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Disposable Pipette Extraction Followed by Direct MS/MS Analysis of Beta Amyloid Peptides (Aβ38, Aβ40, and Aβ42) in Cerebrospinal Fluid Samples

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Alzheimer's disease (AD) is a neurodegenerative disorder characterized by extracellular accumulation of amyloid-β (Aβ) peptides in the brain. This study demonstrates the direct association of the disposable pipette extraction (DPX) with tandem mass spectrometry (MS/MS) for the analysis of Aβ peptides in cerebrospinal fluid (CSF) samples. Different parameters were optimized in order to improve detectability in the MS/MS including mobile phase percentage of ammonium hydroxide, mobile phase flow rate and acquisition mode. Also, this method used an electrospray ionization (ESI) low-flow probe and direct infusion of an organic solution in the MS/MS. The DPX-MS/MS method showed adequate linearity for determining Aβ peptides in CSF-linearity ranged from 0.1 to 1.5 ng mL^{-1}. The coefficients of determination were higher than 0.99; the precision coefficient of variation (CV) ranged from 0.3 to 12.7%; and the accuracy relative standard deviation (RSD) ranged from −13.6 to 13.2%.

Keywords: disposable pipette extraction, MS-MS analysis, amyloid beta peptides, Alzheimer's disease

Introduction

Amyloid beta peptides have been consolidated as reliable biomarkers for Alzheimer's disease (AD).[1] However, quantitative analysis of amyloid-β (Aβ) peptides in biological samples remains a challenge because both the sample and the physical-chemical properties of these peptides are complex.[1]

Different methods such as enzyme-linked immunosorbent assay (ELISA) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) have been reported for analyzing Aβ peptides in biological samples. Immunoassays are sensitive, but also time-consuming and expensive, require highly specific antibodies and reagents, present cross-reactivity among peptides and poor dynamic range, and are very sensitive to matrix interferences.[2] Nevertheless, MS/MS-based approaches can overcome many of the problems associated with immunoassay methods and are considered a reliable tool for Aβ peptides analysis in clinical routine care.[2] In this context, over the last decades many studies have been reported the analysis of Aβ peptides in biological samples such as cerebrospinal fluid, plasma, and blood samples by using MS-based approaches.

Determination of Aβ peptides in peripheral fluids (e.g., blood) is challenging because human serum albumin binds 95% of Aβ in blood plasma, thus their concentration (approximately 30 pg mL^{-1}) is 100-fold lower than in cerebrospinal fluid (CSF).[3] CSF is proved to be a source for detecting biomarkers related to AD because CSF is in close proximity to the brain, and therefore biochemical changes in the brain directly affect the composition of biomarkers in CSF.[4]

Developments in sample preparation techniques such as immunoprecipitation,[3,5-13] 96-well plate μSPE (micro solid-phase extraction),[14-20] online SPME (online solid-phase microextraction),[21-23] and offline SPME[2,24] have enabled not only biological samples to be enriched with Aβ peptides, but also interferents to be removed from the sample matrix (sample cleanup).[1] Immunoprecipitation is a highly selective enrichment strategy that uses anti-Aβ peptide antibodies covalently bound to a solid support such

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as biopolymeric particles, magnetic beads, or membranes which allows the preconcentration of Aβ peptides in challenging matrices such as plasma samples.[8] Variations of the SPME technique (96-well plate and offline and online modes) have been widely used for the quantification of Aβ peptides. When compared to the immunoassays, the 96-well plate SPME technique is less selective which makes the Aβ peptides quantification in blood samples still a challenge. However, the 96-well plate SPME technique has been successfully applied for preconcentration of Aβ peptides in CSF samples by using different polymeric sorbents containing hydrophilic-lipophilic-balanced (HLB) particles,[16] anion exchange groups (MAX)[18] or strong cation exchange groups (MCX).[14,15,17,19,20,23] The MCX sorbent presents some advantages over the HLB and MAX sorbents because the elution solution (alkaline pH) ionized the Aβ peptides under the positive ESI mode (MS/MS system).[1]

In 2014, Korecka *et al.*,[23] reported in the literature an enrichment strategy combining 96-well plate SPME with online SPME for the analysis of Aβ peptides in CSF samples. This strategy provided a better sample cleanup, increasing both the extraction efficiency of the method and the analytical column lifetime when compared to the enrichment procedure that used only Oasis® MCX 96-well plate SPME reported by Lame *et al.*[20] Regarding the offline SPME, an innovative fiber-in-tube SPME capillary was developed to preconcentrate Aβ peptides in both artificial CSF and human plasma samples.[21] In this work, an innovative fiber-in-tube SPME combining the tensile strength of nitinol supports and the selectivity/and robustness of crosslinked zwitterionic polymeric ionic liquid (PIL) coating was synthesized in the inner surface of a fused silica capillary. In another study, this same capillary was directly coupled to an MS/MS system equipped with a low-flow electrospray ionization (ESI) probe which provided sensitive detection of Aβ peptides at trace levels in CSF samples without the need for chromatographic separation.[2] Later, the same research group also developed a strong cation-exchange (sulfopropyl methacrylate-*co*-ethylene glycol dimethacrylate) monolithic capillary that was successfully applied to determine the Aβ38, Aβ40, and Aβ42 concentrations in CSF samples from patients with AD.[24]

Regarding sample preparation techniques, disposable pipette extraction (DPX) is a modification of conventional solid-phase extraction developed by Dr William Brewer.[25] DPX reduces the extraction time and quantity of solvents employed during analysis.[25] DPX is based on a dynamic mixture between the matrix and the sorbent (extraction phase), which allows analytes to be rapidly and effectively extracted and provides vigorous sample clean-up.[26]

Different solid phases are commercially available for DPX tips, and they can be used to extract numerous analytes in different samples. Examples of these phases include (*i*) reversed-phase sorbent (DPX-RP) for nonpolar and slightly polar compounds; (*ii*) strong cation-exchanger sorbents (DPX-SCX) for cations and nonpolar compounds; and (*iii*) weak anion exchanger sorbent (DPX-WAX) for anions and nonpolar compounds.[27] The Oasis MCX cartridges (Waters Corp., Milford, MA) contain a mixed-mode cation-exchange sorbent based on a poly(divinylbenzene-*co*-*N*-vinylpyrrolidone) backbone added with sulfonic acid groups that allow cations to be retained. This water-wettable sorbent is promoted because it can retain both polar and nonpolar compounds and remain stable from pH 1 to 14.[28]

Despite the advances in MS/MS-based methods, Aβ peptide quantification remains challenging because these analytes are present at extremely low endogenous concentrations in CSF, tend to aggregate, and undergo nonspecific binding to surfaces.[2] Although MS/MS method exhibits high-throughput performance, the chromatography separation increases the selectivity electrospray ionization (ESI) that decreases the matrix effect.[29]

This study describes the development of the DPX-MS/MS method for determining Aβ peptides in CSF samples by using a commercial extraction phase (Oasis MCX®) and direct analysis in the MS/MS system without requiring the chromatographic separation. On the basis of concentrations described in previous papers, the DPX-MS/MS method presented limit of quantification (LOQ) adequate for analyzing Aβ peptides in CSF samples obtained from AD patients.

Experimental

Reagents and analytical standards

Synthetic human Aβ peptides ($Aβ_{1-38}$, $Aβ_{1-40}$, and $Aβ_{1-42}$) and nitrogen-15 stable-isotope-labeled human Aβ peptides [$^{15}N_{51}$] $Aβ_{1-38}$, [$^{15}N_{53}$] $Aβ_{1-40}$, and [$^{15}N_{55}$] $Aβ_{1-42}$ were purchased from rPeptide (Athens, USA). Polypropylene Protein LoBind® tubes and LoRetention pipette tips were acquired from Eppendorf (Hamburg, Germany). Acetonitrile (ACN), formic acid (> 98%), ammonium hydroxide (ACS reagent 28-30% NH_4OH basis) and guanidine hydrochloride (> 99%) were purchased from Sigma-Aldrich (São Paulo, SP, Brazil). Aqueous solutions were prepared with ultrapure water from a Milli-Q, Millipore system (18.2 MΩ cm) (São Paulo, Brazil). Oasis MCX® was purchased from Waters Corporation (São Paulo, Brazil).

Preparation of Aβ peptide stock solutions

Stock solutions of the Aβ peptides Aβ38, Aβ40, and Aβ42 and stable-isotope-labeled internal standards (ISs) were prepared at 0.1 mg mL^{-1} in 0.1% aqueous NH_4OH solution (to prevent the peptides from aggregating *in vitro*). The standard solutions were prepared by diluting the stock solutions with ACN/aqueous NH_4OH solution (20:80, v/v). All the prepared solutions were aliquoted and stored at −80 ºC in polypropylene protein LoBind tubes to avoid stability issues.[30-34]

Artificial and biological samples

A blank artificial CSF matrix was prepared on the basis of published study[35] as follows: 234 mM sucrose, 3.6 mM KCl, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 12.0 mM glucose and 25.0 mM $NaHCO_3$.[35] The composition of the artificial CSF that was chosen is commonly used by many laboratories for behavioral and physiological studies.[36-39]

Rat plasma samples were provided by the Department of Basic and Oral Biology, Dental School of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, Brazil and approved by the Local Committee (process No. 15668/2014). After collected, the samples were transported on ice and stored at −80 °C.

Sample pre-treatment

An aliquot of artificial CSF sample (150 µL) containing 5% of rat plasma and spiked with 30 µL of the ISs (0.5 ng mL^{-1}) was added to an Eppendorf containing 120 µL of guanidine hydrochloride solution (5 mol L^{-1}). This tube was vortexed for 60 s and shaken at 30 °C for 1 h. Then, 150 µL of 10% aqueous formic acid was added, and the tube was vortexed. Next, 450 µL of diluted sample was submitted to the DPX procedure.

DPX procedure

DPX device was prepared consisting of a polypropylene tip (1 mL) containing 60 mg of MCX phase freely accommodated between two polytetrafluoroethylene (PTFE) porous filters (manually inserted) and connected to a polypropylene syringe (5 mL). The MCX tips were initially conditioned with methanol (1 × 200 µL) and 10% aqueous formic acid (1 × 200 µL). The sorption process was assessed by repeatedly drawing and ejecting the CSF sample through the tip (5 × 450 µL). The clean-up step was performed with 200 µL of 10% aqueous formic acid/methanol (95:5, v/v). The analytes were eluted with 300 µL of methanol/10% NH_4OH. The extract from the elution process was dried and reconstituted in 50 µL of the mobile phase (0.3% NH_4OH in water/ACN 50:50, v/v).

MS/MS conditions

The analyses were carried out on a Waters ACQUITY UHPLC instrument coupled to the Xevo® TQD tandem quadrupole (Waters Corporation, Milford, MA, USA) mass spectrometer equipped with a Z-spray source and low-flow ESI probe operating in the positive mode with selected reaction monitoring (SRM). The source and the operating parameters were optimized as follows: capillary voltage, 3.50 kV; desolvation temperature, 550 °C; and desolvation gas flow, 800 L h^{-1} (N_2, 99.9% purity). Argon (99.9999% purity) was used as collision gas. Fragments, cone energy, and collision energy were optimized for each analyte, as shown in Table 1. The mass transition with the highest intensity was used to quantify the analytes, whereas the mass transition with the second highest intensity was used as qualifier. Instrument control, peak detection, and integration were acquired by using the MassLynx V4.1 Data System.

Table 1. Ion transitions and instrument settings for each studied analyte

Analyte	Precursor ion (*m/z*)[a]	Product ion (*m/z*)	DP / V	CE / V
Aβ38	1033.2	1000.3	45	23
$^{15}N_{51}$-Aβ38[b]	1046.3	1012.1	45	22
Aβ40	1082.5	1053.5	55	25
$^{15}N_{53}$-Aβ40[b]	1096.3	1066.6	45	23
Aβ42	1128.5	1078.2	55	26
$^{15}N_{55}$-Aβ42[b]	1142.9	1090.7	50	24

[a]Precursor $[M + H]^{4+}$; [b]internal standard; DP: declustering potential; CE: collision energy.

The mobile phase consisted of (A) 0.3% NH_4OH in water and (B) ACN (50:50, v/v) with total analysis time of 2 min and direct infusion in the mass spectrometer of methanolic solution containing 10% NH_4OH. Instead of a chromatographic column, this DPX-MS/MS method used a zero-dead volume union connecting the autosampler and the mass spectrometer. Five microliters of sample were injected into the chromatographic system by the autosampler.

Analytical validation

Analytical validation was based on the international guidelines issued by the Food and Drug Administration.[40]

Artificial CSF containing 5% of rat plasma was used as a surrogate matrix. Linearity, accuracy, precision, carryover, and matrix effect (ME) were evaluated.

Linearity was evaluated by using six calibration curves (in triplicate), with a linear range from 0.1 to 1.5 ng mL^{-1}. The calibration curves were plotted by linear regression of the ratio between the Aβ peptides and the internal standard peak areas *vs.* the Aβ peptide concentrations (ng mL^{-1}). Coefficients of determination (R^2) greater than 0.99 were established as acceptance criteria.

Precision and accuracy were evaluated on the same day (intra-day) and three different days (inter-day); results are expressed as relative standard deviation (RSD) for accuracy and coefficient of variation (CV) for precision. For this assay, 15% of variation was defined as the acceptance criterion. The lower limit of quantification (LLOQ) was defined as the lowest concentration in the calibration curve that could be measured with acceptable precision and accuracy (within ± 20%).

To evaluate carry-over, three aliquots of the same blank artificial CSF were injected into the analytical system: one aliquot was injected before and two aliquots were injected after analysis of the upper limit of quantification (ULOQ). Carryover in the blank sample after injection of the ULOQ should not be higher than 20% of the analyte signal at the LLOQ and 5% of the IS signal.

The matrix effect was determined by using the normalized matrix factor (MF), by dividing the peak area of the analytes by the IS analytical response. The MF was expressed as a ratio of the MF in post-extraction spiked samples to the MF of the same analyte in standard solutions, multiplied by 100. The CV of the calculated MF should not exceed 15%.

Results and Discussion

DPX-MS/MS method

Some studies[41] detailed that the Aβ peptides signals obtained in the MS under alkaline conditions are about 10-fold higher than those obtained using an acid mobile phase. In this context, we observe that a higher percentage of NH_4OH in the mobile phase increased the analyte signal, improving the mass spectrometer ionization in the ESI+ source (Figure 1a).[42] Then, we evaluated that higher flow rates elevate system backpressure and impair ion formation in the ESI, thereby decreasing detectability (Figure 1b).

To improve detectability, we also evaluated the use of a low-flow ESI probe. Figure 1c shows the chromatographic peak areas of the Aβ peptides obtained with the DPX-MS/MS method by using conventional ESI (ESI source needle internal diameter (i.d.) = 120 μm) or the low-flow ESI probe (i.d. = 60 μm). The ESI source needle i.d. directly influences droplet size in the spray: larger diameters produce larger droplets, whereas smaller diameters produce finer droplets.[43] The low-flow ESI source produces finer droplets, which increases analyte ionization efficiency. Therefore, the low-flow ESI probe ensured more sensitive and reproducible analyses.

We also evaluated the comparison between the SIM (selected ion monitoring) and SRM acquisitions modes. Although the proposed method was more sensitive when SIM mode was used, quantification was irreproducible probably because the charged molecular ions did not provide adequate linearity for quantitatively determining the Aβ peptides. In turn, SRM mode proved to be better for quantitative determination of the analytes in complex samples (such as CSF) than SIM mode (Figure 1d). It can be explained because in the SIM mode is used only a single quadrupole, while in the SRM mode the triple quadrupole MS/MS allows enhanced sensitivity and more accurate and reproducible for quantitative analysis.

Because further adjustment of the mobile phase composition to improve MS/MS detectability was not possible (considering the preservation of the equipment), we evaluated the direct infusion in the MS probe of an organic solution containing NH_4OH (at constant flow rate of 10 μL min^{-1}) to enhance MS/MS ionization. According to the analytical signals obtained for all the three Aβ peptides, the direct infusion improved the sensitivity in MS/MS (Figure 1e) and also, ammonia in methanol provided higher detectability than ammonia in ACN (Figure 1f). This result is in accordance with some reports in the literature[44-46] that used direct infusion of different bases including ammonia and trimethylamine to raise the effluent pH to enhance ionization in MS sources. To perform the direct infusion, we used an infusion pump to deliver a constant amount of NH_4OH in methanol (10 μL min^{-1}) into the MS/MS probe (Figure 2). At the same time, the CSF samples extracts are injected into the autosampler under conditions previous described. This procedure is similar to the post-column infusion commonly used to access the matrix effects during analytical validation of chromatographic methods.

Sample pre-treatment optimization

Measuring Aβ peptides in CSF is still challenging-the concentrations of these peptides in CSF are low, not to mention that Aβ peptides are poorly soluble in water, can bind non-specifically to other peptides/proteins or to the walls of tubes and pipette tips, and tend to aggregate.[23]

During sample pre-treatment optimization, we evaluated the addition of rat plasma and high concentration

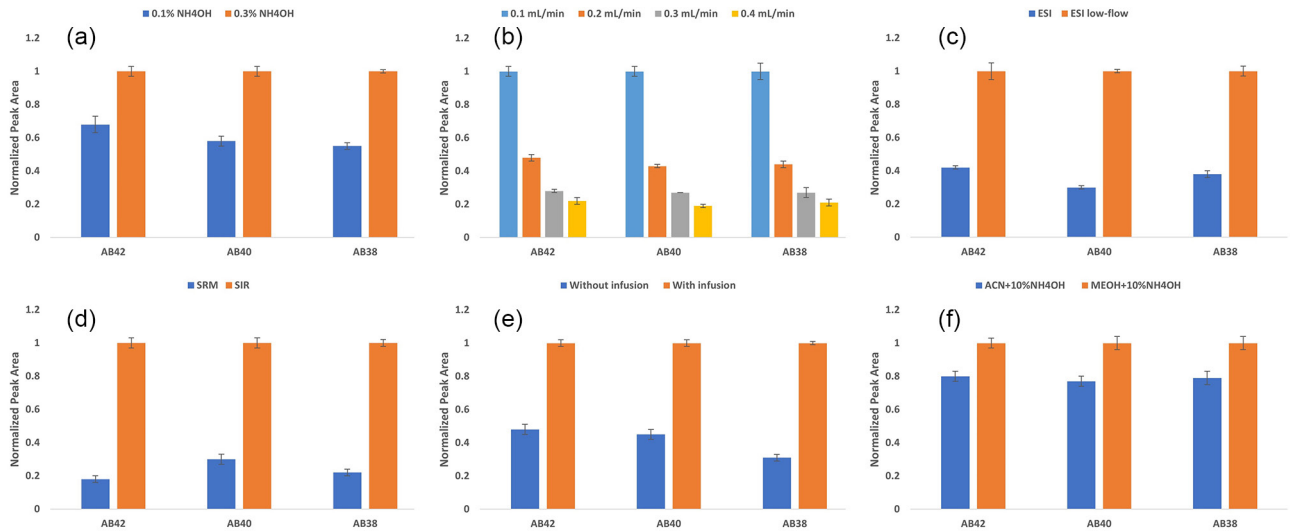

Figure 1. Optimization of the DPX-MS/MS method: (a) percentage of NH_4OH in the mobile phase; (b) mobile phase flow rate; (c) comparison between ESI and low-flow ESI; (d) acquisition mode; (e) direct infusion; (f) direct infusion solution.

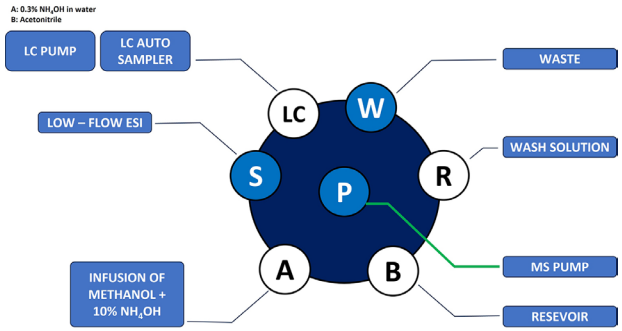

Figure 2. Scheme of direct infusion in the MS/MS system.

of guanidine hydrochloride in the artificial CSF samples in order to (*i*) decrease Aβ peptides (human) nonspecific binding to the CSF albumins,[20] (*ii*) cause the denaturation of Aβ peptides from various aggregated and oligomeric and polymeric forms to monomeric forms,[47,48] and (*iii*) improve MS/MS detection by minimizing matrix effects.

It is known that human serum albumin (HSA) binds highly to Aβ in biological fluids inhibiting plaque formation in peripheral tissue. Recently our research group[21] has published data indicating that approximately 28% (Aβ38), 33% (Aβ40) and 40% (Aβ42) was bound to HSA in artificial CSF samples. Based on this, we evaluated the addition of rat plasma in the artificial CSF samples to favor the binding of the HSA present in the artificial CSF sample with the rat Aβ peptides instead of the human Aβ peptides. In this way, we can increase the free fraction of Aβ peptides (human) for quantification.[20] Rat plasma was selected as a carrier because rat Aβ peptides differ from human Aβ peptides in terms of amino acid composition resulting in different molecular weights from human Aβ peptides. Consequently, rat Aβ peptides do not interfere in the MS/MS transitions of human Aβ peptides. Addition of different percentages of rat plasma (2.5, 5.0, or 7.5%) to artificial CSF sample was evaluated, and the best extraction efficiency was obtained with 5.0% of rat plasma (Figure 3a).

As shown in Figure 3b, addition of 5 mol L^{-1} guanidine to the artificial CSF sample provided the best result because it minimizes enzymatic activities and non-specific interactions between Aβ peptides and proteins present in the sample, allowing the concentrations of free Aβ peptides to be measured.

Moreover, the use of polypropylene protein LoBind® tubes and low retention pipette tips minimize the adsorption of the Aβ peptides to the walls of the plastic materials during the sample pre-treatment step allowing higher recovery rate of the analytes at the end of each extraction (Figure 3c).

Optimization of DPX conditions

Sorbent mass is an important parameter that influences the amount of analytes extracted. In this context, we evaluated masses of 60 and 100 mg of sorbent phase, but the results obtained were not satisfactory using masses higher than 60 mg because it makes more difficult the dynamic mixture of the sorbent phase in the artificial CSF sample. Also, by using smaller mass of sorbent, small amounts of sample and organic solvent were required in the extraction step. Based on this and considering the dimensions of the DPX tip available (1 mL), the DPX device was prepared containing 60 mg of sorbent mass (Oasis MCX®).

The DPX variables (sample pH and volume, sorption time and number of cycles, cleanup, and elution solution

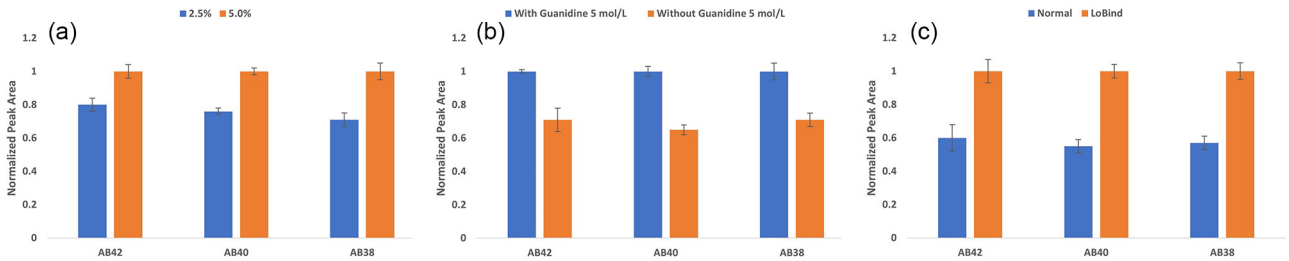

Figure 3. Optimization of the sample preparation (final concentration of 100 ng mL^{-1}): (a) percentage of rat plasma; (b) addition of guanidine hydrochloride 5 mol L^{-1}; (c) protein LoBind tubes and low retention pipettes tips.

and volume) were optimized to obtain good extraction efficiency and are discussed below (Figure 4).

Influence of medium modification on extraction efficiency

Sample pH directly affects analyte sorption during extraction. The Oasis MCX® sorbent, a mixed-mode polymeric sorbent, has been optimized to achieve higher selectivity and sensitivity for extracting basic compounds with cation-exchange groups. Aβ peptides have an isoelectric point of 5.2, therefore, these analytes are positively charged in acidic conditions and can interact with the sulfonic groups in the extraction phase through an ion-exchange mechanism. Thus, we evaluated CSF sample dilution with formic or phosphoric acid (Figure 4a), to achieve the best extraction efficiency when the sample was diluted with 10% aqueous formic acid solution (1:1, v/v). Dilution with formic acid also minimizes enzymatic activities and non-specific interactions of the Aβ peptides with the tube surface and CSF proteins.

Sample volume

The CSF volume was also optimized and the results obtained showed that a sample volume higher than 150 µL did not improve the extraction efficiency, possibly due to more pronounced matrix effect (Figure 4b).

Sorption time and number of draw-eject cycles

DPX is based on sorption of the analytes between the biological sample and extraction phase during their dynamic mixture, which favors sorption interactions of the analytes, thus making extraction more efficient. In this context, we evaluated the sorption time between the MCX phase and Aβ peptides at different times, and observed that extraction for 30 s provided the highest extraction efficiency for all the Aβ peptides (Figure 4c).

Assessing how draw-eject cycles affected the extraction efficiency revealed that recovery of the Aβ peptides increased linearly from one up to five extraction cycles (5 × 450 µL, Figure 4d).

We did not evaluate extraction times higher than 30 s or a larger number of draw-eject cycles (> 5) as they would increase the analysis time.

Clean-up

After Aβ peptides were adsorbed in the extraction phase, we evaluated different solutions to exclude endogenous

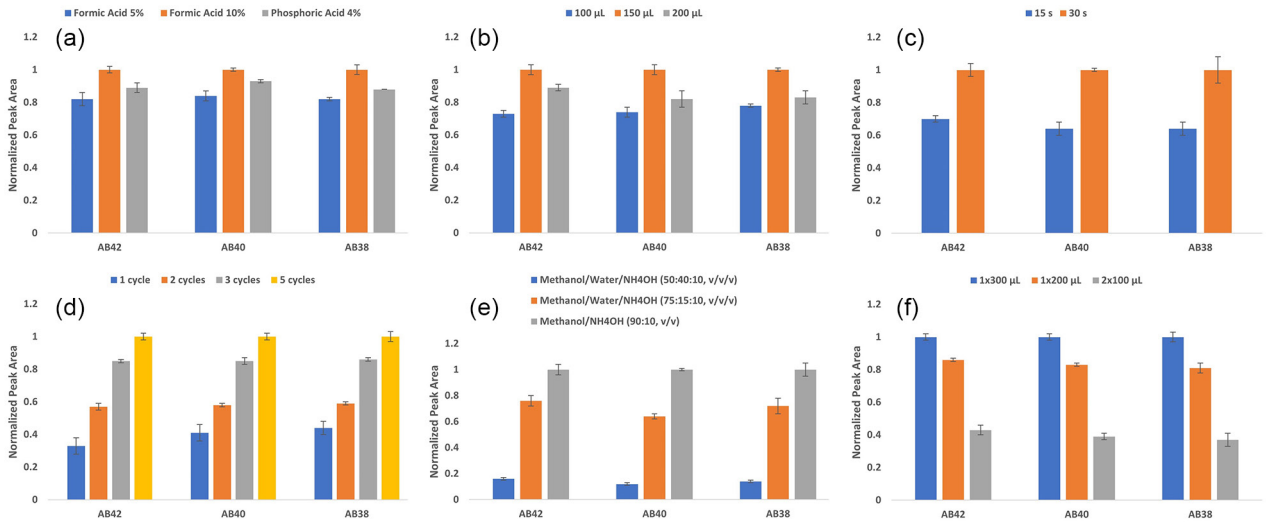

Figure 4. Optimization of the DPX-MS/MS method: (a) sample pH; (b) sample volume; (c) sorption time; (d) number of draw-eject cycles; (e) elution solution composition; (f) elution solution volume.

interferents (proteins and phospholipids) from the MCX phase. This procedure decreased the matrix effect in the CSF samples and favored analyte ionization during MS/MS analysis. On the basis of our published works,[2,24] we evaluated different solutions and carried out the clean-up with 200 µL of 10% aqueous formic acid/methanol (95:5, v/v), which was able to remove endogenous interferents from the MCX phase without the Aβ peptides being eluted.

Elution

To ensure that the Aβ peptides were completely eluted from the MCX phase, we evaluated different solutions (methanol/water/NH_4OH and methanol/NH_4OH) and draw/eject cycles (1 × 300 µL, 1 × 200 µL, and 2 × 100 µL) during the elution process. The extraction efficiency improved upon increasing percentage of methanol in the elution solution and total volume of the elution solution (Figures 4e and 4f, respectively). Furthermore, repeated cycles did not improve the extraction rate because of the total amount of MCX phase inserted in the DPX tip (Figure 4f). We also evaluated ACN, but this solvent provided lower efficiency than methanol. Thus, we employed 300 µL of elution solution (methanol/NH_4OH, 90:10, v/v) to elute the Aβ peptides.

At the end of each extraction, we discarded the MCX phase and ultrasonicated the tips with water and methanol, dried them, and filled them up with new unused MCX phase.

Analytical validation

The DPX-MS/MS method presented adequate linearity for all the Aβ peptides from the LLOQ to 1.5 ng mL^{-1} and coefficients of determination higher than 0.99. Table 2 shows the intra- and inter-assay accuracy of the proposed method. In addition, the reproducibility of the pipette extraction device can be evaluated according to the CV values obtained in each quality control (QC) sample. It is possible due to the fact that the sorbent phase was not reused between each performed extraction.

Considering the CSF concentrations of Aβ peptides reported in the literature,[15,16,19-21,23,24] the linear range of the DPX-MS/MS method was adequate, and this method can be used in future analysis for determining the concentrations of Aβ peptides in CSF samples from AD patients.

The matrix effect and the residual carry-over of the DPX MS/MS method were not significant and presented CV < 15%.

Figure 5 shows the analyte signals obtained by using the DPX-MS/MS method for analysis of an artificial CSF sample spiked with Aβ42, Aβ40, and Aβ38 at the LLOQ. Although the peaks for the three Aβ peptides are detected in the same time, the use of two different ion transitions in the MS/MS (SRM mode) for each analyte, allowed the highly selective detection and quantification of the Aβ peptides in the CSF sample without the chromatographic separation.[49]

Comparison of the proposed methods with literature methods

Table 3 illustrates de comparison between the DPX-MS/MS with other methods reported in the literature.

Although the 96-well plate used in the µSPE methods reported in the literature[15,19,20,23] presents a high analytical

Table 2. Analytical validation parameters for the DPX-MS/MS method

Analyte	Linearity / (ng mL^{-1})	QC / (ng mL^{-1})	Accuracy (RSD) / %		Precision (CV) / %	
			Intra-assay (n = 3)	Inter-assay (n = 3)	Intra- assay (n = 3)	Inter- assay (n = 3)
Aβ38	0.1-1.5	0.1	5.1	9.8	6.2	7.1
		0.2	10.0	10.3	1.2	0.9
		0.5	1.5	–4.5	8.1	10.2
		0.8	–9.6	–11.9	3.0	7.1
		1.0	2.4	6.7	8.6	6.3
		1.5	5.6	4.0	7.9	11.3
Aβ40	0.1-1.5	0.1	–0.3	–0.9	0.8	10.3
		0.2	13.2	8.0	0.3	0.6
		0.5	2.6	–0.3	4.1	4.0
		0.8	–9.0	–13.6	5.8	6.7
		1.0	2.5	6.2	8.6	5.6
		1.5	8.5	0.2	12.7	11.1
Aβ42	0.1-1.5	0.1	7.0	4.6	5.8	4.4
		0.2	12.1	12.1	0.3	7.6
		0.5	–2.7	0.2	6.8	4.9
		0.8	–8.7	–11.5	3.5	5.7
		1.0	12.1	5.4	10.0	9.7
		1.5	–3.3	1.4	6.8	7.0

QC: quality control; RSD: relative standard deviation; CV: coefficient of variation.

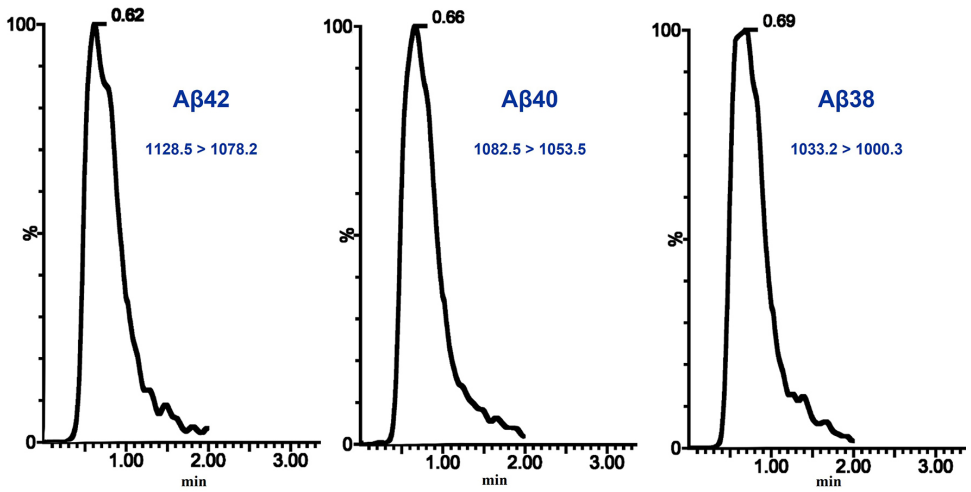

Figure 5. Analyte signals obtained by using the DPX-MS/MS method for analysis of an artificial CSF sample spiked with Aβ42, Aβ40, and Aβ38 at the LLOQ.

Table 3. Comparison between the DPX-MS/MS method with the literature

Analyte	Sample volume / µL	Sample preparation	Analytical system	LOQ / (ng mL^{-1})	Reference
Aβ38 Aβ40 Aβ42	200	online fiber in-tube SPME	MS/MS	0.2	2
Aβ38 Aβ40 Aβ42	200	MCX µSPE	LC-MS/MS	0.2	20
Aβ38 Aβ40 Aβ42	300	offline fiber in-tube SPME	LC-MS/MS	0.3	21
Aβ38 Aβ40 Aβ42	200	MCX µSPE	LC-MS/MS	0.3	19
Aβ38 Aβ40 Aβ42	218	MCX µSPE	LC-MS/MS	0.6	15
Aβ38 Aβ40 Aβ42	300	HLB µSPE	LC-MS/MS	0.4	16
Aβ42	250	MCX µSPE	2D-LC-MS/MS	0.6	23
Aβ38 Aβ40 Aβ42	200	in-tube SPME	LC-MS/MS	0.6	24
Aβ38 Aβ40 Aβ42	150	MCX-DPX	MS/MS	0.1	this work

LOQ: limit of quantification; SPME: solid-phase microextraction; LC-MS/MS: liquid chromatography-tandem mass spectrometry; MCX: strong cation exchange groups; HLB: hydrophilic-lipophilic-balanced; DPX: disposable pipette extraction; µSPE: micro solid-phase extraction.

frequency allowing the simultaneous preparation of 96 samples, these methods also present an elevated cost *per* extraction-two times more expensive than the DPX procedure. Also, the DPX-MS/MS method developed in this work used lower CSF sample volume when compared to the µSPE methods.

The in-tube SPME LC-MS/MS[24] method presents some advantages over the DPX-MS/MS method such as: lower cost of the extraction device (sixteen times cheaper), shorter sample preparation time (90% less time consuming) and reusability of the extraction phase. However, a higher CSF sample volume is needed and also the LOQ obtained was higher. Besides that, the sample cleanup of the sorbent phase (Oasis MCX®) used in the DPX-MS/MS method allows the removal of phospholipids present in the biological matrix resulting in lower matrix effect.

Compared to the offline methods,[21] the online SPME method[2] reduced the number of biological sample preparation steps, which diminishes the sources of error and the analysis time and cost. However, the cost associated with the extraction device (nitinol fibers) is also higher than the DPX tip.

Comparing the direct association of the DPX technique with MS/MS system in contrast to the LC-MS/MS methods,[15,16,19-21,23,24] it is observed a decrease in the toral run time and the reduced organic solvent consumption which puts DPX close to the principles of Green Analytical Chemistry.

Conclusions

The DPX-MS/MS method presented adequate analytical validation parameters for determining Aβ peptides in CSF samples because MS/MS provides high detectability and selectivity (SRM mode). Three procedures favored the detectability and sensitivity of the DPX MS/MS method: (*i*) direct infusion (organic solvent + additive) in the mass spectrometer, (*ii*) use of the ESI low-flow probe with reduced mobile phase flow rate, and (*iii*) sample pre-treatment with the addition of rat plasma and concentrated guanidine solution (to decrease the effect of the biological matrix). Optimizing the DPX variables (sample pH and volume, sorption time, number of cycles, and elution solution/number of cycles) improved the sensitivity of the method and required small volumes of biological sample and organic solvent. Also, the low time required to perform the extractions and its low cost and ease of operation can be highlighted. Future experiments will consist on the application of the DPX-MS/MS method for the determination of Aβ peptides in CSF samples obtained from AD patients for AD diagnosis and treatment monitoring.

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Author Contributions

Caroline F. Grecco was responsible for conceptualization, formal analysis, investigation, methodology, visualization, writing original draft; Eduardo Crevelin for formal analysis, methodology; Vitor Tumas for conceptualization, resources; Jaime Eduardo Cecilio Hallak for conceptualization, resources; Maria Eugenia Queiroz for conceptualization, methodology, resources, supervision, writing review and editing.

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