Volumetric absorptive microsampling (VAMS) and LC–MS/MS analysis for simultaneous monitoring of 16 antiepileptic drugs: workflow development and validation.

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AIMS OF OUR STUDY

1. IS VAMS SAMPLING (MITRA®) SUITABLE FOR THERAPEUTIC DRUG MONITORING OF MANY DIFFERENT ANTIEPILEPTIC DRUGS ?

2. IS MITRA® SAMPLING SUITABLE FOR USE WITH COMMERCIAL KITS DEVOTED TO ROUTINE ANALYSIS IN CLINICAL LABS ?

Drugs included in the study

|	DRUG	ABBREVIATION	FORMULA	IUPAC NAME
1	CARBAMAZEPINE	CBZ	$C_{15}H_{12}N_2O$	benzo[b][1]benzazepine-11-carboxamide
2	CARBAMAZEPINE-EPOXIDE	CBZ-EPOX	$C_{15}H_{12}N_2O_2$	1a,10b-Dihydro-6H-dibenz(b,f)oxiren(d)azepine-6-carboxamide
3	OXCARBAZEPINE	OXC	$C_{15}H_{12}N_2O_2$	5-oxo-6H-benzo[b][1]benzazepine-11-carboxamide
4	10-HYDROXYCARBAZEPINE	10-OH-OXC	$C_{15}H_{14}N_2O_2$	5-hydroxy-5,6-dihydrobenzo[b][1]benzazepine-11-carboxamide
5	PHENYTOIN	PHT	$C_{15}H_{12}N_2O_2$	5,5-diphenylimidazolidine-2,4-dione
6	PHENOBARBITAL	PHB	$C_{12}H_{12}N_2O_3$	5-ethyl-5-phenyl-1,3-diazinane-2,4,6-trione
7	PRIMIDONE	PRM	$C_{12}H_{14}N_2O_2$	5-ethyl-5-phenyl-1,3-diazinane-4,6-dione
8	LACOSAMIDE	LCM	$C_{13}H_{18}N_2O_3$	2-acetamido-N-benzyl-3-methoxypropanamide
9	TOPIRAMATE	TPM	$C_{12}H_{21}NO_8S$	[(3aS,5aR,8aR,8bS)-2,2,7,7-tetramethyl-5,5a,8a,8b-tetrahydrodi[1,3]dioxolo[4,5-a:5',3'-d]pyran-3a-yl]methyl sulfamate
10	LEVETIRACETAM	LEV	$C_8H_{14}N_2O_2$	(2S)-2-(2-oxopyrrolidin-1-yl)butanamide
11	ZONISAMIDE	ZNS	$C_8H_8N_2O_3S$	1,2-benzoxazol-3-ylmethanesulfonamide
12	LAMOTRIGINE	LTG	$C_9H_7Cl_2N_5$	6-(2,3-dichlorophenyl)-1,2,4-triazine-3,5-diamine
13	FELBAMATE	FBM	$C_{11}H_{14}N_2O_4$	(3-carbamoyloxy-2-phenylpropyl) carbamate
14	ETHOSUXIMIDE	ETX	$C_7H_{11}NO_2$	3-ethyl-3-methylpyrrolidine-2,5-dione
15	RUFINAMIDE	RUF	$C_{10}H_8F_2N_4O$	1-[(2,6-difluorophenyl)methyl]triazole-4-carboxamide
16	PERAMPANEL	PMP	$C_{23}H_{15}N_3O$	2-(2-oxo-1-phenyl-5-pyridin-2-ylpyridin-3-yl)benzonitrile

METHOD DEVELOPMENT AND VALIDATION

To validate test we performed assays about selectivity, carry over, calibration curve, accuracy and precision, stability, dilution integrity and incurred samples reanalysis.

The method was considered to be selective: analyzing the blank samples there were no unexpected endogenous interferences >20% of LLOQ for all compounds, nor >5% of the IS signals.

Calibration curves linearity was evaluated as analyte/IS peak area ratio versus nominal analyte concentration using a 1/x weighting factor. Regression correlation coefficients (R^2) were ≥0.989 in all cases.

Examples of linear calibration curves
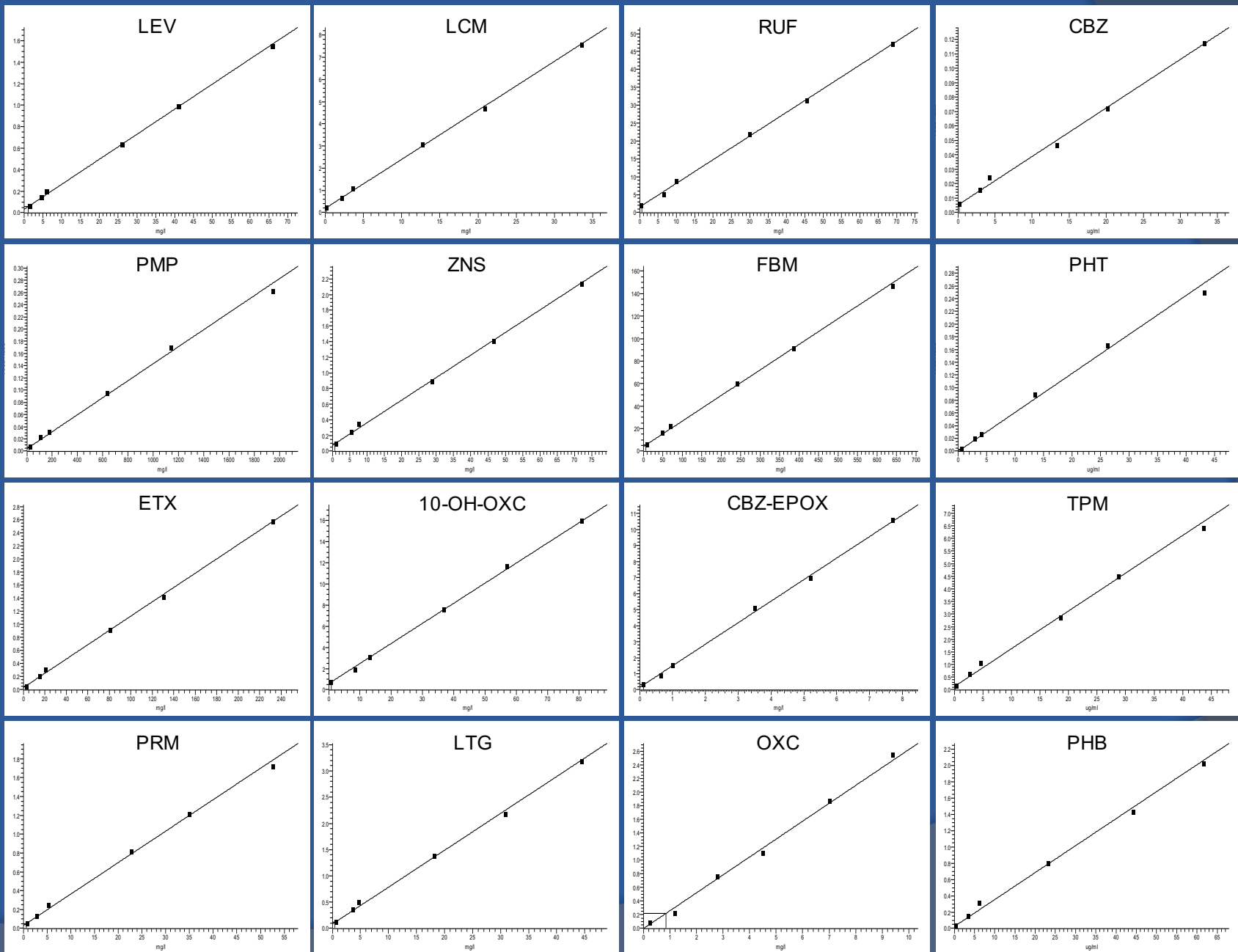
QC low, QC medium and QC high within- and between-run accuracy was assessed between 1% and 15% and between 1% and 13% respectively; precision within- and between-run ranged between 92% and 113% and between 89% and 110% respectively.
LLOQ within- and between-run accuracy was measured between 0.3% and 19% and 3% and 19% respectively; precision was evaluated between 84% and 117% and between 95% and 110% respectively.

No carry-over was observed in repeated blank samples injected after the highest calibrators
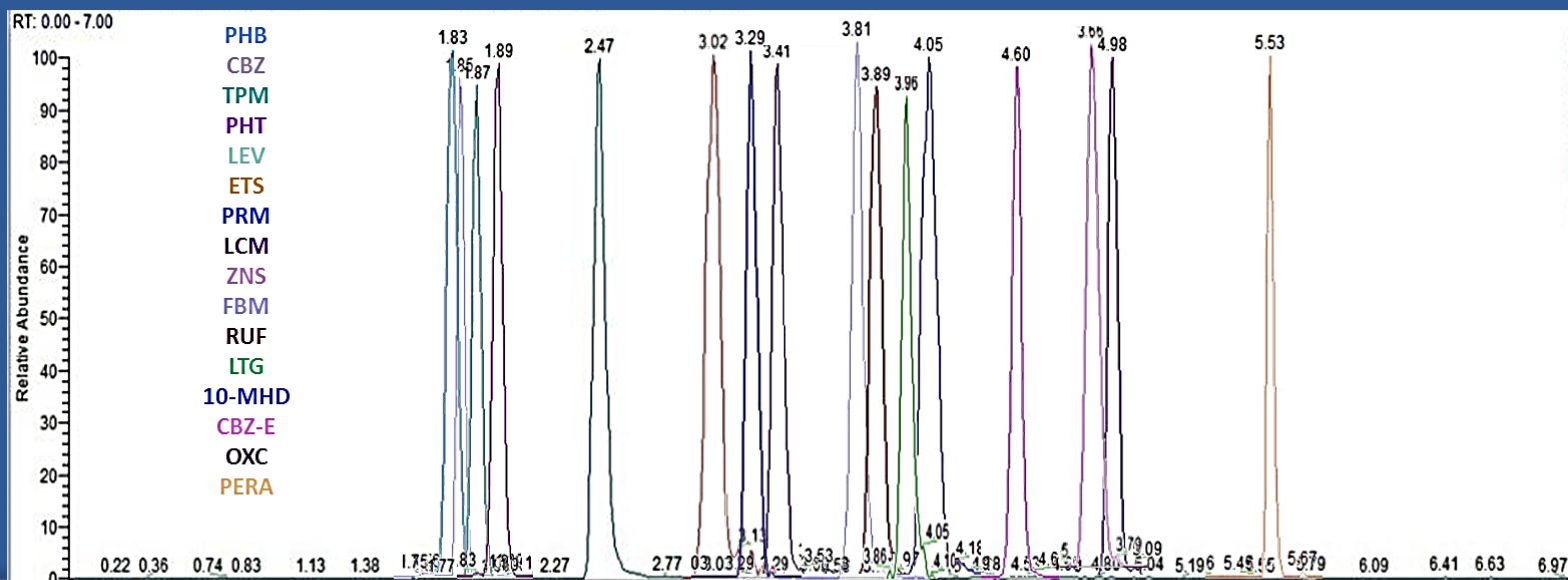
SAMPLE PREPARATION

- Let tips in whole blood from 2 to 4 seconds more than the «red-ding» time
- Allow tips to dry

- <u>Dip tips in water for 10 seconds</u>

- Add organic extraction solution
- Shake in a 96 wells plate for 1 hour at 600 rpm
- Take surnatants
- Centrifuge for 5 minutes at 15000 g
- Add acqueous mobile phase
- Inject 5/10 µl into LC-MS/MS
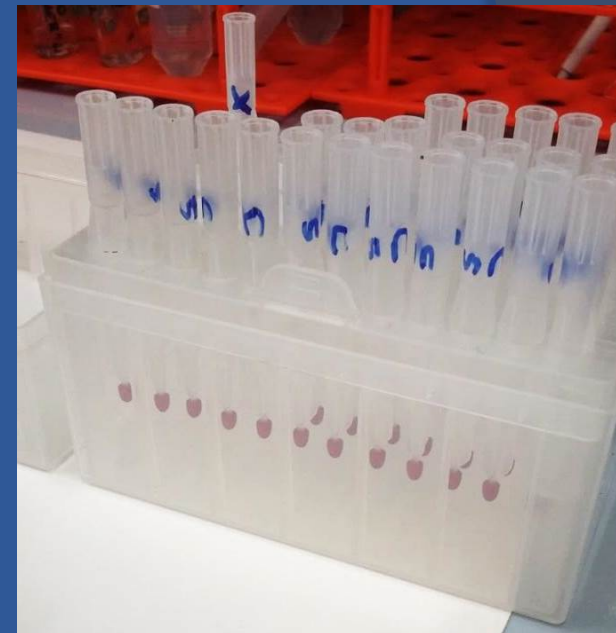
Evaluation of matrix effect (ME), extraction recovery (RE) and process efficiency (PE)

		LEV	PMP	LCM	RUF	ZNS	LTG	TPM	FBM	ETX	OXC	10-OH-OXC	CBZ	CBZ-E	PHT	PHB	PRM
QC LOW	ME%	98	102	94	91	93	99	92	93	99	100	97	104	90	86	91	95
	RE%	101	98	103	106	94	102	96	105	99	87	104	86	105	100	96	100
	PE%	99	99	97	96	88	102	88	97	98	87	101	89	94	86	87	94

		LEV	PMP	LCM	RUF	ZNS	LTG	TPM	FBM	ETX	OXC	10-OH-OXC	CBZ	CBZ-E	PHT	PHB	PRM
QC HIGH	ME%	102	103	100	97	104	89	104	99	111	93	105	104	96	98	108	94
	RE%	95	100	102	112	97	112	99	100	102	94	105	94	105	101	102	102
	PE%	97	103	102	109	102	99	103	100	113	87	110	98	101	99	111	96

matrix effect (ME) 91% - 111%
extraction recovery (RE) 86% - 112%
process efficiency (PE) 86% - 113%

B. K. Matuszewski et al. Anal. Chem. 2003

Stability test at different temperatures for 10 days
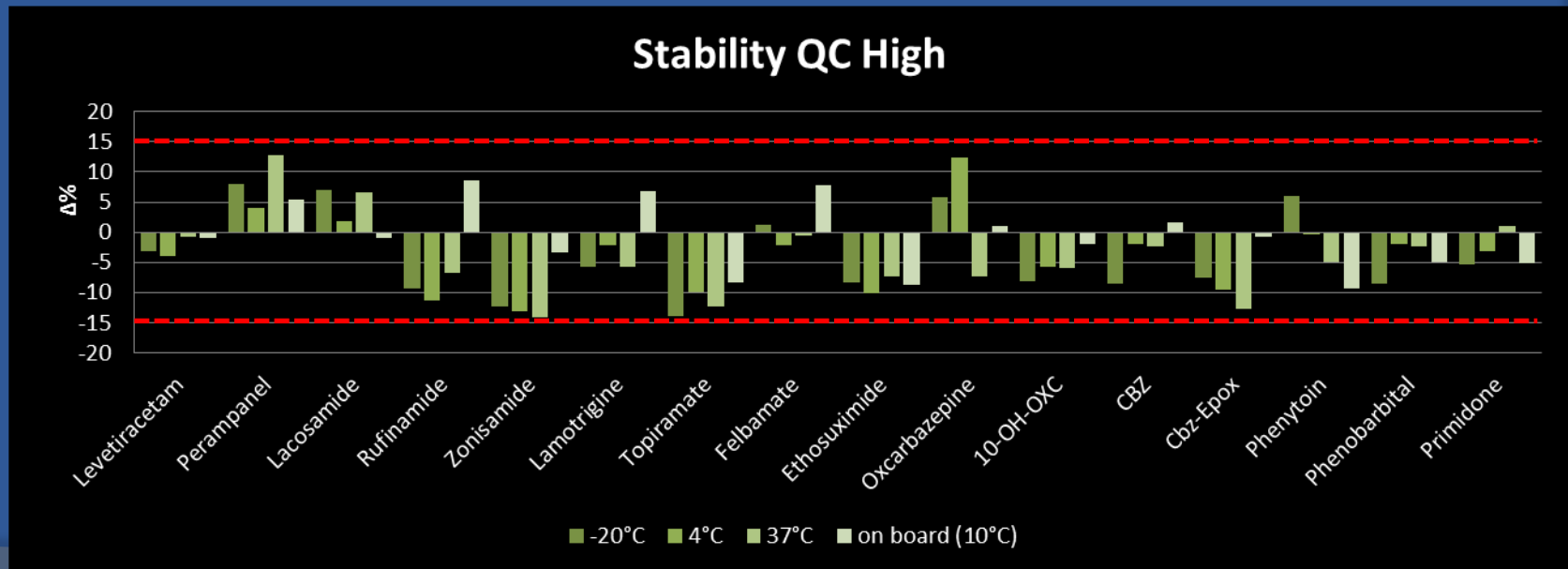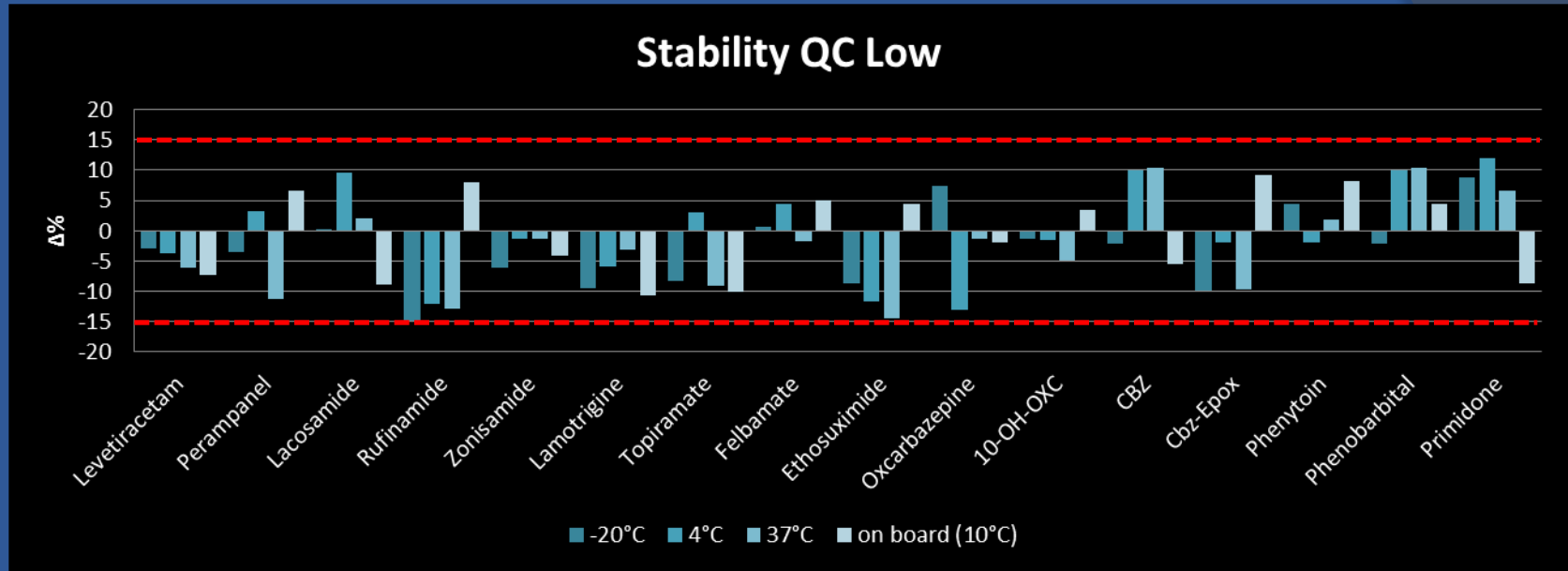
How relay on same lot of calibrators and QC?
1) Preparing a lot of Mitra devices to be stored
2) Preparing a frozen matrix

3) Preparing both! 😉

Evaluation of a frozen matrix, freeze and thaw cycles for 4 month (% difference)

	T°C	LEV	PMP	LCM	RUF	ZNS	LTG	TPM	FBM	ETX	OXC	10-OH-OXC	CBZ	CBZ-E	PHT	PHB	PRM
QC LOW	-20°C	-3	13	11	-7	6	7	6	10	12	8	7	-6	4	4	-6	-4
QC HIGH	-20°C	10	6	8	5	0	13	-1	9	4	7	2	-2	5	13	-2	10

Evaluation of HCT bias by mean of the absolute recoveries

For absolute recoveries we considered 3 hematocrits levels (HCT 35%, 45% and 55%) and L, M and H QC levels of every drug per Hct level.

Then we performed 3 different series of extractions:

Condition A (blood spiked with drugs but not with IS)

Spike blood (3 different HCT, 3 different drug levels). Sample and dry 3 samplers per each HCT and drug level. Extract with IS and analyse.

Condition B (non spiked blood)

Take non spiked blood. Sample and dry 3 samplers per HCT level
Extract with IS and drug spiked (3 different levels) in extractant at the calculated concentration if one was to assume 100% extraction efficiency off a spiked tip.
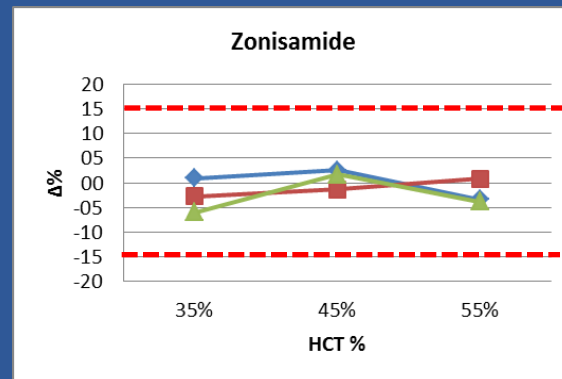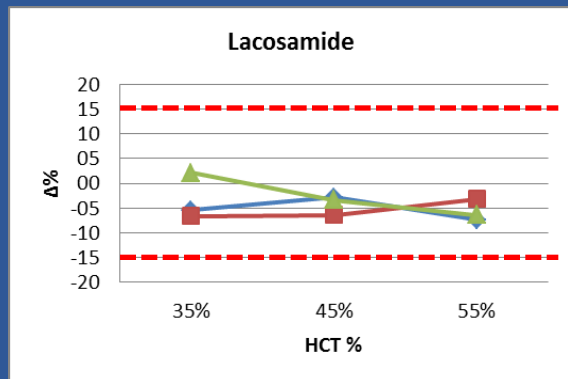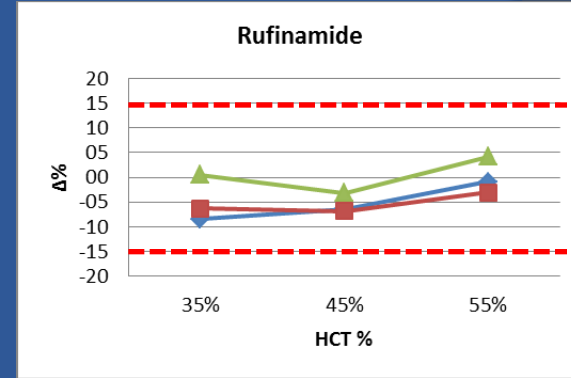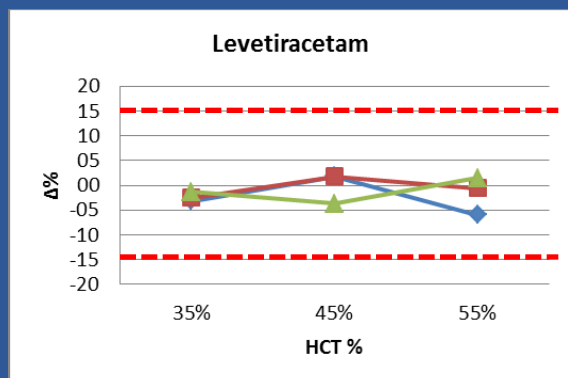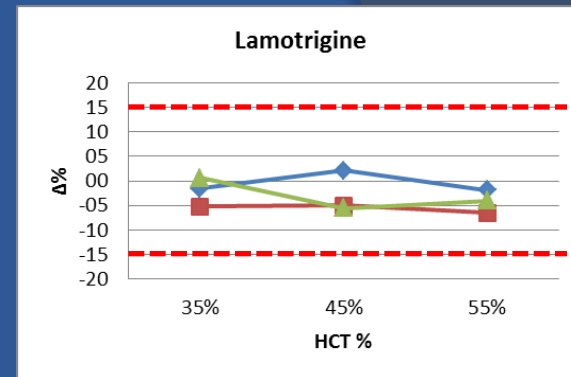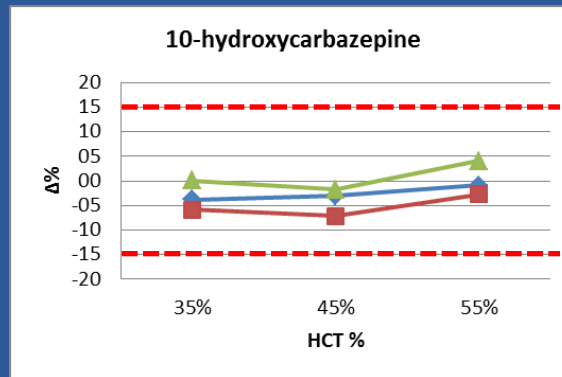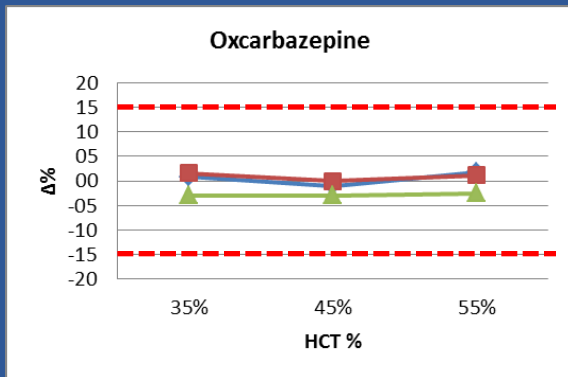
Condition C (blank)

Use a blank. Take non spiked blood (double blank). Sample and dry 1 sampler. Extract with extractant no IS

Then we calculated the peak area ratio of condition A and B having adjusted for any contribution from condition C.

Abu-Rabie P. et al. Anal. Chem. 2015.
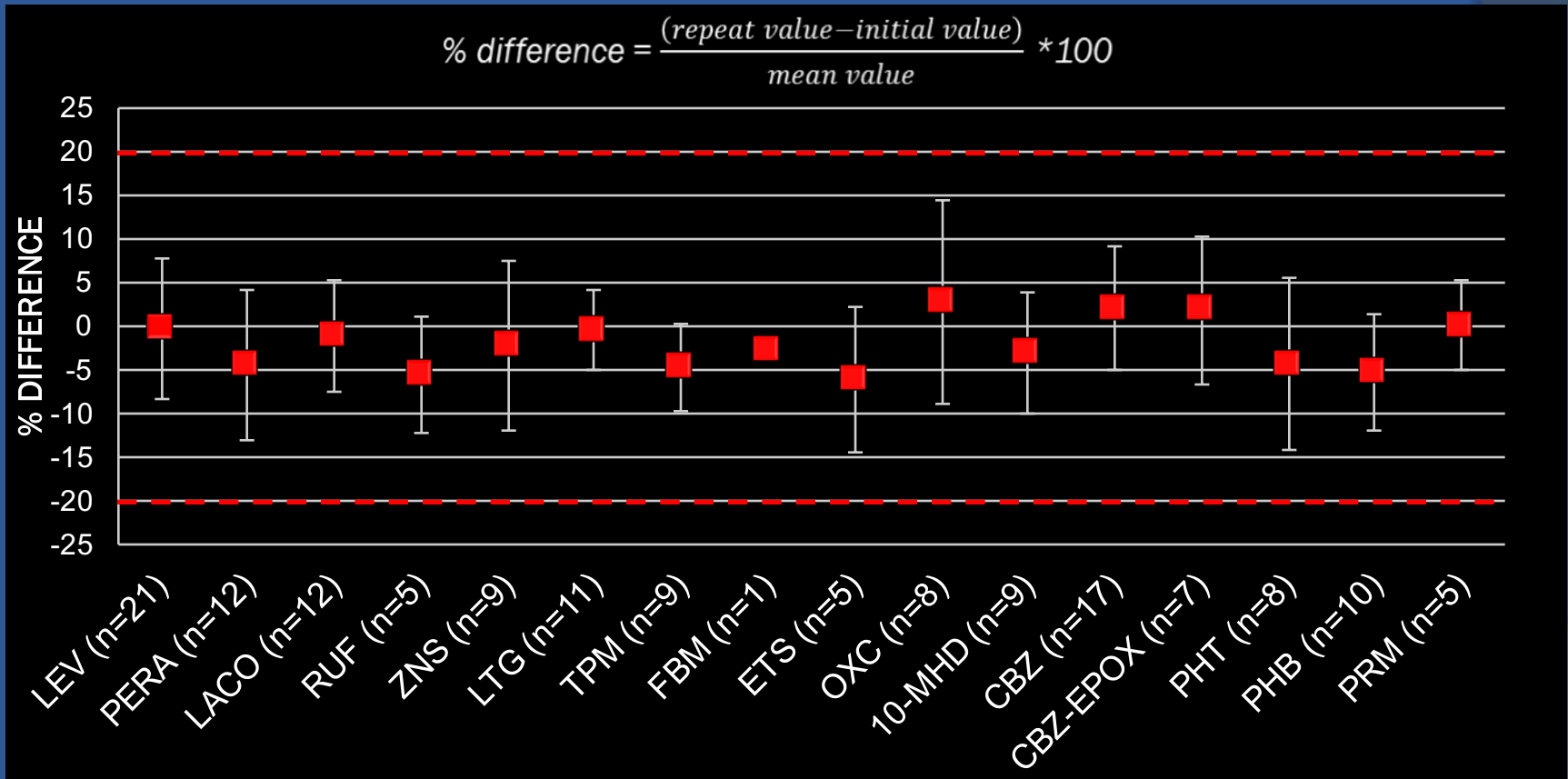
NO HCT bias (>±15%) for first generation AEDs
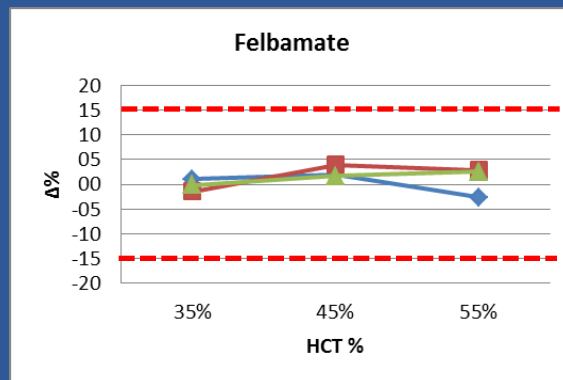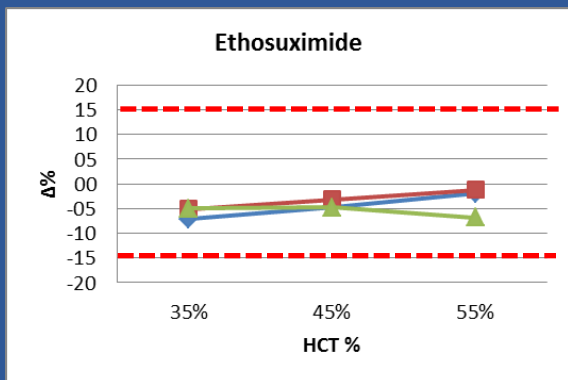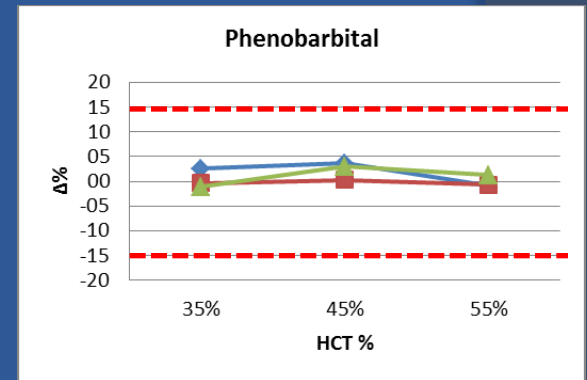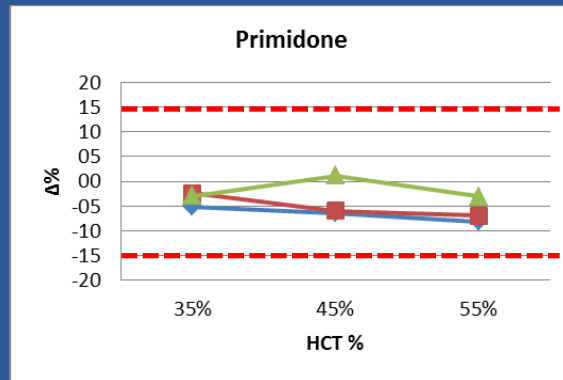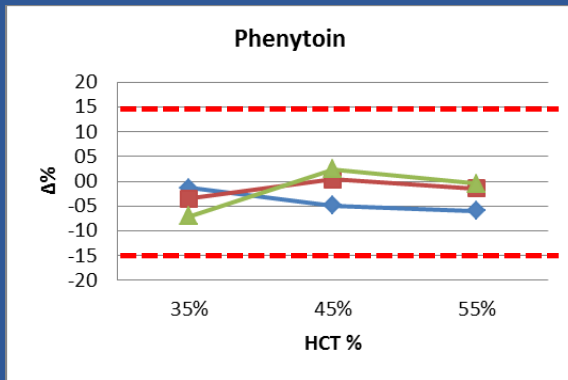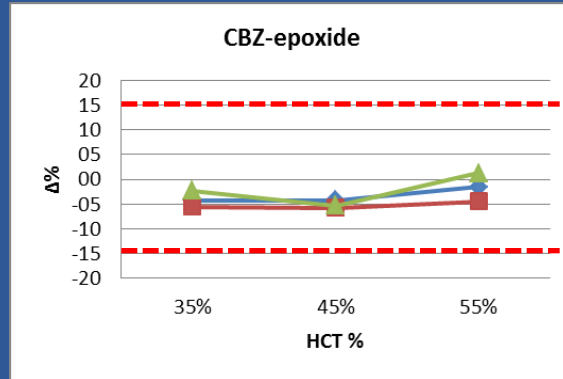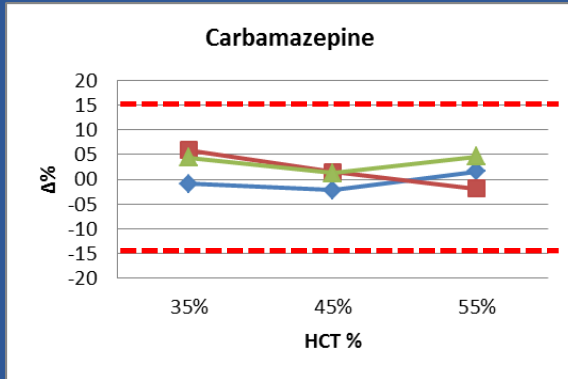
# NO HCT bias (>±15%) for new generation AEDs
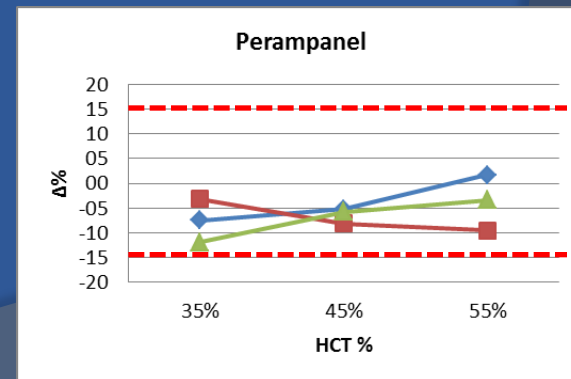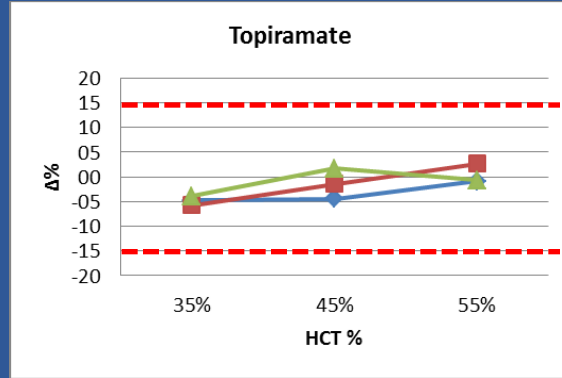
Incurred Sample Reanalysis

The percentage difference between the initial concentration and the concentration measured during the repeat analysis should not be greater than 20% of their mean for at least 67% of the repeats.

$$\% \text{ difference} = \frac{(repeat\ value - initial\ value)}{mean\ value} * 100$$

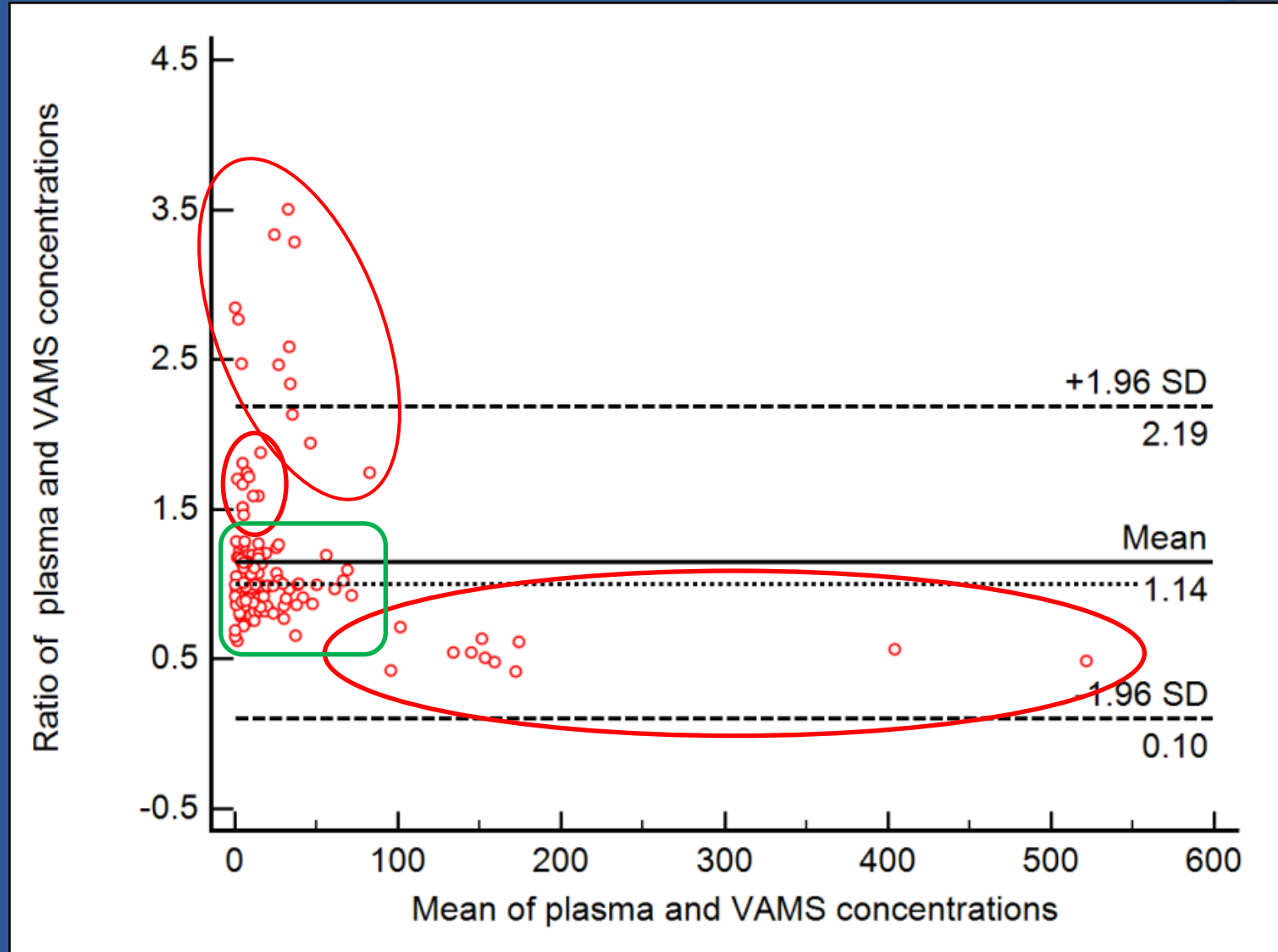## 100% of the repeats had less than 15% variation

Does new method correlates with in plasma AED monitoring?
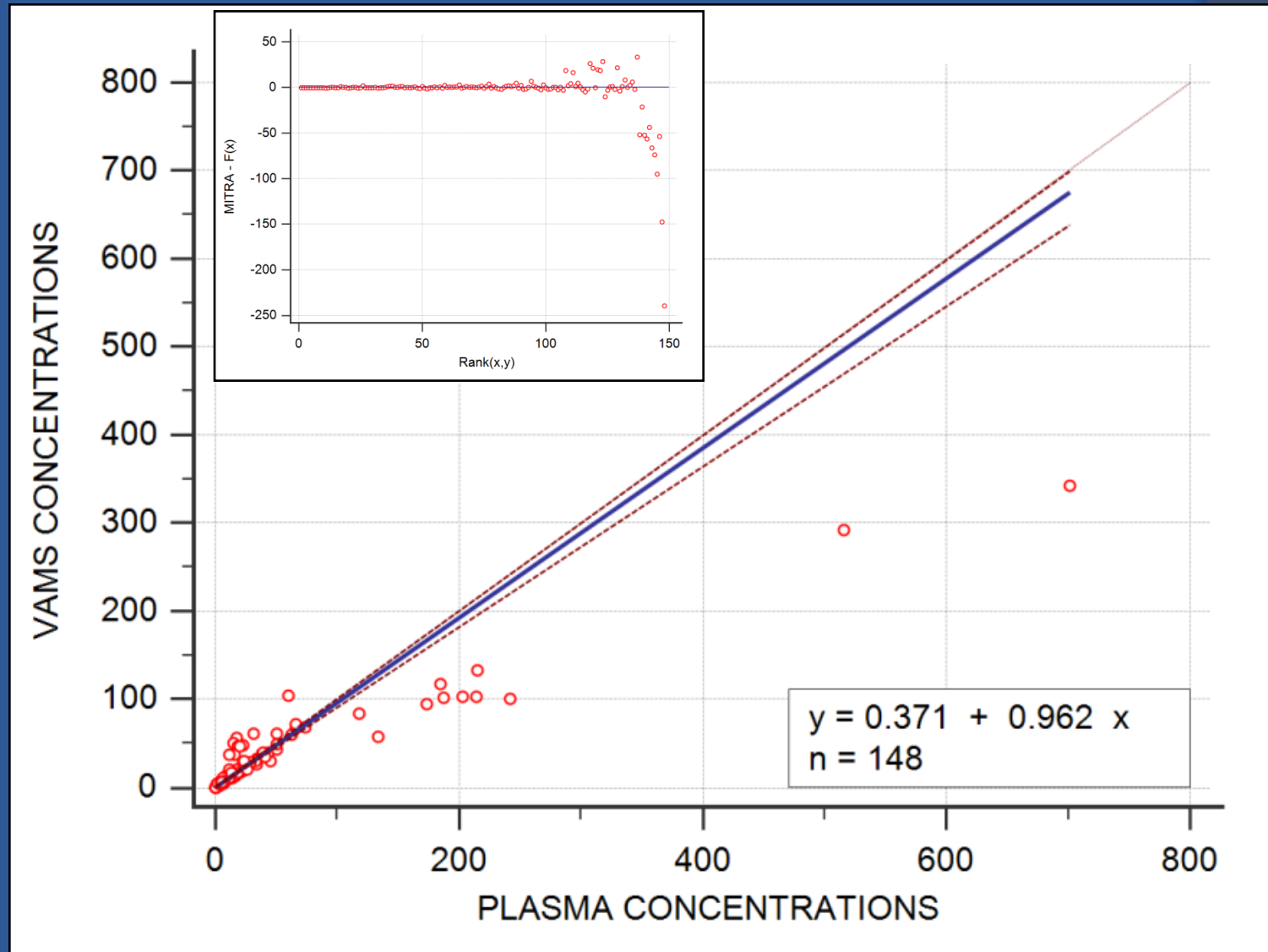
Yes! but…..

CLINICAL VALIDATION

Bland-Altman comparison of patient's samples
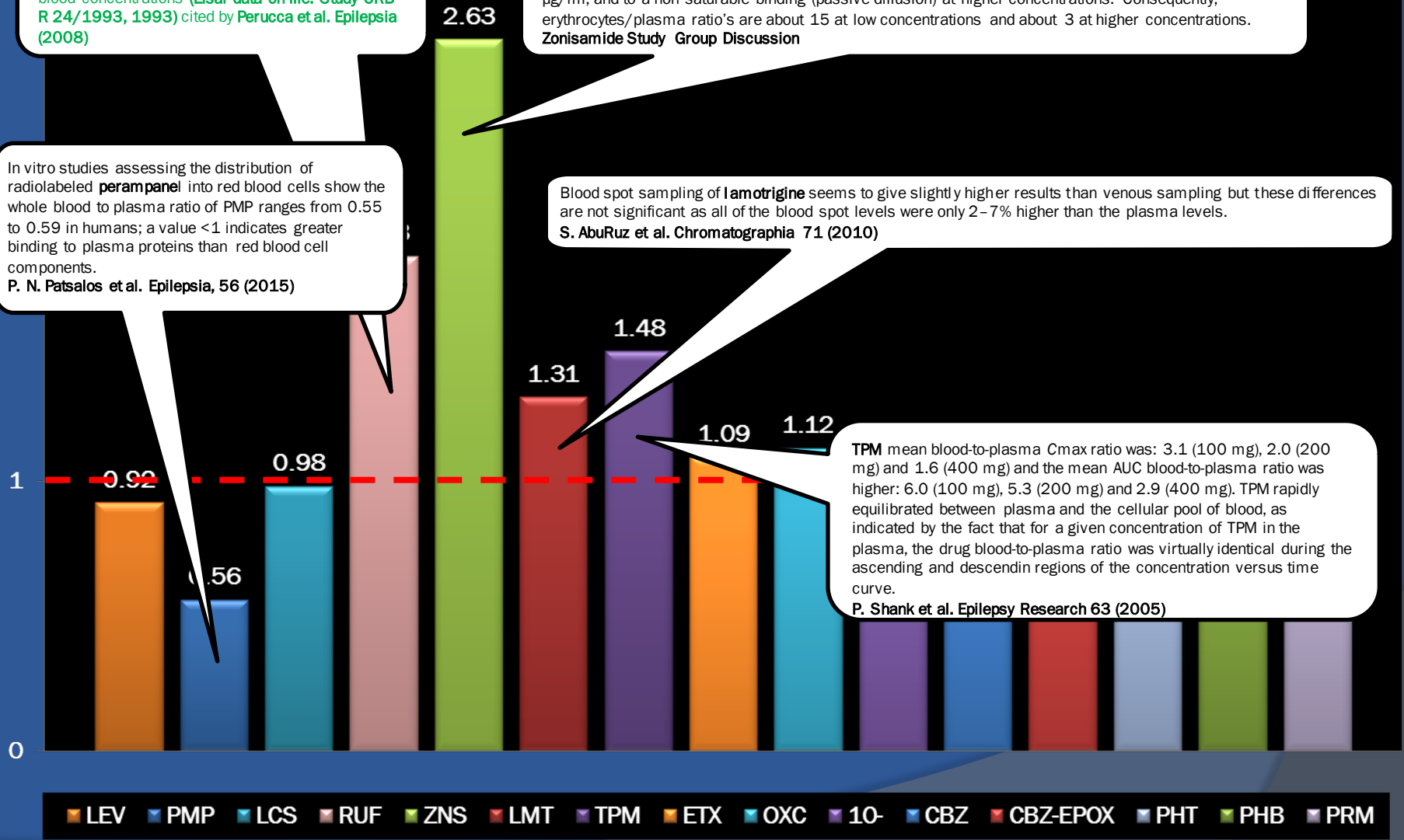
WHOLE BLOOD/PLASMA RATIO

In vitro studies have shown that **rufinamide** partitions nearly equally and reversibly between human plasma and red blood cells at therapeutic blood concentrations **(Eisai data on file: Study CRB R 24/1993, 1993)** cited by Perucca et al. Epilepsia (2008)
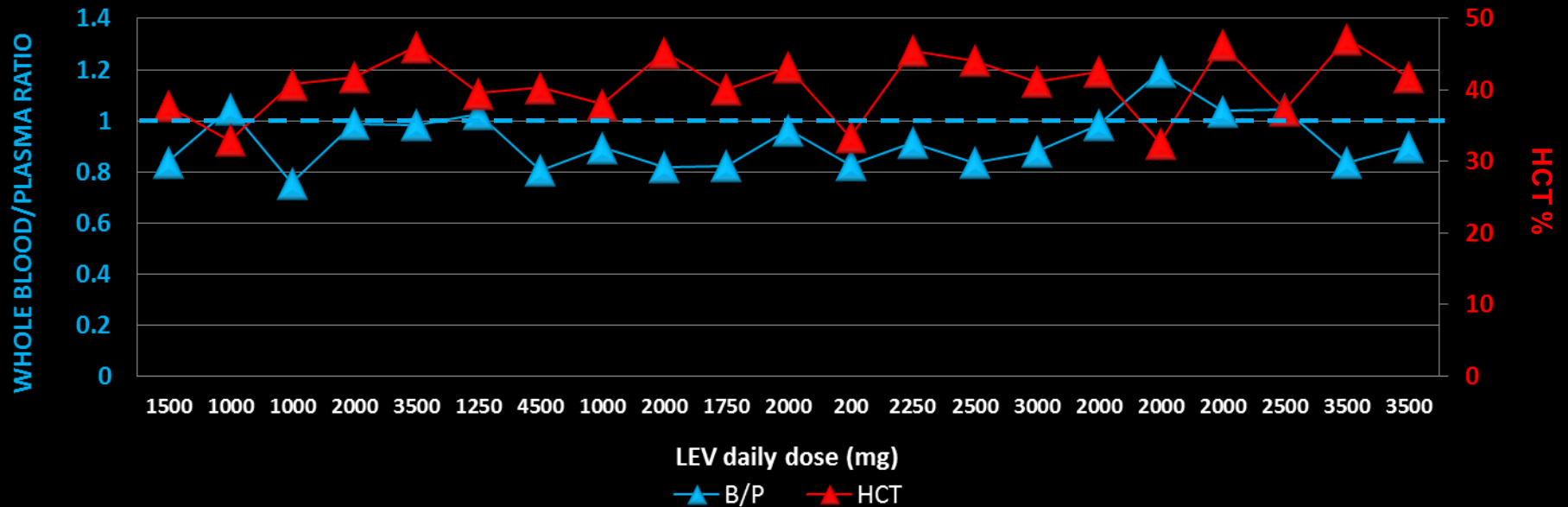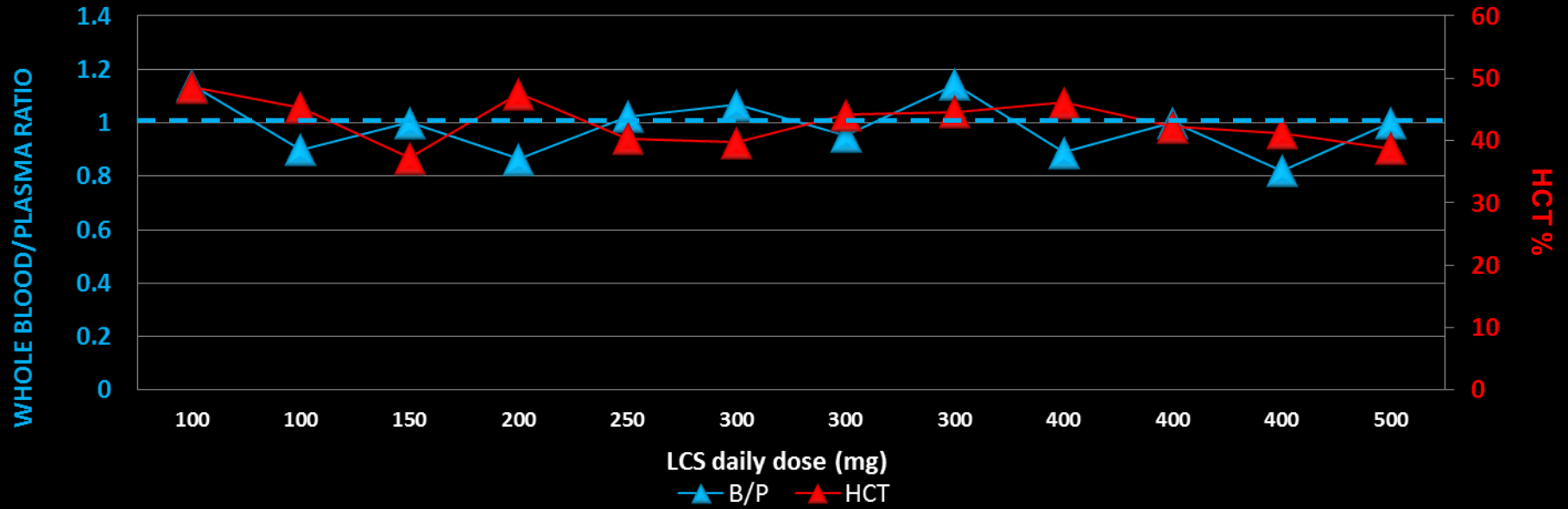
A major distribution compartment of **zonisamide** are the erythrocytes. The binding to erythrocytes is non linear, due to a combined saturable binding to erythrocyte carbonic anhydrase at concentrations below 3 µg/ml, and to a non saturable binding (passive diffusion) at higher concentrations. Consequently, erythrocytes/plasma ratio's are about 15 at low concentrations and about 3 at higher concentrations. **Zonisamide Study Group Discussion**

In vitro studies assessing the distribution of radiolabeled **perampanel** into red blood cells show the whole blood to plasma ratio of PMP ranges from 0.55 to 0.59 in humans; a value <1 indicates greater binding to plasma proteins than red blood cell components. **P. N. Patsalos et al. Epilepsia, 56 (2015)**

Blood spot sampling of **lamotrigine** seems to give slightly higher results than venous sampling but these differences are not significant as all of the blood spot levels were only 2–7% higher than the plasma levels. **S. AbuRuz et al. Chromatographia 71 (2010)**
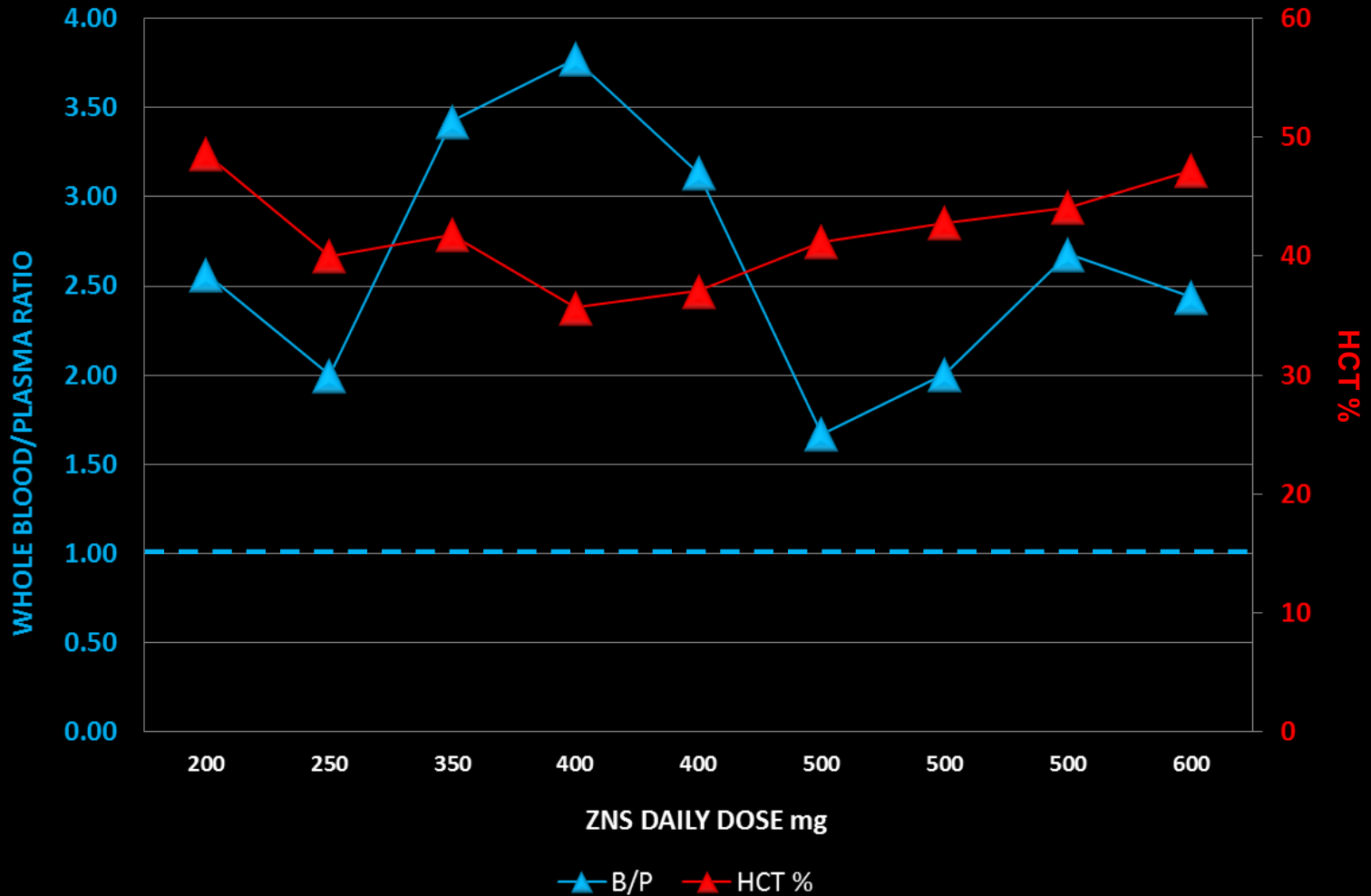
TPM mean blood-to-plasma Cmax ratio was: 3.1 (100 mg), 2.0 (200 mg) and 1.6 (400 mg) and the mean AUC blood-to-plasma ratio was higher: 6.0 (100 mg), 5.3 (200 mg) and 2.9 (400 mg). TPM rapidly equilibrated between plasma and the cellular pool of blood, as indicated by the fact that for a given concentration of TPM in the plasma, the drug blood-to-plasma ratio was virtually identical during the ascending and descendin regions of the concentration versus time curve. **P. Shank et al. Epilepsy Research 63 (2005)**

Bar values: 0.92, 0.56, 0.98, 2.63, 1.31, 1.48, 1.09, 1.12

Legend: LEV, PMP, LCS, RUF, ZNS, LMT, TPM, ETX, OXC, 10-, CBZ, CBZ-EPOX, PHT, PHB, PRM
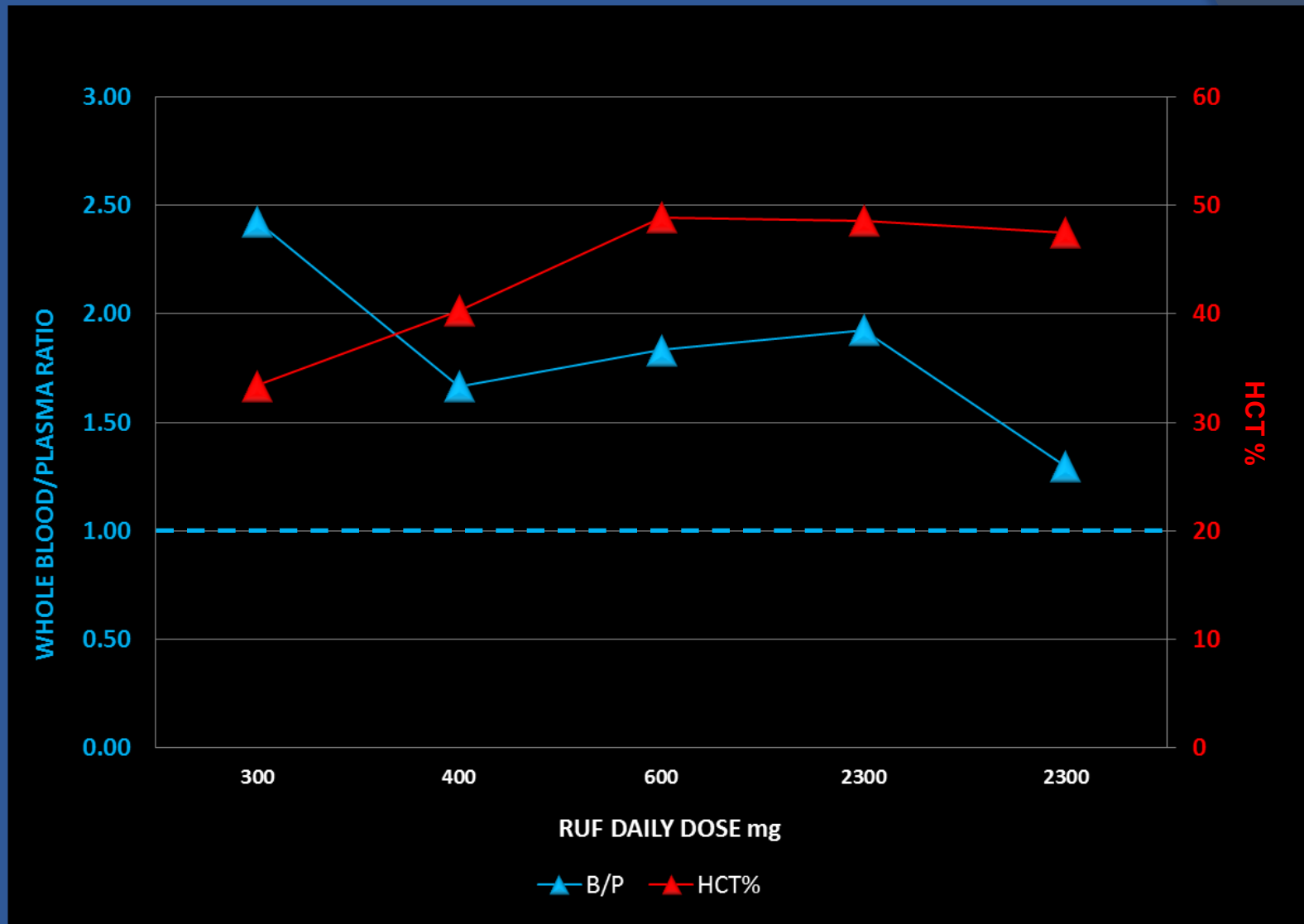
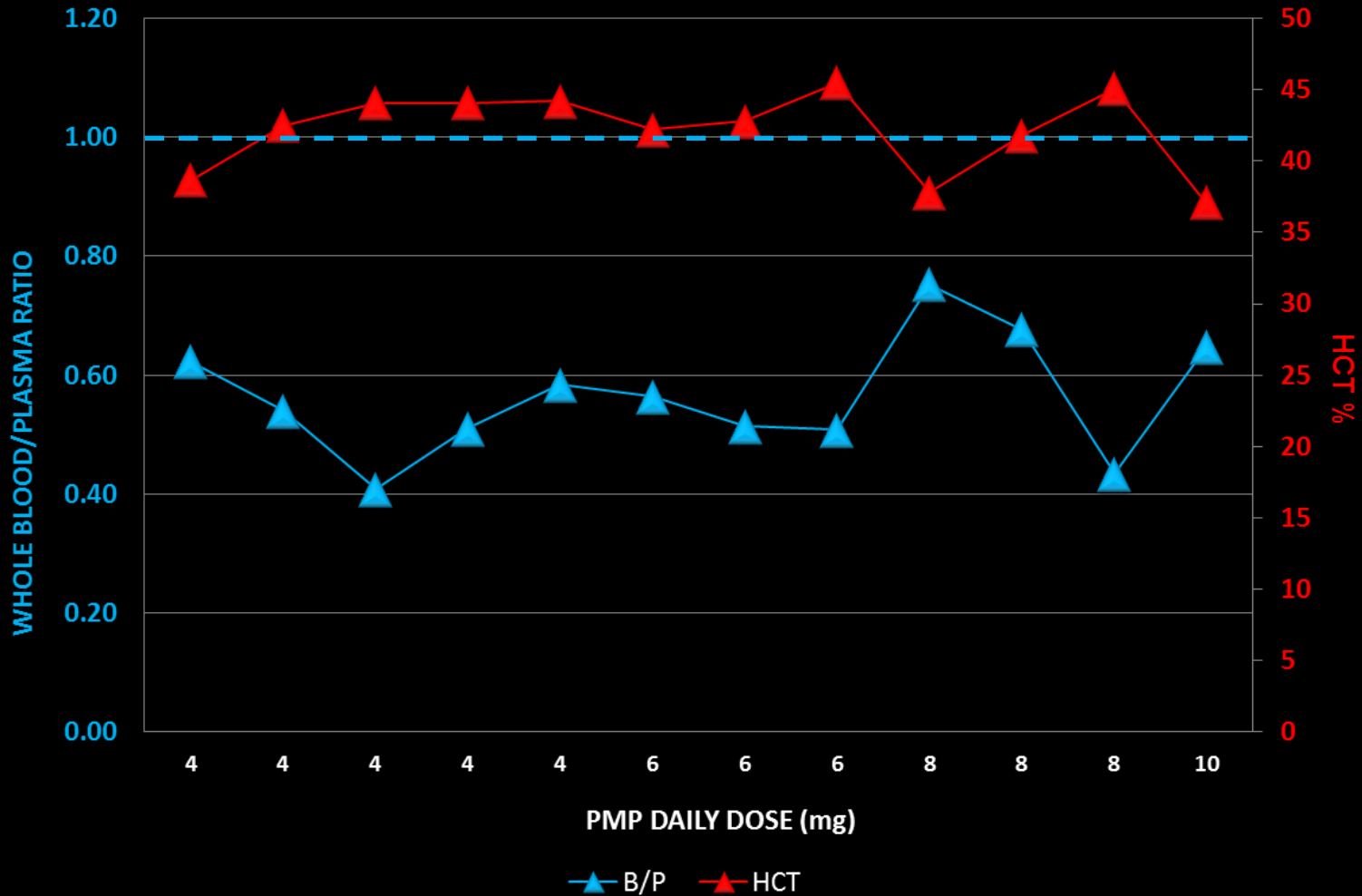# Drugs with whole blood/plasma ratio ≈ 1

ZNS whole blood/plasma ratio = 2,63

RUF whole blood/plasma ratio = 1,83

PMP whole blood/plasma ratio = 0,56

Results:

- Six point calibration curves demonstrated linear and stable, ranging from a LLOQ lower than the lower limit of therapeutic ranges and a ULOQ higher 2-3 fold than the upper limit of therapeutic ranges, depending on the specific drug.
- CVs for three different levels of quality control are consistently under 15%.
- Recoveries varies from 86% to 106%, no matrix effect was found for any of the drugs considered.
- HCT bias was tested for the three levels control at different hematocrit concentrations but no bias (>±15%) was observed.
- Samples demonstrated stable (% variation less than ±15) at different temperatures and times.
- Scavenged whole blood samples from patients had a maximum difference percentage less than ±15% respect to plasma at least for those drugs not entering red blood cells. Drugs like zonisamide contribute to total concentration with their red cells bound fraction.

Conclusions:

- VAMS was successfully applied for the first time to the therapeutic drug monitoring of 16 different antiepileptic drugs in epilepsy patients.

- A simple LC–MS/MS workflow for analysis of VAMS samples was developed and validated.

- Our results established that VAMS is simple, accurate and delivers the benefits of DBS while overcoming the issues of hematocrit and homogeneity and also overcomes issue on stability.

- AED concentrations in whole blood on VAMS device were compared to those in venous blood by routinely used technique with good results. Our results suggest that when dealing with molecules entering red blood cells it may be necessary to recalculate concentration by dividing the result for a factor representing the blood/plasma ratio.

Any question?

Thanks to:

- Special thanks to Annachiara D'Urso for performing all the experiments...

- For believing in our project
- For providing us with Mitra samples
- For technical assistance
- For invitation to share our data

- For providing us with LC-MS/MS reagents
- For tecnhical assistance

You are also invited to have a look to our poster #L03 today at 18.30