Human C-peptide Quantitation by LC-MS Isotope-Dilution Assay in Serum or Urine Samples

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Abstract

In this communication we report a simple and efficient approach to C-peptide quantitation using isotope dilution mass-spectrometry analysis. The method facilitates quantitation of C-peptide levels at least one order of magnitude lower compared to concentration levels achieved with an IDA method reported previously. The improvement was due to more intensive sample preparation procedure that, in turn, makes it possible to increase the sample load without a corresponding increase in matrix effects. We also show the results of a comparison study with a second laboratory using a similarly previously reported method for C-peptide quantitation.

Keywords: C-peptide/Mass spectrometry; Isotope dilution assay; Ion Exchange chromatography; Sample preparation

Introduction

C-peptide is a 31- amino acid central part of the pro-insulin molecule. Equal amounts of insulin and C-peptide are released from cleavage of proinsulin by specific beta cell endopeptidases within the pancreatic islets of Langerhans. The exact biological role of C-peptide is not completely understood. C-peptide has generally been considered a byproduct of insulin biosynthesis, but recent data suggest that it may have biological significance [1-3]. In contrast to serum insulin, which is cleared by the liver, C-peptide is cleared at a much slower rate by the kidney and thus represents a more useful indicator of intrinsic insulin secretion (due to 1:1 stoichiometric ratio with insulin) [4-6]. Low C-peptide concentrations in plasma can indicate early insulin secretory failure in the preclinical stages of diabetes. Additionally, for diabetes patients who take insulin, C-peptide measurement allows indirect assessment of endogenous insulin production. The reference interval for human C-peptide concentration in plasma is 0.5-10 ng/mL (0.15-3 nmol/L) [7,8]; its concentration in urine is approximately one order of magnitude higher [9,10].

Mass-spectrometric (MS) analysis of C-peptide in processed blood (plasma samples) was first performed in 1996 [7] and was one of the first successful applications of the MS isotope dilution assay (IDA) for quantitative analysis of endogenous peptides. The authors used reversed phase chromatography in LC-MS quantitation, and employed solid phase extraction for sample preparation. The same general principle was utilized by other investigators who contributed to further method development [10-16], mainly aimed at increasing analysis throughput.

To overcome the negative effects of poor ionization efficiency for C-peptide in MS analysis, a method that utilized two-dimensional reversed phase reversed phase chromatography was introduced in 2006 [14,15]. A relatively small peak heart-cut fraction, less than 0.3mL containing the C-peptide peak was eluted using a shallow linear gradient during the first dimension separation; this fraction was then transferred to a second dimension reversed phase column prior to MS analysis. Two-dimensional chromatography was thus realized without complete sample mapping [17]. Although, in each dimension the same separation mechanism based on hydrophobic interaction was used, the signal to noise ratio was greatly improved, presumably due to the fact that different hydrophobic interaction stationary phases were employed sequentially using different ion-pairing agents [15], which is resulted in different column selectivities. Importantly, the most abundant fragment ion of C-peptide is Y1 [m/z 147.1], which represents a yield of only 1.5% using collision induced fragmentation (the standard fragmentation conditions).

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fragmentation technique implemented in multiple-reaction monitoring MRM)). Therefore, to achieve optimal sensitivity, MRM mode was avoided and selective-ion monitoring mode was used to quantitate the precursor ions of C-peptide and its stable labeled internal standard. The resulting decreased selectivity of the mass spectrometry analysis was compensated for by the improved 2D LC separation.

An alternative approach to sample preparation has been reported recently where the authors proposed the use of ion exchange chromatography [11]. The main idea of this approach was to take advantage of the high acidity of the peptide of interest to perform the appropriate purification step. High C-peptide acidity results from the fact that there are no positive charged amino acid residues in the peptide sequence except for the N-terminal amino group. As result, C-peptide is not retained on cation exchanger stationary phases even under strong acidic conditions. This property facilitated the use of an isolation scheme that utilized the negative absorption of C-peptide on cation exchanger resin; the detailed conditions were selected based on the theoretical analysis of C-peptide electric charge vs. pH curve [18-21].

In the present paper we report an improved version of this previously developed method [11] which allows for measurements of lower C-peptide concentration in biological samples. The sample preparation procedure is summarized in the table 1.

The general purification scheme consisted of three steps and started with methanol precipitation, where methanol was added to serum samples (mixed with a standard) in ratios up to 4:1 (typically, 40µL of IS, 100ng/mL, were mixed with 160µL of serum sample then 0.8 mL of methanol was added). After centrifugation the supernatant was immediately applied to SepPak C18 disposable cartridges (Millipore,
The New York and Columbia, MO, laboratories, respectively. The two
methods showed excellent correlation ($R^2=0.9647$); results from the
MO laboratory were slightly lower (~4%) overall compared to results
from the NY laboratory.

The improved method for LC-MS quantitative analysis of
C-peptide in human plasma as well in urine described here facilitates
quantification of C-peptide in patient samples with C-peptide
concentrations as low as 20pg/ml, or 7pMol/L. The sample preparation
procedure increased analytical sensitivity at least 10x compared to
results reported previously. The method is fast and robust and allows
for high throughput. The sample preparation procedure does not
employ traditional concentration techniques such as ultrafiltration
and lyophilization which are very time consuming and normally result
in considerable sample loss. Method comparison data demonstrated
excellent correlation with another previously described LC-MS method.

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