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The development of a peptide SRM-based tandem mass spectrometry assay for prenatal screening of Down syndrome

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ABSTRACT

Two new biomarkers, serum amyloid-P (SAP) and plasma C1-inhibitor protein are elevated in the maternal circulation of mothers carrying Down syndrome foetuses. Much emphasis of late\ has been put on the lack of translational tests being developed following the identification of new biomarkers. We have created a single-reaction-monitoring (SRM) tandem mass spectrometry-based assay for the quantitation of these biomarkers and compared these results with an in-house developed immunofluorescence-based technique (IF). This MS-based assay is a rapid 5 min test and a simple "one pot reaction," requiring only 5 μl of plasma. To evaluate the potential of SRM-based quantitation in a clinical setting, SAP and C1-inhibitor were quantitated in 38 normal and Down syndrome affected pregnancies. Plasma SAP levels in the Down's group were significantly raised at 10–14 weeks ($p<0.0015$) and 14–20 weeks ($p<0.0001$). Plasma C1-inhibitor levels were also observed significantly elevated in the Down's group (10–14 weeks, $p<0.0193$, 14–20 weeks, $p<0.0001$). Analysis using the IF technique did not show any significant elevation of plasma SAP levels or C1-inhibitor levels. This rapid and sensitive assay demonstrates the potential of multiplexed tandem MS-based quantitation of proteins in chemical pathology labs and in a more cost-effective, accurate manner than conventionally used antibody methods.

1. Introduction

Down syndrome (DS) is the most common chromosomal abnormality in humans. Antenatal screening for Down syndrome (DS) in the first trimester involves a combination of nuchal translucency scanning and analysis of the plasma biomarkers β subunit of human chorionic gonadotropin (β-hCG) and pregnancy associated plasma protein-A (PAPP-A). Using results from these tests and combining them with an algorithm that takes into account maternal age, a risk factor of an affected pregnancy is produced. Those at higher risk are then offered a diagnostic test from foetal material obtained using amniocentesis or chorionic villus sampling, which have around a 1% risk of miscarriage. Current UK antenatal screening tests are performed in the late first or second trimester of pregnancy or a combination of both [1]. With the discovery of free foetal DNA (ffDNA) in maternal blood [2] there have been rapid research and development for a safer non-invasive alternative test to

Abbreviations: SRM, Multiple reaction monitoring; DS, Down syndrome; SAP, serum amyloid P; C1-inhibitor, plasma C1-protease inhibitor; PLGS, ProteinLynx Global Server; ffDNA, free foetal DNA; min, minutes; h, hours

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amniocentesis and chorionic villus sampling (CVS). A recent study has achieved a 98.6% detection rate in a DS high risk group using massively parallel sequencing of maternal plasma cell free DNA [3]. However, currently DNA sequencing is expensive and could not be considered economically viable as a stand-alone screening test. In addition, ffDNA testing would also miss other chromosomal abnormalities that current screening and diagnostic programmes detect. Therefore, there is still a requirement to improve the specificity of initial screening tests whilst maintaining low running costs. There have been many proteomic based studies investigating maternal plasma for novel markers for Down syndrome [4]. Recently, two new biomarkers were found to be elevated in the plasma of mothers who are pregnant with Down syndrome affected foetuses [5]. Kolialexi et al. demonstrated that 2nd trimester plasma serum amyloid P (SAP) levels were 2-fold elevated in 8 mothers carrying Down syndrome affected pregnancies. Research in our laboratory demonstrated that C1-inhibitor was also significantly elevated at both 10–14 and 14–20 weeks gestation (Heywood et al., unpublished). Subsequently, using triple quadrupole mass spectrometry with isotope labelled peptide internal standards, we have developed a quantitative assay for the rapid and accurate measurement of specific "marker" peptides generated from the tryptic digestion of C1-inhibitor and SAP, and which was used as an indirect quantitation of both proteins, in 5 μl of maternal plasma in 38 patients. In addition, a comparative and complementary antibody immuno-fluorescence (IF) based assay was also created to quantify C1-inhibitor and SAP, a similar technique to that currently used to quantitate the DS screening biomarkers (PAPP-a, β-hCG). This IF-based assay was then compared to the MS-based assay to test the accuracy and specificity of SRM-based quantitation versus the conventional and widely used IF-based screening methods.

2. Materials and methods

2.1. Samples

2.1.1. Plasma samples

Blood samples (EDTA) were taken with ethical consent from 38 pregnant women attending the Fetal Medicine Unit, University College Hospital London for invasive diagnostic testing for a range of clinical indications. Nineteen plasma samples were obtained from normal pregnancies and nineteen from DS affected pregnancies. Plasma was obtained by centrifuging at 1,500*g* for 10 min and further separated by centrifuging at 16,000*g* for 15 min. Protease inhibitor cocktail (Sigma Aldrich, UK) was then added to 1 ml aliquoted plasma fractions and fractions were stored at −80 °C prior to analysis. The study was approved by the UCLH A Research Ethics committee (001/95).

2.2. Sample preparation: The in-solution digestion of plasma proteins prior to analysis by UPLC MS/MS

Approximately 50 pmol of peptide AQUA internal standard (Sigma, Aldrich, UK) was added to 20 μl of 100 mM Tris, pH 7.8 containing 6 M urea (5 μl of 10 nmol/ml 50 mM ammonium bicarbonate pH 8.2 solution). Approximately 5 μl of plasma was then added to the solution, vortexed and was left to shake at room temperature for 1 h. Protein disulphide bridges were reduced by the addition of 3 μl of 100 mM Tris–HCl, pH 7.8 containing 5 M dithioerythritol (DTE) and incubated at ambient room temperature for 60 min. Free thiol groups were carboamidomethylated followed by incubation with 6 μl of 100 mM Tris–HCL, pH 7.8 containing 5 M iodoacetamide. The solution was then diluted with ddH_2O to a final volume of 200 μl, vortexed and 2 μg of sequence grade trypsin (Sigma, Aldrich UK) was added to the solution. Samples were incubated for 12–16 h at 37 °C in a water bath. Digests were spun at 12,000*g* for 10 min prior to analysis by LC–MS.

2.3. Development of tandem LC/MS method for absolute quantitation of C1-inhibitor and SAP in tryptically digested plasma

Fig. 1 shows a schematic representation summarising the SRM-based assay used in the quantitation of C1-inhibitor/SAP. Previous ESI-QToF analyses of tryptic peptides derived from C1-inhibitor and SAP protein standards showed that the peptides *m/z* 593.54 (FQPTLLTLPR) from C1-inhibitor and *m/z* 578.79 (VGEYSLYIGR) from SAP produced both the largest response and optimum product ion scans for C1-inhibitor and SAP analyses, respectively. These peptides were thus chosen as the "marker" peptides for each protein and peptide standards were obtained commercially to be used for optimization and development of the SRM-based assay (GenScript Corp., NJ, USA). An internal standard for the SAP peptide was also synthesised, which included a single missed cleavage site with a short peptide "tag" (VGEYSLIGR[$^{13}C_2^{15}N$]-HKVTS) to compensate for any inefficiencies of the trypsin protease reaction ("Aqua™ peptide," Sigma-Aldrich, Dorset, UK). Confirmation of the identification of the marker peptides for SAP *m/z* 578.79 and C1-inhibitor *m/z* 593.54 in plasma was verified by comparison of their retention time with the synthesised versions of the peptides when spiked into blood. In this way a third level of identification was attained in addition to precursor and product ion mass and enabling unequivocal identification of each peptide/protein. Quantitative data were taken from transition 593.54>455.980 for C1 inhibitor, 578.79>286.199 for SAP and 580.31>286.199 for the deuterated internal standard. Calibration curves were constructed by spiking 5 μl of normal female plasma with 50 pmol of the SAP isotope labelled internal standard and increasing amounts of the synthesised marker peptides used to quantitate the SAP and C1-inhibitor proteins (1, 2.5, 5, 7.5, 10 and 15 μmol/l). Endogenous levels of SAP and C1-inhibitor levels in the normal female plasma were determined by the intercept on the *x*-axis of the unspiked plasma (0 calibrator point) and this correction value was subtracted from each subsequent calibrator point (1–15 μmol/l). Quantitation of SAP and C1-inhibitor was performed by ratioing the peak area of the native peptide to the peak area of isotope-labelled internal standard for SAP (580.31 *m/z*) and the concentration determined by comparison to the calibration curve. Results were expressed as μmol/l.

A Waters Acquity UPLC coupled to a Xevo™ TQ-S triple quadrupole mass spectrometer (Waters Corp., Manchester, UK) was used to develop a rapid 5 min test for the quantitation