Targeted Metabolomics Analysis of Cerebrospinal Fluid Using the Absolute*IDQ*™ Kit

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Introduction

The composition of cerebrospinal fluid (CSF) is dependent upon the metabolic production rates in the brain, the metabolite concentrations in the blood, and transport processes at the blood-CSF barrier. Metabolomic analysis of CSF provides biochemical insights into central nervous system (CNS) disorders and could result in the identification of biomarkers for disease, disease progression, or response to therapy [1-3]. In many cases the concentration ratio of CSF/serum is important for assessing how observed changes in the metabolite concentrations in CSF are related to effects on the blood-CSF barrier, thus methods applicable to both body fluids are of great value.
The Absolute*IDQ*™ Kit, a commercially available assay, was originally validated for plasma. In this application note, we have tested and optimized the kit for the analysis of human CSF. The Absolute*IDQ* kit is based on a targeted metabolomics approach [4, 5] and can simultaneously identify and quantify a large number of endogenous metabolites in plasma samples. The metabolite panel of the kit includes amino acids and acylcarnitines as well as a high number of glycerophospholipids and sphingolipids, which are important components of the lipids in brain and CSF [1].
As a first step in testing the Absolute*IDQ* Kit for CSF, the kit method was evaluated using pooled human CSF. Median concentrations and detection limit (LOD) for the metabolites in the kit were determined. Second, the method was applied to CSF and serum from different patient groups with psychiatric and neurological disorders.

Methods

Optimization using pooled human CSF

The cell-free CSF and the serum were frozen at -80°C until processing. Before being loaded onto the filter of the kit plate, they were vortexed and centrifuged at 10,000 x g. The Absolute*IDQ* kit was prepared as described in detail in the Kit User Manual with some variations. The concentration of most metabolites is significantly lower in CSF compared with plasma. Therefore the amount of CSF added onto the filter paper in the upper kit plate was tested in the range from 10 µl to 60 µL (10 µL is the standard volume for plasma). The addition of 30 µL of CSF yielded best results for ease of preparation and coefficient of variation. Addition of higher amounts (60 µL) resulted in significantly elevated ion suppression. The paper filter in the upper kit plate has a limited capacity, so the 30 µL must be added in two steps: 1) add 15 µL then dry for 30 min under nitrogen flow 2) add the second 15 µL then dry for 30 min under nitrogen flow.
The extracts were analyzed using a 4000 QTrap® or API 4000™ (Applied Biosystems/MDS Sciex) triple quadrupole mass spectrometer. The standard flow injection method of the Absolute*IDQ* kit comprising two 20 µL injections (one for positive and one for negative detection mode) was applied for all measurements. Multiple reaction monitoring (MRM) detection was used for quantification.

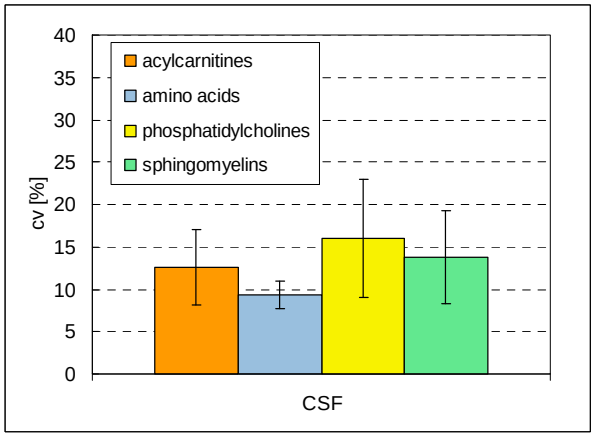

Figure 1: Comparison of CV values
Pooled CSF samples (n=5) were analyzed and mean values of the coefficient of variation (CV) and standard deviations were calculated for the different metabolite classes. Analytes with values above LOD were used.

Coefficient of variation (CV)

For pooled human CSF, the intra-day CVs of the different metabolite classes were compared (Figure 1). The mean values of the CVs were below 10% for the amino acids and were therefore comparable to plasma. For acylcarnitines, phosphatidylcholines and sphingomyelins the mean CV values were slightly higher due to the much lower concentrations, but they were still below 20%.

For several phosphatidylcholines that exhibited concentrations below 40 nM, CV values in the range between 20-30% were found.

Calculation of concentrations for CSF

The zero samples used for the kit contain internal standards but no analytes. They are processed identically as CSF or plasma. The zero samples are used to calculate the limit of detection (LOD) defined as three times the median value of the zero samples. Some of the internal standards of the kit can interfere with the analyte MRM pairs. This can result in a certain degree of background concentration for these analytes reflected in the zero samples. Due to much lower metabolite concentrations in CSF compared with plasma, the concentration of the zero samples needs to be subtracted from the calculated concentration of the CSF samples to compensate for this effect *(Final conc. = Calc. conc. – Conc. zero sample*). The concentrations of metabolites and zero samples are automatically calculated by the Met*IQ* Software. However, the subtraction of the zero samples is currently not implemented since the effects are relatively small for plasma/serum.
PBS buffer or Ringer solution is recommended for the three zero samples on each kit plate. Their salt content is similar to CSF/serum and it was found that they have comparable ion suppression for most metabolites. CSF and zero samples require adding 30 µL to each well of the kit plate, whereas QC samples (pooled human plasma) and standards require addition of 10 µL. A new operating procedure (OP) is available as software patch for Absolute*IDQ* kit users and takes the loading of 30 µL for CSF and zero samples into account during calculation of the concentrations.

Median concentrations and LOD

The LOD for the different metabolite classes and the median concentration values in pooled human CSF are listed in the tables 1-4 below. Median values were calculated from at least three independent (inter-day) analyses of five replicates. PBS buffer was used for zero samples and the concentration was subtracted as described above. It should be noted that CSF from different sources could exhibit significantly different concentration values since factors like age, gender, environment and medical conditions significantly influence the metabolome.

Acylcarnitines

Five acylcarnitines exhibited concentrations above LOD in the pooled CSF. Generally, the mean concentrations of the acylcarnitines were about 5-20 times lower as in plasma. The concentration of acylcarnitines can significantly increase as a result of enzymatic disorders or in certain disease states.

Table 1: Median acylcarnitine concentrations in CSF

Analyte	LOD [nM]	Concentration [nM]
C0	990	1620
C2	45	510
C3	27	69
C4	12	27
C5	9	17

Amino acids

All amino acid concentrations in CSF are in the µM range and clearly above LOD. The high glutamine and the low proline concentrations were noteworthy.

Table 2: Median amino acid concentrations in CSF

Analyte	LOD [µM]	Concentration [µM]
Arg	1.6	24.7
Gln	1.5	432
Gly	2.1	8.0
His	2.3	16.2
Met	0.5	5.9
Orn	1.9	6.7
Phe	0.3	12.9
Pro	0.6	1.2
Ser	1.2	24.5
Thr	1.2	24.5
Trp	3.9	24.6
Tyr	1.2	11.7
Val	1.2	20.5
xLeu	0.7	19.5

Hexose

The hexose concentration was found to be 3.4 mM in pooled CSF in accordance with values from literature.

Table 3A: Median phosphatidylcholine diacyl (PC aa) concentrations in CSF

Analyte	LOD [nM]	Concentration [nM]
PC aa C30:0	54	54
PC aa C32:0	12	316
PC aa C32:1	18	219
PC aa C32:2	6	31
PC aa C34:1	24	2082
PC aa C34:2	27	238
PC aa C36:1	15	298
PC aa C36:2	48	274
PC aa C36:3	12	126
PC aa C36:4	15	252
PC aa C36:5	3	8
PC aa C38:1	24	25
PC aa C38:3	12	103
PC aa C38:4	18	256
PC aa C38:5	6	64
PC aa C38:6	9	80
PC aa C40:3	1	5
PC aa C40:4	3	17
PC aa C40:5	15	23

Glycerophospholipids and Sphingomyelins

The phosphatidylcholine and sphingomyelin concentrations in CSF (Tables 3 and 4) were in the nanomolar range for most analytes and dramatically lower compared with plasma concentrations (about 100-1000 fold). The determination of lipid concentrations in the kit is semi-quantitative due to the lack of specific standards. Despite the relatively low concentrations of these metabolites, 35 of the phosphatidylcholines and 10 sphingomyelins were above LOD in the pooled CSF. They can give valuable information on the lipid status in CSF. The concentrations of the 15 lyso-phosphatidylcholines, which were also determined in the kit, were below LOD for pooled CSF. However, in certain disease states a significant increase of lipids in the CSF can be expected (2, 6-7). This means more lipids could be above LOD as shown in Tables 3 and 4.

Dilution series of pooled human plasma

To further investigate the performance of the kit at the lower lipid concentrations observed in CSF, a dilution series of pooled human plasma was conducted. The plasma was diluted with PBS buffer down to 512-fold dilution, and the percentage of plasma was plotted versus the calculated concentration as shown in Figure 2. Generally, good linearity was observed for most glycerophospholipids and sphingolipids in the concentration ranges found in pooled human CSF. For example, the PC aa C36:1 (median concentration of 298 nM in CSF) exhibits good linearity down to 100 nM and the SM C18:1 (median concentration 82 nM in CSF) down to 30 nM. The FIA-MS/MS method exhibited an excellent dynamic range with metabolite concentrations ranging from below 20 nM (as for several lipids) to 3.4 mM (as for hexose).

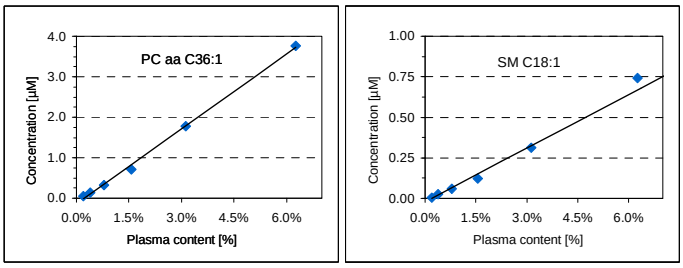

Figure 2: Dilution series of plasma
Pooled human plasma was diluted with PBS buffer and analyzed (n=6). The metabolites PC aa C36:1 and SM C18:1 were selected as representative examples.

Table 3B: Median phosphatidylcholine acyl-alkyl (PC ae) concentrations in CSF

Analyte	LOD [nM]	Concentration [nM]
PC ae C32:1	3	27
PC ae C34:0	3	22
PC ae C34:1	3	87
PC ae C34:2	3	68
PC ae C34:3	3	9
PC ae C36:1	9	38
PC ae C36:2	3	26
PC ae C36:3	3	15
PC ae C36:4	3	26
PC ae C36:5	3	35
PC ae C38:1	3	10
PC ae C38:2	3	14
PC ae C38:3	3	8
PC ae C38:4	3	24
PC ae C38:5	3	25
PC ae C38:6	6	12

Internal Standards

The kit's internal standards (IS) are essential for quantification. Therefore the signal intensity of the MRM pairs of the IS was evaluated against the values obtained for human plasma. This provided information about ion suppression in human CSF. In general, comparable intensities were found for the IS in CSF and plasma. Only the IS of the amino acid tryptophan and the acylcarnitine C8 showed an increase to about 150% of the plasma values. Both analytes should be considered as semi-quantitative for CSF, though they are listed as

quantitative in the Analytical Specifications for plasma samples.

Table 4: Median sphingomyelin concentrations in CSF

Analyte	LOD [nM]	Concentration [nM]
SM (OH) C14:1	9	14
SM C16:0	11	315
SM C16:1	3	38
SM (OH) C16:1	6	17
SM C18:0	27	393
SM C18:1	3	82
SM (OH) C22:1	6	27
SM (OH) C22:2	6	30
SM C24:0	54	76
SM C24:1	18	234

On-board stability

The on-board stability is defined as the time frame in which the kit plate can be processed after the sample material has been added (Kit plate stored at 4°C in the dark). The CSF showed similar stability as plasma (i.e. on-board stability is 12 h).

Analysis of patient samples/CSF Analytics

After the Absolute*IDQ* kit was evaluated and optimized with pooled human CSF, CSF and serum from patients with psychiatric and neurological diseases were analyzed. In many cases, CSF concentrations were clearly below serum concentrations. The results for the metabolites above LOD were evaluated based on standard methods in CSF analytics. All metabolites were evaluated for: 1) dependency of the CSF concentrations on the serum concentrations and 2) the blood/CSF barrier function. There is a relationship between CSF and serum concentrations for acylcarnitines, hexose and partly for amino acids, glycerophospholipids and sphingolipids. The blood/CSF barrier function, which is well described by the CSF/serum albumin ratio, influences the CSF concentration of acylcarnitines, hexose, glycerophospho-lipids and sphingolipids and partly of amino acids. A reasonable comparison of disease groups is only possible if these dependencies are taken into account. A publication including the presentation of the ratio evaluation for the blood/CSF barrier-dependent parameters is in preparation.

Conclusions

The data presented in this application note reveal that the Absolute*IDQ* Kit validated for human plasma can be successfully applied for human CSF. Some slight changes are necessary in the kit preparation and data analysis. These changes partly compensate for the lower metabolite concentrations found in CSF.
Compared to plasma, significantly lower concentrations were found in CSF especially for the glycerophospho-lipids and sphingolipids. However, 45 of these lipids were above LOD in pooled CSF and should give valuable information on the lipid status.
In summary, the data show the potential of a targeted metabolomics analysis of CSF and its impact on the study of CNS disorders.

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