3M MEDICAL DEPARTMENT, CORPORATE TOXICOLOGY

- ST-43: Standard Procedure for Liver Subcelular Fractionation -

OR-5

Study Objective:

Perfluorochemical compounds such as perfluorooctane sulfonate (PFOS) have been linked with reduction in serum cholesterol levels and liver has been identified as the primary target site. The exact cellular mechanism of how perfluorochemical compounds interact with liver tissues is unknown and it is necessary to look into the sub-cellular fractions.

The purpose of this study is therefore to (1) isolate liver sub-cellular fractions and (2) to quantitate the PFOS contents in each fraction. The correlation between sub-cellular PFOS contents and serum biochemical markers will be further investigated.

This protocol is part of an ongoing study in the 3M Strategic Toxicology Laboratory designed to understand the effects of perfluorooctanesulfonamides and their metabolites, including PFOS, on intracellular fatty acid transport and metabolism, mitochondrial function, and cholesterol synthesis. This protocol provides a means of combining our resources and focus analytical capabilities on the same set of tissues.

Study Location:
3M Strategic Toxicology Laboratory
3M Center, Building 270-SB-314
Saint Paul, MN 55144-1000

Sponsor:
3M Specialty Chemicals
3M Center, Building 236
Saint Paul MN 55144-1000

Study Director:
Andrew M. Seacat Ph.D.
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Study Toxicologist:
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Advanced Research Toxicologist
M Medical Dept. / Corporate Toxicology
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Methods & Materials:

Regulatory Compliance:
This is an exploratory in nature and thus classified as non-GLP as explained in TOX SOP 0950, Strategic Toxicology Lab GLP Program Procedure.

Test Material:
The sponsor has provided samples of PFOS to the investigators. Analytical documentation of the starting material will be the responsibility of the sponsor. A chemical composition specification sheet will be kept on file.

Animals:

Species: Rat
Strain: Sprague Dawley
Source: Harlan Laboratories, Inc.
Age at initiation of treatment: 10-12 weeks old
Weight at initiation of treatment: approximately 250-300g

Animal Handling Cares and Specimen Collection:

Please see Study No. DT-15

Specimen Handling / Processing:

For perfluorosulfonamide metabolite analysis, liver samples will be fractionized according to the procedures described below. The isolated sub-cellular fractions will be packed in dry ice and shipped to Dr. Kris Hansen at 3M Environmental Lab.

Kris Hansen, Ph.D.
3M Environmental Technology and Safety Services
935 Bush Avenue
St. Paul, MN 55133-3331
Ph.: 651-778-6081, Fax: 651-778-6176.

Procedures:

Buffer needed:	0.25M sucrose in 0.05M Tris-HCl, pH 7.4
Equipment needed:	Tissue grinder Centrifuge Polypropylene centrifuge tubes (1.5 ml and 50 ml)
Animal:	Rat livers from both control and PFOS-treated groups.

(NOTE - All procedures should be carried out at 0 - 4°C.)

1. Suspend and homogenize the liver in 0.25 M Sucrose in 0.05 M Tris-HCl buffer (pH 7.4) using 20% weight/volume ratio, or approximately 5 volumes of the liver weights. The homogenization should be carried out on ice at all time to prevent the enzymes and tissue proteins from degradation.

   Homogenize on ice at low speed using a 50 ml Tenebrock tissue grinder with the hollow handle of the pestle filled with crushed ice. Homogenize at least 10 strokes, or until the suspension is visibly homogeneous.

   Record the total volume and the corresponding weights of the homogenates. Obtain two 1.5 ml microcentrifuge tubes and label them "1". Aliquot 1 ml of the homogenate into each tube. Store the tubes frozen for further analysis.

   Transfer the remaining of the homogenates into two 50-ml centrifuge tubes.

   Note: ***For this step and steps below following the centrifugation, make sure to measure and record the volumes and weights for each supernatant fraction.***

2. Spin the whole homogenate at 700 x g for 10 minutes at 4°C.

   Supernatants: Decant supernatant fluid into new centrifuge tubes on ice. Weigh and record the total volume. Make two 1 ml aliquot of the supernatants into two 1.5 ml microcentrifuge tubes labeled "2". Store frozen.

   Pellet: The resulting pellet contains debris and unbroken cells. Add 1 ml of buffer per 1 gram of original sample, re-suspend the pellet in 0.25 M Sucrose in 0.05 M Tris-HCl buffer (pH 7.4) and label it as "**Plasma membrane**". Make two 1 ml aliquot of the suspension into two 1.5 ml microcentrifuge tubes labeled "3". Store frozen.

3. Spin the supernatants at 2500 x g for 10 minutes at 4°C.

   Supernatants: Decant supernatant fluid into new centrifuge tubes on ice. Weigh and record the total volume. Make two 1 ml aliquot of the supernatants into two 1.5 ml microcentrifuge tubes labeled "4". Store frozen.

   Pellet: The resulting pellet contains nuclei and unbroken cells. Add 1 ml of buffer per 1 gram of original sample, re-suspend the pellet in 0.25 M Sucrose in 0.05 M Tris-HCl buffer (pH 7.4). Label it as

"**nuclei**". Make two 1 ml aliquot of the suspension into two 1.5 ml microcentrifuge tubes labeled "**5**". Store frozen.

4. Centrifuge the supernatant fluid obtained from step 3 at 10,000 x g for 15 minutes at 4°C.

   Supernatant: Decant supernatant fluid into a new centirfuge tube. Weigh and record the total volume. Make two 1 ml aliquot of the supernatants into two 1.5 ml microcentrifuge tubes labeled "**6**". Store frozen.

   Pellet: The resulting pellet contains mitochondria. Add 1 ml of buffer per 1 gram of original sample, re-suspend the pellet in 0.25 M Sucrose in 0.05 M Tris-HCl buffer (pH 7.4). Label it as "**mitochondria**". Make two 1 ml aliquot of the mitochondrial suspension into two 1.5 ml microcentrifuge tubes labeled "**7**". Store frozen.

5. Centrifuge the supernatant fluid obtained from step 4 at 30,000 x g for 10 minutes at 4°C.

   Supernatants: Decant supernatant fluid into new centrifuge tubes on ice. Weigh and record the total volume. Make two 1 ml aliquot of the supernatants into two 1.5 ml microcentrifuge tubes labeled "**8**". Store frozen.

   Pellet: The resulting pellet contains lysosomes. Add 1 ml of buffer per 1 gram of original sample, re-suspend the pellet in 0.25 M Sucrose in 0.05 M Tris-HCl buffer (pH 7.4) and label it as "**lysosome**". Make two 1 ml aliquot of the lysosome suspension into two 1.5 ml microcentrifuge tubes labeled "**9**". Store frozen.

6. Centrifuge the supernatant fluid obtained from step 5 above at 105,000 x g for 60 minutes at 4°C.

   Supernatants: Decant supernatant fluid into new centrifuge tubes on ice. Weigh and record the total volume. Label the supernatant as "**cytosol**". Make two 1 ml aliquot of the supernatants into two 1.5 ml microcentrifuge tubes labeled "**10**". Store frozen.

   Pellet: The resulting pellet contains microsomes. Add 1 ml of buffer per 1 gram of original sample, re-suspend the pellet in 0.25 M Sucrose in 0.05 M Tris-HCl buffer (pH 7.4) and label it as "**microsome**". Make two 1 ml aliquot of the microsomal suspension into two 1.5 ml microcentrifuge tubes labeled "**11**". Store frozen.

Data:

- 3M Study Number = _____
- Date = _____
- Animal #, sex, and strain = _____
- Dose group = _____
- Weight of liver sample (g) = _____
- Volume of sucrose-Tris HCl added (mL) = _____

Fraction #		Fraction weight before aliquot (g)	*(Minus)* amount saved for LC-MS (mL)	*(Minus)* amout saved for gel electr. (mL)	Note
Whole homogenate					
Plasma membrane sup.	700 x g sup				
Plasma membrane cells	700 x g pellet				
Nuclei sup.	2500 x g sup				
Nuclei cells	2500 x g pellet				
Mitochondrial sup.	10K x g sup				
Mitochondrial cells	10K x g pellet				
Lysosomal sup.	30K x g sup				
Lysosomal cells	30K x g pellet				
Cytosol	105K x g sup				
Microsomal cells	105K x g pellet				

Responsibilities:

Andrew Seacat and Sue Chang will be responsible for performing the experiment and sending specimens for analysis.

Signatures:

Andrew M. Seacat, Ph.D.
Toxicology Specialist
Study Director

Date

Sue Chang, M.S.
Advanced Research Toxicologist
Study Toxicologist

Date

Sponsor Representative

Date