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# Small Samples, Large Molecules and Bigger Benefits

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# Outline

- What is microsampling?
- Drivers for microsampling
- Microsampling techniques
- Bioanalytical practicalities
- Case studies
  - Small molecule assay validation
  - Small molecule toxicology study
  - Large molecule assay validation
  - Large molecule toxicology study
- Conclusions

# Microsampling

- Microsampling – our definition is taking less than 50  $\mu\text{L}$  of blood (or other matrix) from the test subject for analysis
  - Normally prepared into plasma
  - Can also prepare into serum
- Our favoured approach is to use a capillary microsampling technique for the collection of the blood and subsequent plasma preparation
- Materials used are standard glass capillaries – otherwise there are no other specific equipment
- The main application is preclinical studies where blood volumes are limited

# Animal Welfare Drivers for Microsampling

- Animal welfare
  - Reduce animal numbers
    - Minimise satellite groups – potential for up to 90% reduction in satellite animal numbers
    - Eliminate satellite groups entirely
      - Use main study animals for all sampling
  - Reduce animal stress
    - Sample collection can be quicker than conventional bleeds
    - Animals spend less time in warming chambers

# Scientific Drivers for Microsampling

- Generate complete (rather than composite) profiles from rodent studies
- Better correlation between toxicology end points and exposure data
  - TK samples taken from main study animals
- More studies are performed in transgenic, knockout or disease model animals
  - In total only 300  $\mu\text{L}$  of blood can be taken from a 20 g mouse over 28 days
- Release blood volume for other investigations
  - Bioanalysis
  - Haematology / clinical pathology
  - Pharmacodynamic markers / biomarkers
  - Anti-drug antibodies
  - Immunotoxicology

# Microsampling Techniques

- Many ways to collect/store the samples
  - Dried Blood Spots (DBS)
  - Dried Matrix Spots (DMS)
  - Microvettes
  - Capillary Microsampling (**CMS**)
    - Different approaches
- No single technique will provide the universal solution
- Animal technicians and laboratory staff need to be trained to accommodate multiple techniques

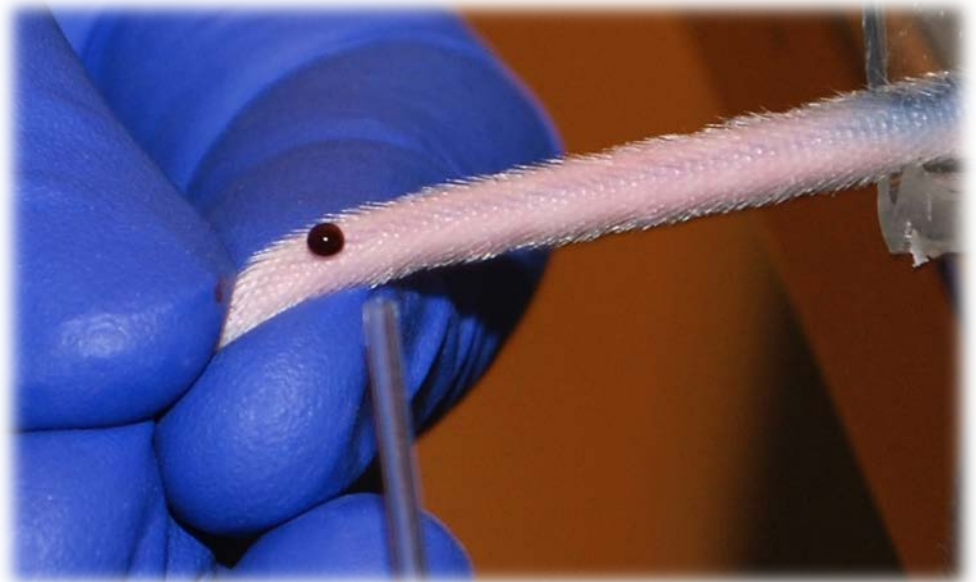
# Capillary Microsampling

- Discussed at scientific meetings for 2-3 years
- Under investigation within pharmaceutical companies for over 6 years
- (Perceived) barriers to implementation
  - Impact on the toxicological findings of taking multiple samples from the main study animals
  - Sample collection
  - Sample handling in the laboratory
  - Assay validation procedures
  - Regulatory acceptance



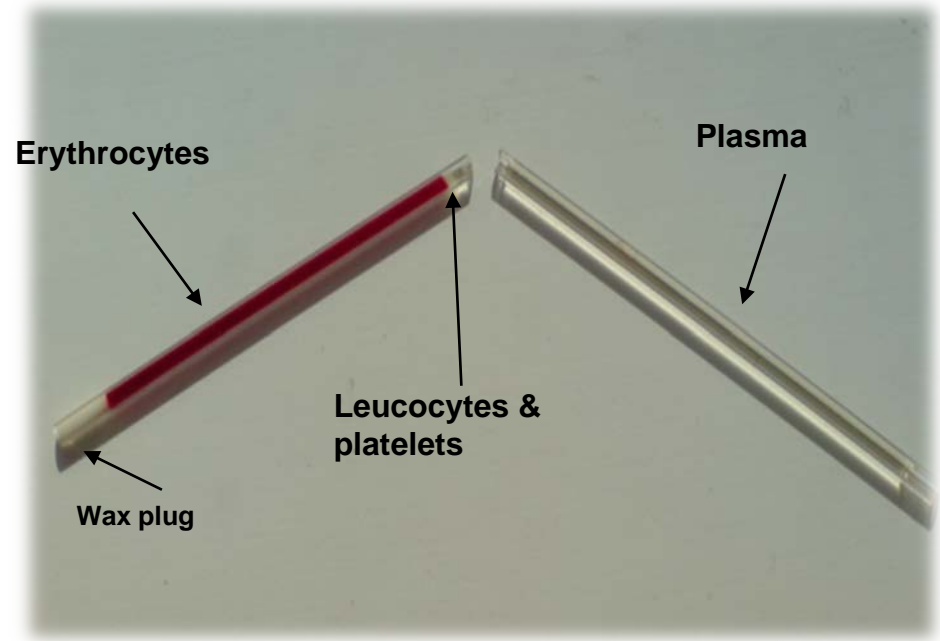
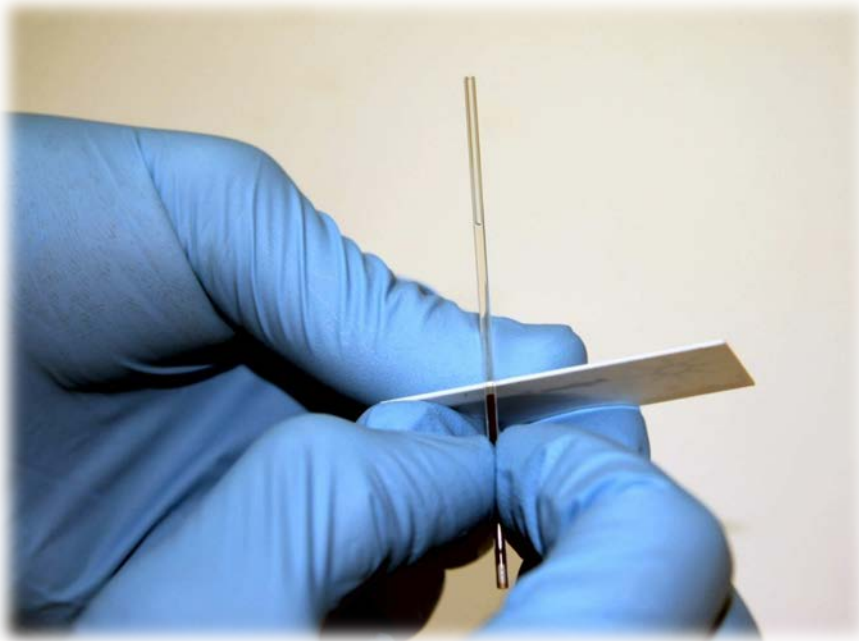
# Capillary Microsampling

- Procedure routinely adopted at Charles River
- Sampling done via tail incision using a scalpel at the distal end of the tail
- Use  $K_2EDTA$  haematocrit tube (32  $\mu L$ ) to collect blood sample



# Capillary Microsampling

- The tube is sealed with wax and centrifuged
- The tube is cut to separate the plasma fraction



# Capillary Microsampling

- Transfer the plasma sample to an exact volume (8  $\mu$ L) microcapillary
- The plasma sample in the microcapillary can be placed in an eppendorf tube and stored frozen at this stage, or it can be diluted with an exact volume of diluent prior to storage



# Capillary Microsampling

- Advantages

- Two samples can be collected from the animal at the same time (eg plasma and serum)
- The exact volume of the sample is known at the collection stage (= volume of microcapillary tube: 4 or 8  $\mu\text{L}$ )
- Different volumes of haematocrit tubes and microcapillary can be used to obtain the optimal volume for the assay
- Duplicate microcapillary samples can be prepared from the same haematocrit tube
- The plasma sample is eluted through the other end of the tube as the blood was originally collected – no potential for contamination

# Capillary Microsampling

- Disadvantages

- Special training for animal room staff for sample collection and transfer
- How to deal with partial plasma sample volumes if an incomplete blood sample is taken
  - Ensure details of microcapillary used are recorded on collection (8  $\mu\text{L}$  or 4  $\mu\text{L}$ )
  - Check for air bubbles in sample – affects the final volume
- Training for the laboratory staff to deal with samples supplied in microcapillaries

# Bioanalytical Practicalities

- Where are we just now?
  - Our systems are sufficiently sensitive
    - UPLC, API5000, API6500
  - We can work with small samples
    - 1-2  $\mu\text{L}$  of matrix or larger volumes of diluted matrix
  - The matrices are common
    - Plasma, serum, blood
  - Staff have been appropriately trained
    - Animal technicians to take samples – global ‘validation’ study
    - Bioanalysts to process samples

# **CMS IN PRACTICE – SMALL MOLECULE ASSAY VALIDATION EXPERIENCE**

# CMS in Practice – Validation

- The main issue is to mimic the handling of the study samples during the validation process
- QCs are prepared in plasma, transferred to microcapillaries and then placed in sample tubes and stored as per the study samples
- Stability will have to be proven for plasma samples in the microcapillaries and also post-dilution samples
  - Assess potential for the analyte sticking to glass
  - Assess potential for sample thawing during transportation/handling
- Samples which are over range can be analysed after dilution of the sample with a matched solution of plasma and diluent



# CMS in Practice – Validation

- Matrix: rat plasma K<sub>2</sub>EDTA
- Range: 0.02 – 20 µmol/L
- Calibration standards prepared as bulk spiked plasma, take 8 µL and add directly to diluent
- QC samples prepared as bulk spiked plasma, then taken up in the 8 µL microcapillaries
  - QC samples stored frozen in the microcapillary, or diluent added and stored frozen as the diluted sample

# CMS in Practice – Validation

- Developed method:
  - 8  $\mu\text{L}$  microcapillary sample + 92  $\mu\text{L}$  water
  - 25  $\mu\text{L}$  aliquot of diluted sample transferred to matrix tube (equivalent to 2  $\mu\text{L}$  plasma)
  - Internal standard added
  - SPE (Waters 10 mg MCX 96 well) on Tomtec Quadra 3
  - Reconstituted in 500  $\mu\text{L}$  methanol/water (25/75, v/v)
  - Inject 10  $\mu\text{L}$  onto LC-MS/MS API 4000<sup>TM</sup>

# CMS in Practice – Validation

- Acceptable method development data
- For the first few validation batches the data generally met the acceptance criteria but:
  - Occasional high values for accuracy and precision
  - Occasional anomalous results with no apparent reason
  - Different analysts gave different results
  - Assay was deemed not robust
- Was microsampling the problem?
- An investigation was carried out

# CMS in Practice – Validation

- Investigation results:
  - Water was not an appropriate diluent
  - Mixing variability depending on method (hand v shaker)
  - Changed diluent to 5% BSA in PBS and increased the volume to 360  $\mu\text{L}$
  - 100  $\mu\text{L}$  aliquot of diluted sample was taken for further processing (still equivalent to approx 2  $\mu\text{L}$  plasma)
  - No changes were required to the SPE extraction
  - More consistent data were achieved

# CMS in Practice – Validation

Replicate	LLOQ 0.0200 µmol/L	Low QC 0.0500 µmol/L	Mid QC 0.800 µmol/L	High QC 16.0 µmol/L	Dilution QC 200 µmol/L
1	0.0187	0.0522	0.834	16.2	228
2	0.0178	0.0534	0.835	16.9	222
3	0.0200	0.0520	0.816	16.4	212
4	0.0197	0.0517	0.823	15.8	208
5	0.0212	0.0488	0.848	16.3	213
6	0.0201	0.0562	0.830	16.7	209
Mean	0.0196	0.0524	0.831	16.4	215
CV(%)	6.1	4.6	1.3	2.4	3.7
Accuracy (%)	98.0	104.8	103.9	102.5	107.5

# CMS in Practice – Validation

- Lessons learned
  - Development of the assay should include looking at the diluent and the volume to be used for ‘washing’ the microcapillary and to investigate the dilution thoroughly
  - Use of water, surrogate matrix (PBS with BSA) or plasma can be used as diluent
  - Standardise mixing using a shaker (with a set speed and time)
  - Good idea to perform the initial Accuracy and Precision batch with and without microcapillaries
    - Identifies any issues specifically with microcapillaries

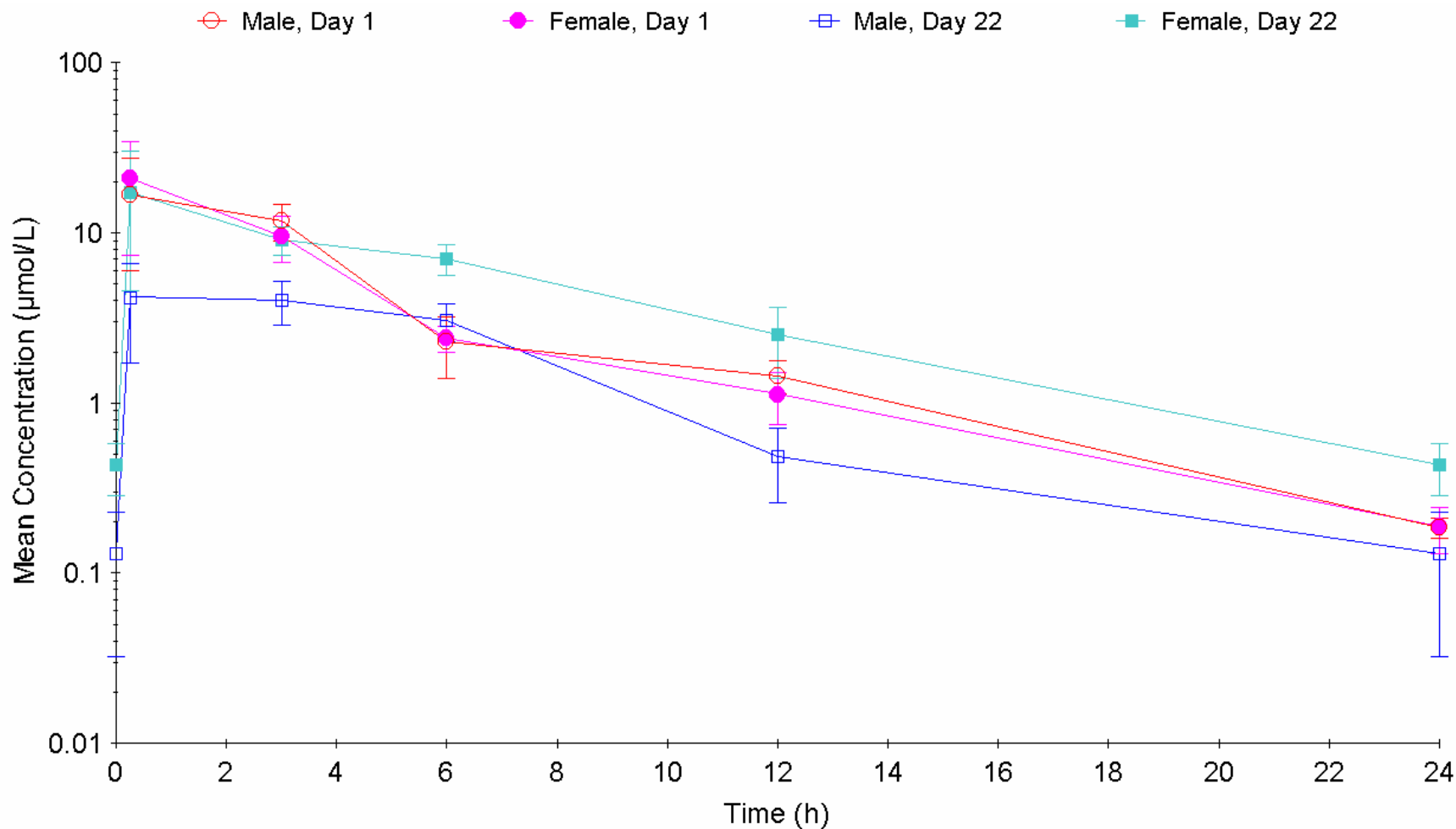
# **CMS IN PRACTICE – SMALL MOLECULE TOXICOLOGY STUDY**

# CMS in Practice – Toxicology Study

- Oral Toxicity Study in the Juvenile Rat
  - Juvenile rats were dosed from 14 days old
  - Animal weights were 30 g at the start of dosing, increasing to 70 g at the end of the study
  - Satellite animals for TK: 3M + 3F per dose group
  - Serial samples were taken from each animal on Day 1 and Day 22 (0.25 hr, 3 hr, 6 hr, 12 hr and 24 hr after dosing)



## Mean plasma concentrations ( $\pm$ SD) in male and female rats on Days 1 and 22 following oral administration of 150 mg/kg/day



# **CMS IN PRACTICE – LARGE MOLECULE ASSAY VALIDATION EXPERIENCE**

# CMS in Practice – Large Molecule Immunoassays

- Current procedures for analysis
  - Samples are analysed in duplicate wells on 96-well plates
  - 25-100  $\mu\text{L}$  of sample is added to each well
  - However, samples are typically diluted 10- to 100-fold in buffer prior to adding to the 96-well plate
  - TK/PK samples frequently have to be diluted further to bring them within the limited assay range
  - So only 0.5-20  $\mu\text{L}$  of actual matrix sample is required
  - Procedure is suitable for microsample analysis

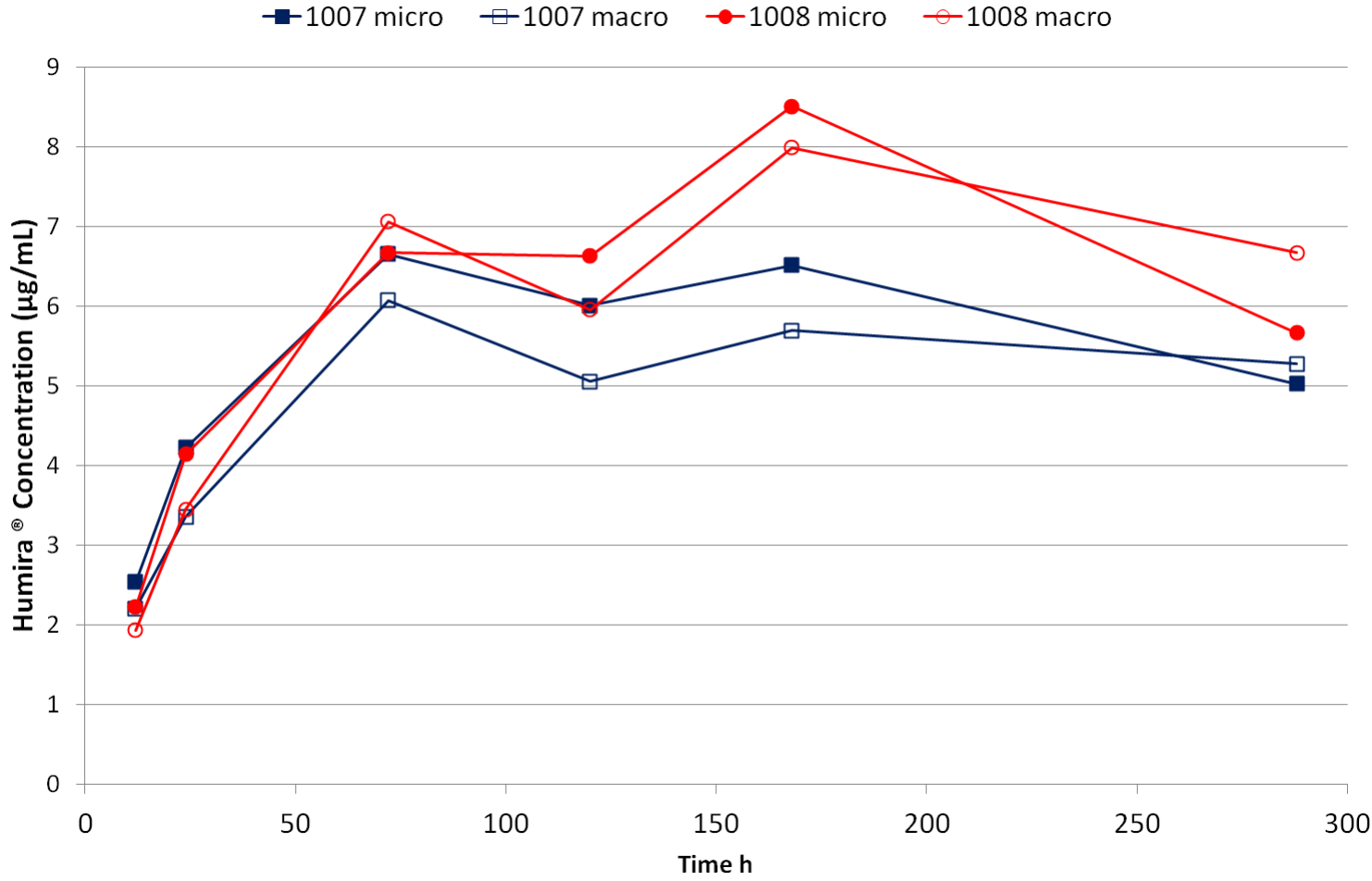
# CMS in Practice – Large Molecule Immunoassay Validation

- Aim – to use the capillary microsampling technique in a rat PK study
- Single oral dose of Humira (monoclonal antibody, MW ~144 kDa)
- 2 male rats
- Sample collection via tail vein
- Take blood samples – micro (2 x 32  $\mu$ L) and macro (400  $\mu$ L) – from the same animal at each timepoint (8 timepoints over 288 hours)
- Compare results between micro and macro samples

# CMS in Practice – Large Molecule Immunoassay Validation

- Determination of Humira in rat plasma (K<sub>2</sub>EDTA)
  - Microsamples were 2 µL
  - Macrosamples were 10 µL
  - All plasma samples diluted 100-fold prior to analysis
  - All samples run in duplicate
  - Electrochemiluminescence immunoassay
  - Calibration curve range 0.1-50 µg/mL (in 100% matrix)
  - Lower limit of quantification 0.5 µg/mL (in 100% matrix)

# CMS in Practice – Large Molecule Immunoassay Validation



# CMS in Practice – Large Molecule Immunoassay Validation

- The macro and micro sample C<sub>max</sub> times and concentrations were comparable within any single animal
- All differences between macro and micro sample results were within the expected variability of a large molecule immunoassay ( $\leq 30\%$  variability)
- Capillary microsampling gave comparable pharmacokinetic results to traditional sampling methods

# **CMS IN PRACTICE – LARGE MOLECULE TOXICOLOGY STUDY**

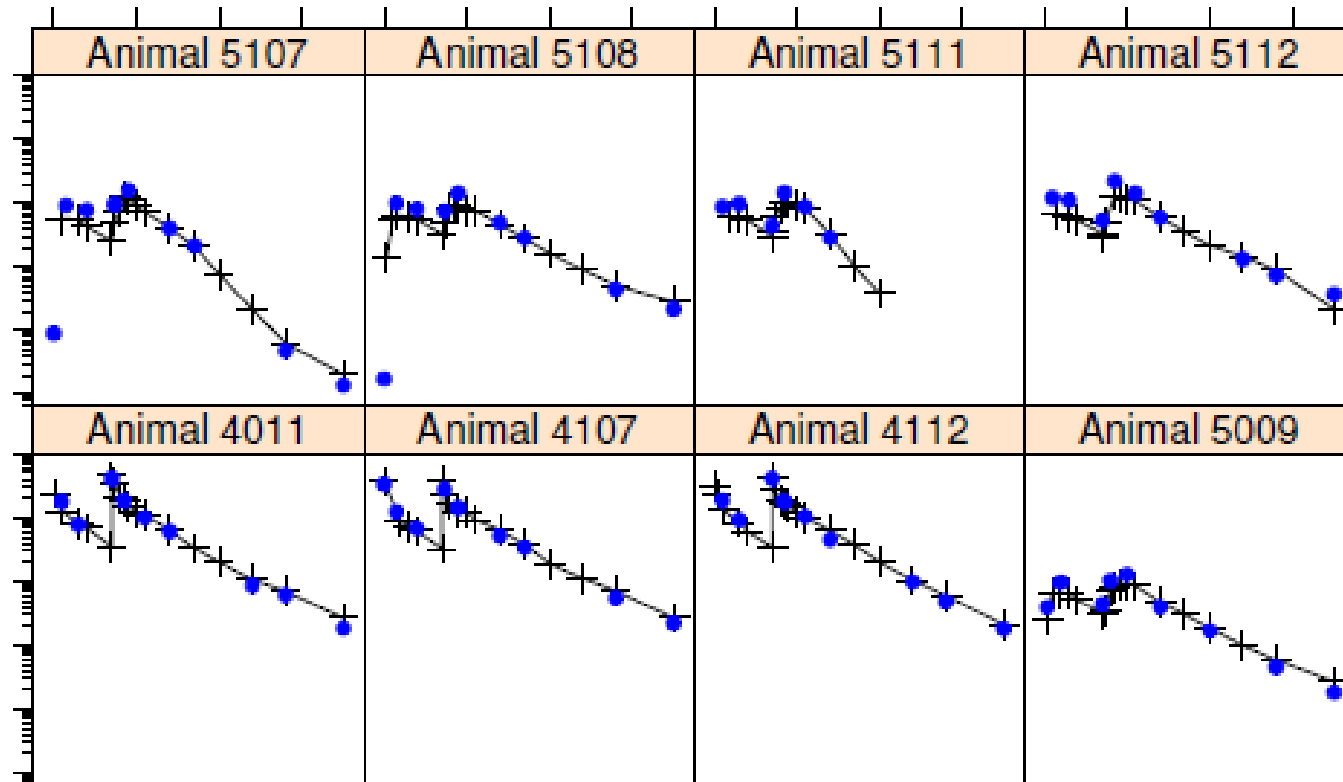


# CMS in Practice – Large Molecule Toxicology Study

- A Dose Range Finding study in rat with an antibody-like construct
  - A fully automated (TECAN-platform) electrochemiluminescence immunoassay
- PK Macrosampling
  - Sparse sampling (2 animals/sex/group/time point)
  - 10 PK timepoints per animal
  - 400  $\mu$ L of blood/sample (4 mL of blood/animal in total)
- PK Microsampling
  - Full PK profiles from all study animals
  - 22 PK timepoints per animal
  - 32  $\mu$ L of blood/sample (0.7 mL of blood/animal in total)
- In vivo phase conducted at Charles River, Montreal, with bioanalysis conducted by Novartis

# CMS in Practice – Large Molecule Toxicology Study

—+— PK microsampling ~36 ul of blood  
• PK macrosampling ~ 200-400 ul of blood



Data from Igor Vostiar, Novartis

# **MICROSAMPLING IN PRACTICE – CONCLUSIONS**

# Conclusions

- Capillary microsampling for bioanalysis has been successfully implemented across Charles River sites
- Bioanalytical experience has grown through the initial validation studies – this will continue to evolve
- Toxicology studies with capillary microsampling conducted at Charles River have proven the value of the technique
  - 12 small molecule studies
  - 1 large molecule study
- Variety of species: mouse, rat and rabbit

# Conclusions

- Microsampling is here to stay and will continue to evolve and adapt to our processes and needs
- Increased uptake will be driven by study requirements and corporate strategies
- The last barrier appears to be:
  - Can taking multiple samples from the main study animals effect the study end points?
- NC3R's, EBF etc will help us to drive the acceptance of this approach more universally

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