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Short communication

# Plasma PCSK9 measurement by liquid chromatography–Tandem mass spectrometry and comparison with conventional ELISA

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ABSTRACT

The combination of liquid chromatography-tandem mass spectrometry (LC–MS/MS) and trypsin proteolysis is an effective tool for accurate quantitation of multiple proteins in a single run. However, expensive samples pre-treatment as immunoenrichment are often required to analyze low abundant proteins. Plasma proprotein convertase subtilisin/kexin type 9 (PCSK9), a circulating regulator of low-density lipoprotein metabolism, was studied as an example of a low abundant plasma protein. We investigated post-proteolysis solid-phase extraction (SPE) as an alternative strategy to improve its detection. After optimization of pretreatment, including denaturation, reduction, alkylation, tryptic digestion and selective SPE concentration, 91 ± 7% of PCSK9 was recovered from human plasma samples and coefficients of variation were less than 13.2% with a lower limit of quantification of 37.5 ng/ml. This LC–MS/MS method was compared with standard enzyme-linked immunosorbent assay in 30 human plasma samples with a broad range of PCSK9 concentrations. Both methods were significantly correlated ($r = 0.936$, $p < 0.001$) with less than 7% of the values out of the 95% confidence interval and similar concentrations were measured using either LC–MS/MS or ELISA methods (514.2 ± 217.2 vs. 504.2 ± 231.0 ng/ml, respectively-p = NS). This method involving SPE is an effective measurement tool for low abundant plasma protein analysis that could be easily included in multiplexed assays.



*Abbreviations:* PCSK9, proprotein convertase subtilisin/kexin type 9; rPCSK9, recombinant PCSK9; ELISA, enzyme-linked immunosorbent assay; LDL, low-density lipoprotein; LDL-C, LDL cholesterol; AB, ammonium bicarbonate; DTT, dithiotreitol; IA, iodoacetamide; SDC, sodium deoxycholate; IS, internal standard; SD, standard deviation; CV, coefficient of variation.

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## 1. Introduction

Enzymatic proteolysis of proteins by endopeptidases and subsequent targeted analysis of signature peptides by liquid chromatography-tandem mass spectrometry (LC–MS/MS) has recently shown to be a powerful method to assay plasma proteins [1–3]. Unlike usual immunoassays, LC–MS/MS methods can easily be multiplexed for accurate quantification of several proteins in a single run [3,4]. This method can also provide additional information such as protein polymorphisms [5,6]. In addition, mass spectrometry can be also used for stable isotope enrichment measurements in kinetic studies after administration of a stable labeled precursor [2,4,7]. However, for proteins with low plasma concentrations, a preparation of the samples before proteolysis (e.g. by immunoaffinity concentration or immunodepletion) is required to allow accurate mass spectrometry analysis [8–10]. Although effective, this preparation is expensive, complex, and often time consuming to allow combined analysis of several proteins in single runs.

Here, we describe a simple method based on a post-proteolysis concentration of peptides by solid-phase extraction (SPE). This method was developed and validated on the proprotein convertase subtilisin/kexin type 9(PCSK9), a low abundant plasma protein (normal range ~100–200 ng/ml or 1.3–2.6 nM) known to be a key regulator of low-density lipoprotein [11–13].

## 2. Experimental section

### 2.1. Chemicals and reagents

UPLC/MS-grade acetonitrile, water, methanol and 99% formic acid were purchased from Biosolve (Valkenswaard, Netherlands). Ammonium bicarbonate (AB), dithiothreitol (DTT), iodoacetamide (IA), sodium deoxycholate (SDC), trypsin, ammonium hydroxide (NaOH), trifluoroacetic acid (TFA), and 37% hydrochloric acid (HCl) were obtained from Sigma Aldrich (Saint-Quentin Fallavier, France). Synthetic peptides GTVSGTLIGLEFIR, GTVSGTLIGLEFI-[$^{13}C_6$, $^{15}N_4$]-R and recombinant PCSK9 (rPCSK9) were purchased from Thermo Scientific Biopolymers (Darmstadt, Germany).

### 2.2. Human samples

Plasma samples were obtained from 30 patients (63% males, age 26–60 years) with molecular diagnosis of familial hypercholesterolemia (FH), and on stable chronic lipid lowering treatment, from the SAFEHEART study, selected to cover a broad range of PCSK9 plasma concentrations (183–1170 ng/ml, i.e. 2.4–15.6 nM) assessed with a commercial ELISA (R&D Systems, Lille, France). All patients gave a written informed consent for any study on lipoprotein metabolism.

### 2.3. LC–MS/MS

Peptide candidate (GTVSGTLIGLEFIR) was selected to maximize sensitivity, specificity, and stability [4,6], dissolved and then diluted in 50% acetonitrile containing 0.1% formic acid as recommended by the manufacturer to produce eight standard solutions at 200, 100, 50, 25, 10, 5, 1 and 0.5 nM (PCSK9 equivalent: 15,000-7500-3750-1875-750-375-75-37.5 ng/ml). Labeled GTVSGTLIGLEFI-[$^{13}C_6$, $^{15}N_4$]-R peptide was used as internal standard (IS) and 25 μl of IS solution (20 μM in 50% acetonitrile containing 0.1% formic acid) were extemporaneously added to 50 ml of a reduction buffer (pH 8) containing 55 mM AB, 0.5% SDC, and 5.5 mM DTT. Plasma samples (100 μl) and standard solutions (100 μl) were added to 900 μl of reduction buffer containing IS. Samples were reduced for 30 min at 60 °C then alkylated with 10 μl of fresh IA solution (1 M in 1 M NaOH) for 60 min at room temperature (protected from light). Samples were digested for 14 h with 50 μl of trypsin solution (0.1 mg/ml in 1 mM HCl), and 50 μl of 2% TFA were added to stop the digestion. Samples (1 ml) were cleaned, desalted and concentrated using 30 mg Oasis HLB 1 cc Cartridges (Waters, Milford, MA, USA). The cartridges were conditioned with 1 ml of methanol and equilibrated with 1 ml of water. After sample loading, washings with 1 ml 5% methanol and 1 ml 20% methanol were performed. Samples were finally eluted with 500 μl of 80% methanol, dried in a stream of nitrogen (40 °C) and reconstituted with 100 μl of 25% acetonitrile containing 0.1% formic acid. Samples were finally centrifuged for 5 min at 4 °C (20,000*g*) and the clear supernatant was injected into the LC–MS/MS system.

Analyses were performed on a Xevo® Triple-Quadrupole mass spectrometer with an electrospray ionization (ESI) interface equipped with an Acquity H-Class® UPLC™ device (Waters Corporation, Milford, MA, USA) as described in Table 1. Chromatographic peak area ratios between GTVSGTLIGLEFIR and IS constituted the detector responses. Standard solutions were used to plot calibration curves. PCSK9 plasma concentrations were expressed in nM assuming 1 mol of peptide equivalent to 1 mol of protein. Concentrations could be thus converted to ng/ml assuming a molecular weight of 75 kDa [14].

### 2.4. Optimization of sample preparation

The method was developed using a pool of plasma from eight donors spiked either with rPCSK9 (final concentration 10 nM or 750 ng/ml) or AB buffer (50 mM, pH 8) for basal level evaluation. Solutions of synthetic peptide (10 nM in AB buffer) were used to assess the full recovery and basal concentrations were subtracted to measured concentrations in spiked plasma samples. For trypsin proteolysis, 3 concentrations (5 μg or 0.02 mg/ml, 25 μg or 0.1 mg/ml and 50 μg or 0.2 mg/ml) and 6 incubation times (1, 2, 4, 6, 14 and 24 h) were assessed. For solid-phase extraction, wash and elution conditions were optimized with increasing levels of methanol or acetonitrile in water. Several mixtures of acetonitrile in water containing 0.1% of formic acid were tested to reconstitute sample after drying. Each condition was tested in 3 experimental replicates and results were expressed as mean ± standard deviation (SD). After selection of the optimal experimental conditions, the assay was conducted on 6 individual plasma and serums (3 males and 3 females for each) spiked either with rPCSK9 (final concentrations 0.5, 2.5, 7.5 and 15 nM, i.e. 37.5, 187.5, 562.5 and 1125.0 ng/ml) or AB buffer (50 mM, pH 8) for basal level evaluation.

### 2.5. Method evaluation

The performance of the method was first assessed for repeatability and accuracy by using pools of plasma spiked with rPCSK9: 0 nM (basal level), 2.5 nM (187.5 ng/ml), 7.5 nM (562.5 ng/ml) and 15 nM (1125 ng/ml). The inter- and intra-assay repeatability was assessed in 3 independent experiments with 6 replicates per samples, and was expressed by the coefficient of variation (CV, %). Both expected and measured concentrations were compared.

Cross-validation was performed by comparing results obtained by standard ELISA and LC–MS/MS on plasma samples from 30 patients. For quantitative experiments, plasma samples spiked with 5 nM (375 ng/ml) of rPCSK9 (n = 3) or AB buffer (n = 3) were used to check the completion of the enzymatic reaction. Paired results were analyzed using a Student's *t*-test. Pearson correlation tests and a Bland-Altman plots were generated to compare both methods [15]. Graphics and statistical analyses were achieved with GraphPad Prism software (version 6.0, GraphPad Software Inc., La Jolla, CA, USA).