Preparation of intravenous cholesterol tracer using current good manufacturing practices

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Abstract Studies of human reverse cholesterol transport require intravenous infusion of cholesterol tracers. Because insoluble lipids may pose risk and because it is desirable to have consistent doses of defined composition available over many months, we investigated the manufacture of cholesterol tracer under current good manufacturing practice (CGMP) conditions appropriate for phase 1 investigation. Cholesterol tracer was prepared by sterile admixture of unlabeled cholesterol or cholesterol-d7 in ethanol with 20% Intralipid®. The resulting material was filtered through a 1.2 micron particulate filter, stored at 4°C, and tested at time 0, 1.5, 3, 6, and 9 months for sterility, pyrogenicity, autoxidation, and particle size and aggregation. The limiting factor for stability was a rise in thiobarbituric acid-reacting substances of 9.6-fold over 9 months (P < 0.01). The emulsion was stable with the Z-average intensity-weighted mean droplet diameter remaining at 60 nm over 23 months. The zeta potential (a measure of negative surface charge protecting from aggregation) was unchanged at ~36.2. Rapid cholesterol pool size was 25.3 ± 1.3 g. Intravenous cholesterol tracer was stable at 4°C for 9 months postproduction.** CGMP manufacturing methods can be achieved in the academic setting and need to be considered for critical components of future metabolic studies.—Lin, X., L. Ma, S. B. Racette, W. P. Swaney, and R. E. Ostlund, Jr. Preparation of intravenous cholesterol tracer using current good manufacturing practices. J. Lipid Res. 2015. 56: 2393–2398.

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Reverse cholesterol transport is the process by which cholesterol is removed from cells, transferred to HDL, excreted into bile, and removed in the stool (1). Long postulated to be important in protection from coronary heart disease, the direct study of reverse cholesterol transport in humans has been difficult, in part because of lack of well-characterized intravenous tracers labeled with stable isotopes. The process of tracer manufacturing seems to be important, as judged by animal studies in which slightly aggregated but filterable cholesterol tracer was cleared selectively from the reticuloendothelial system and gave aberrant kinetic results (2).

Due to detection limits of GC/MS and the diluting effect from unlabeled cholesterol in the plasma, up to 100 mg of cholesterol tracer may be needed to study cholesterol turnover (3). Because cholesterol has almost no water solubility, it is usually complexed to an intravenous lipid feeding emulsion containing triglycerides and lecithin (4). Because cholesterol is not a drug, it has not usually been subjects to Food and Drug Administration (FDA) regulation. The safety of studies involving cholesterol tracers is overseen by local Institutional Review Boards. We proposed previously that intravenous cholesterol tracers be prepared under regulations promulgated by the American Board of Hospital Pharmacy (5). However, this approach is focused on short-term stability, too complex for most pharmacies, and lacks the rigor of FDA oversight.

In this work, we developed a method for preparation of intravenous cholesterol tracer that is compliant with current good manufacturing practices (CGMPs) as defined by the FDA for phase 1 drugs (6). The final product was also tested and characterized over time. It remains

Abbreviations: CGMP, current good manufacturing practice; TBARS, thiobarbituric acid reacting substances; FDA, Food and Drug Administration; PDI, polydispersity index; PFAT5-50, volume occupied by particles ranging from 5.0 µm to 50 µm relative to the volume of fat in the sample; USP, United States Pharmacopeia.

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suitable for use for 9 months at 4°C. The measured zeta potential, reflecting surface charge opposing particle aggregation, supports stability in storage. Infusion into human subjects allows measurement of parameters of cholesterol metabolism.

MATERIALS AND METHODS

Preparation of cholesterol tracer under CGMP conditions

The [25,26,26,27,27,27-3H]cholesterol (cholesterol-d7, product number 677574) was synthesized from plant precursors by Sigma (St. Louis, MO). Plant sourcing reduces the potential risk of rare prion diseases associated with cholesterol extracted from animal central nervous system. Cholesterol-d7 was dissolved in ethanol, emulsified by adding to 4 vol of 20% Intralipid®, and aggregates were removed by filtration. Details are found in a Master Production Record that is filled out with ongoing annotations during the manufacture of each production lot. A copy of the generic Master Production Record is included as a supplement.

Cholesterol-d7 was heated at 100°C for 1 h to remove water of hydration and to destroy adventitious viruses (7,8), dissolved at 37°C in ethanol (Pharmco-AAPER) at 20 mg/ml, and passed through a solvent-resistant Millex®-FG syringe (EMD Millipore, St. Charles, MO). The filtered ethanolic cholesterol was added dropwise to warmed Intralipid® (20%, Fresenius Kabi, Bad Homburg, Germany), and then filtered through a blood transfusion filter (Pall Corporation, Port Washington, NY), followed by a 5.0/1.2 μm double filter (Pall Corporation) fitted together (Fig. 1). Glass dose vials were filled with 10 ml of cholesterol tracer, each using a Multi-Ad® fluid dispensing system (B. Braun, Bethlehem, PA), covered with a vial seal and stored at 4°C. Each vial (10 ml) contained approximately 38 mg cholesterol, 1.67 g ethanol, and 8.33 g Intralipid®. The preparation was performed in the Biologic Therapeutics Core Facility, an FDA-inspected CGMP facility at Washington University School of Medicine (FEI 3007743644).

Release testing of cholesterol tracers

Release testing included sterility, endotoxin levels, and tracer concentration (Fig. 1). Sterility testing used aerobic (VersaTREK REDOX 1® media) and anaerobic (VersaTREK REDOX 2® media) cultures with the VersaTREK® culture system. All sterility testing was performed at the Barnes-Jewish Hospital Microbiology Laboratory using approved testing materials and procedures. Samples of 1 ml each were added to 90 ml aerobic and anaerobic culture bottles. All cultures were held for 14 days before reading for possible bacterial growth. The final ethanol concentration in each culture bottle was 0.2%, whereas less than 2.5% (by volume) was reported not to inhibit bacterial growth (9). The prespecified passing criterion for sterility testing was no growth under either condition. Endotoxin levels were measured according to an approved standard operating procedure using a limulus amoebocyte lysate chromogenic endotoxin quantitation kit (Lonza, Alendale, NJ). The sample volume was 0.5 μl for each assay because the recommended volume of 50 μl interfered with the assay due to the presence of ethanol in the sample. The passing limit of endotoxin levels was ≤35 EU/ml. Cholesterol tracer concentration was determined by enzymatic assay using Infinity™ cholesterol liquid stable reagent (Thermo Scientific, Waltham, MA). The passing range of cholesterol concentration was 3.0–5.4 mg/ml.

Stability over time

To assess the stability of the cholesterol preparation over time, intravenous infusates were prepared with unlabeled cholesterol (Sigma) in the Biologic Therapeutics Core Facility according to the standard operating procedure for the preparation of intravenous tracer. Different vials were examined at time 0, 6 weeks, 3, 6, and 9 months post production for appearance of oil droplets by visual inspection; potential aggregation by filterability and particle size distribution; and oxidation by thiobarbituric acid re-acting substances (TBARSs) for the measurement of triglyceride oxidation and 7-ketocholesterol for cholesterol autoxidation. TBARS assay was performed according to a published protocol (10). The prespecified passing criterion for TBARSs was less than 10-fold over that at time 0. The level of 7-ketocholesterol was measured by GC/MS using 7-ketocholesterol-d4 (Avanti Polar Lipids, Alabaster, AL) as an internal standard (11). The passing

Fig. 1. Flow chart showing the preparation and testing of intravenous infusate of deuterated cholesterol tracer. Cholesterol or cholesterol-d7 was dissolved in ethanol and then filtered through a 0.2 μm filter. Warm cholesterol in ethanol was added dropwise to warm Intralipid®6 forming a lipid emulsion containing ethanol, Intralipid®, and cholesterol. The lipid emulsion was filtered in two sequential steps, the first step with a 40 μm blood transfusion filter, the second one with a double filter consisting of a 5.0 μm and a 1.2 μm filter attached together (the 5.0 μm before the 1.2 μm filter). The filtrate was the final product, which was tested before release from storage for human use as an infusate.
Preparation of cholesterol tracer

RESULTS

Stability of intravenous infusate of unlabeled cholesterol

Based on visual examination of vials at 6 weeks, 3, 6, and 9 months, no oil droplets, aggregates, or clumps were found. TBARSs at time 0 were very low and increased over time with significant time effect (Fig. 2A). The increase from time 0 to 9 months was 9.7-fold, which is less than the prespecified 10-fold increase failing criterion. There was a significant time effect for autoxidation of cholesterol, as measured by 7-ketocholesterol concentration. However, multiple-comparison analysis did not show differences across the different time points (Fig. 2B). More importantly, 7-ketocholesterol concentration was much less than 2.0% of cholesterol amount present.

Filterability did not decrease over time (Fig. 2C) and was greater than the passing criterion of 95% or higher at all time points. PFAT50 was more than 0.4% at all time points, exceeding the passing criterion. Although PFAT50 appeared to increase over time, the difference did not reach statistical significance (Fig. 2D).

Release criteria testing in the stability study

Release criteria were achieved for all three tests at all time points (Table 1). There was no aerobic or anaerobic growth during sterility testing. Cholesterol concentration ranged from 3.6 to 5.2 mg/ml, and endotoxin levels ranged from 0.02 to 0.36 EU/ml (passing limit = <35 EU/ml).

Z-average of particle size, PDI, and zeta potential

To assess smaller particles (0.0003–10 μm), Z-average was measured on Intralipid® alone at time 0, intravenous cholesterol-d7 infusion at 1.5 and 9.5 months post production, and unlabeled cholesterol infusion at 23.4 months. Z-average of all cholesterol preparations appeared larger numerically than that of Intralipid® alone. More importantly, Z-average of the cholesterol preparations was larger at 9.5 months and 23.4 months than at 1.5 months (Fig. 3A, solid lines and circular dots). PDI was lower at 9.5 months than 1.5 months or 23.4 months (dotted lines and triangles). There was no time effect for zeta potential (Fig. 3B), but a trend toward decreasing values was observed, a potentially favorable effect.

Cholesterol pool size in humans

The characteristics of human subjects are listed in Table 2. The cholesterol pool size from five healthy subjects was 25.3 ± 1.3 g (Table 2).

DISCUSSION

CGMP manufacturing of metabolic tracer preparations used in clinical research has the potential to substantially improve study reproducibility and patient safety. The FDA has issued guidance on CGMP methods for phase 1 drug manufacturing (6), and we used that guidance because metabolic physiological studies most closely resemble phase 1 trials. To our knowledge, this is the first reported research directed toward developing CGMP manufacturing methods for cholesterol and metabolic studies.

Although studies of human physiology generally are exempt from CGMP requirements and subject only to regulation by local Institutional Review Boards, the preparation of isotopically labeled tracers using protocols that adhere strictly to CGMP guidelines is advantageous...
The ability to use a single tracer lot for up to 9 months is an advantage in performing clinical studies. In contrast to indirect particle sizing techniques that relied on filterability and phase contrast microscopy (5), we assessed particle size directly using dynamic light scattering (USP Method I) and light obscuration (USP Method II, for particles >5.0 μm), in accordance with USP guidelines (12). PFAT<sub>5-50</sub>, an estimate of larger globule content, was higher than 0.4% at time 0 and across all time points in our study, exceeding the regulatory limit for pure Intralipid<sup>®</sup> of less than 0.05%. However, PFAT<sub>5-50</sub> did not increase over time, suggesting that no significant aggregation of particles occurred during the test period and that the product was stable with respect to quality control, scientific outcomes, and subject safety.

Most intravenous tracers used in metabolic studies continue to be prepared under non-CGMP conditions and lack systematic testing for composition and stability. In this study, we evaluated the stability over time by examining the oxidation of key components (TBARSs for triglycerides from Intralipid<sup>®</sup> and 7-ketocholesterol for cholesterol) and particle aggregation (filterability and PFAT<sub>5-50</sub>). We were surprised to find that the limiting factor in storage of tracer was not related to cholesterol or particle aggregation, but rather to an increase in TBARSs, reflecting autoxidation of triglyceride fatty acids. Nevertheless, the stability of intravenous infusate of cholesterol over time. Intravenous infusate of cholesterol was prepared using unlabeled cholesterol as described in the Materials and Methods. The stability over time (time 0, 6 weeks, 3 months, 6 months, and 9 months postproduction) was assessed on oxidation by TBARS assay [a measure of triglyceride peroxidation (A)] and 7-keto-cholesterol [a measure of cholesterol autoxidation (B)], and on potential aggregation by filterability [percent of postfiltration cholesterol concentration to prefiltration cholesterol concentration (C)] and Accusizer [PFAT<sub>5-50</sub> percent of particles from 5 to 50 μm to total fat (v/v) (D)]. Values shown are individual results with means. **Significance is indicated by P < 0.01 compared with values at time 0.

**Fig. 2.** Stability of intravenous infusate of cholesterol over time. Intravenous infusate of cholesterol was prepared using unlabeled cholesterol as described in the Materials and Methods. The stability over time (time 0, 6 weeks, 3 months, 6 months, and 9 months postproduction) was assessed on oxidation by TBARS assay [a measure of triglyceride peroxidation (A)] and 7-keto-cholesterol [a measure of cholesterol autoxidation (B)], and on potential aggregation by filterability [percent of postfiltration cholesterol concentration to prefiltration cholesterol concentration (C)] and Accusizer [PFAT<sub>5-50</sub> percent of particles from 5 to 50 μm to total fat (v/v) (D)]. Values shown are individual results with means. **Significance is indicated by P < 0.01 compared with values at time 0.

### TABLE 1. Release criteria testing results in the stability study

| Tests                        | Time 0 | 6 Weeks | 3 Months | 6 Months | 9 Months |
|------------------------------|--------|---------|----------|----------|----------|
| Sterility testing            |        |         |          |          |          |
| Aerobic                     | No growth | No growth | No growth | No growth | No growth |
| Anaerobic                   | No growth | No growth | No growth | No growth | No growth |
| Endotoxin concentration (EU/ml) | 0.02  | 0.093   | 0.037    | 0.05     | 0.36     |
| Cholesterol concentration (mg/ml) | 3.6 ± 0.1 | 3.6 ± 0.1 | 4.4 ± 0.1 | 4.8 ± 0.1 | 5.1 ± 0.1 |

The passing limit of endotoxin levels was ≤35 EU/ml. The passing range of cholesterol concentration was 3.0–5.4 mg/ml. Each value of cholesterol concentration represents the mean ± SE. The number of vials tested per time point was four for sterility, one for endotoxin, and three for cholesterol concentration.
Having good stability over time does not guarantee product safety. To release the cholesterol tracer from storage for use in human subjects, the product needed to have the correct tracer concentration, to be sterile, and to be low in endotoxin. These three criteria were tested at time 0, 6 weeks, and 3, 6, and 9 months post production. Aerobic and anaerobic cultures had no growth at any time points, cholesterol concentration was similar over time, and the endotoxin level remained very low. Taken together, these results indicated that the intravenous infusates were safe and stable for up to 9 months post production.

To ensure the maximum safety of human subjects, an additional filtration step using an in-line 1.2 μm filter was performed during infusion. This precautionary step appeared not to affect the actual amount of tracer given because little residual tracer was recovered in the in-line filter (less than 0.6% of total). In addition, this step might not be necessary considering the fact that no significant adverse reactions have been reported from commercial parenteral fat emulsions that have been administered to patients for decades, even though they contained a significant number of particles larger than 5 μm (19). A recommendation that fat emulsions compounded in pharmacies have less stable. Consistently, the filterability at all time points was above 95%.

Zeta potential is an indicator of the degree of repulsion between similarly charged particles in the formulation, and yet another key indicator of stability of a lipid emulsion (14). The zeta potential of Intralipid® was high in negative value. Adding cholesterol to Intralipid® tended to increase the absolute zeta potential (even more negative) (Fig. 3B). Furthermore, the zeta potential appeared to be unchanged over time, up to 23.4 months (Fig. 3B). Although incorporation of drugs may render lipid emulsions physically unstable during storage (15), cholesterol is a notable exception. As a component of cell membranes, cholesterol increases the packing of fatty acid tails of membrane phospholipids. Cholesterol, therefore, has been added together with phospholipids to provide stability of phospholipid vesicles in vitro and in vivo (16). Therefore, the inherent component of intravenous infusate, cholesterol tracer, may have an added advantage of providing stability to the final product.

The stability of our cholesterol tracers may also have been enhanced by ethanol, which was used in the tracer preparation to enable water-insoluble cholesterol to be mixed with Intralipid®. Ethanol has been used to make ethosomes, novel liposomes, for drug delivery to the skin (17). More importantly, ethanol enhances the stability of ethosomes by providing a net negative surface charge, which prevents aggregation of vesicles due to electrostatic repulsion (18).

Each subject received approximately 1.67 g ethanol from the infusion of 10 ml infusate. Assuming a 70 kg person with 40 kg of body water, the peak concentration of blood ethanol would be about 0.004%, a very low value. We did not observe any adverse effects of ethanol administration except for occasional vein irritation during infusion, which was corrected by limiting the infusion rate to 0.5 ml/min or less.

| TABLE 2. Participant characteristics and body cholesterol pool size |
|-----------------------------------------------|
| Women/Men (n)                          | 2/3             |
| Age (y)                               | 62.2 ± 3.9      |
| Weight (kg)                           | 86.7 ± 4.0      |
| BMI (kg/m²)                           | 28.2 ± 1.5      |
| Lipids                                |                 |
| Total cholesterol (mg/dl)             | 217.6 ± 12.6    |
| LDL cholesterol (mg/dl)               | 132 ± 9         |
| HDL cholesterol (mg/dl)               | 62 ± 7          |
| Triglyceride (mg/dl)                  | 114 ± 17        |
| Glucose (mg/dl)                       | 96 ± 4          |
| Body cholesterol pool size (g)        | 25.3 ± 1.3      |

Values are mean ± SE.
than 0.4% PFAT$_{5,50}$ is based on the finding that such admixtures are unstable and tend to aggregate further over time (20). In contrast, we found no systematic change in PFAT$_{5,50}$ with storage of our cholesterol tracer and observed a “stabilizing” zeta potential.

The intravenous cholesterol tracer was infused into healthy subjects to measure cholesterol pool size. The average cholesterol pool size was 25.3 ± 1.3 g, consistent with published data (4).

In conclusion, we have prepared and tested deuterated cholesterol tracer under CGMP conditions. The product was chemically stable for 9 months post production and physically stable for up to 23.4 months. The shelf life of the preparation, limited by the chemical stability, was declared to be 9 months post production. Our strict compliance with CGMP guidelines should make the product safe and useful in metabolic studies of cholesterol transport and metabolism.

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