Core J Foam Cell Sterol Extraction and Quantitation

Jeff McDonald, Bonne Thompson
9/26/2007

Synopsis: This protocol describes the method used to extract and analyze sterols from thioglycolate-elicited intraperitoneal macrophages. Cells are not cultured, but are frozen as a cell pellet after removal from the animal. Lipids are extracted via a modified Bligh-Dyer method, sterol esters are cleaved by hydrolysis, and samples are cleaned up using silica SPE columns. Sterols are then separated using a reverse phase binary liquid chromatography (LC) gradient and quantitated using a MRM method with positive electrospray ionization mass spectrometry (ESI-MS) and normalized to mass of DNA.

I. Sample Preparation

Cells of ten animals were pooled, counted and divided into aliquots of 1E7 cells each. Aliquots were pelleted by centrifugation and snap frozen in liquid nitrogen.

Reagents:

- PBS
- Chloroform/Methanol (1:1 *v:v*)
- Ethanol
- 10M KOH
- BHT (1 mg/mL in ethanol)
- Toluene
- Hexane
- 30% Isopropanol in Hexane
- 5% H_2O in Methanol

A. Lipid Extraction

1. Thaw cell pellets several minutes at room temperature.
2. Add 1 mL PBS to each pellet, vortex to suspend, and transfer to glass 16x100mm tube.
3. Add another 1 mL PBS to original tube, vortex to wash, and pool with cells in glass tube.
4. Add 1 mL PBS to cells in glass tube to bring volume to 3 mL total.
5. Add 6.6 mL $CHCl_3$/MeOH (1:1 *v:v*).
6. Add 10 µL each of two surrogate standard mixes.
7. Cap with Teflon-lined cap, shake well, and centrifuge at 2600 rpm (1360 rcf) for five minutes.
8. Remove organic phase (lower layer) to 4 mL glass vial using a Pasteur pipette.
9. Dry the organic phase under N_2 with gentle heating (37˚C).

B. Hydrolysis of Lipids

1. Prepare fresh hydrolysis solution each time lipids are hydrolyzed:
    - 46.4 mL EtOH
    - 3 mL 10M KOH
    - 600 µL BHT (1mg/mL in ethanol)
2. Add 1 mL hydrolysis solution to dried lipids, vortex, and cap with Teflon-lined cap
3. Place in oven at 90˚C for two hours.
4. Remove from oven, allow to cool to room temperature.
5. Add 1 mL each of $CHCl_3$ and PBS.
6. Vortex well, then allow phases to separate (about 5 minutes).
7. Remove organic phase (lower layer) to 2 mL glass vial using a Pasteur pipette.
8. Dry the organic phase under N_2 with gentle heating (37˚C).

C. Silica SPE

1. Dissolve dried lipids in 1 mL toluene.
2. Assemble 100 mg silica SPE column on a vacuum chamber.
3. Condition the column using 2 mL hexane. Draw solvent through slowly with vacuum.
4. Apply lipids dissolved in toluene.
5. Use 1 mL toluene to wash out vial, applying wash to column. Draw lipids into column using vacuum. Discard eluate.
6. Wash column with 1 mL hexane, drawing it through column with vacuum. Discard eluate.

7. Elute sterols using 8 mL 30% isopropanol in hexane. Draw through the column slowly with vacuum, collecting eluate.
8. Transfer eluate to 8 mL glass vial
9. Dry the eluate under N_2 with gentle heating (37˚C).
10. Dissolve dried lipids in 400 µL 5% H_2O in methanol.
11. Transfer dissolved lipids to an autosampler vial with 500 µL insert containing 10 µL internal standard mix.

II. Positive ESI Liquid Chromatography Mass Spectrometry (ESI- LC/MS)

The LC/MS protocol outlined below is for the analysis of sterols in cultured cell and medium extracts (part I). Sterols are resolved by reverse-phase HPLC using a binary solvent system and gradient elution is performed on a C18 RP-HPLC column. The HPLC is coupled to a triple quadrupole MS with an ESI source. The MS is operated in multiple reaction monitoring (MRM) mode with transitions optimized for each sterol of interest. Sterols are quantified using the internal standards, surrogate, and relative response factor (RRF) of each sterol of interest.

A. Solutions

1. **Mobile Phase A**
   Methanol with 5mM ammonium acetate (sparged with Helium for 5 minutes)

2. **Mobile Phase B**
   15% High Purity water in methanol with 5mM ammonium acetate (sparged with Helium for 5 minutes)

3. **Surrogates**
   Two deuterated surrogates, 10 µL each, are added to cells before lipid extraction:

Table 1: Surrogate composition

SURROGATE MIX 1	**SOURCE**	**CONCENTRATION [PPM]**
27-Hydroxycholesterol (D_5) in MeOH	Avanti Polar Lipids	3.992
24,25-Epoxycholesterol (D_6) in MeOH	Avanti Polar Lipids	3.824
7α-Hydroxycholesterol (D_7) in MeOH	Avanti Polar Lipids	4.055
7-Oxocholesterol (D_7) in MeOH	Avanti Polar Lipids	4.025
4β-Hydroxycholesterol (D_7) in MeOH	Avanti Polar Lipids	1.948
SURROGATE MIX 2		
Cholesterol (D_7) in MeOH	Avanti Polar Lipids	156.4
Desmosterol (D_6) in MeOH	Avanti Polar Lipids	72.0

4. **Internal Standard**
   6α-Hydroxycholesterol (D_6) 3.921 ppm from Avanti Polar Lipids

B. Compounds of interest

We are monitoring the following compounds via Selected Reaction Monitoring

Table 2: Compounds monitored via Selected Reaction Monitoring

COMPOUND	**MRM PAIR**
22r-Hydroxycholesterol	420/385
24-Hydroxycholesterol	420/385
25-Hydroxycholesterol	420/367
26-Hydroxycholesterol	420/385
24,25-Epoxycholesterol	418/383
7α-Hydroxycholesterol	385/367
7-Ketocholesterol	401/383
5/6β Epoxycholesterol	420/385
5/6α Epoxycholesterol	420/385

4β-Hydroxycholesterol	420/385
Zymosterol	385/367
Desmosterol	402/367
7-Dehydrocholesterol	385/367
3keto cholestene	385/367
Lathosterol	404/369
Cholesterol	404/369
Lanosterol	444/409
Cholestanol	404/387
24-Dihydrolanosterol	429/411
3,16dioxo cholestenoic acid	429/411
TriOH cholesterol	401/383
4-chol-27acid-3one	415/397
4-chol-22OH-3one	401/383
4-chol-24OH-3one	401/383
4-chol-25OH-3one	401/383
4-chol-2OH-3one	401/383
20-Hydroxycholesterol	385/367
4-chol-26(25r)OH-3one	401/383
4-chol-26(25s)OH-3one	401/383
3keto,26cholestene	401/383
8(14) cholesten 3β,15α diol	385/367
3β,15α cholestanol	422/369
8(14) cholesten 3βOH 15one	401/383
cholestan 3oh 15one	403/385
7α hydroxycholestenone	401/383
8(14) cholesten 3β,15β diol	385/367
3β,15β cholestanol	422/369
7ketocholestanone	401/383
dihydroxyketocholesterol	401/383
19-Hydroxycholesterol	420/385
4,6 Chlestadiene -3-one	383/365
Lathosterone	385/367
5-chol-3-one	385/367
cycloartenol	444/409
Bsitosterol	432/397
Bsitosterone	413/413
3,16dioxo cholestenoic acid	429/411
TriOH cholesterol	401/383
4-chol-27acid-3one	415/397
4-chol-22OH-3one	401/383
4-chol-24OH-3one	401/383
4-chol-25OH-3one	401/383
4-chol-2OH-3one	401/383
20-Hydroxycholesterol	385/367
4-chol-26(25r)OH-3one	401/383
4-chol-26(25s)OH-3one	401/383
3keto,26cholestene	401/383
8(14) cholesten 3β,15α diol	385/367
3β,15α cholestanol	422/369
8(14) cholesten 3OH 15one	401/383
cholestan 3oh 15one	403/385
7α hydroxycholestenone	401/383
8(14) cholesten 3β,15β diol	385/367
3β,15β cholestanol	422/369
7ketocholestanone	401/383
dihydroxy ketocholesterol	401/383

19-Hydroxycholesterol	420/385
4,6 Chlestadiene -3-one	383/365
Lathosterone	385/367
5-chol-3-one	385/367
cycloartenol	444/409
Bsitosterol	432/397
Bsitosterone	413/413
DEUTERATED COMPOUND	**MRM Pair**
7β-Oxocholesterol (D_7)	408/390
7β-Hydroxycholesterol (D_7)	391/373
4β-Hydroxycholesterol (D_7)	426/391
7α-Hydroxycholesterol (D_7)	391/373
25-Hydroxycholesterol (D_3)	423/370
27- Hydroxycholesterol (D_5)	425/390
24,25 Epoxycholesterol (D_6)	424/389
Cholesterol (D_7)	411/376
Desmosterol (D_6)	408/373

C. Instrumentation

1. Column Information

Company: Phenomenex
Packing: Reverse Phase C18
Particle Size: 3µm
Diameter: 2mm
Length: 250mm

This column is maintained at 30°C.

2. HPLC conditions

Total Flow: 0.25 mL/min

Table 3: HPLC Gradient

TIME (MIN)	% MOBILE PHASE B
0	100
2	100
15	0
25	0
25.5	100
30	100

3. API 4000 Q Trap Conditions

CUR: 15.00
CAD: Medium
IS: 5500.00
GS1: 60.00
GS2: 20.00
DP: Variable Depending on MRM pair (45.00-120.00)
EP: 10.00
CE: Variable Depending on MRM pair (10.00-65.00)
CXP: 10.00