HPLC Procedure

Specimens were assayed in batches. The same control plasma was processed at the beginning and the end of each batch to validate the homogeneity of the profiles determination within the batch.

Chromatographic conditions for lipoprotein profiles:

- Column: Superose 6H 300 x 10 mm (GE Healthcare)
- Column temperature: Ambient (20–25°C)
- Reaction coil temperature: ca. +40°C
- Detection Wavelength: 550 nm
- Mobile Phase: NaCl 0.15M -Natrium azid 0.02%
- Flow Rate: 0.5 mL/min (Isocratic)
- Injected Volume: 10 μL
- Running time: 50 min for unesterified cholesterol and total cholesterol
- Annex Pumps: enzymatic reagents flow rate 0.100 mL/min
Approximate retention times for dual detection for total cholesterol/unesterified cholesterol:

- VLDL: 21 min
- LDL: 28 min
- HDL: 35 min

**Analytical procedure for lipoprotein profiles**

After injection and fractionation, the separate lipoprotein peaks (VLDL, LDL, HDL) were integrated baseline to valley on the chromatograms using the software processing method. A line was plotted with reference to the baseline of the chromatogram, then the software cut after each valley and identified the peaks in relation to their retention time. If a haemoglobin peak was present at a retention time of approximately 42 min, that peak was also integrated.

The sum of the areas of all the integrated peaks was performed. The percentage of each integrated peak was calculated. To calculate the total or unesterified cholesterol content in each lipoprotein fraction, the percentage of each peak was multiplied by the apparent total content of total or unesterified cholesterol measured previously by the automated chemistry analyzer. If Hb was present, its signal contributed equally to the cholesterol signal in plasma and in the HPLC profile.

**Acceptance criteria for analysis on HPLC profile**

Plasma controls were injected at the beginning and at the end of each analytical batch, and every 11 samples. Chromatographic analysis was validated if internal plasma controls were within the range of specifications for the following parameters: lipoprotein retention times,
height of the peaks, and total and unesterified cholesterol levels in each lipoprotein fraction (range: ±2 SD of the reference values). For total and unesterified cholesterol levels, the observed values were compared to the reference values. For the other parameters, each control was compared to the first control injected at the beginning of the sample sequence. (For each run, controls were compared as follows: control 1 versus control 2, control 1 versus control 3, etc.). The patterns had to be similar to meet the acceptance criteria for the analytical run.

**Chromatographic conditions for the test items gel permeation analysis**

- Column: Superdex 200, 300 x 10 mm (GE HEALTHCARE)
- Column temperature: Ambient (20 to 25°C)
- Dual Detection Wavelength: 280 nm for peptides and 220 nm for lipids
- Mobile Phase: Phosphate buffered saline solution
- Flow Rate: 0.5 mL/min (Isocratic)
- Injected Volume: 25 μL
- Preparation of formulations: diluted to 1mg/mL in PBS
- Running time: 50 min

**Analytical procedure for formulation profiles**

After injection and separation, the lipids and peptides (retention time approximately 25 min) were integrated baseline to valley on the respective chromatograms (at 220nm and 280 nm). The total areas of all the integrated peaks were compared.
Cholesterol extraction and analysis in ligatured carotids from mice

Carotid tissue was placed in a glass tube; 1.8 ml of CHCl₃/MeOH (2:1) was added and mixed overnight at 4°C. The carotid was then removed and the organic solution (CHCl₃/MeOH) was divided in equal volume into two glass tubes. For unesterified cholesterol, 100 μl of β-sitosterol (internal standard) was added and the solution dried; 200 μl of ethanol was added for solubilisation of the sample which was then analysed by HPLC. For total cholesterol, 100 μl of β-sitosterol (internal standard) was added and the solution dried; 1 ml of 0.1M methanolic KOH was added and incubated at 60°C for at least 30 minutes. A Bligh and Dyer for cholesterol extraction was performed: 1 ml of chloroform was added followed by 1 ml of water, the mixture was vortexed and then left until phase separation. The lower phase was collected and dried; 200 μl of ethanol was added for solubilisation of the sample which was then analysed by HPLC.

HPLC analysis: In brief, 50 μl was injected on a C18 Zorbax SB-C18 4.6 x 250 mm column. The flow rate was 1.5 ml/min at 60% of eluent A: ACN/EtOH/H₂O (85/10/5) and 40% of eluent B: ACN/EtOH (86/14). The run time was 55 min with a retention time for cholesterol at 22.85 minutes and a retention time for β-sitosterol of 32.2 minutes. The system used a Waters 1525 binary pump and a UV detector set at 214 nm.