Journal of Chromatography B, 878 (2010) 590–602

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

# Quantitative assay for six potential breast cancer biomarker peptides in human serum by liquid chromatography coupled to tandem mass spectrometry

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ARTICLE INFO

*Article history:*
Received 24 August 2009
Accepted 11 January 2010
Available online 18 January 2010

*Keywords:*
Bradykinins
$ITIH_{4[666–687]}$
$C4a_{[1337–1350]}$
Fibrinogen-$\alpha_{[605–629]}$
quantification
LC–MS/MS
Biomarkers
Breast cancer

ABSTRACT

An assay to quantify several possible breast cancer peptide biomarkers in human serum has been developed and validated, using liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS). The peptides include bradykinin, $Hyp^3$-bradykinin, des-$Arg^9$-bradykinin and fragments of fibrinogen α-chain (Fib-$\alpha_{[605–629]}$), inter-α-trypsin inhibitor heavy chain 4 ($ITIH_{4[666–687]}$) and complement component 4a ($C4a_{[1337–1350]}$). $Ile^{13}$-$ITIH_{4[666–687]}$, d20-$C4a_{[1337–1350]}$ and Sar-D-$Phe^8$-des-$Arg^9$-bradykinin were used as internal standards. Bovine plasma, with 2 mM captopril and 2 mM D-L-mercaptoethanol-3-guanidino-ethylthiopropanoic acid (MEGETPA) to prevent rapid degradation of the bradykinins, was used as analyte-free matrix. Recoveries for solid-phase extraction (SPE) on mixed-mode weak cation exchange sorbents were between 62 and 90%. Multiple reaction monitoring (MRM) on a triple quadrupole mass spectrometer equipped with a heated electrospray source (H-ESI), operating in the positive ion-mode, was used for detection. The assay was fully validated and stabilities of the peptides were extensively explored. Bradykinin (10–500 ng/ml), $Hyp^3$-bradykinin (4–200 ng/ml), des-$Arg^9$-bradykinin (2–100 ng/ml), Fib-$\alpha_{[605–629]}$ (120–3000 ng/ml), $ITIH_{4[666–687]}$ (0.4–10 ng/ml) and $C4a_{[1337–1350]}$ (1–25 ng/ml) were simultaneously quantified with deviations from the nominal concentrations below 22% and intra- and inter-assay precisions below 15 and 20%, respectively, for all peptides at all concentrations. The method has been successfully applied to several serum samples from breast cancer patients and matched controls.



## 1. Introduction

Protein and peptide profiling in biological fluids of cancer patients has become an attractive approach in the search for novel cancer biomarkers. Different mass spectrometry methods have been used to compare peptide or protein patterns of patients with different types of cancer with healthy controls [1,2]. As a result, potential diagnostic values have been ascribed to specific proteins or peptides that show either decreased or increased expression.

However, despite initial enthusiasm, criticism on the robustness of this approach has grown as results from different studies showed large clinical and analytical variations [3–5]. This suggests that differences in proteomic profiles between controls and cancer patients are more likely caused by other factors than the presence of cancer. Proposed sources of bias are variations in (1) sample collection, handling and storage; (2) disease related factors (extent and type of the malignancy); (3) patient related variables (e.g. gender, age, and ethnicity); (4) clinical and analytical experiments or (5) statistical analysis [3–5]. Furthermore, it remains unclear whether peptide patterns differ between plasma and serum and how they are altered after the blood collection procedure by *ex vivo* proteolysis. On the other hand, *ex vivo* generated peptides have also been proposed to exhibit diagnostic information [2]

These discrepancies and uncertainties have given rise to several studies on the effect of variations in sample handling on the peptide profile [6–8], which suggest careful evaluation of the sample preparation procedure and use of a standard protocol in all peptide biomarker studies. More knowledge on possible confounding factors as well as the development of more sensitive and specific

*Abbreviations:* $C4a_{[1337–1350]}$, Complement component $4a_{[1337–1350]}$; d20-$C4a_{[1337–1350]}$, Stable isotope (deuterium-leucine) labeled analog of complement component $4a_{[1337-1350}$; Fib-$\alpha_{[605-629]}$, Fibrinogen α-$chain_{[605-629]}$; $Hyp^3$-bradykinin, $Pro^3$-hydroxylated bradykinin; $ITIH_{4[666-687]}$, Inter-α-trypsin inhibitor heavy chain $4_{[666-687]}$; $Ile^{13}$-$ITIH_{4[666-687]}$, Structural analog of inter-α-trypsin inhibitor heavy chain $4_{[666-687]}$ with $Val^{13}$ replaced by $Ile^{13}$; MEGETPA, D-L-mercaptoethanol-3-guanidino-ethylthiopropanoic acid.

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doi:10.1016/j.jchromb.2010.01.011

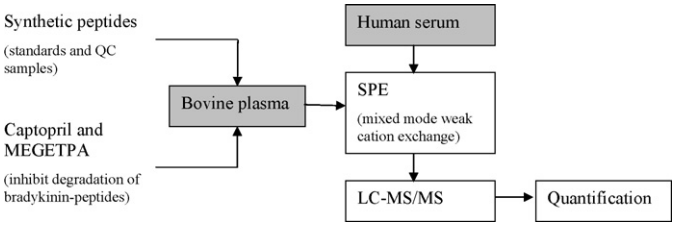


**Fig. 1.** Simplified overview of the different steps of the analytical method.

analytical procedures is therefore required to further explore the potential of peptides as biomarkers for cancer.

For this purpose, an LC–MS/MS assay to quantify potential breast cancer peptide biomarkers in human serum has been developed (Fig. 1). The peptide analytes were selected from a large set of signature peptides identified by Villanueva *et al.* [2] and include the fragments with the highest significant relation to breast cancer fibrinogen α-chain (Fib-$\alpha_{[605-629]}$), inter-α-trypsin inhibitor heavy chain 4 ($ITIH_{4[666-687]}$), complement component 4a ($C4a_{[1337-1350]}$) and bradykinin. Furthermore, two bradykinin analogs, $Hyp^3$-bradykinin and des-$Arg^9$-bradykinin, were added to the set of peptide analytes as they showed significant correlation to breast cancer in the same study and were easily detectable in human serum samples. More details of the selected peptides are listed in Table 1.

The selected $ITIH_4$ fragment has been identified more often, being either significantly [10] or not significantly [11] related to breast cancer. The Fib-α fragment has also been recognized by others in plasma [1,8] and has been proposed as a tumor marker for oral cancer [12] as well as a marker for HER2-positive breast cancer [13]. In the latter case, the Fib-α fragment was decreased in breast cancer versus controls, in contrast to the Villanueva study [2], possibly due to the use of plasma instead of serum.

However, little is known about the absolute concentration levels of these potential breast cancer biomarker peptides. Quantitative MS assays have only been reported for bradykinin and the $ITIH_4$ fragment [14,15]. Reported bradykinin serum or plasma concentrations measured by immunoassays vary largely between the low nanomolar and the low picomolar range [16] due to its rapid degradation and paracrine activity. Other assays, using LC–MS, have been applied to quantify bradykinin in rat muscle tissue dialysates [15] or bradykinin's stable metabolite bradykinin [1–5] in human blood [17]. An LC–MS/MS method for the quantification of the peptide fragment of $ITIH_4$ in human serum and plasma together with seven other proteolytically derived peptides from the same protein has recently been developed in our laboratory [14]. However, analysis of plasma and serum samples from cancer patients revealed that this method was unable to detect the peptide in plasma, whereas the concentrations in serum were usually below the lower limit of quantification (LLOQ) of the method (6 ng/ml).

**Table 1**
Average molecular weights and amino acid sequences of the six potential breast cancer biomarker peptides selected for quantification.

| Peptide[a] | Average mass (Da) | Sequence |
|---|---|---|
| Bradykinin | 1060.2 | RPPGFSPFR |
| $Hyp^3$-bradykinin | 1076.2 | RPP$^o$GFSPFR |
| des-$Arg^9$-bradykinin | 904.1 | RPPGFSPF |
| Fib-$\alpha_{[605-629]}$ | 2659.8 | DEAGSEADHEGTHSTKRGHAKSRPV |
| $C4a_{[1337-1350]}$ | 1626.8 | NGFKSHALQLNNRQ |
| $ITIH_{4[666-687]}$ | 2358.6 | SSRQLGLPGPPDVPDHAAYHPF |

[a] Amino acid sequences of precursor proteins derived from NCBI Protein database [9].

Here, an assay is presented for the quantification of $ITIH_{4[666-687]}$ with an LLOQ as low as 0.4 ng/ml, while simultaneously enabling quantification of five other peptide fragments with biomarker potential for breast cancer. The assay is likely to help clarify the usefulness of proteolytically derived peptides as diagnostic (bio)markers. Furthermore, preliminary results of the analysis of serum samples from breast cancer patients and matched controls are presented.

## 2. Experimental

### 2.1. Chemicals and reagents

The peptide fragments Fib-$\alpha_{[605-629]}$ (96.5%) and $C4a_{[1337-1350]}$ (98.2%) were synthesized by Genscript Corporation (Piscatway, NJ, USA). The $ITIH_{4[666-687]}$ peptide fragment (80%), its $Ile^{13}$-structural analog (107%) and the stable isotope-labeled analog of the C4a-fragment, d20-$C4a_{[1337-1350]}$ (95%) in which the leucine amino acid residues were replaced by d10-leucine, were supplied by H. Hilkmann (The Netherlands Cancer Institute, Amsterdam, The Netherlands). The proteolysis-resistant bradykinin analog Sar-D-$Phe^8$-des-$Arg^9$-bradykinin (≥95%) was obtained from Phoenix Europe GmbH (Karlsruhe, Germany).

Acetonitrile (gradient grade), methanol (HPLC grade) and LC–MS grade water were from Biosolve (Valkenswaard, The Netherlands), acetic acid (extra pure) from Riedel-de Haën (Seelze, Germany), formic acid (p.a.) from Merck (Darmstadt, Germany) and trifluoroacetic acid (99.5%) from Acros Organics (Geel, Belgium). Bovine plasma with sodium-citrate as anticoagulant was obtained from Innovative Research (Novi, MI, USA) whereas blank human serum was obtained from the Sanquin Bloodbank (Utrecht, The Netherlands). The carboxy-peptidase N inhibitor D-L-mercaptoethanol-3-guanidino-ethylthiopropanoic acid (MEGETPA, Plummer's inhibitor) was supplied by Calbiochem (San Diego, CA, USA) and the ACE inhibitor captopril (>98% HPLC) as well as bradykinin (88.7%), $Hyp^3$-bradykinin (97.0%) and des-$Arg^9$-bradykinin (84.3%) by Sigma-Aldrich (Steinheim Germany).

### 2.2. Equipment

LC–MS/MS analyses were performed using an Accela high-speed chromatographic system coupled to a TSQ Quantum Ultra triple quadrupole mass spectrometer equipped with a heated electrospray ionization (H-ESI) probe (both from Thermo Scientific, San Jose, CA, USA). An Acquity UPLC ethylene-bridged hybrid (BEH) 300 $C_{18}$ reversed-phase column (50 mm × 2.1 mm i.d., 1.7 μm particle size) was protected by an Acquity BEH $C_{18}$ VanGuard pre-column (5 mm × 2.1 mm ID, 1.7 μm particle size), both from Waters (Waters Chromatography, Milford, MA, USA).

SPE was performed on a Speedisk 96 positive pressure processor (Mallinckrodt Baker, Deventer, The Netherlands), using Oasis WCX 96 wells extraction plates (Waters) which contain 30 mg carboxylic acid bonded to poly(divinylbenzene-co-N-vinylpyrrolidone) copolymer (30 μm). Evaporation of the SPE eluates in the 96 wells plates was performed on a MiniVap (Porvair Sciences, Norfolk, UK).

### 2.3. LC–MS/MS conditions

Acetic acid (0.1%, v/v) in water and methanol were used as eluent A and B, respectively. The flow rate was set to 400 μl/min and column temperature to 30°C. A linear gradient was used, with eluent B rising from 0 to 32% in 0.3 min, holding for 0.4 min and then rising to 90% in 1.3 min. Eluent B was maintained at 90% for 0.7 min after which it returned to 0%, holding for 1.5 min for re-equilibration. Total run time was 4.2 min for each injection.

**Table 2**
MS/MS settings for the different peptide analytes.

| Peptide | Parent ion (*m/z*) | Ion type | Product ion (*m/z*) | Ion type | Coll. energy (V) | Q1 Resolution (FWHM) | Tube lens voltage (V) |
|---|---|---|---|---|---|---|---|
| Bradykinin | 531.1 | $[M+2H]^{2+}$ | 70.0 | P(i) | −34 | 1.40 | 112 |
| $Hyp^3$-bradykinin | 539.1 | $[M+2H]^{2+}$ | 70.0 | P(i) | −36 | 1.40 | 116 |
| Des-$Arg^9$-bradykinin | 453.0 | $[M+2H]^{2+}$ | 642.8 | $b_6^+$ | −17 | 1.40 | 91 |
| Sar-D-$Phe^8$-des-$Arg^9$-bradykinin | 488.6 | $[M+2H]^{2+}$ | 713.8 | $b_6^+$ | −20 | 1.40 | 90 |
| Fib-$\alpha_{[605-629]}$ | 533.0 | $[M+5H]^{5+}$ | 605.0 | $y_{23}^{4+}$ | −15 | 1.40 | 97 |
| $C4a_{[1337-1350]}$ | 543.0 | $[M+3H]^{3+}$ | 531.4 | $y_4^+$ | −23 | 0.20 | 102 |
| | | | 644.4 | $y_5^+$ | | | |
| | | | 772.7 | $y_6^+$ | | | |
| d20-$C4a_{[1337-1350]}$ | 549.7 | $[M+3H]^{3+}$ | 531.4 | $y_4^+$ | −24 | 0.20 | 100 |
| | | | 644.4 | $y_5^+$ | | | |
| $ITIH_{4[666-687]}$ | 590.5 | $[M+4H]^{4+}$ | 540.1 | $y_{15}^{3+}$ | −15 | 0.70 | 91 |
| | | | 743.0 | $b_7^+$ | | | |
| | | | 809.6 | $y_{15}^{2+}$ | | | |
| $Ile^{13}$-$ITIH_{4[666-687]}$ | 594.0 | $[M+4H]^{4+}$ | 544.8 | $y_{15}^{3+}$ | −16 | 0.70 | 92 |

The autosampler temperature was maintained at 5 °C. An interior and exterior needle wash was performed before and after each injection with methanol containing 0.2% (v/v) formic acid to reduce carry-over effects.

The H-ESI source of the mass spectrometer was operated in the positive ion mode and heated to 250 °C. The nitrogen sheath, ion sweep and auxiliary gasses were set at 50, 0 and 55 arbitrary units (AU), respectively, while the ion tube temperature was maintained at 360 °C and the spray voltage was set at 3.5 kV. Resolutions of the Q1 and Q3 quadrupoles were set at 1.40 FWHM, to measure all the different natural isotopes of the peptides. For $C4a_{[1337-1350]}$ and $ITIH_{4[666-687]}$ Q1 was set at higher resolution to reduce interferences at the lower concentration levels (Table 2). Argon was used as collision gas and the collision pressure was set at 1.7 mTorr for all peptides. Individual parameters for the different compounds are listed in Table 2.

### 2.4. Preparation of standards and quality control samples

All stock and working solutions were prepared in methanol/water 1:1 (v/v), 0.1% formic acid. Stock solutions were prepared at concentrations of 250 μg/ml for Fib-$\alpha_{[605-629]}$, $C4a_{[1337-1350]}$ and $ITIH_{4[666-687]}$ and of 100 μg/ml for bradykinin, $Hyp^3$-bradykinin and des-$Arg^9$-bradykinin. The stock solutions were combined and diluted to obtain a working solution containing 75, 12.5, 5, 2.5, 0.625, 0.25 μg/ml of Fib-$\alpha_{[605-629]}$, bradykinin, $Hyp^3$-bradykinin, des-$Arg^9$-bradykinin, $C4a_{[1337-1350]}$ and $ITIH_{4[666-687]}$, respectively. A similar working solution was obtained by dilution of independently prepared stock solutions of the same peptides for the preparation of the quality control samples at mid (MQC) and high (HQC) concentration. For the preparation of the quality control samples at the low (LQC) and the LLOQ concentration a third working solution was prepared, containing 6.25, 2.5, 1.25 μg/ml of bradykinin, $Hyp^3$-bradykinin and des-$Arg^9$-bradykinin, respectively, while concentrations for the other peptides were identical to levels in the other working solutions. The internal standard (IS) working solution contained 2.5, 0.625 and 1.25 μg/ml of Sar-D-$Phe^8$-des-$Arg^9$-bradykinin, $Ile^{13}$-$ITIH_{4[666-687]}$ and d20-$C4a_{[1337-1350]}$, respectively. All stock and working solutions were stored at −30 °C, except for the bradykinin-peptides, stored at −80 °C.

### 2.5. Sample preparation

A volume of 20 μl of IS working solution was added to a 500 μl aliquot of plasma or serum, diluted 1:1 (v/v) with 100 mM potassium phosphate buffer (pH 6). The samples were loaded on the Oasis WCX cartridges that were conditioned with 1 ml methanol and 1 ml 100 mM phosphate buffer (pH 6). After washing of the cartridges with 1 ml of 100 mM phosphate buffer (pH 6) followed by 1 ml of 20% methanol, the analytes were eluted with 1 ml of acetonitrile water trifluoroacetic acid 80:20:0.1 (v/v/v) in 1 ml 96-well collection plates. After evaporation (40 °C, 60 L/min nitrogen) the samples were reconstituted in 150 μl methanol/water 1:3 (v/v), 0.1% acetic acid.

### 2.6. Validation

Validation was based on the FDA guidelines for Bioanalytical Method Validation [18].

#### 2.6.1. Linearity

Calibration standards were prepared in bovine plasma containing 2 mM captopril and 2 mM MEGETPA. For Fib-$\alpha_{[605-629]}$, $C4a_{[1337-1350]}$ and $ITIH_{4[666-687]}$ eight calibration standards were analyzed, ranging from 120 to 3000, 1 to 25 and 0.4 to 10 ng/ml, respectively. For bradykinin, $Hyp^3$-bradykinin and des-$Arg^9$-bradykinin nine calibration standards were measured in concentrations from 10 to 500, 4 to 200 and 2 to 100 ng/ml, respectively. All standards were analyzed in three separate runs, calculating target peptide IS ratio's for each concentration level. Sar-D-$Phe^8$-des-$Arg^9$-bradykinin was used as IS for the bradykinin-peptides, d20-$C4a_{[1337-1350]}$ for $C4a_{[1337-1350]}$ and Fib-$\alpha_{[605-629]}$ and $Ile^{13}$-$ITIH_{4[666-687]}$ for $ITIH_{4[666-687]}$.

Standard curves were constructed by least squares linear regression analysis using a weighting factor of $1/x^2$ (with $x$ as the concentration in ng/ml), except for Fib-$\alpha_{[605-629]}$, for which a linear log–log calibration was applied.

#### 2.6.2. Precision and accuracy

The precision and accuracy of the method were determined by analysis of quality control samples at four different concentration levels in three separate runs ($n = 18$ at each level). Deviations of the accuracies were expressed as [((overall mean concentration − nominal concentration)/nominal concentration) × 100%]. Intra- and inter-assay precisions were expressed as relative standard deviations (RSD). Accuracy should be within 15% and precision should be less than 15%. At the LLOQ accuracy should be within 20% and precision should be less than 20% [18].

To ensure accurate and precise quantification in human samples with the use of standards prepared in bovine plasma, one single run with six replicates spiked at LQC and HQC concentration levels in human serum was performed. Additionally, six blank human serum samples from the same batch were analyzed. As for bovine plasma, captopril and MEGETPA were added to the human serum (2 mM each) to prevent rapid degradation of the bradykinin-peptides. Deviations from the nominal concentrations were calculated by correcting the mean measured concentrations in the spiked

samples by the mean measured concentrations of the blank samples.

*2.6.3. Specificity and selectivity*

Blank bovine plasma with 2 mM captopril and MEGETPA, used for the preparation of standards and QC samples, was analyzed to guarantee absence of any interference.

Peak areas of endogenous and exogenous compounds co-eluting with the analytes or internal standards should be less than 20% of the peak area of the LLOQ standard and less than 5% of the response of the IS. Furthermore, six blank and six LLOQ samples in blank human serum from six different individuals were analyzed to determine specificity and selectivity in the human matrix.

*2.6.4. Recovery and ion suppression*

The extraction recovery was determined by comparing the peak areas of blank bovine plasma samples spiked before extraction with blank bovine plasma samples spiked after extraction. The peak areas of the bovine plasma samples spiked before and after extraction were also compared to the peak areas of reference samples in elution solvent to determine total recovery and ion suppression in the H-ESI source, respectively. All samples were analyzed in triplicate at three different concentrations (low, mid and high QC).

*2.6.5. Stability*

The stabilities of the peptides in different matrices under various conditions were extensively examined at various concentrations during all stages of the method. On one hand, for accurate quantification, stability of the *synthetic* peptides in all possible environments is concerned, while on the other hand, for robust application of the method, examination of the stabilities of the *endogenous* peptides in the human clinical samples is crucial. Therefore, stability experiments were performed in both bovine plasma and human serum.

Bovine plasma, containing 2 mM captopril and 2 mM MEGETPA, was spiked with all analytes at LQC and HQC concentrations. Human serum samples were spiked at LQC concentration with only $ITIH_{4[666–687]}$, while at HQC level with all peptides, except for bradykinin. In both matrices, stabilities of the peptides were assessed after one hour on ice, after three freeze–thaw cycles (−80 °C) and after different periods of storage at −80 °C. The stabilities of the spiked peptides were determined by comparing their response to the response of freshly prepared samples. For the endogenous peptides, not added to the human serum samples, absolute concentrations were measured and compared to the concentrations measured on the day of preparation of the samples.

Other stability experiments include the examination of the in-process-stabilities in the dried extract after SPE and one week storage at 5 °C as well as the stabilities in the final extract after 48 h storage in the autosampler. Both experiments were performed at LQC and HQC concentrations and deviations from the initial concentrations were determined in relation to freshly prepared samples.

Furthermore, the stabilities of the peptides in the stock and working solutions were examined. Stabilities of the peptides in the working solutions were assessed after 24 h at ambient temperature and after one month storage at −30 °C, whereas stabilities of the peptides in the stock solutions were assessed after six months storage at −30 °C for Fib-$\alpha_{[605–629]}$, $C4a_{[1337–1350]}$ and $ITIH_{4[666–687]}$ and after two months storage at −80 °C for bradykinin, $Hyp^3$-bradykinin and des-$Arg^9$-bradykinin. Relative deviations were calculated by comparing MS response ratios to freshly prepared samples at identical concentrations.

All stability tests were performed in triplicate. The analytes were considered stable when 85 to 115% of the initial concentration was found.

*2.7. Analysis of patient samples*

Six serum samples from post-menopausal patients with advanced breast cancer were analyzed, as well as six samples from healthy controls, matched for age and sample storage duration. All samples were collected prior to any therapy and sample collection, handling, storage and transportation followed a uniform standard protocol. After blood collection in 9.5 ml BD Vacutainer® SST™ tubes (Beckton-Dickinson, Breda, The Netherlands), the samples were left for 30 min at ambient temperature. Following coagulation, samples were centrifuged (15 min; 3000 rpm) and the serum was immediately separated into aliquots of 1 ml and directly stored at −80 °C until analysis. Transport of the samples was performed on dry ice. All samples were collected after approval by the local medical ethics committee and receiving individuals' written informed consent.

## 3. Results and discussion

*3.1. Method development*

*3.1.1. Sample pre-treatment*

Protein precipitation with acetonitrile resulted in poor recoveries, especially for the fragment of the fibrinogen α-chain and bradykinin, and therefore SPE was chosen as the preferred sample pre-treatment technique. All peptides showed good recoveries on different types of silica or polymer-based SPE sorbents if dissolved in phosphate buffered saline (PBS). However, extraction from plasma or serum samples on the same sorbents did not result in recovery of bradykinin, possibly as a result of protein binding in the biological environment. Only negatively charged weak cation-exchange SPE columns appeared to be able to retain and elute bradykinin, which exhibits a high $pK_a$ value due to its C- and N-terminal arginine residues. As the other peptides did not exhibit these specific characteristics for weak-cation exchange, mixed-mode weak-cation exchange SPE was the only option for extraction with good recoveries of all selected peptides.

*3.1.2. MS/MS optimization*

The ESI- and product ion mass spectra of the bradykinin peptides, the other peptide analytes and the internal standards are shown in Figs. 2–4, respectively. Addition of 0.1% (v/v) acetic acid to the mobile phase improved the response of most peptides compared to other mobile phase additives.

For clinical relevance, the LLOQ for $C4a_{[1337–1350]}$ and $ITIH_{4[666–687]}$ had to be as low as possible and MS settings had to be particularly optimized for these two analytes. Therefore, multiple product ions were selected for MRM analysis and increased Q1 resolution was applied in order to reduce interferences at the LLOQ concentration. Heating of the ESI-probe up to 250 °C slightly improved the signal of the peptides at the applied flow rate of 400 μl/min.

*3.1.3. Liquid chromatography*

Achieving fast analysis with good chromatographic performance for a mixture of strongly varying peptides, each exhibiting its own specific characteristics, was quite challenging. The Fib-$\alpha_{[605–629]}$ peptide fragment showed very poor retention and peak shape, especially at lower pH, higher temperature, lower ionic strength or at higher percentages of organic solvent in either the initial mobile phase or the injection solvent. Although the peptide is too small to theoretically expect secondary structures, the existence of a somewhat "folded" conformation with different retention behavior could be hypothesized. The location of many acidic amino acid residues at the N-terminal site and several basic amino acid residues at the C-terminal site, as well as the above

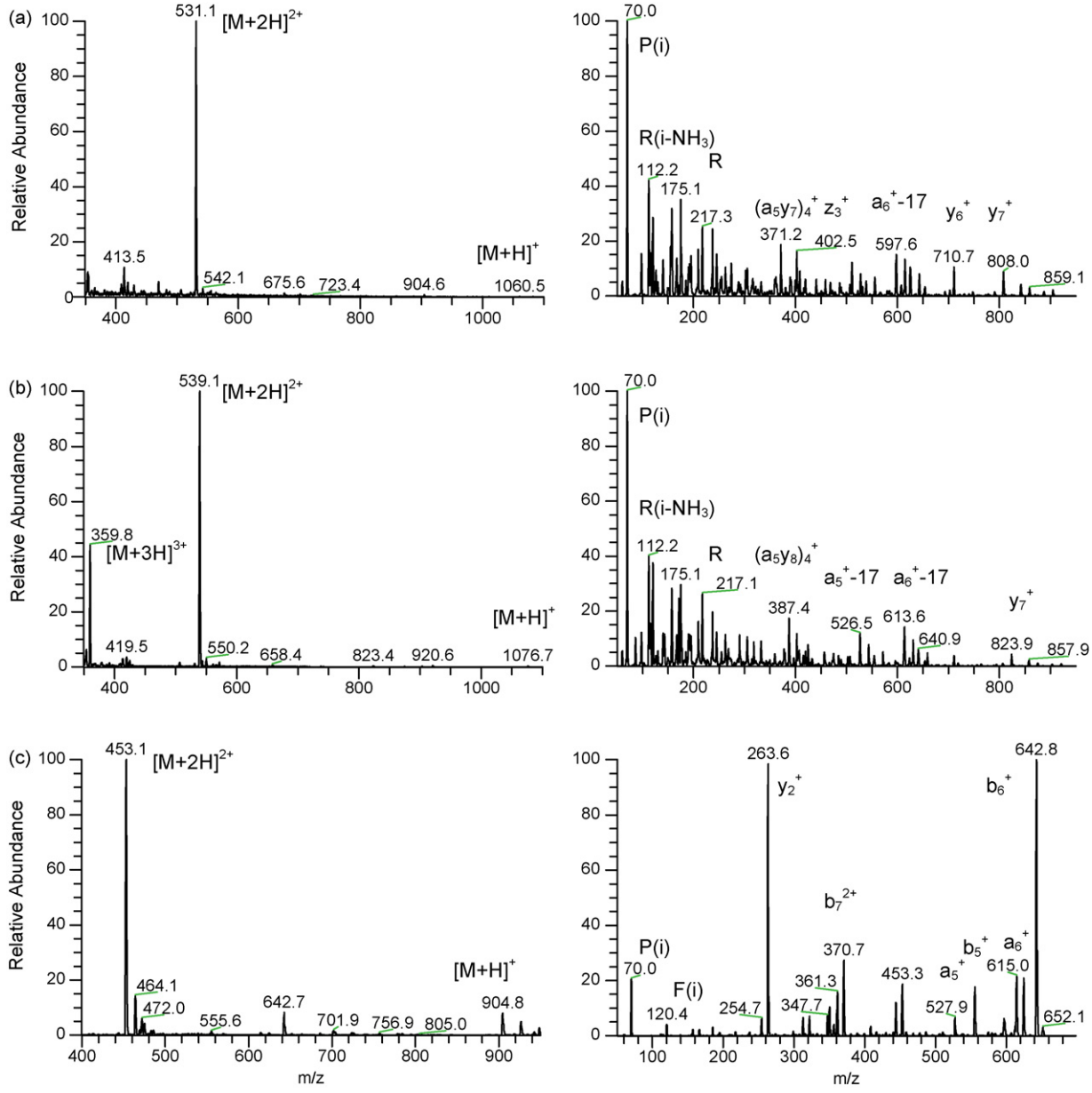


**Fig. 2.** ESI (left) and product ion (right) mass spectra of bradykinin (a); Hyp$^3$-bradykinin (b) and des-Arg$^9$-bradykinin (c). MS settings were as listed in Table 2.

mentioned conditions favoring the occurrence of the unretained fraction, make the idea of an unretained, "unfolded" and positively charged, conformer imaginable.

For the plasma or serum extracts a good compromise could be achieved as the ionic strength was high enough to favor the speculated retained conformer under acidic conditions as long as the initial mobile phase was 100% aqueous and column temperature did not exceed 30 °C.

During initial experiments at higher pH values, an additional peak with slightly more retention was observed for the fragment of C4a. This peak could be identified after mass spectrometric exploration as a degradation product with a mass difference of only +1, caused by de-amidation at the C-terminal asparagine amino acid residue. Non-enzymatic de-amidation of asparagine and glutamine residues is well known to occur, especially in more basic solutions and also at neutral pH when asparagine is followed by a glycine residue [19]. To prevent interference by the de-amidated product, use of higher pH should be avoided while sufficient chromatographic separation could be achieved by holding the percentage of methanol in the mobile phase at 32% for a short period. Furthermore, the increased Q1 resolution (0.2 FWHM) for the quantification of C4a$_{[1337-1350]}$ resulted in less interference of the degradation product.

Methanol was preferred as organic modifier, as it improved signal-to-noise (S/N) ratios at the LLOQ concentration of the peptides compared to a mobile phase with acetonitrile. Representative MRM chromatograms of an LLOQ and a blank bovine plasma sample are shown in Fig. 5.

### 3.1.4. *Bradykinin stability*

In human plasma bradykinin is rapidly degraded, predominantly by angiotensin I converting enzyme (ACE) at the 7–8 and 5–6 positions and carboxypeptidase N at the 8–9 position to produce the fragments BK1-7, BK1-5 and des-Arg$^9$-bradykinin respectively [20]. This rapid degradation was observed in both bovine and human samples spiked with bradykinin. Even on ice, the $t_{1/2}$ of bradykinin in bovine plasma was not more than 30 min while no response was observed anymore after three hours storage of a

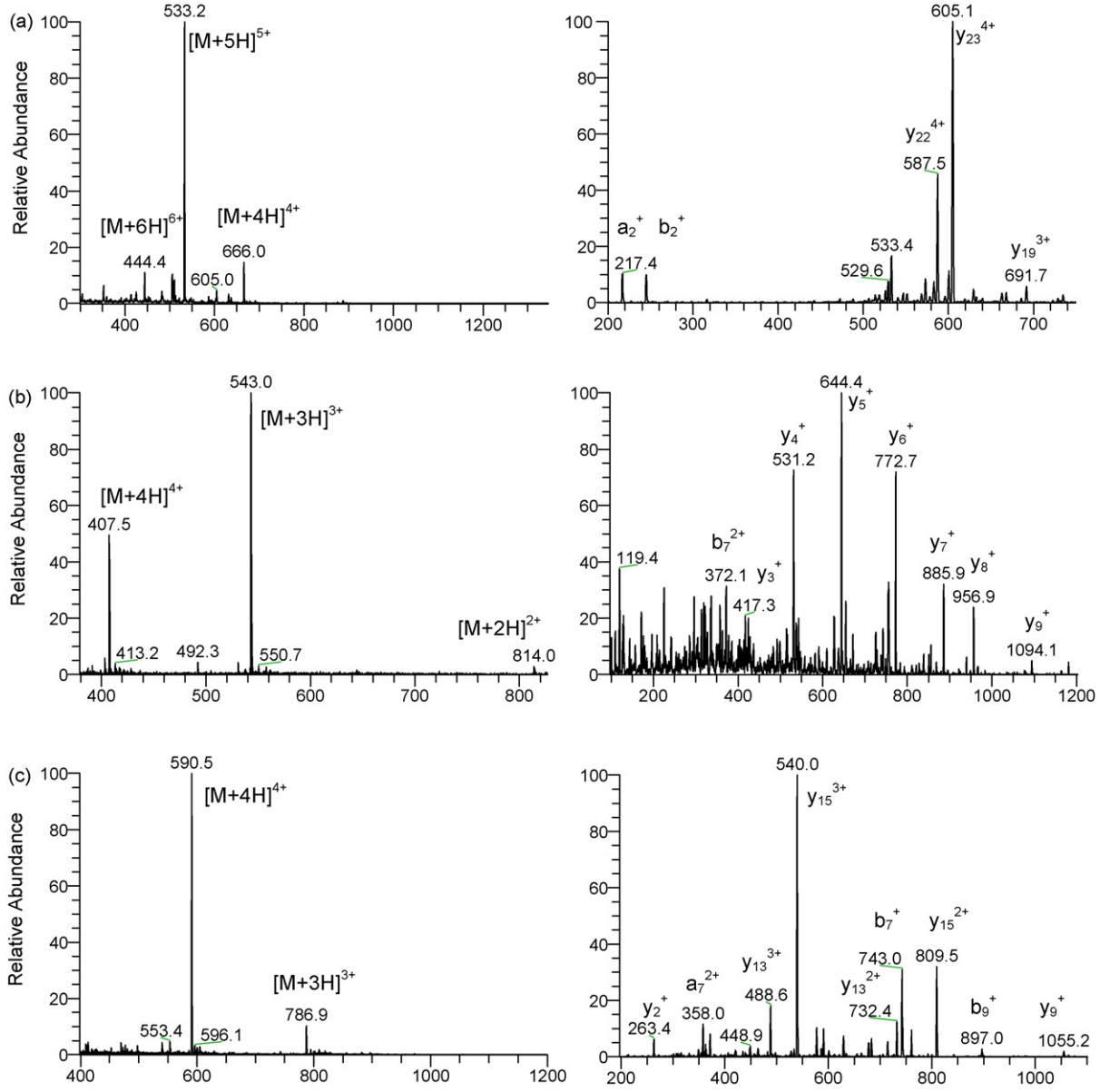


**Fig. 3.** ESI (left) and product ion (right) mass spectra of Fib-$\alpha_{[605–629]}$ (a); C4a$_{[1337–1350]}$ (b) and ITIH$_{4[666–687]}$ (c). MS settings were as listed in Table 2.

1 μg/ml solution (Fig. 6). However, despite a similar rapid degradation just after the addition of bradykinin, the response in a human serum sample did not completely disappear but reached a more or less constant level (Fig. 6). This response was comparable with the response in directly prepared human serum samples of the same batch that were not spiked with synthetic bradykinin. Therefore, it seems likely that *ex vivo* formation of bradykinin occurs along with its degradation, resulting in a somehow constant endogenous amount of bradykinin.

Nevertheless, the rapid decrease in spiked bradykinin concentration would seriously complicate accurate quantitative analysis. Therefore, inhibitors of ACE (captopril) and carboxypeptidase N (MEGETPA) were added to the blank bovine plasma used for the preparation of the calibration standards and QC samples. Fig. 7 shows their inhibitory effect on the degradation of bradykinin. The degradation of bradykinin could almost be completely inhibited by the addition of 2 mM captopril and 2 mM MEGETPA, thereby also enabling simultaneous stabilization of Hyp$^3$-bradykinin and quantification of des-Arg$^9$-bradykinin.

### 3.2. Method validation

#### 3.2.1. Linearity

Average determination coefficients ($R^2$), slopes and intercepts obtained after three separate runs are summarized in Table 3. Deviations of the mean calculated concentrations from the nominal concentrations over three runs were all below 11%. For Fib-

**Table 3**
Slope (a), intercept (b) and determination coefficient ($R^2$) of the six peptide analytes ($n = 3$). For Fib-$\alpha_{[605–629]}$ a double-logarithmic curve was used ($\log(y) = a\log(x) + b$).

| Peptide | Slope (ml/ng) | Intercept | $R^2$ |
|---|---|---|---|
| Bradykinin | 0.009 ± 0.002 | −0.005 ± 0.02 | 0.992 ± 0.007 |
| Hyp$^3$-bradykinin | 0.006 ± 0.001 | 0.000 ± 0.003 | 0.994 ± 0.004 |
| Des-Arg$^9$-bradykinin | 0.020 ± 0.001 | 0.020 ± 0.003 | 0.998 ± 0.001 |
| Fib-$\alpha_{[605–629]}$ | 1.363 ± 0.2 | −3.098 ± 0.17 | 0.993 ± 0.005 |
| C4a$_{[1337–1350]}$ | 0.049 ± 0.005 | −0.015 ± 0.002 | 0.993 ± 0.004 |
| ITIH$_{4[666–687]}$ | 0.271 ± 0.03 | 0.018 ± 0.03 | 0.994 ± 0.003 |

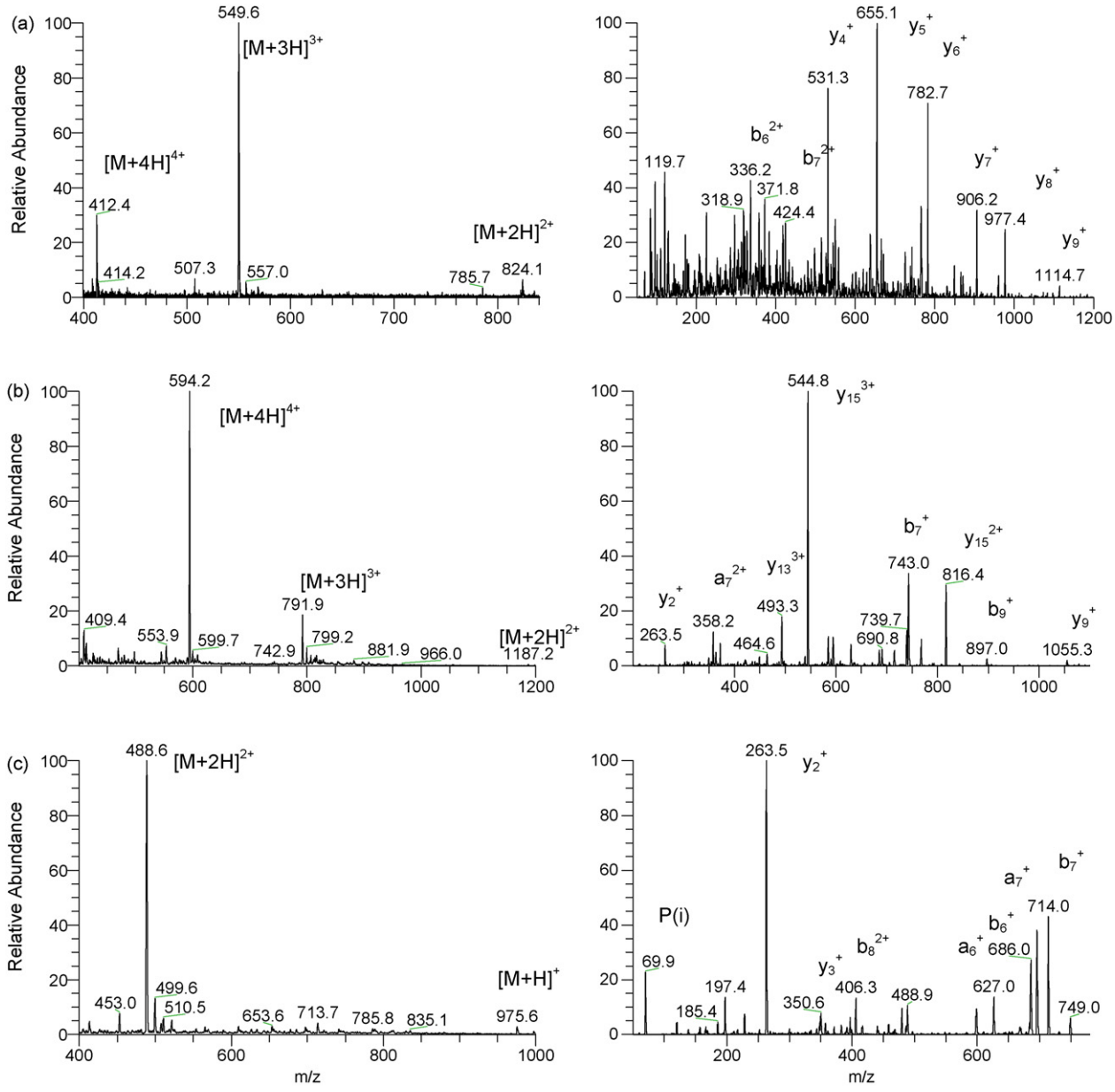


**Fig. 4.** ESI (left) and product ion (right) mass spectra of d20-C4a$_{[1337–1350]}$ (a); Ile$^{13}$-ITIH$_{4[666–678]}$ (b) and Sar-D-Phe$^{7}$-des-Arg$^{9}$-bradykinin (c). MS settings were as listed in Table 2.

$\alpha_{[605–629]}$, linearity and measured concentrations showed a better fit with a linear log–log calibration, which was therefore employed for this particular peptide.

### 3.2.2. Precision and accuracy

The assay performance data for all peptides are presented in Table 4. For all peptides, accuracies and precisions of the lowest plasma QC samples were within the ±20% range, defining LLOQs of 0.4, 1.0, 2.0, 4.0, 10.0 and 120.0 ng/ml for ITIH$_{4[666–687]}$, C4a$_{[1337–1350]}$, des-Arg$^{9}$-bradykinin, Hyp$^{3}$-bradykinin, bradykinin, and Fib-$\alpha_{[605–629]}$, respectively. Intra- and inter-assay precisions and deviations from the nominal concentrations were below 13% for the bradykinin peptides. In contrast to these three peptides, the LLOQs of ITIH$_{4[666–687]}$ and C4a$_{[1337–1350]}$ had for clinical relevance to be as low as possible and were probably near the limit of detection (LOD) of the method, requiring optimal conditions before analysis. Nevertheless, accuracies and precisions were within the acceptable limits at all concentration levels. For Fib-$\alpha_{[605–629]}$ only the HQC sample showed a large deviation of the mean measured concentration from the nominal concentration, possibly due to the lack of an analogous internal standard. As this peptide exhibited a different retention behavior compared to the other peptides, accurate and precise quantification of Fib-$\alpha_{[605–629]}$ may therefore be simply facilitated with the use of a structural or stable isotope-labeled analog as IS.

Table 5 shows the results for the accuracy and precision of the measured concentrations in human serum samples, corrected for the endogenous concentrations in the blank matrix. Except for the bradykinin peptides at their LQC concentration, quantification in human serum could be accurately and precisely performed for all peptides, also after correction for the concentrations in the blanks. Due to the contribution of the variations in the blanks, higher RSD values were obtained, but only for C4a$_{[1337–1350]}$ this value was above 15%. As RSD values for the measured concentrations in both the blank and the QC samples were below the 15% limit, precise quantification of this analyte in human serum is expected. These

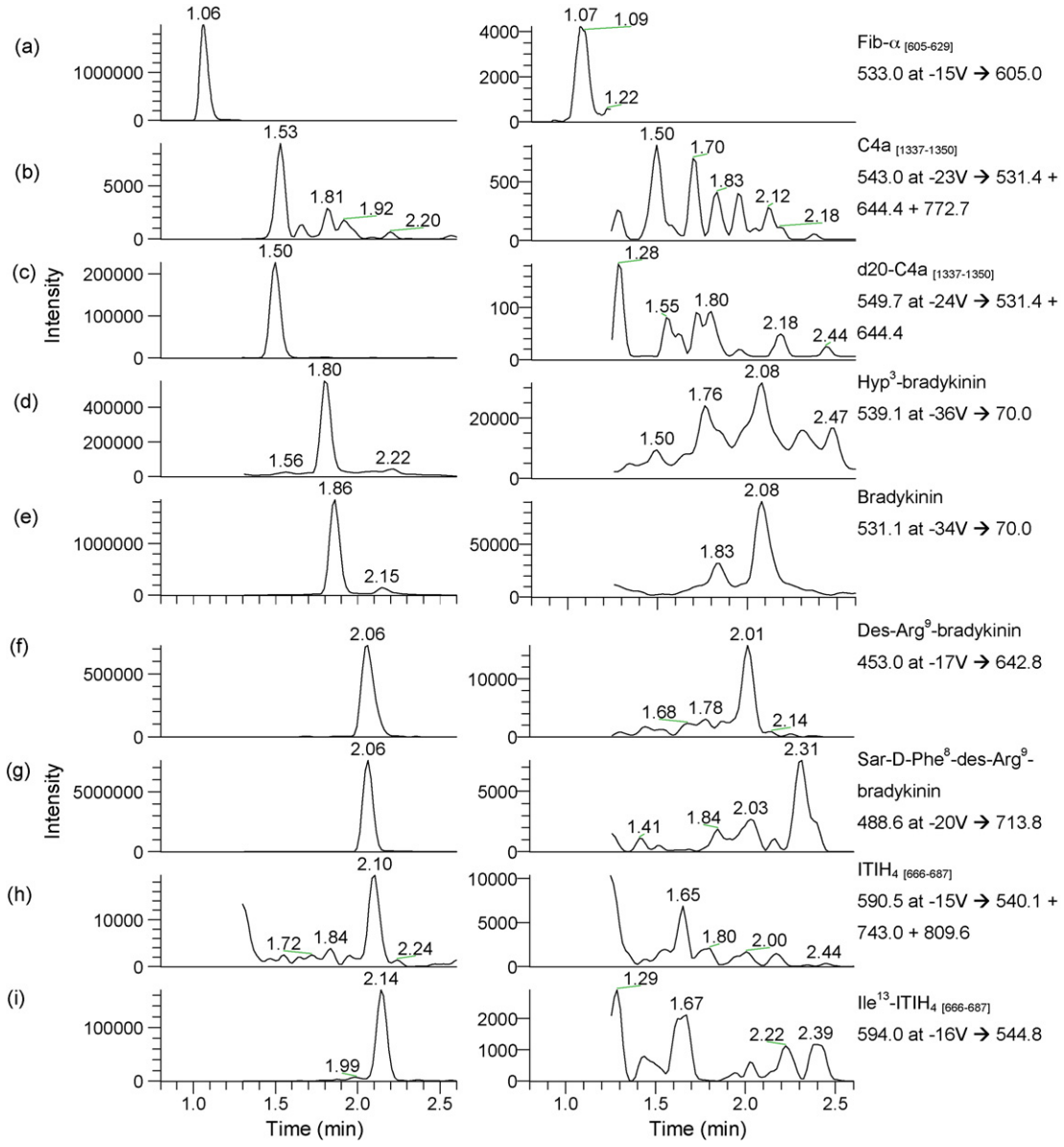


**Fig. 5.** MRM chromatogram of an LLOQ (left) and blank bovine plasma sample (right). (a) Fib-$\alpha_{[605-629]}$; (b) $C4a_{[1337-1350]}$; (c) d20-$C4a_{[1337-1350]}$; (d) $Hyp^3$-bradykinin; (e) Bradykinin; (f) Des-$Arg^9$-bradykinin; (g) Sar-D-$Phe^7$-des-$Arg^9$-bradykinin; (h) $ITIH_{4[666-687]}$ and (i) $Ile^{13}$-$ITIH_{4[666-678]}$. MS settings were as listed in Table 2.

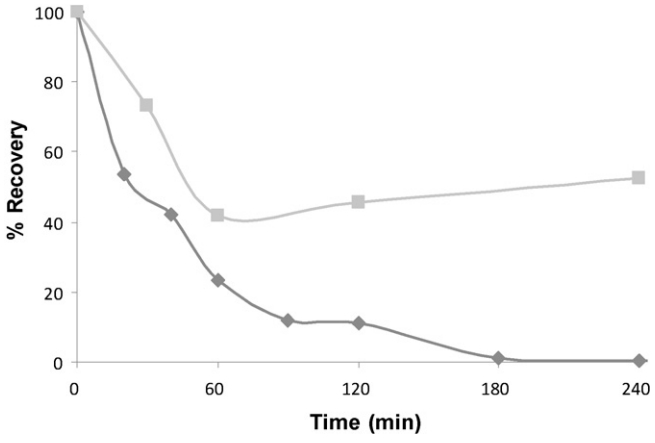


**Fig. 6.** The degradation of bradykinin in bovine plasma (◆) and in human serum (■) on ice, expressed as % of the measured concentration at $t = 0$.

results implicate that no different behavior of the peptides in either a bovine or human matrix is supposed and quantification in human serum can be performed using bovine plasma for the preparation of calibration standards.

Results obtained for the bradykinin peptides spiked at LQC concentration could not be correctly interpreted, as the added inhibitors, required to prevent rapid degradation, affected the endogenous "background" concentrations in the blanks and the QC samples in different extents by affecting both the degradation and *ex vivo* formation of these peptides. Nevertheless, as no effects of the human matrix were observed for the quantification of Fib-$\alpha_{[605-629]}$, $C4a_{[1337-1350]}$, $ITIH_{4[666-687]}$ and the bradykinins at HQC level, also accurate and precise quantification of the bradykinins at lower concentrations using bovine plasma standards is assumed.

### 3.2.3. *Specificity and selectivity*

As the method is developed for the quantification of endogenous peptides in human samples, bovine plasma was selected as analyte-free matrix for the preparation of standards and QC sam-

**Table 4**
Assay performance data of the six peptide analytes at four concentration levels ($n = 18$).

| Peptide | Nominal concentration (ng/ml) | Measured concentration (ng/ml) | Intra-assay precision (%) | Inter-assay precision (%) | Deviation (%) |
|---|---|---|---|---|---|
| Bradykinin | 9.99 | 10.51 ± 0.96 | 7.8 | 9.1 | 5.2 |
| | 24.98 | 24.38 ± 1.90 | 5.3 | 7.8 | −2.4 |
| | 199.9 | 196.3 ± 16 | 6.4 | 8.0 | −1.8 |
| | 399.7 | 422.1 ± 20 | 3.8 | 4.7 | 5.6 |
| Hyp$^3$-bradykinin | 3.38 | 3.36 ± 0.41 | 9.8 | 1.5 | −0.5 |
| | 8.45 | 8.01 ± 1.1 | 8.8 | 13.4 | −5.2 |
| | 67.59 | 59.45 ± 7.6 | 6.0 | 12.8 | −12.0 |
| | 135.2 | 126.7 ± 14 | 3.8 | 10.7 | −6.3 |
| Des-Arg$^9$-bradykinin | 1.95 | 2.08 ± 0.1 | 3.7 | 5.9 | 6.5 |
| | 4.88 | 5.02 ± 0.2 | 3.2 | 4.4 | 3.0 |
| | 39.01 | 39.40 ± 1.0 | 1.8 | 2.4 | 1.0 |
| | 78.02 | 78.93 ± 1.6 | 1.1 | 2.0 | 1.2 |
| Fib-α$_{[605–629]}$ | 118.1 | 129.9 ± 11 | 8.0 | 9.4 | 10.0 |
| | 295.2 | 281.2 ± 26 | 8.6 | 8.8 | −4.8 |
| | 1181 | 1325 ± 123 | 8.4 | 9.1 | 12.2 |
| | 2362 | 2923 ± 446 | 8.8 | 14.0 | 22.3 |
| C4a$_{[1337–1350]}$ | 0.99 | 1.12 ± 0.2 | 14.8 | 14.3 | 13.1 |
| | 2.47 | 2.50 ± 0.4 | 12.7 | 14.5 | 0.8 |
| | 9.88 | 9.18 ± 1.2 | 13.1 | 13.4 | −7.1 |
| | 19.76 | 19.34 ± 2.4 | 10.8 | 12.4 | −2.1 |
| ITIH$_{4[666–687]}$ | 0.40 | 0.39 ± 0.08 | 14.6 | 19.9 | −2.6 |
| | 1.01 | 0.96 ± 0.1 | 10.5 | 12.7 | −4.7 |
| | 4.02 | 3.83 ± 0.4 | 9.4 | 9.5 | −4.7 |
| | 8.04 | 7.95 ± 0.6 | 5.4 | 7.3 | −1.2 |

**Table 5**
Assay performance data of the peptide analytes in human serum at LQC and HQC concentration with correction for endogenous concentration in the matrix ($n = 6$).

| Peptide | Concentration in blank serum (ng/ml) | Added concentration (ng/ml) | Measured concentration (ng/ml) | Precision (%) | Deviation (%) |
|---|---|---|---|---|---|
| Bradykinin | 106.7 ± 12 | 399.7 | 530.3 ± 19 | 11.4 | 6.0 |
| Hyp$^3$-bradykinin | 27.1 ± 0.9 | 135.2 | 151.2 ± 6 | 5.4 | −8.2 |
| Des-Arg$^9$-bradykinin | 7.1 ± 0.2 | 78.02 | 92.2 ± 1.3 | 3.3 | 9.1 |
| Fib-α$_{[605–629]}$ | 541.9 ± 50 | 295.2 | 799.9 ± 71 | 12.9 | −12.6 |
| | 541.9 ± 50 | 2362 | 3029 ± 250 | 9.6 | 7.1 |
| C4a$_{[1337–1350]}$ | 1.0 ± 0.2 | 2.47 | 3.56 ± 0.5 | 20.3 | 2.7 |
| | 1.0 ± 0.2 | 19.76 | 21.8 ± 1.6 | 16.5 | 5.0 |
| ITIH$_{4[666–687]}$ | × | 1.01 | 1.10 ± 0.1 | 12.8 | 9.2 |
| | × | 8.04 | 8.91 ± 0.8 | 8.4 | 10.7 |

ples. Comparison of the amino acid sequences of the proteolytic human peptides with the corresponding amino acid sequences of bovine proteins ensured that these fragments would not be present in the bovine matrix [21]. Furthermore, analysis of blank bovine plasma did not reveal any interfering peaks for any peptide (Fig. 4). Between Sar-D-Phe$^7$-des-Arg$^9$-bradykinin and des-Arg$^9$-bradykinin some cross-interference could be observed, but this response remained below 20% of the LLOQ response of des-Arg$^9$-bradykinin.

Analysis of six blank human serum samples from six different sources showed responses for all peptides in all samples. Quantification of the LLOQs was therefore not possible. As the developed method is intended for the quantification of endogenous peptides, these analyses merely show the necessity for an analyte-free matrix.

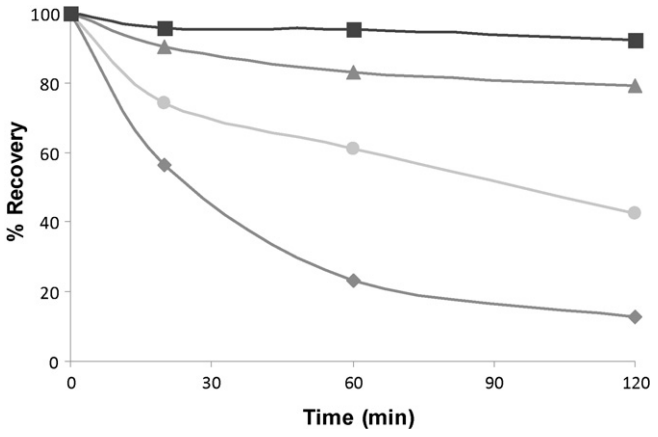


**Fig. 7.** The inhibition of bradykinin degradation in bovine plasma (initial concentration 100 ng/ml) by the ACE-inhibitor captopril at a concentration of 1 mM (●), 2 mM (▲) and in combination with the N-carboxypeptidase inhibitor MEGETPA (■), compared to a bovine plasma sample without inhibitors (◆). All samples were kept on ice.

### 3.2.4. Recovery and ion suppression

The SPE recoveries from bovine plasma varied between 62 and 90% for all peptides, including the internal standards and are shown in Table 6. Comparing the peak areas of the peptides spiked after extraction of blank bovine plasma samples with peak areas of stan-

**Table 6**
SPE-recoveries from bovine plasma of the six peptide analytes and the three internal standards ($n = 3$ at each concentration level and $n = 9$ for the internal standards).

| Peptide | LQC | MQC | HQC |
|---|---|---|---|
| Bradykinin | 82.9 ± 9.5 | 74.2 ± 5.2 | 82.3 ± 9.6 |
| Hyp$^3$-bradykinin | 84.9 ± 12.3 | 76.9 ± 6.9 | 80.6 ± 8.2 |
| Des-Arg$^9$-bradykinin | 88.0 ± 13.6 | 80.9 ± 5.2 | 83.3 ± 8.5 |
| Fib-α$_{[605–629]}$ | 80.6 ± 1.7 | 66.5 ± 6.1 | 78.4 ± 6.5 |
| C4a$_{[1337–1350]}$ | 62.3 ± 11.6 | 75.2 ± 7.0 | 66.8 ± 13.3 |
| ITIH$_{4[666–687]}$ | 90.3 ± 19.3 | 62.4 ± 9.1 | 77.5 ± 7.0 |
| d20-C4a$_{[1337–1350]}$ | | | 75.4 ± 4.1 |
| Ile$^{13}$-ITIH$_{4[666–687]}$ | | | 76.4 ± 13.6 |
| Sar-D-Phe$^7$-des-Arg$^9$-bradykinin | | | 86.2 ± 15.6 |

**Table 7**
Stabilities of the six peptide analytes in the stock and working solutions (methanol/water 1:1 (v/v), 0.1% formic acid) under different storage conditions ($n = 3$).

| Peptide | Stock solutions | | Working solutions | | | |
|---|---|---|---|---|---|---|
| | % DEV[a] | RSD | % DEV[b] | RSD | % DEV[c] | RSD |
| Bradykinin | −8.1[d] | 5.7 | 11.1 | 14.5 | −0.1 | 17.0 |
| Hyp$^3$-bradykinin | −6.8[d] | 6.7 | 4.1 | 15.1 | 9.3 | 10.6 |
| Des-Arg$^9$-bradykinin | 9.7[d] | 6.8 | 6.8 | 8.8 | 0.5 | 7.4 |
| Fib-α$_{[605–629]}$ | 5.7 | 20.5 | 1.9 | 20.1 | 1.6 | 20.0 |
| C4a$_{[1337–1350]}$ | −5.4 | 9.4 | −9.4 | 11.1 | −8.8 | 13.9 |
| ITIH$_{4[666–687]}$ | −1.9 | 12.0 | −0.9 | 9.8 | −2.1 | 12.9 |

[a] Relative deviations after six months storage at −30 °C.
[b] Relative deviations after one month storage at −30 °C.
[c] Relative deviations after 24 h at ambient temperature.
[d] Relative deviations after two months storage at −80 °C.

dard solutions spiked with the peptides at the same LQC, MQC and HQC concentrations showed a reduction of the MS response by 4–25% over all concentration levels for all peptides, except for Fib-α$_{[605–629]}$. For this peptide the response in the plasma extracts was higher than in the standard solution, likely caused by destabilization of the supposed "folded" confirmation of Fib-α$_{[605–629]}$ in a solution with low ionic strength, resulting in an increased fraction of unretained peptide.

### 3.2.5. Stability

The results for the stability experiments of all peptides in the stock and working solution are summarized in Table 7. All analytes were stable in the working solution after 24 h at ambient temperature and after one month storage at −30 °C. The stock solutions of the bradykinin peptides were stored at −80 °C, as they were thought to be susceptible to degradation. However, no significant deviations from the initial concentrations could be observed after two months storage. The stock solutions of the other peptides were stored at −30 °C and showed stable responses after six months storage.

Table 8 shows the results of the stability assessments for the analytes in, or extracted from, bovine plasma, containing 2 mM captopril and 2 mM MEGETPA. All peptides were stable in this matrix for at least one hour when kept on ice. Only for the fibrinogen fragment the stability could not be assessed due to large variations in the response, most likely caused by the absence of d20-C4a$_{[1337–1350]}$ as IS at the time of measurement. Nevertheless, as this peptide did not show any significant deviations after three freeze–thaw cycles, short-term instability of this peptide would not be expected. In addition, all other peptides showed to give a stable response after three freeze–thaw cycles. Furthermore, stable responses were observed for the analysis of the dried extracts after one week storage at 5 °C, although variations were slightly higher than for directly analyzed QC samples. Quantification of the QC samples after 48 h storage at 5 °C with freshly prepared standards revealed some instabilities for Fib-α$_{[605–629]}$, most likely caused by the lack of a proper internal standard that better corrects for little response variations. Storage of the bovine plasma samples at −80 °C for six months showed no significant deviations for all peptides.

For the application of the method in human samples, insight in the (in)stability of the peptides in the human matrix under different conditions is required. Table 9 summarizes the results of stability determinations of the peptides in human serum samples. The serum samples were spiked at HQC concentrations for all peptides, except for bradykinin as instability of this peptide might complicate the stability assessment of des-Arg$^9$-bradykinin. Therefore, Hyp$^3$-bradykinin stability served as an indication of the stability of bradykinin.

As can be seen in Table 9, for the *spiked* peptides at HQC concentration only Hyp$^3$-bradykinin showed a large negative deviation compared to freshly prepared samples after both short-term and long-term storage, as well as after three freeze–thaw cycles. This could be expected since no inhibitors were added to the human serum and rapid degradation of bradykinin and Hyp$^3$-bradykinin occurs, as discussed before. These inhibitors were not added to specifically assess the stability of the endogenous peptides. There-

**Table 8**
Stabilities of the six peptide analytes in, or extracted from, bovine plasma, containing 2 mM captopril and 2 mM MEGETPA ($n = 3$).

| Peptide | Short term stability[a] | | Long term stability[b] | | Freeze–thaw stability[c] | | In-process stability[d] | | Stability of final extract[e] | |
|---|---|---|---|---|---|---|---|---|---|---|
| | % DEV | CV | % DEV | CV | % DEV | CV | % DEV | CV | % DEV | CV |
| LQC | | | | | | | | | | |
| Bradykinin | 0.7 | 6.8 | −1.9 | 3.0 | −3.5 | 11.1 | 8.1 | 4.3 | −14.1 | 4.7 |
| Hyp$^3$-bradykinin | 4.6 | 9.5 | −2.6 | 7.8 | −7.9 | 9.4 | 8.5 | 6.6 | −7.1 | 6.9 |
| Des-Arg$^9$-bradykinin | −2.6 | 4.9 | 3.3 | 10.9 | 2.7 | 4.9 | 6.5 | 3.5 | 3.8 | 3.8 |
| Fib-α$_{[605–629]}$ | 34.4 | 42.4 | −4.4 | 9.5 | −1.5 | 9.8 | 8.6 | 10.0 | 0.2 | 13.0 |
| C4a$_{[1337–1350]}$ | −4.9 | 14.2 | 11.3 | 15.5 | −3.7 | 11.5 | −5.1 | 15.0 | −8.3 | 15.3 |
| ITIH$_{4[666–687]}$ | −5.8 | 9.6 | −6.2 | 15.9 | 5.1 | 13.5 | 9.1 | 19.2 | 7.3 | 14.9 |
| HQC | | | | | | | | | | |
| Bradykinin | 7.2 | 2.2 | −11.3 | 15.0 | −1.3 | 5.7 | 0.5 | 2.9 | 7.5 | 7.9 |
| Hyp$^3$-bradykinin | 7.8 | 2.5 | −13.4 | 14.6 | 5.8 | 2.9 | −1.0 | 3.6 | 7.3 | 6.5 |
| Des-Arg$^9$-bradykinin | 3.7 | 2.3 | −0.8 | 6.1 | −0.1 | 4.8 | −1.5 | 1.3 | 4.3 | 1.8 |
| Fib-α$_{[605–629]}$ | 7.1 | 14.1 | −6.6 | 14.0 | −11.1 | 12.2 | 2.2 | 13.4 | 22.4 | 12.2 |
| C4a$_{[1337–1350]}$ | 7.8 | 14.3 | 2.6 | 12.3 | −3.7 | 15.0 | −7.3 | 16.5 | −5.4 | 14.6 |
| ITIH$_{4\ [666–687]}$ | 5.2 | 7.1 | 3.6 | 7.2 | −3.0 | 4.5 | 4.6 | 15.6 | 8.0 | 14.0 |

[a] Samples placed on ice for 1 h.
[b] After six months storage at −80 °C.
[c] After three freeze–thaw cycles (−80 °C).
[d] Dried extracts after one week storage at 5 °C.
[e] Re-injection after 48 h in autosampler (5 °C).

**Table 9**
Stabilities of the six peptide analytes in human serum samples ($n = 3$).

| Peptide | Short term[a] | | Long term[b] | | Freeze–thaw[c] | | $n$ |
|---|---|---|---|---|---|---|---|
| | % DEV | CV | % DEV | CV | % DEV | CV | |
| Endogenous concentration | | | | | | | |
| Bradykinin | −6.3 | 12.1 | −0.8 | 13.9 | 13.9 | 13.2 | 6 |
| $Hyp^3$-bradykinin | −13.0 | 7.1 | −13.5 | 9.7 | 3.3 | 8.9 | 3 |
| Des-$Arg^9$-bradykinin | −4.1 | 11.5 | −3.2 | 8.2 | 11.4 | 9.7 | 3 |
| Fib-$\alpha_{[605–629]}$ | 16.4 | 11.0 | −12.1 | 11.3 | −9.1 | 10.7 | 3 |
| $C4a_{[1337–1350]}$ | 12.3 | 13.7 | −4.1 | 7.3 | 2.9 | 7.0 | 3 |
| LQC | | | | | | | |
| $ITIH_{4[666–687]}$ | −5.3 | 14.2 | 10.8 | 10.8 | −1.7 | 14.4 | 3 |
| HQC | | | | | | | |
| $Hyp^3$-bradykinin | −38.5 | 5.5 | −47.0 | 15.2 | −37.9 | 13.9 | 3 |
| Des-$Arg^9$-bradykinin | −3.2 | 2.5 | −5.1 | 4.4 | −3.1 | 3.1 | 3 |
| Fib-$\alpha_{[605–629]}$ | 11.9 | 11.6 | 0.7 | 15.0 | 8.5 | 14.1 | 3 |
| $C4a_{[1337–1350]}$ | 5.4 | 14.7 | 1.7 | 12.8 | 1.2 | 13.2 | 3 |
| $ITIH_{4[666–687]}$ | 8.7 | 12.1 | −10.8 | 14.9 | −1.6 | 19.6 | 3 |

[a] Samples kept on ice for 1 h.
[b] The endogenous peptide concentrations were measured after five months storage at −80 °C and compared to the measured concentrations at the time of preparation. Initial concentrations were 727.7 ± 67.4; 11.2 ± 0.8; 30.0 ± 2.4; 113.6 ± 14.6 and 8.0 ± 0.2 for Fib-$\alpha_{[605–629]}$, $C4a_{[1337–1350]}$, $Hyp^3$-bradykinin, bradykinin and des-$Arg^9$-bradykinin, respectively. The spiked LQC and HQC samples were measured after three months storage at −80 °C and compared to freshly prepared QC samples.
[c] After three freeze–thaw cycles (−80 °C).

fore, only $ITIH_{4[666–687]}$ was added at LQC level, while for the other peptides the endogenous peptide concentration was measured. After 1 h storage on ice as well as after three freeze–thaw cycles no significant changes in the endogenous concentrations were observed. Furthermore, no significant deviations were observed compared to the initial concentrations after five months storage at −80 °C.

### 3.3. Internal standards

As the ACE- and carboxypeptidase-N inhibitors that were added to the bovine plasma affect the *ex vivo* generation of bradykinin from high-molecular-weight kininogen and the further *ex vivo* degradation of bradykinin itself, they were not added to human samples. However, for accurate quantification, an ACE and carboxypeptidase-N resistant bradykinin-like internal standard was then required. Sar-D-$Phe^8$-des-$Arg^9$-bradykinin is a bradykinin analog resistant for both enzymes and was therefore selected as internal standard for the bradykinin-like peptide analytes. Furthermore, an $Ile^{13}$-analog of $ITIH_{4[666–687]}$ was available in our laboratory and was initially used as internal standard for the four non-bradykinin-like peptides. However, this IS could not sufficiently correct for response variations of Fib-$\alpha_{[605–629]}$ and $C4a_{[1337–1350]}$ and a third IS was added to the assay the stable-isotope labeled analog of the C4a fragment. This IS enabled quantification of $C4a_{[1337–1350]}$ and significantly improved the quantification of the fibrinogen α-chain fragment.

### 3.4. Analysis of patient samples

All peptides were detected in the serum samples from six breast cancer patients. The measured concentrations are shown in Table 10 together with the measured concentrations of the matched controls, obtained after single measurement. An MRM chromatogram of one serum sample from a breast cancer patient is shown in Fig. 8. These analyses included a too small amount of samples to provide information on the diagnostic value of the various peptides, but showed the applicability of the method for the quantification of all peptides. Further assessment of their clinical value with a larger set of clinical samples is intended.

The concentrations of des-$Arg^9$-bradykinin were sometimes higher than the upper limit of quantification (ULQ) of the method, while for the other peptides measured concentrations were within the validated concentration ranges. For $ITIH_{4\,[666–687]}$ on the other hand, concentrations in the serum samples from the matched controls were below the LLOQ in five of the six samples. The found concentrations for bradykinin were much higher than the (low ng–pg/ml) concentrations mentioned in the literature [16]. On the other hand, the higher normalized intensity values mentioned by Villanueva et al. [2] for bradykinin and des-$Arg^9$-bradykinin compared to the other peptides stimulate the assumption that higher concentrations for these peptides can very well be expected. Furthermore, the various factors affecting the *ex vivo* formation of bradykinin are still unclear and an induction of the *ex vivo* formation by the sample pre-treatment procedure, allowing measurement of an endogenous "background" response, might be possible.

**Table 10**
Individual measured concentrations in serum samples from breast cancer patients and matched controls.

| Peptide | Concentrations (ng/ml) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sample type | #1 | | #2 | | #3 | | #4 | | #5 | | #6 | |
| | BC | CO | BC | CO | BC | CO | BC | CO | BC | CO | BC | CO |
| Bradykinin | 144 | 74 | 123 | 58 | 94 | 57 | 43 | 87 | 104 | 162 | [a] | 108 |
| $Hyp^3$-bradykinin | 18 | 41 | 15 | 61 | 32 | 54 | 19 | 11 | 5.0 | 16 | [a] | 12 |
| Des-$Arg^9$-bradykinin | 136 | 50 | 134 | 46 | 122 | 66 | 100 | 151 | 85 | 63 | 133 | 68 |
| Fib-$\alpha_{[605–629]}$ | 407 | 268 | 154 | 163 | 140 | 174 | 105 | 189 | 287 | 351 | 232 | 213 |
| $C4a_{[1337–1350]}$ | 3.0 | 2.9 | 5.1 | 4.6 | 3.3 | 3.2 | [a] | [a] | 2.1 | 1.6 | 4.1 | 2.6 |
| $ITIH_{4[666–687]}$ | 0.6 | [a] | 0.9 | 0.5 | 0.6 | [a] | 0.4 | [a] | 0.5 | [a] | 2.7 | [a] |

BC: Breast cancer patient; CO: Matched control.
[a] Concentration was below LLOQ.

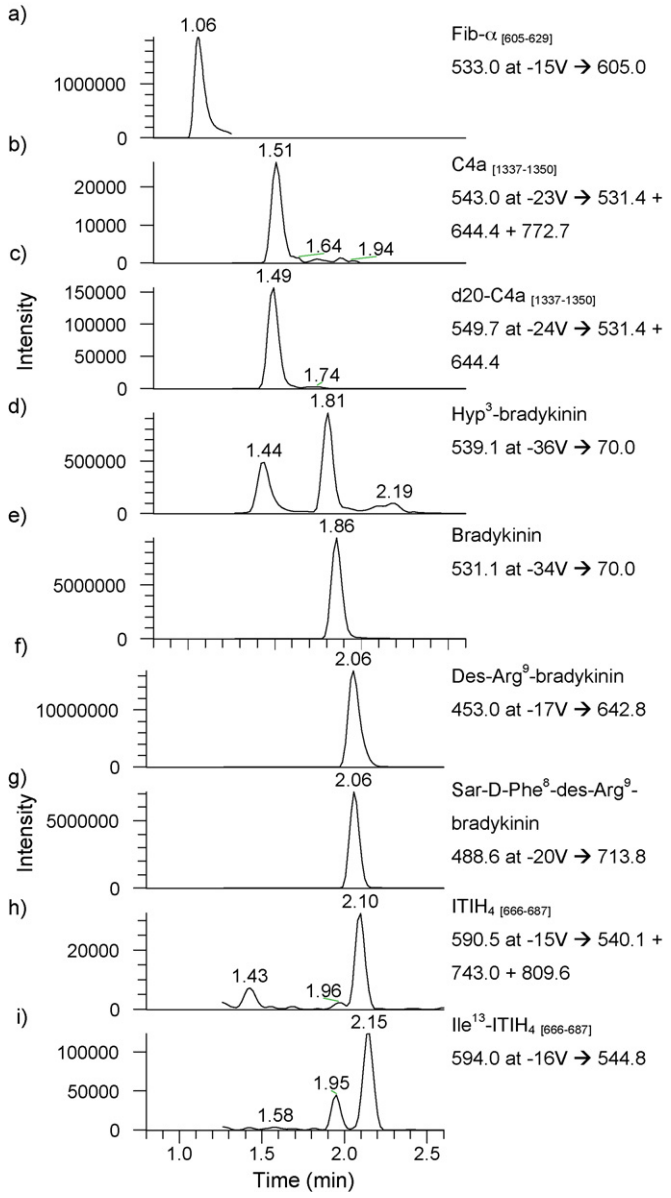


**Fig. 8.** MRM chromatogram of a serum sample from a patient with breast cancer. (a) Fib-$\alpha_{[605-629]}$ (154 ng/ml); (b) $C4a_{[1337-1350]}$ (5 ng/ml); (c) d20-$C4a_{[1337-1350]}$; (d) $Hyp^3$-bradykinin (15 ng/ml); (e) Bradykinin (123 ng/ml); (f) Des-$Arg^9$-bradykinin (136 ng/ml); (g) Sar-D-$Phe^7$-des-$Arg^9$-bradykinin; (h) $ITIH_{4[666-687]}$ (0.9 ng/ml) and (i) $Ile^{13}$-$ITIH_{4[666-678]}$. MS settings were as listed in Table 2.

Another factor concerning the bradykinin quantification is the eventual addition of inhibitors to the clinical samples. As described above, the ACE- and carboxypeptidase-N inhibitors were not added to the clinical samples to prevent interference with the *ex vivo* protease activity, which has been proposed to contribute to differences in peptide expressions between healthy controls and cancer patients [2]. The observation of a more or less stable bradykinin response in human plasma and serum, as well as the difficulty for accurate quantification in spiked human serum samples strengthened the idea that the addition of any kind of inhibitors to clinical samples would not be favorable. However, all issues addressed above, reveal that the effect of the sample handling procedure on potential biomarker analytes requires further attention and emphasizes the necessity of identical treatment of all samples during all stages from collection to analysis. Only similar sample handling would theoretically yield comparable results that can be used as an absolute measure, independent on whether the analytes are generated *in* or *ex vivo*. Future research is therefore required to define or improve the diagnostic values of the measured concentrations. This includes the optimization of the sample handling procedure, either optimizing or inhibiting *ex vivo* activity, for each proteolytic peptide specifically.

Nevertheless, the analyses of clinical serum samples showed the usefulness of the presented assay for future research on the diagnostic and prognostic potential of the peptide analytes which may ultimately result in a biomarker assay for breast cancer.

## 4. Conclusions

An LC–MS/MS method has been developed and validated for the quantification of multiple proteolytically derived peptide fragments with the potential to serve as biomarkers for breast cancer. Bradykinin, $Hyp^3$-bradykinin and des-$Arg^9$-bradykinin can be quantified in the ranges 10–500, 4–200 and 2–100 ng/ml, respectively. Although these concentrations were higher than reported in literature, measured concentrations in serum samples from breast cancer patients were within these ranges and even higher for des-$Arg^9$-bradykinin in some samples. The assay allows simultaneous quantification of $ITIH_{4[666-687]}$ (0.4–10 ng/ml) and $C4a_{[1337-1350]}$ (1–25 ng/ml) with high sensitivity required to quantify these peptides in patient samples. The method furthermore enables quantification of a fragment of the fibrinogen-alpha chain (120–3000 ng/ml) which showed relative high concentrations in human serum samples.

These results indicate the applicability of this method to further explore the potential of the peptide analytes as useful biomarkers. Especially the effect of the sample handling procedure and the instability of these proteolytically derived analytes on the robustness of the method deserve further attention. The developed assay will provide the required support for future research on the diagnostic and prognostic potential of the six selected peptide fragments.

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