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Detecting molecular forms of antithrombin by LC-MRM-MS: defining the measurands

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Abstract

Background: Antithrombin (AT) is a critical regulator of coagulation, and its overall activity is typically measured using functional tests. A large number of molecular forms of AT have been identified and each individual carries multiple molecular proteoforms representing variable activities. Conventional functional tests are completely blind for these proteoforms. A method that ensures properly defined measurands for AT is therefore needed. We here assess whether mass spectrometry technology, in particular multiple reaction monitoring (MRM), is suitable for the quantification of AT and the qualitative detection of its molecular proteoforms.

Methods: Plasma proteins were denatured, reduced and alkylated prior to enzymatic digestion. MRM transitions were developed towards tryptic peptides and glycopeptides using AT purified from human plasma. For each peptide, three transitions were measured, and stable isotope-labeled peptides were used for quantitation. Completeness of digestion was assessed using digestion time curves.

Results: MRM transitions were developed for 19 tryptic peptides and 4 glycopeptides. Two peptides, FDTISEK and FATTFYQHLADSK, were used for quantitation, and using a calibration curve of isolated AT in 40 g/L human serum albumin, CVs below 3.5% were obtained for FDTISEK, whereas CVs below 8% were obtained for FATTFYQHLADSK. Of the 26 important AT mutations, 20 can be identified using this method, while altered glycosylation profiles can also be detected.

Conclusions: We here show the feasibility of the liquid chromatography multiple reaction monitoring mass spectrometry (LC-MRM-MS) technique for the quantitation of AT and the qualitative analysis of most of its molecular proteoforms. Knowing the measurands will enable standardization of AT tests by providing in-depth information on the molecular proteoforms of AT.

Keywords: antithrombin; defined measurands; molecular proteoforms; quantitative protein analysis; triple quadrupole mass spectrometry.

Introduction

Antithrombin (AT) is a 58-kDa glycoprotein that is typically present in blood at concentrations of 112–140 mg/L [1]. AT is a physiologically critical regulator of coagulation by inhibition of thrombin and other coagulation factors. The protein contains two functional sites; a heparin-binding site and a reactive site. When heparin is bound to AT, thrombin inactivation is reported to be up to 1000-fold more efficient [2]. Over 300 genetic defects have been described in AT, and hereditary AT deficiencies have been associated with venous thromboembolism (VTE) [3]. In the general population, the prevalence of AT deficiency varies from 1:500 to 1:5000 [4, 5], and in about 0.5% of patients with a first VTE, AT deficiency is detected [6]. Because thrombin also plays a critical role in arterial thrombosis, it was recently suggested that AT deficiencies should also be relevant in arterial thrombosis [7]. Although initial literature does not support this hypothesis [8], recent findings have provided evidence for the role of AT deficiency in arterial thrombosis [2, 9, 10]. AT deficiencies may be subdivided in two types: quantitative type I deficiencies in which the AT concentration is reduced and qualitative type II deficiencies in which the AT activity is impaired. Type II deficiencies can be further classified based on the functional site that is affected, i.e. the reactive site (RS, type IIa) or the heparin binding site (HBS, type IIb) or

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Table 1: Common type-II mutations in AT.

No	AA no	From	To	NCBI snpDB	Peptide without mutation		Peptide with mutation		Mass difference	Detected by LC-MRM-MS?
					Sequence	Mass, Da	Sequence	Mass, Da		
1	39	I	N	rs121909558	HGSPVDICTAKPR	1436.7	HGSPVDNCTAKPR	1437.7	1.0	Yes, addition of N-glycan
2	56	R	C	rs28929469	DIPMNPMCIYR	1408.6	DIPMNPMCIYCSPEK	1853.8	445.2	Yes, loss of peptide
3	73	P	L	rs121909551	IPEATNR	799.4	ILEATNR	815.5	16.0	Yes, loss of peptide
4	79	R	C	rs121909547	R	174.1	CVWELSK	920.4	746.3	Yes, loss of peptide VWELSK
	79	R	S	rs121909547	R	174.1	SVWELSK	848.5	674.4	Yes, loss of peptide VWELSK
	79	R	H	rs121909552	R	174.1	HVWELSK	897.5	723.4	Yes, loss of peptide VWELSK
5	131	L	F	rs121909567	LGACNDTLQQLMEVFK	1865.9	LGACNDTFQQLMEVFK	1899.9	34.0	Yes, loss of peptide
	131	L	V		LGACNDTLQQLMEVFK	1865.9	LGACNDTVQQLMEVFK	1851.9	−14.0	Yes, loss of peptide
6	147	T	A	rs2227606	TSDQIHFFFAK	1339.7	ASDQIHFFFAK	1309.6	−30.0	Yes, loss of peptide
7	148	S	P	rs121909569	TSDQIHFFFAK	1339.7	TPDQIHFFFAK	1349.7	10.0	Yes, loss of peptide
8	150	Q	P	rs765445413	TSDQIHFFFAK	1339.7	TSDPIHFFFAK	1308.7	−31.0	Yes, loss of peptide
9	161	R	Q	rs121909563	LNCR	561.3	LNCQLYR	965.5	404.2	No
	161	R	H		LNCR	561.3	LNCHLYR	974.5	413.2	No
10	219	N	D	rs121909571	AAINK	515.3	AAIDK	516.3	1.0	No
	219	N	K		AAINK	515.3	AAIK	401.3	−114.0	No
11	269	E	K	rs758087836	ELFYK	698.4	K	146.1	−552.3	Yes, loss of peptide
12	273	K	E		ELFYK	698.4	ELFYEADGESCSASIMYQEGK	2413.0	1714.6	Yes, loss of peptide
13	283	M	I		ADGESCSASMMYQEGK	1749.7	ADGESCSASIMYQEGK	1731.7	−18.0	Yes, loss of peptide
	283	M	V		ADGESCSASMMYQEGK	1749.7	ADGESCSASVMYQEGK	1717.7	−32.0	Yes, loss of peptide
14	316	I	N		GDDITMVLILPKPEK	1667.9	GDDITMVLNLPKPEK	1668.9	1.0	? – little mass difference
15	334	E	K		ELTPEVLQEWLDELEEMMLV VHMPR	3065.5	ELTPK	586.3	−2479.2	No
16	424	G	D	rs121909566	AFLEVNEEGSEAAASTAVVI AGR	2290.1	AFLEVNEEGSEAAASTAVVI ADR	2348.1	58.0	No
17	425	R	C	rs121909554	AFLEVNEEGSEAAASTAVVI AGR	2290.1	AFLEVNEEGSEAAASTAVVI AGCSLNPNR	2975.4	685.3	Yes, loss of SLNPNR
	425	R	H	rs121909549	AFLEVNEEGSEAAASTAVVI AGR	2290.1	AFLEVNEEGSEAAASTAVVI AGHSLNPNR	2952.5	662.3	Yes, loss of SLNPNR
	425	R	P		AFLEVNEEGSEAAASTAVVI AGR	2290.1	AFLEVNEEGSEAAASTAVVI AGPSLNPNR	2912.5	622.3	Yes, loss of SLNPNR
18	426	S	L	rs121909550	SLNPNR	699.4	LLNPNR	725.4	26.1	Yes, loss of peptide
19	434	F	C		VTFK	493.3	VTCK	506.3	13.0	No
	434	F	L		VTFK	493.3	VTLK	459.3	−34.0	No
	434	F	S		VTFK	493.3	VTSK	433.3	−60.0	No
20	436	A	T	rs121909546	ANRPFLVFIR	1231.7	TNRPFLVFIR	1261.7	30.0	Yes, loss of peptide
21	437	N	K		ANRPFLVFIR	1231.7	AK	217.1	−1014.6	Yes, loss of peptide
22	438	R	G		ANRPFLVFIR	1231.7	ANGPFLVFIR	1132.6	−99.1	Yes, loss of peptide
	438	R	M		ANRPFLVFIR	1231.7	ANMPFLVFIR	1206.7	−25.1	Yes, loss of peptide
23	439	P	A		ANRPFLVFIR	1231.7	ANR	359.2	−872.5	Yes, loss of peptide
	439	P	L	rs121909555	ANRPFLVFIR	1231.7	ANR	359.2	−872.5	Yes, loss of peptide
	439	P	T		ANRPFLVFIR	1231.7	ANR	359.2	−872.5	Yes, loss of peptide
24	441	L	P		ANRPFLVFIR	1231.7	ANRPFPVFIR	1215.7	−16.0	Yes, loss of peptide
25	457	R	T		EVPLNTIIFMGR	1388.7	EVPLNTIIFMGTVANPCVK	2102.1	713.3	Yes, loss of VANPCVK
26	461	P	L	rs121909564	VANPCVK	786.4	VANLCVK	802.4	16.0	Yes, loss of peptide

Besides the amino acid modification, the peptide sequence is shown without and with the mutation, and the resulting mass difference is indicated. Based on these results, it is indicated whether theoretically the mutation can be observed using LC-MRM-MS analysis.

other pleiotropic effects (type IIc). An overview of 26 clinically relevant type II mutations is presented in Table 1.

AT carries four sites of N-glycosylation at asparagines Asn^{128}, Asn^{167}, Asn^{187} and Asn^{224}. The glycosylation profile of AT has been studied quite in-depth using mass spectrometric techniques and was shown to be relatively homogeneous, with the fully sialylated biantennary glycan being by far the most abundant glycan, whereas other glycans are present at abundances of only 1–5% [11]. Using heparin binding affinity, two distinct proteoforms of AT can be identified: α- and β-AT [12]. Although all four glycosylation sites are occupied in α-AT, the glycan at one of the N-glycosylation sites, N^{167}, is completely missing in β-AT [13, 14]. Because of its 2.5-fold increased heparin binding affinity, it is speculated that β-AT may be the more active form, thus providing the higher anticoagulant activity (e.g. [15, 16]). Recently, it has been reported that overall differential glycosylation caused by type II congenital disorders of glycosylation was observed in 27% of cases with AT deficiency without genetic defects in the encoding gene [7, 17, 18], thus indicating an important role of AT glycosylation for its activity.

Currently, two main types of AT tests are in use; activity-based functional tests and antigen-based immunoassays [19]. Typically, when AT deficiency is suspected, a functional test is performed first. If the functional test shows a deficiency, an immunoassay follows to assess the type of AT deficiency. However, this strategy requires the use of two tests for a diagnosis, does not point towards the particular molecular defect and does not determine the relative quantities of α-AT over β-AT. The interlaboratory precision of the current activity tests is good with CVs of 4–7% for healthy individuals and 8–12% for patients [20]; however, differences in the detection of type II deficiencies have been observed between commercially available functional tests [2, 21, 22]. Such results may lead to missed diagnoses of AT deficiency. Therefore, as was recently recognized [7], alternative techniques for the detection and quantitation of AT and its deficiencies, which ensure a properly defined measurand by taking the molecular forms of AT into account, are required.

One promising technique for such an assay is mass spectrometry (MS), because bottom-up proteomics strategies have long been recognized as a powerful tool for the identification of proteins [23]. The technology is also highly suited for the identification of relevant molecular forms in which the protein product of a single gene can be found, including changes due to genetic variations, alternatively spliced RNA transcripts and post-translational modifications [24, 25]. Quantitative clinical chemistry proteomics (qCCP) using MS was recently introduced in the clinical chemistry laboratory as a technique that lacks most of the drawbacks of immunoassays and allows for multiplexing of tests [26, 27]. Therefore, we believe MS could be an excellent technology for the quantitation of AT and the detection of its proteoforms – both genetic mutations as well as altered glycosylation. Our group recently developed and provisionally validated an MS-based method for the multiplexed quantitation of six apolipoproteins, including apolipoprotein E phenotyping using LC coupled to multiple reaction monitoring (MRM) MS [28], showing the potential for a mass spectrometry based test for absolute protein quantitation, with analytical performance specifications meeting clinical requirements.

In this study, we aim to assess the potential of MS technology for the quantitation of AT and the qualitative detection of its molecular deficiencies (pathological proteoforms). To allow for metrological traceability, the proper characterization of the different AT proteoforms is necessary as the measurands need to be accurately defined. Therefore, four method requirements were aimed at to assess as many clinically relevant proteoforms of AT variation as possible: (1) absolute quantitation of the AT concentration, (2) identification of clinically relevant AT mutations, (3) relative quantitation of α- and β-AT and (4) identification of altered AT glycosylation profiles. To our knowledge, this is the first attempt at a MS-based characterization of most clinically relevant proteoforms of AT.

Materials and methods

Materials

Iodoacetamide, Tris (Trizma preset crystals, pH 8.1), sodium deoxycholate (DOC), $CaCl_2$, EDTA, HCl, cysteine and formic acid were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Tris(2-carboxyethyl)phosphine (TCEP) was from Thermo Scientific (Rockford, IL, USA), and ammonia was obtained from MerckMillipore (Amsterdam, The Netherlands). Sequencing-grade modified porcine trypsin and chymotrypsin were from Promega (Madison, WI, USA), whereas GingisRex was obtained from Genovis AB (Lund, Sweden). HPLC-grade methanol was from Biosolve (Valkenswaard, The Netherlands). Human serum albumin (HSA) solutions of 40 g/L in physiological saline (Albuman®) were from Sanquin (Amsterdam, The Netherlands). Oasis HLB, MCX and WAX cartridges were obtained from Waters (Milford, MA, USA). Synthetic peptides (both stable isotope labeled and non-labeled) were obtained from our in-house peptide synthesis facility and were at least 89.4% pure by HPLC. Human-derived AT and β-AT protein standards were obtained from J. Amiral and were prepared through affinity chromatography on heparin-Sepharose gel by elution with a salt gradient from 0.30 to 2.00 M NaCl. The protein concentrations were determined with Lowry's method [29].

Digestion of standard proteins using four proteases

Human-derived AT standard was digested according to the manufacturer's recommendations using trypsin, chymotrypsin or GingisRex. In short, 25 µg AT was dissolved in 8 µL MQ water, and added to 40 µL DOC (0.40% w/v) and TCEP (2.3 mmol/L) in 100 mmol/L Tris for trypsin digestion, to 40 µL TCEP (2.3 mmol/L) and $CaCl_2$ (10 mmol/L) in 100 mmol/L Tris for chymotrypsin digestion and to 40 µL TCEP (2.3 mmol/L), $CaCl_2$ (5 mmol/L) and EDTA (2 mmol/L) in 100 mmol/L Tris for GingisRex digestion. The samples were denatured, and cysteines were reduced for 30 min at 56 °C and subsequently alkylated by addition of 20 µL 4.6 mmol/L iodoacetamide (30 min at RT in the dark). For trypsin digestion, 0.3 µg trypsin in 24 µL resuspension buffer was added, and the sample was incubated at 37 °C for 3 h. For chymotrypsin digestion, 0.3 µg chymotrypsin in 24 µL 1 mmol/L HCl in 100 mmol/L Tris was added and the sample was incubated at 25 °C for 20 h. For GingisRex digestion, 0.3 µg GingisRex in 24 µL 5 mmol/L $CaCl_2$, 2 mmol/L EDTA in 100 mmol/L Tris as well as 1.5 µL of 20 mmol/L freshly prepared cysteine were added and the sample was incubated at 37 °C for 20 h. Each of the digestions was quenched using 106 µL 0.6% (v/v) formic acid, and 150 µL was transferred to an LC-MS vial for analysis.

LC-MS/MS analysis for peptide identification and transition development

An Agilent 1290 infinity LC system coupled to an Agilent 6495 triple quadrupole (QqQ) mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) was used for the LC-MRM analysis. The column oven temperature was set to 50 °C and the autosampler to 8 °C. A 10 µL volume of digested sample was injected onto a Zorbax SB-C18 2.1×50 mm analytical column with 1.8 µm particles (Agilent Technologies), and peptides were separated using a gradient of 5% (v/v) methanol in 0.05% formic acid in water (Eluent A) and 95% (v/v) methanol in 0.05% formic acid (v/v) in water (Eluent B) at a flow rate of 0.2 mL/min. The gradient increased linearly from 0% to 95% eluent B in 12 min, followed by a 4-min wash at 100% eluent B.

For peptide identification, first the MS was operated in full scan MS mode with a range of 250–1500 *m/z* to generate a full MS1 scan of the digested samples. Then the instrument was operated in product ion scan (PIS) mode to generate fragmentation spectra for *m/z* that were suspected to be AT peptides based on in silico digestion experiments using the online tool Peptide Mass (http://web.expasy.org/peptide_mass/) to identify peptides based on fragmentation pattern. Fragmentation spectra for all tryptic peptides and glycopeptides are shown in Supplementary Figure 10. Three transitions were developed per peptide and transitions were selected towards fragment ions that were highest in abundance and peptide specific. It has to be noted that glycan oxonium ions were used for the transitions of the glycopeptides, which has previously been shown to be a suitable strategy [30]. Collision energies were optimized for each transition individually and most intense, stable transitions were selected to be quantifying transitions.

Semiautomated digestion of plasma samples

Samples were prepared on a Bravo liquid handling platform equipped with a 96LT disposable tip head (Agilent Technologies) and a temperature-controlled heated lid (Inheco, Martinsried, Germany), similarly to our previously reported protocol for the digestion of serum samples [28]. To allow for accurate quantitation, stable isotope-labeled (SIL) peptides were included for the non-mutation prone peptides FATTFYQHLADSK and FDTISEK as well as the mutation prone peptides ANRPFLVFIR and TSDQIHFFFAK. In these peptides, heavy C and N is incorporated in the C-terminal lysine or arginine residues, resulting in a mass shift of 8 Da for K and 10 Da for R residues. Eight microliters of 20-fold diluted plasma was added to a 40-µL mixture of DOC (0.40% w/v), TCEP (2.3 mmol/L) and 42 fmol of each of the SIL peptides (FATTFYQHLADSK, FDTISEK, ANRPFLVFIR and TSDQIHFFFAK) in 100 mmol/L Tris in a 96-well plate. The samples were denatured and reduced for 30 min at 56 °C under gentle shaking (700 rpm). Then 20 µL 4.6 mmol/L iodoacetamide was added to induce alkylation (30 min at RT in the dark) of reduced cysteine residues. Trypsin digestion was performed at 37 °C at a 1:35 w/w trypsin-to-protein ratio in 92 µL total volume and gentle shaking (700 rpm). During all heated incubations, the sample plate was sealed and temperature controlled using the heated lid. After 3 h, the digestion was quenched by addition of 106 µL 0.6% (v/v) formic acid in 5% (v/v) methanol in water resulting in precipitation of the DOC. Next, the sample plate was centrifuged for 10 min at 2000*g*, and 150 µL supernatant was transferred to a clean 96-well plate for solid phase extraction (SPE).

Solid phase extraction

To desalt and concentrate AT peptides and glycopeptides, a manual SPE step was included. To aid conditioning, equilibration and elution of solvent from the cartridges, a positive pressure-96 processor (Waters) was used. First, the SPE plate was conditioned using 0.1 mL MeOH, followed by equilibration with 0.4 mL MQ water. One hundred fifty microliters of the tryptically digested samples was then pH adjusted using 25 µL 5% NH_3 (aq) in water (pH 12), mixed and loaded onto the plate. The plate was washed using 0.5 mL water and subsequently eluted using 0.1 mL 80% MeOH in water containing 2% formic acid. Eluates were dried using N_2 at 37 °C for approximately 2 h until dryness. Samples were reconstituted in 50 µL eluent A prior to analysis. To evaluate the relative recoveries of endogenous and SIL peptides from the SPE, mixtures of 0.15 µM synthetic peptides (n = 6) were subjected to SPE, dried and reconstituted. Both the original sample and the sample that underwent SPE were analyzed by liquid chromatography multiple reaction monitoring mass spectrometry (LC-MRM-MS).

LC-MRM-MS analysis

The same LC-MS setup was used for LC-MRM-MS as for the peptide identification, with the exception that the MS was operated in dynamic MRM mode. For each peptide, three transitions were measured in a scheduled MRM list with a 1.0-min retention time window, 500-ms cycle time and unit resolution for Q1 and Q3.

Data analysis

LC-MS/MS data were processed using Mass Hunter Workstation software, version B.07.00 (Agilent Technologies). Signal intensities were

obtained from the peak areas, and all transitions (both quantifying and qualifying) were evaluated individually. Initial data quality control was performed by assessing the ion ratios between quantifying and qualifying transitions, which were required to be within 15% accuracy. The system suitability test was passed for all analyses performed in this study.

System suitability test

To ensure that the LC-MS instrumentation is performing accurately during the sample analysis, a system suitability testing (SST) procedure was designed. In the SST, a sample consisting of eight (non-labeled) synthetic peptides, each at 0.15 µmol/L in 5% MeOH, was prepared. Two microliters of this sample was then analyzed five times prior to a test run as well as five times after a test run. Each of these five samples was followed by a blank sample to assess the carryover. Criteria for accurate performance were pre-defined, partially based on Briscoe et al. [31] and CLSI protocol C-62A [32], and were as follows: a carryover <1%, <15% difference in instrument counts between the samples before and after and a CV <10% between each of the five injections. Furthermore, the ion ratios between the quantifying transitions and the qualifying transitions should be on target ±15%.

Results

The choice of protease for AT digestion is essential, as it determines which peptides should be detected in the mass spectrometer, and thus which proteoform characteristics can be identified. Traditionally, trypsin is the protease most commonly used for both protein identification [33] and protein quantitation. However, the use of trypsin would result in a glycopeptide with a short peptide backbone for glycosylation site N^{167}: KANK, including one missed cleavage due to steric hindrance by the glycan moiety [11]. The use of this protease would thus likely hamper the application of reverse phase chromatography to distinguish α- and β-AT. Therefore, the potential use of alternative proteases was evaluated using in silico digests of AT with six other proteases commonly used for protein identification [33]: LysC, LysN, AspN, ArgC, chymotrypsin and GluC. Based on the results, shown in Supplementary Table 1, none of the proteases produce readily measurable glycopeptides (backbone between 7 and 15 AAs) for all four sites of glycosylation. However, peptide backbones for site N^{167} with 13 and 9 amino acids were obtained for ArgC and chymotrypsin, respectively. Therefore, it was decided to initially evaluate GingisRex, a more specific version of ArgC, and chymotrypsin besides trypsin for the development of a quantitative method for plasma AT characterization.

Comparison of trypsin, chymotrypsin and GingisRex for the characterization and digestion of AT

To evaluate which of the three proteases is most suitable for the quantitation of AT and its proteoform characteristics, we first set out to assess which peptides are observed in digests of each of the proteases. Digests of AT standard were prepared with each of the three proteases, and peptides and glycopeptides were identified by first obtaining an MS1 spectrum and then fragmenting the potential peptides and glycopeptides to confirm identification. The protein sequence coverage thus obtained for each of the three proteases is shown in Figure 1. For GingisRex and chymotrypsin protein sequence, coverages of 30.1% and 31.3% were obtained, whereas a sequence coverage of 53.5% was obtained with trypsin. This is further reflected by the number of the 26 most important mutation sites that were included in the coverage: 11 for GingisRex, 4 for chymotrypsin and 18 for trypsin.

Because the protein quantitation using a bottom-up type proteomics approach relies on the stable conversion and consistent enzymatic digestion of protein into peptides, a preliminary digestion time course experiment was performed with all three proteases. Single samples of human-derived AT standard in 40 g/L HSA were digested

Figure 1: Protein coverage for human plasma-derived AT obtained using three different proteases.
To assess the optimal protease, AT was digested using trypsin, chymotrypsin and GingisRex, and peptides were identified using MS scans followed by product ion scans for confirmation. Parts of the sequence highlighted in bold could be identified. Red amino acids indicate glycosylated asparagines, and green amino acids indicate the presence of a known polymorphism.

for 0, 1, 3, 6 and 20 h. The results of these experiments are shown in Supplementary Figure 1. In general, the peptides generated by chymotrypsin are much less stable during the digestion, as indicated by the decline in peptide recovery for the longer digestion times than the peptides from trypsin and GingisRex, which are much more stable. However, the abundance of the GingisRex glycopeptide KANKSSKLVSANR containing site N^{167} is very low, and only four (larger) peptides are observed from GingisRex, of which only one is not containing one of the 26 mutation sites. This limits the absolute quantitation of AT, for which at least two of such peptides are desired. Given these results, it was decided to further use trypsin as the protease of choice during method development.

The LC-MRM-MS method for quantitation of AT and its proteoform characteristics from plasma

Based on the sequence coverage for AT determined in this study, transitions were developed for all tryptic peptides and glycopeptides observed in the AT digest and collision energies were optimized. A list of the 19 tryptic peptides and 4 tryptic glycopeptides monitored is shown in Table 2. A chromatogram showing the application of this LC-MRM-MS method to a tryptic AT standard digest is shown in Figure 2. Using this method, 20 out of the 26 most important mutations can theoretically be observed (Table 1), and all four sites of glycosylation could theoretically be monitored. However, the glycopeptide KANK at site N^{167} is very short, with limited retention (see Figure 2, small signal eluting before 1 min). Therefore, the abundance of the signal would likely be around the limit of detection when plasma samples were analyzed.

System suitability

To ensure that the LC-MS instrumentation is performing accurately during the sample analysis, an SST procedure was designed, in which synthetic peptides were analyzed five times prior to the sample run and five times upon completion of the run. The results of a typical system

Table 2: Transitions for the peptides and glycopeptides from AT monitored in the LC-MRM-MS method developed.

AA	Peptide	Precursor *m/z*	Quantifier		Qualifier 1		Qualifier 2		Rt	Affected by mutations?
			m/z	CE	*m/z*	CE	*m/z*	CE		
33–45	HGSPVDICTAKPR	479.9	732.4	18	593.3	18	572.4	18	5.9	Yes
46–56	DIPMNPMCIYR	705.3	591.3	12	953.4	28	839.4	28	9.4	Yes
72–78	IPEATNR	400.7	590.3	18	461.2	18	687.3	15	4.7	Yes
90–102	FATTFYQHLADSK	510.3	655.8	12	605.4	12	219.1	12	8.0	No
140–146	FDTISEK	420.2	692.3	12	577.3	12	263.1	12	6.1	No
147–157	TSDQIHFFFAK	447.6	796.4	12	620.3	12	576.8	12	9.0	Yes
172–177	LVSANR	330.2	447.2	8	546.3	12	360.2	8	3.3	No
202–208	LQPLDFK	430.7	619.3	12	522.3	20	409.2	20	8.3	No
269–273	ELFYK	350.2	310.2	8	457.2	12	570.3	10	7.0	Yes
274–289	ADGESCSASMMYQEGK	584.2	624.3	12	755.3	15	973.4	15	6.4	Yes
295–307	VAEGTQVLELPFK	715.9	1131.6	25	746.4	25	391.2	25	9.8	No
308–322	GDDITMVLILPKPEK	557.0	748.8	15	635.0	15	288.0	15	10.1	yes
357–364	IEDGFSLK	454.7	794.9	20	665.9	20	215.0	20	7.9	No
383–391	LPGIVAEGR	456.3	701.4	20	531.3	20	268.2	20	7.5	No
392–402	DDLYVSDAFHK	437.2	803.4	15	704.3	15	231.1	15	7.9	No
426–431	SLNPNR	350.7	500.3	8	386.2	12	613.3	8	3.1	Yes
436–445	ANRPFLVFIR	411.6	699.4	10	586.3	10	534.3	10	9.0	Yes
446–457	EVPLNTIIFMGR	695.4	1161.3	20	580.8	20			10.3	No
458–464	VANPCVK	394.2	617.3	8	503.3	12	688.3	12	4.8	Yes
–	*TSDQIHFFFAK-SIL*	*450.1*	*804.4*	*12*	*624.3*	*12*	*580.8*	*12*	*9.0*	–
–	*FDTISEK-SIL*	*424.2*	*700.3*	*12*	*585.3*	*12*	*263.1*	*12*	*6.1*	–
–	*FATTFYQHLADSK-SIL*	*513.0*	*659.8*	*12*	*609.4*	*12*	*219.1*	*12*	*8.0*	–
–	*ANRPFLVFIR-SIL*	*414.8*	*699.4*	*10*	*586.3*	*10*	*544.3*	*10*	*9.0*	–
124–139	GP-LGACNDTLQQLMEVFK	1018.9	366.0	25	274.0	25	204.0	25	10.9	Yes
165–168	GP-KANK	889.4	366.0	25	274.0	25	204.0	25	0.9	No
183–201	GP-SLTFNETYQDISELVYGAK	1096.6	366.0	25	274.0	25	204.0	25	10.8	No
221–225	GP-WVSNK	710.5	366.0	25	274.0	25	204.0	25	4.7	No

SIL peptides are indicated in italics.

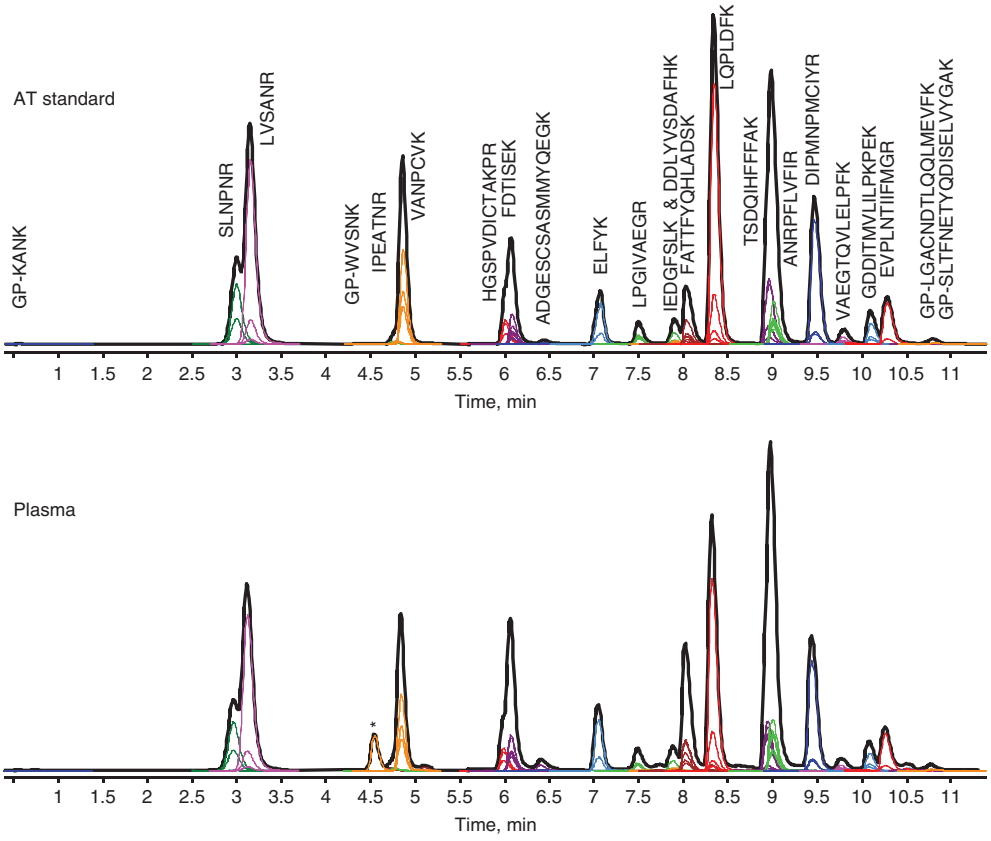

Figure 2: LC-MRM-MS chromatograms AT peptides and glycopeptides in human plasma-derived AT standard and plasma.
The total ion chromatogram is shown in black, whereas the different colors represent the transitions monitored for different peptides, with one color representing one peptide. Signals have been annotated with their respective peptide.

suitability test are shown in Supplementary Figure 2, where it can be seen that the retention time is stable, absolute abundances do not deviate more than 10% within five samples and no more than 15% between the analyses prior and after the sample measurements, and that the carryover is below 1%. The SST was passed every time samples were analyzed (including SPE test, optimization of the digestion as well as the calibration curves).

AT peptide enrichment and desalting

Because DOC is used as a surfactant and Tris is used as the buffer during digestion, which are non-volatile and would interfere with MS detection, a sample clean-up strategy was optimized. The hydrophilic glycopeptide KANK has no retention on an online loading column, and therefore off-line SPE was evaluated. At neutral pH, glycopeptide KANK is net neutral, as it contains two positively charged lysine residues, but also two negatively charged sialic acids. Moreover, peptide ANRPFLVFIR contains two positively charged arginine residues and is therefore positively charged with an estimated pI of 12.1. Because of the large variation in chemical properties of the different peptides, three stationary phases were considered, using four different conditions (see Supplementary Figure 3F): MCX, WCX and Oasis HLB with retention at both high and low pH. First, these conditions were evaluated using a tryptic digest of 20 µmol/L purified AT, for which the results are shown in Supplementary Figure 3. Glycopeptide KANK only showed retention on the MCX material and Oasis HLB when high pH conditions were applied. Of these two conditions, peptide ANRPFLVFIR was irreversibly retained on MCX material but could be recovered from Oasis HLB using acidic conditions (2% FA) (see Supplementary Figure 3H). Highly similar results were obtained when the experiment was repeated using a plasma sample (Supplementary Figure 4). However, it has to be noted that the ion abundances for glycopeptide KANK were very low, potentially due to the lower physiological AT concentration of ~2 µmol/L, but also due to remaining impurities in the sample. Because peptides and glycopeptides started

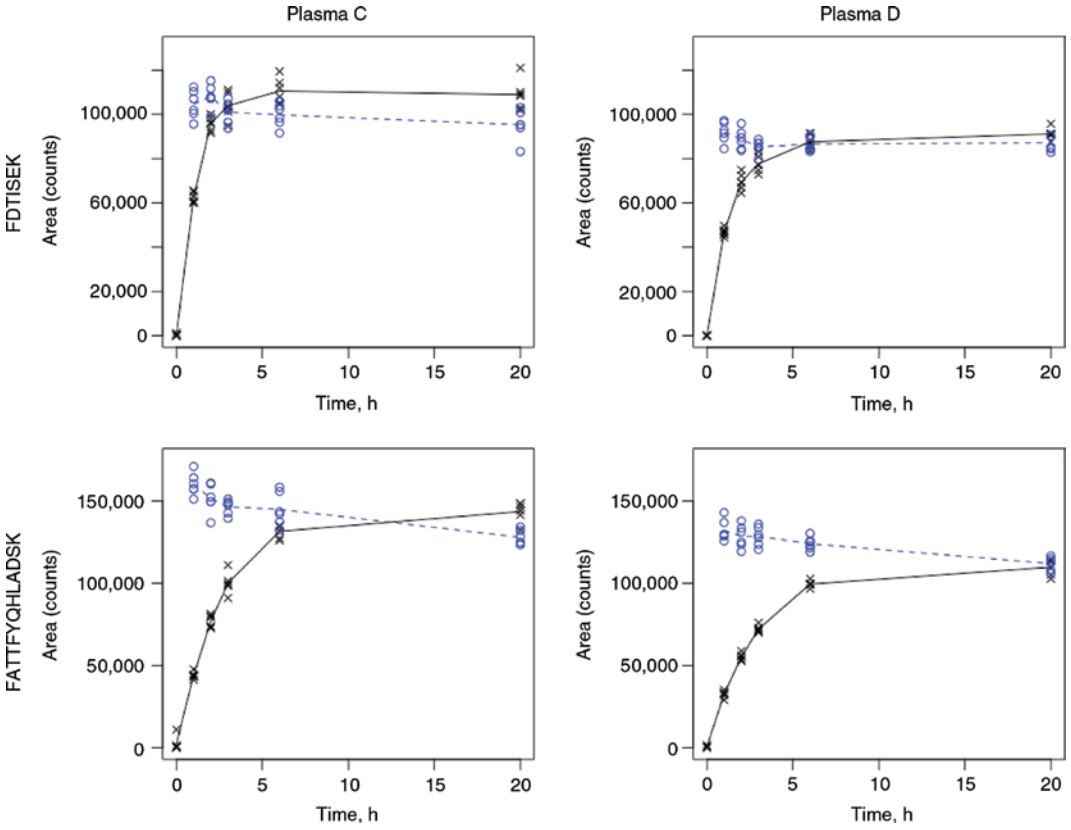

Figure 3: Digestion characteristics of quantifying peptides FDTISEK and FATTFYQHLADSK in two plasma samples.
Two plasma samples were digested in duplo, and each sample was analyzed in triplicate for a total of 6 data points. Area counts for the endogenous peptides are represented by black crosses and a full line, whereas area counts for the SIL peptides are represented by open blue circles and a dotted line.

eluting from the Oasis HLB material with 5% methanol, it was decided that the optimal SPE procedure is to load the sample in 5% NH_3(aq), wash with water and elute using 80% MeOH containing 2% FA. This procedure was further used throughout the experiments. The relative recoveries of endogenous and SIL peptides were evaluated using a mixture of synthetic endogenous and SIL peptides. Recoveries of the endogenous and SIL peptides were not significantly different (Supplementary Figure 5).

Optimization of the tryptic digestion

It is well known that the tryptic digestion and particularly its completion is an important aspect of quantitative clinical chemistry methods for protein quantitation. Therefore, we assessed the completeness of the digestion using a digestion time curve. Human-derived AT standard was spiked in 40 g/L HSA and in two randomly selected native plasma samples. Samples were prepared in duplo with digestion times of 0, 1, 2, 3, 6 and 20 h. Each of the samples was analyzed in triplicate. The results of the digestion time curve for quantifying peptides FDTISEK and FATTFYQHLADSK for the two plasma samples are shown in Figure 3. The digestion curves of the peptides reach a plateau by 3 and by 6 h, respectively, whereas there is limited degradation of the SIL peptide (less than 10% by 20 h), indicating these peptides are suitable for accurate quantitation. Results of the other peptides and glycopeptides monitored for one of the plasma samples are shown in Supplementary Figure 6. Clearly, the rate of formation of peptides during digestion may vary widely among peptides, as has been observed previously for other proteins [34]. However, this does not pose a problem, as these other peptides besides FDTISEK and FATTFYQHLADSK are only used for relative quantitation.

Calibration of AT test using AT standard purified from plasma

To evaluate whether the developed method provides precise and more or less accurate results, three calibration curves were prepared: human-derived AT standard

Table 3: Quantitation of AT using LC-MRM-MS in four plasma samples.

	FDTISEK	FATTFYQHLADSK	TSDQIHFFFAK	ANRPFLVFIR
Plasma A				
Concentration, μmol/L	1.47	1.72	1.73	1.81
%CV	2.08	7.63	5.37	1.16
Plasma B				
Concentration, μmol/L	1.24	1.60	1.62	1.51
%CV	2.14	3.10	2.62	2.28
Plasma C				
Concentration, μmol/L	1.72	2.15	2.28	2.22
%CV	2.69	4.53	2.61	2.66
Plasma D				
Concentration, μmol/L	1.54	1.81	2.03	1.90
%CV	3.41	5.67	1.43	2.16

Calibration curves of human-derived AT standard in 40 g/L HSA were prepared in duplicate, and samples were analyzed in quadruplicate.

as well as human-derived β-AT standard were individually spiked in 40 g/L HSA at concentrations of 0, 0.2, 0.5, 2.0, 5.0 and 20 μmol/L. The calibration curves were prepared and analyzed in duplicate. Furthermore, four randomly selected plasma samples were included in quadruple to preliminarily assess the precision. Results of the calibration curve for the human-derived AT standard are shown in Supplementary Figure 7. Calibration curves of both AT and β-AT show good linearity, but it has to be mentioned that ~10% lower quantities are found for the β-AT curve (data not shown), most likely indicating that the concentrations of the purified protein materials as determined by Lowry's method were different. When the concentrations of the four plasma samples were calculated based on the average calibration curve obtained for AT, CVs below 3.5% were obtained for peptide FDTISEK and below 8% for peptide FATTFYQHLADSK (Table 3).

To assess the relative concentration of β-AT in a sample, the glycopeptide KANK was measured in mixtures of β-AT standard in AT standard in 40 g/L HSA at 0%, 5%, 10%, 20%, 50%, 75% and 100% β-AT at an AT concentration of 2 μmol/L (Supplementary Figure 8). Unfortunately, the area counts of glycopeptide KANK are too low, thus indicating that the method is not yet suitable to distinguish and quantify β-AT from α-AT.

To further assess whether the calibration curve prepared in HSA is commutable to plasma samples, calibration curves of AT were prepared in 40 g/L HSA and in two randomly selected plasma samples. The resulting calibration curves are shown in Supplementary Figure 9. Because the slopes of the calibration curves in plasma are close to 1 relative to the calibration curve in HSA, these results indicate that the calibration curve in HSA can be used for quantitation of AT in plasma samples.

Discussion

Standardization of any medical test starts with defining the measurand(s) at the top of the traceability chain. AT measured in an activity-based assay measures overall AT-activity originating from different molecular proteoforms. Novel AT tests that recognize the molecular forms of AT are required. MS, which starts to find its way in protein clinical chemistry, is a promising technology as it allows the identification of specific molecular forms and facilitates multiplexed testing. In this study, we assessed the potential of LC-MRM-MS for the quantitation of AT and its proteoforms. Of our initial four aims to (1) absolutely quantify the plasma AT concentration, (2) identify as many clinically relevant AT mutations as possible, (3) relatively quantify α- and β-AT and (4) identify altered AT glycosylation profiles, we were successful in three, with the exception that we are so far not able to separately quantify α- and β-AT. Using SIL peptides as internal standards, we were able to quantify AT in four plasma samples with a CV below 3.5% for peptide FDTISEK and below 8% for peptide FATTFYQHLADSK. It has to be noted that a digestion time of 3 h was used to accommodate regular working hours, whereas the digestion time courses indicate that peptide FATTFYQHLADSK formation is only completed after 6 h. This could contribute to the lower precision. So far, the trueness of this quantitation remains to be evaluated.

Of the 26 clinically relevant mutations identified, we are theoretically able to identify 20 by loss of specific peptides (Table 1). Potentially, this number could be increased even further if transitions could be specified for peptides LNCQLYR and LNCHLYR, which would be formed upon mutation of R^{161}. A strategy in which qualitative analysis of peptides is used to evaluate the mutation status of a protein has been shown to be successful for ApoE [28,

35], but the strategy remains to be evaluated for AT. Similarly, we are now able to assess the relative quantity of the "typical" sialylated biantennary N-glycans present on sites N^{128}, N^{187} and N^{224}. Reduced quantities of these glycopeptides will provide strong indications of altered N-glycosylation profiles.

Unfortunately, the currently developed method is not successful in distinguishing α- and β-AT, as the glycopeptide KANK, originating from site N^{167}, has no retention on the reverse phase LC phase due to its small number of amino acids and results in ion counts too low for accurate relative quantitation. Alternative strategies are to be considered to specifically target α- and β-AT. One such alternative approach could be the derivatization of glycosylated peptides, which would alter their chemical properties. Although glycopeptide derivatization is not yet widely applied, several potential strategies have been reported [36–38]. Similarly, the use of ion mobility MS could be evaluated, as previous preliminary studies have shown glycopeptides separation in the ion mobility phase [39, 40]. Another alternative strategy could be the use of immunocapturing in combination with either a bottom-up protein quantitation approach, as described here, which would allow for the digestion and analysis of a concentrated AT sample, or a top-down intact protein analysis strategy using high resolution accurate MS. This latter strategy was recently applied for the analysis of α-1-antitrypsin [41].

A two-phased approach was used in the establishment of the LC-MRM-MS test for AT. First, the method was developed using the guidance provided by the CLSI C62-A guideline [32]. We specifically introduced a system suitability check to ensure that the mass spectrometer is in optimal condition to perform the measurements. Second, lab-developed tests that are intended to be used in clinical practice remain to be thoroughly evaluated according to, e.g. the cyclical Test Evaluation framework [42]. Under the assumption that the clinical utility of AT proteoforms will be demonstrated in the future, it remains to be proven that the LC-MRM-MS test for AT profiling is robust enough to withstand the rigor required in clinical chemistry [43].

Overall, this proof-of-principle study shows the potential and challenges of bottom-up proteomics for the quantification of plasma AT and for the in-depth characterization of clinically relevant proteoforms. We anticipate that knowing the measurands (proteoforms) at the top of the metrological traceability chain will enable future standardization of AT tests on one hand, as well as recognition of clinically relevant AT proteoforms with specific functional defects on the other hand. These technological developments will further contribute to precision diagnostics to facilitate better patient care.

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