ABSTRACT FORM ECAT SYMPOSIUM 15 – 16 SEPTEMBER 2022

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Title:

Next-generation antithrombin diagnostics by mass spectrometry

Abstract:

Introduction
Hereditary deficiency of antithrombin, a natural anticoagulant, causes thrombophilia with high risk for venous thromboembolism. Activity testing is recommended for diagnosing antithrombin deficiency but may lead to diagnostic uncertainty around the decision limit, depending on the brand or principle of the test. Antithrombin exists in various molecular forms, caused by genetic mutations and post-translational modifications (e.g. α- and β-antithrombin), which vary in heparin affinity and activity levels (1). Molecular testing has the potential to refine the diagnosis of antithrombin deficiency with mass spectrometry (MS) allowing full molecular characterization of the protein. We established and validated an MS-based test that simultaneously quantifies antithrombin and detects specific proteoforms caused by genetic variation and glycosylation. The method comprises of immunocapture followed by MS analysis (2) and has been implemented on a liquid handler to enhance throughput.

Objective
To enable precision diagnostics for antithrombin deficiency, an MS-based test for molecular characterization of antithrombin proteoforms, including the identification of α- and β-antithrombin, was developed and analytically validated. With this all-in-one-test, information on (dys)functional antithrombin proteoforms is obtained, which should ideally enable clinicians to make better informed decisions.

Method
Antithrombin was captured from fifty microliters of 200x diluted citrate plasma using a biotinylated llama VHH-antibody coupled to streptavidin coated 96-well plates. After denaturation and alkylation, antithrombin was digested using trypsin (sequence-grade, Promega). Proteotypic peptides were quantified relative to stable isotope labelled internal standard peptides using liquid-chromatography coupled to multiple-reaction-monitoring MS. External calibration was based on native citrate plasmas, which were value assigned using an antithrombin standard (Hyphen Biomed). The sample preparation was implemented on a BRAVO liquid handling platform and can be executed in less than 4 hours. The method was developed in agreement with CLSI guideline C62 and analytical validation included precision, linearity, measuring range intervals and carry-over.

Results
A total of 23 peptides, including 4 glycopeptides, are monitored to enable identification of the most common genetic variations. Detection of the glycopeptide KANK enables discrimination between α- and β-antithrombin. Total imprecision of five different samples for the three quantifying peptides (LVSANR, EVPLNTIIFMGR and HGSPVDICTAKPR) varied between 4.7-8.2%, 4.9-8.0% and 6.3-8.8% respectively. The test showed good linearity in the clinical range with reference values for healthy individuals between 1.07 and 1.49 µmol/L for peptide LVSANR. Clinical application of the test has already resulted in identification of an antithrombin deficient patient, who could not be diagnosed using the regular AT-activity test.

Conclusion
The here presented next-generation test for antithrombin enables molecular characterization at the protein level. The plate-based strategy allows for high-throughput needed for clinical trials and provides good precision within predefined analytical performance specifications. This performance allows identification of mutations in a clinical setting highlighting that the test is ready for translation. We envision clinical validations in specific target groups to unravel the value of antithrombin proteoforms in the context of antithrombin deficiency and thrombophilia.

(1) Peterson et al. J Biol Chem 1985 260:610-615
(2) Kruijt et al. Manuscript submitted 2022