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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 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 variability 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 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 euthanized and often require the use of addition "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 researchers have turned to microsampling as an alternative to composite studies. With advances in bioanalytical techniques drug levels can be determined from samples of 20 µL or less. This small sample volume or microsample reduces the dependency on satellite animals to complete a study. Additionally, microsampling is faster and less stressful than traditional composite studies. At Labcyte, we are describing a method used by researchers at F. Hoffmann-La Roche AG of using Echo Liquid Handlers for microsampling.

Materials & Methods

STEP 1:

Dosing the Mice

Animals are treated with compounds dissolved in a formulation buffer either intravenous or per os orally.

STEP 2:

Blood Sample Collection

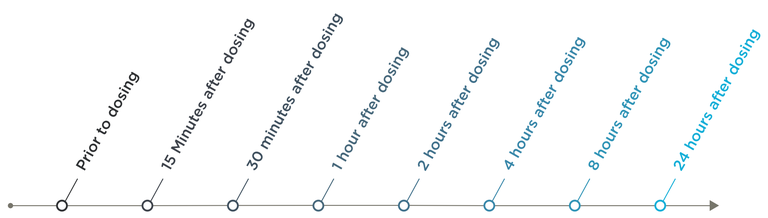


FIGURE 1: Blood is collected from the mouse at eight individual time points:

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 ul (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 -20C° until further sample preparation.

Materials & Methods

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

Echo® 550 Acoustic Liquid Handler

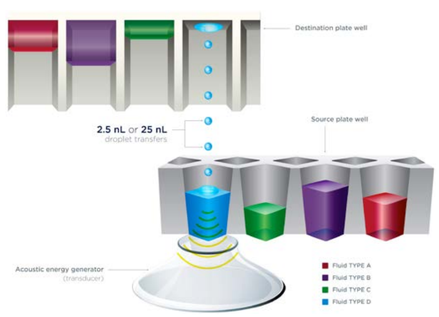


FIGURE 1: An acoustic transducer is positioned below a source plate. Water flowing between the transducer and well bottom couples the transducer to the well enabling sound energy to propagate into the fluid contained in the well. Acoustic energy focused at the fluid surface enables droplets of a predetermined volume (2.5 nL or 25nL) to be ejected. Larger volume transfers are achieved by serially ejecting droplets. Ejected droplets are collected in an inverted destination plate positioned immediately above the source plate. Droplets can be transferred to either a dry well or a well already containing fluid.

STEP 4:

Preparation of Samples for Transfer Using the Echo Liquid Handler

The contents of the 384-Well LDV microplate are transferred using the Echo® Liquid Handler to an 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 plate. In a subsequent run on the Echo system 490 nL of blank plasma is transferred to each well of the standard curve and 500 nl of each unknown plasma sample is transferred to adjacent wells of the ABgene plate. This is followed by a transfer of 12.5 nL 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.

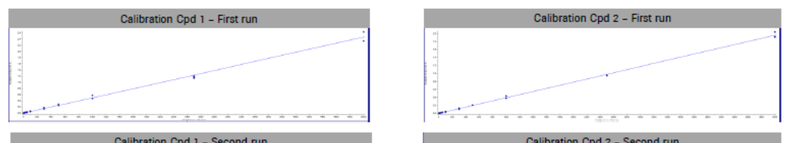
Results

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% H_2O + 10% ACN + 0.2% HCOOH
- Mobile Phase B: 100% ACN + 0.2% HCOOH
- Mobile Phase C: 90% H_2O + 10% MeOH + 0.2% HCOOH (rarely used)



Quality Control Sples Cpd 1		
Added Conc.	Accuracy % First run	Sec. Run
15	107	120
30	94	99
300	104	99
1000	99.4	101
15	111	110
30	89.4	112
300	104	112
1000	109	119

Study Sples Cpd 1	Concentration ng/mL First run	Second run	Difference %
1	6210	5980	3.7
2	7020	6560	6.6
3	4210	3610	14.3
4	8900	8690	2.4
5	6980	6510	6.7
6	5040	5010	0.6
7	9820	8660	11.8
8	8480	8020	5.4
9	5900	5450	7.6
10	3960	4180	-5.6
11	5040	5250	-4.2
12	4170	4570	-9.6

Quality Control Sples Cpd 2		
Added Conc.	Accuracy % First run	Sec. Run
15	93	83.7
30	112	102
300	97.7	97.8
1000	101	94.3
15	94.7	110
30	92.7	121
300	102	115
1000	106	120

Study Sples Cpd 2	Concentration ng/mL First run	Second run	Differenc e %
1	2490	2420	2.8
2	2830	2950	-4.2
3	3290	2940	10.6
4	1680	1610	4.2
5	3910	3550	9.2
6	4040	3950	2.2
7	3010	2810	6.6
8	3100	3120	-0.6
9	2370	2190	7.6
10	1150	1250	-8.7
11	2140	2070	3.3
12	2030	1990	2.0

FIGURE 3: Reproducibility with study samples. Prepared with 1 µL plasma and analyzed at 2 different days.

Manual vs. Labcyte	Sample	Measured Conc. ng/mL		Difference Labcyte vs. Manual	
		Manual	Labcyte	ng/mL	%
Study samples	1	156	155	-1	-0.6
	2	752	762	10	1.3
	3	171	196	25	14.6
	4	933	1110	177	19.0
	5	926	1160	234	25.3
	6	1880	2070	190	10.1
	7	2020	2170	150	7.4
	8	906	981	75	8.3
	9	2320	2580	260	11.2
	10	2050	2500	450	22.0
	11	2130	2410	280	13.1
	12	1460	1680	220	15.1
	13	5210	6690	1480	28.4
	14	5750	7610	1860	32.3
	15	24700	29400	4700	19.0
	16	24000	30200	6200	25.8
	17	20000	22700	2700	13.5
	18	26900	30500	3600	13.4
	19	51200	61700	10500	20.5
	20	34900	37000	2100	6.0
	21	51900	58300	6400	12.3

FIGURE 4: Sample preparation Manual versus Labcyte Echo Liquid Handler. 50 µL plasma volume used for manual sample preparation and 1 µL plasma volume used for Labcyte Echo Liquid Handler sample preparation.

Summary

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 R's of animal welfare: Replace, Refine, Reduce.
- Use of fewer animals in study, Less stress on test animals, Less animal mortality.
- Use as little at 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.

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