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HPLC analysis of Bodipy Cholesterol Efflux from RAW 264.7 Macrophages Reveals a Stimulation by Oxysterols.

Esha Garg, Kelly Fellows, Shruthi Mohan, Murali Ramanathan and Richard W. Browne

Department of Biotechnical and Clinical Sciences and Department of Pharmaceutical Science

Abstract

Background: High density lipoprotein (HDL) is the key element in cellular reverse cholesterol transport. The capacity of HDL to promote cholesterol efflux ("HDL functionality") can be measured in a cell culture assay using RAW 264.7 macrophages loaded with bodipy cholesterol (Bodipy-Ch; a fluorescently tagged cholesterol analog). Here, we describe a high pressure liquid chromatography-fluorescent detection (HPLC-FL) assay developed and validated for analysis of Bodipy-Ch. Using this method we demonstrate that oxysterols stimulate cholesterol efflux capacity.

Methods: RAW 264.7 macrophages were loaded with Bodipy-Ch and cultured in 5 mM MEM – HEPES containing 2.8% ApoB depleted human serum as the HDL source. Efflux of Bodipy-Ch into media was measured with and without treatment of cells with 1 uM of five different oxysterols; 24-hydroxycholesterol (24HC), 25-hydroxycholoesterl (25HC), 27-hydroxycholesterol (27HC), 7α-hydroxycholesterol (7αHC) and 7-ketocholesterol (7KC). Dibutryl cAMP was used as positive control. For HPLC-FL analysis, media sample protein was precipitated with isopropyl alcohol, centrifuged and the supernatant injected onto the HPLC-FL system with detection of Bodipy-Ch at Ex 482nm/Em 515nm. Method validation was accomplished according to the FDA guidelines for bioanalytical method validation.

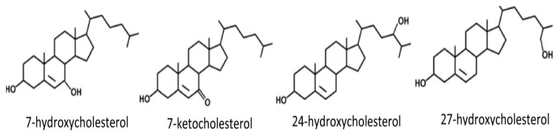
Results: The HPLC assay demonstrated linearity between 5 to 250 ng/mL of Bodipy-Ch in media and fully passed FDA guidelines for linearity, accuracy, precision and recovery. 24HC, 25HC and 7αHC significantly increased efflux of cholesterol while 27HC and 7KC did not.

Conclusion: The Bodipy-Ch HPLC assay described fulfills FDA guidelines for a valid bioanalytical method. Results indicate that only certain oxysterols stimulate cellular cholesterol efflux consistent with their demonstrated activity as nuclear transcription factor agonists.

Introduction

HDL is responsible for reverse cholesterol transport in which excess cholesterol is effluxed from cells for ultimate delivery and excretion at the liver. The efflux capacity of HDL can be measured in an HDL functionality assay where bodipy-cholesterol is used to tag cholesterol present in cells. Here we used a bodipy cholesterol loaded RAW 264.7 macrophage cell line and measure bodipy-cholesterol efflux into MEM-HEPES containing HDL isolated from human serum (ie apoB depleted serum).

Oxysterols are oxygenated forms of cholesterol that are metabolic intermediates of bile acid and steroid synthesis as well as powerful regulatory molecules.



Oxysterols regulate cellular cholesterol efflux capacity via agonism of cellular liver X receptors (LXRs) and increased expression of downstream genes including ATP binding cassette transporters (ABC Transporters) including ABCA1.

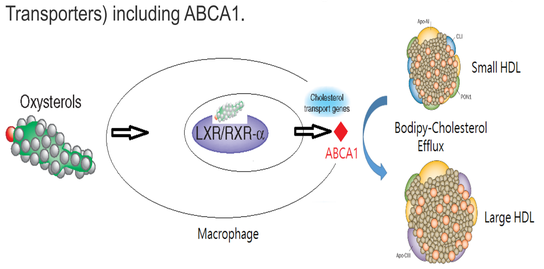


Figure 1: Oxysterols agonize LXR/RXR increasing the expression of cholesterol transport genes including ABCA1 thereby increasing cholesterol/bodipy cholesterol efflux onto HDL acceptor particles

Here, we describe an HPLC-Fluorescence (HPLC-FL) assay for the measurement of bodipy cholesterol as the substrate for HDL functionality assay. HPLC-FL offers increased sensitivity and specificity over standard fluorescence microplate assay. We have validated this assay according to USFDA guidelines for bioanalytical method validation. We have applied this assay to investigate the ability of different oxysterol compounds to stimulate cellular cholesterol efflux

Methods and Materials

HDL Functionality assay: RAW 264.7 macrophages were loaded with bodipy cholesterol and then incubated in cell culture media (10 mM MEM – HEPES) containing 2.8% ApoB depleted human serum. ApoB depleted human serum was prepared by precipitating 500 ml of serum with 125 µl PEG-6000 (45 g/dL).

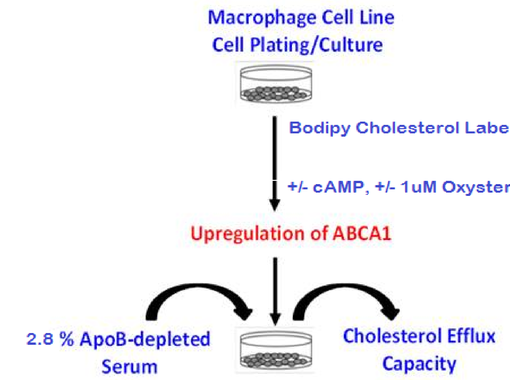


Figure 2: Bio-analytical assay

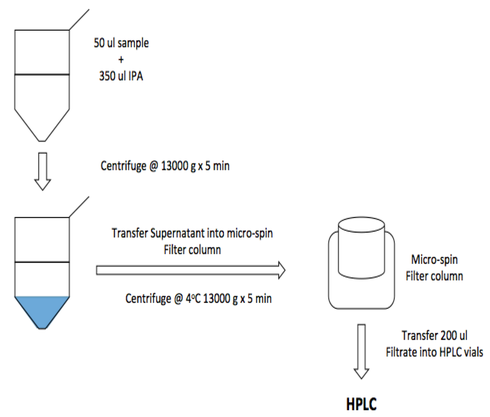


Figure 3: Process of sample preparation and analyze using HPLC.

HPLC Sample Preparation: Samples and bodipy cholesterol calibrators were prepared by "crush and shoot method" in which 50 ml of each sample was precipitated with 350 ml of isopropyl alcohol followed centrifugation (13,000 X g for 5 min) and filtration using 0.22µm-microspin columns at (13,000g for 5 min at 4°C). Filtrate (200 ml) from each sample was transfered to HPLC vials for analysis.

HPLC Analysis: The HPLC system was used was a Shimadzu 20A series HPLC system with RF10-AXL fluorescence detector. A 25 cm X 4.6 mm i.d, 5 micron Kinetex C-18 (ODS) analytical column using an isocratic mobile phase of 100% acetonitrile at a flow rate of 2.0 mL/min was used. Fluorescent detection was made at excitation/emission wavelengths of Ex 482nm/Em 515nm. Time for analyzing each sample was 6 minutes with injection volume of 50 µl. Calibration spanned 10 to 250 ng/mL using unweighted linear regression.

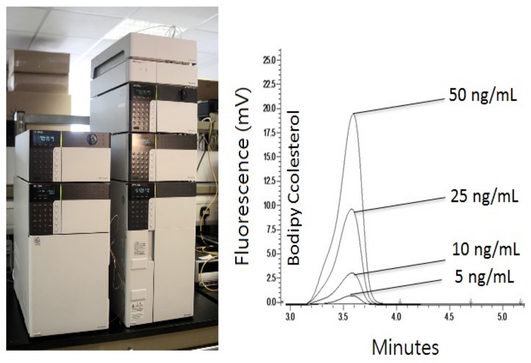


Figure 4: HPLC – Fluorescence system (left) and chromatogram (right)

Results

Linearity:

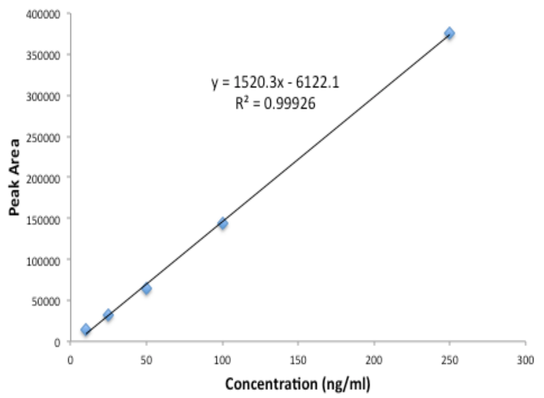


Figure 5: Calibration curve of Bodipy Cholesterol by HPLC with fluorescence detection at Ex=482nm and Em=515nm. The linear dynamic range is 5 to 250 ng/mL.

Accuracy and Precision:

Table1: Accuracy and imprecision of quality control sample results. Six replicates of each of four QC levels were analyzed each day for three consecutive days. Result showed acceptable performance according to FDA guidelines.

No	LLQC	LQC	MQC	HQC
Concentration (ng/ml)	10	40	100	200
Samples	18	18	18	18
CV%	10.32	4.86	9.37	5.41
DV%	16.98	9.15	21.06	8.55

Effect of oxysterols on the efflux of cholesterol:

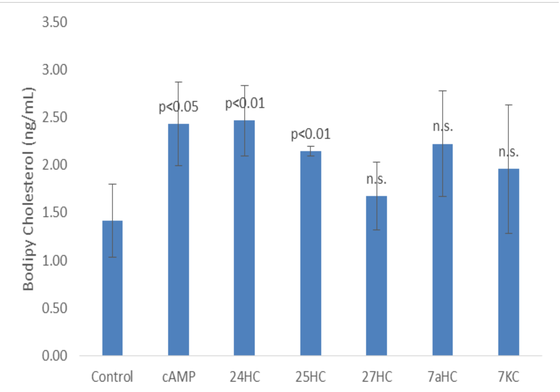


Figure 6: Levels of effluxed cholesterol in macrophage culture media in oxysterol treated cells compared to untreated (control) and cAMP (+ control) treated cells. Levels are reported as the mean of three replicates +/- 2 standard deviations. Statistical significance (p-values) were generated using student's t-test.

Discussion

Advantages:

- HPLC shows higher sensitivity compared to fluorescence microplate.
  - * Lower limit of quantification is 5 ng/ml for HPLC versus 25 ng/ml for fluorescence microplate.
- HPLC analysis shows wider linear dynamic range.
  - * 5 ng/ml to 250 ng/ml by HPLC versus 25 ng/ml to 100 ng/ml by fluorescence microplate.
- HPLC allows for smaller number of cells and less required cell culture media volume to analyze.
- Cell culture media often contains phenol as a pH indicator, phenol is fluorescence. This HPLC analysis removes phenol interference from fluorescence bodipy cholesterol measurement.

Disadvantages:

- Sample preparation and protein removal requires additional time and consumables.
- Loading of bodipy cholesterol into macrophage cell lines has been shown to be highly variable.

Conclusion

- The HPLC method described here provides greater sensitivity and linear dynamic range for the measurement of Bodipy-cholesterol efflux from cells in culture.
- Using this method we have demonstrated that oxysterols, oxidized metabolites of cholesterol, effectively stimulate cholesterol efflux from RAW 264.7 macrophages.
- This efflux stimulating activity of oxysterol has not been previously described. Further studies will be required to determine the mechanism of this efflux stimulation.