Quality Controlled Targeted Metabolome Analysis in Plasma Samples Using the Absolute*IDQ*® p180 Kit together with Waters® ACQUITY UPLC® I-Class and Xevo® TQ-S mass spectrometer

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1 Introduction

Metabolic signatures give crucial insights about pathophysiological mechanisms and shed new light on our understanding of diseases [1-3]. Blood is a preclinical and clinical important biofluid, which can be used for a systemic readout of metabolites for monitoring of all major pathways. Mass spectrometry provides high selectivity and detection sensitivity for metabolic phenotyping. Hence, it is considered as one of the fundamental analytical workhorses presently used in metabolomics [4]. Automation and standardization of the metabolomics workflow are of utmost importance to obtain reliable analytical results and improve interlaboratory comparability. Here we present a standardized quantitative Kit for the simultaneous analysis of more than 180 endogenous metabolites in low-volume (10 µL) human and rat plasma samples (Figure 1). It is excellently suited for monitoring the metabolic status of various species in translational medicine.

188 Metabolites from five key analyte classes

Acylcarnitines:	40
Amino Acids:	19 proteinogenic + ornithine and citrulline
Biogenic Amines:	21
Hexose:	1 (sum of hexoses)

Phospho- and Sphingolipids:

- Phosphatidylcholines	76 (plasmalogens included)
- Lyso-Phosphatidylcholines	14
- Sphingomyelins	15

*Figure 1: Metabolite panel of the Absolute*IDQ*® p180 Kit.*

Five different key metabolite classes are covered with our p180 Kit, which combines UPLC-MS/MS of amino acids and biogenic amines followed by FIA-MS/MS of sphingolipids, hexoses, acylcarnitines and glycerolipids into a single workflow.
The Absolute*IDQ*® p180 Kit was validated on a Waters® ACQUITY UPLC® I-Class system coupled to a Xevo® TQ-S mass spectrometer for human plasma and rat plasma, considering EMA guidelines.

2 Materials and Method

High throughput analysis with minimal amounts of sample volume (10 µL) is achieved by an easy to use and rapid sample preparation with a specially designed 96-well filter plate. Blank and zero samples, 7 calibration standards, 3 levels of quality control samples (QC, human plasma based) and plasma samples were subjected to UPLC®-ESI-MS/MS analysis in multiple reaction monitoring (MRM) mode, followed by FIA-MS/MS. A total of 7 min and 2 min analysis time per sample is required for UPLC®-ESI-MS/MS and FIA-MS/MS analysis, respectively. A Waters® ACQUITY UPLC® I-Class system was coupled to a Xevo® TQ-S mass spectrometer (Waters Inc.). The ACQUITY UPLC® I-Class system was equipped with a BEH C18 (2.1 x 75 mm, 1.7 µm) UPLC® column for chromatographic separation of amino acids and biogenic amines. More than 22.000 MRM chromatograms from >230 metabolites and internal standards were acquired from a single filter plate (96-well format). Quantitation of LC data was performed with TargetLynx (Waters) based on an external 7 point calibration. FIA data was converted and imported into the Biocrates® Met*IDQ*™ software. The multifunctional Met*IDQ*™ software enables automatization of the entire workflow by assisting the user with the sample registration, data processing of LC and FIA data, technical validation and statistical analysis.

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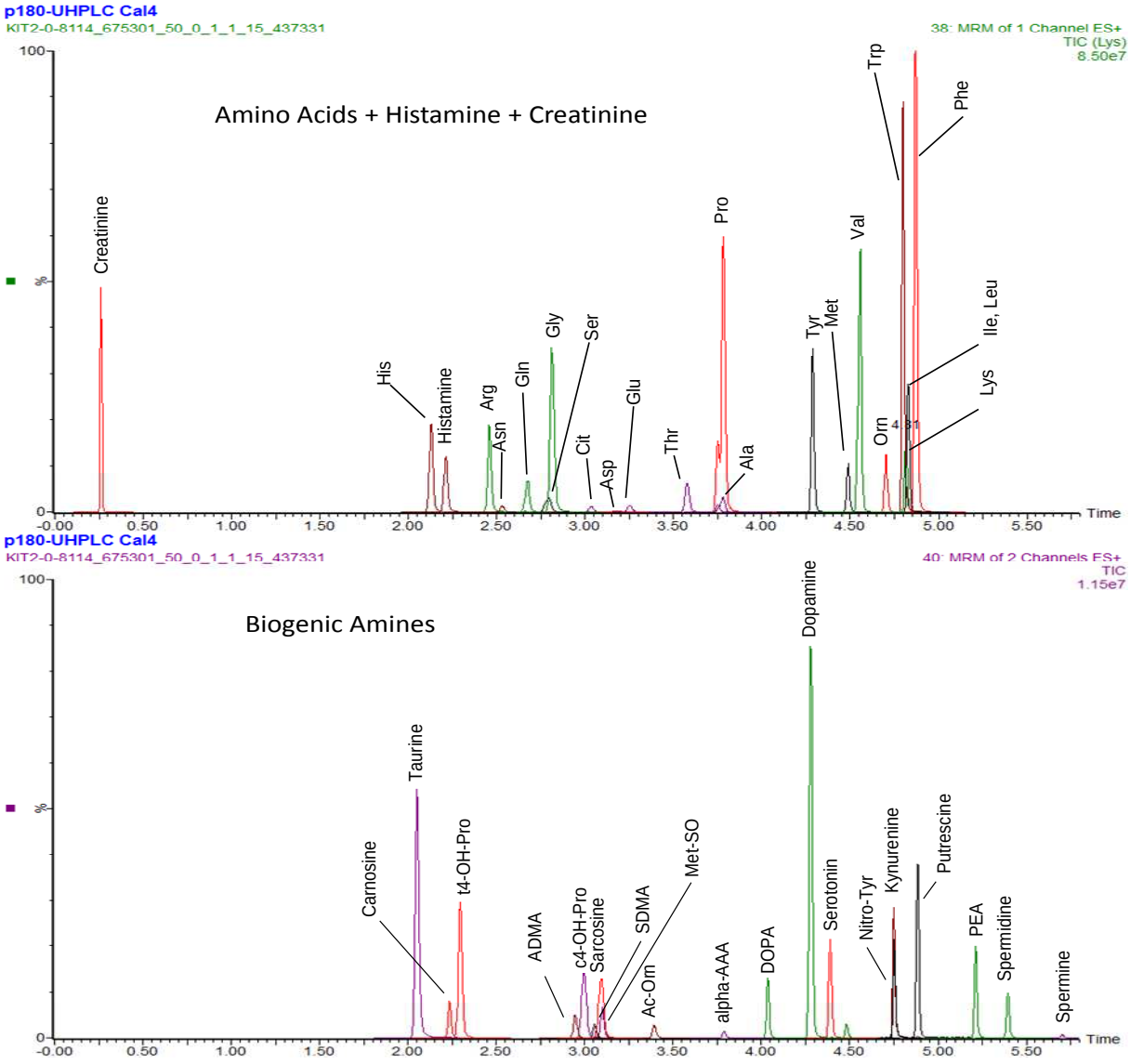

Figure 2: UPLC®-MS/MS chromatograms of a calibration standard level 4. The upper panel shows amino acids, histamine and creatinine, whereas biogenic amines are presented in the lower chromatogram. All analytes are measured within one UPLC®-MS/MS run.

3 Results and Discussion

Optimization of injection volumes and extract dilutions was performed in the beginning. The optimum injection volumes and extract dilutions deviated from those that were previously used for a Waters® ACQUITY UPLC® H-Class system with loop-injector in combination with a Xevo® TQ-MS mass spectrometer. This was due to the difference in sample injection and detection sensitivity of the MS. Best peak profiles for analysis of amino acids and biogenic amines (UPLC®-MS/MS based metabolites) on the ACQUITY UPLC® I-Class system comprising a Sample Manager with Flow-Through-Needle (SM-FTN) injector were obtained with an injection volume of 2 µL. Moreover, a 1:2

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diluted extract (dilution with Milli-Q water) was used for the Xevo® TQ-S mass spectrometer. Various extract dilutions were further tested for the FIA-MS/MS analysis on the Xevo® TQ-S system. A high extract dilution decreases ion suppression caused by high abundant metabolites, which is highly relevant as no chromatographic separation is intended for FIA-MS/MS. The optimum extract dilution depends on the detection sensitivity of the MS instrument. Therefore an adequate extract dilution had to be determined to achieve a good compromise between acceptable ion suppression and signal intensity. The high instrument detection sensitivity of the Xevo® TQ-S enabled the optimum extract dilution of 1:50 and an injection volume of 10 µL.

Validation: Summary

Human plasma and rat plasma were used as matrices for validation of the Absolute*IDQ*® p180 Kit on the Waters® ACQUITY UPLC® I-Class system coupled to a Xevo® TQ-S mass spectrometer. The following Table 1 gives an overview of the validity of amino acids and biogenic amines in these matrices. To determine the validity of the analytes in respective matrix, intraday (within batch) and interday (batch-to-batch) plates were evaluated in terms of precision and accuracy, as well as detection sensitivity and selectivity. Excellent intra- and inter-batch accuracy (between 85-115%) and coefficient of variation as a measure for precision (CV <15%) were obtained for most amino acids and biogenic amines in the course of the validation. Leucine and 6 biogenic amines did not fulfill the optimum accuracy and/or precision requirements in at least one of the tested biological matrices. Thus, these analytes are termed “valid with restrictions” in Table 1. However, precise values were obtained for all of them except spermine in rat plasma.

Furthermore, a broad spectrum of key metabolites present in biological specimens can be determined by FIA-MS/MS analysis with the p180 Kit. These metabolites belong to key analyte classes, such as acylcarnitines, sugars (hexoses) and lipids (14 lysophosphatidylcholines (lysoPC), 76 phosphatidylcholines (PC) and 15 sphingomyelins (SM)).

Table 1: Analytical validity of amino acids and biogenic amines in human plasma and rat plasma.

Amino Acid	Validity in human plasma		Validity in rat plasma	
	Valid	Valid with restrictions	Valid	Valid with restrictions
Ala	x		x	
Arg	x		x	
Asn	x		x	
Asp	x		x	
Cit	x		x	
Gln	x		x	
Glu	x		x	
Gly	x		x	
His	x		x	
Ile	x		x	
Leu		x[2)]		x[2)]
Lys	x		x	
Met	x		x	
Orn	x		x	
Phe	x		x	
Pro	x		x	
Ser	x		x	
Thr	x		x	
Trp	x		x	
Tyr	x		x	
Val	x		x	

1) Restriction criteria: interday/intraday precision
2) Restriction criteria: interday/intraday accuracy

Biogenic Amine	Validity in human plasma		Validity in rat plasma	
	Valid	Valid with restrictions	Valid	Valid with restrictions
Ac-Orn	x		x	
ADMA	x		x	
SDMA	x		x	
alpha-AAA		x[2)]	x	
Carnosine	x		x	
Creatinine	x		x	
DOPA	x		x	
Dopamine	x		x	
Histamine		x[2)]		x[2)]
Kynurenine		x[2)]		x[2)]
Met-SO	x		x	
Nitro-Tyr		x[2)]		x[2)]
c4-OH-Pro	x		x	
t4-OH-Pro	x		x	
PEA		x[2)]		x[2)]
Putrescine	x		x	
Sarcosine	x		x	
Serotonin	x		x	
Spermidine	x		x	
Spermine		x[2)]		x[1) 2)]
Taurine	x		x	

1) Restriction criteria: interday/intraday precision
2) Restriction criteria: interday/intraday accuracy

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Table 2: Analytical validity of acylcarnitines in human plasma and rat plasma.

Compound		Validity in human plasma		Validity in rat plasma	
MetIDQ Short Name	Biochemical Name	Valid	Relative quant	Valid	Relative quant
C0	Carnitine	X		X	
C2	Acetylcarnitine	X		X	
C3	Propionylcarnitine*	X		X	
C3:1	Propenoylcarnitine		X		X
C3-OH	Hydroxypropionyl carnitine*		X		X
C4	Butyrylcarnitine*	X		X	
C4:1	Butenylcarnitine		X		X
C4-OH (C3-DC)	Hydroxybutyrylcarnitine*		X		X
C5	Valerylcarnitine*	X		X	
C5:1	Tiglylcarnitine		X		X
C5:1-DC	Glutaconylcarnitine		X		X
C5-DC (C6-OH)	Glutarylcarnitine* (Hydroxyhexanoyl-carnitine)		X		X
C5-M-DC	Methylglutarylcarnitine		X		X
C5-OH (C3-DC-M)	Hydroxyvalerylcarnitine* (Methylmalonylcarnitine)		X		X
C6 (C4:1-DC)	Hexanoylcarnitine* (Fumarylcarnitine)	X		X	
C6:1	Hexenoylcarnitine		X		X
C7-DC	Pimelylcarnitine*		X		X
C8	Octanoylcarnitine*	X		X	
C9	Nonaylcarnitine*		X		X
C10	Decanoylcarnitine*	X		X	
C10:1	Decenoylcarnitine*		X		X
C10:2	Decadienylcarnitine		X		X
C12	Dodecanoylcarnitine*	X		X	
C12:1	Dodecenoylcarnitine*		X		X
C12-DC	Dodecanedioylcarnitine*		X		X
C14	Tetradecanoylcarnitine*	X		X	
C14:1	Tetradecenoylcarnitine*		X		X
C14:1-OH	Hydroxytetradecenoyl-carnitine*		X		X
C14:2	Tetradecadienylcarnitine		X		X
C14:2-OH	Hydroxytetradecadienyl-carnitine		X		X
C16	Hexadecanoylcarnitine*	X		X	
C16:1	Hexadecenoylcarnitine*		X		X
C16:1-OH	Hydroxyhexadecenoyl-carnitine*		X		X
C16:2	Hexadecadienylcarnitine		X		X
C16:2-OH	Hydroxyhexadecadienyl-carnitine		X		X
C16-OH	Hydroxyhexadecanoyl-carnitine*		X		X
C18	Octadecanoylcarnitine*	X		X	
C18:1	Octadecenoylcarnitine*		X		X
C18:1-OH	Hydroxyoctadecenoyl-carnitine		X		X
C18:2	Octadecadienylcarnitine		X		X

* Concentrations are isotope corrected

An overview of the validity of acylcarnitines in human and rat plasma is given in Table 2. Since not all FIA metabolites are commercially available as external and internal standards, the accurate determination of lipids in particular and a subset of acylcarnitines is limited. Thus, the validity of these analytes is termed "relative quantitative". Nevertheless, excellent precision values were obtained for all metabolite classes, which are highly suited for comparative studies of different sample cohorts. Figure 3 shows the average interday precision of FIA-MS/MS metabolites in different plasma samples on 4 days: 3 levels of quality controls (QCs, low, medium and high levels), human plasma samples (5 individual, 1 pooled) and rat plasma samples (4 individual, 1 pooled). Excellent average precision was obtained for all metabolite classes. Individual analytes exhibited endogenous concentrations close to their detection limits, which explains higher CV values up to 26%.

The validity of metabolites of the Absolute*IDQ*® p180 Kit on the ACQUITY UPLC® I-Class system coupled to a Xevo® TQ-S mass spectrometer was compared to their performance on an ACQUITY UPLC® H-Class system with a Xevo® TQ-MS instrument.

During validation a much higher detection sensitivity and reproducibility of the Xevo® TQ-S system was observed. A total of 12 amino acids and biogenic amines could be quantitated with higher precision and accuracy compared to the analysis on the Xevo® TQ-MS. Thus following metabolites are valid for at least one biological matrix on the Xevo® TQ-S, but valid with restriction on the Xevo® TQ-MS: Asp, Cit, AcOrn, ADMA, SDMA, alpha-AAA, Creatinine, DOPA, Dopamine, MetSO, t4-OH-Pro and Spermidine. The higher detection sensitivity of the Xevo® TQ-S in comparison to the Xevo® TQ-MS was also reflected in the lower injection volume and higher extract dilution that could be used for the Xevo® TQ-S instrument. In fact, 2 µL instead of 5 µL were used. Further, dilution of the extract for FIA analysis was 1:50 for the Xevo® TQ-S and 1:4 for the Xevo® TQ-MS.

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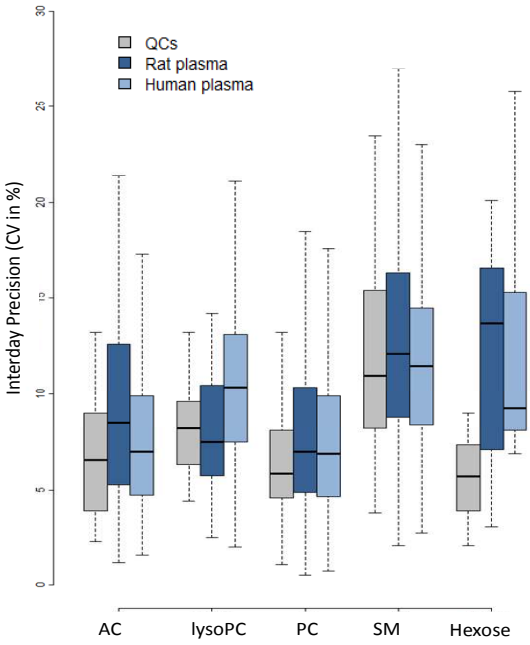

Figure 3: Average interday precision of FIA metabolite classes in quality controls (QCs), human and rat plasma samples.

Reference to SRM 1950 material (NIST): analysis of amino acids, creatinine and glucose

The NIST 1950 standard reference material was analysed and the obtained concentration values were compared to the specified values in order to demonstrate the excellent performance of the Absolute*IDQ*® p180 Kit/ Xevo® TQ-S system. Excellent accuracy and precision were obtained for amino acids, creatinine and glucose (Figure 4). These findings are important in the context of inter-laboratory comparability.

Comparison of Xevo® TQ-S versus Xevo® TQ-S micro mass spectrometer: analysis of biogenic amines

The detection sensitivity of the Xevo® TQ-S mass spectrometer versus Xevo® TQ-S micro MS was compared by the analysis of biogenic amines, which is a class of typically low-abundant compounds in human and rat plasma. Both MS instruments were coupled to an ACQUITY UPLC® I-Class system and the same extract dilution (1:2) was used, which allowed a fair comparison.

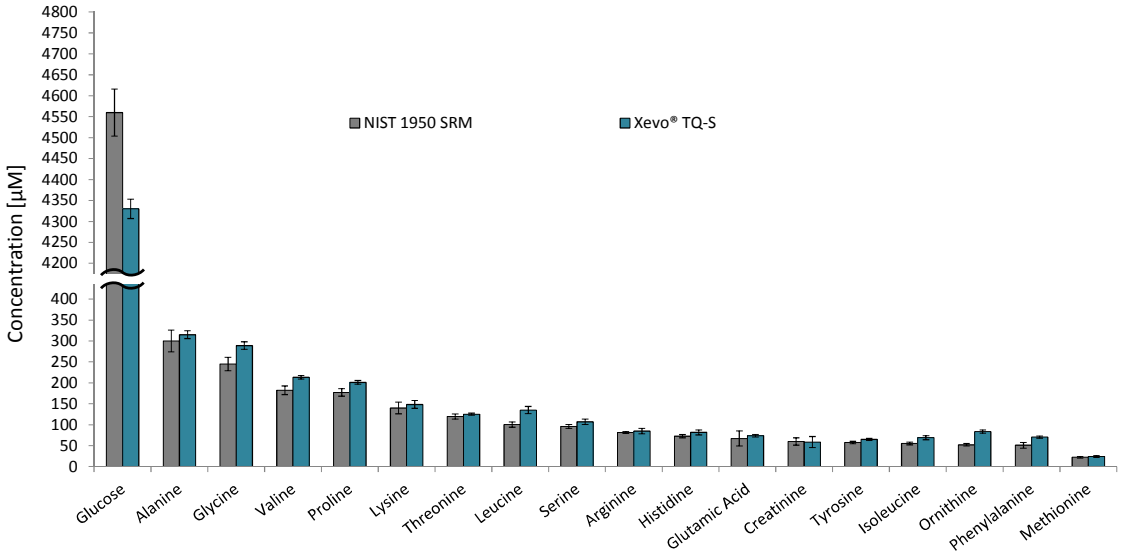

Figure 4: UPLC®-Xevo® TQ-S concentration values obtained for the NIST standard reference material 1950 in comparison to the specified concentration values.

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An enhanced detection sensitivity of the Xevo® TQ-S system was observed during validation of biogenic amines. A total of 7 metabolites could be clearly detected during the analysis with the Xevo® TQ-S instrument, whereas these biogenic amines were close to the detection limit or completely absent with the Xevo® TQ-S micro MS. This refers to the following compounds for at least one biological matrix: alpha-AAA, Carnosine, DOPA, Dopamine, Histamine, Nitro-Tyr and Serotonin. The improved detection sensitivity of the Xevo® TQ-S MS is illustrated in Figure 5 for alpha-AAA and DOPA.

Overall, the Xevo® TQ-S instrument performed extremely well for very low abundant metabolites as it enabled quantitative and semi-quantitative analyses of a higher number of biogenic amines with overall better precision for this compound class.

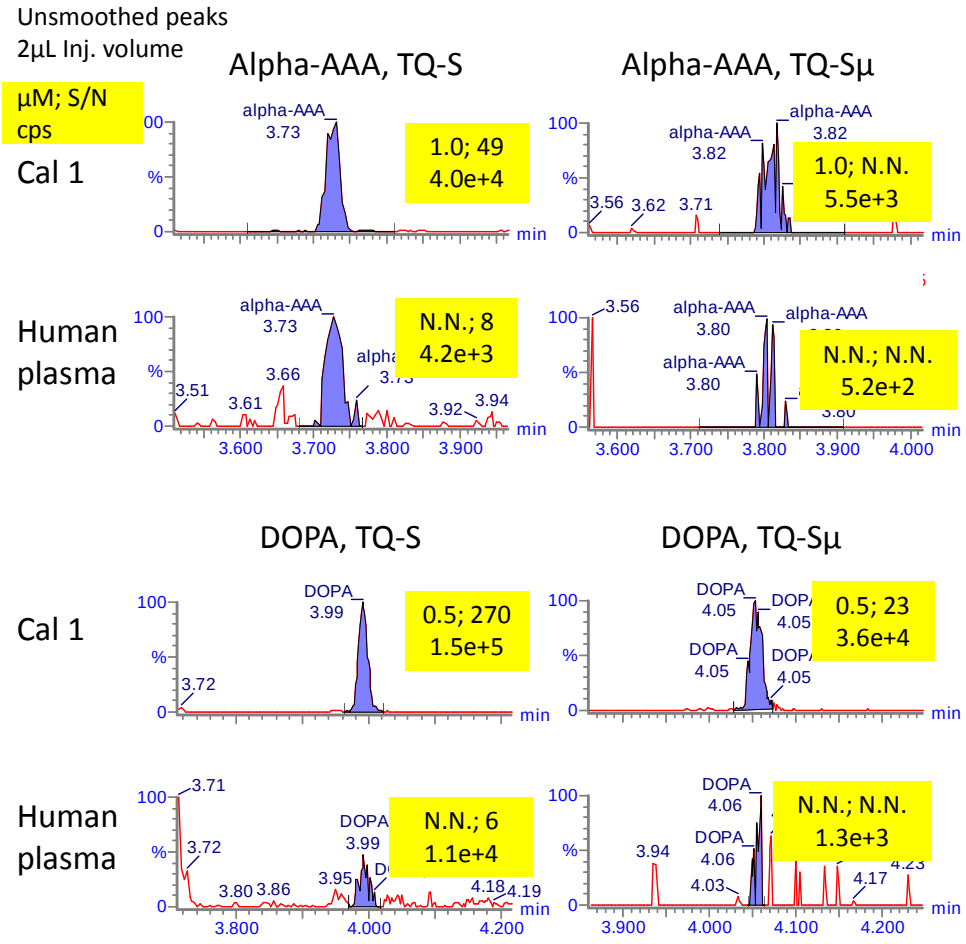

Figure 5: UPLC®-MS/MS analysis of alpha-AAA and DOPA in calibration standard 1 and human plasma using a Xevo® TQ-S versus Xevo® TQ-S micro mass spectrometer. The first, second and third digits in the yellow boxes represent concentration level in µM, signal-to-noise ratio and signal intensity (cps), respectively. N.N., not named.

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4 Conclusions

Using the Absolute*IDQ*® p180 Kit together with Waters® ACQUITY UPLC® I-Class coupled to a Xevo® TQ-S system, accurate and precise results are obtained for a broad range of metabolites in human and rat plasma. The total analysis time from sample thawing to the final technically validated concentration table is 25 hours for 82 samples. Hence, high sample throughput is achieved.

Targeted metabolome analysis with the Absolute*IDQ*® p180 Kit on the Xevo® TQ-S system gives reliable analytical results in an automated and highly standardized workflow. This is of utmost importance to enable inter-laboratory comparability and inter-instrument robustness.

In summary, the developed and validated Kit on a Waters® ACQUITY UPLC® Xevo® TQ-S instrument is a powerful tool for the targeted quantitative analysis of the blood metabolome. This platform can be especially recommended for the analysis of very low abundant classes of metabolites such as biogenic amines.

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