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Development of a Method for Absolute Quantification of Equine Acute Phase Proteins Using Concatenated Peptide Standards and Selected Reaction Monitoring

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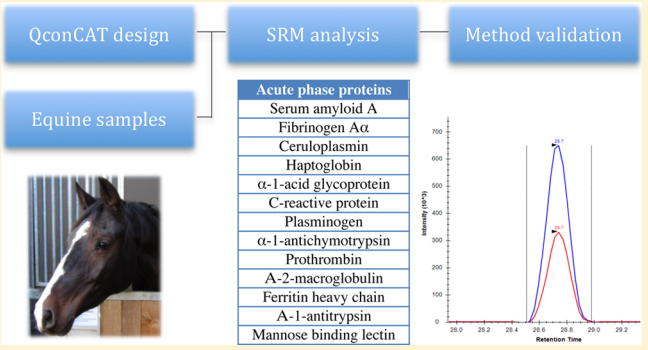
Supporting Information

ABSTRACT: The aim of this study was the development of a quantitative assay that could support future studies of a panel of acute phase proteins (APPs) in the horse. The assay was based on a quantification concatamer (QconCAT) coupled to selected reaction monitoring methodology. Thirty-two peptides, corresponding to 13 putative or confirmed APPs for the *Equus caballus* (equine) species were selected for the design of a QconCAT construct. The gene encoding the QconCAT was synthesized and expressed as an isotope-labeled chimaeric protein in *Escherichia coli*. The QconCAT tryptic peptides were analyzed on a triple-quadrupole instrument, and the quantotypic properties were assessed in equine serum, wound tissue, and wound interstitial fluid. Reasonable quantotypic performance was found for 12, 14, and 14 peptides in serum, wound tissue, and interstitial fluid, respectively. Seven proteins were quantified in absolute terms in serum collected from a horse before and after the onset of a systemic inflammatory condition, and the observed protein concentrations were in close agreement with previous data. We conclude, that this QconCAT is applicable for concurrent quantitative analysis of multiple APPs in serum and may also support future studies of these proteins in other types of tissues and body fluids from the horse.



KEYWORDS: *QconCAT, acute phase proteins, veterinary proteomics, selected reaction monitoring, quantotypic peptides, wound healing, equine, validation*

INTRODUCTION

Proteomics has become a quantitative science, and its relevance and importance in biomedical and clinical research is increasingly evident.[1] Several mass-spectrometry-based technologies have been developed to quantify the complexity, interconnectivity, and dynamic nature of proteomes. Several such approaches rely on stable isotope labeling of peptides or proteins, supporting either relative[2] or absolute[3−5] quantification of specific proteins in complex biological samples.

Relative quantification provides a dimensionless term and is valuable for the discovery of differentially expressed proteins, whereas validated methods for absolute quantification are essential for clinical applications[6,7] as well as for correlating protein expression data across different biological samples and across multiple experiments, instruments, and laboratories.[8]

A common feature of absolute quantification strategies is the analysis of proteotypic peptides as surrogates for the intact protein. Isotope-labeled peptides for absolute quantification can be produced by chemical synthesis[5,9] or by biological expression via a quantification concatamer (QconCAT).[3,10] In the QconCAT strategy, a chimaeric protein is designed as a concatamer of tryptic standard peptides, metabolically labeled with stable isotopes labeled amino acids[3] or simple heavy atom

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centers[11] in *Escherichia coli*. The QconCAT protein is spiked into biological samples to allow absolute quantification of the analytes. Because all isotope-labeled peptides are present in a single copy within the chimaeric protein, they will inherently be present at the exact equimolar quantities within the analyte sample. It has been estimated that QconCATs are both technically and economically efficient for multiplexed absolute quantification of more than about five proteins.[4,10,12,13]

An important consideration for quantification experiments, whether with chemically synthesized peptides or QconCATs, is completeness of digestion.[14] In QconCAT methodology, quantification is impaired if either the concatamer or the analyte proteins are incompletely digested to different extents. The impact of primary structure on tryptic cleavage is influenced by flanking residues at least three positions proximal to or distal from the cleavage site.[15] One solution to this problem is the insertion of short peptide sequences of three to four amino acids in length, juxtaposed between each standard peptide, to mimic the native primary sequence context of the analyte. The interspersion of the short native peptide sequences has previously been used to resolve issues of dibasic sequences.[4] Here we also apply this approach in the design of a QconCAT that reconstructs the primary sequence context with interspersed hexapeptide sequences between each standard peptide to equalize the rate of digestion of standard and analyte.

The QconCAT was designed to target seven well-known (serum amyloid A (SAA),[16] fibrinogen (FIB),[17] ceruloplasmin (Cp),[18] haptoglobin (Hp),[19] α-1-acid glycoprotein (A1AG),[20] C-reactive protein (CRP),[21] and plasminogen (PLG)[22]) and six potential acute phase proteins (APPs) (α-1-antichymotrypsin (A1ACT), prothrombin (PT), α-2-macroglobulin (A2M), ferritin (FTH), α-1-antitrypsin (A1AT), mannose-binding lectin (MBL)) in the horse. APPs are a functionally heterogeneous group of proteins with the common feature of concentration changes by at least 25% during inflammation.[23] This makes this group of proteins particularly appropriate for disease monitoring. More than 30 APPs have been described in human,[24] but there is extensive variability in the APP response patterns between different species,[25,26] and of the more than 30 identified human APPs, only the seven well-known APPs previously mentioned have been verified as APPs in the horse. Here we report the design of the QconCAT construct termed equine acute phase 1 (eAP1) and present the validation of the quantotypic properties of 32 standard peptides representing the 13 proteins. The eAP1 is the first quantification concatamer to support mass-spectrometry-based quantification of equine protein. We evaluate its applicability for absolute protein quantification in three different types of samples from *Equus caballus* (equine) that are relevant for monitoring wound healing, namely, serum, wound tissue, and wound interstitial fluid (obtained with microdialysis).

EXPERIMENTAL METHODS

Selection of Proteins and Peptides for the Method

Previously published data[24,27,28] have contributed to the selection of equine APP candidates, and proteins were selected to represent a variety of biological functions known from the human APP, including host defense, coagulation and fibrinolysis, inhibitors of serine proteases, transport proteins, and proteins with antioxidant activities. In total, 13 proteins were represented in the QconCAT (Table 1). Proteotypic peptides were selected from those proteins included in the Equine PeptideAtlas (www.peptideatlas.org).[29] Selection was based on the PeptideAtlas empirical suitability scores of each peptide, which takes into account both observations from previous shotgun proteomics experiments and the physicochemical properties of the peptides.[30] Only unique and true tryptic peptides with a length of 7−20 amino acids and which mapped to single genome locations were chosen. The criteria for peptide selection for QconCATs described by Brownridge et al. 2012[3] were followed whenever possible. Exclusion of peptides expected to compromise the quantification reduced the list of candidate peptides dramatically and even left some proteins with target peptides that include amino acids that may compromise quantitative detection by LC−MS/MS. For proteins that were not yet included in the Equine PeptideAtlas, proteotypic peptides were selected based on an in silico digest of the protein, and quantotypic peptides were selected according to the same criteria as above. Three to five candidate peptides were initially selected from each protein (Supplementary File 1 in the Supporting Information).

Preparation and Analysis of Synthetic Candidate Peptides

Synthetic candidate peptides (SpikeTides, JPT Peptide Technologies, Berlin, Germany) were used for optimization of the selected reaction monitoring (SRM) assay conditions. Between 50 and 60 nmol of synthesized crude peptides were resuspended in 250 μL of 80% (v/v) 0.1 M ammonium bicarbonate (AmBic) and 20% (v/v) acetonitrile for 30 min in a 96-well plate. SpikeTides were combined by adding 2 μL aliquots from each peptide stock to an Eppendorf tube with 200 μL in 0.1% (v/v) formic acid. The combined peptide samples were micropurified (Poros 50 R2 reverse-phase column material; Applied Biosystems, Foster City, CA, packed in GEloader tips; Eppendorf, Hamburg, Germany) and dried in a vacuum centrifuge, resuspended in 0.1% (v/v) formic acid, and stored at −20 °C until analysis. Each injection of the combined peptide sample contained ∼100 fmol of each peptide. The samples were loaded on an EASY-nano liquid chromatography (LC) (Thermo Scientific, Waltham, MA) with a Biosphere C18 precolumn (ID 100 μm × 2 cm, 5 μm, 120 Å, NanoSeparation, Nieuwkoop, The Netherlands) and separated on an in-house packed C-18 analytical column (3 μm ReproSil-Pur C18-AQ material packed in a PicoTip emitter, ID 75 μm × 10 cm, New Objective, Woburn, MA). The samples were separated at a flow rate of 250 nL/min using either a 13 or 50 min gradient from 5 to 35% solvent B (0.1% formic acid, 90% acetonitrile), followed by a 10 min wash in 100% solvent B. The Easy-nLC was coupled in-line to a QTRAP 5500 (AB SCIEX, Framingham, MA) mass spectrometer operated under Analyst TF v. 1.6.1. The QTRAP was in positive ion mode with 2500 V ion spray voltage, curtain gas setting of 30, ion source gas setting of 5, and an interface heater temperature of 150 °C.

Creation and Optimization of the Selected Reaction Monitoring Assay for Peptide Analysis

The SRM method was developed and optimized using Skyline v. 1.4 (https://skyline.gs.washington.edu).[31] After each SRM−mass spectrometry (MS) scan, data were imported to Skyline where transitions were filtered according to quality, and refined SRM methods were constructed by including only the best and most intense transitions for each peptide. A minimum of three transitions with product ion m/z greater than the precursor, the highest intensities, and the best signal-to-noise ratios were selected. The collision energy settings were optimized with parameters set to five steps on each side of the value predicted by default equation and the step size set to 1. Finally, a 60 min scheduled method was created with a 2 s cycle time and a detection window of 4 min. The Savitzky−Golay smoothing

Table 1. Information about the Peptides Included in the Quantification Concatamer eAP1[a,b]

protein	N-	peptide sequence	C-	peptide ID	WT	IF	SE
GluFib	MGTK	EGVNDNEEGFFSAR		GluFib			
CRP (*F6PZG1*)[c]	LTR	DYSLFSYATK	KQN	CRP_p1	B	B	B
	MSK	QAFVFPK	ESE	CRP_p2	B	B	B
SAA (*F7BJA9*)[c] (*hepatic*[1]) (*F6PJF6*)[c] (*extrahepatic*[2]) (*common*[3])	AAR	GTWDMIR[1]	AYH	SAA_p1	C	C	C
	SSR	EWFTFLK[2]	EAG	SAA_p2	B	B	B
	DMR	EANYIGADK[3]	YFH	SAA_p3	B	B	B
	AQR	GPGGAWAAK[3]	VIS	SAA_p4	B	B	B
	DSR	ADQAANEWGR[3]	SGK	SAA_p5	B	B	B
FIBα (*O97641*)[c]	RLK	VSLLSDLLPADFK	SQL	FIBα_p1	A	A	B
	LFR	TFPGEGSLDGLFHR		FIBα_p2	A	A	B
		HPDEAAFFDSFSSK	TQS	FIBα_p3	A	A	B
Cp (*F6PQ46*)[c]	HNK	GAHPLSIEPIGVR	FSK	Cp_p1	A	A	A
	GVK	TESSTVTPTSPGETR	TYI	Cp_p2	A	A	A
Hp (*F6XWM5*)[c]	RLR	SEGDGVYALNSEK	QWV	Hp_p1	A	A	A
	VQR	IIGGLLDAK	GSF	Hp_p2	A	A	A
	VGR	VGYVSGWGR	NAN	Hp_p3	A	A	A
A1AG (*F7CQ86*)[c]	NFR	ASPITDATLDR	ISG	A1AG_p1	A	B	B
	LGR	EHVGYLWLTK	DPR	A1AG_p2	A	B	B
	MDK	SEIIYADEK	QDQ	A1AG_p3	A	A	B
A1ACT (*F6ZLR1*)[c]	LPK	FSISSNYELETILTQLGIEK	VFT	A1ACT_p1	C	C	C
	AEK	LINDYVEK	KTE	A1ACT_p2	B	A	B
PT (*F7BFJ1*)[c]	STK	NLSPSLESCVPDR	GQQ	PT_p1	A	A	A
	EGR	TTDEDFPLFFDVK	TFG	PT_p2	A	A	A
PLG (*F6USP9*)[c]	FTR	KPLSASSIEECEAK	CTE	PLG_p1	A	A	A
	RGR	VSVTQSGLTCQR	WSE	PLG_p2	A	A	A
A2M (*gi*|*194211675*)[d]	LPK	FEVQVTVPK	IIT	A2M_p1	A	A	A
	IAR	LLVYTILPDGEVVGDSAK	YEI	A2M_p2	A	A	A
FTH (*Q8MIP0*)[c]	FAK	YFLHQSHEER	EHA	FTH_p1	B	B	B
	LEK	NVNESLLELHK	LAT	FTH_p2	B	B	B
A1AT (*B5BV01*)[c]	QGK	LQHLEDTLTK	GIL	A1AT_p1	A	A	A
	LHK	AVLTIDEK	GTE	A1AT_p2	A	A	A
MBL (*F7CI62*)[c]	PGK	LGPPGSPGPR	GLP	MBL_p1	B	B	B
	DVK	TEGQFVDLTGR	ALT	MBL_p2	B	B	B

[a]GluFib: [Glu1]-fibrinopeptide B; CRP: C reactive protein; SAA: serum amyloid A; FIBα: fibrinogen Aα; Cp: ceruloplasmin; Hp: haptoglobin; A1AG: alpha-1-acid glycoprotein; A1ACT: alpha-1-antichymotrypsin; PT: prothrombin; PLG: plasminogen; A2M: alpha-2-macroglobulin; FTH: ferritin heavy chain; A1AT: Alpha-1-antitrypsin; MBL: mannose-binding lectin. [b]Sequence is given for each peptide including the N- and C-terminal juxtaposed three natural occurring amino acids. The peptides are classified as either Type A (Analyte-to-standard ratio >0.05), Type B (Analyte-to-standard ratio <0.05), or Type C (Analyte and standard signal missing) in three equine matrices: wound tissue (WT), interstitial fluid from a wound (IF), and serum (SE). [c]UniProt (www.uniprot.org). [d]NCBI (www.ncbi.nlm.nih.gov).

application in Skyline was applied to the generated peaks. Peptides that could not be detected by SRM or for which three coeluting transitions could not be identified were excluded from the method. From the initial 56 peptides, 32 peptides were taken forward for the QconCAT construct (Supplementary File 2 in the Supporting Information).

Construction and Production of the Quantification Concatamer

On the basis of the SRM-derived performance data, at least two peptides for each target protein were assembled into the QconCAT, termed equine acute phase proteins 1 (eAP1), flanked with their three natural juxtaposed amino acids. Thus, each quantification peptide was interspersed with a six amino acid sequence recapitulating the true primary sequence context of the natural peptide. In addition, [Glu1]-Fibrinopeptide B (GluFib) and a hexahistidine-tag were incorporated into the QconCAT at the N- and C-termini, respectively (Supplementary File 3a in the Supporting Information). The codon-optimized eAP1 gene (Supplementary File 4a,b in the Supporting Information) was synthesized and ligated into the expression vector pET-21a by PolyQuant (Bad Abbach, Germany). The eAP1 plasmid is freely available for research applications and is distributed upon request.

The eAP1 gene was expressed in *Escherichia coli*.[3] The bacterial cell pellets were lysed using sonication, and the eAP1, present in inclusion bodies, was recovered by centrifugation (6000*g*, 8 min, 4 °C) solubilized in buffers containing 6 M guanidinium hydrochloride and purified by metal affinity chromatography.[3] Positive-ion MALDI-TOF (Ultraflex; Bruker, Bremen, Germany) mass spectra (reflector mode, m/z range 900−4500) were obtained of the purified and digested eAP1. Peptide monoisotopic masses verified that all expected peptides were readily discernible (data not shown).

Equine Tissue and Body Fluid Samples for Validation of the QconCAT-SRM-Based Method

The protocol was approved by the Danish Animal Experiments Inspectorate, Ministry of Justice (license no. 2010/561-1882), and all procedures were carried out according to the Danish Animal Testing Act. From two different experimental wounds located on the shoulder of a horse, a wound tissue biopsy was

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collected 2 days after wounding, and interstitial fluid was sampled 14 days after wounding following a previously described procedure.[32] The biopsy, sampled from the margin of the wound avoiding the epithelium was immediately washed in phosphate-buffered saline to remove blood. The wound tissue biopsy and microdialysate were snap-frozen in liquid nitrogen and stored at −80 °C until further processing. Blood was collected from the jugular vein in a serum tube with clot activator (BD Vacutainer; Belliver Industrial Estate, Plymouth, U.K.) and centrifuged within 4 h (2000*g*, 10 min, room temperature). Serum was stored at −80 °C until further processing.

Equine Serum and Microdialysate for Quantification in Absolute Terms

Serum was recovered from a second horse that developed systemic inflammation due to cellulitis in one hindlimb. The horse developed cellulitis 8 days after the experimental wounds were created. The horse was treated with butorphanol tartrate (Torbugesic, ScanVet, Denmark), firocoxib (Equioxx, Merial, Duluth, GA), gentamicin (Genta Equine, Franklin Pharmaceuticals, Ireland) and benzylpenicillin (Benzylpenicillin Panpharma, FarmaPlus, Norway), and the clinical signs of cellulitis resolved within 4 days. The blood concentrations of the equine inflammatory marker SAA[27] peaked after 2 days and decreased to reach normal reference values after 13 days. Serum from this horse was obtained the day before the surgical procedure (day 0) and 14 days after the wounding procedure. These samples allowed absolute quantification of the target proteins under normal conditions and after onset of systemic inflammation of the same individual.

Protein Digestion and Analysis

Wound biopsies were pulverized in liquid nitrogen using an in-house designed mortar. An amount of 14 mg powder was further homogenized in 432 μL of 25 mM AmBic solution for 5 × 90 s (30 Hz) (TissueLyzer II, Qiagen, Hilden, Germany) and cooled for 1 min on ice in between each homogenization interval. Further processing and storing of the biological samples and eAP1 was performed in 0.5 mL low-binding tubes (Eppendorf, Hamburg, Germany). A preliminary mass spectrometric analysis of the eAP1 combined with different volumes of the sample material was used to decide the adequate amount of sample. A volume of 11 μL of microdialysate, 0.25 μL of serum, 48 μL of eAP1, and a volume corresponding to 1 mg wound tissue were pipetted out for further processing. A 25 mM AmBic solution was added to all samples to a final volume of 160 μL. Denaturation of proteins was attained by the addition of 10 μL of a 1% w/v in 25 mM AmBic solution of Rapigest (Waters, Milford, MA), followed by heating for 10 min at 80 °C. Disulfide bonds were reduced by the addition of 10 μL of 9.2 mg/mL dithiothreitol in 25 mM AmBic for 10 min at 60 °C, and cysteinyl residues were alkylated in 10 μL of 33.3 mg/mL iodoacetamide in 25 mM AmBic for 30 min in the dark. A volume of 10 μL of a 4.4 pmol/μL GluFib standard (Waters) in 25 mM AmBic was added to the eAP1 samples to enable absolute quantification. All samples were digested by adding trypsin (50:1 protein:trypsin ratio) at 37 °C. An additional aliquot of 10 μL trypsin was added after 4 h, and hence the overnight digestion at 37 °C was made in a final (25:1 protein:trypsin) ratio. SDS-PAGE of 10 μL of digest was used for quality check of the digestion. Samples were cleaned by incubation with 1 μL of trifluoroacetic acid for 45 min in 37 °C, followed by centrifugation at 13 000*g* for 20 min, and the supernatant fractions were carefully removed. The eAP1 digest was spiked into the sample and diluted to the appropriate end concentration by addition of the digested analyte. Following micropurification (StageTip C18, 20 μL tips, Thermo Scientific, Waltham, MA) and resuspension in 0.1% formic acid, samples were processed on the EASY-nLC (Thermo Scientific) and analyzed by SRM on the QTRAP 5500 (AB SCIEX, Framingham, MA) with settings as previously described. The quantotypic properties of the eAP1-derived peptides were based on triplicate sample processing and SRM analysis. An amount corresponding to 1.2 nL serum, 48 ng wound tissue, and 52 nL of microdialysate was analyzed with 7 fmol heavy-labeled eAP1 added to the matrix. This corresponds to ∼0.1 μg protein for body fluids and 0.2 μg protein for tissue.

Linearity of Calibration Curves

An accurately quantified amount of unlabeled GluFib (Waters) was added to the sample containing eAP1 just before digestion. The eAP1 digests were diluted to defined concentrations of the unlabeled GluFib by addition of analyte digest (interstitial fluid). The calibration curve was based on the following amounts of unlabeled GluFib: 0.15; 0.3; 1.5; 3; 7.5; 15; 22.5; 30 fmol. These specific amounts were chosen to ensure evenly distributed values in the entire dynamic range investigated.

The analyte samples were analyzed with the addition of 7 fmol eAP1-derived labeled peptides on column. Furthermore, calibration curves were established for each peptide without missed cleavage and analyte-to-standard (A/S) ratio >0.05 through serial dilution of the heavy-labeled peptides from 400 amol to 80 fmol to assess linearity. In addition, the concentration of SAA in the analyte sample was adequate to establish a calibration curve spanning the entire dynamic range investigated (Supplementary File 5 in the Supporting Information).

Data Analysis and Statistics

The QconCAT labeling-protocol generates a standard protein that is labeled to better than 98−99%, leaving only 1 to 2% of the standard unlabeled.[33] The small amount of unlabeled standard has the consequence of reducing the dynamic range of quantification. In these studies, an analyte intensity (the added area under the product-ion curves) of <5% of the standard intensity was set as a cutoff, below which quantification was deemed insecure (data in Supplementary File 6a-f in the Supporting Information).

The standard deviation and coefficient of variation (CV) were determined for the mean A/S ratio obtained by triplicate processing and SRM analysis of a sample. The R statistical software version 3.01 (Boston, MA) was used. The library "plyr"[34] was used to calculate means. standard deviation, and CV. Graphics were generated by use of the R library "ggplot2" (http://had.co.nz/ggplot2/) (Supplementary File 5a−f in the Supporting Information).

Quantification in absolute terms was based on the mean of the A/S ratios for quantotypic peptides mapping to the same protein. The known amount of the heavy-labeled peptide enabled calculation of the amount of the analyte peptide in fmol/μL digest. The concentration of each specific protein was calculated from femtomol signals to μg protein/mL of serum by taking into account the molecular weight of each specific full-length protein.

RESULTS AND DISCUSSION

Target Proteins and Peptides

The QconCAT described here is the first quantification concatamer to support mass-spectrometry-based quantification of equine proteins. Some equine APPs such as SAA, FIB, and Hp

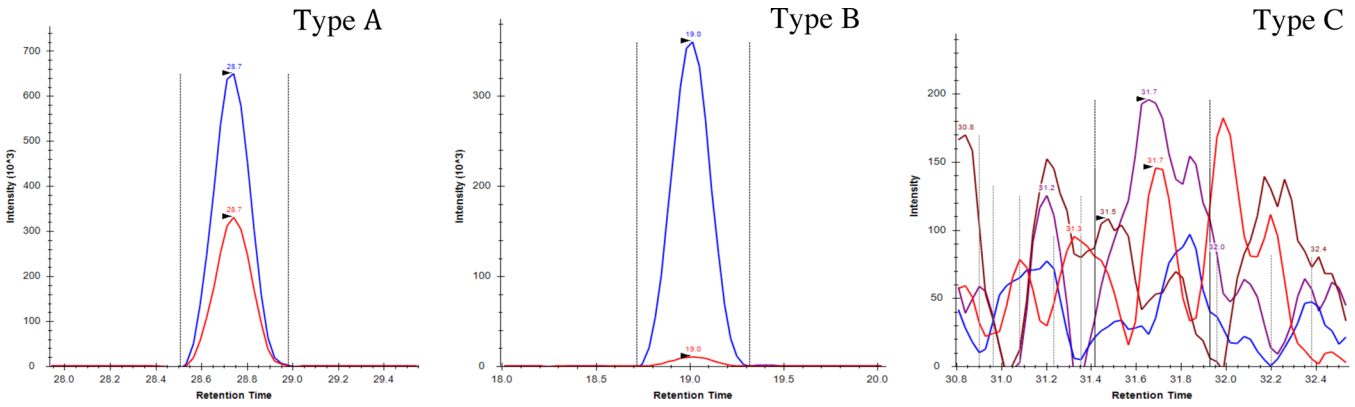

Figure 1. Selected reaction monitoring chromatograms exemplifying Types A−C peptide quantification. Type A: analyte-to-standard ratio >0.05; Type B: analyte-to-standard ratio <0.05; Type C: missing signal from both analyte and standard peptide. Blue: intensity of doubly charged standard peptide; brown: intensity of triply charged standard peptide; red: intensity of doubly charged analyte peptide; purple: intensity of triply charged analyte peptide.

are well-documented inflammatory markers for inflammation and are widely used in clinical diagnostics,[27] but the equine APP response is not yet fully elucidated. In part, this is due to the lack of adequate and robust methods for monitoring the APP concentrations in complex biological samples.[23] The peptides included in eAP1 represent seven equine APPs and six proteins with proposed APP properties, included to support future investigations of the APP response in the horse.

The recent release of the Equine PeptideAtlas[29] provided information about proteotypic peptides for the majority of the proteins studied herein. Not all proteotypic peptides are equally well suited for stable quantification, why proteotypic peptides suitable for quantification are often referred to as quantotypic peptides.[33,35] Many factors contribute to explaining why some proteotypic peptides are not suitable for quantification including inconsistent enzyme susceptibility at specific cleavage sites, inconsistent trajectory in LC−MS/MS analysis or post-translational modifications.[3] These constraints reduced the list of candidate peptides dramatically, and inclusion of peptides prone to variable cleavage was unavoidable.

Thirty-two peptides were assembled into eAP1 (Supplementary File 1 in the Supporting Information), with at least two quantotypic peptides representing each protein of interest. Two independently selected proteotypic peptides per protein are sufficient for greatly quantification of the target protein,[3] and inclusion of three or more peptides per protein would provide additional support to the protein quantification data but would impact the size of the QconCAT. A standard of around 50−70 kDa (heavy labeled), encoding 450−550 amino acids, has been suggested as optimal in terms of downstream handling.[36] The eAP1 described in the present study is ∼64.1 kDa and encodes 594 amino acids.

Bacterial high-level expression of the codon-optimized gene led to aggregation of eAP1, facilitating recovery as inclusion bodies.[33] After purification, a single major band was observed by SDS-PAGE of mobility consistent with the expected size of eAP1 (Supplementary File 3b in the Supporting Information). The peptide order in the QconCAT has previously been shown to affect the translation success,[37]but we confirmed by in-gel digest and MALDI-TOF peptide mass fingerprinting (data not shown) that eAP1 has the expected molecular mass, and thus we conclude that the eAP1 was successfully translated.

Quantotypic Properties of eAP1 Peptides

Biological samples such as tissues and body fluids vary considerably in complexity and in the relative abundances of the individual proteins. The aim of establishing a QconCAT-SRM method for APPs is to provide a method that can assist in evaluating the abundance and dynamic fluctuations of these proteins in natural samples under a wide range of physiological conditions. We chose to investigate the quantotypic properties of this subset of standard peptides in a range of samples that reflect biological processes in wound healing and inflammation. This allowed us to monitor the effects of the very different protein matrices of tissues/body fluids on the quantotypic properties of these peptides. The test samples included interstitial fluid as well as granulation tissue from a wound. Additionally, we chose to include studies of quantotypic properties in serum because this allowed correlating our observations in the mass spectrometer to previous observations on some of the proteins from other laboratories where other biochemical assays have been used.

The quantotypic properties of the eAP1-derived peptides were evaluated based on triplicate processing of digestion and SRM analysis with 7 fmol of the eAP1-derived peptides. The peptides were categorized as "Type A" (A/S ratio >0.05), "Type B" (A/S ratio <0.05), or "Type C" (missing standard and analyte signal) for a particular protein loading (Figure 1).[36] Of the 32 peptides composite of the eAP1 13, 19, and 17 peptides were "Type A" in serum, wound tissue, and microdialysate, respectively. Only two peptides were "Type C" in all three matrices (Table 1, Figure 2). The classification of a peptide is affected of both the concentration of the heavy standard and the analyte, so decreasing the load on column of the standard or the use of an enrichment strategy might promote the "Type B" peptides to "Type A". Furthermore, the abundance of some analyte peptides may vary dependent on the physiological condition at the time point of sampling and, for instance, increase or decrease in their abundance in response to inflammation, which particularly is relevant when analyzing APPs as in this study.[16]

Validation and consistency of the quantification method was evaluated by several criteria that included tryptic digestion efficiency, compliance between isotope ratio of sibling peptides (those peptides in the eAP1 construct derived from the same protein), reproducibility of the quantification, and the monotonicity and linearity of the calibration curves (Figures 2 and 3).

The eAP1 performed well in all three types of matrix (Figure 2). The relative abundance of peptides was close to similar in between the matrices except for FIBα and A1AG. For FIBα, the analyte signal was below the limit of quantification in serum, but this was expected because FIB is processed to form the fibrin clot during

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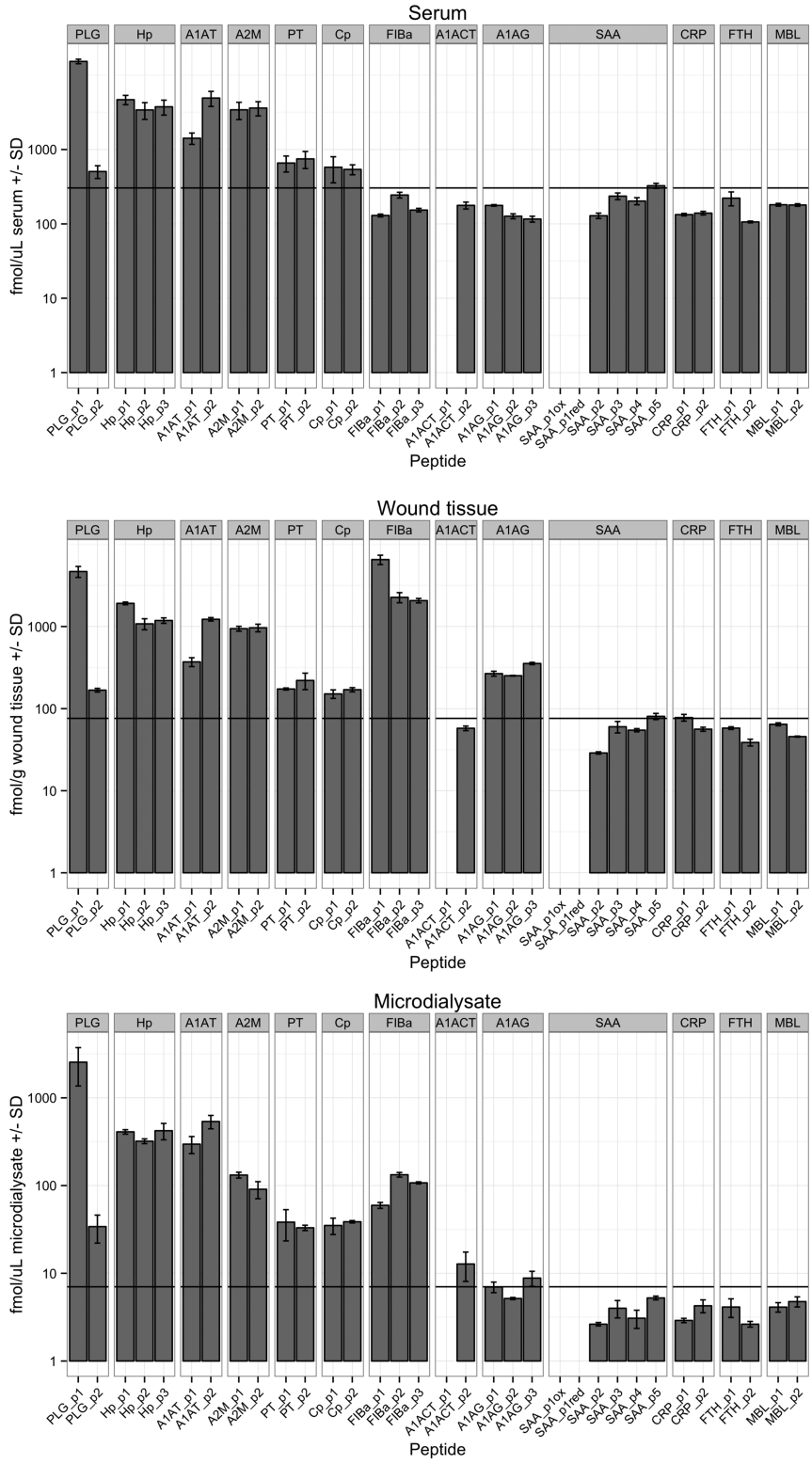

Figure 2. Analyte-to-standard ratio for each peptide in serum, wound tissue, and interstitial fluid collected by microdialysis from a wound (microdialysate). Horizontal line marks the lower limit of quantification (analyte-to-standard ratio <0.05). PLG: plasminogen, Hp: haptoglobin, A1AT: alpha-1-antitrypsin, A2M: alpha-2-macroglobulin, PT: prothrombin, Cp: ceruloplasmin, FIBa: fibrinogen Aα, A1ACT: alpha-1-antichymotrypsin, A1AG: alpha-1-acid glycoprotein, SAA: serum amyloid A, CRP: C reactive protein, FTH: ferritin heavy chain, MBL: mannose-binding lectin. The analyte-to-standard ratios are mean values from triplicate sample processing and analysis. The *y* axis is log-transformed.

the coagulation process to recover serum.[38] A1AG was relatively more abundant in wound tissue compared with serum and interstitial fluid (Figure 2), which is theorized to be due to a difference in abundance in body tissues and fluids.

The ideal protein standard would be an identical protein, isotopically labeled, accurately quantified, occupying exactly the same tertiary and quaternary structural space, and displaying an identical range of post-translational modifications as the native

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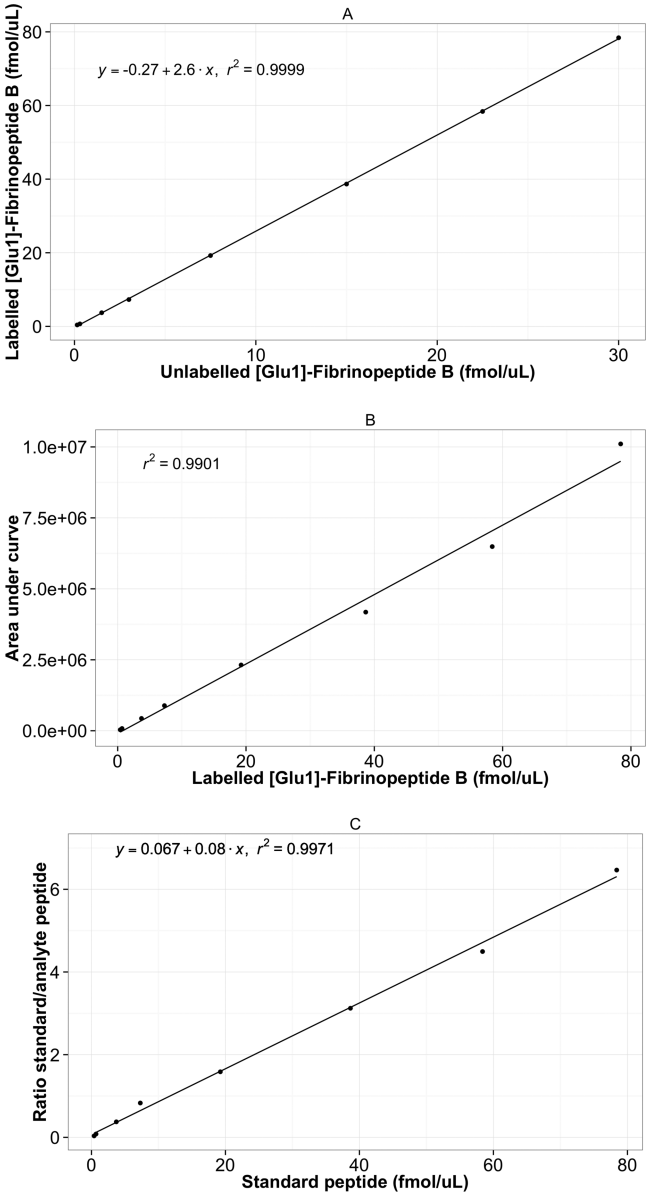

Figure 3. (A) Correlation of unlabeled and labeled [Glu1]-fibrinopeptide B peptide. The equation and r^2 for the best line of fit are given. (B) Correlation of the labeled [Glu1]-fibrinopeptide B and the area under the curve from the selected reaction monitoring analysis. (C) Calibration curve for the peptide Hp_p3 mapping to the protein haptoglobin. Standard peptide refer to the heavy-labeled peptide derived from the quantification concatamer eAP1. For the best line of fit the r^2 is given.

protein. However, as for other protein standards, the QconCAT protein is expressed in a different organism and thus may not fold in the same structure or carry the same PTMs, and such variances can alter the kinetics of proteolysis and could compromise the anticipation of strict stoichiometry between standard and analyte peptide.[33] Despite these considerations accumulating studies conclude that the results generated by use of a QconCAT with interspersed native amino acids is a close approximation.[4,39]

An essential requirement for SRM-based absolute quantification is complete and consistent tryptic release of the peptides from analyte and standard.[14]

Occasional missed cleavage was evident for four peptides (Table 2). The peptide VSVTQSGLTCQR was observed with a

Table 2. Peptides Observed with Missed Cleavage by LC−MS/MS Analysis[a]

Protein	Peptide ID	Sequence with missed cleavage
Haptoglobin	Hp_p1	LR **SEGDGVYALNSEK**
Alpha-1-acid glycoprotein	A1AG_p3	MDK **SEIIYADEK** QDQLPK **SEIIYADEK** QDQLPK MDK **SEIIYADEK**
Alpha-1-antichymotrypsin	A1ACT_p2	**LINDYVEK** K
Plasminogen	PLG_p2	GR **VSVTQSGLTCQR**

[a]Proteotypic peptide included in the quantification concatamer eAP1 is given in bold text. The peptide in bold text including the flanking amino acids given in the table is the peptide observed by LC−MS/MS.

missed cleavage in the N-terminal. This is likely caused by the position of the arginine residues in the interspersed sequence (RGR), a pattern that can result in missed cleavage.[40] An acidic residue in P2′ can impair the interaction of trypsin at the cleavage site especially if the residue in P1 is lysine.[15] This may explain why SEIIYADEK (A1AG_p3) and SEGDGVYALNSEK (Hp_p1) were observed with a missed cleaved site. The peptide LINDYVEK (A1ACT_p1) was observed with a missed cleavage around the dibasic site at the C-terminus. Peptides with dibasic sites tend to be cleaved at one or the other of the basic residues[40] and are therefore normally not recommended for absolute quantitation studies.[3] Despite this shortcoming, we decided to evaluate the quantotypic traits of this peptide because better-suited alternative peptides were not available.

The relative occupancy of the route of digestion of a protein is a consequence of the intrinsic digestibility of each bond. Because the peptides included in eAP1 have been selected based on their proteotypic characteristics and the cleavage context is optimized by including the native three amino acids N- and C-terminal to the peptide, the order of the peptides in the artificial eAP1 protein is assumed to have an insignificant influence on the digestion efficiency.[14] This was also concluded in a previous study.[37] Furthermore, it is critical to remove barriers to digestion by reduction of disulfide bonds, blocking disulfide reformation with alkylating agents, and by the use of chaotropes to unfold proteins.[14] These steps were all included in this study. In addition, a digestion enhancer detergent (Rapigest) was employed to minimize complications from incomplete proteolysis. The peptides evident with missed cleavage were excluded for absolute quantification.

Assessment of quantification performance by comparing the results from sibling peptides showed good compliance between the obtained ratios, when peptides with missed cleavage were excluded. The only exception was the peptides representing A1AT, which showed a two- to four-fold difference between the A/S ratios. A1AT both have a type 1 and 2 isoform with high sequence homology.[29] The peptide A1AT_p1 only maps to the A1AT type 1 isoform, whereas A1AT_p2 maps to both isoforms, which likely explains the observed difference in the ratios between the two sibling peptides. The amount of FIBα_p1 was markedly higher than what is observed for its sibling peptides (Figure 2). A likely explanation is that this standard peptide may be insufficiently cleaved from the expressed concatamer. Additionally, the SRM transitions of FIBα_p1 were observed with markedly lower intensities than what was seen for FIBα_p2

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and FIBα_p3 peptides. In conclusion, FIBα_p1 was classified as not well-suited as a target peptide for quantification of FIBα.

Reproducibility of the Quantification

The CV for triplicate sample preparation and SRM analysis of a sample were determined for all "Type A" peptides with consistent quantotypic properties (Table 3). The CV ranged from 7.0 to 38.4% in serum, 0.9 to 22.7% in wound tissue, and 3.1 to 46.5% in microdialysate. This is in accordance with the CV values demonstrated using a comparable sample preparation and analysis pipeline in a multilaboratory study to assess reproducibility.[8] As with all isotope dilution methods, the most accurate quantification is obtained when the ratio of the A/S is close to unity. To achieve unity for each peptide would require multiple analytical runs at different loadings of the QconCAT.[3,12] In this study an A/S ratio of 1:20 to 20:1 was accepted, but a ratio closer to 1:1 would likely reduce the CV. A CV less than 0.2 across multiple samples is the golden standard for clinical validation trials,[41] but a higher CV may be better tolerated when the change in expression of a protein in response to disease is high. Because the APPs are characterized as proteins with a 25% change in concentration in response to inflammation, a CV above 25% might in some cases hamper the detection of the biological variation. However, according to what is so far known about fluctuation of the confirmed equine APPs, SAA, FIB, Cp, Hp, A1AG, CRP, and PLG, included in this study, these proteins abundance change by more than 100% in response to inflammation,[22,27] hence biological variation substantially exceeds the technical variation observed for these protein measures. For the other proteins included in the assay, knowledge about the fluctuation in concentration in response to inflammation is needed to conclude if the technical variation will obscure the biological variation. The SRM analysis step alone varies from 3 to 15%;[41] and hence SRM analysis of technical triplicates may likely bring down the variance, as previous demonstrated.[42]

Table 3. Coefficient of Variation (CV) for Triplicate Processing and Selected Reaction Monitoring Analysis of Equine Serum, Wound Tissue, and Wound Interstitial Fluid Collected by Microdialysis[a,b]

		CV %		
protein	peptide ID	serum	wound tissue	microdialysate
fibrinogen Aα	FIBα_p2		14.1	6.1
	FIBα_p3		6.2	2.6
ceruloplasmin	Cp_p1	38.4	11.9	21.2
	Cp_p2	15.3	5.8	3.1
haptoglobin	Hp_p2	25.3	15.4	6.2
	Hp_p3	22.7	7.6	21.1
plasminogen	PLG_p1	7.0	15.3	46.5
prothrombin	PT_p1	24.3	2.8	38.8
	PT_p2	25.9	22.7	7.0
alpha-2-macroglobulin	A2M_p1	26.1	6.8	7.6
	A2M_p2	21.7	10.6	22.0
alpha-1-antitrypsin	A1AT_p1	17.4	12.3	22.1
	A1AT_p2	22.9	4.8	17.4
mean CV		22.4	11.0	17.1

[a]CV is not given for fibrinogen in serum because fibrinogen is processed to form the fibrin clot during the coagulation process and consequently not present in serum. [b]Peptides included are peptides included in the quantification concatamer eAP1 with analyte-to-standard ratio >0.05 with 7 fmol on column, and without missed cleaved sites.

Linearity of Calibration Curves

Calibration curves were established to assess the linearity of the correlated heavy labeled standard and unlabeled light analyte peptide. Figure 3a,b shows the correlation between the unlabeled and labeled GluFib. This curve facilitated concentration determination of the labeled standard and clearly demonstrated consistent linearity over the entire dynamic range of 400 amol to 80 fmol/μL of labeled standard ($r^2 > 0.99$).

Calibration curves spanning 400 amol to 80 fmol/μL of labeled standard demonstrated good agreement in standard-to-analyte ratios for sibling peptides and r^2 values ranging from 0.985 to 0.998 (Table 4, Supplementary File 5 in the Supporting Information). As representative for the observed linearity of the calibration curves, Figure 3c shows the calibration curve for the peptide Hp_p3 mapping to haptoglobin. For the peptides mapping to Cp and PT, linear correlation ceased above 7 fmol/μL of labeled standard. The standard-to-analyte ratios obtained with a load of 20−80 fmol indicated that the abundance of the analyte peptides is low relative to the analyte peptides of the other proteins. In the horse, the concentration of Cp in serum is low relative to other equine APPs,[27] which likely explains the high standard-to-analyte ratio observed herein. The r^2 from 400 amol to 7 fmol/μL ranged from 0.983 to 0.995, and the standard to analyte ratios was linear within this dynamic range. There was no linear correlation for peptides mapping to A1AG. The concentration of A1AG has previously been assessed by another method and was found to be markedly lower than the concentrations for Cp and FIBα.[27] The lack of linear correlation for A1AG may be explained by a very low amount of analyte peptide in the sample. From these studies, we concluded that correlated peptide signals for SAA_p1, SAA_p2, SAA_p3, FIBα_p2, FIB_p3, Cp_p1, Cp_p2, Hp_p2, Hp_p3, PT_p1, PT_p2, PLG_p1, A2M_p1, A2M_p2, A1AT_p1, and A1AT_p2 were linear over more than two orders of magnitude and that the selected peptides were suited for absolute quantification assays.

Biological Relevance

Plasminogen. PLG is considered an APP in the horse with a two to three times increase in concentration in response to injury.[22] The sibling peptides of PLG included in the eAP1 were observed in remarkably diverse A/S ratio in all three biological matrices (Figure 2). Although the standard peptide PLG_p2 is occasionally observed with a missed cleavage (Table 2), the A/S ratio was lower than that for PLG_p1.[14] An explanation for the inconsistency might be found in the biological nature of PLG. This protein is an important component in the fibrinolytic cascade and is during this process converted to plasmin with the consequent release of an activation peptide from the N-terminal[43,44] The PLG_p1 peptide quantified here is located in the released activation peptide. The PLG_p2 peptide originates from the circulating Angiostatin fragment of PLG,[45] and a catabolic difference for these two PLG fragments may explain the observed difference in ratio between peptide PLG_p1 and PLG_p2. Because of the missed cleavage of PLG_p2, only PLG_p1 was classified as a quantotypic peptide suitable for absolute quantification.

Haptoglobin. Hp is an equine APP and has previously been used to assess both systemic and local inflammatory conditions.[19,46] The APP composition directly in the microenvironment could likely increase the diagnostic specificity and also provide insight into local disease processes.[19,47,48] In this

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Table 4. Standard-to Analyte Ratio Obtained by Selected Reaction Monitoring Analysis of Analyte Sample with Addition of an Increasing Amount of Heavy Labelled Standard Peptide[a,b]

	amount heavy labeled standard peptide (fmol/μL)									
peptide ID	0.4	0.7	4	7	20	40	60	80	r^2	dynamic range
SAA_p3	0.03	0.04	0.16	0.31	0.53	1.16	1.58	2.57	0.985	0.4−80
SAA_p4	0.06	0.10	0.54	1.12	2.29	4.19	6.10	8.52	0.998	0.4−80
SAA_p5	0.02	0.05	0.31	0.54	1.13	2.18	2.98	4.43	0.995	0.4−80
FIBα_p2	0.06	0.16	0.82	2.42	3.20	7.73	9.80	13.52	0.989	0.4−80
FIBα_p3	0.11	0.27	1.12	3.25	4.23	10.98	14.21	19.62	0.998	0.4−80
Cp_p1	0.23	0.55	2.17	5.52	6.75	12.76	15.34	18.90	0.983[a]	0.4−7
Cp_p2	0.20	0.43	1.92	4.31	6.83	11.94	14.02	17.85	0.995[a]	0.4−7
Hp_p2	0.03	0.04	0.19	0.46	0.78	1.72	2.29	3.20	0.996	0.4−80
Hp_p3	0.04	0.08	0.38	0.83	1.59	3.12	4.49	6.47	0.997	0.4−80
A1AG_p1	7.16	11.54	24.21	28.82	29.24	31.44	31.17	31.98		
A1AG_p2	7.25	15.55	29.16	42.73	46.44	45.18	50.18	51.96		
PT_p1	0.27	0.60	2.21	4.92	7.05	13.18	16.31	19.73	0.985	0.4−7
PT_p2	0.26	0.68	3.23	8.30	11.05	23.11	27.08	32.64	0.993[a]	0.4−7
PLG_p1	0.01	0.00	0.03	0.06	0.10	0.22	0.31	0.50	0.985[a]	0.4−80
A2M_p1	0.03	0.08	0.39	1.12	1.50	4.05	5.48	8.40	0.989	0.4−80
A2M_p2	0.04	0.09	0.53	1.47	1.77	5.21	7.26	9.82	0.991	0.4−80
A1AT_p1	0.05	0.09	0.49	1.15	1.85	4.21	5.55	8.13	0.994	0.4−80
A1AT_p2	0.02	0.05	0.22	0.53	0.86	1.95	2.62	4.13	0.989	0.4−80

[a] r^2 and dynamic range of the A1AG peptides are not given because the obtained ratios are not linear in the dynamic range investigated. [b] r^2 is given for the best line of fit over the entire dynamic range investigated.

study, two peptides, Hp_p2 and Hp_p3, were ranked as reliable quantotypic peptides for absolute quantification of the Hp concentration in serum, wound tissue, and wound interstitial fluid (Figure 2). Hp is composed of two α and two β subunits,[49] and Hp_p2 and Hp_p3 map to the α and β subunits, respectively.

Alpha-1-antitrypsin. In the horse, different isoforms of the proteinase inhibitor A1AT have been reported.[50] The eAP1 peptide A1AT_p2 map to the common part of the A1AT isoforms, whereas A1AT_p1 only map to the isoform A1AT 1 (www.peptideatlas.org). Both peptides were classified as consistently observed quantotypic peptides (Figure 2) and will support further investigations of A1AT isoforms in equine health and disease.

Alpha-2-macroglobulin. The A2M protein has previously been investigated in various equine disorders both in regards to its function and as a marker of disease.[51−53] The APP properties of A2M in the horse have not been reported. Here we demonstrate a method for absolute quantification of A2M both systemically and locally that may assist the determination of the APP properties of the protein.

Prothrombin. PT is a focal point of the coagulation cascade under which it is converted to thrombin with a consequent release of Fragment 1.2.[54] The peptides in the eAP1 representing PT map to this fragment. Fragment 1.2 is a potential marker for clinical states associated with increased thrombosis,[54] and thus the presence and analysis of the eAP1 PT peptides in serum and other body fluids and tissues may be relevant for clinical applications. Both peptides were classified as consistently observed quantotypic peptides (Figure 2).

Ceruloplasmin. Cp is classified as an APP in horses and increases two to three times in response to inflammation.[18,55] In the eAP1, two peptides quantify Cp as "Type A" in all three biological matrices (Figure 2) and were found to be reliable peptides for absolute quantification of Cp.

Fibrinogen. For several years, FIB has been the most commonly used systemic marker for inflammation in horses. The normal plasma concentration is relatively high (1000 to 4000 mg/L), and in response to inflammation, it increases two to four times.[16,17,46] Fibrinogen comprises three pairs of polypeptide chains designated Aα, Bβ, and γ and are cleaved to produce fibrin by the action of thrombin. Upon cleavage of fibrinogen Aα (FIBα) to yield fibrin, only a short peptide is released.[38] For this SRM assay, peptides that represent FIBα in the eAP1 construct also map to fibrin. The peptides were classified as "Type A" in microdialysate and wound tissue but "Type B" in serum (Figure 2). The missing analyte signal in serum was expected because FIB is processed to form the fibrin clot during the coagulation process.[38] Fibrin is important in the wound-healing process as a hemostatic barrier and provides a structural frame for repair.[56] The relative higher amount of FIBα in the wound tissue biopsy compared with the amount in microdialysate may reflect a high content of fibrin in the wound tissue (Figure 2). On the contrary, the amount observed in wound interstitial fluid likely represents FIBα only because the fibrin polymers are expected to be too large for diffusion into the microdialysate probes due to the size limit of the membrane pores. The peptides FIBα_p2 and FIBα_p3 were ranked as reliable quantotypic peptides.

Alpha-1-antichymotrypsin. In this study, no reliable quantotypic peptides were found for A1ACT because one candidate peptide (A1ACT_p1) was not detected (Figure 2) and the other peptide (A1ACT_p2) was observed with missed cleavage (Table 2).

Alpha-1-acid glycoprotein. Two (A1AG_p1 and A1AG_p2) of the three peptides mapping to A1AG were evaluated as good quantotypic candidates. The two peptides were "Type A" in wound tissue but "Type B" in microdialysate and serum (Table 1, Figure 2), which may be due to a difference in abundance in body tissues and fluids. A1AG has been classified as an APP in the horse,[20] and in humans it has an anti-inflammatory and immunomodulating role in disease processes.[57] However, there was no linear correlation for peptides mapping to A1AG. The lack of linear correlation for A1AG may be explained by a very low amount of analyte peptide in the sample.

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Table 5. Absolute Concentration of Three Confirmed and Four Putative Acute Phase Proteins in Serum (mg/L)[a]

		analyte-to-standard			absolute concentration			
					noninfl.[b]		infl.[c]	
protein[d]	peptide ID	noninfl. A/S[e]	infl. A/S	fold change	conc.[f]	ref. interval	conc.	ref. interval
SAA	SAA_p5	0.03	18.7	>374	<18	0.5−20	6459	100−12 000
Cp	Cp_p1	0.09	0.17	1.9	294	300−400	624	400−900
	Cp_p2	0.10	0.24	2.4				
Hp	Hp_p2	0.64	2.21	3.5	1341	200−2600	4662	3000−4200
	Hp_p3	2.16	7.76	3.6				
PLG	PLG_p1	8.21	22.95	2.8	18 480		51 639	
PT	PT_p1	0.18	0.22	1.2	244		315	
	PT_p2	0.10	0.14	1.4				
A2M	A2M_p1	0.91	1.58	1.7	3411		5957	
	A2M_p2	0.79	1.38	1.7				
A1AT	A1AT_p1	0.35	0.72	2.1	763		1303	
	A1AT_p2	0.98	1.53	1.6				

[a]Serum was obtained from a horse before and 6 days after onset of systemic inflammation. The obtained concentrations are compared to reference intervals reported in the literature. [b]Noninfl: Serum from a horse without signs of systemic inflammation. [c]Infl. Serum from a horse with systemic inflammation. [d]SAA: Serum amyloid A; Cp: Ceruloplasmin; Hp: Haptoglobin; PLG: Plasminogen; PT: Prothrombin; A2M: Alpha-2-macroglobulin; A1AT: Alpha-1-antitrypsin. Molecular weight (Da) used for calculations (Uniprot): SAA: 14 104; Cp: 121 951; Hp: 38 335; PLG: 91 715; PT: 70 466; A2M: 164 040; A1AT: 46 974. [e]A/S: Analyte-to-standard ratio. [f]Conc: The absolute concentration obtained by use of the validated quantification concatamer method, ref. interval: Reference intervals given in the literature (SAA;[27] Cp;[27,55] Hp;[19,46,61,62]).

Serum Amyloid A. In the horse, three isoforms of SAA exist; SAA1 and SAA2 are produced hepatically and SAA3 is produced extrahepatically.[48] The peptide SAA_p1 in the eAP1 construct originates from the hepatically produced isoforms, SAA_p2 originates from the extrahepatically produced isoform,[29] and the three other peptides represent a common sequence for the three isoforms. SAA_p1 contains a methionine, which might cause a splitting of the observed peptide signal because of variable oxidation,[3] and consequently both the oxidized and nonoxidized forms were included in the presented quantitative analysis (SAA_p1red, SAA_p1ox). For these two peptide states, the signal obtained was highly inconsistent for both the analyte and standard peptide and consequently ranked as "Type C" (Figure 2). The other peptides were classified as "Type B" (Figure 2). SAA is categorized as a major APP in the horse because it is present in very low concentrations in healthy individuals and increase 100−1000 times in response to inflammation, reaching concentrations of 2000 mg/L or more.[27] This is in good agreement with the findings in this study because the amount of SAA in serum from a healthy individual analyzed with a load of 7 fmol heavy labeled peptides on column was below the defined limit of detection in serum from healthy individuals, and the amount was markedly above the limit of detection in serum from an individual with systemic inflammation (Table 5).

C-Reactive Protein, Ferritin, and Mannose-Binding Lectin. In the horse, CRP has been found to possess APP properties with an increase in concentration of two to three times in response to inflammation.[27] On the contrary, the APP properties of FTH and MBL are not known in the horse. The three proteins were all represented in the eAP1 construct by two peptides classified as "Type B" (Figure 2), and their quantotypic properties in regard to compliance of sibling peptides may likely be evaluated with a lower concentration of the standard peptide on column.

Quantification in Absolute Terms

Absolute versus relative quantification of a specific protein within complex biological samples is greatly advantageous in clinical research and diagnostics because it opens the possibility for interlaboratory comparison, relation of the amount to a reference value in clinical samples, and thereby clinical diagnostics.[1,58]

To investigate the compliance of the results obtained with the eAP1, with previous reported results acquired using other methods, the concentration of known equine APPs was measured in serum from one horse under normal conditions and 6 days after clinical signs of systemic inflammation (Table 5). Under normal conditions, the A/S ratio was below 0.05, which led to the conclusion that the absolute concentration was below 18 mg/L. The concentration increased to 6459 mg/L on day six after clinical signs of systemic inflammation. From previous research and personal observations from biochemical blood analyses, the concentration of SAA is found within the range of 0.5−20 mg/L and 100−12,000 mg/L under normal and inflammatory conditions, respectively.[27] Other SRM studies also find a slightly higher protein concentration estimates when compared with the concentration measures obtained by other assays.[59,60] The obtained concentration only relies on the ratio for the peptide SAA_p5 because the ratios between the sibling peptides differed (Supplementary File 6f in the Supporting Information). In this study, the quantotypic properties of the peptides mapping to SAA were not possible to evaluate because the samples were from healthy individuals and therefore all "Type B". Consequently, information about the peptides was extracted from the equine PeptideAtlas, where the consistency of observation for the peptides was calculated from multiple observations in previous MS/MS discovery runs. The peptide SAA_p5 was found to be the most consistently observed. Hp and Cp were found to increase from 1341 to 4662 mg/L and 294 to 624 mg/L, respectively. Hp levels have previously been observed to be within 200−2200 mg/L in healthy individuals and 3000−4200 mg/L in horses with systemic inflammation,[19,46,61,62] and Cp normally is observed to be within 300−400 mg/L in healthy and 400−900 mg/L in horses with systemic inflammation.[18,55] The CVs for Hp and Cp in serum are high, which is why the exact absolute concentration given should be interpreted with caution. However, even with the high CV the results demonstrate a clear increase in the concentration of both proteins in response to inflammation, and the fold change observed corresponds with

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previously reported fold changes.[27] In conclusion, we demonstrated a close correlation between our SRM-based measures with existing measures obtained by other assays.

The absolute concentration was determined for the four putative APPs with reliable quantotypic peptides: plasminogen, prothrombin, α-2-macroglobulin, and α-1-antitrypsin (Table 5). The fold change in response to inflammation for the A/S ratio was in good agreement for sibling peptides and above 100% for all peptides. To our knowledge, this is the first time these proteins have been reported in absolute terms in serum from a horse.

Analysis of serum samples from healthy and diseased individuals on a large scale may further add to the reliability of the method evaluated here and demonstrate the biological variation between individuals

CONCLUSIONS

In conclusion, our results demonstrated that the expressed eAP1 provides a useful method for simultaneous absolute quantification of a panel of well-known and potential equine APPs. Assessment of tryptic digestion efficiency, compliance of sibling peptides, quantotypic classification, and reproducibility with 7 fmol on column showed robust quantotypic performance for PLG, Hp, A1AT, A2M, PT, and Cp in all three matrices and for FIBα in wound tissue and interstitial fluid. All proteins were represented by two peptides except PLG, which was represented by one peptide. The peptides that could not support quantification using this current approach may be quantified with a lower amount of the heavy labeled peptides on column, be successfully substituted by other proteotypic peptide candidates in future QconCAT constructs, or may be quantified after further optimization of the sample processing. The CVs ranged from 2.6 to 46.5%. A high CV may be better tolerated when the change in expression of a protein in response to disease is high. According to what is so far known about fluctuation of the confirmed equine APPs SAA, FIB, Cp, Hp, A1AG, CRP, and PLG, included in this study, these protein abundances change by more than 100% in response to inflammation, and hence biological variation exceeds technical variation for these proteins. The reliability of the method was supported by correlation of absolute concentrations of SAA, Cp, and Hp, found in equine serum from a healthy individual and in response to inflammation, using this QconCAT-SRM-based approach, to previous studies of the proteins using other biochemical analyses. This is the first report to demonstrate the absolute protein levels of APPs quantified by SRM-based LC−MS/MS in the horse. These targeted QconCAT-SRM-based assays can circumvent the considerable challenges associated with developing species-specific enzyme-linked immunosorbent assays; hence, we expect that SRM-based MS will be a valuable future approach for both research and diagnostics in the horse.

ASSOCIATED CONTENT

Supporting Information

Supplementary File 1. Proteotypic candidate peptides representing the confirmed and putative acute phase proteins in the horse of interest in this study. These peptides were tested for their quantotypic properties by selected reaction monitoring. Supplementary File 2. Information about the selected reaction monitoring-assay used to analyze the peptides included in the quantification concatamer eAP1. Supplementary File 3. (A) Schematic illustration of the quantification concatamer eAP1 composition. (B) Results from SDS-PAGE analysis of the fractions obtained during purification of the quantification concatamer eAP1. Supplementary File 4. (A) Report giving the details on optimization of the quantification concatamer eAP1. (B) The codon optimized gene and the amino acid sequence for the quantification concatamer eAP1. Supplementary File 5. Calibration curves for the peptides included in the quantification concatamer eAP1 with Supplementary File 6a. Supplementary File 6a−f. Data from analysis of (a) a serum sample from a horse, (b) wound interstitial fluid collected by microdialysis from a horse, and (c) a wound tissue sample from a horse by use of the created selected reaction monitoring method in three technical replica. Data obtained to establish a calibration curve for (d) [Glu1]-Fibrinopeptide B and (e) the peptides included in the quantification concatamer with properties suitable for absolute quantification. Supplementary File 7a−f. R-script for analysis of data in (a) Supplementary File 6a, (b) Supplementary File 6b, (c) Supplementary File 6c, (d) Supplementary File 6d, (e) Supplementary File 6e, and (f) Supplementary File 6f. This material is available free of charge via the Internet at http://pubs.acs.org.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest

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ABBREVIATIONS

A1ACT, α-1-antichymotrypsin; A1AG, α-1-acid glycoprotein; A1AT, α-1-antitrypsin; A2M, α-2-macroglobulin; AmBic, ammonium bicarbonate; APP, acute phase proteins; Cp, ceruloplasmin; CRP, C-reactive protein; CV, coefficient of variation; eAP1, equine acute phase proteins 1; Equine, *Equus caballus*; FIB, fibrinogen; FIBα, fibrinogen Aα; GluFib, [Glu1]-Fibrinopeptide B; FTH, ferritin; Hp, haptoglobin; LC, liquid chromatography; MBL, mannose-binding lectin; MS, mass spectrometry; QconCAT, quantification concatamer; PLG, plasminogen; PT, prothrombin; SAA, serum amyloid A; SRM, selected reaction monitoring; A/S, analyte to standard ratio

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