

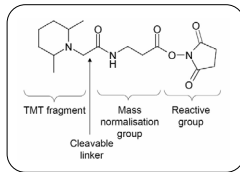
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Overview

1

A new 6-plex isobaric mass tagging technology was used and proof-of-principle studies were carried out using standard protein mixtures and human cerebrospinal fluid (CSF) samples. The Tandem Mass Tags[®] (TMT[®]) [1] comprise a set of structurally identical tags which label peptides on free amino-terminus and epsilon-amino functions of lysine residues (Scheme 1).



Scheme 1. Structure of the Tandem Mass Tag[®] (TMT[®]). The reporter ion provides the abundance of a peptide upon tandem mass spectrometry (MS/MS) in individual samples being mixed. The cleavable linker enables the release of the reporter ion from the whole tag. The mass normalization group balances mass differences from individual ion reporter to ensure the same overall mass of the label reagents. The reactive group labels amino groups.

On collision-induced dissociation each TMT[®] label releases a specific reporter ion of 126, 127, 128, 129, 130 or 131 Da in the MS/MS whose ratio is directly proportional to the concentration of the proteins in the respective samples. Using an artificial four protein mixture with theoretical abundance ratios 1:2:3:3.5:10, we observed protein ratios of 1.3:2.4:2.8:3.0:4.8:9.7 respectively (Figure 1) giving a mean quantitative precision of approximately 10%. The mean relative standard deviation (RSD) was 13.6% (range 6.7% to 20.3%) for the quantitation on each reporter ion determined across two independent experiments analyzed twice by MALDI TOF/TOF MS/MS ($n = 4$). In subsequent studies on ESI Q-TOF we have routinely observed RSD's of less than 10% for all reporter ions.

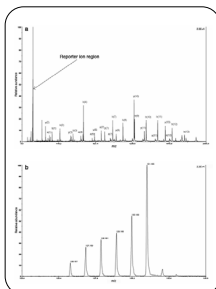


Figure 1. Tandem mass spectrum of peptide identified at $m/z = 1944.902$ Th, identified as LSPNFPTQLQEEQCHI of β -lactoglobulin, labeled with TMT[®] and iodoacetamide.

These quantitative values were obtained through a purity correction (Figure 2) that takes especially into account the isotopic purity of each TMT[®] label (natural occurrence of isotopes and impurities of incorporated isotopes).

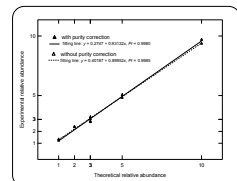


Figure 2. Calibration curve of the model protein mixture with six concentrations. The theoretical ratios are 1:2:3:3.5:10.

Introduction

2

The measurement of the different levels of protein expression in cells, tissues or body fluids is a major task in proteomics and is widely used for biomarker discovery. Proteins that are found to exhibit significant regulation in disease state samples are potentially biochemical markers of the considered pathologies with application ranging from diagnosis and prognosis to the monitoring of a biological response or therapeutic intervention.

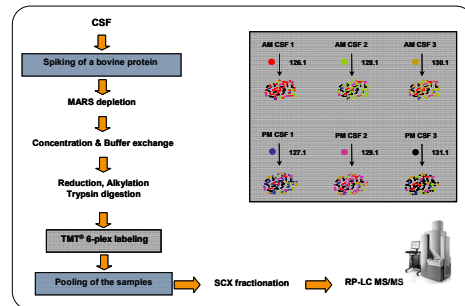
In this study, a new 6-plex isobaric mass tagging technology (so-called Tandem Mass Tag[®] or TMT[®]) was used for the quantitative comparison of proteins in *ante-mortem* (AM) and *post-mortem* (PM) cerebrospinal fluids (CSF). According to the fact that protein leakage associated with necrosis may share similarity with those occurring from brain lesions of neurological disorders, PM CSF has been shown to be a valuable source for the discovery of new potential brain damage biomarkers.

Methods

3

PM CSF samples from different patients ($n = 4$) were collected by ventricular puncture at autopsy, 6 h after death on average. Control AM CSF samples were collected by routine diagnostic lumbar puncture from living healthy patients ($n = 4$).

After immunoaffinity depletion liquid chromatography (LC), triplicates of AM and PM CSF pooled samples were reduced, alkylated, digested by trypsin and labeled respectively with the six isobaric variants of the TMT[®] with reporter ions from $m/z = 126.1$ to 131.1 Th (Scheme 2). The six samples were combined and fractionated off-line by strong cation exchange (SCX) LC. The contents of the SCX fractions were deposited on MALDI target plates following reversed-phase nano-LC and analyzed by MS/MS (4800 Proteomics Analyzer from Applied Biosystems). The analysis of the SCX fractions was also carried out by on-line nano-LC ESI MS/MS (Micromass Q-ToF from Waters).



Scheme 2. Analytical workflow followed for the CSF analysis.

The resulting peak files were searched against UniProt-Swiss-Prot/TrEMBL database (Swiss-Prot Release 54.4 of 23-Oct-2007; 287050 entries and TrEMBL Release 37.4 of 23-Oct-2007; 4988379 entries) using Phenyx (Gene Bio) and Mascot (version 2.2.03, Matrix Sciences) operating on a local server.

Results

4

In this CSF study, 91 proteins were identified with stringent criteria (e.g. at least 2 different peptide sequences matched per protein), of which 87 could be quantified. Reporter peak intensities were corrected for isotopic distribution and normalized according to an internal protein standard to calculate the average ratio of individual proteins in PM CSF/AM CSF.

The proteins that were found to be more concentrated in the PM CSF were classified in 3 categories. Seven proteins were shown to be massively increased in PM CSF (ratio PM CSF/AM CSF > 20). Seven proteins had a ratio between 10 and 20. Sixteen proteins had a ratio PM CSF/AM CSF between 3 and 10. Therefore, 30 proteins can be estimated to be highly concentrated in PM CSF. Interestingly, nearly all of these 30 proteins have already been identified in previous studies on PM CSF [2], strongly supporting the validity of the findings reported here.

Several of these proteins have been previously described as brain damage biomarkers.

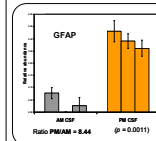


Figure 3. MS/MS quantification from intra-run triplicate AM and PM CSF samples for GFAP using isobaric tagging.

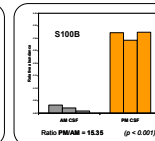


Figure 4. MS/MS quantification from intra-run triplicate AM and PM CSF samples for protein S100B using isobaric tagging.

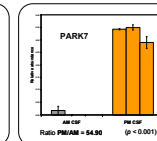


Figure 5. MS/MS quantification from intra-run triplicate AM and PM CSF samples for PARK7 using isobaric tagging.

The concentration comparison of glial fibrillary acidic protein (GFAP), protein S100B and PARK7 in AM and PM CSF is shown in Figures 3-5, respectively. The level of GFAP was previously assessed in PM CSF by 2D gel electrophoresis immunoblot assays with a specific antibody. A strong increase was detected in deceased patients compared to healthy controls [3]. In the same study, PARK7 was determined to increase in deceased patients by comparison of spot intensities on 2D electrophoretic gels of AM and PM CSF.

In order to confirm the MS/MS-based quantitative measurements, ELISA validations on four different AM and PM samples were carried out.

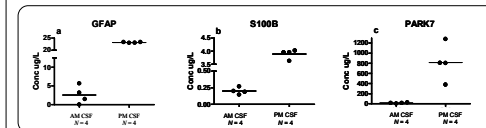


Figure 6. Immunovisualization of GFAP (a), protein S100B (b) and PARK7 (c) in AM and PM CSF.

ELISA for GFAP, S100B and PARK7 on 4 different AM and PM CSF samples confirmed the elevated concentration of these proteins in PM CSF. The ELISA revealed a dramatic and significant ($p = 0.0286$) increase of their concentration in PM CSF (Figure 6). The ratios PM CSF/AM CSF found by ELISA correlated impressively with those obtained by MS/MS using TMT[®] (9.52 vs. 8.44, 19.45 vs. 15.35 and 58.0 vs. 54.9).

4

In an additional work carried out in the Sanchez's group, PARK7 was measured in plasma in 3 independent studies on a total of more than 600 stroke patients and 150 control individuals (Figure 7). The study revealed the significant increase in plasma of the PARK7 biomarker concentration within 3h of stroke onset [4]. PARK7 may be useful plasma biomarker for the early diagnosis of stroke.

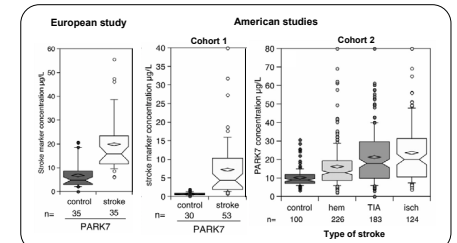


Figure 7. PARK7 concentrations in stroke and control plasma samples (subtypes of stroke are hemorrhagic (hem), transient ischemic attack (TIA) and ischemic (isch)).

Conclusions

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The TMT[®] label procedure provides the simultaneous relative quantitation of proteins between up to 6 different extracts from MS/MS experiments. The relative quantitation for protein ratios from 1 to 10 has been shown to present accuracy and relative standard deviation in similar or better ranges than several other quantitative methods used in proteomics.

When comparing *ante-* and *post-mortem* cerebrospinal fluid samples with TMT[®], it clearly appeared that some of the proteins that are present in *post-mortem* CSF are almost absent in *ante-mortem* CSF. Some of these proteins have already been shown to have a great potential in the diagnosis or prognosis of brain-related diseases. The MS-based quantitative values for GFAP, S100B and PARK7, were further validated by ELISA, which confirmed the excellent accuracy of the TMT[®] relative quantitation, even when high concentration differences exist between the samples. This work therefore demonstrated the usefulness of the TMT[®] reagents for the discovery of biomarkers in a shotgun proteomics approach [5].

Acknowledgments and references

The authors thank Nadia Walter, Catherine Fouda, Xavier Robin, Yoann Couté and Olivier Lassout for technical and scientific assistance and Proteome Sciences plc for financial support and providing the TMT[®] reagents.

[1] Anal Chem. 2003, 75, 1895-904.

[2] J. Proteome Res. 2006, 5, 1674-1681.

[3] Proteomics 2004, 4, 2234-2241.

[4] Clin. Chem. 2005, 51, 2043-2051.

[5] Anal Chem. 2008, 80, 2921-31.