Development of Human C-peptide LC-MS Isotope-Dilution Assay: Optimization of C-peptide isolation from biological fluids with Ion-Exchange Chromatography

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Abstract

Background: Human C-peptide is an effective marker of insulin secretion in diabetes diagnostics. Little metabolism of C-peptide by the liver enables its concentrations to be three to five times higher than insulin in the plasma. Commercial immunoassay analyses are well established, however, they have questionable specificity with results not always comparable across laboratories. Isotope-dilution assays (IDAs) provide an alternate method for C-peptide analysis and offer greater specificity. The greater specificity of IDA makes it an ideal reference for which other analysis methods can be harmonized against; however, there is still need for further development.

Methods: C-peptide isolation from serum was performed by a multi-step procedure (utilizing IDA techniques) of alcohol precipitation, ion exchange chromatography (IEx), and lyophilization. Analysis was done on a LC-MS system consisting of a paired Shimadzu Prominence HPLC system with a Varian Pursuit C18 reverse phase column and API 4000 MS/MS system. IEx was performed on a Hitrap™ SP column. Fragments were monitored in selected ion & multiple reaction monitoring modes.

Results: It was identified that upon passage of C-peptide through ion exchange chromatography, C-peptide levels were amplified, while background was reduced. Post-lyophilization (after IEx as well) displayed the greatest amplification of C-peptide and reduction of background.

Conclusions: The multi-step process utilizing ion exchange chromatography displayed an improvement in C-peptide isolation compared to standard IDA methods of C-peptide analysis. The new isolation scheme can result in increased sensitivity and specificity, enhancing C-peptide analysis and providing for a more reliable assessment of an individual's C-peptide levels.

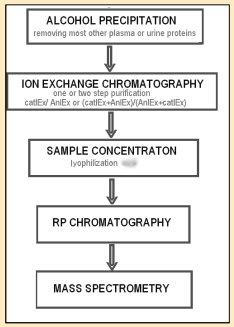
Introduction

Human C-peptide is an effective marker of insulin secretion in diabetes diagnostics. Little metabolism of C-peptide by the liver enables its concentrations to be three to five times higher than insulin in the plasma. Commercial immunoassay analyses are well established, however, they have questionable specificity with results not always comparable across laboratories (1).

Isotope-dilution assays (IDAs) provide an alternate method for C-peptide analysis with greater specificity (2). This greater specificity makes it an ideal reference method to which other methods can be harmonized. Low analyte concentrations as well as the presence of undesired compounds (especially in plasma) limit the ability of C-peptide quantification. The incorporation of Ion-exchange chromatography for sample purification can assist in eliminating these limitations by reducing background and increasing sensitivity.

Methods

1. Alcohol precipitation with methanol removing most undesired proteins
2. Ion-exchange chromatography (IEx)- Using one or two-step purification. One-step using cation exchange or two-step using cation exchange (Hitrap™ SP) & anion exchange (Hitrap™ Q) sequentially.
3. Lyophilization for concentration of sample
4. Analysis performed on an API-4000 triple quadrupole mass spectrometer (ABI/Sciex) paired to a Shimadzu Prominence LC system (Shimadzu Scientific Instruments) with a Varian Pursuit C18 reverse phase (RP) column (50 x 2.1mm, 300 A) using a linear methanol gradient elution.



Results

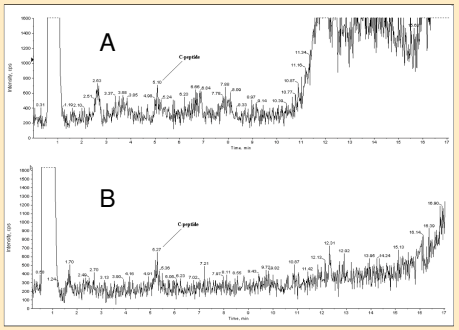


Fig. 1- **A**. The chromatogram depicts C-peptide analysis using standard IDA methods without IEx (alcohol precipitation and RP chromatography still utilized). Large background interference is present. **B**. C-peptide analysis after use of one-step IEx. A considerable reduction in background is apparent.

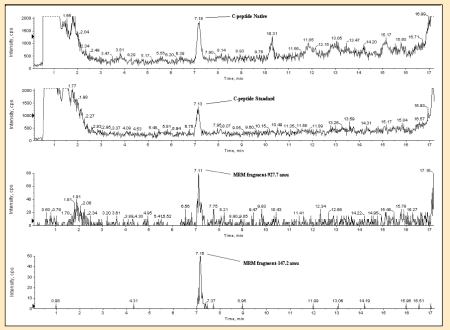


Fig. 3-Purified and concentrated sample seen in Q1 and Q3. Background is reduced considerably with amplitude increased as well. MRM transition monitoring fragments of 147.2 and 927.7 shown on lowest panel

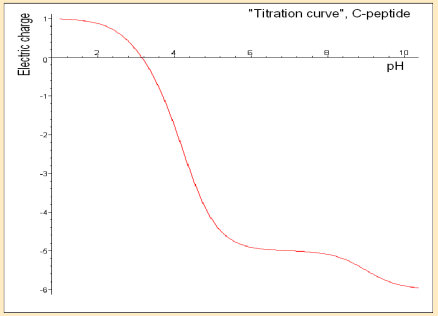


Fig. 2- The titration curve for C-peptide. The curve is the basis for IEx, with equilibration pHs of IEx columns dependent on titration curve of C-peptide

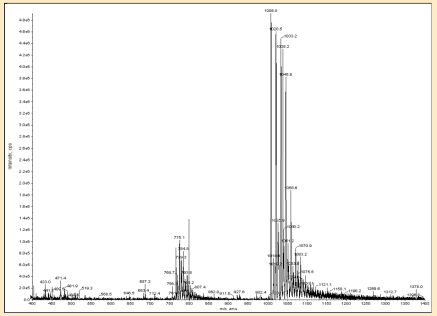


Figure 4- Mass spectrometry profile of C-peptide standard.
Profile displays heterogeneity of the isotope-labeled C-peptide 3050 standard [+30]. The solid phase synthesized peptide contains14 residues labeled with 13C and 15N.

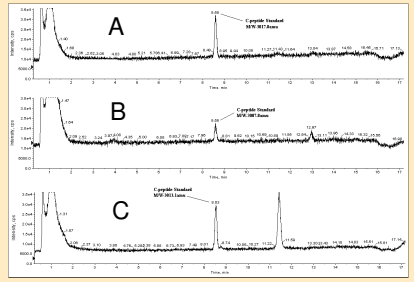


Figure 5- LC/MS profiles of various C-peptide standards. **A.** Profile of C-peptide standard with molecular weight of ~3018 amu. **B.** Profile of C-peptide standard with molecular weight of ~3008 amu (same as native C-peptide). **C.** Profile of C-peptide standard with molecular weight of ~3014 amu. The profiles display the chromatographic variation among C-peptide standards used with IDA procedures.

Conclusions

The use of ion-exchange chromatography for isolation & purification of C-peptide was shown to be effective.

IEx was able to increase sensitivity and specificity decreasing several of the challenges facing C-peptide analysis. High sample volumes are also capable of being processed by the developed method, which is important when C-peptide concentrations are low.

The two-step ion-exchange chromatographic procedure, although not fully developed, offers a general approach to more complex mixtures.

The use of IEx use in IDA methods facilitates accurate value assignment to calibrators which can in turn be used to standardize routine C-peptide assay methods.

Acknowledgements
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References

1. Little, et al. "Standardization of C-peptide measurements." *Clinical Chemistry*. (2008) 54:1023-26
2. Rogatsky, E, et al. "Sensitive quantitative analysis of C-peptide in human plasma by 2-dimensional liquid chromatography-mass spectrometry isotope-dilution assay." *Clinical Chemistry*. (2006) 52:872-879