# notes on methodology

## Tritiated glycerol triether as an oil phase marker in man

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**Summary** $^3$H-labeled glycerol triether has been suggested as a marker of the oil phase during digestion and absorption of a lipid test meal. This study examines the behavior of this isotope in the human alimentary tract. The results suggest that it is completely recovered from the gastrointestinal tract, and thus it remains solely with the oil phase of emulsions in vivo and with the oil phase of intestinal aspirates. $^3$H-labeled glycerol triether may thus be an appropriate marker of the oil phase for use in human studies of lipid absorption.

**Supplementary key words** triglyceride · absorption studies · inert marker

Tritiated glycerol triether (1, 2) has been used in animal studies as an inert oil phase marker to measure indirectly the digestion and absorption of lipids (3, 4). Various criteria (5) govern the use of inert markers in such studies, and, to date, although there are reports in the literature (6) where $^3$H-labeled glycerol triether has been used as an oil phase marker in man, its use in experiments to measure the absorption of lipids in human subjects has not been validated.

The object of this study was to investigate the use of tritiated glycerol triether (1-hexadecyl-2,3-didodecyl glycerol with $^3$H at positions 9 and 10 of the hexadecyl moiety) as a marker of the oil phase of intraluminal contents in the alimentary canal of human subjects.

*Experimental.* Normal male and female human volunteers between the ages of 30 and 65 yr were studied in two series of experiments.

The first series was concerned with the recovery of tritiated glycerol triether from the human gastrointestinal tract. 50 mg of the unsaturated precursor of glycerol triether 1-(9-*cis*-hexadecenyl)-2,3-didodecyl glycerol was catalytically reduced over platinum oxide with 2 Ci of tritium by the Radiochemical Centre at Amersham.

Seven subjects were given orally a gelatin capsule containing 3 ml of triolein and about 5 $\mu$g of the labeled glycerol triether (approximately 200 $\mu$Ci). Tritiated glycerol triether was prepared for each patient as described by Morgan and Hofmann (3). The tritiated triether (about 80% pure) was purified to greater than 99% by thin-layer chromatography on silica gel H, in the solvent system ether–hexane 15:85 (v/v), until a single homogeneous component (peak) was isolated from the solvent front. Impurities and the products of radiolysis remained at the origin of the chromatogram. Peaks were detected by passing the plate through a Packard radiochromatogram scanner. The silica gel around the purified component was scraped off and tritiated triether was extracted with 5 × 5 ml of diethyl ether; the ether extract was added to 4 ml of triolein. Ether was evaporated in a fume hood on a hot water bath under a stream of nitrogen. About 3 ml of the remaining oil, containing dissolved, purified, tritiated glycerol triether, was weighed into gelatin capsules. Three 0.1-ml portions were taken as standards and weighed into liquid scintillation counting vials, and the radioactivity was measured. From these results the radioactivity administered to the patient was calculated.

After healthy volunteers (patients or staff fed a standard hospital diet, 2500 calories and 80 g of fat per day) had swallowed the gelatin capsules containing triolein, their feces were collected on a daily basis for 5 days. A urine sample was taken over the first 24 hr, and five 20-ml samples of blood were obtained over a period of 24 hr in order to check that no detectable triether activity had been absorbed.

Stools were homogenized, using a Silverson emulsifier, in water, and about 100 ml was refluxed in 20 vol of chloroform–methanol 2:1 (v/v) for 8 hr (7). The resulting chloroform–methanol extract was filtered and concentrated by rotary evaporation. 3 × 0.1 ml of the concentrated extract was pipetted into a scintillation counting vial. 100 $\mu$l of the concentrated fecal extract was applied to a thin-layer plate (silica gel H, 0.5 mm thick) and chromatographed with reference standards of glycerol triether and cholesteryl oleate, which have been shown to have the same $R_F$ value as triether in the solvent system hexane–diethyl ether–methanol–glacial acetic acid 85:20:3:3 (v/v/v/v) (3). Whole blood, serum, and urine were extracted in 20 vol of chloroform–methanol 2:1. After 24 hr the extract was centrifuged and filtered, and an aliquot was removed to a counting vial. To each counting vial was added 10 ml of a toluene-based scintillator, and samples were counted for 10 min on a Packard Tri-Carb liquid scintillation counter, model 3375. Quenching was corrected by external standardization.

After administration of the test capsule, volunteers were observed clinically for at least 5 days. No clinical abnormality associated with this test was noted.

The second series of experiments, using 11 normal volunteer subjects, was designed to determine whether glyc-

erol triether would separate from triglyceride in an aqueous emulsion. These experiments utilized emulsions containing $^{14}$C-labeled triolein (tri[1-$^{14}$C]oleyl glycerol) and tritiated glycerol triether.

Emulsions were prepared with unlabeled and $^{14}$C-labeled triolein (5 mM final concentration) in which was dissolved tritiated glycerol triether. Sodium taurocholate was added to a final concentration of 2.5 mM, and the mixture was insonated in a MSE 100-W ultrasonic disintegrator for 10 min. Emulsions for intragastric infusions were prepared in water. Those for intraduodenal infusions were prepared in isotonic glucose–saline solutions, the glucose concentration being 56 mM because this concentration is consistent with maximum absorption of water (8).

In six subjects a gastric sampling tube was placed in the stomach under fluoroscopic control. The emulsions were divided into two portions after insonation and were kept moving continuously, under nitrogen, using a magnetic stirrer. The first portion of 450 ml (at room temperature) was infused as a single dose into the patient's stomach, and aspirated samples of 20 ml were collected on ice at zero time and at 15-min intervals for either 60 or 90 min from the start. Control 20-ml samples were removed at similar periods of time from the second portion of the emulsion, which was kept stirring under nitrogen on the bench.

In a similar manner, using a polyvinyl double-lumen tube (4 mm ID, 45 cm between infusion and collection points; Portex Ltd.), emulsions (250 ml) were instilled again as a single dose into the duodenum of each of five subjects, and 20-ml samples were aspirated from the jejunum. In every case these emulsions were divided into two portions as described above, and samples from each of the portions were collected.

The 20-ml samples removed from the emulsion and aspirates from patients were treated in the following manner. Duplicate 1-ml aliquots were removed and extracted in chloroform–methanol 2:1. The extracts were placed in a scintillation counting vial and evaporated to dryness, and 10 ml of Packard Instagel was added. The ratio of $^{3}$H to $^{14}$C was calculated after counting.

10-ml aliquots were passed through Millipore pressure filters in order to separate the aqueous phase (9). 1-ml aliquots of the filtrates were extracted in chloroform–methanol 2:1, and isotope ratios in the extracts were calculated. 5 ml was ultracentrifuged for 100 min at 20,000 rpm at 25°C in a Beckman model L2-65B ultracentrifuge, using a 50-SW rotor, to isolate the aqueous phase, which was then extracted in chloroform–methanol 2:1. Isotope ratios ($^{3}$H/$^{14}$C) were then calculated.

For some samples the aqueous layer from the chloroform–methanol separation was also counted to determine whether or not triether would migrate out of the organic phase into the aqueous phase during the separation procedure.

TABLE 1. Isotope recoveries from feces of normal subjects

| Patient | [$^{3}$H]Glycerol Triether | | |
|---|---|---|---|
| | Dose Administered | Dose Recovered | Recovery |
| | *μCi* | *μCi* | % |
| 1 | 274.08 | 327.57 | 119.5 |
| 2 | 158.00 | 129.56 | 82.0 |
| 3 | 201.00 | 160.00 | 80.0 |
| 4 | 164.00 | 155.80 | 95.0 |
| 5 | 171.00 | 165.87 | 97.0 |
| 6 | 175.00 | 164.50 | 94.0 |
| 7 | 183.00 | 164.70 | 90.0 |
| Mean recovery ± SD | | | 93.9 ± 13.0 |

Each patient was fed a capsule containing triolein marked with tritiated glycerol triether. The recovery from feces over 5 days was calculated.

The statistical method employed in comparing the $^{3}$H/$^{14}$C ratios was measurement of the coefficient of variation (C.V.) as described by Hill (10). The C.V. = (SD/mean) × 100, and enables variables having widely different means to be compared.

*Results.* The results of the first series of experiments are shown in **Table 1.** The mean recovery of tritiated glycerol triether from the feces of seven subjects was 93.9%. About 90% of the recovered dose was excreted on the 2nd and 3rd day after administration of the test capsule. There were no detectable tritium counts from glycerol triether in samples of urine, blood, or serum, which would suggest that no triether was absorbed. Thin-layer chromatography of the fecal extract demonstrated that all of the tritium was associated with one spot, which had the same $R_F$ value as the reference standards, namely triether.

The isotope ratios over a period from 0 to 600 min in 13 different emulsions were calculated. These remained effectively constant (C.V. 0.32–1.66%). Isotope ratios measured in gastric aspirates also remained effectively constant (C.V. 1–10%) except in one case where the ratio increased (C.V. 38%).

**Table 2** demonstrates the ratios in jejunal aspirates. Here the range in coefficient of variation was large (10.15–50.87%). However, this increase in ratio is anticipated because absorption of $^{14}$C-labeled lypolytic product occurs from the jejunum.

**Table 3** confirms by both Millipore pressure filtration and ultracentrifugation studies that [$^{3}$H]triether remains with the oil phase and does not enter the aqueous (micellar) phase in significant amounts.

*Discussion.* The results of these experiments indicate that tritiated glycerol triether is almost completely recovered from the gastrointestinal tract in normal subjects, the mean recovery being greater than 93%. The fact that tri-

TABLE 2. Isotope ($^{3}H/^{14}C$) ratios in jejunal aspirates

| Control and Patient | 0 min | 15 min | 30 min | 45 min | 60 min | 75 min | 90 min | 120 min | Coefficient of Variation |
|---|---|---|---|---|---|---|---|---|---|
| C1 | 0.404 | 0.400 | 0.400 | | 0.398 | | 0.390 | 0.399 | 1.16 |
| P1 | 0.463 | | 0.574 | | 0.448 | | 0.583 | 1.135 | 44.21 |
| C2 | 0.835 | | | | 0.830 | | 0.830 | 0.833 | 0.30 |
| P2 | | | | | 0.837 | 0.847 | 1.001 | 1.007 | 10.15 |
| C3 | 0.410 | | 0.370 | | 0.370 | | 0.370 | 0.373 | 4.65 |
| P3 | | | 0.405 | | 0.418 | | 0.447 | 0.422 | 4.15 |
| C4 | 0.410 | | 0.370 | | 0.370 | | 0.370 | 0.373 | 4.65 |
| P4 | | | 0.367 | | 0.534 | | 0.624 | 0.531 | 20.83 |
| C5 | 0.313 | | 0.312 | | 0.313 | | 0.319 | 0.318 | 1.03 |
| P5 | 0.319 | | | | 0.450 | | 0.451 | 0.621 | 26.9 |
| C6 | 0.313 | | 0.312 | | 0.313 | | 0.319 | 0.318 | 1.03 |
| P6 | 0.328 | | | | 0.620 | | 1.091 | 1.220 | 50.87 |

Emulsions containing $^{14}C$-labeled triolein and tritiated glycerol triether were prepared in isotonic glucose–saline and divided into two portions. Samples from one portion left stirring on the bench were removed at timed intervals as controls. The second portion of 450 ml was infused into the duodenum, and samples were aspirated from the jejunum at timed intervals. Isotope ratios ($^{3}H/^{14}C$) were calculated in the samples. Ratios from the controls remained effectively constant. Those from jejunal samples increased, which is consistent with the absorption of $^{14}C$-labeled lipid.

tium remained solely with glycerol triether in the fecal extract suggests that triether is not degraded in the human alimentary canal by either digestive or bacterial enzyme systems, nor is it absorbed to any significant extent. This confirms the results of the earlier studies in animals by Morgan and Hofmann (4).

Isotope ratios ($^{3}H/^{14}C$) measured in the second series of experiments did not drop appreciably. The variation in the ratios was within the limits of experimental error except in those samples from patients in whom the $^{3}H/^{14}C$ ratio increased due to the absorption of $^{14}C$-labeled fat. In the stomach and duodenum there is little digestion and absorption of fat, and hence the ratio ($^{3}H/^{14}C$) should remain constant. This would also be true of the emulsions themselves, assuming that glycerol triether does not leave the oil phase and enter into the aqueous phase. However, if triether were to enter into the aqueous phase the ratio ($^{3}H/^{14}C$) would fall in the oil phase extract. On the other hand, if absorption of $^{14}C$-labeled triolein occurred from the oil phase the ratio ($^{3}H/^{14}C$) would increase.

In one subject the ratio ($^{3}H/^{14}C$) increased in the stomach. This could have been caused by the triolein and triether passing out of the stomach and into the duodenum, where triglyceride becomes partly digested and $^{14}C$-labeled lipolytic products are absorbed from an aqueous phase, leading to an increase in the isotope ratio in the oil phase if reflux occurred back into the stomach. Fats are absorbed from the jejunum, and loss of $^{14}C$ occurs from the lumen when the lipolytic products are absorbed, resulting in an increased ratio of $^{3}H$ to $^{14}C$ (11, 12).

TABLE 3. [$^{3}H$]Glycerol triether counts in the oil and aqueous phases from Millipore filtration and ultracentrifugation studies

| Specimen | | Millipore Filtration | | | Ultracentrifugation | | |
|---|---|---|---|---|---|---|---|
| | | $^{3}H$ Counts in the Oil Phase | $^{3}H$ Counts in the Aqueous Phase | Percentage of $^{3}H$ Counts in the Aqueous Phase | $^{3}H$ Counts in the Oil Phase | $^{3}H$ Counts in the Aqueous Phase | Percentage of $^{3}H$ Counts in the Aqueous Phase |
| Emulsion | 1 | 151,698 | 1,880 | 1.2 | 145,630 | 2,475 | 1.7 |
| | 2 | 228,918 | 2,060 | 0.9 | 215,183 | 3,013 | 1.4 |
| | 3 | 240,024 | 2,640 | 1.1 | 228,027 | 3,648 | 1.6 |
| Gastric aspirate | 1 | 70,321 | 422 | 0.6 | 66,102 | 661 | 1.0 |
| | 2 | 52,476 | 367 | 0.7 | 48,278 | 531 | 1.1 |
| | 3 | 57,251 | 401 | 0.7 | 54,103 | 757 | 1.4 |
| Jejunal aspirate | 1 | 36,781 | 184 | 0.5 | 34,467 | 310 | 0.9 |
| | 2 | 31,028 | 93 | 0.3 | 28,669 | 258 | 0.9 |
| | 3 | 33,124 | 264 | 0.8 | 30,805 | 246 | 0.8 |

Samples from the oil and aqueous phases from emulsions containing triglyceride and tritiated glycerol triether and from emulsions aspirated from stomach and jejunum were counted to determine the percentages of tritium in the phases. The oil phase was separated from the aqueous phase by Millipore filtration and by ultracentrifugation. There were negligible tritium counts in the aqueous phases obtained from both procedures.

In all of the experiments of the second series the aqueous phase was isolated using Millipore pressure filtration. There were no significant tritium counts in the aqueous phase, further supporting the concept that triether had not separated from the oil phase. Ultracentrifugation studies gave results similar to those obtained using Millipore filtration. This finding would suggest that there is little micellar solubilization of triether.

It has been suggested that glycerol triether separates from triglyceride emulsions in the stomach and duodenum (13). However, the results of our work show no evidence of separation.

We are currently using [$^3$H]glycerol triether in the measurement of fat absorption in fecal and perfusion studies. Preliminary results are encouraging and indicate that suitable measurements of fat absorption can be made using this isotope.

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