Cryopreserved Hepatocyte Uptake Transporter Assay

Introduction

Compounds move into human hepatocytes from the plasma by a number of mechanisms, including those mediated by membrane-bound transport proteins. Primary hepatocytes have been used successfully to assay the uptake pattern of endogenous and exogenous compounds. Cryopreserved human hepatocytes have uptake transporter activities similar to those of freshly isolated hepatocytes, offering an important model system.[1, 2]

Purpose

This protocol is designed to assist researchers in determining the rate of uptake of test articles via cryopreserved hepatocytes.

Principle of the Procedure

A test article is incubated with cells for a given amount of time, allowing for uptake of the compound into the cell. The suspension is then centrifuged through an oil layer, which allows the cells to pass through but excludes the solution containing the free test article. The tube is cut to separate the cells from the free test article. The concentration of the test article in the cells is assessed by scintillation counting for radioactively labeled test articles or by LC/MS methods. The assay is run at both 37℃, where active transport occurs, and 4℃, where transport is limited. The uptake transport rate is calculated by subtracting the 4℃ background value from the 37℃ active uptake value.

Materials*

Item	Manufacturer	Name/Catalog/Model #
Biosafety cabinet	NuAire, Inc.	NU425FM600
CO_2 water-jacketed incubator	Forma Scientific	3110
Plastic multi-well plates	Costar	
Cryopreserved human hepatocytes	BioreclamationIVT	M/F00995
Pump	GAST	DPA104AA
0.4 mL centrifuge tubes	Fisher Scientific	02-681-229
Plastic tubing cutter	Fisher Scientific	22-088245
Rotator	Fisher Scientific	11-671-50Q
Microcentrifuge	Eppendorf	Model 5415
Chemicals/ Media		
*InVitro*GRO™ HT medium	BioreclamationIVT	Z99019
*InVitro*GRO™ KHB medium	BioreclamationIVT	Z99074
2N Sodium hydroxide (NaOH)	Sigma Chemical Co.	S-0899
2N Hydrochloric acid (HCl)	JT Baker	5616-02
Silicone oil	Sigma Chemical Co.	175633
Mineral oil	Sigma Chemical Co.	M8662
Methanol	Sigma Chemical Co.	270474
Taurocholic acid	Sigma Chemical Co.	T4009
Estrone-3-sulfate	Sigma Chemical Co.	E-0251
1- *Methyl*-4-phenylpiridinium iodide	Sigma Chemical Co.	D048
^{3}H-Taurocholic acid	PerkinElmer	NET322
^{3}H-Estrone-3-sulfate	PerkinElmer	NET203
^{3}H- *Methyl*-4-phenylpiridinium iodide	PerkinElmer	NET914

*Items listed in this Materials section are provided for your convenience; suitable materials and equipment from other manufacturers may be substituted as appropriate. The use of radioactive materials for detection by scintillation requires special training, safety and licensing. Please refer to your suppliers' MSDS for specific safety information for all chemicals.

Procedure

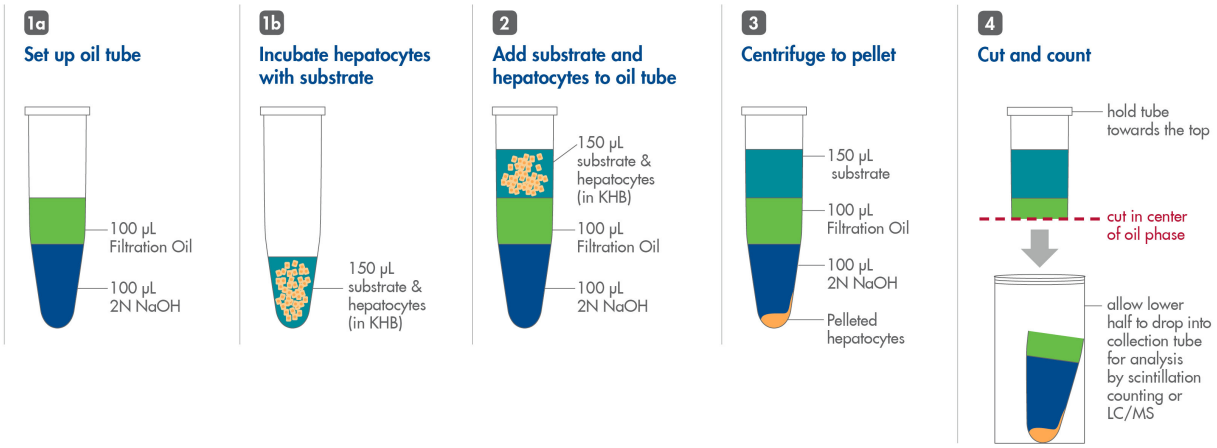
General information

Samples should be run in at least triplicate although quadruplicate samples are recommended. Cells should be >80% viable at the time of assay to reduce background signal. The samples at 37 ℃ are typically assayed first. These samples should not be equilibrated to 37 ℃ for an extended amount of time in order to reduce the possibility of viability differences with samples at 4 ℃.

Reagent preparation

Prepare compounds to be tested in solutions of *InVitro*GRO™ KHB at the appropriate concentrations. When using the radiolabeled compounds, mix with non-radiolabeled (cold) test article to provide the appropriate concentration and specific activity. The stock test article solution will be three times (3X) the desired final incubation concentration. Equilibrate a 48-well plate and an aliquot of the stock test article solution at both 4 ℃ and 37 ℃. Prepare 0.4 mL centrifuge tubes by filling the bottoms with 100 µL of 2N NaOH. Layer 100 µL of filtration oil on top of the NaOH. The filtration oil is prepared as a mixture of 5 parts silicone oil to 1 part mineral oil, resulting in an oil mixture with a density of 1.015 g/mL.

Diagram of Assay Set-up



Assay

1. Thaw cryopreserved human hepatocytes according to BioreclamationIVT's thawing protocol. Count cells and resuspend at a concentration of $2x10^6$ viable cells/mL in *InVitro*GRO KHB.

2. Split cell suspension into two equal volumes and equilibrate one at 37 ℃ and the other at 4 ℃ for 15 minutes.

3. Add 100 µL of 37℃ equilibrated cells to 4 wells of a 37℃ equilibrated 48-well plate. Set timer for the appropriate incubation time.

4. Add 50 µL of 37℃ equilibrated 3X substrate solution to the wells containing the cells. Start timer, return to incubator, and place on a rotator at moderate speed.

5. Remove plate from incubator with approximately 20 seconds remaining in incubation and transfer to 0.4 mL tubes, layering carefully on top of the oil layer. Spin immediately at 13,000xg for 15 sec. This will move the cells through the oil layer into the NaOH section, leaving the free test article in the aqueous layer on top of the oil.

6. Repeat steps 3-5 with cells, test articles, and plate equilibrated to 4℃.

7. Incubate tubes 2 hours to overnight at ambient temperature; optionally, freeze the tubes in a -80℃ freezer or on dry ice just prior to cutting. The freezing of the layers will reduce sample volume loss due to incidental splashing and minimize contamination with the free test article layer.

8. Cut tubes in the middle of the oil layer, allowing the bottom section to drop into a suitable receptacle. If test article is radiolabeled, resuspend the pellet in 2N HCl to neutralize the solution. Add scintillation cocktail and read on scintillation counter. For LC/MS analysis, extract with a compatible method.

9. The rate of uptake is calculated as the uptake at 37℃ minus the uptake value at 4℃.

References

1. Li, A. P. Primary hepatocyte cultures as an *in vitro* experimental model for the evaluation of pharmacokinetic drug-drug interactions. *Adv. Pharmacol. Series* **1997**, *43*, 103–130.
2. Jigorel E. et. al. Functional expression of sinusoidal drug transporters in primary human and rat hepatocytes. *Drug Metabolism and Disposition* **2005**, *33(10),* 1418-1422.

Cautions

- Treat all products containing human- and monkey-derived materials as potentially infectious, as no known test method can offer assurance that products derived from human or monkey tissues will not transmit infectious agents.
- All products are for research use only. Do not use in animals or humans. These products have not been approved for any diagnostic or clinical procedures.
- Please refer to the manufacturers' MSDS for specific safety information for all chemicals.

Tables

Table 1: Uptake transporters, substrates, typical substrate conditions

Human		
Uptake Transporter	**Substrate**	**Concentration / Time**
OATP (1B1, 1B3, 2B1)	Estrone-3-Sulfate	2 µM / 3 min
	Estradiol 17-β-Glucuronide	0.1 µM / 3 min
NTCP	Taurocholic Acid	1 µM / 3 min
OCT1	1-Methyl-4-Phenylpiridinium	1 µM / 3 min