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Relative quantification of albumin and fibrinogen modifications by liquid chromatography tandem mass spectrometry in the diagnosis and monitoring of acute pancreatitis

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ABSTRACT

The increasing availability of liquid chromatography tandem mass spectrometry (LC–MS/MS) in clinical laboratories provides the opportunity to replace or complement present underperforming immuno- and chemometric assays. Amylase and lipase show limited specificity and sensitivity for pancreatic inflammation and lack the capacity of monitoring the disease due to their short half-lives. Previous findings suggested that cleavage products of the pancreatic enzyme carboxypeptidase A could be a more suitable indicator for defining and classifying pancreatic inflammation. The plasma proteins albumin and β-fibrinogen were digested with trypsin and truncated forms (des-Leu-albumin, and des-Gln-β-fibrinogen) quantified against their non-truncated forms by LC–MS/MS. Four hundred fifty eight samples from 83 patients were used to evaluate the novel method and affirm its suitability for detecting acute pancreatitis. A robust, selective, precise and accurate LC–MS/MS method was set up to measure the proportion of truncated proteins. Reference ranges for the proportion of the truncated albumin and β-fibrinogen were from 2% to 9% and 3% to 25%, respectively. Acute pancreatitis patients had values above these ranges and were distinctly separated from reference control individuals. The longer circulating half-lives of albumin and fibrinogen compared to pancreatic enzymes themselves provide the potential to diagnose pancreatitis more specifically over a longer time period, to monitor the course of the disease, and to track recurrent complications. The wide range of the proportion and the differential half-life of both truncated proteins could also be used for assessing the severity of pancreatitis.

1. Introduction

Severe acute pancreatitis is a life-threatening disease and successful treatment relies on a quick and accurate diagnosis. Pancreatic amylase and lipase are well established diagnostic tests; however, they are not very sensitive or specific for acute pancreatitis. A confirmation and differentiation of the initial diagnosis requires imaging techniques like ultrasound, computer tomography (CT) or magnetic resonance imaging (MRI). These procedures are time-consuming and costly, the results are occasionally of limited quality and their interpretation is operator dependent. A final diagnosis needs to correctly consolidate the complementary information of symptoms and features, laboratory tests and high quality imaging results. This process is laborious and unsuitable for an ongoing monitoring of the disease.

Efforts to improve the testing regime have been numerous, but "the clinical performance of current biomarkers for acute pancreatitis has been disappointing" [1]. Potential indicators of the severity of acute pancreatitis have been described [2,3]; however, the classification of severity is still under debate [4]. Chromatographic approaches have been reported for biomarkers of chronic pancreatitis [5] and pancreatic cancer [6,7]. Interestingly modified forms of albumin and β-fibrinogen have been identified in the plasma of patients with pancreatitis [8,9]. These truncated proteins

Abbreviations: LC–MS/MS, liquid chromatography tandem mass spectrometry; CT, computer tomography; MRI, magnetic resonance imaging; DLA, des-Leu-albumin; DQF, des-Gln-β-fibrinogen; CpA, carboxypeptidase A; ALB, albumin; FIB, β-fibrinogen; EDTA, ethylenediaminetetraacetic acid; ERCP, endoscopic retrograde cholangiopancreatography; MW, molecular weight; VS, validation samples; DW, ratio of the dwell time; TP, truncated peptide; FP, full length peptide; SD, standard deviation; CV, coefficient of variation.

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known as des-Leu-albumin (DLA) and des-Gln-β-fibrinogen (DQF) lack their C-terminal residues and result from the action of the exoprotease, carboxypeptidase A (CpA). Unlike amylase and lipase CpA is produced exclusively in the pancreas and direct measurements of its activity has previously shown elevated plasma levels in patients with acute pancreatitis [10,11].

The short half-life [9] of several hours for the currently used marker enzymes, CpA, amylase and lipase restricts the clinical utility of their measurement. In contrast albumin and fibrinogen have much longer circulatory half-lives (18 and 3–4 days, respectively) and are available at readily detectable concentrations in plasma. A tryptic digest of plasma yields the full length albumin peptide LVAASQAALGL (ALB), the truncated variant LVAASQAALG (DLA), the full length β-fibrinogen peptide IRPFFPQQ (FIB) and its truncated variant IRPFFPQ (DQF).

The aim of this study was to enhance a tentative method [12] for quantifying des-Leu-albumin and develop a second parallel method for quantifying des-Gln-β-fibrinogen. Liquid chromatography tandem mass spectrometry (LC–MS/MS) parameters were optimised for a simple routine assay set-up. The final assay was explored using 449 plasma samples from 83 individuals with a focus on acute pancreatitis. Ultimately we are after a smart diagnostic tool covering pancreatic diseases with a set of biomarkers which allow a quick differential diagnosis, grading of severity and monitoring.

2. Materials and methods

2.1. Blood samples

Four hundred and twenty six ethylene diaminetetraacetic acid (EDTA) plasma and nine serum samples were collected from 60 patients while 23 healthy individuals donated one EDTA plasma sample each. Series of up to 39 samples were obtained from the patients with suspected gastrointestinal diseases with symptoms similar to pancreatitis. Twenty-nine of these admitted patients were classified as gastrointestinal controls and regarded as normal with respect to their pancreatic function and morphology. Twenty-six patients have been diagnosed with various forms of chronic and acute pancreatitis and five patients with pancreatic cancer. Five pancreatitis patients have been additionally classified as gastrointestinal controls (normal) at least at some stage during the sampling period, e.g. three patients had samples taken before Endoscopic Retrograde CholangioPancreatography (ERCP) and developed pancreatitis after the ERCP examination. After collection samples were stored at −80 °C until analysis. Diagnosis of pancreatitis is based on clinical symptoms and features suggestive of pancreatitis, laboratory results (pancreatic amylase activity > 159 U/L or lipase activity > 210 U/L) and morphological indication from various imaging techniques (Ultrasound, CT, MRI and ERCP).

For the initial clinical survey of the assay acute pancreatitis patients diagnosed with biliary (12 patients), alcoholic (2 patients) or ERCP-induced (4 patients) pancreatitis, were selected. The first sample from a series of samples from these patients was used. It was taken at admission (15 patients), one day after admission (2 patients) and two days after admission (1 patient).

2.2. Amylase and lipase activity

Pancreatic amylase (Reagent-Kit no. GK22-30) and lipase (Reagent-Kit no. 7D80) activities were measured on an Abbott c8000/c16000 analyser (Abbott Laboratories, North Chicago, USA) according to manufacturer's protocols. The reference range is 8–53 U/L and 10–70 U/L, respectively.

2.3. Sample preparation

2.5 μL of plasma was diluted with 7.5 μL of ultrapure water in 0.2 mL Polymerase Chain Reaction (PCR) tubes, heated to 95 °C to denature albumin and fibrinogen as well as deactivate trypsin inhibitors or any proteases present. After addition of 2 μg trypsin and 2 μL of 0.5 mol/L ammonium bicarbonate (pH 7.8) samples were incubated at 37 °C overnight. Finally, samples were diluted to 500 μL with 4% acetonitrile/0.1% formic acid, centrifuged and the supernatant transferred to glass vials for analysis.

Calibrators were prepared from commercial peptides (Mimotopes PTY LTD, Melbourne, Australia) dissolved in 4% acetonitrile/0.1% formic acid. Three calibrators at concentrations of 1 μmol/L DLA and 3 μmol/L ALB (25%), 2 μmol/L DLA and 2 μmol/L ALB (50%), and 3 μmol/L DLA and 1 μmol/L ALB (75%) were prepared and stored at −80 °C until analysis. The concentrations of the fibrinogen peptides within these calibrators were: 100 nmol/L DQF and 300 nmol/L FIB (25%), 200 nmol/L DQF and 200 nmol/L FIB (50%), and 300 nmol/L DQF and 100 nmol/L FIB (75%).

Labelled peptides of albumin (h-ALB: molecular weight (MW) = 1020 g/mol) and des-Leu-albumin (h-DLA: MW = 907 g/mol) for monitoring of ion suppression/enhancement were supplied by New England Peptide (Gardner, USA). The N-terminal Leucine of these peptides had six ^{13}C and one ^{15}N isotopes.

Three patient samples (VS1–VS3) and one purified human albumin sample (VS4) that was pre-treated [13] with CpA were digested and kept frozen in aliquots until used as validation samples (VS) for the LC–MS/MS analysis.

2.4. Chromatography and mass spectrometry

Six microliter tryptic digest was separated using a Phenomenex Jupiter 4 μm Proteo 90 Å (150 × 2 mm) column (Phenomenex, Torrance, USA) on a Shimadzu Prominence HPLC system (Shimadzu, Tokyo, Japan). The temperature was set at 40 °C and flow rate of 0.25 mL/min was used. After equilibration at a concentration of 4% acetonitrile/0.1% formic acid, a three-step gradient starting with an initial increase to 12% acetonitrile within 0.1 min, following an effective linear separation step of three minutes ranging from 12% to 28% acetonitrile and a third step raising the acetonitrile fraction to 80% within 0.5 min with constant 0.1% formic acid was applied before the column was finally washed with 80% acetonitrile/0.1% formic acid for one minute. An ABSCIEX 4000 tandem mass spectrometer (ABSCIEX, Mt Waverley, Australia) fitted with an electrospray ionisation source was used. Optimised source parameter settings were: temperature 200 °C; ion spray voltage 5000 V. Declustering potentials and collision energies were optimised for each transition and ranged from 40 to 65 V and 18 to 35 V, respectively. The dwell time was set to 20 μs for albumin peptides and 200 μs for β-fibrinogen peptides. The parent and fragment ions are summarised in Table 1.

For the method set-up two different albumin ions (ALB, ALB2, DLA, and DLA2) and two different β-fibrinogen ions (FIB, FIB2, DQF, and DQF2) were used. Initial source and compound settings for peptides were pre-selected from Skyline software (MacCoss Lab, Seattle, USA) [14]. Peptide solutions were used to optimise the LC–MS/MS acquisition parameters. Peptide LVAAS was included as it had been reported as a by-product of the albumin digest [12]. Total protein LC–MS analysis has been described previously [13]. Data analysis was performed with SigmaPlot 11 (Systat Software Inc., San Jose, USA). Bag plots were calculated with R 3.1.1 (The R Foundation for Statistical Computing, Vienna, Austria).