A Simplified and Reliable Capillary Microsampling Procedure to Support Preclinical Regulatory Studies in Rodents

Abstract #901

Mader, Robert[1]; Iqbal, Sobia[2]; Fraschini, Marie-Claude[1]; Burnett, Josephine[2]; Kilford, Peter[2]; Bravo, Juan[1]

[1] Debiopharm International SA, Lausanne, Switzerland, [2] Covance Laboratories Ltd, Harrogate, United Kingdom

Introduction

Regulatory rodent toxicity studies require a proof of exposure (non-exposure for control animals). In general, the animals undergoing toxicity evaluation are usually not bled for exposure determination, because the blood volume in rodents, and especially so in mice, is very limited. Importantly reducing the circulating blood volume by taking multiple samples over time to obtain a toxicokinetic (TK) profile will alter the animals' physiology and therefore also potential toxic effects of the compound under investigation. Moreover, animal welfare laws dictate restrictions on the maximum volume that can be drawn from each animal. For these reasons, additional TK animals are added to each group of toxicity animals. The number of blood samples required to obtain a meaningful TK profile often exceeds the acceptable maximum blood volume available of an individual animal. Therefore, composite TK profiles are constructed by using different animals for different time points and different sampling occasions. Thus, these additional animal groups can become relatively large, sometimes surpassing the group size of principal toxicity animals. The following table shows recent data from Covance UK (Harrogate), collected from 2010 to 2014 in the frame of 4 week GLP toxicity studies.

Species	Overall study count	Average number and range of TK animals per study	Average number of control TK animals per group per sex	Average number of treated TK animals per group per sex
Rats	53	60.1 [24 - 96]	3.7	8.8
Mice	36	110.3 [32 - 240]	9.0	15.4

A major drawback of the use of TK animals is the fact that, in contrast to large non-rodent species, such as dog, monkey or mini-pig, no correlation of individual toxicity to individual exposure can be made.

Recent advancements in the sensitivity of bioanalytical instrumentation (LC-MS/MS) now allow an important reduction of the plasma volume required for analysis and thus also the reduction of the blood volume that has to be drawn from the animals. This has led to proposals of new approaches to TK sampling, such as Dried Blood Spot (DBS) and plasma microsampling.

Here, we propose a modified microsampling procedure that does not require an error prone transfer into a volumetric capillary and has shown its advantages and applicability in a 4 week regulatory GLP study in mice. Though we could not get rid of separate TK animals we could importantly reduce the group size, obtain full TK profiles from individual animals and added a proof of exposure (single time point at Tmax) to the main toxicity animals in order to potentially correlate observed individual toxicity to exposure.

Method

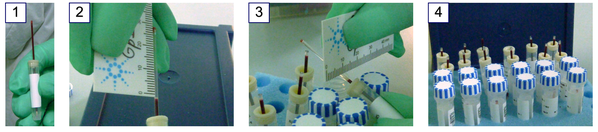
The microsampling procedure was used in the frame of a 4 week GLP toxicity study in mice. Each of the four groups (control and 3 dose levels) consisted of 12 main toxicity animals and additional 4 mice for TK per sex. Control and high dose groups also included 6 recovery animals per sex each.

In the animal facility:

- Prior to sampling, keep the animals in a heating cabinet to widen the tail veins for a few minutes, no anesthesia required
- Draw blood from the lateral caudal vein using a butterfly needle with tubing cut away
- Take up the developing blood drop using a EDTA coated 60 µL capillary
- Plug the capillary twice on the blood entry side with wax in a horizontal position
- Put the capillary into a holding tube for centrifugation (pierced lid and u-bottom for upright position, Fig. 1)

In the sample management facility:

- Centrifuge with low force (1500g) but extended time (10min) at RT (the wax plug may leak when cooling)
- Centrifugation results in a sharp boundary and excellent separation of good quality plasma (nearly colorless)
- Measure the length of the plasma column to confirm sufficient plasma volume for bioanalysis (Fig. 2)
- Cut the capillary in horizontal position (Fig. 3)
- Introduce the plasma containing part of the capillary (in horizontal position) into a storage tube for freezing, storage and shipment (Fig. 4)
- Pay attention not to cross-contaminate the outside of the capillary, because all contents of the storage tube are part of the sample

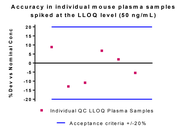


In the bioanalytical lab:

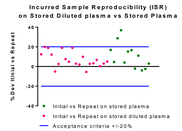
- Using a pipet, flush the capillary contents into the storage tube and homogenize
- Take an aliquot (10 µL) and dilute with 90uL of diluent
- Take an aliquot (25 µL) of diluted plasma for SPE micro elution prior to analysis
- Freeze the remaining volume for ISR (Incurred Sample Reanalysis) or other re-analysis

Bioanalytical Validation

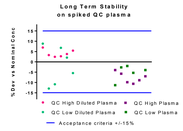
The validation process is comparable to that for 'standard' plasma samples with the addition of the final dilution step. Sample work-up and analysis is performed on the diluted plasma as well as the long term storage for potential re-analysis.



Mouse plasma samples were spiked with 50 ng/mL (the Lower Limit Of Quantification) and processed including the dilution step. The graph shows the deviation (in %) of the actual concentration from the nominal value. The validated detection range of the bioanalytical method is 50 ng/mL to 100'000 ng/mL.



Re-analysis of samples shows good reproducibility. An aliquot of each sample was analyzed and the remaining plasma or diluted plasma stored frozen for 8 days, before performing a second analysis. The graph shows the deviation (in %) of the second analysis from the first.



The long term stability is determined after 90 days of storage at –70°C at low (150 ng/mL) and high (80'000 ng/mL) concentrations in diluted plasma as well as in undiluted plasma. An additional time point at 180 days confirmed that there is no loss in undiluted plasma as the graph suggests.

Method development

Although the method appears to be straight forward on paper, we have identified a few very critical steps that determine reliability and success of the method. In particular we modified the following parameters:

- Work with capillaries held horizontally when both ends are open to avoid losses
- Plug the capillaries on the end with which the blood has been taken up
- Plug the capillaries twice to increase the strength of the wax plug
- Centrifuge at room temperature to avoid contraction of the wax plug and leakage
- Centrifuge at lower speeds and longer duration to avoid leakage
- Measuring the length of the plasma column is a good indicator of sufficient plasma available for bioanalysis

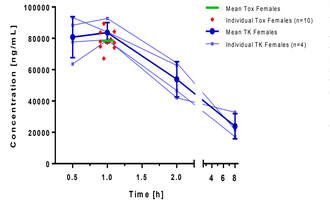
The optimization of these parameters reduced sample loss due to the procedure to 5%

	Sample count	% of total
Total Samples	184	100%
No Blood	5	3%
Small Sample	25	14%
Leaks	9	5%

In the present mouse study, a total of 184 samples was planned. Due to the difficulties of tail vein sampling in mice several samples were not sufficient to be processed for bioanalysis, either because no blood at all could be taken or the developing blood drop on the butterfly needle was too small to provide sufficient plasma (> 10 µL).

Toxicokinetic Data

The data presented below is taken from a mouse 4 week GLP study. The compound was administered orally and microsampling was performed on day 1 (not shown) and day 27. In addition to the TK animals, the main toxicity animals were bled once at 1h post-dose to allow the correlation of the individual exposure to the observed toxicity.



Individual and mean toxicokinetic profiles of TK animals and individual and mean plasma concentrations of main toxicity animals at Tmax. Blood sampling in main toxicity animals was acceptable because tail vein sampling does not require anesthesia. The individual concentrations are scattered around the one hour time point for better illustration. In the present case, the exposure of the main toxicity animals is presumably very comparable to that of the TK animals. However, the advantage of this approach will become apparent when individual animals show important toxicity. In such cases one can determine if the toxicity is related to individual (increased) exposure. In larger rodents, e.g. rats, it could also be envisaged to refine the exposure estimation by taking an additional microsample before the next dose to determine the trough level.

Conclusions

- Extensive training of personnel is required. Though the method is straight forward on paper, the details dictate success (percentage of samples that can be analyzed)
- Applicable for any drug provided that the assay sensitivity, the plasma and the diluted plasma sample stability are satisfactory
- Amenability to GLP (however: labeling of primary tube is currently not feasible)
- The method applies 2 out of the 3 R's
  - Important ***reduction*** in required animal numbers per study: in the present mouse study by a factor of 3.4-fold compared to Covance study average
  - ***Refinement***: Better data due to serial sampling in rodents
  - ***Refinement***: Better data due to the potential (at least in rats) of (serial) sampling in the toxicity animals and not in a separate TK group, allowing correlation of tox effects and exposure.
  - ***Refinement***: Better data due to the potential to obtain a backup sample by filling a second capillary (in the presented study, the backup is the diluted plasma)

The poster is available for download by QR or at:
http://www.debiopharm.com/medias/publications.html
For more information:
robert.mader@debiopharm.com / sobia.iqbal@covance.com