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A multiplex targeted Mass spectrometry approach for the quantification of synuclein proteoforms in human biological fluids

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1. Overview

Innovative multiplex and targeted mass spectrometry method development for the quantification of the alpha, beta and gamma synuclein in biological samples as CSF and plasma in the context of neurodegenerative diseases.

2. Introduction

In the synucleopathy field, one of the main goals remains to discover biomarkers allowing to discriminate between Parkinson's disease, Lewy Body dementia or Multi System Atrophy. For this purpose, alpha synuclein has been intensively studied due to its major presence in aggregates such as Lewy bodies, hallmark of these neurodegenerative diseases [Vinnakota *et al*, 2018; McLean *et al*, 2012]. Alpha synuclein and its proteoforms are present at different levels in brain and CSF indicating that they might be relevant biomarkers. [Schmid *et al*, 2013; Otto *et al*, 2019; Zetterberg *et al*, 2019]. In this context, our objective is to develop a mass spectrometry multiplex method to quantify proteoforms of the synucleins family (alpha, beta and gamma) in human biological fluid (CSF, plasma).

Alpha synuclein sequence:

(ac)MDVFMKGLSKAKEGVVAAAEKTKQGVAEAAGKTKEGVLYVG
SKTKEGVVHGVATVAEKTKEQVTNVGGAVVTGVTAVAQKTVEG
AGSIAAATGFVKKDQLGKNEEGAPQEGILEDMPVDPDNEAYEMP
SEEGYQDYEPEA

Beta synuclein sequence:

MDVFMKGLSMAKEGVVAAAEKTKQGVTEAAEKTKEGVLYVGSKT
REGVVQGVASVAEKTKEQASHLGGAVFSGAGNIAAATGLVKREEF
PTDLKPEEVAQEAAEEPLIEPLMEPEGESYEDPPQEEYQEYEPEA

Gamma synuclein sequence:

MDVFKKGFSIAKEGVVGAVEKTKQGVTEAAEKTKEGVMYVGAKT
KENVVQSVTSVAEKTKEQANAVSEAVVSSVNTVATKTVEEAENIAV
TSGVVRKEDLRPSAPQQEGEASKEKEEVAEEAQSGGD

Figure 1. LC-MRM tryptic peptides (in color) followed in biofluids, (ac): acetylated

3. Methods

Targeted MRM approach without immunocapture was developed (Figure 2). Protein precipitation followed by a protein clean up and proteolytic digestion were performed for the sample treatment before LC-MRM analysis. 16 peptides were monitored from the synuclein family, as well as hemoglobin peptide which are used to check blood contamination in CSF (Table 1). Data analysis was realized with the Skyline software.

HPLC conditions (Nexera Mikros)

Column: ZORBAX SB-Aq (1 x 150 mm, 3,5 µm, Agilent T.)
Mobile phase A: water + 0,1% FA; B: ACN + 0,1% FA
Flow rate: 50 µL/min
Time program: B conc.0%(1.5 min) -30%(1.5-31.5 min) - 90%(31.6-33.6min) - 5%(33.6-40min)
Injection vol.: 10 µL
Column temperature: 35°C

MS conditions (LCMS-8060)

Ionization: ESI, Positive mode, scheduled MRM

4. Results

4-1. LC-MRM Method development for synuclein

We choose to follow most of the proteotypic alpha synuclein peptides except the C-ter peptide, representing around 70% of protein sequence. All the peptides and transitions were selected based on observed sensitivity and selectivity in biological sample compared to the spiked internal labelled standards. C-ter peptide containing a long residue chain (40 aa long) and composed of many acidic residues, MS sensitivity analysis was under the LOD/LOQ in biological sample. The same procedure was used to select the beta and gamma synuclein peptides to be followed. MS parameters (collision energy, applied voltage on quadrupoles) were optimized accordingly (Table 1). Separation conditions (LC) were optimized using different mobile phase composition (ACN, water, FA and DMSO). Sample preparation (protein precipitation, protein clean up and digestion) was also optimized:

• Protein precipitation protocol was previously optimized and described by [Bros et al, 2015].

• Protein clean up (RPW cartbridges) was optimized using different amount of ACN (washing and elution step)

• Digestion was optimized (concentration of the Tryspsin/lysC, time of digestion, use of other enzyme: as Lys C, Lys N and Glu-C) to enhanced the sensitivity of the alpha synuclein peptides detection (data not shown).

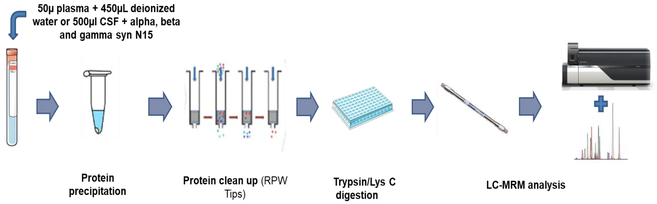

Figure 2. Workflow of the LC-MRM analysis of synuclein in biological sample

Table 1. MRM transition and MS parameters of synuclein

Protein name	Peptide sequence of synculein	Position on sequence	Charge (z)	Precursor ion	Product ion	Collision Energy (V)	Retention time (min)
alpha and beta synuclein common peptide	(ac)MDVFMK	1 - 6	2+	406.69	639.3 524.3 425.2 535.2 666.3	13	27.2
	EGVVAAAEK	13 - 21	2+	437.49	588.7 456.5 527.6 598.7	19	11.1
	QGVAEAAGK	24 - 32	2+	415.96	702.8 546.6 485.5 556.6 627.7	18	8.5
Proteotypic alpha synuclein peptide	EGVLYVGSK	35 - 43	2+	476.55	823.0 666.8 562.6 661.8 805.9	21	16.1
	EGVVHGVATVAEK	46 - 58	2+	648.35	618.35 522.27 579.29 678.36	30	13.8
	EQVTNVGGAVVTGVTAVAQK	61 - 80	2+	643.73	875.0 617.7 736.4 679.3 656.7 792.4	31	22
	TVEGAGSIAAATGFVK	81 - 96	2+	740.34	1022.2 764.9 693.8 622.7 551.7 450.6	29	22.1
Proteotypic beta synuclein peptide	TREGVVQGVASVAEK (missed 1)	44 - 58	3+	510,61	604,3 533,3 572,3 543,8 543,3 542,8	17	15.8
	EGVVQGVASVAEK	46 - 58	2+	636,84	987,6 760,4 703,4 669,4	25	17.7
	EQASHLGGAVFSGAGNIAAATGLVK	61 - 85	3+	776,08	1014,6 957,6 843,5 851,4 950,5 834,4	20	25
Proteotypic gamma synuclein peptide	EGVVGAVEK	13 - 21	2+	444,25	602,4 503,3 513,3	18	12.8
	QGVTEAAEK	24 - 32	2+	466,74	804,4 747,4 648,3 547,3 515,3 586,3	22	9.2
	TKENVVQSVTSVAEK (missed 1)	44 - 58	3+	540,29	820,4 634,3 572,3	19	17
	ENVVQSVTSVAEK	46 - 58	2+	695,36	1047,6 948,5 820,4 733,4 756,4	28	20
	EQANAVSEAVVSSVNTVATK	61 - 80	3+	668,68	1005,6 906,5 819,5 732,4 700,3 829,4 900,4	24	22.7
	TVEEAENIAVTSGVVR	81 - 96	2+	837,44	1015,6 788,5 717,4 618,4 786,9	35	21.3
Hemoglobin (sub alpha unit) proteotypic peptide	TYFPHFDLSHGSAQVK	42 - 57	3+	611,97	412,2 646,3 793,4	44	22.1

4-2. LC-MRM analysis in biological samples

7 peptides all along the alpha synuclein in blood plasma and 5 in CSF were detected, allowing to cover 70 % of the protein sequence in plasma and 55% in CSF. Despite that three of the alpha synuclein peptides seen are common with the beta synuclein, one unique peptide for the total beta synuclein was detected in CSF and plasma. For the gamma synuclein two peptides were observed in plasma covering 35% of the protein sequence. For the first time at our knowledge, 5 peptides all along the gamma synuclein were observed in CSF covering more than 50% of the total gamma synuclein sequence. The LC-MRM chromatograms obtained are illustrated in figure 3, 4 and 5.

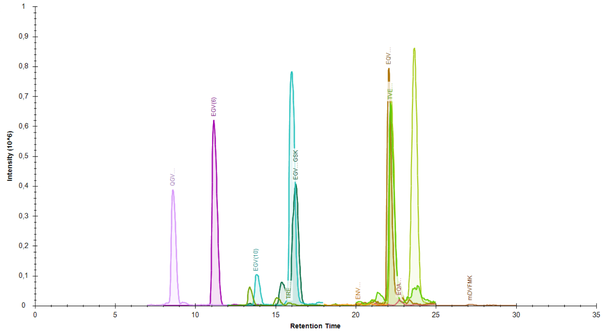

Figure 3. Represent the LC-MRM chromatograms extracted from Skyline software of the synuclein peptides in plasma. From left to right, peptide name are QGVAEAAGK, EGVVAAAEK, EGVVHGVATVAEK, TREGVVQGVASVAEK, EGVLYVGSK, ENVVQSVTSVAEK, EQVTNVGGAVVTGVTAVAQK, TYFPHFDLSHGSAQVK, TVEGAGSIAAATGFVK, EQANAVSEAVVSSVNTVATK, (ac)MDVFMK respectively.

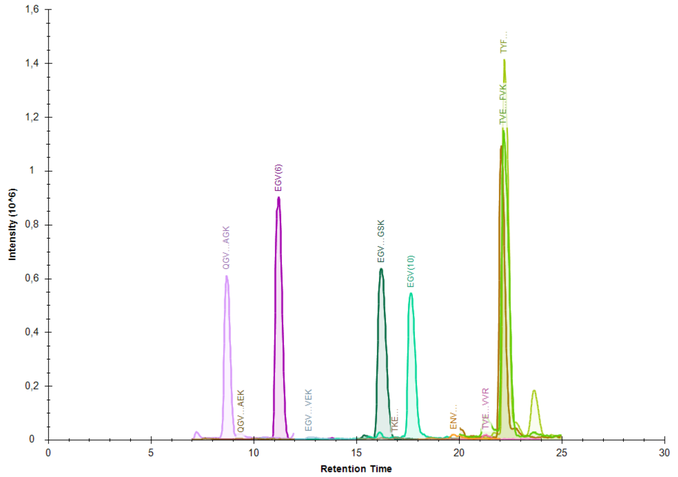

Figure 4. Represent the LC-MRM chromatograms extracted from Skyline software of the synuclein peptides in CSF. From left to right, peptide name are QGVAEAAGK, QGVTEAAEK, EGVVAAAEK, EGVVGAVEK, EGVLYVGSK, EGVVQGVASVAEK, ENVVQSVTSVAEK, TVEEAENIAVTSGVVR, EQVTNVGGAVVTGVTAVAQK, TVEGAGSIAAATGFVK, TYFPHFDLSHGSAQVK respectively.

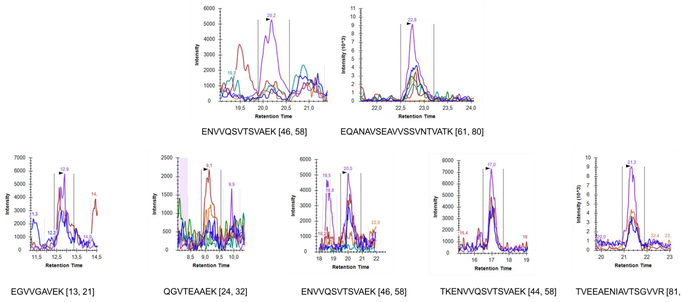

Figure 5. Represent a zoom on LC-MRM chromatograms extracted from Skyline software of the gamma synuclein peptides. In first line we have the gamma synuclein peptides detected in plasma and in CSF in the second line.

5. Conclusions

• Synuclein LC-MRM method was developed without immunocapture and anytical validation following EMA guidelines is currently ongoing (LOD, LOQ, LOB, repeatability, reproducibility, matrix effect…).

• Clinical validation will be performed based on a well stratified patients cohort (n= 200) with synucleinopathies and Alzheimer's disease.