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The potential for isotope dilution-LC-MS/MS to improve laboratory measurement of C-peptide: Reasons and critical determinants

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ABSTRACT

Human C-peptide is secreted in equimolar amounts with insulin by pancreatic beta-cells. Measurement of C-peptide plays an important role in the diagnosis and treatment of diabetes where it is used to evaluate the function of islet cells. However, C-peptide measurement results across different laboratories vary considerably and there is an urgent need to improve comparability between laboratories. As it is sensitive and specific, isotope dilution-liquid chromatography-tandem mass spectrometry (ID-LC-MS/MS) has made a major contribution and will continue to play a significant role in the standardization of C-peptide measurement. Here, we reviewed the application of ID-LC-MS/MS in C-peptide measurement by discussing the biochemical properties of C-peptide, common sample preparation procedures, and the sensitivity problems often encountered with ID-LC-MS/MS C-peptide measurement. Collectively, these factors are crucial for the development of ID-LC-MS/MS methods for C-peptide measurement. We also discussed the advantages, disadvantages, and progress of implementing ID-LC-MS/MS as a routine measurement tool for C-peptide in clinical laboratories. Finally, we summarized the existing reference system and the status of C-peptide measurement in clinical laboratories to convey the necessity of improving the comparability of C-peptide measurement in clinical laboratories using ID-LC-MS/MS.

Introduction

Proinsulin is cleaved to generate equal amounts of insulin and C-peptide, the latter is a single-chain peptide of 31 amino acids. C-peptide detection is useful in the diagnosis, treatment, and prognosis of type 1 and type 2 diabetes where its levels indicate the status of endogenous insulin secretion by pancreatic islet beta-cells [1–3].

Given its clinical importance, C-peptide is one of the most frequently ordered diabetes-associated laboratory tests. Nevertheless, an international comparison of C-peptide measurement procedures, conducted by the C-peptide standardization committee, identified significant variation between different laboratories and different measurement procedures where the results of C-peptide immunoassays are often incomparable. The committee also identified that using a WHO International Reference Reagent (IRR) for C-peptide measurement does not improve the comparability of results between laboratories [4]. Most notably, the performance of the routine immunoassays is easily impacted by the cross-reactions. Consequently, there is an urgent need to improve analytical performance and standardize C-peptide measurement, which is meaningful for both effective patient care and use of medical resources.

Owing to discrepancies between laboratories, it is necessary to develop well-performing measurement procedures to calibrate or improve existing clinical measurement procedures for C-peptide. A methods comparison program conducted in 2008 found that the comparability of clinical immunoassays among laboratories could be dramatically improved when a calibrator serum, assigned by ID-LC-MS/MS, was used [5]. Therefore, as the reference measurement procedure for C-peptide, ID-LC-MS/MS plays a very important role in the process of improving the comparability of C-peptide measurement across different

Abbreviations: ID-LC-MS/MS, isotope dilution-liquid chromatography-tandem mass spectrometry; EAQ, external quality assessment program; %CV, coefficient of variation expressed as a percent; PI, The isoelectric point; JCTLM, the Joint Committee for Traceability in Laboratory Medicine.

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laboratories.

In this article, we reviewed the critical determinants for the successful implementation of the ID-LC-MS/MS to measure C-peptide. Key parameters discussed include the biochemical properties of C-peptide, sample preparation procedures, as well as the sensitivity problem associated with C-peptide ionization, fragmentation efficiency, and ion transmission. We also summarized the existing reference system for C-peptide to document the achievements in the standardization of C-peptide measurement. Furthermore, to convey the need for standardizing C-peptide measurements, we also presented the utility and current status of C-peptide measurements in clinical laboratories.

The clinical utility of C-peptide measurement

In type 1 diabetes, lower C-peptide levels are observed along with absolute insulin deficiency. In the early stage of type 2 diabetes mellitus, insulin resistance is characterized by compensatory secretion of insulin and C-peptide, nevertheless, as the function of the islet beta cells fails, the secretion of insulin and C-peptide also decreases. Hence, both C-peptide and insulin correlate with both the type and the duration of diabetes. Moreover, their levels are helpful in differentiating type 1 and type 2 diabetes, especially in long-standing diabetes, as there may be a substantial overlap of C-peptide and insulin levels between type 1 and type 2 diabetes at the time of diagnosis [6]. However, three key attributes make C-peptide a more informative biomarker for the diagnosis and treatment of diabetes than insulin.

(1) *Stability*. The stability of C-peptide differs from insulin which is rapidly cleared by the liver, C-peptide is cleared by the kidneys at a much slower rate, with a half-life of 20–30 min versus 3–5 min for insulin [2,7,8]. Hence the level of serum or plasma C-peptide is higher than insulin and provides a more reliable test window to estimate fluctuations in beta-cell responses.
(2) *Cross-reactivity*. Exogenous insulin and insulin analogs are commonly employed treatments in diabetic patients. However, patients frequently produce anti-insulin antibodies against these drugs resulting in cross-reactivities when detecting insulin using immunoassays. Since C-peptide is not used in a treatment capacity regularly, and does not share similar antigenic determinants with insulin, the cross-reactivities are much lower than for insulin. Thus C-peptide testing more accurately reflects islet function [9].
(3) *Convenience*. Since more than half of C-peptide produced is cleared by the kidneys, C-peptide (40–150 ng/mL) is more abundant in urine than in serum or plasma (0.5–10 ng/mL) [7,10,11], a characteristic that can be leveraged to create a less intrusive test to replace serum or plasma-based tests. However, the results of urinary C-peptide tests should be interpreted with caution when patients have renal impairments [12].

In addition to its role in the diagnosis and treatment of type 1 and 2 diabetes, C-peptide has also been found to help diagnose latent autoimmune diabetes in adults (LADA) [13–15] and maturity-onset diabetes of the young (MODY) [16,17]. Researchers first thought C-peptide had no biological activity. However, an increasing number of studies confirmed that C-peptide exerts various *in vivo* physiological actions, including relaxing blood vessels, improving erythrocyte deformability, and promoting the use of sugars and amino acids in muscle tissues [18–20]. Therefore, not only does C-peptide have clinical significance in reflecting the function of beta cells, but it also performs significant physiological functions in its own right. This illustrates the clinical importance of improving the standardization and performance of C-peptide measurement.

The status of C-peptide measurement in clinical laboratories

Currently, most clinical laboratories use various immunological-based assays to measure C-peptide. These methods are rapid, highly automated, and have good reproducibility. However, different immunoassays often use different antibody reagents with different cross-reactivities, which may lead to incomparable results. The C-peptide standardization committee implemented an international comparison of C-peptide measurement procedures which sent 16 different heparin plasma samples to 15 laboratories located in 7 countries. The samples were analyzed using 10 different immunoassays with the results showing significant variation between different laboratories and different measurement procedures. Moreover, using WHO International Reference Reagent (IRR) for C-peptide to recalibrate those procedures also failed to promote the comparability of results among laboratories [4].

The external quality assessment (EQA) program is a useful tool to evaluate the performance of routine measurements and promote the standardization of biomarker measurement in clinical laboratories [21]. The National Center for Clinical Laboratories (NCCL) of China has been dedicated to conducting the EQA program for Chinese clinical laboratories since 1982. The EQA program for C-peptide was first carried out by the NCCL of China in 2007 with 247 participating laboratories. This number increased to 2158 in 2020.

Here, we list the ten most commonly used calibrator producers in the 2020 EQA C-peptide program in China, which account for 91.6% of all participating laboratories (Table 1, Fig. 1). These calibrators are manufactured for immunoassays and have good repeatability (all the within-group %CV are<8%, except for the Siemens Centaur). We regarded a result as an outlier if it was more than 3 times the standard error from the target value, which sets as the mean value of all participants after removing the outliers. It is worth noting that the within-group %CV was much less than the total %CV (19.9%) among all participating laboratories. The reason may be that the different calibrators have various degrees of traceability to the higher-order reference system.

As mentioned above, the results of the clinical immunoassay for C-peptide vary considerably between different laboratories. Previous studies have confirmed that using ID-LC-MS/MS to calibrate existing measurements can dramatically improve the comparability between laboratories [5,22], making it a suitable reference measurement procedure.

ID-LC-MS/MS in C-peptide measurement

ID-LC-MS/MS has been accepted as the reference method of many clinical indicators because of its specificity, accuracy, and sensitivity, with all of these reference methods listed on the JCTLM website

Table 1
The top ten calibrator producers used by participants in the 2020 EQA program in China for C-peptide. The %CV presents the coefficient of variation between laboratories using the same calibrator (within-group %CV), e.g. the CV between laboratories using Roche's calibrator was 6.63%.

Calibrator producer	Number of laboratories	%CV	Proportion
Roche	946	6.63	44.4%
Siemens Centaur	268	13.53	12.6%
Abbott	214	5.17	10.0%
Snibe Maglumi#	157	6.06	7.4%
Mindray	129	6.36	6.1%
AUTOBIO#	106	7.64	5.0%
TOSOH	38	6.49	1.8%
Chivd BeyondBiotech#	34	6.91	1.6%
YHLO#	30	7.18	1.4%
ORIENTER#	29	6.25	1.4%
Others	180	N/A	8.4%
Total	2131	19.9%	100%

# denotes Chinese manufacturers, N/A denotes not applicable.

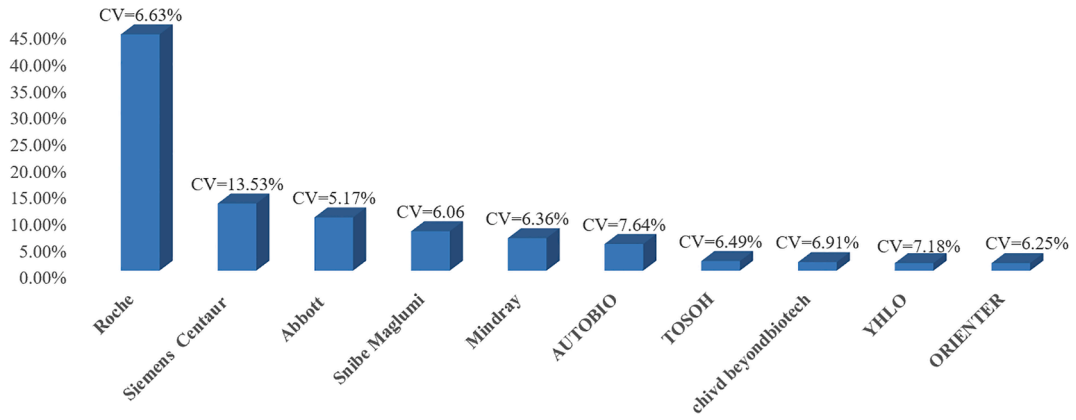

Fig. 1. Manufacturers of different calibrators ranked by percentage of total participants in the 2020 EQA program for C-peptide in China.

(https://www.bipm.org/jctlm/home.do). It is a highly useful technology for the measurement of small molecules, however, it has not been extensively applied to measure biological macromolecules, such as proteins and peptides. The contributing reasons include:

(1) *Sample processing*. The molecular weights of polypeptides and proteins are usually too large to generate suitable *m/z* during MS measurements, which often necessitates additional procedures, such as enzymatic digestion to cleave polypeptides into small specific peptides to represent the original analytes [23]. However, this approach has many limitations. Firstly, it is tedious and expensive, and second, not every protein and polypeptide can produce specific small peptides that adequately represent the original analyte. Third, the specificity of the process needs to be carefully considered; for example, quantifying insulin through its enzyme-digested products is not accurate, since proinsulin, which contains the whole amino acid sequence of insulin, may generate the same digestion products.
(2) *Sample complexity*. There are numerous proteins, peptides, lipids, salts, and other components in serum and plasma. It is important and demanding to isolate sufficiently 'clean' analyte to perform successful ID-MS measurements.
(3) *Expense*. Owing to longer amino acid sequences, it is more difficult and expensive to synthesize the isotopic internal standard materials of biological macromolecules than small molecules [24].

Compared to many other peptides, C-peptide is well-suited for detection by ID-LC-MS/MS. First, the molecular weight of C-peptide (3020.3 Da) is not as large as other polypeptides and proteins. Without extra enzymatic cleavage, the *m/z* of its precursor ions can be located within the mass spectrum measurement range through the formation of multiply charged ions, like the type $[M + nH]^{n+}$. Second, as a single peptide chain of 30 amino acids, the cost of synthesizing isotope internal standards is less than for other biological macromolecules, and commercialized internal standards like $[_2H^8\text{-Val}^{7,10}]$C-peptide (Bachem. Bubendorf, Switzerland) are available. Also, as an acidic peptide, either negative electrospray or positive electrospray ionization modes can be used to produce the precursor ions of C-peptide. Indeed, the convenience of this method has made sample analysis without digestion the method of choice for most published ID-LC-MS/MS reports involving C-peptide measurements. Nonetheless, quantifying C-peptide through its enzyme-digested products has advantages, since this approach can assist sensitivity and decrease the price of the isotopic internal standard. However, owing to C-peptide sharing its amino acid sequence with proinsulin, it is necessary to consider the contribution of proinsulin when quantifying C-peptide following enzymatic digestion. Owusu et al. [25] developed an ID-LC-MS/MS measurement procedure for C-peptide based on endoproteinase Glu-C digestion. However, while this greatly promoted the measurement sensitivity and required less serum than most ID-LC-MS/MS measurements, the procedure requires up to two days to complete a test and the sample preparation process is tedious.

Overall, the properties of C-peptide make it relatively advantageous versus other macromolecules for ID-LC-MS/MS measurement. Hence, as early as 1997, researchers were detecting plasma C-peptide using ID-LC-MS/MS [26]. Table 2 summarizes the published ID-LC-MS/MS measurement procedures for C-peptide. Nevertheless, despite the suitability of ID-LC-MS/MS to measure C-peptide, there are still many factors that need to be carefully considered when developing the ID-LC-MS/MS method, in particular the biochemical properties of C-peptide.

Biochemical properties of C-peptide

The biochemical properties of an analyte are of great importance in the successful development of any measuring method. Three critical properties of C-peptide that should be considered in order to build a successful ID-LC-MS/MS method are as follows:

(1) *The isoelectric point (pI)*. The C-peptide sequence includes 5 acidic amino acids (3 glutamic and 2 aspartic acid residues) and no basic amino acids [27–29]. Therefore, C-peptide is an acidic molecule with a pI of about 3. Many LC-MS methods take advantage of this property for purification purposes, which we discussed below in the sample preparation section [27,30].
(2) *Adsorption*. Similar to insulin, C-peptide is easily adsorbed to the surface of plastic and glass vials, which is exacerbated with increasing C-peptide levels. Consequently, C-peptide can be readily lost during analysis, but this problem can be resolved by adding carrier proteins, such as bovine serum albumin (BSA) as non-specific binding competitors [22,31–33].
(3) *Stability*. Serum C-peptide can be stably preserved at −80℃ for at least 5 years [34]. However, significant degradation occurs after 24 h of storage at room temperature (18-26℃) or after 7 days of refrigeration (2-8℃) [35].

Common sample preparation techniques for C-peptide

Sample preparation is a key component of LC-MS/MS analysis of biological samples for many reasons including: purifying and concentrating the analytes, promoting sensitivity, reducing matrix effects, and avoiding ion enhancing or suppression effects that would otherwise impact the specificity and sensitivity of the assay. Therefore, it is necessary to ensure that sample preparation procedures can sufficiently isolate C-peptide from the sample matrix. The existing sample preparation procedures for C-peptide commonly include protein precipitation, solid-phase extraction (SPE), immuno-affinity purification, ion-

Table 2
Summary of the published ID-LC-MS assays for C-peptide.

Sample	Internal standard	Sample preparation	Instruments and ionization	Detecting principle	Performance (precision and accuracy)	Advantages	Disadvantages	RF
Serum	$[^2H_{14}]$Cp	SPE(C_{18})/IF	VG-Trio 2000, ESI(+)	ID-LC-MS	tCV < 10%	Quantifying insulin and Cp simultaneously. First ID-LC-MS method to measure Cp.	Demands excessive serum volume(1 mL). the specificity of SIM mode is not comparable to MRM. Performance not verified.	[26]
Urine	$[^2H_{14}]$Cp	UF	VG Quattro II, ESI(-)	ID-LC-MS/MS	tCV < 5%	SP is simple and time-saving	Limited to urine. The recovery of SP is just 60%.	[22,32,33]
Serum	$[_2H^8$-$Val^{7,10}]$Cp	SPE(C_{18}) and SPE (Oasis®MCX)	VG Quattro II, ESI(-)	ID-LC-MS/MS	tCV < 2.9%, SRE (accuracy): 94.6%-103.6%; trueness: 98.1%	Accurate and sensitive (LOD: 0.03 ng/ml)	Demands excessive serum (at the LOD, 2.5 mL serum). The SP procedure is tedious and time-consuming	[38,40]
Plasma	[M + 18]Cp and [M + 30] Cp	SPE(C_{18})/2-DC	API 4000 TQ-MS, ESI(+)	ID-LC-MS	tCV < 2% SRE (accuracy): 99%-102%	Accurate and sensitive, precision is<2%	The SP procedure is complex and tedious. Applying SIM mode, specificity may not be as good as MRM	[41]
Plasma*	$[_2H^8$-$Val^{7,10}]$Cp	IEC and PP	API 4000 TQ-MS, ESI(+)	ID-LC-MS/MS	tCV < 4.5%	The SP procedure is efficient and semi-automatic. monitoring two product ions to enhance the specificity	Requires extra instrumentation like ion-exchange chromatography	[27,29]
Serum*	$[_2H^8$-$Val^{7,10}]$Cp	IF and CM	TS-Q Quantum TQ-MS, ESI(+)	ID-LC-MS/MS	tCV < 4% SRE (accuracy): 99.4% to 103%.	Most sensitive method among published methods. Calibrator can be traced to the SI unit.	The SP procedure is expensive and time-consuming	[36]
Serum	$[^{13}C_6$,^{15}N $Leu_{30}]$ Cp	IF /2-DC	API4000 and API365 TQ-MS, ESI(+)	ID-LC-MS/MS	tCV < 10%, SRE (accuracy): 91%-104%	Almost fully automatic, can detect insulin and Cp simultaneously, and is more accurate and specific than immunoassays	Compared to immunoassays it is more expensive and tedious.	[35]
Plasma	$[^{13}C_6$, $Leu_{26,30}$] Cp	PP and SPE(Oasis® MCX)	AQ Exactive HFX HR-MS ,ESI(+)	ID-LC-MS/MS	tCV < 13.3% SRE (accuracy): 93%-94%	SP procedure is simple and inexpensive, detecting Cp, insulin, and insulin analogs simultaneously	SP procedure is not automatic. The CV is relatively large.	[30]
Serum	custom synthesized isotopic C-peptide	PP/ SPE(Waters MCX)/ digestion with endoproteinase Glu-C	Waters Xevo TQ-S MS/MS ESI(+)	ID-LC-MS/MS	tCV < 7.7% SRE (accuracy): 98.2 % (±9.1 %)	This procedure promoted the sensitivity of detection by digesting Cp. The amount of sample (200 μL) is less than most ID-LC-MS/MS procedures	The SP is quite complicated and time-consuming (takes about two days to complete a test). The recovery of this procedure is not very good.	[25]
Serum	custom synthesized isotopic C-peptide	IF and CM	Bruker Microflex LRF (Bremen, Germany)	MALDI-TOF MS	tCV < 10% SRE (accuracy): 39%-66%	This procedure is time-saving, and high-throughput.	The spike recovery is not very high. Compared to immunoassays it is more expensive and tedious.	[45]
Urine	$[^{13}C_6$ $Leu_{26,30}]$ Cp	PP/ SPE (Waters MCX)	Orbitrap Exploris 480 HR-MS (Thermo) ESI(+)	ID-LC-MS/MS	N/A	This procedure is non-invasive. The SP procedure is simple and inexpensive. Detecting Cp, insulin, and insulin analogs simultaneously	This procedure is focused on qualitative results only	[51]

* denotes reference measurement procedures in the JCTLM list. We abbreviated some terms to make the table more concise.
Abbreviations: Cp: C-peptide, SPE: solid-phase extraction, IF: immunoaffinity purification, 2-DC: two-dimensional liquid chromatography, IEC: ion-exchange chromatography CM: chemical modification, PP: protein precipitation, UF: ultrafiltration, tCV: total CV, SRE: spike-recovery experiment, TQ-MS: triple-quadrupole mass spectrometer, HR-MS: high-resolution mass spectrometer, SP: sample preparation, LOD: limit of detection, RF: reference, SIM: single ion monitoring, MRM: multi reaction monitoring. MALDI-TOF MS: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

exchange chromatography, two-dimensional liquid chromatography, and derivatization. Generally, to achieve sufficient enrichment and purification, it is essential to combine multiple preparation methods.

The simplest method is protein precipitation, which can isolate C-peptide from most interfering substances including various proteins. Most published ID-LC-MS/MS methods use organic reagents, such as methanol and acetonitrile for general protein precipitation, but precipitation with salt solutions, such as $ZnSO_4$, is more convenient as the extra steps to reduce organic reagent concentrations are not required before further solid-phase extraction.

Based on high-specificity binding reactions between antigens and antibodies, immuno-affinity purification not only ensures high fidelity recovery of C-peptide, but also removes most of the interfering substances [26,36]. There are two commonly used immuno-affinity purification approaches, these being magnetic beads and column-based procedures. Notably, the generally small size of magnetic beads and the

assay design allows efficient contact with the analytes in the sample solution, which facilitates purification efficiency. Indeed, this technology has recently been used for the immunoaffinity purification of proteins and peptides from biological fluids (Table 2). Compared to column-based immunoaffinity purification, magnetic beads are more readily adaptable to small-scale samples and are easier to handle in routine experiments [37]. However, the significant downsides of immunoaffinity purification are the high cost of antibodies and the time-consuming nature of the procedure.

The pI of C-peptide is around 3, which allows for a simple, but effective, purification procedure using ion-exchange chromatography [27]. For example, applying a starting solution with a pH_1 slightly above the C-peptide pI to a strong anion ion-exchange resin will trap C-peptide on the column while most of the impurities whose pI is above the pH_1 will flow through. Subsequent application of a pH_2 solution, which is slightly lower than the C-peptide pI, will elute the C-peptide from the column while most interfering substances with a pI lower than pH_2 will remain adsorbed. This procedure using a pI window (pH_2-pH_1) results in a highly purified sample suitable for MS measurement as illustrated in Fig. 2. Reverse-phase chromatography has also been used to successfully isolate C-peptide from a complicated matrix. Rogatsky et al [38] used a two-step chromatographic process which they called 2D chromatography to purify C-peptide from most interferences. The disadvantage of ion-exchange and reverse-phase chromatography procedures beyond more conventional SPE procedures, is their requirement for additional expensive instrumentation.

Like ion-exchange chromatography, the ion-exchange SPE procedure based on the acidic properties of C-peptide has also been used by many researchers. Most published studies have utilized cation-exchange-based SPE, such as the MCX column (Waters) to purify C-peptide [30,39,40]. However, since C-peptide is acidic, in theory an anion-exchange column may be more suitable for its purification. Nevertheless, the focus of the reported cation-exchange studies may not only include consideration of C-peptide, but also the biochemical properties of the many related analytes they want to detect, including insulin and its analogs present in the matrix; this may be the reason why the studies chose cation-exchange SPE procedures.

There are various sample preparation procedures available for C-peptide, and researchers should choose the best procedure based on their application. In addition to sample preparation, there are also additional ways to promote sensitivity in C-peptide measurements by ID-LC-MS/MS, which we discuss these in the next section.

Optimizing sensitivity for measuring C-peptide by ID-LC-MS/MS

The sensitivity for measuring C-peptide by ID-LC-MS/MS is impacted by various factors. For example, the highly acidic nature and high molecular weight of C-peptide can result in a 60-fold loss in sensitivity when measuring its levels via ID-LC-MS/MS [38,41]. Optimizing sensitivity is therefore essential for the development of ID-LC-MS/MS-based methods for C-peptide. There are many ways to enhance sensitivity including optimizing sample preparation to concentrate C-peptide and reduce the levels of interfering substances, which can improve the signal-to-noise ratio (S/N) and sensitivity. On the other hand, increasing ionization, ion transmission and fragmentation efficiency can also greatly improve sensitivity. In the following sections, we discuss how to optimize sensitivity through promoting ionization, ion transmission, and fragmentation efficiency.

Ionization

Electrospray ionization (ESI) can not only perform very mild ionization, but can also generate multiply charged ions like $[M + nH]^{n+}$, making it possible to measure high molecular mass compounds in lower *m/z* ranges. Hence, ESI technology has been widely utilized for the ionization of polar, non-volatile, high molecular mass, and thermolabile compounds, like proteins and peptides. All published LC-MS/MS methods for C-peptide have adopted ESI mode (Table 2), with most using positive mode ESI ionization in multiple reaction monitoring mode (MRM) and selecting the high abundance of 3^+ charged ion as the precursor ion.

Peptide ionization efficiency is correlated with many factors, for example, minor alterations in orifice potential and capillary voltage can greatly improve ionization [42]. The pH of the eluent can also influence the formation of specific ions, as at lower pH values higher charged ions are generally more pronounced [43,44]. Thus, a lower pH mobile phase is generally beneficial for C-peptide ionization. On the other hand, Stoyanov et al. reported the LC-MS analysis of C-peptide using methanol as the mobile phase produced a higher signal than acetonitrile [27].

Ion transmission

Changing some instrument parameters can greatly enhance the intensity of ion transmission, e.g. Chen et al. applied lower Q1 resolution to enhance product ion response [35]. Our primary investigations to build a new ID-LC-MS/MS reference measurement procedure for C-peptide on the SCIEX QTRAP 6500 instrument confirmed that adjusting the Q_1 resolution from unit to low can enhance the product ion signal nearly 4-fold (Fig. 3). We hypothesize that when the Q_1 resolution is unit, only a part of a precursor's isotopic distribution is captured due to the isolation window being too narrow. After adjusting the Q_1 resolution to expand the window, more of the precursor's isotopic distribution is allowed to enter the collision cell. This appears to improve sensitivity and may be helpful in the development of ID-LC-MS/MS-based methods

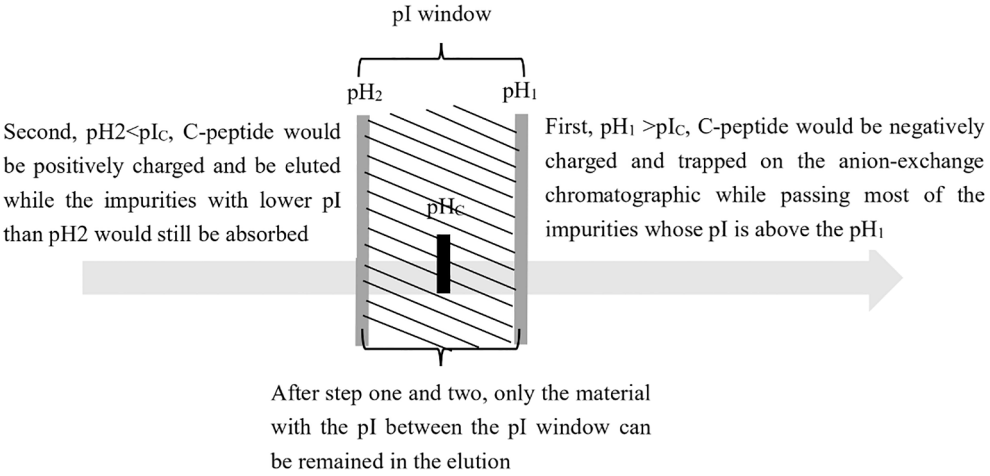

Fig. 2. A two-step ion-exchange chromatographic procedure for C-peptide. In this example anion-exchange chromatography is used to isolate mixture components with pIs that belong to the selected pI window (pI_C represents the pI of C-peptide). First, making the pH_1 slightly above the pI_C, the analytes with greater pI values ($pI > pH_1$) will be positively charged and thus pass through the anion-exchange resin. Second, by changing the pH of the elution to pH_2, the negatively charged analytes with lesser pI values ($pI < pH_2$) will be retained on the column, while analytes like C-peptide with their pI above pH_2 ($pI > pH_2$) will be eluted. In theory, similar procedures can be used to purify multi-component system ampholytes (proteins or peptides) via interacting with cation or anion exchangers.

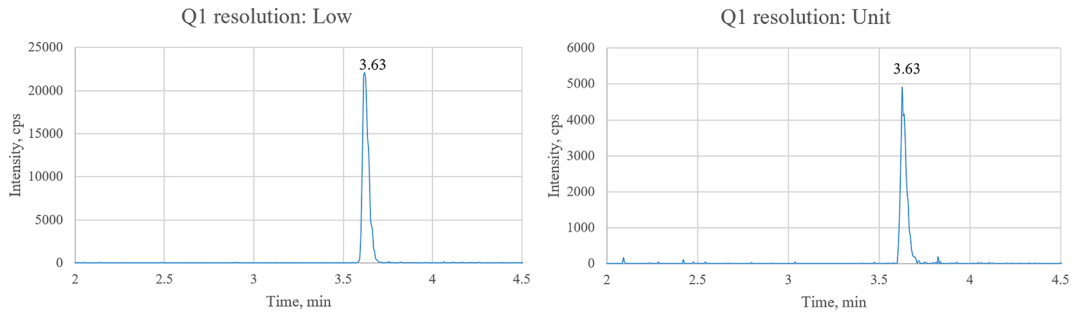

Fig. 3. LC-MS/MS of C-peptide standard solution. The right and left panels show the response of C-peptide when Q1 resolution was set as low and the unit, respectively. By changing Q1 resolution from unit to low, the intensity of C-peptide was enhanced nearly four times.

for many other proteins or peptides. However, it is not suitable for all ID-LC-MS/MS methods, since confirmation is needed to determine how this modification will impact specificity. Particularly, it is necessary to consider whether the changed resolution is sufficient to distinguish the analyte and its isotopic internal standard on the LC-MS/MS, and if analyte responses are enhanced more than the interfering substances.

Fragmentation efficiency

As mentioned above, the fragmentation efficiency of C-peptide is negatively impacted by its highly acid nature [38,41]. Below, we summarize two common approaches implemented in published ID-LC-MS/MS measurement procedures for overcoming the problem of low fragmentation efficiency of C-peptide.

(1) *Mode*. Single-ion monitoring (SIM) electrospray mode is a good choice to analyze C-peptide which can avoid low fragmentation efficiency, but the specificity of the measurement should be carefully considered [23].
(2) *Derivatization*. This approach is commonly used for target compounds in GC–MS measurement procedures, but derivatization is also quite useful to enhance signals in LC-MS/MS for C-peptide [45]. In 2014, Kinumi et al. used 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) to modify the N-terminal amino group of C-peptide, which dramatically increased fragmentation efficiency by adding a positive charge to the peptide. This currently represents the most sensitive published ID-LC-MS/MS measurement procedure for C-peptide [36].

ID-LC-MS/MS in the clinical laboratory

Through assessing the function of pancreatic beta cells, C-peptide detection plays an important role in the diagnosis and treatment of diabetes. In the clinical setting, there are many advantages in the ID-LC-MS/MS-based detection of C-peptide compared to immunoassay. Here, some clinical laboratories have made successful use of highly automated ID-LC-MS/MS methods to detect C-peptide. In 2016 Chen et al. [35] reported a routine ID-LC-MS/MS procedure to simultaneously measure C-peptide and insulin. The procedure involved online immune-affinity extraction and two-dimensional high-performance liquid chromatography (HPLC) to purify samples before MS injection. The performance of the assay meets clinical requirements with a recovery rate of 95–106% and a linear range of 0.11 to 27.2 ng/mL. The most impressive advantage of this method is the almost fully automated sample preparation procedure, which satisfies the high-throughput requirement of clinical laboratories. However, like previous studies, the values obtained by ID-LC-MS/MS were lower than results from immunological methods. The causes are unknown, but are likely related to the fact that the immunological results might be overestimated due to cross-reactions between C-peptide anti-serum and proinsulin or C-peptide metabolite fragments.

After developing the reference measurement procedure for C-peptide in 2011, Stoyanov et al. continued to improve sample preparation procedures leading to the successful development of a semi-online process in 2016 based on anion ion-exchange chromatography and reverse-phase chromatography [29]. It is worth noting that even though this sample preparation procedure is not completely automatic, it represents an improvement over the former measurement procedure. Importantly, this also indicates that ID-LC-MS/MS measurement procedures can be simplified and used in clinical laboratories by exploring automatic sample preparation procedures.

More recently, in 2020, Thomas et al.[30] established an ID-LC-MS/MS procedure to simultaneously assess insulin, insulin analogs, and C-peptide. The simple sample preparation procedure of this method is based on protein precipitation and cation-exchange SPE. This method is inexpensive and simple, but presently requires several manual operations and, therefore, may be difficult to implement in an automated or semi-automated high-throughput setting.

As mentioned above, ID-LC-MS/MS achieves high-throughput, automated detection of serum or urine C-peptide while possessing many other unique advantages. For example, one single injection can simultaneously detect different compounds with high sensitivity and specificity. Importantly, its measurements are not affected by the cross-reactivities seen in immunoassays. Nonetheless, the current obstacles are high initial cost and the requirement for professional operators. It is expected, however, that the unique advantages of ID-LC-MS/MS will promote further development of the technology and the associated costs are expected to gradually decrease, as such it will be more widely used in clinical laboratories in the future. Moreover, as the reference measurement procedure for C-peptide, ID-LC-MS/MS also played a key role in the standardization of C-peptide measurement and the invention of certified reference materials.

The standardization of C-peptide measurement

Standardization and harmonization activities are two principal approaches to improve the comparability of measurements among clinical laboratories. Both activities aim to establish the unbroken metrological traceability chain where measurements can be traced to reference systems consisting of reference methods and materials. Standardization means the measurement results of a well-defined measurand in clinical laboratories can be traced to the International System of Units. However, under some conditions, the results cannot be traced to the SI unit and can only be traced to a reference system agreed on by convention, which is known as harmonization. Both standardization and harmonization aim to reduce the variation between laboratories [46]. In principle, as a clearly defined compound, C-peptide measurements can be traced to the SI unit through standardization activities, which typically include the following steps: (1) developing reference systems consisting of reference measurement procedures and reference materials that can be traced to the SI unit, (2) tracing the results in clinical laboratories to the established reference system through a commutable calibrator, (3) verifying comparability of daily measurements by measuring a set of authentic patient samples across different methods and different

laboratories.

The existing reference system of C-peptide measurement

Reference systems are inclusive of reference methods, reference materials, reference laboratories, and reference services. The Joint Committee for Traceability in Laboratory Medicine (JCTLM) was established in 2002 to improve the standardization and harmonization of clinical measurements across the world. The database established by JCTLM contains lists of higher-order reference materials, higher-order reference measurement procedures, and accredited reference laboratory services [47].

There are two reference procedures for C-peptide in the JCTLM list, both are based on ID-LC-MS/MS. The first method developed by Stoyanov et al.[27] in 2011 used protein precipitation and ion-exchange chromatography to effectively isolate C-peptide from the complex matrix. The total relative CV is<4.5% and linear range is 0.01–3 ng (r^2 = 0.998) on the column. The second method developed by Kinumi et al. [36] uses an immunoaffinity purification and precolumn derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) to purify C-peptide before ID-LC-MS/MS detection, which results in a 20-fold improvement in sensitivity. This procedure adopted C-peptide CRM 6901-b as the calibrator to ensure the results trace to the SI unit. The linear range is 0.003–2.9 ng on the column, which covered the reference interval of C-peptide. The total relative SDs at low and high concentrations were both<4.0%, and the mean recoveries of spike experiments of two different levels are between 99 and 103%.

With the help of ID-LC-MS/MS, the primary reference materials tracing to SI units (NMIJ CRM6901-b; NMIJ CRM6901-c) in the JCTLM list were produced successfully by the National Institute of Metrology of Japan (NMIJ) and can be used to calibrate the reference measurement procedures [48]. Notably, there are no qualified secondary calibrators in the JCTLM list. As shown in Fig. 4, due to the lack of the secondary calibrator, it is unable to establish an unbroken traceability chain to trace the results of routine methods to higher-order reference measurements procedure and SI units. Hence, the qualified second calibrator is a major issue that needs to be resolved before realizing the standardization of C-peptide measurements.

Like enzyme measurement, C-peptide measurement can also be traced to higher-order reference measurement procedures by calibrating manufacturers' commercial systems with a panel of native clinical samples assigned by accredited reference laboratories performing reference measurement procedures [49,50]. However, no accredited laboratories providing reference services for C-peptide measurements are currently listed in the JCTLM database. Hence, worldwide efforts are required to build more reference measurement procedures and provide reference services to manufacturers and/or clinical laboratories. It should be noted that the production and assignment of the reference materials, the development of the reference measurement procedures and reference measurement services often happen in different sites and different laboratories. To achieve standardization of C-peptide measurements, it is necessary to strengthen the cooperation of these agencies and develop unbroken traceability chains.

Conclusion

Accurate C-peptide measurement is crucial for diabetic patient care. However, the results between laboratories are not comparable, which impacts the application of C-peptide measurements in the clinical setting. The standardization of C-peptide measurements has been ongoing for nearly forty years since the WHO began providing the first International Reference Reagent. ID-LC-MS/MS is sensitive and plays an important role in the process of standardization of C-peptide measurement where it has unique advantages. However, adopting ID-LC-MS/MS to detect C-peptide requires consideration of the three specific difficulties involving sample preparation, sensitivity, and the biochemical properties of C-peptide, which have been discussed in this article. As MS techniques have matured for small molecule measurement in clinical laboratories, there has been a growing trend to use ID-LC-MS/MS systems to measure larger biomacromolecules, although this comes with added difficulties. Along with the development of MS technology, it is expected this approach will have more widespread clinical application in the future.

Owing to the lack of a certified secondary calibrator, routine

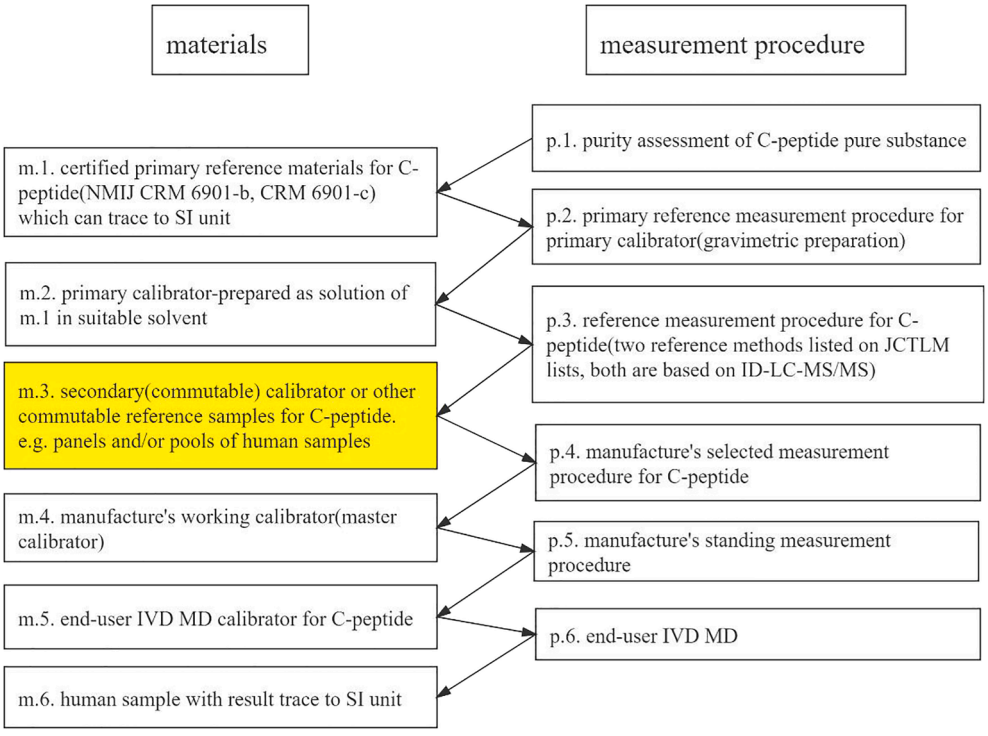

Fig. 4. The unbroken traceability chain of C-peptide. The three most important components of an unbroken traceability chain to the SI unit usually contain primary reference materials, reference measurement procedures, and the secondary calibrator. For C-peptide, there are primary reference materials and reference measurement procedures, but no qualified secondary calibrators in the JCTLM list. Therefore, an unbroken traceability chain cannot be established to trace the results of routine methods to higher-order reference measurement procedures and SI units.

measurement results still cannot trace to higher-order reference measurement systems of C-peptide. However, in addition to building an unbroken traceability chain of C-peptide measurement, clinical laboratories should participate in the EQA program and trueness verification programs to improve their measurement procedure. In concert, reference measurements procedures, such as that using ID-LC-MS/MS, and reference laboratories should be established across the world so that the standardization of routine measurements can be achieved via assigning clinical samples to calibrate commercial instruments of manufacturers.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Yuhang Deng researched literature and wrote the article. Chuanbao Zhang provided the initial idea of this review, Weiyan Zhou, Haijian Zhao, and Qingxiang Liu contributed to the scientific discussions about this review. All authors reviewed and edited the manuscript and approved the final version of the manuscript.

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