Exploration of micro-LC/HRMS for quantification of low abundant endogenous truncated amyloid beta peptides in human cerebrospinal fluid

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Introduction

Glutaminyl cyclase (QC) might be involved in the Alzheimer's disease (AD) pathology, as it is essential for the formation of pyroglutamate-amyloid beta (pE-Aβ) peptides, which are regarded as important toxic culprits (see Figure 1). pE-Aβ formation depends on the QC activity and on the availability of enzyme substrates, which are the N-terminally truncated Aβ peptides Aβ 3-40/42 and Aβ 11-40/42.

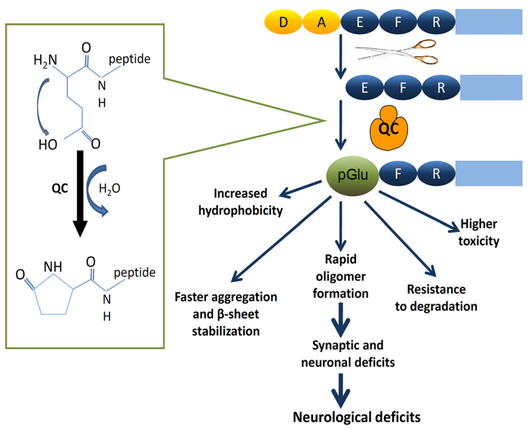

Figure 1: Generation of pyroglutamate-Aβ

The first N-terminal amino acids are cleaved off, exposing glutamate at position 3 and 11 of the N terminus of Aβ. Subsequently, glutamate is post-translationally modified to N-terminal pyroglutamate (pE) by QC activity [2].

A QC-inhibitor, PQ912, is currently in clinical development [1].

To monitor cerebrospinal fluid (CSF) levels of the QC substrates in clinical trials, a specific and sensitive LC-MS/MS method allowing simultaneous quantification of the N-terminally truncated Aβ variants in human CSF was established. The targeted MRM method using conventional chromatographic conditions after immuno-affinity-enrichment was found to be promising for the detection of endogenous levels of QC substrates in human CSF [3].

To further improve sensitivity and robustness for the lower abundant truncated Aβ peptides, a dedicated micro-2D-LC system was evaluated in comparison to conventional LC flow conditions in combination with HRMS.

Material and Methods

Reference Items:

Reference peptides and stable isotope labelled internal standards were obtained from Innovagen, JPT Peptide Technologies and rPeptide. Anti-Aβ-antibodies were purchased from Biolegend.

CSF samples:

Human CSF samples from early AD patients were collected during SAPHIR clinical trial (Eudra CT 2014-001967-11). The study was approved by local ethical review board and all subjects gave written informed consent for the use of their CSF for biomarker research.

Sample preparation and LC-MS Analysis

The Aβ peptides were immunoprecipitated from 300 µL human using anti-Aβ antibodies and anti-mouse IgG as described in [3]. The method was slightly modified and adapted for analysis in 96 well format. After 2 hours incubation on a shaker, the immuno-complex was coupled to magnetic beads. Beads were separated after three washing steps and peptides of interest eluted using 40 µL acetonitrile/water (75/25, v/v) containing 1% ammonium hydroxide. After dilution with water containing 1 % ammonium hydroxide, an aliquot of 50 µL was injected onto micro flow LC connected to a LTQ Orbitrap XL (Thermo Fisher Scientific). Instrumental details are summarized in Table1.

	Micro-flow setup	Regular flow setup
Ion Source	Prolab Microflow	HESI II
Loading Pump	Agilent 1200	
Analytical Pump	Prolab Zirconium Ultra	Agilent 1200
Trapping column	10 x 1 mm ID, C18 (Maisch)	
Analytical column/ Dimensions	BEH C18 (Waters) 150 x 0.3 mm ID	150 x 2.1 mm ID
Mobile phase A	Water + 0.2 % NH4OH	
Mobile phase B	Acetonitril/methanol/2-propanol, 70/20/10 (v/v/v) +0.1% NH4OH	
Trapping	2 minutes loading with 200 µL/min	
Analytical run time	12 minutes	8 minutes
Analytical flow rate	4 µL/min	400 µL/min
Injection volume	20 µL or 50 µL	20 µL

Table 1: Instrumental Set-up for LC-MS analysis

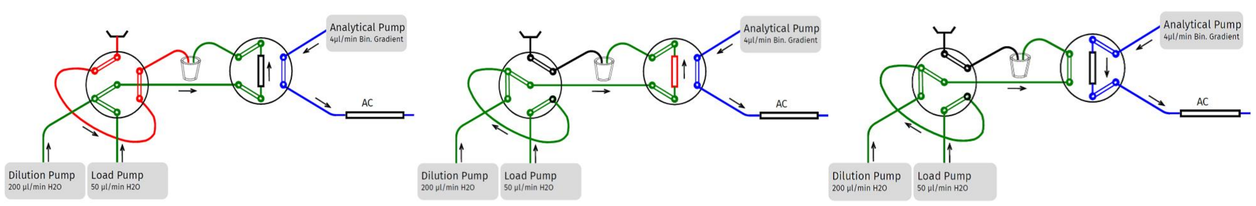

Figure 2: Schema of Micro-flow-2D-LC Set-up

Immunoassay Analysis

The human Aβ 3-Plex kit analyses Aβ(x-38), Aβ(x-40) and Aβ(x-42). The peptides are captured by C-terminal specific antibodies for the different Aβ species and detected by labeled 6E10. Assays were performed according to instructions of the manufacturer (MSD®).

Conclusion

A robust and highly sensitive assay for simultaneous quantification of endogenous QC substrates together with the full-length Aβ variants in human CSF was qualified and successfully applied in clinical trial sample analysis.

The application of micro-LC in instead of conventional LC revealed a gain in sensitivity of a factor of 20. Using this LC-Set-up in combination with HRMS, endogeneous QC substrates Aβ 3-40, Aβ 11-40 and Aβ 11-42 could be quantified along with full length peptides Aβ 1-40/42 in human clinical CSF. Aβ 3-42 was not detectable.

For the full-length Aβ peptides the mass spec data were highly correlating (r> 0.87) with immunoassay data.

Results

Assay Development and Optimization

A suitable LC-MS/MS approach for absolute quantification of Aβ 1-40 and Aβ 1-42 was previously described by Lame et al. [4] and is currently under validation as a clinical standard reference method [5]. We applied and further adapted this analytical approach including also the N-terminally truncated peptides Aβ 3-40, Aβ 11-40, Aβ 3-42 and Aβ 11-42. In contrast to Lame et al. [4], and due to the much lower abundance of the N-terminally truncated peptides, immuno-affinity enrichment instead of solid phase extraction was applied.

A targeted MRM method using conventional chromatographic conditions was established first [3]. As the detected level of Aβ 11-42 in CSF were found to be close to LLOQ, a dedicated micro-LC-Set-up (see Figure 2) was evaluated to improve assay sensitivity. Comparing conventional LC flow vs micro-flow on the same mass spec instrument, an increase in sensitivity by a factor of 20 was achieved (see Figure 3), which was close to the theoretically expected value of a factor 49 (ratio of the squares of the inner diameters).

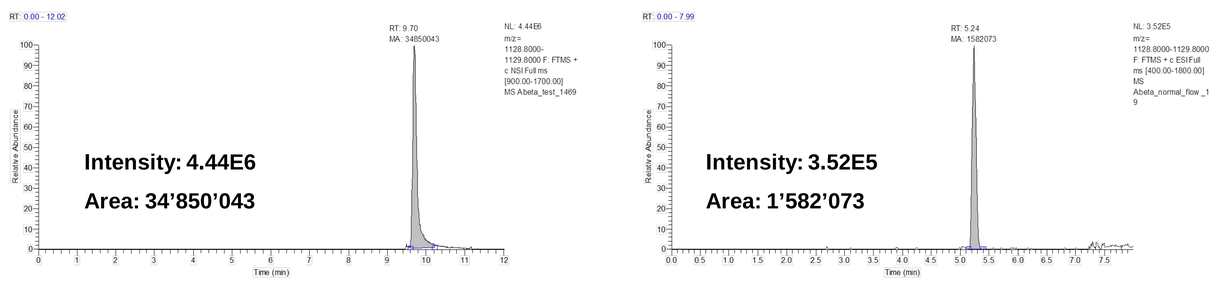

Figure 3: Extracted ion chromatogram of Aβ 1-42 of a standard sample, micro-flow set-up (left), regular flow set-up (right)

Application for Clinical Sample Analysis

A chromatogram of all detectable Aβ variants human CSF is shown in Figure 4.

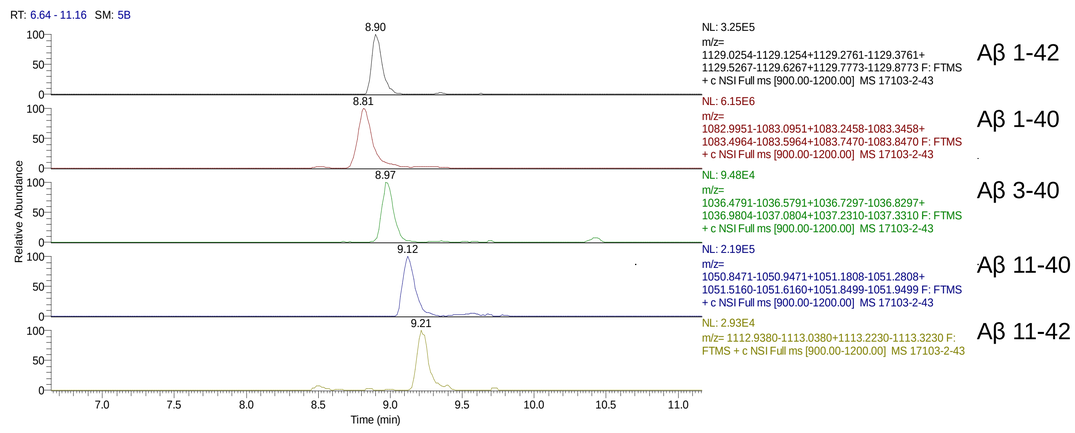

Figure 4: Extracted ion chromatograms of QC substrates detected in human CSF

Internal standardization with stable isotope-labeled peptides reveals the chance for absolute quantification, but we and others [5] discovered, that determining the exact peptide content of Aβ peptides appears to be difficult, due to potential chemical modifications, non-specific binding and tendency for aggregation. Therefore, a relative quantification approach, considering the relative response of analyte versus internal standard, was applied for group comparison within this study. Nevertheless, calibration curves from spike-in of reference peptides into artificial CSF were prepared for each analytical batch and found to be linear within the anticipated concentration range (see examples in Figure 5).

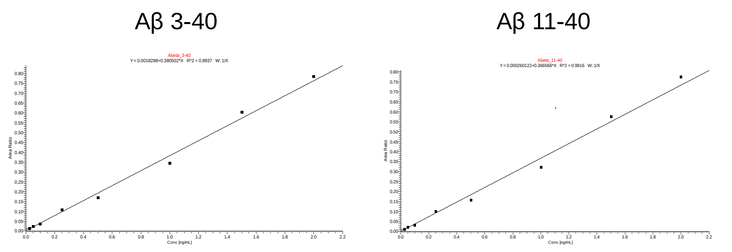

Figure 5: Calibration Range for Aβ 3-40 and Aβ 11-40

Analyte	CV%		
	QC1	QC2	QC3
Aβ 1-40	9.5	5.2	6.8
Aβ 3-40	2.2	7.2	6.2
Aβ 11-40	4.6	5.5	8.8
Aβ 1-42	3.5	4.4	7.3
Aβ 11-42	3.2	4.6	7.2

Table 2: Inter-assay precision of QC samples

LLOQs for the low abundant Aβ variants were qualified with 25 pg/mL. Considering a peptide content less than 50% for the truncated peptides the LLOQs can be considered as even better.

Finally, a cohort of clinical samples were analyzed within 4 valid analytical batches. Intra- and Inter-assay precision was assessed from repeated analysis of QCs samples prepared from 3 different pools of CSF samples. Inter-assay precision ranged between 2 and 10 % (see Table 2).

LC-HRMS assay vs Immunoassay

The comparison of LC-HRMS data with immunoassay for the full-length Aβ revealed a high correlation as shown in Figure 6 (Pearsons r = 0.87), supporting that both methods are valid for quantification of Aβ. Similar results were previously reported, also by other groups [3,5].

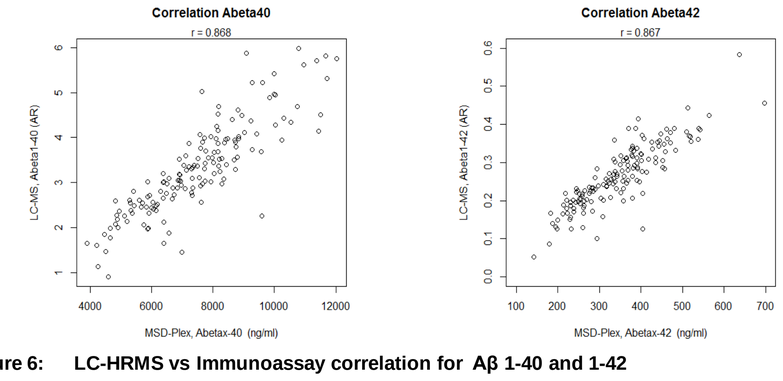

Figure 6: LC-HRMS vs Immunoassay correlation for Aβ 1-40 and 1-42

References

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Acknowledgements

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