Reduced Animal Usage and Decreased Study Cost through Microsampling Approaches During Preclinical Safety Testing

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Abstract
Pharmacokinetic and pharmacodynamic safety testing relies heavily upon small animal models during the preclinical phase. Promising lead compounds are typically administered to an animal cohort and blood is drawn across various time points and/or compound dose courses. Current assay approaches demand significant blood sample volumes, often requiring multiple animals per data course or in extreme cases as many as one animal per data point, adding to experimental variabilty and overall study cost. Assay miniaturization can reduce animal usage by enabling smaller blood draws and, therefore, an increased number of data points generated per animal. This approach we refer to as “microsampling”. We demonstrate that assay miniaturization using Labcyte Echo® Acoustic Liquid Handling Technology enables blood sampling to be reduced by 5 to 50-fold resulting in 89% reduction in animal needs. Combined with reductions in compound consumption and decreases in study length, microsampling approaches can afford up to 80% reduction in overall study cost, and fulfills the ethical need to reduce animal usage overall.

Introduction
Serial microsampling of animals reduces the overall number of animals sampled and euthanized for toxicity studies. Traditional composite studies in mice can require up to 700 μL of blood for each time point in a pharmacokinetic study. A draw of 700 μL from a mouse would require the animal to be anesthetized and require the use of anesthetic “satellite” animals to complete the study. Thus, in a 9-point time course analysis of mouse plasma, nine mice would be sacrificed. In response to programs like the EU 3Rs initiative to reduce the use of animals in research the study. Thus, in a 9-point time course analysis of mouse plasma, nine mice would be sacrificed. For toxicology studies. Traditional composite studies in mice can require up to 700 μL of blood for each time point in a pharmacokinetic study. A draw of 700 μL from a mouse would require the animal to be anesthetized and require the use of anesthetic “satellite” animals to complete the study. Thus, in a 9-point time course analysis of mouse plasma, nine mice would be sacrificed. In response to programs like the EU 3Rs initiative to reduce the use of animals in research.

Materials & Methods

STEP 1:
Dosing the Mice
Animals are treated with compounds dissolved in a formulation buffer either intravenous or per orally.

STEP 2:
Blood Sample Collection
Mice are prepared for blood collection in the following manner:
1. The tail vein is punched with a cannula (outer diameter 0.6 mm) and blood is collected directly into a Minivette® POCT 20μl K3E, which is EDTA treated and has a sample pickup volume of 20 μL (Sarstedt cat.no. 17.2113.020). Once the Minivette is filled, blood is immediately transferred into an Eppendorf PCR Tube (0.2 ml, Eppendorf cat.no. 0030124332).
2. The tube with the blood is loaded into an Eppendorf Centrifuge 5424R and spun down at 4°C for 5 min with 11,000 rpm.
3. The blood separates into the three layers and after centrifugation 8 μl of plasma is aspirated from the top plasma layer using a pipette making sure not to disturb or aspirate the buffy coat layer underneath. The plasma is then transferred into a fresh Eppendorf PCR Tube and stored at -20°C until further sample preparation.

STEP 3:
Preparation of Samples for Transfer Using the Echo Liquid Handler
The plasma samples of the treated animals (unknown plasma samples) are transferred into a Labcyte 384-Well Low Dead Volume Microplate (LP-0200). After the samples are transferred they are sealed with adhesive seal immediately to prevent evaporation. In addition to the unknown plasma samples, other wells of the same microplate are filled with blank plasma samples, pure DMSO, an internal standard and the analytical compound dissolved in DMSO at different concentrations:
- 1000 μg/ml, 500 μg/ml, 50 μg/ml, 5 μg/ml, 0.5 μg/ml, 0.05 μg/ml

Running the Echo Dose Response protocol using the Echo Liquid Handler produces a 14-point dilution of the analytic compound in the Abgene 384-well Storage plate (ThermoFisher Scientific, cat.no. AB-1056).

Using the compound concentrations provided in Step 3, a protocol to assemble a standard curve is created in the Echo® Dose Response Software Application using the following parameters:
- 14 dilution points
- 2-fold dilutions
- Top concentration: 15,000 ng/ml
- 2% DMSO max. (10 nl)
- Automatic backfill

Running the Echo Dose Response protocol using the Echo Liquid Handler produces a 14-point dilution of the analytic compound in the Abgene 384-well Storage plate. In a subsequent run on the Echo-system 490 μl of blank plasma is transferred to each well of the standard curve and 500 μl of each unknown plasma sample is transferred to adjacent wells of the Abgene plate. This is followed by a transfer of 12.5 μL of the internal standard to each well of the standard curve and to each well containing unknown plasma sample.

The samples are now prepared for downstream analysis.

STEP 4:
Downstream Sample Preparation
For protein precipitation, 30 μL of MeOH is added into the wells of the Abgene plate using a bulk reagent dispenser. This corresponds to a 60x dilution, which is high compared to a traditional workflow (usually 3x). The plate is sealed with an adhesive seal. Matrix effects in the mass spec analysis coming from traces of formulation buffers in the samples (from dosing) are eliminated at those higher dilution levels.

Results

STEP 6:
LC-MS / MS Analysis
At Roche researchers use a Shimadzu UFLC system for the liquid chromatography. The mass spectrometry analysis is done on an AB SCIEX QTRAP 5500 or 6500+ system. The sealed plate is loaded into the cassette of the injector module, which automatically pierces the seal with a needle. Depending on the compound 1-10 μl of a sample is injected every 3 minutes. The mobile phases are:
- Mobile Phase A: 90% H2O + 10% ACN + 0.2% HCOOH
- Mobile Phase B: 100% ACN + 0.2% HCOOH
- Mobile Phase C: 90% H2O + 10% MeOH + 0.2% HCOOH (rarely used)

LC chromatograms display the study samples. Prepared with 1 µl plasma and analyzed at 2 different days.

Discussion

High precision, low volume fluid transfers by the Echo Liquid Handler make it possible for researchers to perform serial sampling for pharmacokinetic (PK) and pharmacodynamic (PD) studies — dramatically reducing study costs and animal use while increasing data quality.

- Microsampling better addresses the 3 Rs of animal welfare: Replace, Refine, Reduce.
- Use of fewer animals in study, Less stress on test animals, Less animal mortality.
- Use as little as 1 µL of plasma from mice vs 50 μL with no loss of data quality.
- Save up to 80% of study costs, 89% of animal costs, and 75% of test compound used.
- Removes the animal to animal variability seen in composite collection studies by decreasing the number of subjects used within a study.
- Utilize the same animals for PK and PD studies for better correlation between drug level and drug action.