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Quantification of serum C-peptide by isotope-dilution liquid chromatography–tandem mass spectrometry: Enhanced detection using chemical modification and immunoaffinity purification

Tomoya Kinumi*, Ryoko Mizuno, Akiko Takatsu

Bio-Medical Standard Section, National Metrology Institute of Japan (NMIJ), National Institute of Advanced Industrial Science and Technology (AIST), 1-1-1 Umezono, Tsukuba, Ibaraki 305-8563, Japan

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ABSTRACT

A method was developed to quantify human serum C-peptide by isotope-dilution mass spectrometry (ID MS). This new approach used immunoaffinity purification and chemical modification to improve the sensitivity which covered the wide range of reference interval of serum C-peptide. The immunoaffinity purification was performed using monoclonal antibody against human C-peptide that was immobilized on magnetic beads, and the purified C-peptide was chemically modified using 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) prior to liquid chromatography–tandem mass spectrometry (LC–MS/MS). With this method, the LC–MS/MS peak area increased 23-fold compared with the conventional purification by solid-phase extraction and without chemical modification. The limit of quantification was estimated to be 0.003 ng on column, which was lower than previously reported. The validation study showed that (1) the response in the 0.003–2.9 ng range on column was linear (regression coefficient, $r^2 = 0.9994$), (2) the relative standard deviation (RSD) within and between days was inferior to 4.0%, and (3) the spike and recovery test showed the mean recoveries ranging between 99% and 108%. Comparison with an established commercial immunoassay showed high correlation ($r^2 = 0.9994$) at serum concentration of 0.19–8.49 ng/mL. These assessments suggest that this ID MS-based approach can quantify human serum C-peptide with high sensitivity and precision in the reference interval and find a potential use in the reference measurement procedure of serum C-peptide, allowing traceable measurement. This method may also generally be applied to peptide quantification in biological fluids with high sensitivity.

1. Introduction

Human C-peptide is an acidic peptide that is secreted from the pancreatic β-cells into the blood [1]. Because it is produced by the proteolytic cleavage of proinsulin, the amount of secreted C-peptide is equimolar to that of insulin. More than 85% of C-peptide is metabolized by the kidneys, and excreted to urine [2]. The half-life of C-peptide in plasma (20–30 min) is longer than that of insulin (3–5 min), making it five times more concentrated than insulin [3–5]. As a result, C-peptide has become an important marker in blood plasma for insulin secretion in diabetes patients, and provides a means to differentiate between type 1 and type 2 diabetes [5–7].

The measurement of C-peptide has been routinely performed by immunoassays. However, the lack of reference measurement procedure affects the comparability between immunoassays during routine C-peptide measurements. Recently, isotope-dilution mass spectrometry (ID MS) has been developed to establish a reliable analytical method that ensures traceable measurement. Because serum is a complex mixture containing numerous proteins, peptides, lipids, salts and other ingredients, it is important to establish a proper method to isolate the analyte from the biological fluid for a successful ID MS measurement [8]. Several methods have been developed for C-peptide purification/enrichment prior to mass spectrometry. Kippen et al. first reported serum C-peptide measurement that utilized a solid phase extraction cartridge and immunoaffinity purification using an immobilized antibody column [9]. Others have reported chromatography-based purification methods such as two-step solid phase extraction and two-dimensional chromatographic method [10,11]. Recently, Stoyanov et al. figured out selective purification by strong cation

* Corresponding author. Tel.: +81 29 861 4028; fax: +81 29 861 4137.
E-mail address: t.kinumi@aist.go.jp (T. Kinumi).

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exchange chromatography that exploited the acidic nature of C-peptide [12].

Although these methodologies can deal with the wide range of serum C-peptide concentrations covering the reference interval (0.5–10 ng/mL), their lack of sensitivity at low concentrations requires large amounts of serum, and sometimes produces less reliable results. Human C-peptide consists of 31 amino acids and its isoelectric point is approximately 3 [12]. Because its large size and acidic nature both make C-peptide difficult to ionize by positive electrospray ionization, liquid chromatography–tandem mass spectrometry (LC–MS/MS) using positive electrospray ionization have resulted in low quality measurement, especially at low concentrations [13,14].

In this study, the ID MS-based quantification method utilized both immunoaffinity purification and chemical modification to achieve a highly sensitive analysis of serum C-peptide. C-peptide was purified through an immunoaffinity technique that used antibodies immobilized on magnetic beads (Ab-beads), and was chemically modified using 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) prior to LC–MS/MS analysis to improve the ionization efficiency in electrospray ionization. We have previously developed a certified reference material of pure human C-peptide (CRM 6901) [15]. The certified value of this material has traceability to SI (Système International d'Unité) and was used as a standard in this study to ensure the reliability of measurement. Performance assessments show that this improved analytical approach provides a sensitive and precise ID MS-based quantification of human serum C-peptide.

2. Experimental

2.1. Materials

The certified reference material NMIJ CRM 6901-b, which was produced from the synthetic peptide was used as a standard material for human C-peptide. Monoclonal antibody against human C-peptide (2I2, clone # 7E10) was purchased from Hytest Ltd. (Turku, Finland). Dynabeads® MyOne Tosylactivated magnetic beads were purchased from Invitrogen Dynal (Oslo, Norway). IgG-free and protease-free grade bovine serum albumin (BSA) was obtained from Jackson Immuno Research (West Grove, PA, USA). Isotopically labeled C-peptide (D_8-Val_2 C-peptide) was synthesized according to standard 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry and purified by reversed-phase chromatography. AQC was synthesized according to the literature [16]. Eight batches of human pooled serum were provided by Tsukuba Medical Center Hospital (Ibaraki, Japan). C-peptide concentrations in each pooled serum were measured by chemiluminescence enzyme immunoassay using Lumipulse (Fujirebio, Tokyo, Japan). Other chemicals were purchased from Wako Pure Chemical (Osaka, Japan).

2.2. Instrumentation

LC–MS/MS analysis was performed using a Prominence HPLC system with a 6-port valve (Shimadzu, Kyoto, Japan) and a TSQ Quantum triple quadrupole mass spectrometer (Thermofisher Scientific, USA) equipped with an electrospray ion source.

2.3. Sample solution and Ab-bead preparation

Standard C-peptide solutions were prepared gravimetrically by diluting the reconstituted CRM 6901-b in 0.1% BSA solution in phosphate-buffered saline (BSA/PBS). A D_8-Val_2 C-peptide stock solution was also prepared in BSA/PBS. Standard C-peptide and D_8-Val_2 C-peptide stock solutions were stored in polypropylene vials at −80 °C until the day of analysis. Sample blends were prepared gravimetrically by mixing a serum sample with the D_8-Val_2 C-peptide solution. Calibration blends were prepared gravimetrically by mixing standard C-peptide and D_8-Val_2 C-peptide solutions. Ab-bead suspensions (1 mL) were prepared according to the manufacturer's instructions using 100 μg of antibody and 25 μL of magnetic bead suspension.

2.4. Sample preparation by immunoaffinity purification and chemical modification

Sample blends and calibration blends were mixed with 1.5 mL of 0.05% Tween 20, 20 mM Tris–HCl (pH 7.4), and 150 mM NaCl (TBST) for 1 h by rotating mixer. The solutions were incubated with the Ab-bead suspension (40 μL) using rotating mixer at 4 °C overnight or at room temperature for 1 h. Ab-bead containing the mixture was placed on a magnetic stand and successively washed with TBST (1 mL, three times) and 20 mM Tris–HCl (1 mL, pH 7.4) and 150 mM NaCl (TBS). Captured materials were collected by suspending the beads three times in 100 μL of 0.1% trifluoroacetic acid and dried under a nitrogen flow at room temperature. The resulting pellets were dissolved in 0.2 M borate buffer (60 μL, pH 8.8), and 10 mg/mL AQC in acetonitrile (20 μL) was added to the solution. After mixing, the samples were heated at 55 °C for 1 min in a heat block and filtered using a 0.45 μm membrane filter (Ultrafree MC Durapore PVDF, Millipore Corporation, Bedford, MA, USA).

2.5. LC–MS/MS measurement

Each filtered sample (35 μL) was injected into a C8 trap cartridge (Unison UK-C8 guard-column, 2.0 i.d. × 5 mm, Intakt Corporation, Kyoto, Japan) equipped in the HPLC system. After injection, the trap cartridge was washed with 5% solvent B (0.1% formic acid/acetonitrile) against 95% solvent A (0.1% formic acid/water) for 2 min and then automatically switched to an HPLC flow line by switching a 6-port switching valve. HPLC separation was conducted on a C18 reversed-phase column (Capcellpak C18 ACR, 1.5 i.d. × 150 mm, 5 μm, Shiseido, Tokyo, Japan) at a flow rate of 0.2 mL/min with a linear gradient profile from 5% B to 65% B in 15 min. Mass spectrometry was performed under a selected reaction monitoring (SRM) mode with a 70 eV collision-induced dissociation (CID) energy using argon (1.5 mTorr) and positive ion detection. The SRM transitions were m/z 1064.55 → 171.06 (for +3 charge of AQC-modified natural C-peptide), and m/z 1069.73 → 171.06 (for +3 charge of AQC-modified D_8-Val_2 C-peptide).

3. Results and discussion

3.1.1. Chemical modification and LC–MS/MS measurement

Because the highly acidic nature of C-peptide hampers its efficient ionization under positive electrospray ionization conditions, its chemical modification is expected to improve the sensitivity [12]. Several kinds of modification reagents have been developed to introduce functional groups in peptides [17]. AQC was first developed for the high-sensitivity amino acid analysis by fluorescence detection using reversed-phase chromatography. Later, the application of AQC to LC–MS/MS achieved a greater sensitivity during amino acid analysis [18]. Although it has been exclusively used for amino acid analysis, this study is the first to apply AQC to peptide analysis. AQC modifies the N-terminal amino group of C-peptide, and the aminoquinolyl group is expected to increase the ionization efficiency by adding a positive charge to the peptide during electrospray ionization.

The AQC-modified C-peptide was analyzed by SRM using LC–MS/MS, but its molecular weight (3190) is too big to induce