for Encapsin and 7.6 for the new product) [14]. The degree of substitution simply implies the number of hydroxypropyl groups that have been substituted onto the hydroxl groups of the cyclodextrin molecule [14]. The higher the degree of substitution the greater the solubility of HP-β-CD and complexes made with HP-β-CD in aqueous solutions. A difference in the degree of substitution should have little effect on binding of the guest, in our case the free fatty acid (FFA) tracer. In fact it is possible that binding of the FFA tracer may be improved with this new HP-β-CD as a higher degree of substitution will increase the surface area to which the guest can bind [14] and this may favour FFA binding due to the long hydrocarbon chain structure of FFAs.

Therefore, the results from this study suggest that the viability of HP-β-CD as an alternative carrier for FFA tracers is dependent on perfecting a mixing protocol for the tracer carrier complex. A molecular ratio of 1:3 with corresponding concentration ratio of 3.5:10.5 (mM) for FFA to HP-β-CD is suitable. However, success of the complexing is dependent on effective thermal incubation at 65^{o}C during the entire solution preparation procedure. In addition the use of polypropylene plastic based containers is recommended.

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