Supporting a paediatric study using wet and dry samples

Analytical Considerations

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Supporting a paediatric study using wet and dry samples

Introduction

- Collaboration between GSK and Leicester Hospital
  - Support Paediatric Study
  - Wet and Dry samples in Parallel
  - Measuring Midazolam and 1-Hydroxymidazolam
  - ...In whole liquid blood
  - ...and VAMS

- Part 1: Analytical Considerations
  - Method Development
  - Method Validation
  - Study support

- Part 2: Clinical Aspects (Hitesh Pandya)

Performing PK Studies in Children: The Problem

PK studies in children beset with ethical and technical challenges.

PK studies in children require

(i) Relatively large volumes of blood
(ii) Repeated vene-puncture to obtain blood

Together (i) & (ii) are generally unacceptable to parents, researchers and ethics committees

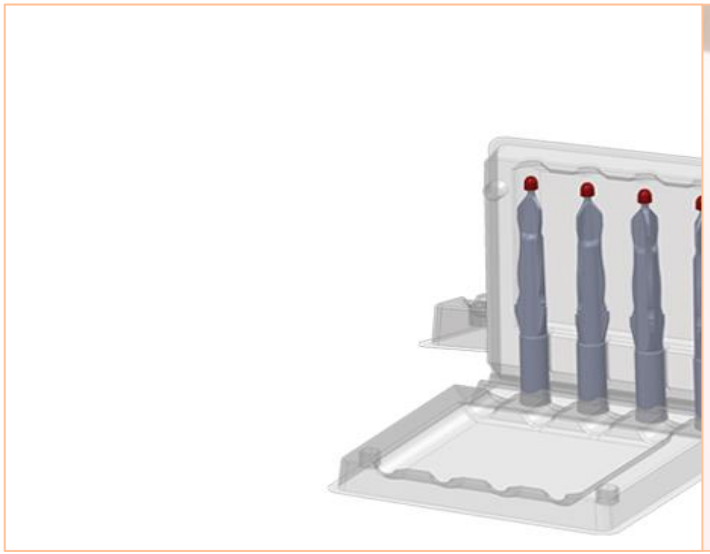
'POP-PK' modelling techniques partly resolve the issue of obtaining multiple samples from patients for PK studies

Dried Blood Spots (DBS)
A Solution to PK Studies in Children?

‘CUBS’ Study: Caffeine PK study in neonates using DBS:
Patel et al BJCP 2013

- High proportion of spoilt samples
- Haematocrit bias

……..Mitra (VAMS) ……the 4Ms study



The 4Ms Study: Why Midazolam ?

- Used in children => ICU and peri-operatively
  - sedative, anxiolytic & agent

- Side effects => cardio-respiratory depression, withdrawal symptoms => Increased morbidity and mortality

Have we got the dose of Midazolam right for critically ill children?

Systemic Inflammatory Response Syndrome (SIRS =>Critical Illness) alters Midazolam PK

Ince I et al Ther Drug Monit 2012; 34:381–9

4M's: Study Hypotheses

1. *In children administered IV drug, Blood adsorbed onto VAMS tips and traditional wet samples provide equivalent blood Midazolam and 1-OH Midazolam (active metabolite) concentration measurements*

2. *Critical illness alters midazolam PK*

4M's:
Patient Population and Blood Sampling

- Children between 1 month and 16 years, administered IV Midazolam (Bolus dose +/- Continuous infusion)

- Blood Sampling
    1. *Opportunistic = Extra blood collected when sampling blood for routine clinical tests*
    2. *Scavenged samples = Blood collected for routine clinical laboratory tests but no longer needed*
    3. *An extra blood sample for research purposes*

    All blood collected in EDTA tubes prior to adsorption onto VAMS tips
    Blood adsorbed onto 3 different VAMS tips for each PK time point

4M's:
Blood Sample Management

- VAMS tips stored in bespoke cartridges containing desiccants at room temperature
- Wet blood samples stored in a dedicated study freezer at -20^{o} C
- Samples stored on clinical site for up to 90 days prior to transfer to GSK laboratories (Ware, UK)
- [Blood midazolam] & [1-OH Midazolam] analysis via HPLC / MS

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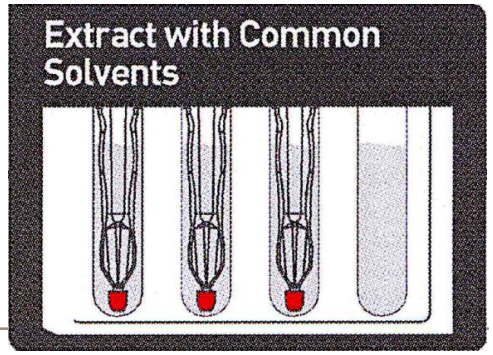
Method Development - Extraction

WET (WHOLE BLOOD)	EXTRACTION STEP	DRY (VAMS)
Thaw from frozen	STORAGE	Stored at ambient
10µL via manual pipette	SUB-ALIQUOT	Not Required
96 Well Micronic (1.4mL)	FORMAT	96 Well Block (2mL)
200µL SIL I.S. added to each tube (post sample)	INTERNAL STD	200µL SIL I.S. added to each well (pre-sample)
60 mins on lateral shaker	EXTRACTION	60 mins on lateral shaker
Not Required	REMOVE SAMPLE	Remove and discard tips
10 mins @ 3000g	CENTRIFUGE	Not Required
96 Well Micronic (1.4mL)	TRANSFER SUPERNATANT	96 Well Micronic (1.4mL)

LC-MS/MS Analysis

Effort

Automation



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Method Development – UPLC Methodology

– UPLC Conditions

Autosampler	Waters Acquity
Strong Solvent Wash	400 µL 4/3/3/0.01 (v/v/v/v:Acetonitrile/isopropanol/water/formic acid
Weak Solvent Wash	2000 µL Water containing 0.1% formic acid (v/v)
Typical Injection Volume	10 µL
Sample Loop Option	Partial Loop
Load Ahead	Disabled
Loop Offline	Disabled
Air Gaps (pre aspirate)	Automatic
Air Gaps (post aspirate)	Automatic
Needle Overfill Flush	Automatic
Chromatography System	Waters Acquity UHPLC
Flow Rate	0.6 mL/min
Analytical Column	50 x 2.0mm i.d. BEH C18 1.7 µm, Waters
Column Temperature	50°C
Column Divert	Eluent from the column was diverted from the mass spectrometer between 0 and 0.6 min
Run Time	2.2 minutes
Mobile Phase A	10mM Ammonium Acetate (native pH)
Mobile Phase B	Acetonitrile

– Gradient Profile

Time (mins)	%A	%B	Curve
0	80	20	6
0.5	80	20	6
0.8	70	30	6
1.4	10	90	6
1.6	10	90	6
1.62	80	20	6
2.2	80	20	6

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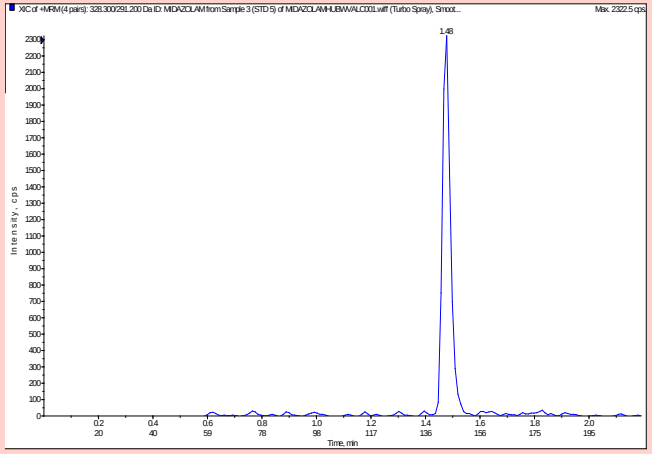
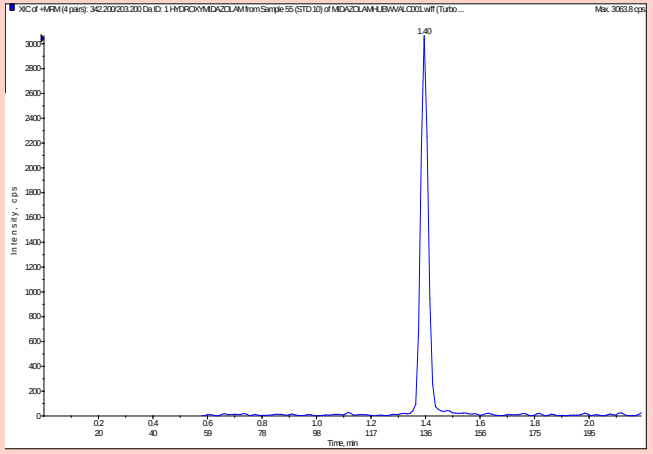
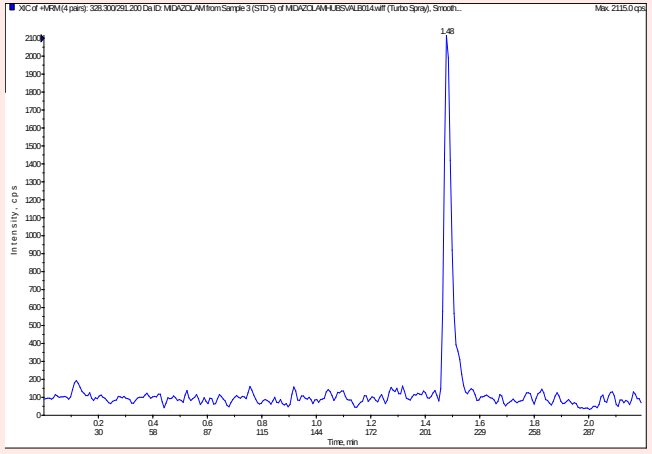
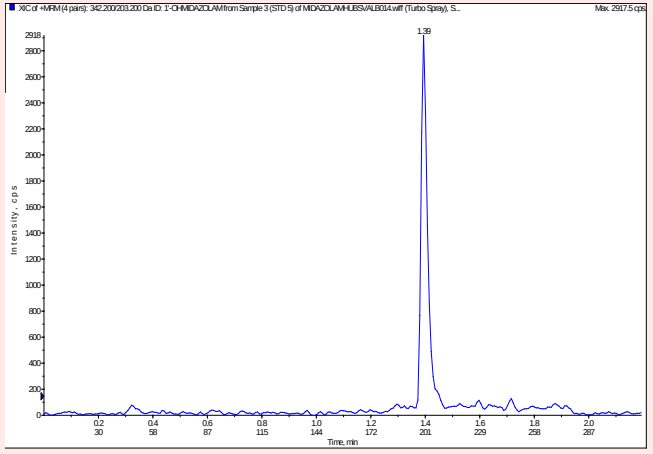
Method Development – LC-MS/MS (API4000; MRM Mode)

LLQ (5ng/mL)	Midazolam (m/z 328/291)	1-Hydroxymidazolam (m/z 342/203)
Liquid Whole Blood		
VAMS		

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Method Validation – 'Typical' Criteria (Quant Bio Method in Reg Environ)

	Liquid Whole Blood		VAMS	
	Midazolam	1-OHMidazolam	Midazolam	1-OHMidazolam
Calibration Model	Linear - Weighted 1/(x*x)			
Validated Range	5 to 5000ng/mL		5 to 5000ng/mL	
Accuracy (% Bias)	-9.3%≤ Bias ≤7.3%	-6.1%≤ Bias ≤4.6%	-11.4%≤ Bias ≤7.5%	-7.6%≤ Bias ≤9.6%
Precision (%CV) Within-run	≤10.3%	≤12.6%	≤10.4%	≤12.8%
Precision (%CV) Between-run	≤1.8%	≤1.8%	≤1.4%	≤5.0%
Stock Stab. in DMF	At least 152 days at 4°C / At least 6 hours at ambient temperature			
Stability in Human Whole Blood (EDTA) or VAMS	At least 131 days at -20°C		At least 131 days at ambient temperature	
Processed Sample Stability	At least 120 hours at 4ºC		At least 120 hours at 4ºC	
Freeze/Thaw Stability	At least 3 cycles from -20°C to ambient		N/A	
Matrix Dilution	10 Fold in human whole blood		10-Fold in methanol extract of VAMS Tips supporting Human Whole Blood	

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Method Validation – 'Additional' Criteria

	Liquid Whole Blood		VAMS	
	Midazolam	1-OHMidazolam	Midazolam	1-OHMidazolam
LT Stability in Human Whole Blood (EDTA) [Extreme conditions prior to long term storage] [Prior to application to VAMS]	At least 131 days at -20°C [At least 120 hours at ambient temperature At least 120 hours at 4°C At least 120 hours at 40°C]		[At least 192 hours at ambient temperature At least 192 hours at 4°C At least 192 hours at 40°C]	
Stab of dried Human Whole Blood (EDTA) on VAMS Tips at extreme conds.	N/A		At least 131 days at ambient temperature At least 43 days at 40°C At least 43 days at -20°C	
Compatibility + Stab of samples LiHep as anti-coagulant	At least 4 hours at 37°C		At least 4 hours at 37°C	
Tolerance of Vaseline content in Human Whole Blood (EDTA)	Assay tolerated up to at least 20mg/mL of Vaseline		Assay tolerated up to at least 20mg/mL of Vaseline	
Tolerance Levels of Co-medications in Human Whole Blood (EDTA)	Assay tolerated up to at least 2000, 1500, 120 and 200 ng/mL (pharmacologically relevant (Cmax) levels) Ciprofloxacin, Furosemide, Nifedipine and Omeprazole, respectively			

Hematocrit Based Assay Bias

- HCT at extreme levels can have a significant effect on DBS assays

- What is the situation with VAMS?

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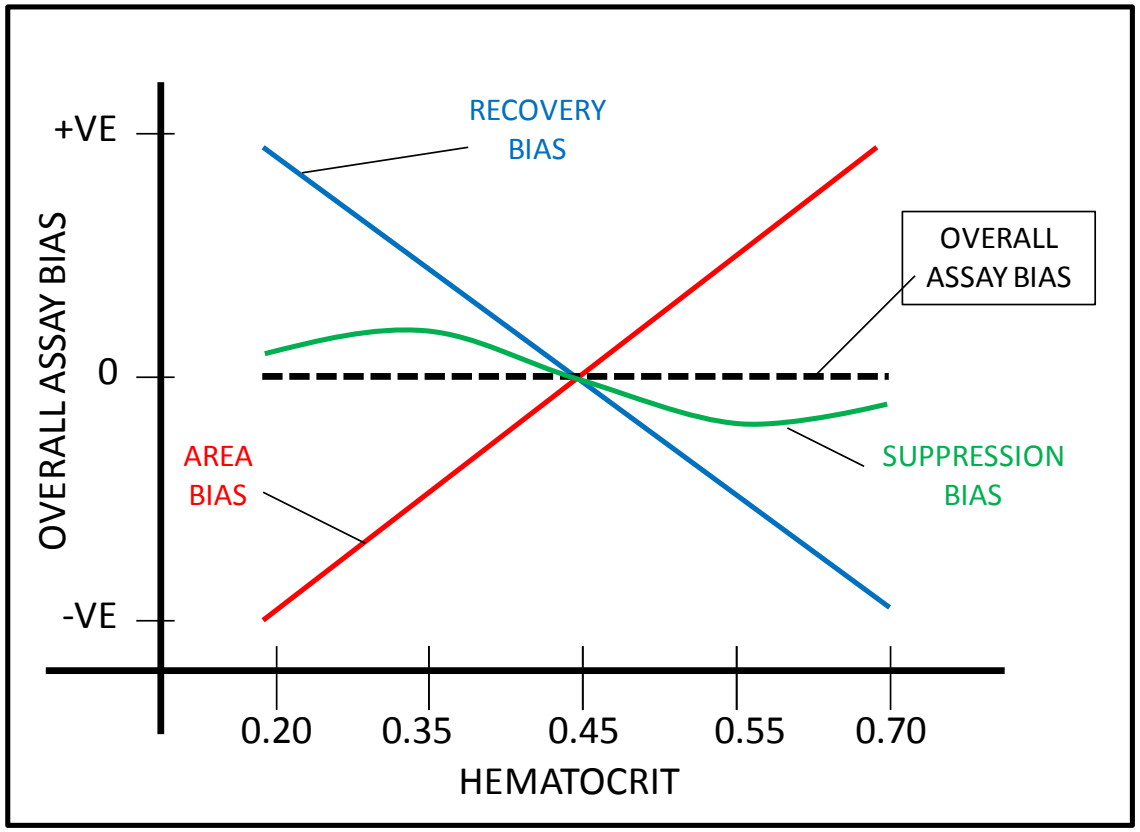
Components of HCT based Overall Assay Bias **(DBS Sub-Punch Assay)**



- **DBS (sub punch)** Area bias effects with HCT well known
- ...HCT based **recovery bias** can also have a very significant effect on the overall assay bias
- Area bias consistent for all assays
- Magnitude of recovery bias dependent on overall assay recovery
- Recovery bias and area bias tend to cancel each other out to produce the combined **overall bias**
- What happens to this 'balance' when area bias is eliminated by using whole spot extraction (or VAMS)?
- *All bias values are normalised to those obtained for a typical HCT value of 0.45.*

Investigation of different approaches to incorporating internal standard in DBS quantitative bioanalytical workflows and their effect on nullifying hematocrit-based assay bias . Abu-Rabie, P. et al. 2015 . Analytical Chemistry 87 (9), pp. 4996-5003

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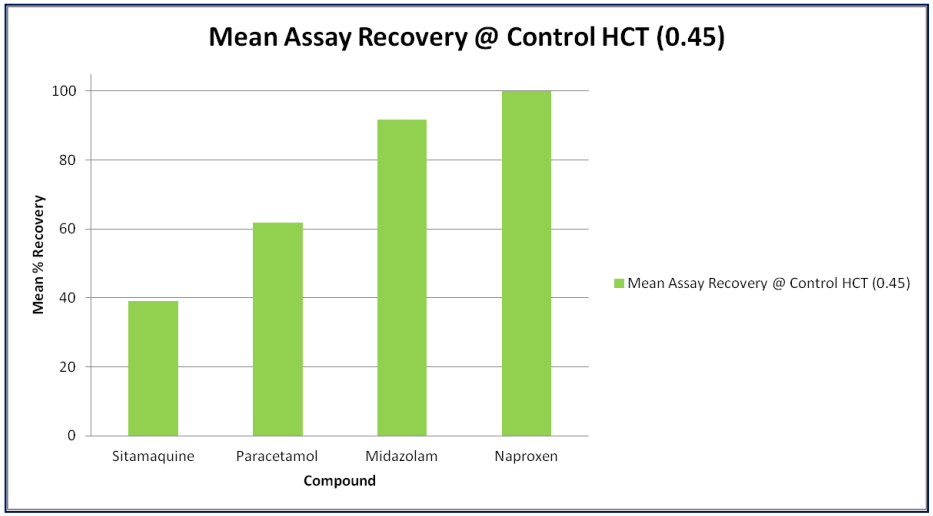
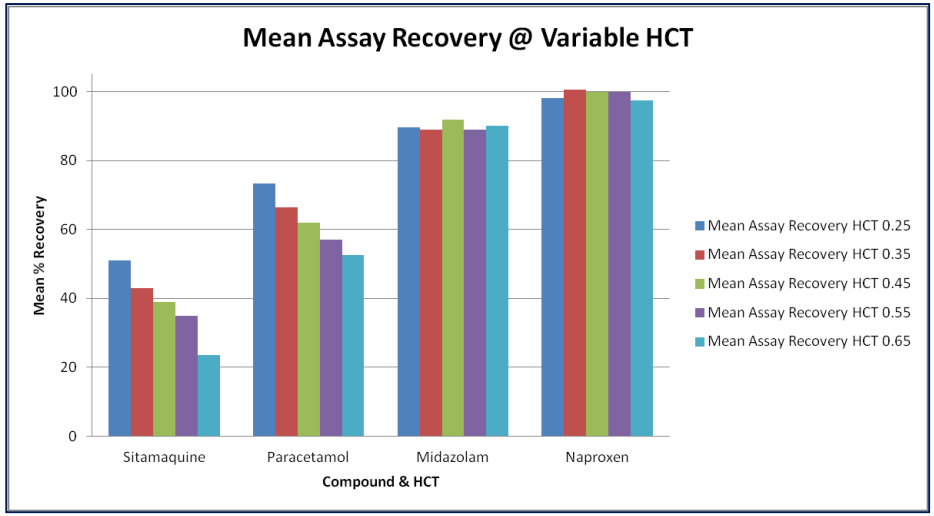
HCT based recovery Bias in Manual DBS Extraction

- **What causes HCT based recovery bias?**
- Manual DBS Extraction
  - IS added with extraction solvent
  - IS **not integrated** with DBS prior to extraction
  - Analyte and IS **not co extracted**.
  - **Any change in recovery with varying HCT affects the analyte only; not the IS**
  - So when we use **PEAK AREA RATIO (PAR)** to quantify drug concentrations...a bias occurs





Abu-Rabie, P. et al. 2015 . Analytical Chemistry 87 (9), pp. 4996-5003

- **The lower the overall assay recovery, the greater the effect changing HCT has on the recovery**

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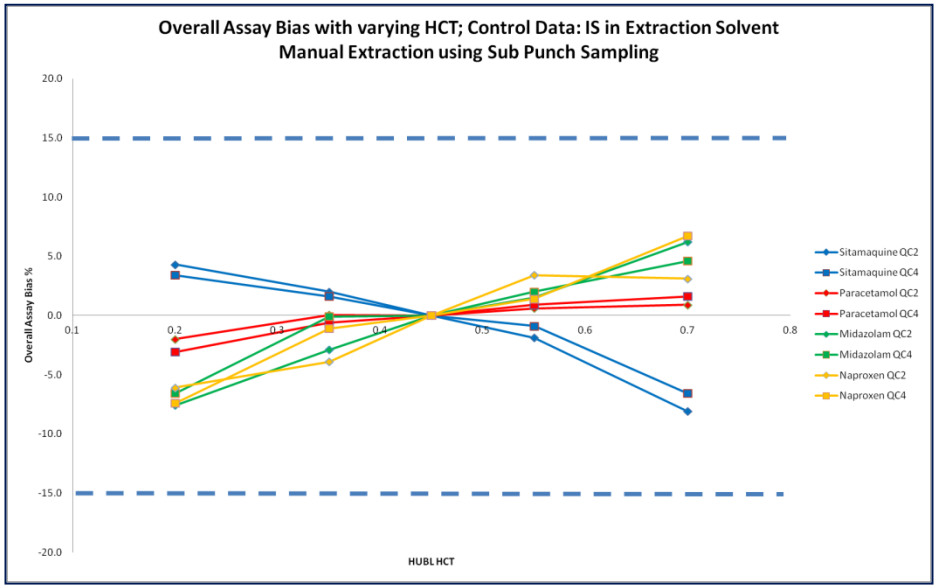
HCT Based Recovery Bias Nullification – **Control data (Sub Punch) IS Added Via Extraction Solvent**

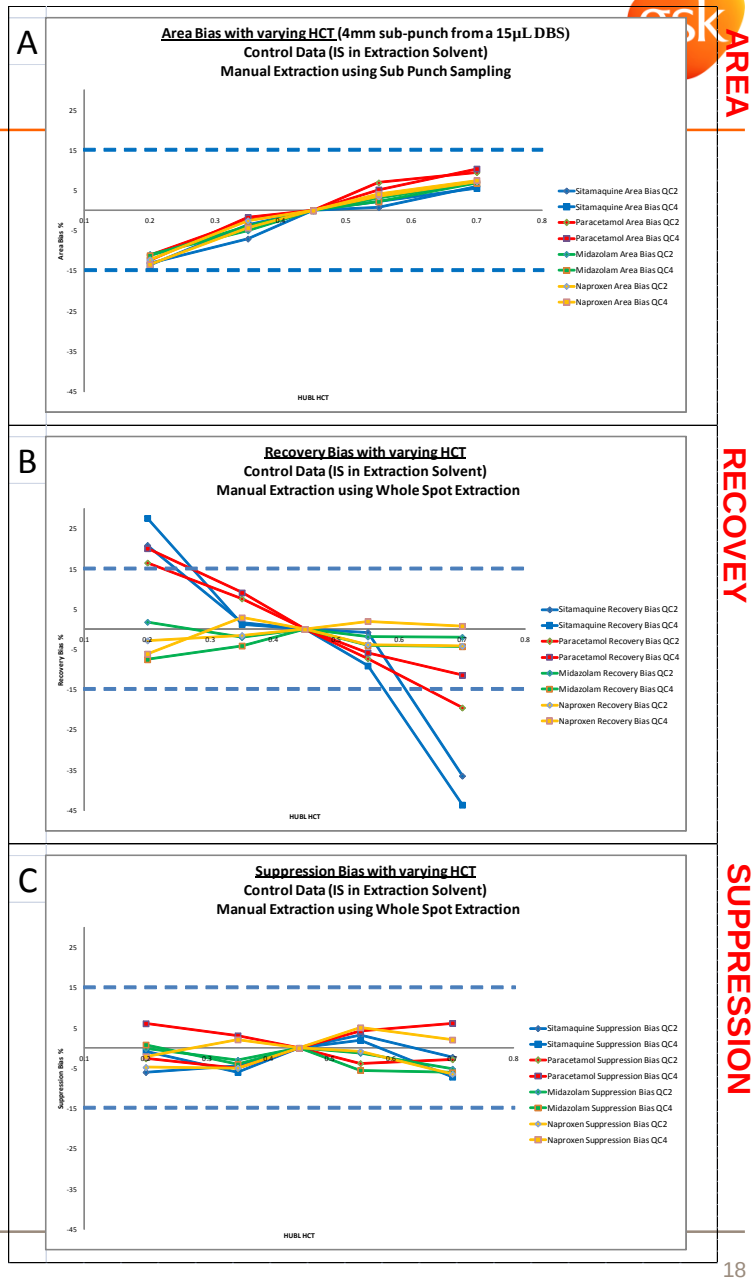
– Figure 1: Overall assay bias for **sub-punch** manual extraction. Control data where IS is added conventionally, via the extraction solvent.

– LC-MS/MS quantitative data derived from validation type runs.

OVERALL ASSAY BIAS



– Figure 2: Area bias, recovery bias, and suppression bias (A, B and C, respectively) contributions to overall assay bias for DBS conventional manual extraction where internal standard is added via the extraction solvent.



Abu-Rabie, P. et al. 2015 . Analytical Chemistry 87 (9), pp. 4996-5003

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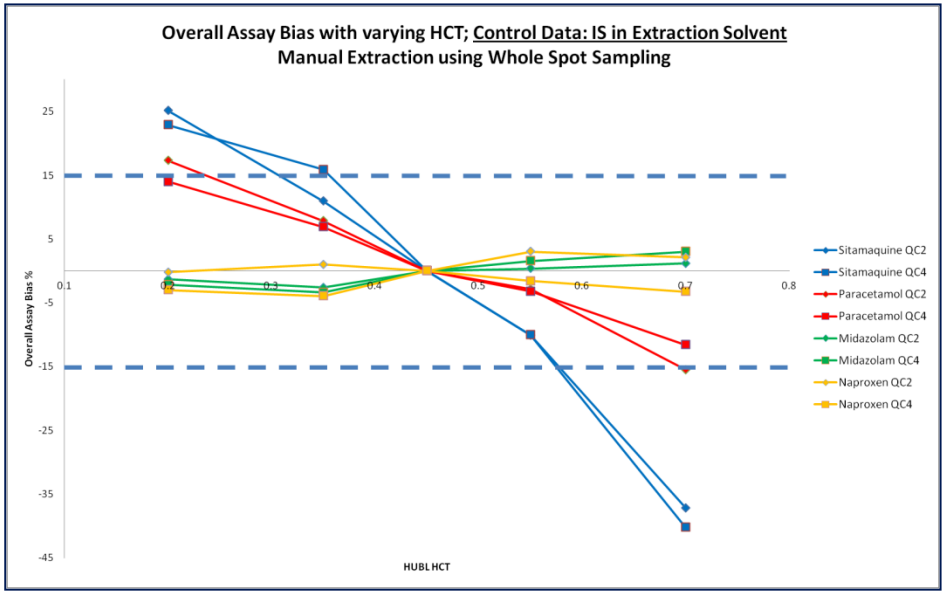
HCT Based Recovery Bias Nullification – **Control data (Whole Spot) IS Added Via Extraction Solvent**

– Figure 1: Overall assay bias for **whole-spot** manual extraction. Control data where IS is added conventionally, via the extraction solvent.

OVERALL ASSAY BIAS



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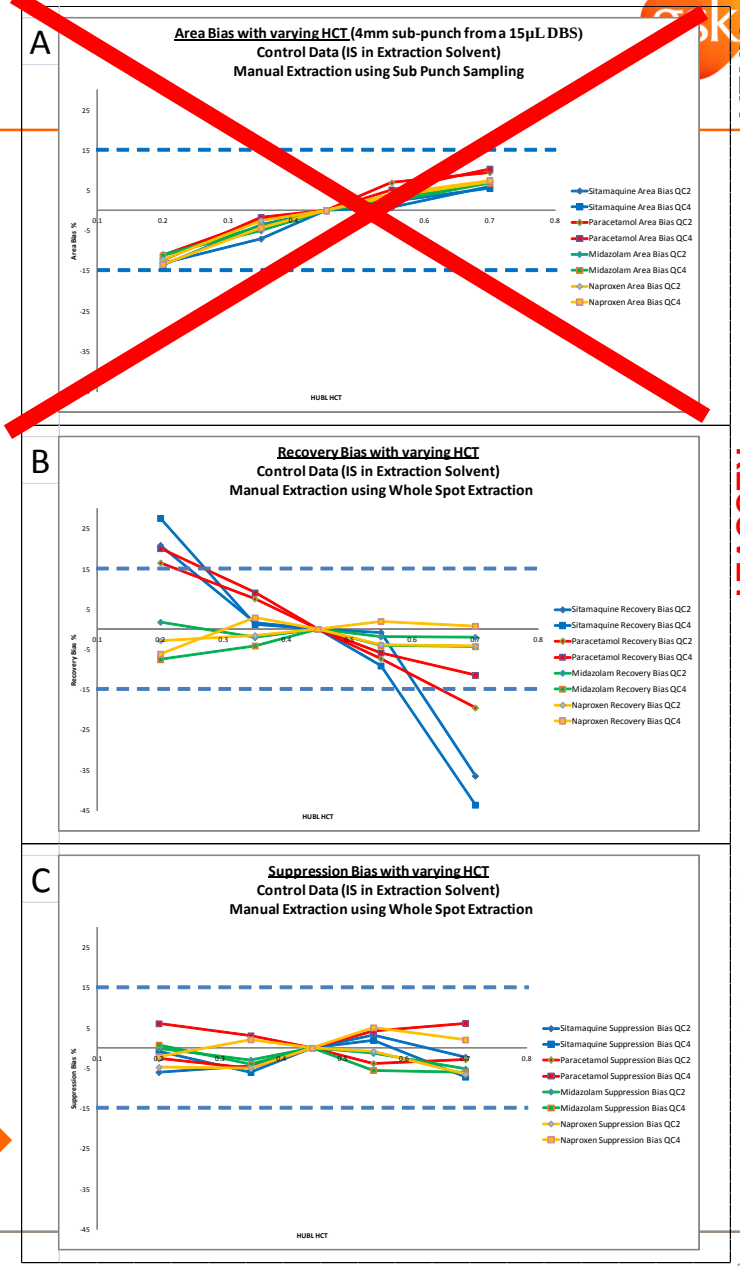

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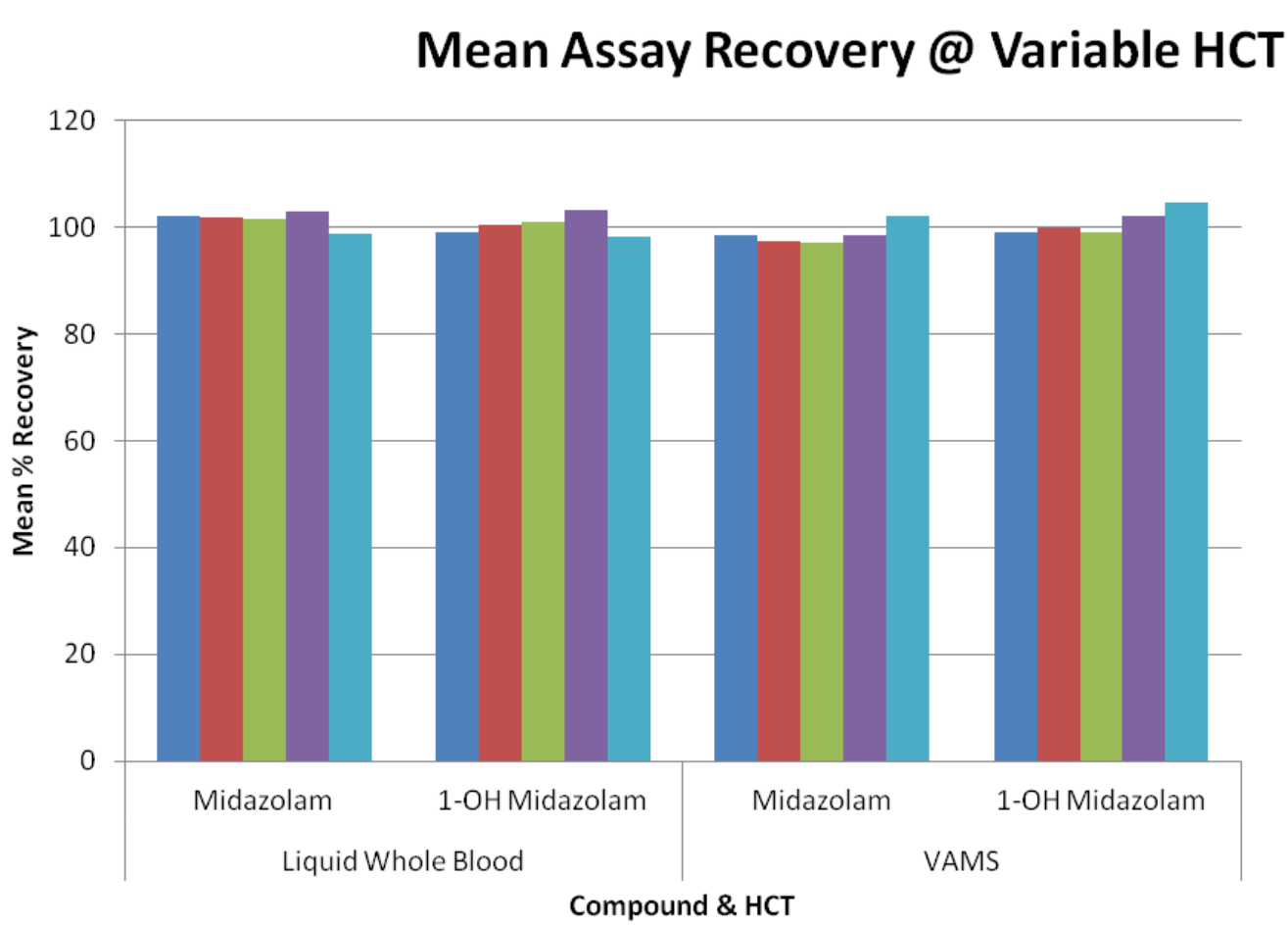
Whole Blood/VAMS Method Validation – Recovery and Hematocrit



TAKE HOME MESSAGE:

1) Understand assay recovery
2) Maximise recovery if at all possible!

Recovery = ES/PES

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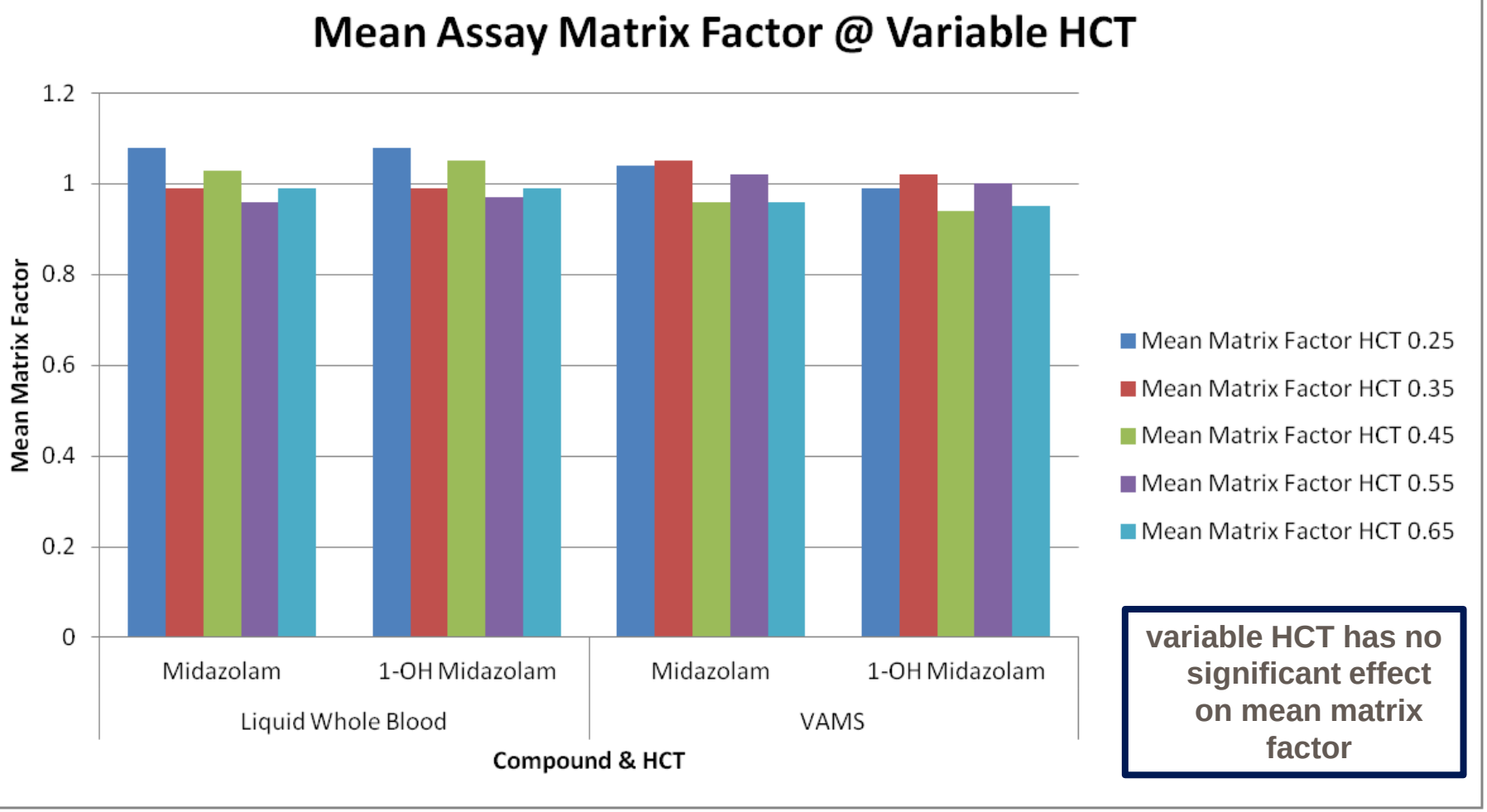
VAMS Method Validation – Matrix Effects and Hematocrit



variable HCT has no significant effect on mean matrix factor

Matrix Factor = PES/MFS

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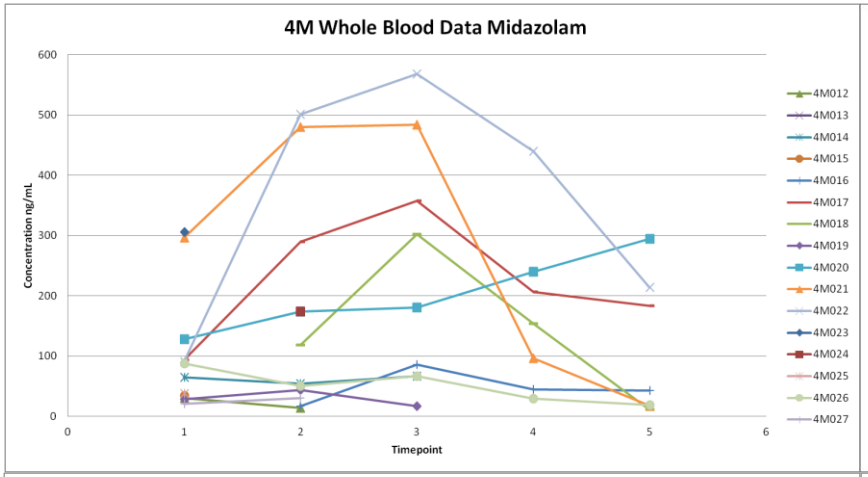
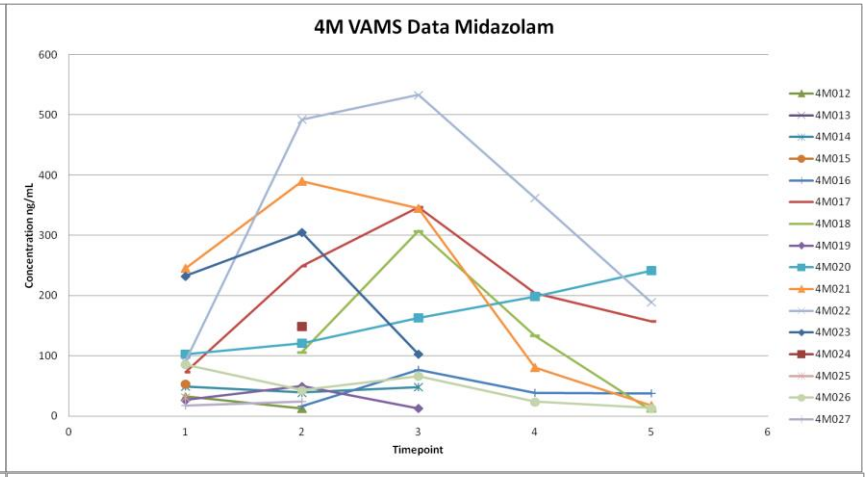
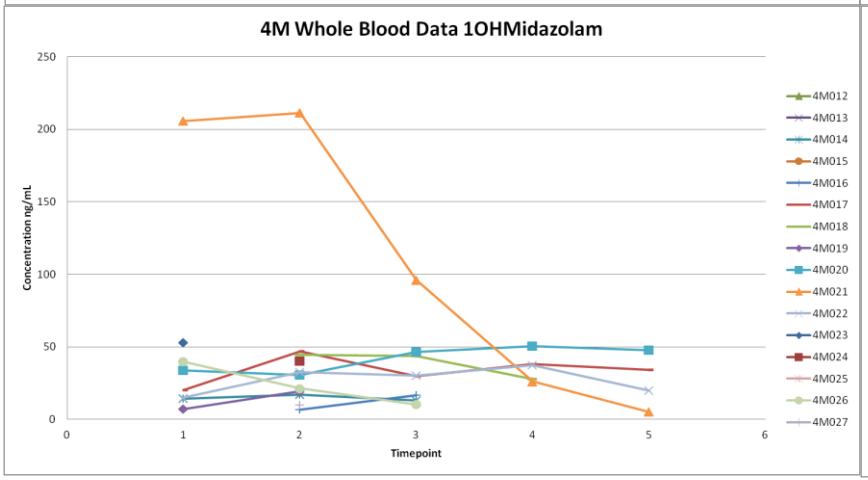
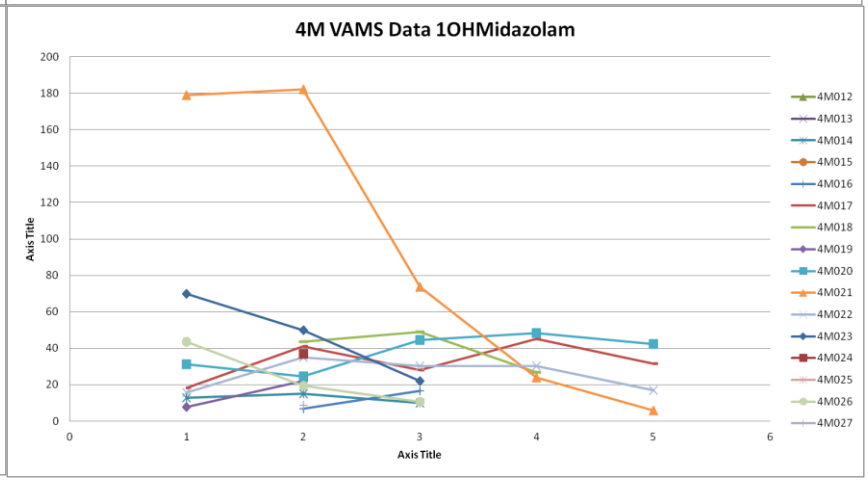
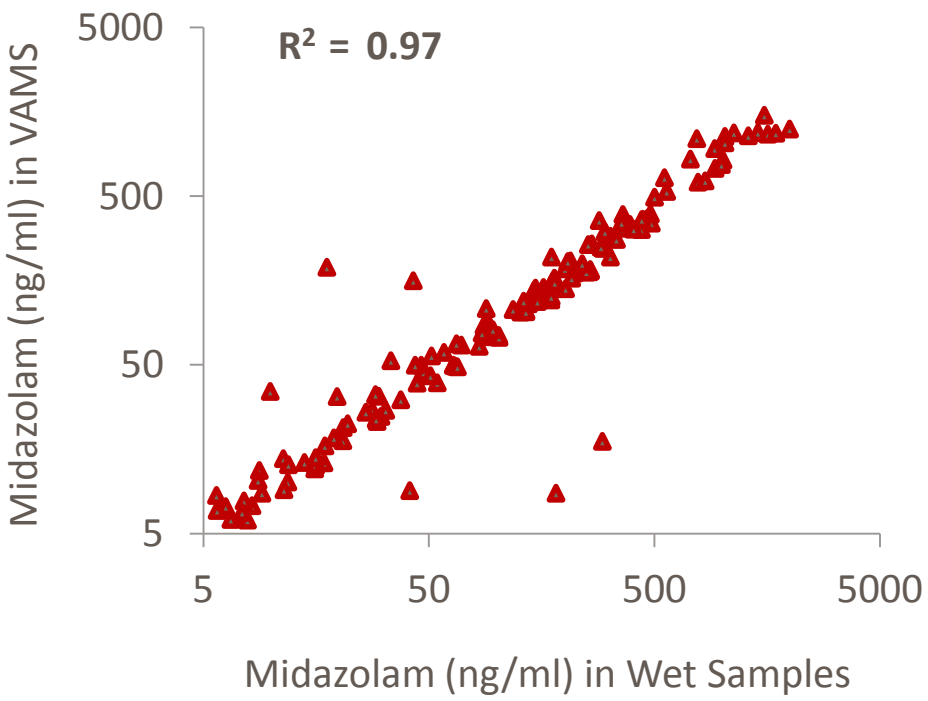
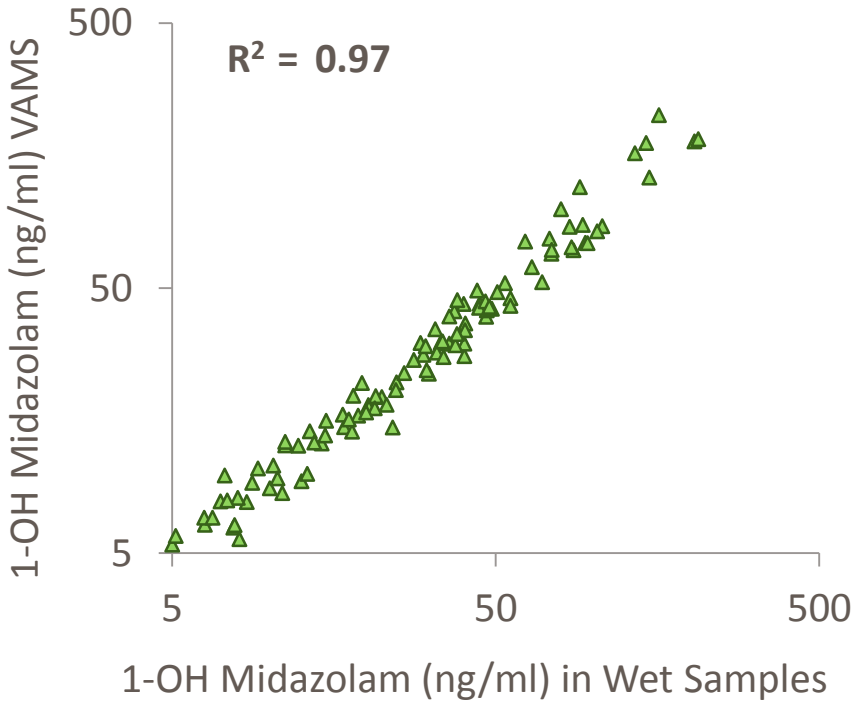
Data Correlation

	– Whole Blood / Wet	– VAMS/ Dry
MIDAZOLAM		
1-OHMIDAZOLAM		

Midazolam Measurements: Correlation between Wet and VAMS

N = 56 patients, *N* = 199 time points





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Clinical – Preliminary Findings/Conclusions

- Collecting blood samples onto VAMS tips is simple
- Training to collect blood onto VAMS tips can be achieved in a relatively short period of time
- Management and storage of VAMS tips is easy (unlike wet samples)
- VAMS tips are superior to dry blood spots in relation to spoilt samples
- Strong correlation in blood midazolam and metabolite concentrations between VAMS and Wet Samples
  - suggest that VAMS tips provide equivalent concentration data to wet samples

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Analytical – Preliminary Findings/Conclusions

- No significant difference in **difficulty/effort** in developing/validating/supporting studies using wet (whole blood) or dry (VAMS) samples
  - Accuracy and Precision
  - Stability
  - Automation
  - More validation criteria
  - Particularly important to assess assay recovery
    - Higher recovery the better
    - Improved methods of IS addition (integration/co-extraction)

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Acknowledgements

- Dr Philip Denniff (GSK)
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- Dr Bikalpa Neupane (University of Leicester)
- Dr James Rudge (Neoteryx)

4M's: A Multidisciplinary Collaboration

Leicester
Dr Hitesh Pandya
Dr Bikalpa Neupane
Dr Hussain Mulla
Dr Eric DeMelo
Dr Sanjiv Nichani
Teresa McNally

Spooner Bio Solutions
Dr Neil Spooner

GSK
Dr Paul Abu Rabie
Dr Stephen White
Dr Oscar Della Pasqua

Neoteryx
Dr Emmet Welch
Dr James Rudge

neoteryx

University of Leicester

University Hospitals of Leicester NHS
NHS Trust

gsk GlaxoSmithKline

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Analytical Considerations

SUPPLEMENTARY INFORMATION

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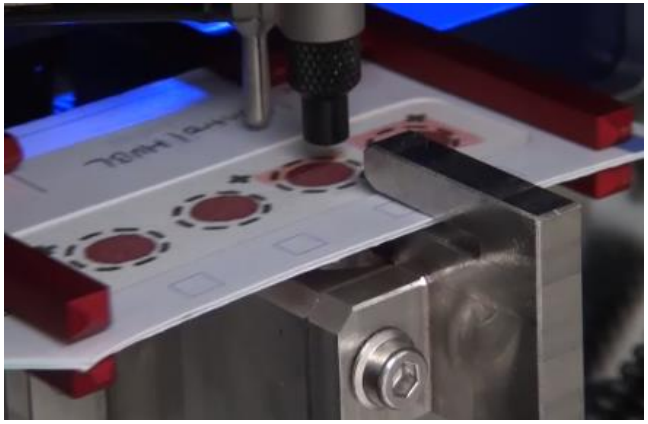
Method Development – UPLC Methodology

Mass Spectrometer	Applied Biosystems API4000
Split Ratio	none
Ionisation Interface and Temperature	TurbolonSpray™ at 600°C
Pause Time	5 msec
Nebuliser Gas	12
Turbo Gas Flow	6 L/min
Gas 1 Setting (Nitrogen)	60 psi
Gas 2 Setting (Nitrogen)	40 psi
Curtain Gas Setting (Nitrogen)	30
Collision Gas Setting (Nitrogen)	8
DP Value	80
CE Value	35 (Midazolam); 48 (OHMidazolam)

Dried Blood Spot Direct Analysis

HCT based recovery Bias – What's the solution?

- **What's the solution?**
- Co-extraction of analyte and IS
  - I.S. must be integrated with sample prior to extraction
  - HCT based recovery bias would still occur
  - ...but IS response would also vary with HCT
  - IS corrects for recovery bias
  - HCT recovery **bias effect** is **nullified (PAR)**
  - **Could this be achieved using the IS spray module?**



Control Data: IS in Extraction Solvent	IS Spray Application to DBS prior to extraction
Control Data: IS Spiked into Whole Blood	IS Spray Application to Substrate prior to spotting

Dried Blood Spot Direct Analysis

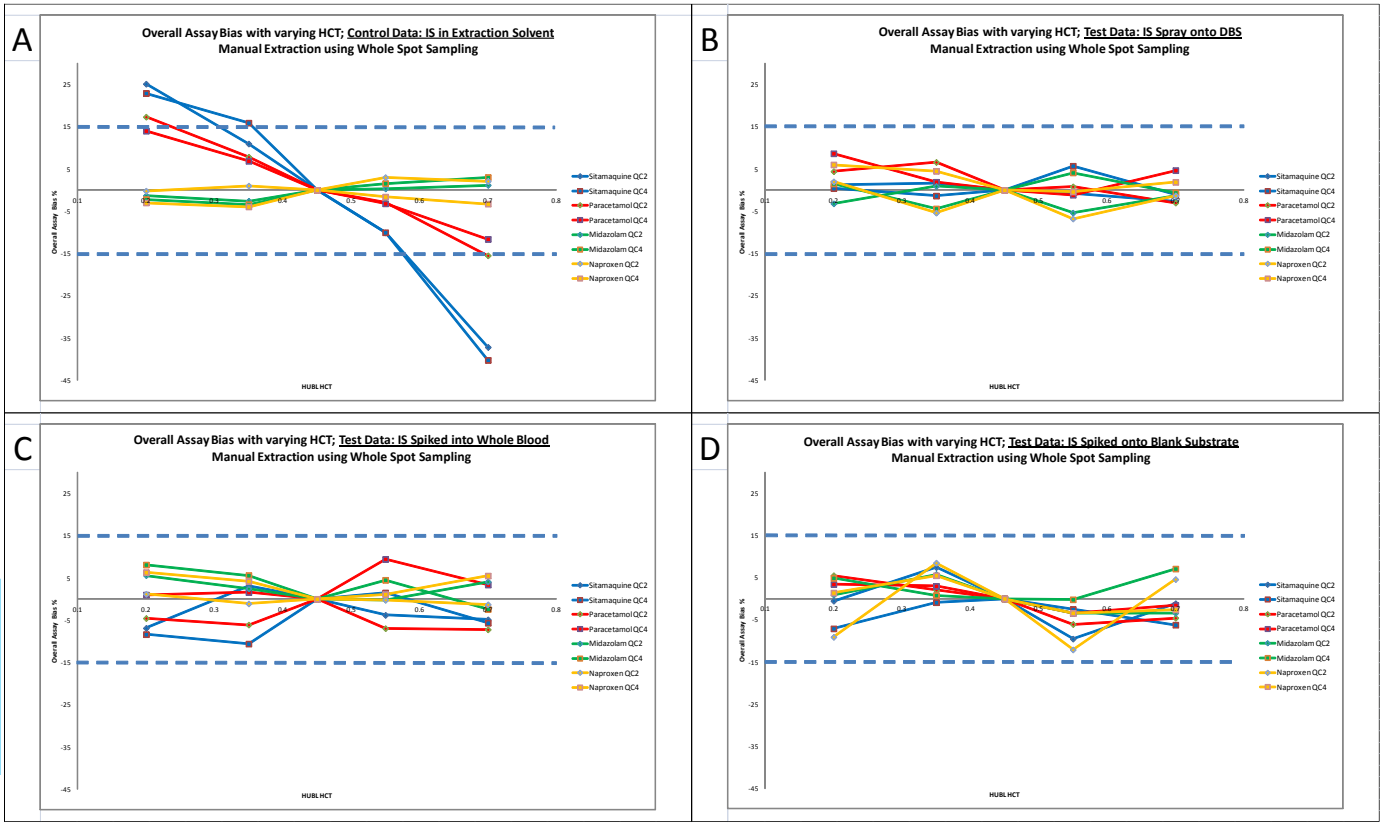
HCT Based Recovery Bias Nullification – **Test data (Whole Spot)**
IS Added Via Extraction Solvent & Alternatives

Control Data: IS in Extraction Solvent

IS Spray Application to DBS prior to extraction

Control Data: IS Spiked into Whole Blood

IS Spray Application to Substrate prior to spotting



– *Figure 2: Overall assay bias for whole-spot manual extraction using four different methods of internal standard (IS) addition. 2A: Control data where IS is added conventionally, via the extraction solvent. 2B: Test data where IS is added by spraying IS onto the DBS prior to extraction. 2C: Test data where IS is spiked into whole blood before spotting. 2D: Test data where IS is sprayed onto blank substrate prior to applying the DBS. The blue dashed lines represent ±15% bias (the limit of total error allowable according to internationally accepted guideline acceptance criteria).*

Investigation of different approaches to incorporating internal standard in DBS quantitative bioanalytical workflows and their effect on nullifying hematocrit-based assay bias .

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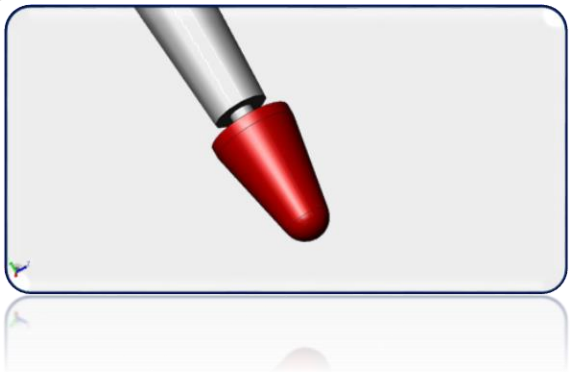
Dried Blood Spot Direct Analysis

Summary 2

- **DBS Regulatory Authority issues**
  - Asking for wet vs. dry data
    - Significant cost implication
  - Currently niche application only

U.S. Food and Drug Administration
Protecting and Promoting *Your* Health

- **Benefit for GSK**
  - Directly apply learning's to other microsampling techniques (VAMS/Mitra)
  - General capability development:
    - Learned about automation; direct analysis techniques; forming collaborations
    - DBS digital microfluidics platform is being applied to biopharm applications

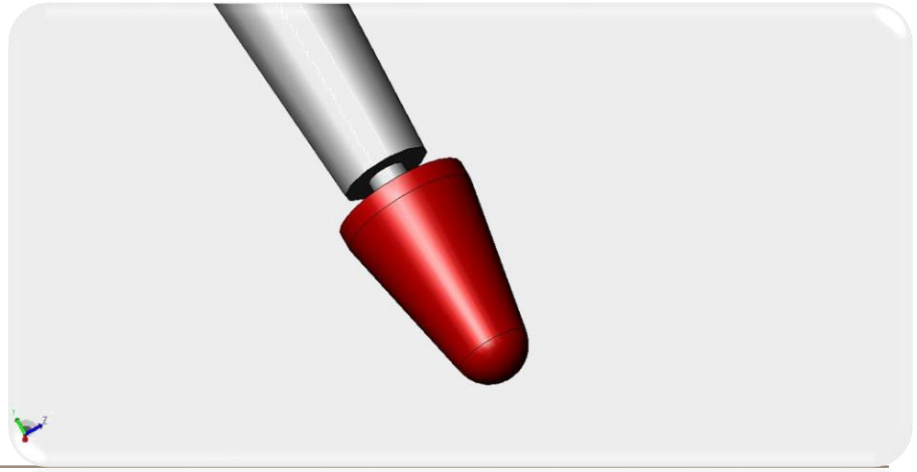


Development of the VAMS™ Sampling Tip

Mitra blood sampler

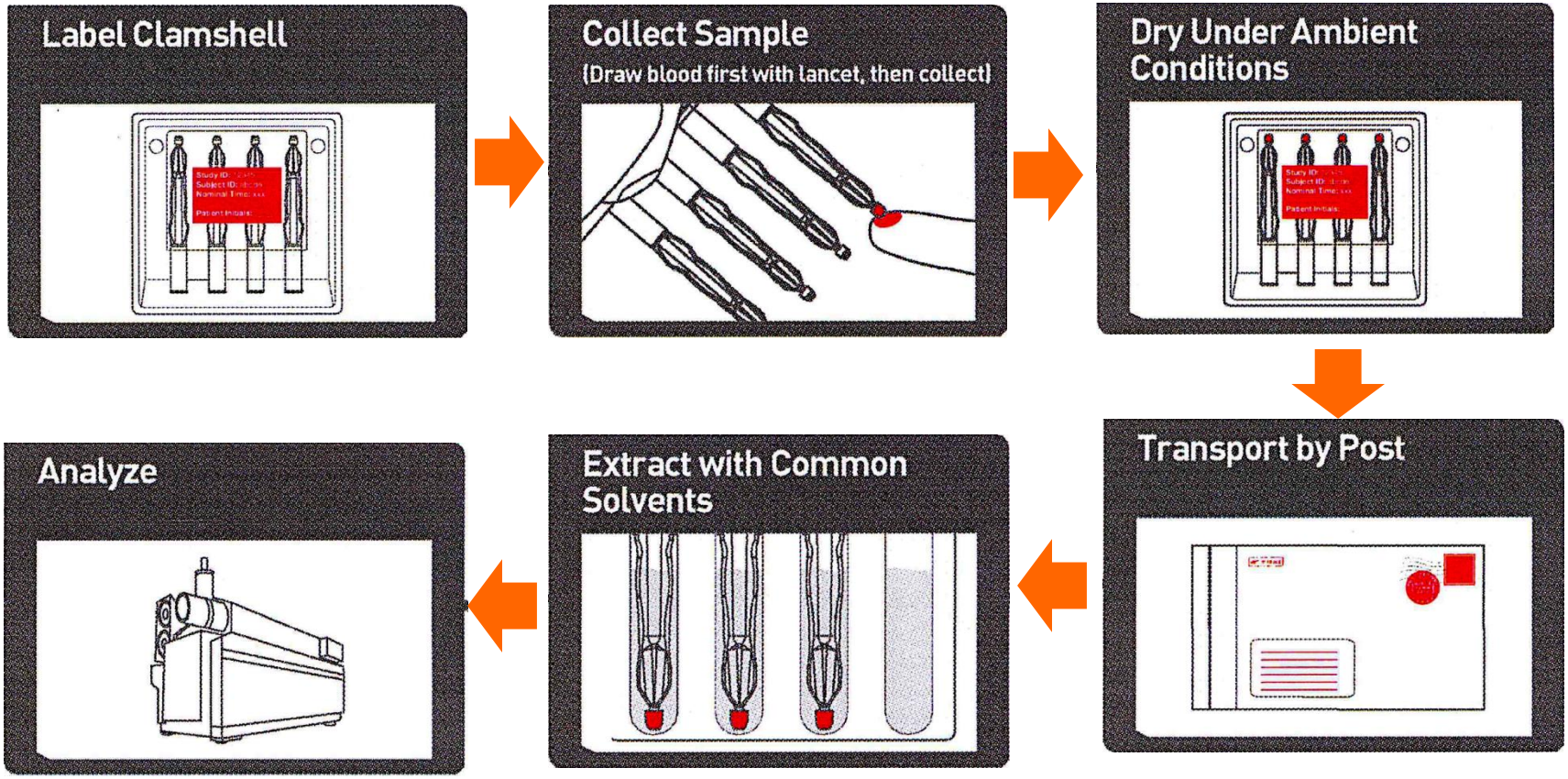
- Hydrophilic porous material
- Each Tip has a fixed, highly reproducible internal porous volume
  - Accurate, precise wicking volume
- Rapid wicking (under 6 seconds)

From humble beginnings....



Volumetric Absorptive Microsampling

Mitra blood sampler



Dried Blood Spot Direct Analysis

HCT based recovery Bias in Manual DBS Extraction

Overall Assay Recovery

HCT	Recovery %							
	Sitamaquine		Paracetamol		Midazolam		Naproxen	
	QC2	QC4	QC2	QC4	QC2	QC4	QC2	QC4
0.2	45.9	52.5	71.0	74.6	94.5	84.0	97.0	94.9
0.35	38.6	41.6	65.5	67.8	90.9	87.1	98.3	104.0
0.45	38.0	41.1	60.9	62.1	92.8	90.8	99.8	101.1
0.55	37.7	37.4	56.5	58.5	91.1	87.1	96.0	103.1
0.7	24.1	23.2	49.0	55.0	91.0	86.9	95.7	101.9

- **What causes HCT based recovery bias?**
- Manual DBS Extraction
  - IS added with extraction solvent
  - IS **not integrated** with DBS prior to extraction
  - Analyte and IS **not co extracted**.
  - **Any change in recovery with varying HCT affects the analyte only; not the IS**
  - So when we use **PEAK AREA RATIO (PAR)** to quantify drug concentrations...a bias occurs

Abu-Rabie, P. et al. 2015 . Analytical Chemistry 87 (9), pp. 4996-5003

Supporting a paediatric study using wet and dry samples

Method Validation – Recovery and Hematocrit

	Liquid Whole Blood		VAMS	
QC Lev 2 / 3 / 4	Midazolam	1-OHMidazolam	Midazolam	1-OHMidazolam
Mean % Recovery @ HCT 0.25	103.8 / 100.6	97.1 / 101.9	99.4 / 97.6	100.6 / 97.8
Mean % Recovery @ HCT 0.35	104.1 / 100.6	101.1 / 100.6	96.0 / 99.7	100.4 / 99.4
Mean % Recovery @ HCT 0.45	103.0 / 100.6 / 102.2	102.9 / 97.6 / 100.9	98.6 / 94.7 / 97.4	101.3 / 97.6 / 97.7
Mean % Recovery @ HCT 0.55	105.6 / 99.6	103.2 / 103.2	97.8 / 101.0	104.5 / 100.6
Mean % Recovery @ HCT 0.65	101.9 / 96.6	100.0 / 97.2	103.3 / 101.2	109.4 / 99.4

Supporting a paediatric study using wet and dry samples

Method Validation – Matrix Effects and Hematocrit

	Liquid Whole Blood		VAMS	
QC Lev 2 / 3 / 4	Midazolam	1-OHMidazolam	Midazolam	1-OHMidazolam
Mean Matrix Factor @ HCT 0.25	1.24 / 1.00	1.31 / 1.02	1.07 / 1.01	0.98 / 1.01
Mean Matrix Factor @ HCT 0.35	0.98 / 0.99	0.99 / 0.99	1.01 / 1.08	1.01 / 1.05
Mean Matrix Factor @ HCT 0.45	1.10 / 1.01 / 0.99	1.02 / 1.01 / 0.99	0.95 / 0.95 / 0.92	0.93 / 0.93 / 0.95
Mean Matrix Factor @ HCT 0.55	0.92 / 0.97	0.96 / 0.97	1.03 / 0.99	1.02 / 0.97
Mean Matrix Factor @ HCT 0.65	0.98 / 1.00	1.03 / 1.00	0.93 / 0.99	0.91 / 1.00

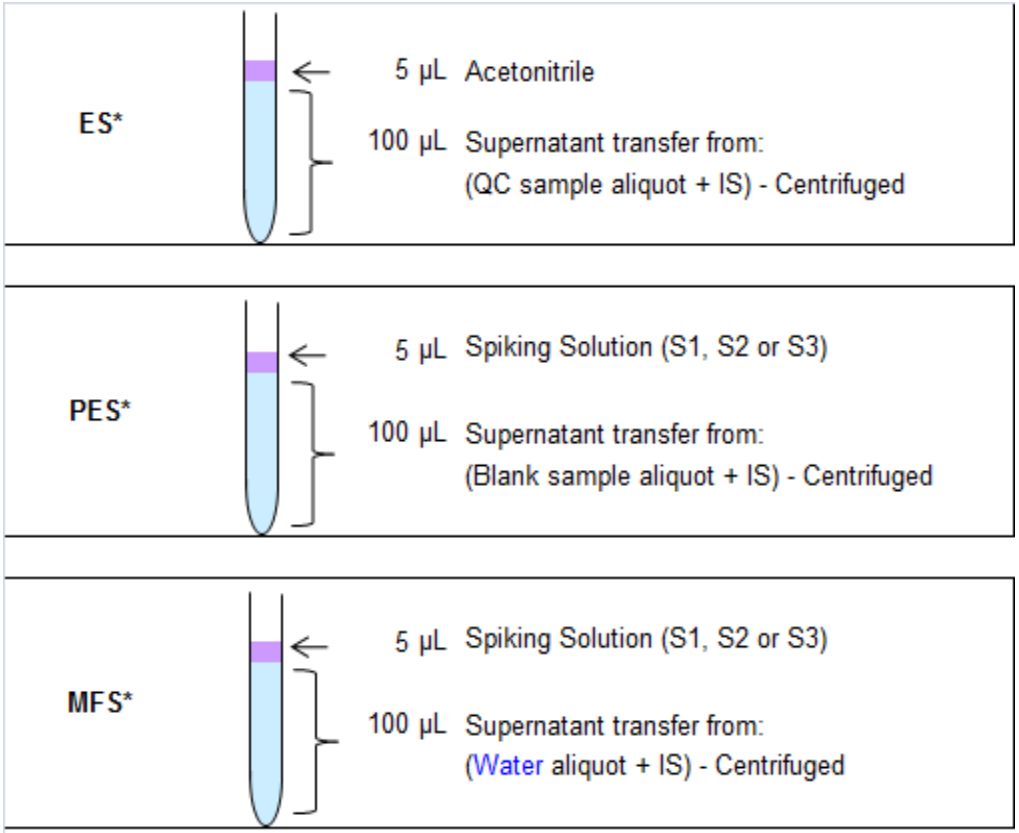
Supporting a paediatric study using wet and dry samples

Recovery and Matrix Effects Calculations

- The recovery of midazolam and 1-hydroxymidazolam from human whole blood samples spiked at 15, 200 and 4000 ng/mL was assessed by comparing the analyte/internal standard peak area ratio of the extracted samples (ES) to those of blank extracts of dried human whole blood supported on VAMS tips spiked to the same concentration after extraction (PES). The recovery was greater than 94.7% for midazolam and 97.6% for 1-hydroxymidazolam at all concentrations. The precision was less than 15% at all concentrations and is therefore acceptable.
- A comparison of the recovery of midazolam and 1-hydroxymidazolam from human whole blood samples of varying HCT values (0.25, 0.35, 0.45, 0.55 and 0.65) spiked at 15 and 4000 ng/mL was made. The recovery was greater than 96.0% for midazolam and 97.7% for 1-hydroxymidazolam at all concentrations. The precision was less than 15% at all concentrations and is therefore acceptable and demonstrates that recovery is unaffected by HCT values ranging from 0.25 to 0.65, inclusive).
- **Recovery = ES/PES**
- The effects of matrix components on the HPLC-MS/MS response of midazolam and 1-hydroxymidazolam in six individual lots of human whole blood was assessed at 3 different concentrations (15, 200 and 4000 ng/mL) by comparing the analyte peak areas of blank extracts of human whole blood supported on VAMS tips spiked after extraction (PES), with the analyte peak areas of matrix free samples (MFS) at the same concentrations. The precision of the calculated matrix effect values between the different lots of matrix was less than 15% at all concentrations and is therefore acceptable.
- A comparison of the effects of matrix components on the HPLC-MS/MS responses of midazolam and 1-hydroxymidazolam from human whole blood samples of varying HCT values (0.25, 0.35, 0.45, 0.55 and 0.65) spiked at 15 and 4000 ng/mL was made. The precision of the calculated matrix effect values between the different lots of matrix and the varying degrees of HCT was less than 15% at all concentrations and is therefore acceptable and demonstrates that the effects of matrix components is unaffected by HCT values ranging from 0.25 to 0.65, inclusive.
- **Matrix Factor = PES/MFS**

Supporting a paediatric study using wet and dry samples

Recovery and Matrix Effects Calculations



** After extracting as per method sheet, transfer supernatant. Add additional solvent if method requires, then add solvent or spiking solution. Briefly vortex mix*

Blood Volumes: EMA Guidelines

- 'Per individual, the trial-related blood loss (including any losses in the manoeuvre) should not exceed 3 % of the total blood volume during a period of four weeks and should not exceed 1 % at any single time'

- Deviations from these recommendations must be justified

  - In neonates the total volume of blood is estimated at 80 to 90 ml/kg body weight
  - 3 % corresponds to 2.4 to 2.7 ml blood per kg body weight

*Doc. Ref. EMEA/536810/2008