PAPER IN FOREFRONT

Development of an antibody-free ID-LC MS method for the quantification of procalcitonin in human serum at sub-microgram per liter level using a peptide-based calibration

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Abstract

The quantification of low abundant proteins in complex matrices by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) remains challenging. A measurement procedure based on optimized antibody-free sample preparation and isotope dilution coupled to LC-MS/MS was developed to quantify procalcitonin (PCT) in human serum at sub-microgram per liter level. A combination of sodium deoxycholate-assisted protein precipitation with acetonitrile, solid-phase extraction, and trypsin digestion assisted with Tween-20 enhanced the method sensitivity. Linearity was established through peptide-based calibration curves in the serum matrix (0.092–5.222 μg/L of PCT) with a good linear fit (R2 ≥ 0.999). Quality control materials spiked with known amounts of protein-based standards were used to evaluate the method's accuracy. The bias ranged from −2.6 to +4.3%, and the intra-day and inter-day coefficients of variations (CVs) were below 2.2% for peptide-based quality controls. A well-characterized correction factor was determined and applied to compensate for digestion incompleteness and material loss before the internal standards spike. Results with metrological traceability to the SI units were established using standard peptide of well-characterized purity determined by peptide impurity corrected amino acid analysis. The validated method enables accurate quantification of PCT in human serum at a limit of quantification down to 0.245 μg/L (bias −1.9%, precision 9.1%). The method was successfully applied to serum samples obtained from patients with sepsis. Interestingly, the PCT concentration reported implementing the isotope dilution LC-MS/MS method was twofold lower than the concentration provided by an immunoassay.

Keywords Procalcitonin · Sepsis · Isotope dilution mass spectrometry · Peptide-based calibration

ABC Highlights: authored by *Rising Stars and Top Experts.*

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Introduction

Human serum and plasma represent a rich source of information on physiological and pathological conditions, making quantification of biomarkers in these matrices a powerful tool to assist clinicians in diagnosis, prognosis, and therapeutic monitoring of patients [1]. Most biomarkers are routinely quantified by immunological methods thanks to automation and easy integration into the clinical laboratory [2]. However, immunoassays do not exist for all clinically relevant biomarkers. These assays often reported a lack of reproducibility across different platforms and sometimes a lack of specificity due to the antibodies' cross-reactivity [3]. Liquid chromatography coupled to mass spectrometry is a powerful alternative analytical technique in bioanalysis because of its good

reproducibility and high specificity by eliminating interferences that may impact immunoassays (e.g., cross-reactivity of antibodies) [3]. However, LC-MS requires a high initial investment for instrument acquisition and highly experienced operators, and, for intended use in clinical practice, presents a limited sample throughput compared to immunoassays [3–5]. However, this last point tends to be addressed by developing high-throughput LC-MS methods, particularly on sample preparation steps, to improve LC-MS adoption into routine practice.

For quantifying low abundant proteins present at sub-microgram per liter concentrations in a complex matrix such as serum, LC-MS-based protocols often require significant efforts to reach the required analytical sensitivity. Indeed, an extended dynamic range of serum protein concentration (>10 orders of magnitude) and a high amount of total protein from 22 to 102 mg/mL constitute a major limiting factor of proteomic analysis of serum/plasma [6]. Selective immunoaffinity capture enrichment is frequently implemented to overcome this challenge and extract the biomarker of interest before quantifying at sub-microgram per liter [7]. However, specific antibodies against a target biomarker are not always available, and the development and production of these antibodies take time and can be costly. Moreover, the use of an antibody during the LC-MS sample preparation may not enable quantifying the total form of the biomarker of interest. Indeed, the antibody may not allow capturing the analyte bound to other proteins (e.g., protein-protein interaction) and thus lead to a bias in quantification. Many proteins exist under different isoforms and proteoforms, and very little is known regarding the antibody's selectivity for each proteoform. In the end, what is captured and measured remains very often unknown. On the contrary, the development of an accurate LC-MS quantification method without using immunoaffinity capture enrichment offers the possibility to perform a targeted analysis with the highest selectivity. However, due to the ion suppression phenomenon, more efforts are needed regarding sample preparation with orthogonal protein purification approaches. These approaches are based on proteins of interest's physicochemical characteristics and often consist of protein precipitation or solid-phase extraction. Several studies reported the quantification by LC-MS of low abundant protein biomarkers in serum/plasma without immunoaffinity capture enrichment [8–15].

Procalcitonin (PCT), a 12-kDa protein, is an excellent example of a clinically relevant protein biomarker found at a very low blood concentration. PCT is a biomarker of sepsis present in the blood at a concentration below 0.05 μg/L in healthy conditions, which may rise to 1000 folds in case of bacterial infection [16–18]. A cutoff of 0.5 μg/L for sepsis diagnosis and a cutoff of 0.25 μg/L for antibiotic therapy implementation are used in clinical practice [19, 20]. These cutoff points, traceable to LIA immunoassay, are widely applied for others PCT immunoassays [21]. Thus, PCT testing requires high accuracy of measurements because patient management can be tailored based on these thresholds. PCT was chosen as a case study to illustrate the analytical challenge of developing an antibody-free quantification method for a low abundant biomarker based on the LC-MS technique and highlight the need to optimize the sample preparation step. To our knowledge, there are only two studies reported to determine the PCT concentration in human serum using the LC-MS strategy, with a limit of quantification (LOQ) of 1.0 μg/L [22] and 0.1 μg/L [23]. However, these studies employed selective immunoaffinity capture enrichment against either PCT protein [22] or PCT-related peptides [23]. Therefore, this work aimed to develop an antibody-free ID-LC-MS/MS method that enables sensitive, selective quantification of a low abundant protein in serum matrix with a peptide-based calibration strategy. The performance of this method was evaluated using both peptide- and protein-based quality control samples. Finally, this method was compared to a commercially available immunoassay method, and the potential of the developed LC-MS method to become a candidate reference measurement procedure is discussed.

Materials and methods

Chemicals and reagents

The peptide standards SALESSPADPATLSEDEAR and FHTFPQTAIGVGAPGK and the stable isotope-labeled (SIL) standards SALESSPADPATLSEDEA(^{13}C$_3$,^{15}N)R (> 99.9% purity ^{13}C,^{15}N) and FHTFPQTAIGVGAP(^{13}C$_5$,^{15}N)GK (> 99.9% purity ^{13}C,^{15}N) were purchased from Pepscan Presto (Lelystad, The Netherlands) as powder. Recombinant PCT protein solution was obtained from Promise Advanced Proteomics (Grenoble, France). PCT concentration (807 mg/g) in 20 mM Tris pH 7.6 and 100 mM NaCl buffer was determined in-house with metrological traceability to the SI units by amino acid analysis. HRMS established an impurity profile. Thanks to the hyphenated purification to which the recombinant PCT was subject, no impurity requiring correcting the raw amino acid results was identified.

Dimethyl sulfoxide (DMSO, cat# 1029502500) was purchased from Merck Millipore (MA, USA). Sodium deoxycholate (SDC, cat# D6750-100G), ammonium bicarbonate (cat# 09830-500G), dithiothreitol (cat# 43819-1G), iodoacetamide (cat# I1149-5G), and Tween-20 (cat# P1379-100ML) were obtained from Sigma-Aldrich (St. Quentin Fallavier, France). Acetonitrile (ACN, cat# 0001204102BS), methanol absolute (cat# 0013684102BS), formic acid (cat# 00069141A8BS), and trifluoroacetic acid (cat# 00202341A8BS) (all LC-MS grade) were purchased from Biosolve Chimie (Dieuze, France). Acetic acid (cat# A113-50, LC-MS grade)

was purchased from FisherChemical (Illkirch, France). Water was purified using a Milli-Q system (Millipore, MA, USA). Trypsin Gold (cat# V5280, MS grade) was obtained from Promega (Madison, USA).

The certified reference materials (CRM) of amino acid L-alanine (CRM 6011-a, 99.9 ± 0.2%), L-leucine (CRM 6012-a, 99.9 ± 0.2%), and L-proline (CRM 6016-a, 99.9 ± 0.2%) were obtained from the National Metrology Institute of Japan (NIMJ, Tsukuba, Japan). The $^{13}C^{15}$N-labeled or 13C-labeled amino acids were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA).

Human serum (pool from different male donors, cat# H4522) was purchased from Sigma (St. Quentin Fallavier, France).

Proteotypic peptide's selection

The proteotypic peptides used for the quantification of PCT with a bottom-up approach were selected after in silico tryptic digestion of PCT, LC-MS/MS analysis of a PCT tryptic digest in a buffer, and in serum matrix. The peptide's uniqueness to the PCT protein was determined by performing a blast of the two peptide sequences against a human protein database and bacterial protein database. Fully tryptic peptides, without misscleavage, with a length between 7 and 20 amino acids, devoid of residues prone to artifactual or post-translational modifications, have been selected.

Purity assessment of SALESSPADPATLSEDEAR standard peptide

The purity assessment of the peptide standards used as calibrators was performed with peptide impurity corrected amino acid strategy (PICAA) [24].

The peptidic content of the SALESSPADPATLSEDEAR primary stock solutions was quantified by amino acid analysis using a 5-point calibration curve. The experiment was performed with three processed replicates per day over 3 days. The peptide's primary stock solutions were gravimetrically diluted in water before adding the labeled amino acids in a 0.1 N HCl solution. The gas-phase hydrolysis was performed at 130 °C ± 5 °C for 40 h under vacuum in the presence of 6 N HCl. The final solution was then evaporated to dryness at room temperature in a centrifugal vacuum concentrator, and the resulting samples were reconstituted with 100 μL of water. The amino acids produced were separated using a Waters BEH column (C18, 150 × 2.1 mm, 1.7 μm) on isocratic mode combining 2% of phase B (0.1% formic acid in acetonitrile) and 98% of phase A (0.1% formic acid in water). Amino acids were then detected on a QExactive Focus high-resolution mass spectrometer (Thermo Scientific, Waltham, MA, USA) using selected ion monitoring mode. Three amino acids (Ala, Leu, and Pro) were used for quantification.

The SALESSPADPATLSEDEAR primary stock solution was analyzed by LC-HRMS and LC-HRMS/MS to identify and quantify peptidic impurities that could compromise the amino acid analysis trueness. Analyses were acquired on a Thermo Scientific™ Q Exactive™ Focus hybrid Quadrupole-Orbitrap mass spectrometer coupled with a Thermo Scientific™ Dionex™ Ultimate 3000 ultra-performance liquid chromatography system (Thermo Scientific, Waltham, MA, USA) in electrospray ionization mode. The SALESSPADPATLSEDEAR peptide and its impurities were separated on a Pepmap column (C18, 250 × 1 mm, 5 μm) (Thermo Scientific, Waltham, MA, USA). The mobile solvents consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The separation was achieved using a linear gradient (from 2 to 35% of solvent B over 15 min, and from 35 to 60% of solvent B over 15 min) at a 100 μL/min flow rate.

Each amino acid's concentration obtained by amino acid analysis was then corrected by each impurity's contribution containing the concerned amino acid (see Supplementary Information, ESM). The final concentration of SALESSPADPATLSEDEAR peptide in the primary stock solution was obtained by averaging the corrected concentrations of the three quantified amino acids alanine, leucine, and proline.

Preparation of calibrators and internal quality materials

All steps of preparation of solution were performed gravimetrically using calibrated precision balances. Therefore, the concentration of peptide solutions was expressed in molar per mass of solution to avoid implication of solution density and each peptide's molecular weight. PCT concentration was provided in micrograms per liter as expressed in clinical use.

Stock solutions and working solutions

Individual primary stock solutions were prepared in DMSO for SALESSPADPATLSEDEAR and SALESSPADPATLSEDEA($^{13}C_3$,^{15}N)R peptides and in 50% (v/v) DMSO in water for FHTFPQTAIGVGAPGK and FHTFPQTAIGVGAP($^{13}C_5$,^{15}N)GK peptides. Solutions were aliquoted and stored at −80 °C ± 10 °C.

The individual primary stock solutions of peptides and SIL peptides were diluted with 10% (v/v) ACN and 0.001% (v/v) Tween-20 in water to obtain individual secondary stock solutions of unlabeled and labeled peptides at 6660 pmol/g (corresponding to a PCT concentration of 85,100 μg/L).

A mixed solution of the two unlabeled peptides at 10.8 pmol/g (corresponding to a PCT concentration of 138 μg/L) was further prepared by mixing their individual secondary solutions in 10% (v/v) ACN and 0.001% (v/v)

Tween-20. A mixed solution of the two SIL peptides at 4.1 nmol/g was further prepared by mixing the individual secondary solutions of SIL peptides in 10% (v/v) ACN and 0.001% (v/v) Tween-20.

A working solution of recombinant PCT at 6.8 μg/L was prepared by diluting in series the recombinant PCT stock solution at 807 mg/L in human serum to preserve its stability and prevent any loss from adsorption.

Peptide calibrators and peptide quality control materials

Peptide calibrators and peptide quality control (QC) material consisted of processed and digested serum samples obtained from healthy subjects (see "Sample preparation procedure") spiked with calibration/QC solutions prepared as followed. Calibration solutions were prepared by mixing an increasing amount of unlabeled peptides mixed solution with a constant amount of SIL peptide mixed solution with a mass ratio of peptides ranging from 0.06 to 2.65 (peptide concentration from 0.007 to 0.4 pmol/g, corresponding to a PCT concentration from 0.092 to 5.222 μg/L). Peptide calibrators were used to establish the linear regression. Two QC solutions were prepared by mixing different amounts of unlabeled peptide mixed solution with a constant amount of SIL peptide mixed solutions to reach a quantity ratio of 0.52 and 1.92 (peptide concentration of 0.08 pmol/g and 0.3 pmol/g, corresponding to a PCT concentration of 1.020 and 3.876 μg/L, respectively). The unlabeled peptide solutions used to prepare calibration solutions and QC solutions were prepared from two different aliquots of the individual primary stock solutions. Peptide QC materials were used to determine the measurement accuracy of the peptide-based calibration model and to detect any calibration issues.

Protein-based quality control materials

Protein QC material consisted of human serum obtained from healthy subjects spiked with recombinant PCT solution prepared as followed. Four recombinant PCT solutions were prepared by diluting the working solution of recombinant PCT in human serum at four different concentrations of 0.484 μg/L, 0.912 μg/L, 3.792 μg/L, and 9.213 μg/L. Protein QC materials were used to determine the recovery of the whole sample workflow and the correction factor to apply for the quantification of PCT with the peptide-based calibration model (see "Trueness and precision for the quantification of the PCT"). It thus informs on the trueness and precision of the entire protocol.

Furthermore, eight PCT serum samples were prepared by adding another working solution of recombinant PCT in human serum at 0.245 μg/L. These samples were used in an independent experience to evaluate the method trueness and precision for PCT quantification at a concentration near the clinical cutoff of 0.25 μg/L.

Analytical workflow

Sample preparation procedure

The sample preparation procedure for PCT quantification is described in Fig. 1. Five hundred microliters of human serum obtained from healthy subjects (for calibration calibrators and peptide QC materials) or serum samples (protein-based QC materials and serum-based pooled patient samples) was weighted before adding 500 μL of 1% (w/w) SDC detergent in water. The samples were sonicated for 5 min and then mixed for 10 min at room temperature. The proteins were precipitated by adding 1 mL of acetonitrile. Samples were mixed for 10 min at room temperature and centrifuge at 14,000×g for 5 min at room temperature. The supernatant was diluted with 14 mL of water to reduce the organic fraction and then loaded on an HLB (hydrophilic-lipophilic balance) Oasis 500 mg cartridge (Waters, MA, USA), previously conditioned with 3 mL of methanol and 3 mL of 60% (v/v) acetonitrile in water and equilibrated with 3 mL of water. Loaded samples were washed with 3 mL of water and 1 mL of 10% (v/v) methanol in water. Samples were eluted with 1 mL of 60% (v/v) acetonitrile in water and evaporated to dryness in a centrifugal vacuum at room temperature.

The dried extracts were dissolved in 10 μL of methanol. The proteins were reduced at 37 °C for 1 h by adding 82 μL of 15.3 mM ammonium bicarbonate buffer and 20.8 mM dithiothreitol in water and alkylated at 20 °C for 1 h in the dark under shaking by adding 18 μL of 500 mM iodoacetamide in water. Before digestion, samples were diluted with 92 μL of 94 mM ammonium bicarbonate buffer, 2.4% of acetonitrile, and 0.00024% of Tween-20 in water (v/v/v) to prevent peptide adsorption. Samples were then digested with 6 μL of 770 μg/mL trypsin gold in 50 mM acetic acid solution, at 37 °C for 6 h under shaking before stopping the digestion by adding 2 μL of formic acid.

Twenty microliters of SIL peptides was then gravimetrically added to the tryptic digest of serum samples. Twenty microliters of each calibration solution or peptide QC solution was added to the processed and digested human serum samples to prepare peptide-based calibrators and peptide-based QC materials. All samples were diluted by adding 1 mL of 0.1% (v/v) trifluoroacetic acid in water before SPE on an HLB Oasis 30 mg cartridge (Waters). The cartridge was previously conditioned and equilibrated with 1 mL of acetonitrile 0.1% (v/v) trifluoroacetic and 1 ml of 0.1% (v/v) trifluoroacetic acid in water. Loaded samples were washed with 1 mL of 0.1% (v/v) trifluoroacetic acid and 1 mL of 10% acetonitrile 0.1% trifluoroacetic. Peptides were finally eluted with 1 mL of 20% (v/v) acetonitrile 0.1% (v/v) trifluoroacetic acid. Extracts were
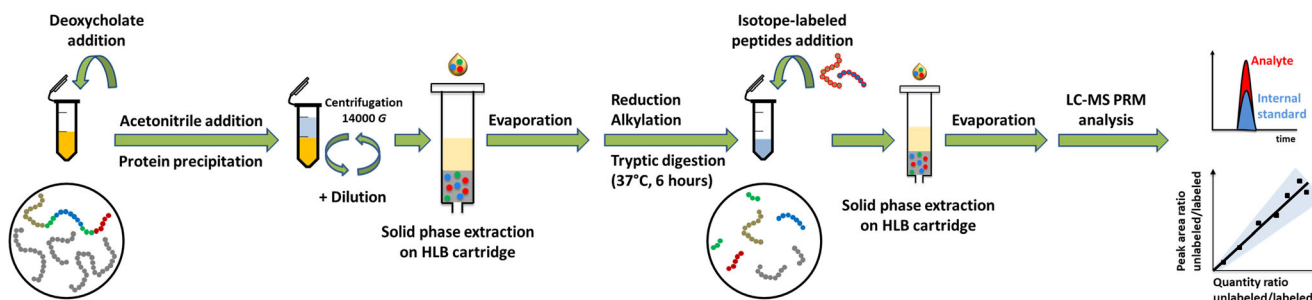
Fig. 1 Sample preparation procedure for the quantification of PCT in human serum at sub-nanogram per milliliter level. Proteins in human serum sample were first denatured using the ionic detergent sodium deoxycholate and precipitated using acetonitrile as precipitating agent. The supernatant was purified on an HLB solid-phase extraction (SPE) cartridge. After evaporation under vacuum, extracted proteins were digested with trypsin. Finally, tryptic peptides were purified on an HLB SPE cartridge, then evaporated to dryness before LC-MS analysis

then evaporated to dryness in a centrifugal vacuum at room temperature and stored at −20 °C until analysis. Prior to LC-MS/MS analysis, the extracts were reconstituted in 100 μL of 2% (v/v) methanol and 0.1% (v/v) formic acid in water.

Each extract was analyzed three times, and the mean of three injections was used to calculate the final concentration. Each serum sample was measured in three processed replicates to evaluate the analytical performance of the developed method, and the comparability of the results between the routine immunoassay Brahms Kryptor Gold and the ID-LC-MS/MS method.

LC-MS/MS conditions

Analyses were acquired on a Thermo Scientific™ Q Exactive™ Focus hybrid Quadrupole-Orbitrap mass spectrometer coupled with a Thermo Scientific™ Dionex™ Ultimate 3000 ultra-performance liquid chromatography system (Thermo Scientific). The mass spectrometer was equipped with an electrospray ion source. After optimization (see ESM Fig. S8), the following conditions were applied: capillary temperature at 250 °C, sheath gas flow rate at 25, aux gas flow rate at 2, spray voltage at 4.2 kV, S-lens RF level at 50, and aux gas heater temperature at 140 °C. After optimization, 20 μL of the peptide extracts was injected on an Acclaim™ PepMap™ 100 C18 analytical column (1 × 150 mm, 3 μm) (Thermo Scientific, Waltham, MA, USA) at a flow rate of 80 μL/min and a temperature of 60 °C. The solvent system consisted of 0.05% (v/v) acetic acid in water (mobile phase A) and 0.05% (v/v) acetic acid in methanol (mobile phase B). Peptides were eluted with the following gradient of mobile phase B: 2% for 2 min, linear from 2 to 22% in 8 min, linear from 22 to 38% in 1 min, linear from 38 to 42% in 14 min, and from 42 to 98% in 1 min.

The selected signature peptides were detected and quantified using parallel reaction monitoring (PRM). Precursor ions (Table 1) were isolated within an isolation window of 1.5 m/z. The collision energy applied is detailed in Table 1. MS2 scans

were collected at a resolution of 35,000 with an automatic gain control target of 2e5, and 1 microscan.

Data analysis

PRM data treatment was achieved with Quanbrowser algorithm of XCalibur 4.1 version software (Thermo Scientific, Waltham, MA, USA). Two transitions per peptide (SALESSPADPATLSEDEAR: $973.4529 \rightarrow 1371.6387$, $973.4529 \rightarrow 1088.5218$; SALESSPADPATLSEDEA($^{13}C_3,^{15}N$)R: $975.4565 \rightarrow 1375.6387$, $975.4565 \rightarrow 1092.5218$; FHTFPQTAIGVGAPGK: $543.2912 \rightarrow 585.3355$, $543.2912 \rightarrow 301.1870$; FHTFPQTAIGVGAP($^{13}C_5,^{15}N$)GK: $545.2958 \rightarrow 591.3493$, $545.2958 \rightarrow 307.2008$) were retrieved from PRM data (Table 1). The final peak areas were obtained by computing the sum of these two transitions. All data were manually checked to ensure correct peak detection and accurate peak integration. Peak detection and area integration were based on the following criteria: the same retention time between unlabeled peptide and SIL peptide (below 0.5% of variation) and the same relative PRM peak area ratios of two transitions between unlabeled peptide and SIL peptide (below 15% of variation).

Evaluation of the method performance for the quantification of PCT in human serum using a peptide-based calibration approach

After defining the calibration curve (linearity), the developed method for quantifying PCT in human serum was evaluated regarding measurement trueness and precision (QC samples) over three independent assays.

1. The calibration model was evaluated according to the correlation coefficient (R2, Pearson) and the residuals' response distribution of the calibration curves. The calibration curves were obtained by plotting the peak area ratio of the unlabeled peptide over its SIL peptide against the corresponding mass ratio of the unlabeled

Table 1 Amino acid sequence, retention time and PRM parameters for the detection of two selected peptides and the SIL peptides homologs

Proteotypic peptides	Retention time (min)	Precursor ions		Collision energy (eV)	Product ions	
		ion	m/z		ion	m/z
SALESSPADPATLSEDEAR	19.1	$[M+2H]^{2+}$	973.4529	35	y13$^+$	1371.6387
					y10$^+$	1088.5218
SALESSPADPATLSEDEA($^{13}C_3$,^{15}N)R	19.1	$[M+2H]^{2+}$	975.4565	35	y13$^+$	1375.6387
					y10$^+$	1092.5218
FHTFPQTAIGVGAPGK	14.9	$[M+3H]^{3+}$	543.2912	20	y7$^+$	585.3355
					y3$^+$	301.1870
FHTFPQTAIGVGAP($^{13}C_5$,^{15}N)GK	14.9	$[M+3H]^{3+}$	545.2958	20	y7$^+$	591.3493
					y3$^+$	307.2008

peptide over its SIL peptide in the freshly prepared calibration solutions as described in Eq. 1.

$$\frac{\text{Peak area of peptide}}{\text{Peak area of SIL–peptide}} = f\left(\frac{\text{Quantity peptide added}}{\text{Quantity SIL–peptide added}}\right) \quad (1)$$

The linear regression was plotted over six calibrator points (corresponding to PCT concentration ranging from 0.09 to 5.12 µg/L) on three different days. Residuals should be randomly distributed around zero. In addition, the peptide quantification of each point of the calibration curve was evaluated by considering the calibration samples as unknown samples. The bias was assessed by calculating a difference between the amount of peptide measured and the gravimetric SI-traceable amount added.

2. Trueness and precision of the peptide quantification were investigated in intermediate precision conditions of measurement by quantifying each peptide in peptide-based QC samples at two different concentrations. Trueness was evaluated by the bias between the measured concentration and the theoretical concentration determined gravimetrically (Eq. 2). Precision was calculated as the coefficient of variation (CV %) of the concentration of the measured peptides within a single run (repeatability, intra-day) and between three assays realized at different days (intermediate precision, inter-day). Bias and precision in repeatability conditions were evaluated by analyzing three peptide-based QC replicates per concentration per day ($N = 3$). Bias and precision in intermediate precision conditions were evaluated by analyzing three peptide-based QC replicates per concentration per day on three different days ($N = 9$). The peptide concentration of each sample was determined using a calibration curve prepared on the same day. The bias and precision of each level of the peptide-based QC sample should be within ±15%,

according to the guideline of bioanalytical method validation from FDA and EMA [25, 26].

$$\text{Bias}(\%) = \frac{C\text{measured} - C\text{theorical}}{C\text{theorical}} \times 100 \quad (2)$$

To assess the trueness and precision of PCT quantification at the protein level, the total recovery and the need to implement a correction factor were evaluated. The total recovery was evaluated by analyzing protein-based QC materials spiked with recombinant PCT over two independent experiments (four different concentrations in three processed replicates). The amount of PCT added was compared with the amount of PCT quantified in these samples using a peptide-based calibration curve (Eq. 3). The correction factor was then calculated by converting the sample preparation total recovery (Eq. 4) and applied to correct the potential quantitative bias occurring during the quantification process (see ESM Fig. S3).

$$\text{Total recovery} = \frac{\text{PCT Concentration quantified}}{\text{PCT Concentration added}} \quad (3)$$

$$\text{Correction factor (Fc)} = \frac{1}{\text{Total recovery}} \quad (4)$$

Method application: quantification of endogenous PCT in serum samples

Three pools of patient serum samples with different PCT concentrations were provided by University Hospital Montpellier (Montpellier, France). Patient blood samples were collected in dry tubes. Each pool was produced by pooling twelve single frozen donations (leftovers) from patients suffering from sepsis or septic shock. After pooling and aliquoting, the three pools were analyzed at the clinical chemistry laboratory of Montpellier Hospital to determine PCT concentration with

Kryptor Gold immunoassay and immediately stored at −80 °C ± 10 °C until analysis. These pools of samples were used to apply the developed method for the quantification of endogenous PCT.

Results

Proteotypic peptides selection

In the bottom-up approach, the identification of a protein of interest is achieved at the peptide level. Therefore, the selected peptides have to be proteotypic peptides (specific to the protein and easily identified in LC-MS). After in silico tryptic digestion of PCT, LC-MS/MS analysis of a PCT tryptic digest in a buffer, and LC-MS/MS analysis of a PCT tryptic digest in serum matrix, two peptides SALESSPADPATLSEDEAR and FHTFPQTAIGVGAPGK could be detected at low concentration and were selected for the detection of PCT. These peptides are located in two different PCT protein regions, which ensures the high specificity of the developed method for full-length PCT (Fig. 2). However, according to PCT's amino acid sequence, the FHTFPQTAIGVGAPGK peptide is followed by a KR dipeptide. The chain containing KKR could potentially affect tryptic proteolysis reproducibility [27–29]. In our peptide-based calibration approach, the SIL peptide could neither correct variations of tryptic digestion completion nor material loss occurring before the digestion. Consequently, FHTFPQTAIGVGAPGK peptide was finally used as a qualitative control to confirm the identification of PCT, while SALESSPADPATLSEDEAR peptide was selected to quantify the PCT protein. For both peptides, the charge state with the highest signal-to-noise ratio was associated with the most sensitive measurements and was selected.

Purity assessment of SALESSPADPATLSEDEAR standard peptide

The SALESSPADPATLSEDEAR peptide was selected to quantify the PCT protein. Therefore, the characterization of the commercial synthetic SALESSPADPATLSEDEAR

peptide standard was performed to ensure the accuracy of the PCT quantification method and its ability to provide results that are traceable to the SI units.

Impurities profiling

The LC-HRMS analyses of the SALESSPADPATLSEDEAR primary stock solution showed a major chromatographic peak corresponding to the monoisotopic mass 1944.8933 Da (CV = 0.0001%, 8 injections), in agreement with the theoretical mass 1944.8912 Da (mass error 1.08 ppm) (Fig. 3a). Furthermore, the identification of the SALESSPADPATLSEDEAR peptide was confirmed by LC-MS/MS analysis (see ESM Fig. S2).

Impurity profiling was performed to identify possible peptide impurities that could affect the amino acid quantification of the primary stock solution and thus compromise the accuracy of PCT quantification results. The identification of each impurity was performed by LC-MS/MS. The identity and extracted ion chromatogram of the main impurities of the SALESSPADPATLSEDEAR reference are presented in Fig. 3b and detailed in ESM Table S2. Twelve impurities were identified, including three isomers of the SALESSPADPATLSEDEAR peptide. The main peptide impurities consisted of forms missing one aspartate or one glutamate residue compared to the full peptide sequence or truncated forms of the correct peptide sequence. Punctual isobaric exchange of leucine to isoleucine at the 3rd and 13th position in the peptide sequence is the most probable explanation for the presence of isomers, considering that pure leucine standards may contain isobaric isoleucine as a major impurity. The isomers' identity cannot be discriminated between the LC peaks as standards were not available for isomers. The mass fraction value for each impurity detected by LC-MS/MS was approximated by assuming the same response factor as the main peptide SALESSPADPATLSEDEAR by LC-HRMS with an uncertainty of 80% for the response factor.

Amino acid analysis

The total amino acid analysis allowed quantifying the peptide content in the primary stock solution using three separate gas-
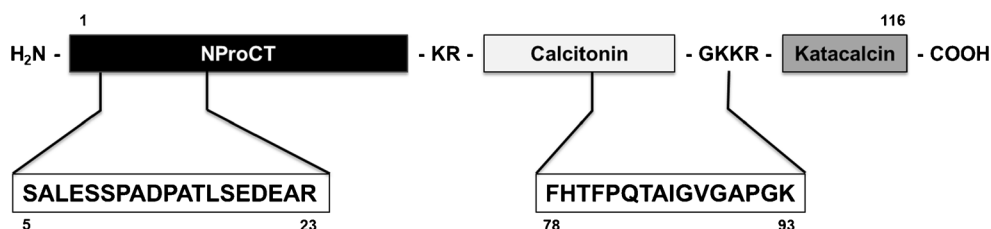
Fig. 2 Schematic presentation of amino acid sequence of PCT. PCT is released in the bloodstream into three forms: NProCT, calcitonin, and katacalcin. SALESSPADPATLSEDEAR peptide was selected to

quantify the PCT protein, while FHTFPQTAIGVGAPGK peptide was used as a qualitative control to confirm the identification of the PCT
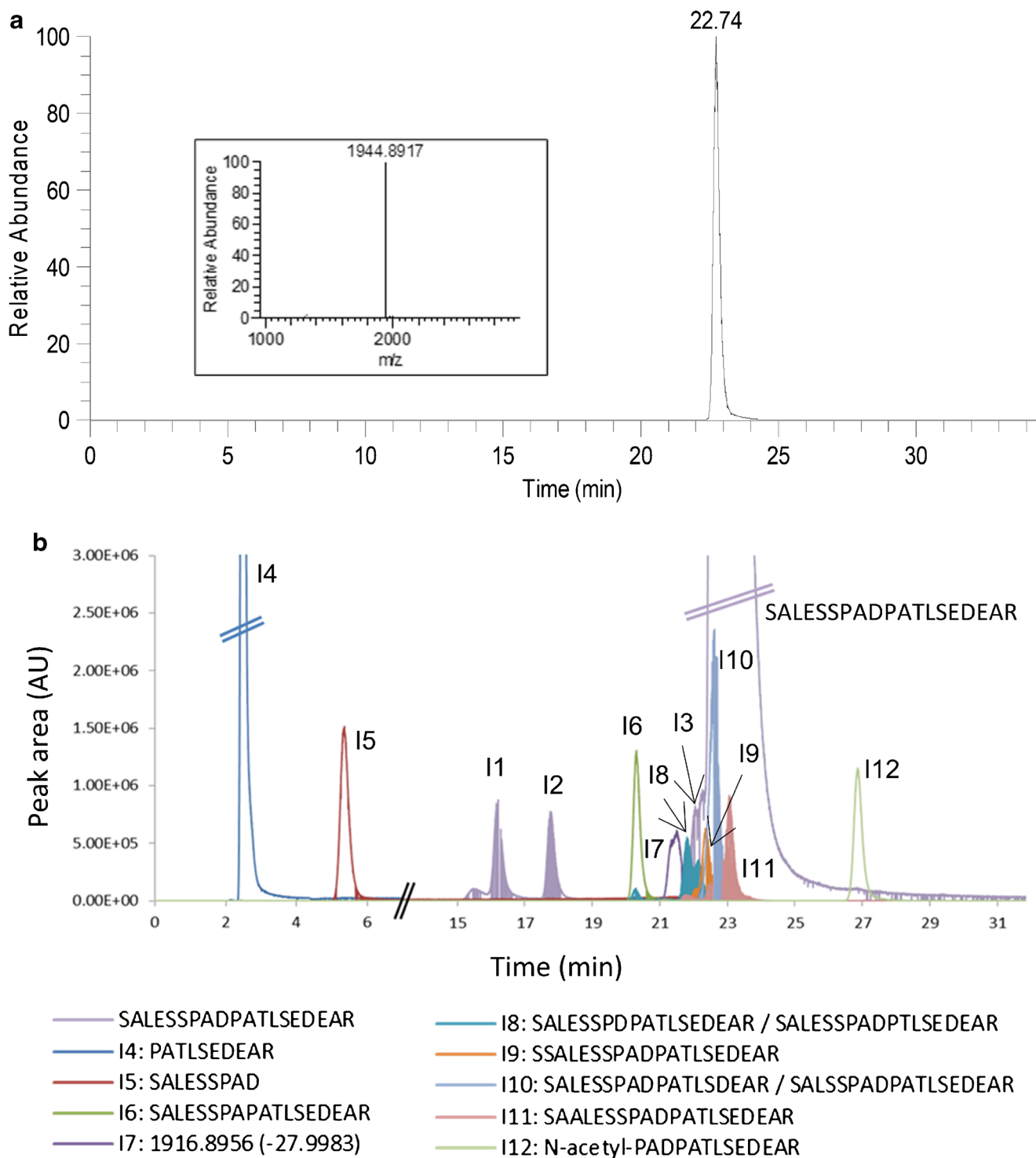
Fig. 3 Purity assessment of the SALESSPADPATLSEDEAR peptide. (**a**) Representative LC-HRMS chromatogram of SALESSPADPATLSEDEAR peptide obtained by injecting 128 ng of the standard peptide. Inset represents deconvoluted spectrum showing a major component with a monoisotopic mass of 1944.8917 Da corresponding to the main SALESSPADPATLSEDEAR peptide. (**b**) Representative overlap of extracted MS chromatogram of SALESSPADPATLSEDEAR peptide and its main impurities in a peptide solution obtained by injecting 128 ng of the standard peptide with the occurrence of isobaric impurities of SALESSPADPATLSEDEAR peptide (impurities I1, I2, and I3). Several artifacts, such as sodium and potassium adducts generated during the ionization process, were excluded from data analysis. AU, arbitrary unit

phase hydrolysis experiments, as detailed in Table S1 (see ESM). Without impurity characterization, the amino acid analysis would overestimate the mass fraction values of the SALESSPADPATLSEDEAR peptide, as impurities also

contain the amino acids used for the amino acid analysis. Therefore, each impurity's contributions were subtracted from the amino acid analysis results (see ESM). The mass fraction value of the SALESSPADPATLSEDEAR peptide and its corresponding expanded uncertainty in the primary stock solution was 1206 ± 39 μg/g (coverage factor $k = 2$). The mass fraction value and the corresponding combined uncertainty of each impurity are summarized in Table S2 (see ESM).

Method optimization

Quantifying a protein at sub-microgram per liter level in a complex matrix is an analytical challenge, particularly without antibody-based enrichment. Therefore, the method optimization should be carried out rigorously for each step of the protocol from sample preparation to LC-MS/MS analysis to reduce any material loss that may occur during the sample preparation, to increase the protein extraction and proteolysis yield, to increase the extraction efficiency of the subsequent tryptic peptides of interest, and to decrease the interferences.

### Sample preparation and tryptic digestion optimization

The first step to be optimized to increase the method's sensitivity is protein extraction from the complex matrix. Protein precipitation or solid-phase extraction are some of the most common techniques to deplete the serum matrix before protein digestion. The extraction and isolation of the protein of interest are improved after protein denaturation. Chemical agents such as urea, thiourea, or guanidine and detergents such as CHAPS (3-[(3-cholamidopropyl) diméthylammonio]-1-propanesulfonate) or SDC are common additives for protein denaturation. However, one of the major limitations of detergent is its negative impact on LC-MS analytical performances. A high concentration of urea/thiourea/guanidine could reduce trypsin activity or introduce chemical modifications during proteolysis (e.g., carbamylation) [30]. Among the detergents, SDC was reported to improve protein denaturation and solubilization, enhancing the number of detected proteins [30–34]. The SDC detergent could be easily eliminated from the sample solution with acidic precipitation.

Moreover, it has been shown that SDC at a concentration below 1% does not compromise the tryptic digestion efficiency compared to other detergents or chaotropic agents such as guanidine [31, 35]. Adding SDC before the sample preparation to improve PCT detection was further investigated to determine the optimal amount of SDC useful for protein denaturation. Figure 4a shows that the addition of SDC ranging from 0.25 to 1.25% in serum samples increases the LC-MS/MS signal of the two targeted PCT peptides by at least 1.7-fold compared to sample preparation conditions without using SDC, with a maximum of 2.8-fold reached with 0.5% SDC

(v/v). This condition was finally selected for protein denaturation.

The broad dynamic range of proteins in serum and the sample matrix complexity were first decreased with a solid-phase extraction (SPE). By differencing proteins according to their physicochemical characteristics, SPE successfully separated the protein of interest and reduced sample complexity to quantify low abundant proteins in a complex matrix [9, 12, 14, 36, 37]. Bronsema and colleagues efficiently employed HLB SPE cartridge to achieve high sensitivity to quantify calcitonin, a proteolyzed PCT product, at picograms per milliliter concentration starting from 500 μL of plasma [9]. Accordingly, after evaluating different SPE phases (see ESM Fig. S5), we integrated the HLB SPE approach in the sample preparation procedure and optimized PCT elution conditions. An additional step of purification was implemented to reduce the complexity of the final solution injected on the LC column. The protein acetonitrile precipitation is a technique to deplete serum proteins, especially high-molecular weight proteins [38, 39]. The partial acetonitrile precipitation allowed achieving a very low LOQ of interferon-gamma-inducible protein-10 at the picomolar level [11]. We compared different acetonitrile contents (30%, 50%, and 70% in water (v/v)) to improve PCT peptide detection and to reduce the complexity of the sample (Fig. 4b and see ESM Fig. S6). Protein precipitation with 50% acetonitrile and 0.5% SDC in water (v/v/v) showed the two PCT peptides' best signals. This result also confirmed the benefit of adding SDC to improve protein denaturation. When precipitation of proteins was performed with 70% (v/v) acetonitrile, most of the proteins precipitated, resulting in lower recovery of PCT peptides in the supernatant. Protein acidic precipitation induced a lower recovery of PCT peptides compared with the other conditions. This was also observed in other studies [39], especially for high-molecular weight proteins where the disruption of the hydration sphere surrounding the proteins is more important in acidic conditions by positively charging the protein. Even if the addition of a protein precipitation step did not show any significant improvement or degradation of the PCT-related peptide signals (Fig. 4b), this step considerably reduced the sample complexity and allowed removing interferences (see ESM Fig. S6). Thus, the protein precipitation step with 50% acetonitrile and 0.5% SDC in water (v/v/v) was included in the sample preparation protocol.

During the sample preparation, nonspecific hydrophobic adsorption of peptides on the microtube surface may occur and affect the quantification method's sensitivity. At the peptide level, several studies reported the ability of Tween-20 to prevent adsorption and to enhance peptide recovery [9, 40–43]. Tween-20 is a non-ionic polysorbate surfactant and a gentle detergent that was not reported to affect trypsin activity [44]. Thus, Tween-20 can be added just before the digestion step to prevent peptide adsorption after their release
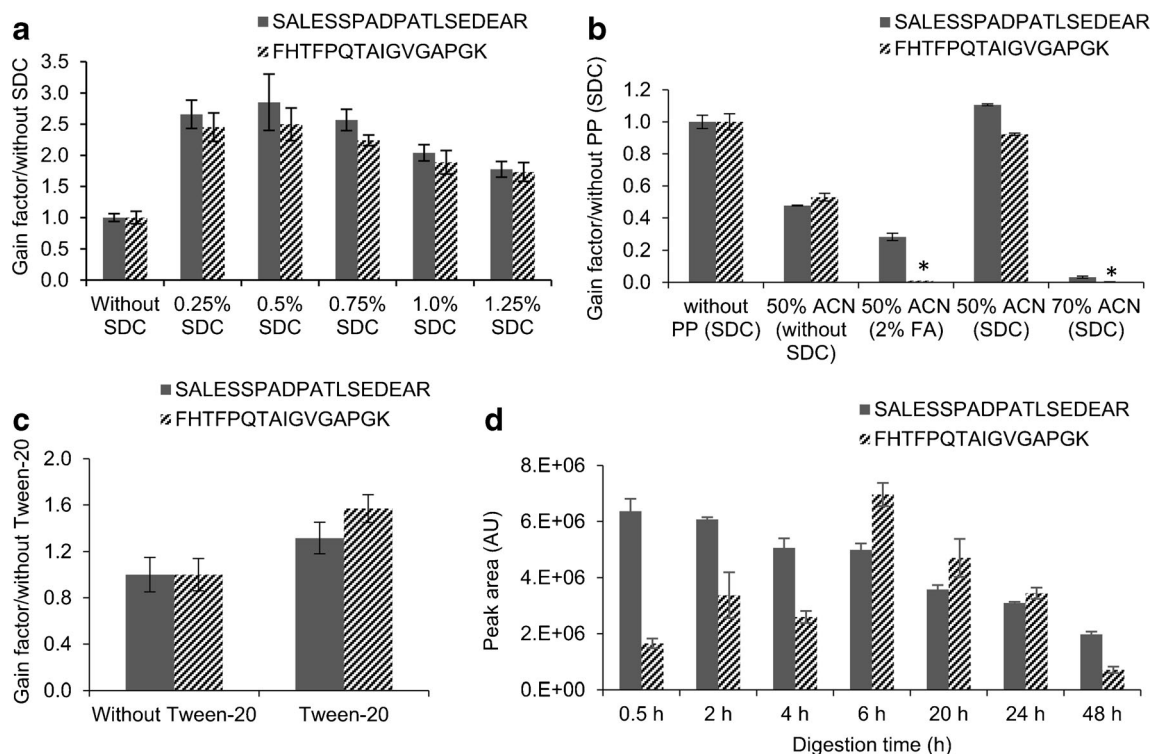
Fig. 4 Protocol optimization for PCT quantification at sub-microgram per liter level. The results were obtained by analyzing two PCT-related peptides by LC-MS/MS in a serum sample spiked with recombinant PCT. (**a**) Optimization of the denaturation step with SDC. Different proportions of SDC ranging from 0.25 to 1.25% were evaluated in two processed replicates ($N = 2$). The results are presented as the signal gain factor (peak area ratio) between the studied condition and the condition without SDC. The experiment was carried out without performing the protein precipitation step. (**b**) Optimization of the protein precipitation (PP) step. Different acetonitrile contents with or without SDC were evaluated in two processed replicates ($N = 2$). The results are presented as the signal gain factor (peak area ratio) between the studied condition and the condition without PP (SDC). Asterisk means that no signal was observed for FHTFPQTAIGVGAPGK peptide. (**c**) Evaluation of the use of Tween-20 to assist the digestion step. The results are presented as the gain factor (peak area ratio) between the studied condition and the condition without using Tween-20, performed in three processed replicates ($N = 3$). (**d**) Kinetics of trypsin digestion from 500 μL of human serum spiked with recombinant PCT. Peak areas of the 2 PCT proteotypic peptides are reported for different digestion times (0.5 h, 2 h, 4 h, 6 h, 20 h, 24 h, and 48 h). For each digestion time, the experiments were carried out once using the final sample preparation protocol in which only the digestion time varied. Samples were analyzed three times. AU, arbitrary unit

without affecting trypsin efficiency. We evaluated the effect of Tween-20 on PCT-related peptides signals. Results are presented in Fig. 4c and show concordance with the literature. The addition of Tween-20 at a final proportion of 0.0001% before digestion increased the PCT peptide signals by 1.3-fold for SALESSPADPATLSEDEAR peptide and 1.6-fold for FHTFPQTAIGVGAPGK peptide, compared to the condition without Tween-20. This increase was more important for the FHTFPQTAIGVGAPGK peptide, as this peptide is less hydrophilic (13% of hydrophilic residues) than the SALESSPADPATLSEDEAR peptide (53% of hydrophilic residues). Thus, the addition of Tween-20 improves the peptide solubility and, thus, the recovery of these peptides. The digestion in the presence of this surfactant was retained for the quantification of PCT.

To accurately quantify a given protein in serum using a peptide-based calibration, complete digestion of the protein resulting in a 1:1 molar ratio between the resulting targeted proteotypic peptides and the original protein amounts is essential.

Indeed, incomplete digestion will result in a negative bias between the actual amounts of the protein and the amounts of proteotypic peptides [33]. Thus, we evaluated the kinetics of the digestion of PCT in human serum. The results showed a maximum signal after 0.5 h for the SALESSPADPATLSEDEAR peptide and 6 h for the FHTFPQTAIGVGAPGK peptide (Fig. 4d). The fact that optimal digestion times are not the same for the two peptides could result from the -KKR- residue chain, with three consecutive tryptic sites at the C-terminal side of the FHTFPQTAIGVGAPGK peptide. A compromise between optimal conditions for the two peptides has to be made. Even though PCT concentration was determined by quantifying the SALESSPADPATLSEDEAR peptide, the FHTFPQTAIGVGAPGK peptide was also monitored as a qualitative control to increase the specificity of the quantification. The lower sensitivity of the FHTFPQTAIGVGAPGK peptide LC-MS/MS analysis due to its low solubility and the potential miss cleavage sites tend to favor the selection of digestion conditions optimal for this

peptide. However, the SALESSSPADPATLSEDEAR peptide intensity was 22% lower with a digestion time of 6 h compared with 0.5 h, explained by peptide degradation. A digestion time of 6 h was selected to enable using the FHTFPQTAIGVGAPGK peptide as a qualitative control since it is required to identify the PCT intact form in human serum. To correct the negative bias in the quantification of SALESSSPADPATLSEDEAR and also to take into account any material loss that could occur during sample preparation steps taking place before the addition of the SIL internal standard (e.g., protein precipitation by ACN, first SPE, peptide degradation), a correction factor was applied, as described hereafter.

LC optimization

The high complexity of the matrix and high dynamic range of protein concentration in serum may induce signal suppression due to the coelution of different peptides during LC-MS/MS analysis. Therefore, we need to resolve the peptides of interest and the proteolytic peptides from other proteins as much as possible. We optimized the LC gradient to improve peptide separation and decrease ionization suppression in the MS source (see ESM Fig. S7). Moreover, for the quantification of PCT, attention was also paid to the effect of different organic solvents and mobile phase additives on the MS response. Even if acetonitrile is the most commonly used organic solvent for peptide analysis by LC-MS, in our study, methanol allowed obtaining a better signal-to-noise ratio, with a sensitivity gain of up to 1.5-fold improvement (Fig. 5a). This observation was also previously reported [45, 46] and could be explained by the high volatility of methanol compared to acetonitrile, facilitating the ion desolvation in the ESI source. In addition, we compared different concentrations of formic acid and acetic acid. The results presented in Fig. 5b indicate that 0.05% (v/v) acetic acid content is the best compromise for detecting the two PCT-related peptides. This observation is in concordance with the literature, in which a low percentage of a weak acid was preferred with methanol [46]. Acetic acid, a weaker acid than formic acid (pKa 4.76 vs. 3.75), contributes to increasing peptide signals. A lower percentage of acetic acid (0.01%) limited the ionization efficiency of SALESSSPADPATLSEDEAR peptide, which could not be detected at all anymore, while the signal of FHTFPQTAIGVGAPGK peptide reached its highest intensity. Indeed at the pH of mobile phases (pH = 4.6), the SALESSSPADPATLSEDEAR peptide (pH$_I$ = 3.3) is mostly negatively charged, while the FHTFPQTAIGVGAPGK peptide is present mainly in its positive forms (pH$_I$ = 10.0).

Moreover, MS conditions were also optimized in terms of MS source parameters and collision energy. The detailed data in MS optimization are presented in Fig. S8 (see ESM). The MS/MS spectra of the two selected peptides and their SIL standard peptide are shown in Fig. S9 (see ESM).

Evaluation of the method performance for the quantification of PCT in human serum using peptide-based calibration approach

Linearity

The linearity of the 6-point peptide-based calibration curve (corresponding PCT concentrations ranging between 0.092 and 5.222 μg/L) was established, with a regression coefficient above 0.9993. The average slope from three independent experiments was 0.9966 (intermediate precision = 0.5%). Residuals were randomly distributed around zero. The bias from the back-calculated concentrations of calibration samples ranged from −8.0 to +3.9% (Fig. 6). The detailed data are summarized in Table S3 (see ESM). The lowest concentration of the calibration range was 0.092 μg/L to cover any PCT or PCT-related peptide loss through the quantification process of PCT samples at 0.245 μg/L, corresponding to a clinical cutoff value.

Trueness and precision for the quantification of the peptide in processed matrix

The trueness and precision were evaluated in intermediate precision conditions of measurement of peptide-based QC samples at two different concentrations (corresponding PCT concentration of 1.020 and 3.876 μg/L) in three processed replicates over three independent days. The results showed satisfying trueness and precision for the peptide-based QC samples. The intra-day bias and precision ranged from −2.6 to 4.3% and 0.4 to 1.5%. The inter-day bias and precision ranged from 1.1 to 2.6% and within ±2.2%, respectively. The data are detailed in Tables S4 and S5 (see ESM).

Trueness and precision for the quantification of the PCT

The trueness and precision were evaluated by analyzing PCT protein-based QC materials at four different concentrations (0.484, 0.912, 3.792, and 9.213 μg/L) in three processed replicates over two independent experiments. The results showed a satisfying precision. The intra-day precision ranged from 4.5 to 14.7%. The inter-day precision ranged from 9.7 to 11.2%. One sample was excluded on day 2 due to material loss during sample handling. Evaluation of the trueness shows a limited recovery of PCT. The mean total recovery from all replicates and all concentration levels of the optimized method was 38.1% (%CV = 8.0%) and 39.5% (%CV = 12.3%) for day 1 and day 2, respectively (Table 2). The mean total recovery from the two independent days was 38.8% (%CV = 10.2%).
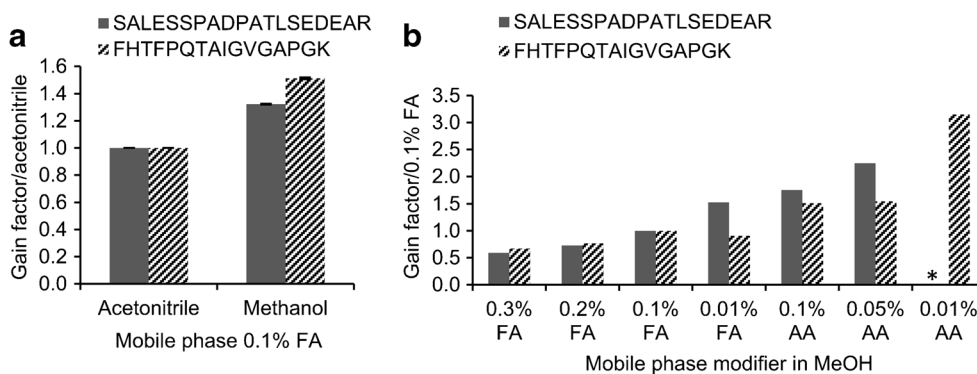
Fig. 5 Optimization of LC mobile phases. The results were obtained by analyzing PCT-related peptides in a serum sample spiked with recombinant PCT using LC-MS/MS. (**a**) Comparison between 0.1% FA in ACN and 0.1% FA in MeOH as organic mobile phase. The results are presented as the gain factor (peak area ratio) between the studied condition and the condition using acetonitrile taken as arbitrary reference. The experiment was performed in three processed replicates of serum samples ($N = 3$).

The relative standard bias was below 1.0%. (**b**) Comparison of different contents of formic acid (FA) or acetic acid (AA) in the mobile phase (% (v/v) in MeOH). The results are presented as the gain factor (peak area ratio) between the studied condition and the condition using 0.1% FA in MeOH. *At 0.01% AA in MeOH, no peptide signal was detected for SALESSPADPATLSEDEAR, because this peptide was mostly in anionic form

Evaluation of the use of a correction factor for the quantification of PCT

The previous data show that even if the sample preparation process was rigorously optimized, the material loss remains high during different steps before SIL peptide addition. These include protein precipitation, SPE protein extraction, and tryptic digestion. Interestingly, the negative bias of 22% due to sub-optimal digestion conditions for the SALESSPADPATLSEDEAR peptide (6 h digestion time) only accounted for approximately one-third of recovery incompleteness. This demonstrates that other material losses occurring during the sample preparation steps are significant. Thus, a correction factor is required in any case, even with a digestion time of 0.5 h. Although the peptide-based calibration approach is widely applied

thanks to its ease of use and reduced cost [47–52], our results agree with a significant quantitative bias using this approach, as reported in other studies [53–55].

Although a correction factor allows taking into account material losses during the sample preparation, a prerequisite is that the correction factor should be robust enough and applicable to PCT quantification in any unknown sample over time. The reported mean total recovery was reproducible with a mean coefficient of variation of 10.4% through two independent experiments with four PCT concentration level samples processed in triplicate. These results support that a correction factor can successfully be applied to accurately quantify PCT in human serum with the peptide-based calibration approach. Therefore, a correction factor of 2.61 was used to correct the experimental bias.
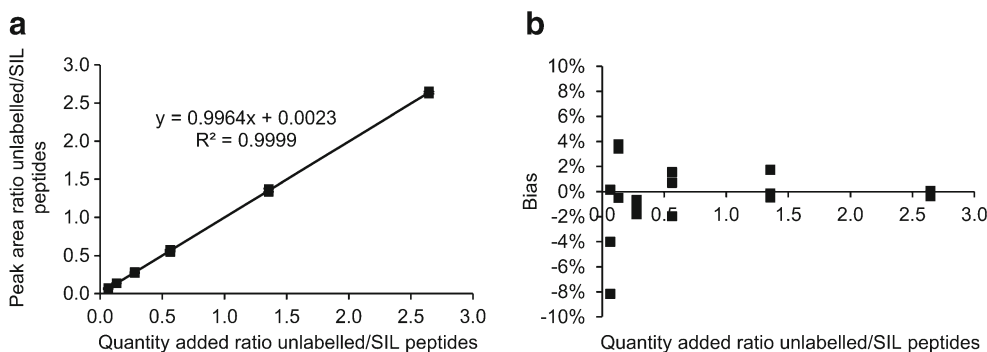
Fig. 6 Six-point peptide-based calibration curve. (**a**) A linear relationship over the PCT concentration range of 0.092–5.222 µg/L (2.5 to 125 fmol on-column) established by spiking unlabeled peptide and SIL peptide (H) in the serum digest for quantifying PCT. (**b**) The bias observed between the back-calculated concentration and the theoretical concentration at each concentration level. Points on the calibration curve are the values

obtained from three independent experiments. The relative standard bias of each calibration point obtained from three independent experiments was below 10.1%. The linear regression's intercept was not significantly different from zero (P-value 0.509, rejecting hypothesis intercept different from zero)

Table 2 Determination of method's total recovery and the correction factor used to compensate for digestion incompleteness and material loss occurring before the addition of the internal standard using protein-based QC materials

	Theoretical spiked PCT concentration (µg/L)	Measured PCT concentration from SALESSPADPATLSEDEAR peptide quantification					
		Day 1			Day 2		
		Concentration (µg/L)	Total recovery (%)	CV (%)	Concentration (µg/L)	Total recovery (%)	CV (%)
QC0	0.48	0.19	39.0	9.3	0.21	44.4	13.2
QC0	0.48	0.18	36.4		0.17	34.9	
QC0	0.48	0.21	43.4		0.18	36.4	
QC1	0.91	0.32	34.7	9.2	0.36	39.4	14.7
QC1	0.91	0.37	40.8		0.37	40.2	
QC1	0.91	0.37	40.9		0.28	30.5	
QC2	3.79	1.40	37.0	4.5	1.76	46.5	38.3
QC2	3.79	1.52	40.0		NA	NA	
QC2	3.79	1.52	40.2		1.01*	26.7	
QC3	9.21	3.04	33.0	5.3	3.91	42.5	8.0
QC3	9.21	3.28	35.6		3.98	43.2	
QC3	9.21	3.37	36.5		3.43	37.2	
Mean total recovery (%)			38.1			39.5	
CV (%)			8.0			12.3	
Correction factor			2.64			2.57	
SD			0.21			0.34	
CV (%)			8.1			13.2	
Final correction factor	2.61						
CV (%)	10.4						

*Samples excluded due to the material loss during sample handling

The proposed method using a correction factor was finally applied in an independent experiment to quantify PCT spiked in serum samples at 0.245 µg/L, a clinical decision cutoff for diagnosis and antibiotic therapy monitoring in sepsis and respiratory tract infection. The correction factor of 2.61 was applied to determine PCT concentration in the spiked samples at 0.245 µg/L in eight processed replicates. The bias and repeatability were −2.0% and 9.1%, respectively, which were lower than 20% as recommended by the guideline of bioanalytical method validation from the FDA and EMA for quantification at the lowest concentration (Table 3) [25, 26].

Fig. S10 shows PRM chromatograms of the two products of the SALESSPADPATLSEDEAR peptide and the FHTFPQTAIGVGAPGK peptide and their SIL peptides by analyzing a pool of human serum obtained from healthy subjects and spiked with recombinant PCT at a concentration of 0.245 µg/L, corresponding to 2.5 fmol of each peptide injected on-column. No signal interfering with the signal of the peptides of interest or their SIL peptides in human serum from healthy subjects could be detected. The coelution and same relative PRM peak area ratios across transitions between FHTFPQTAIGVGAPGK and its SIL peptide confirmed the detection of PCT protein at 0.245 µg/L level.

Method application: quantification of endogenous PCT in serum samples

The developed method was applied to analyze three patient sample pools to evaluate its suitability to measure endogenous PCT in unspiked patient samples. The results obtained by the ID-LC-MS/MS and the results obtained with the Kryptor Gold immunoassay are presented in Table 4. The precision, determined in intermediate precision conditions of measurement, for each patient pool sample was less than 7.1% ($n = 3$), which is in concordance with the previous precision results.

The results obtained by the commercially available immunoassay Kryptor Gold were almost twofold higher than those obtained by ID-LC-MS/MS (average relative difference of −54.3%, $n = 9$, CV = 6.35%). It should be noticed that the difference between the immunoassay and the ID-LC-MS/MS method was consistent across the three materials that were measured with both methods, and an estimated coefficient of

Table 3 Detailed data of PCT quantification at 0.245 μg/L in serum samples spiked with recombinant PCT using a peptide-based calibration curve with a correction factor

	Theorical PCT concentration (μg/L)	Measured PCT concentration (μg/L)	Bias %	CV %
	0.245	0.251	2.6	9.1
		0.211	−13.9	
		0.263	7.5	
		0.225	−9.2	
		0.244	−0.5	
		0.213	−12.9	
		0.245	0.2	
		0.269	9.8	
Overall ($N=8$)	0.245	0.240	−1.9	

correlation of 0.996 was observed over only three concentration levels. This observed relative difference could first be explained by the difference of calibration system between the ID-LC-MS/MS method, and the compared immunoassay may have an impact. As both methods were not calibrated using the same calibrator(s), differences in the method used to determine the calibrators' concentration could induce a proportional bias between the two methods. Secondly, a lack of specificity of the immunoassay compared to the LC-MS technique in the presence of interferences from nonspecific binding of antibodies to compounds having a sequence close to PCT, while the ID-LC-MS/MS method exclusively measures the proteotypic peptides from PCT. However, such interferences are more related to random measurement error and may not explain a correlated systematic bias as the level of interference is unlikely to be proportional to the analyte amount. However, in our study, the data were obtained from a restricted number of clinical samples, which may not represent the global situation over the whole PCT concentration range. A more extensive comparative study with a larger sample size, ideally individual samples, should be performed to confirm this observation.

The large bias observed between ID-LC-MS/MS and immunoassays suggests that the same clinical decision limits cannot be used for both techniques. As it may be confusing for the medical community, this should be carefully considered and evaluated accurately.

Discussion

To our knowledge, only two analytical methods are described for the PCT quantification by LC-MS. Both involved immunocapture in sample preparation [22, 23]. The current work proposed a method based on a thorough optimization of classical sample preparation techniques without using any antibody to quantify PCT at sub-microgram per liter level in human serum by μLC-MS. With a sample uptake of 500 μL of serum, a LOQ of 0.245 μg/L (about 20 pM) was achieved. This concentration is lower than the lower LOQ of 1.0 μg/L reported by Krastin et al., in which 1 mL of serum/plasma sample was subject to an immunocapture enrichment at the protein level and the quantification involved a microflow LC system coupled with a triple quadrupole mass spectrometer.

Table 4 Comparison between a commercially available immunoassay and the ID-LC-MS/MS method for three pools of human serum samples from patients suffering from sepsis

	PCT concentration from the Kryptor Gold immunoassay measurements (μg/L)	PCT concentration from ID-LC-MS/MS measurements (μg/L)	CV (%)	Relative difference between ID-LC-MS/MS and immunoassay (%)
Pool 1	2.93	1.31	7.1	−55.3
	2.93	1.37		−53.3
	2.93	1.50		−48.8
Pool 2	6.48	2.79	7.1	−57.0
	6.48	3.07		−52.6
	6.48	3.21		−50.5
Pool 3	18.3	7.44	5.9	−59.3
	18.3	7.71		−57.8
	18.3	8.34		−54.4

Relative difference = (ID-LC-MS/MS − immunoassay)/immunoassay × 100%

Recently, Dubois reported an analytical method to quantify PCT in serum with a LOQ of 0.1 μg/L starting from 100 μL of serum sample. In this work, the peptide separation was achieved on a capillary liquid chromatographic column of low diameter (300 μm) after immunoaffinity capture enrichment at the peptide level. Capillary flow LC or nanoflow LC systems allow obtaining low LOQ [12, 42, 56–58]. Indeed, the lower the chromatographic column's sectional area, the more concentrated the analyte and the higher the signal-to-noise ratio after electrospray ionization efficiency. However, nanoflow or capillary flow LC could often suffer from perfectible reproducibility of results when analyzing low abundant compounds in highly complex samples due to chromatographic overloading and the lack of stability of the ESI spray over time [59].

Moreover, the proposed method allows performing a targeted analysis with high robustness and specificity. Using antibodies with high specificity, modifications of the PCT sequence or side chains could affect the recognition between the antibody and the epitope, resulting in partial coverage of the total PCT content. Some forms of PCT are not recovered due to a potential antibody specificity restricted to a yet uncharacterized subset of PCT forms. It is expected that less specific techniques will reduce this phenomenon, but determining which forms of PCT are actually captured by antibodies or extracted by SPE is challenging. Although SPE used in the present method may not capture 100% of all isoforms of a protein, SPE is considered less specific than immunoprecipitation. It is therefore expected that loss of protein modified forms, if any, will be less important than by immunoprecipitation. It is also expected that differences in specificity of the antibodies that could be caused by lot to lot variations will mitigate the risk that the same isoforms of PCT are not extracted if different lots of antibodies are used in the long run [60].

However, this work pointed out some limitations that may affect the application of this method. First, the specific sample preparation workflow involving numerous steps and the complete workflow duration compromises applying the method in clinical care where a rapid turnaround is needed to diagnose sepsis and monitor patients with antibiotic treatment.

From a metrological point of view, the need for reliable and comparable PCT measurement results is crucial for this clinically relevant protein biomarker [61]. There is currently no higher-order reference measurement procedure for the SI-traceable quantification of PCT in human serum. While the proposed method allows establishing metrological traceability of results to the SI units, the method was shown to have important limitations that compromise its use as a reference method.

In the proposed method, the quantification of PCT in human serum was performed using a peptide-based calibration. This strategy was described to be a suitable approach for absolute quantification of low abundant protein biomarkers and for medium/high abundance proteins with complex sample preparation [50, 62, 63]. However, in the present study, the quantification of protein-based QC materials (recombinant protein spiked in serum) evidenced the necessity to use a correction factor to quantify PCT in serum samples accurately. Even though the protein extraction recovery and tryptic digestion efficiency appear to be reproducible, the correction factor could be subject to some variations, e.g., in cases where digestion did not perform as usual and/or change reagents required in key steps of the sample preparation. Thus, the correction factor's validity should be monitored over time and between labs to confirm its robustness. In the perspective of a reference method for PCT, the use of such a correction factor appears as highly questionable, and a protein-based calibration should be preferred to a peptide-based calibration.

The specificity of the quantification with a peptide-based calibration approach relies on the SALESSPADPATLSEDEAR peptide because results based on the FHTFPQTAIGVGAPGK peptide appear to be subject to variations of tryptic digestion completion. In healthy conditions, PCT is released in the bloodstream into three cleavage products: the N-terminal peptide NProCT (57 amino acids), the calcitonin (32 amino acids), and katacalcin (21 amino acids) [64]. In bacterial infectious conditions, PCT is released in the bloodstream into three forms: PCT (1-116), PCT (2-116), and PCT (3-116). The SALESSPADPATLSEDEAR peptide is located in the NProCT part of the sequence and the FHTFPQTAIGVGAPGK in the calcitonin-KKR-katacalcin part of the sequence (Fig. 2). Thus, the SALESSPADPATLSEDEAR peptide alone does not allow discriminating between full-length PCT forms and NProPCT, whereas using FHTFPQTAIGVGAPGK allows distinguishing between full-length PCT forms and calcitonin and katacalcin. Quantifying both FHTFPQTAIGVGAPGK and SALESSPADPATLSEDEAR peptides increases the specificity of the developed method for tPCT.

Despite these limitations, the presented work is still of interest. It presents a hyphenated sample preparation procedure and optimized LC-MS conditions allowing the quantification of PCT at sub-microgram per liter concentrations. To develop a candidate reference measurement procedure, the same sample preparation procedure could be applied to an ID-LC-MS/MS method using a protein-based calibration with a SI-traceable recombinant protein primary calibrator and a SIL-protein as an internal standard should be investigated. A recombinant protein and its labeled analog would overcome all material loss and variation occurring during the whole workflow, particularly during the protein digestion step.

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Conclusion

We developed a sensitive LC-MS/MS method to quantify PCT protein in human serum at a sub-microgram per liter level without using immunoaffinity capture enrichment. The method allowed the quantification of PCT concentration with a LOQ of 0.245 μg/L in human serum. PCT quantification was performed using a peptide-based calibration and a correction factor to overcome the bias caused by material loss occurring at the protein level (protein purification, tryptic digestion, protein/peptide adsorption, or degradation). The developed procedure was successfully applied to the quantification of endogenous PCT in three pools of clinical samples. This work established an alternative methodology to immunoaffinity capture enrichment and nano/capillary LC system for protein quantification at a sub-microgram per liter concentration in complex matrices.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00216-021-03361-0.

Abbreviations *AAA,* Amino acid analysis; *ACN,* Acetonitrile; *CRM,* Certified reference material; *CV,* Coefficient of variation; *DMSO,* Dimethyl sulfoxide; *ID-LC-MS/MS,* Isotope dilution liquid chromatography tandem mass spectrometry; *HLB,* Hydrophilic-lipophilic balance; *HRMS,* High-resolution mass spectrometry; *LOQ,* Limit of quantification; *PCT,* Procalcitonin; *PICAA,* Peptide impurity corrected amino acid strategy; *PRM,* Parallel reaction monitoring; *QC,* Quality control; *SDC,* Sodium deoxycholate; *SIL,* Stable isotope labeled; *SPE,* Solid-phase extraction

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Declarations

Conflict of interest The authors declare no competing interests.

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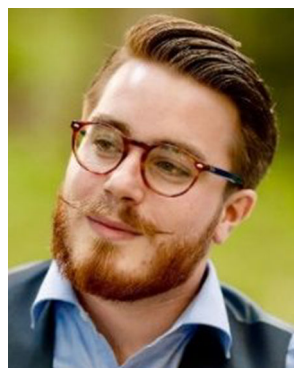
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