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Development and Application of Reference Measurement Procedures for C-peptide and Insulin in Human Serum

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Summary of used abbreviations

ACN	Acetonitrile
ADA	American Diabetes Association
ANOVA	Analysis of variance
BIPM	"Bureau International des Poids e Mesures" (International Bureau of Weights and Measures)
BSA	Bovine serum albumin
CEN	Comité Européen de Normalisation ("European Committee for Standardization")
CI	Confidence interval
CID	Collision induced dissociation
C-peptide	Connecting peptide
CV	Coefficient of variation
CV_{assay}	Total imprecision of the routine immunoassay
$CV_{between}$	Between-run coefficient of variation
CV_{mp}	Coefficient of variation of the method pair (total expected imprecision in a method comparison)
CV_{RMP}	Total imprecision of the reference measurement procedure
CV_{total}	Total coefficient of variation
CV_{within}	Within-run coefficient of variation
ESI	Electrospray ionization
ESI+	Positive electrospray ionization
ESI-	Negative electrospray ionization
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HOMA	Homeostatic model assessment
HPLC	High performance liquid chromatography
IAC	Immunoaffinity chromatography
ID-LC/tandem MS	Isotope dilution-liquid chromatography/tandem mass spectrometry
ID-MS	Isotope dilution-mass spectrometry
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
IgG	Immunoglobulin G
IRP	International reference preparation

IS	Internal standard
ISO	International Organization for Standardization
IUPAC	International Union of Pure and Applied Chemistry
IU	International unit
JCTLM	Joint Committee for Traceability in Laboratory Medicine
LC	Liquid chromatography
LC/MS	Liquid chromatography/mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
MS	Mass spectrometry
Mw	Molecular weight
m/z	Mass to charge ratio
RIA	Radio immunoassay
RML	Reference measurement laboratory
RMP	Reference measurement procedure
rpm	Revolutions per minute
SI-units	“Système International d’Unités“ (International System of Units)
S/N	Signal to noise ratio
SPE	Solid phase extraction
SRM	Selected reaction monitoring
TE	Total error
TFA	Trifluoroacetic acid
Th	Thomson (unit of m/z, where 1 Th=1 u/e)
wESI-	Negative wrong-way-round electrospray ionization mode
wESI+	Wrong-way-round electrospray ionization in the positive mode
WHO	World Health Organization

Definitions

The definitions of the following terms were taken from the “Vocabulaire International de Métrologie – Concepts fondamentaux et généraux et termes associés“ (VIM) (i):

- *Accuracy (measurement ~)*
  Closeness of agreement between a measured quantity value and a true quantity value of the measurand.
  Notes: 1. The concept ‘measurement accuracy’ is not given a numerical value, but a measurement is said to be more accurate when it offers a smaller measurement uncertainty. Measures of measurement accuracy are found in ISO 5725.
  2. The term “measurement accuracy” should not be used for ‘measurement trueness' and the term “measurement precision“ should not be used for ‘measurement accuracy’.

- *Bias (measurement ~)*
  Systematic measurement error or its estimate, with respect to a reference quantity value.

- *Detection limit or limit of detection*
  Measured quantity value, obtained by a given measurement procedure, for which the probability of falsely claiming the absence of a component in a material is β, given a probability α of falsely claiming its presence.
  Notes: 1. IUPAC recommends default values for α and β equal to 0.05.
  2. The abbreviation LOD is sometimes used.

- *Influence quantity*
  Quantity that, in a direct measurement, does not affect the quantity that is actually measured, but affects the relation between the indication and the measurement result.
  Notes: 1. An indirect measurement involves a combination of direct measurements, each of which may be affected by influence quantities.
  2. In the GUM, the concept ‘influence quantity’ is defined as in the 2nd edition of the VIM, covering not only the quantities affecting the measuring

system, as in the definition above, but also those quantities that affect the quantities actually measured. Also, in the GUM this concept is not restricted to direct measurements.

3. It is also called “interference”, but there is no international consensus about the definition of this term.

- *Measurand*

  Quantity intended to be measured.

  Notes: 1. The specification of a measurand requires description of the state of the phenomenon, body, or substance carrying the quantity, including any relevant component and the chemical entities involved.

  2. In the 2nd edition of the VIM and in IEC 60050-300:2001, the measurand is defined as the “quantity subject to measurement”.

  3. The measurement might change the phenomenon, body, or substance such that the quantity being measured may differ from the measurand. In this case adequate correction is necessary.

  4. In chemistry, “analyte”, or the name of a substance or compound, are terms sometimes used for ‘measurand’. This usage is erroneous because these terms do not refer to quantities.

  5. In chemistry, the measurand can be a biological activity.

- *Measurement*

  Process of experimentally obtaining one or more quantity values that can reasonably be attributed to a quantity.

  Notes: 1. Measurement implies comparison of quantities or counting of entities.

  2. Measurement presupposes description of the quantity commensurate with the intended use of the measurement result, a measurement procedure, and a calibrated measuring system operating according to a specified measurement procedure.

- *Metrological traceability*

  Property of a measurement result whereby the result can be related to a stated reference through a documented unbroken chain of calibrations, each contributing to the measurement uncertainty.

Notes: 1. For this definition, a ‘stated reference’ can be a definition of a measurement unit through its practical realization, or a measurement procedure including the measurement unit for a non ordinal quantity, or a measurement standard.

2. Metrological traceability requires an established calibration hierarchy.

3. Specification of the stated reference must include the time at which this reference was used, along with any other relevant metrological information about the reference, such as when the first calibration in the calibration hierarchy was performed.

4. For measurements with more than one input quantity in the measurement model, each of the input quantities should itself be metrologically traceable and the calibration hierarchy involved may form a branched structure or a network. The effort involved in establishing metrological traceability for each input quantity should be commensurate with its relative contribution to the measurement result.

5. Metrological traceability by itself does not ensure adequate measurement uncertainty or absence of mistakes.

6. A comparison between two measurement standards may be viewed as a calibration if the comparison is used to check and, if necessary, correct the quantity value and measurement uncertainty attributed to one of the measurement standards.

7. The abbreviated term “traceability” is sometimes used for ‘metrological traceability’ as well as for other concepts, such as ‘sample traceability' or ‘document traceability’ or ‘instrument traceability’, where the history (‘trace’) of an item is meant. Therefore, the full term is preferred.

- *Precision*

  Closeness of agreement between indications obtained by replicate measurements on the same or similar objects under specified conditions.

  Notes: 1. Measurement precision is usually expressed numerically by measures of imprecision, such as standard deviation, variance, or coefficient of variation under the specified conditions of measurement.

2. The ‘specified conditions’ can be repeatability conditions of measurement, intermediate precision conditions of measurement, or reproducibility conditions of measurement (see ISO 5725-5:1998).

3. Measurement precision is used to define measurement repeatability, intermediate measurement precision, and measurement reproducibility.

4. Sometimes “precision” is erroneously used to mean ‘measurement accuracy (2)’.

- *Repeatability (measurement ~)*
  Measurement precision under a set of repeatability conditions of measurement.

- *Repeatability condition of measurement*
  Condition of measurement in a set of conditions that includes the same measurement procedure, same operators, same measuring system, same operating conditions and same location, and replicate measurements on the same or similar objects over a short period of time
  Note: In chemistry, the term ‘intra-serial precision condition of measurement’ is sometimes used to designate this concept.

- *Reproducibility (measurement ~)*
  Measurement precision under reproducibility conditions of measurement.
  Note: Relevant statistical terms are given in ISO 5725-2:1998.

- *Reproducibility condition of measurement*
  Condition of measurement in a set of conditions that includes different locations, operators, measuring systems, and replicate measurements on the same or similar objects.
  Notes: 1. The different measuring systems may use different measurement procedures.
  2. A specification should give the conditions changed and unchanged, to the extent practical.

- *Selectivity (~ of a measuring system)*

  Capability of a measuring system, using a specified measurement procedure, to provide measurement results, for one or more measurands, that do not depend on each other nor on any other quantity in the system undergoing measurement.

  Notes: 1. In physics, there is only one measurand, the other quantities are of the same kind as the measurand, and they are input quantities to the measuring system.

  2. In chemistry, the measured quantities often involve different components in the system undergoing measurement and these quantities are not necessarily of the same kind.

  3. In chemistry, selectivity of a measuring system is usually obtained for quantities with selected components in concentrations within stated intervals.

  4. Selectivity as used in physics (see Note 1) is a concept close to specificity as it is sometimes used in chemistry.

- *Sensitivity*

  Quotient of the change in the indication and the corresponding change in the value of the quantity being measured.

  Notes: 1. The sensitivity can depend on the value of the quantity being measured.

  2. The change considered in the value of the quantity being measured must be large compared with the resolution.

- *Trueness (measurement ~)*

  Closeness of agreement between the average of an infinite number of replicate measured quantity values and a reference quantity value.

  Notes: 1. A reference quantity value can be a true quantity value of the measurand or an assigned quantity value of a measurement standard with negligible measurement uncertainty.

  2. Measurement trueness cannot be expressed numerically, but measures are given in ISO 5725.

  3. Measurement trueness is inversely related to only systematic measurement error.

4. The term “measurement trueness” should not be used for ‘measurement accuracy‘ and vice versa.

- *Validation*

  Verification, where the specified requirements are adequate for a stated use.

  Note: 1. The ISO 9000 (ii) has another definition to this term: “Confirmation, through the provision of objective evidence, that requirements for a specific intended use or application have been fulfilled”.

- *Verification*

  Provision of objective evidence that a given item fulfils specified requirements, taking any measurement uncertainty into consideration.

  Notes: 1. The item may be, e.g., a process, measurement procedure, material, compound, or measuring system.

  2. The specified requirements may be, e.g., that a manufacturer’s specifications are met.

  3. In legal metrology, verification pertains to the examination and marking and/or issuing of a verification certificate for a measuring instrument.

  4. Verification should not be confused with calibration or validation.

  5. In chemistry, verification of identity of entity involved, or of activity, requires a description of the structure or properties of that entity or activity.

The following definitions were taken from the standard ISO 17511 (iii):

- *Analytical specificity*

  Ability of a measurement procedure to measure solely the measurand.

- *Commutability of a material*

  Degree to which a material yields the same numerical relationships between results of measurements by a given set of measurement procedures, purporting to measure the same measurable quantity, as those between the expectations of the relationships for the same procedures applied to those other types of material for which the procedures are intended.

The term *evaluation* is not officially defined by any organization but it is a generic term for any study that measures the performance capabilities of a method. An alternative term is *study.*

References

i. BIPM, IEC, IFCC, ISO, IUPAC, IUPAP, OIML. ISO:2007 International Vocabulary of Metrology – Basic and General Concepts and Associated Terms (VIM). 3rd ed.

ii. ISO. ISO 9000:2005 Quality management systems – Fundamentals and vocabulary. Definition is also available from internet: www.clsi.org (Accessed June 2007).

iii. ISO and CEN. ISO 17511:2003 In vitro diagnostic medical devices – Measurement of quantities in biological samples – Metrological traceability of values assigned to calibrators and control materials. Available from internet: www.clsi.org or www.nccls.org (Accessed June 2007).

Index

CHAPTER I.

BACKGROUND AND OBJECTIVES FOR THE DEVELOPMENT OF REFERENCE MEASUREMENT PROCEDURES FOR THE DETERMINATION OF C-PEPTIDE AND INSULIN IN HUMAN SERUM

For a more detailed information on this topic see:

- Thienpont LM, Van Uytfanghe K, Rodríguez Cabaleiro D. Metrological traceability of calibration in the estimation and use of common medical decision-making criteria. Clin Chem Lab Med 2004;42:842-50 [Review].

I.1. BACKGROUND

I.1.1. Metrological considerations

It has long been known that for comparability of analytical results in clinical chemistry a generally accepted coherent measurement or reference system is necessary. To fully understand the basis of such a system, the following considerations are necessary.

The full specification of a measurement in the clinical laboratory comprises the description of the following three elements, i.e., the system (e.g., blood serum), the component (also called analyte, e.g., insulin), and the kind-of-quantity (e.g., amount-of-substance concentration) (1,2). All elements together are called the measurand (3). The value of a quantity, determined by a measurement, is expressed by both a number and an unit. As a consequence, in order to obtain comparable measurement results, it is a basic requirement that they are expressed in commonly accepted units. For this purpose, the international metrological society developed the “Système International d’Unités” (1), better known as SI-units. The “International Federation of Clinical Chemistry and Laboratory Medicine” (IFCC) recommended the use of the aforementioned units in clinical chemistry, at least for analytes of which the identity is unambiguously known (4). The units used most often are those for the mass concentrations, i.e., g/L and substance concentrations, i.e., mol/L.

Note that as suggested before, in clinical chemistry many analytes lack an unequivocally defined entity with the consequence that they do not fit into the SI-system. Nevertheless, being clinically relevant analytes, they also require a common measurement unit for expression of laboratory measurement results. In response to that need, the World Health Organization (WHO) developed the concept of the International Unit (IU) (5) using the following principle. An International Standard material is prepared with state-of-the-art purification and identification techniques and its function is tested by the response of a biological system. The IU is then assigned by convention, e.g., one IU is assigned to one mg of preparation. Once defined, the IU is applied to all further International Reference Preparations (IRP). In this way, metrological consistency is achieved.

I.1.2. The reference measurement system: Metrological traceability chain and calibration hierarchy

Traceability is defined as a “property of a measurement result whereby the result can be related to a stated reference through a documented unbroken chain of calibrations, each contributing to the measurement uncertainty” (3). For measurement results expressed in SI-units, this property is the mechanism to track the path of a measurement from the SI-unit to the actual result. The mechanism consists of an unbroken chain of traceable comparisons, whereby each link has a known uncertainty of measurement, defined as the ‘‘parameter characterizing the dispersion of the quantity values being attributed to a measurand, based on the information used" (3).

The structure of such a hierarchical chain of comparisons, further referred to as the metrological traceability chain, is presented in Figure I.1. In general, it can be described as a multilevel hierarchy of alternating measurement procedures and calibration materials. The arrows indicate how these elements are combined: at each level, the calibration material on the left side is used to calibrate the measurement procedure on the right. The latter serves the purpose of assigning a value to the calibration material at the level below. In Figure I.1, the SI-traceability chain starts at the top with the definition of the appropriate SI-unit for the measurand (system, analyte, kind-of-quantity), followed by the primary realization of the SI-unit (via a primary reference measurement procedure (RMP) and a primary calibrator). The traceability chain ends with the end-user’s routine measurement procedure applied to produce a result from a sample. The SI-traceability chain shown in Figure I.1 is the most extensive one. To reduce the uncertainty that accumulates at each of the different levels, one might advocate the omission of pairs of consecutive levels of calibrator and procedure (e.g., the secondary calibrator and the manufacturer’s selected measurement procedure).

The above described traceability chain and calibration hierarchy form the so called reference measurement system. The key to trueness of measurement results in clinical chemistry is the establishment of traceability of measurements to the highest international standards by a comprehensive reference measurement system (6). It should be mentioned that this standard is written in relation to the European legislation, laid down in the EC Directive on In-vitro Diagnostic Medical Devices (98/79/EC) and requiring that manufacturers demonstrate metrological traceability of their products (7).

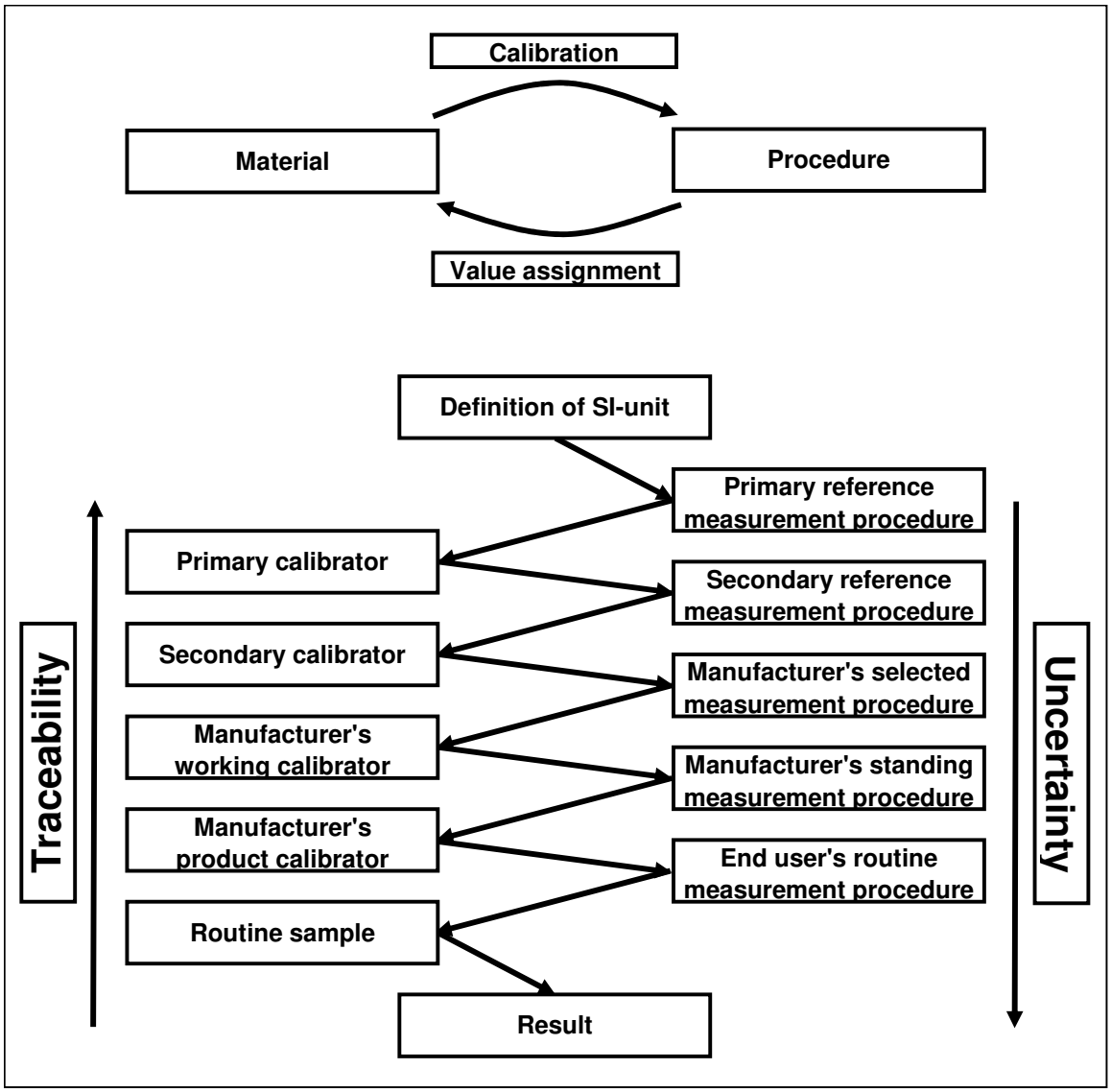

Figure I.1. Extensive calibration hierarchy to ensure metrological traceability to the SI (adapted from EN/ISO 17511) (6).

I.1.3. Reference measurement procedures

The International Vocabulary of Metrology (VIM) (3) defines a RMP as a “measurement procedure accepted as providing measurement results fit for their intended use in assessing measurement trueness of measured quantity values obtained from other measurement procedures for quantities of the same kind, or in characterizing reference materials”. In clinical chemistry the main purpose of RMPs is to make results of field assays metrologically traceable to the highest international standard or SI. In this way, the comparability of laboratory results independent of time, place and used measurement

system is achievable, as well as the use of common reference intervals and decision limits.

The International Organization for Standardization (ISO) recommends that for standardization of physico-chemically well-defined analytes an accuracy-based higher order measurement procedure with traceability to the SI-unit should be preferably used. The prerequisites for a measurement procedure to be qualified as accuracy-based RMP are: it must be directly calibrated (matrix free) with primary calibrators, it is based on a matrix-independent measuring principle (absence of sample related effects) and it has either a smaller imprecision than the routine measurement procedure or performs a greater number of measurements (8). With respect to the method principle to be at the basis of a SI-traceable measurement procedure for organic analytes, isotope dilution-liquid chromatography/tandem mass spectrometry (ID-LC/tandem MS) is a valid one (5,6,9). This is by virtue of the combination of 3 effects: compensation for possible losses of the analyte (e.g., adsorption and degradation of the analyte, incomplete recovery) and incontrollable variables during the measurement procedure (e.g., ion suppression) by the stable isotopically labeled analyte that is added to samples and calibrators in the earliest as possible phase; separation of the analyte from other matrix constituents in the chromatographic step; specificity of the mass spectrometric detection at m/z values characteristic for the analyte, or in case of tandem MS, for a precursor to product ion transition. In other words, an optimized ID-LC/tandem MS measurement procedure is capable of controlling the stability and recovery of the analyte during the entire measurement procedure and producing interference-free quantification of the analyte in accordance with its definition.

For a particular RMP, the analytical requirements should, to a certain extent, be related to the medically required quality of the routine measurement procedure it intends to calibrate (8). It is obvious that to guarantee these performance specifications, a RMP should be performed under conditions of rigorous internal quality control. In addition, RMPs should be performed in competent reference measurement laboratories (RMLs) working under defined performance conditions (10), for example, as is the case in so-called networks of RMLs (11). As already addressed above, the traceability chain is intended to make hierarchically lower routine measurement procedures traceable to the SI. Ideally, this requirement could be realized exactly in the same way as described previously for RMPs, i.e., by using primary calibrators as manufacturer's working calibrators. However, most method principles at the basis of routine measurement

procedures severely hamper direct calibration due to commutability problems. The commutability problems come down to the fact that routine measurement procedures may have a different behavior with a calibrator than with a patient sample. A solution to that problem is to use matrix-matched calibrators, e.g. native samples with values assigned by a matrix-independent RMP, itself being calibrated with the primary calibrator (12). This calibration approach is also called ‘‘split-sample measurement design’’. Up to now, however, guidelines are missing for ‘‘valid’’ calibration using such a design. Indeed, before split-sample measurements are applied for calibration of a routine measurement procedure, it is essential that this procedure is proven sufficiently specific.

The requirements of RMPs in terms of measurement principle and analytical performance specifications can be obtained from specific literature (13) and the ISO 15193 (14). Since RMPs are per definition associated with reference materials (see traceability chain) and are performed in RMLs, it is also of interest mentioning the ISO 15194 (describing reference materials) (15) and the ISO 15195 (specifying the required level of competence of a RML) (16).

I.1.4. Current situation with regard to organizing the implementation of reference measurement systems

The responsibilities for establishing the metrological traceability chain are as follows (6): the international and national institutions such as the International Bureau of Weights and Measures (BIPM), National Metrological Institutes and accredited RMLs are responsible for assigning values to the primary and secondary calibrators; documentation of the traceability of the end-user’s routine measurement procedure is the responsibility of the manufacturer and/or the end-user; everything in between is the responsibility of the IVD manufacturers.

Up to this point it appears, at least on paper, that complying with the traceability requirement of the European legislation should work (7). However, to what extent have the elements of the necessary reference measurement systems been established? The answer to that question is that candidate RMPs, reference materials of ‘a higher order’ and RMLs exist, however, without official endorsement by an agency created by the European legislators. In response to this need, a ‘framework’ has recently been established for international recognition. The Joint Committee for Traceability in Laboratory Medicine (JCTLM) has been created through a “Declaration of Co-operation”

by the International Committee of Weights and Measures (CIPM) and BIPM, IFCC and the International Laboratory Accreditation Cooperation (ILAC), as principal promoters/stakeholders, together with other key stakeholders such as producers of certified reference materials, the IVD industry, proficiency testing organizers, regulatory bodies, etc. Meanwhile, the JCTLM has implemented a process whereby reference materials, RMPs and RMLs can be nominated and are assessed for conformity with appropriate international documentary standards (mainly the above mentioned ISO 15193, 15194 and 15195). The results of this process are published already for reference materials and RMPs on the BIPM and IFCC websites (www.bipm.fr and www.ifcc.org) (17). Those for RMLs will be published soon. In addition, as required by the ISO 15195, proficiency testing surveys for RMLs are currently being organized on behalf of the IFCC, so that technical competence can be demonstrated. This mechanism of interlaboratory comparisons is comparable to that used in networks of RMLs. Future plans are to make an 'International Recognition Arrangement' that will be available to all interested stakeholders who make reference to the JCTLM framework and databases in any relevant documentation they produce. Last but not least, it should be mentioned that up to now the JCTLM has no legal status. Therefore, it is not yet clear whether the decisions made by this organization will be accepted by the European Commission.

I.2. OBJECTIVES OF THE RESEARCH

Due to the world wide diabetes mellitus epidemic there is an increasing interest in the measurement of insulin and C-peptide. Insulin serum concentrations provide important information for the estimation of insulin secretion and resistance. For example, indices such as those derived from homeostatic model assessment (HOMA) are widely used as a measure of insulin resistance, sensitivity and beta-cell function in clinical practice, as well as in research or epidemiological studies. Also the pharmaceutical industry, currently involved in the development of new therapeutic tools for the treatment of diabetes type II patients, i.e., the so-called insulin sensitizers, is strongly interested in the HOMA insulin resistance indices. Likewise, C-peptide determination has multiple applications in clinical practice and research. It is even accepted that C-peptide concentrations provide a more reliable reflection of the pancreatic function than insulin (18-20).

Unfortunately, the demonstrated lack of between-assay comparability counteracts the aforementioned utility of insulin and C-peptide measurements and necessitates either testing under controlled analytical conditions or interpreting results against assay-specific reference intervals (18,21-29). For example, the C-peptide cut-point value of 0.5 µg/L, initially issued by Medicare and Medicaid regulations, had to be replaced by a value ≤110% of the assay-specific lowest reference value (30); cut-off or area under the curve values used in the definition of fulminant type I diabetes mellitus or preservation of beta cell function still need to be accompanied by rigorous specification of the used immunoassay (19,31). Also the calculated indices from HOMA insulin resistance vary to a greater extent since they depend on the absolute insulin concentrations, hence, currently using those indices in big epidemiologic studies with pooling of data is impossible. In other words, the problem of limited comparability of data generally hinders the development of uniform clinical practice guidelines.

The statements given in the background section of this dissertation make it obvious that the solution to the lack of between-assay comparability is an accuracy-based standardization of both insulin and C-peptide assays. Also as explained before, the way to do the standardization is straightforward, i.e., by application of the SI-traceability chain comprising adequate RMPs and reference materials. However, to the best of our knowledge at the start of this dissertation, no such SI-traceability chain existed for insulin, nor for C-peptide. Consequently, the few attempts at standardization that have been

undertaken until now were all based on a different concept which, unfortunately, was likely to fail.

In the case of insulin, in 1996 the American Diabetes Association (ADA) organized a task force on the standardization of insulin assays (26). The efforts started with the assessment of the comparability of insulin measurements between laboratories using 17 insulin assays. Because significant variability in insulin values was obtained for the distributed samples, the task force tried to improve the situation by using the approach of a common calibration material and curve fitting procedure. Unfortunately, these steps did not succeed in reducing the variability, which was in fact no surprise because of the known risk of non-commutability of the calibration material used (32-34). Despite of calibration of immunoassays with such a common material and curve fitting, the assays may still give discrepant results on patient samples because of a different behavior with the latter versus with the calibrator (9). In addition, the approach does not account for the difference in specificity between immunoassays, which may be attributable to the heterogeneity in circulating insulin forms that react to a greater or lesser extent with the different antisera, or to cross-reactivity with, for example, proinsulin. As a consequence of the observed difference in assay bias, the task force concluded from this study that it was not recommendable to use different insulin assays within a population or research study, unless beforehand sufficient specificity and interassay comparability was ensured from, respectively, validation and interlaboratory comparison studies according to the task force's guidelines. In 2004, a follow-up of the study was done by the ADA in conjunction with the National Institute of Diabetes & Digestive & Kidney Diseases (NIDDK) and the Centers for Disease Control and Prevention (CDC). An international work group was convened to evaluate the specificity of different assays, to establish guidelines for assay acceptability, and to develop a standardization program to achieve uniform, accuracy-based values (27). Again the work group started the new project by repeating the 1996 study but with eleven more recent commercially available insulin assays. The report confirmed a second time that (27) "a common insulin reference preparation did not change the among-assay coefficient of variation and failed to improve harmonization of results among assays". The work group also concluded that to provide a metrologically appropriate basis for the measurement of insulin, there was an urgent need of a RMP with an international standard defined in terms of mass.

In parallel to the above study, a similar study in 2004 in the UK also was performed by a new clinical research network for diabetes (35). In this study also eleven

currently available commercial assays performed in different laboratories were involved. The conclusion of this network's recent report in 2007 (28) was that the source of discrepancies in results is likely due to multiple factors and not explainable by a single analytical performance characteristic. The group also observed that not all insulin assays have acceptable performance characteristics at concentrations as low as 12 pmol/L, as is needed for clinical use. Last but not least they appealed upon the development of a RMP for standardization, instead of the common calibrator approach.

For C-peptide, already one decade ago, authoritative associations such as the ADA and the NIDDK also appealed to the professionals and the diagnostics industry for the urgent standardization of immunoassays (26, 36). Once again, in this case it was assumed that the approach of using a common calibration material would help, but it failed (22, 26). Despite this failure, it was repeated by a new working group (members of the NIDDK C-peptide Standardization Committee), but this time a WHO reference reagent and plasma were used to serve as common calibrator. The outcome of this latest study (29, 36) was that normalization of results against the WHO reference reagent did not improve comparability, but against plasma it did. Nevertheless, the study concluded that the improvement in comparability may be insufficient to fulfill the requirements of clinical trials and better be replaced by a valid standardization basis using an accuracy-based RMP. To the best of our knowledge, no further progress in this direction was made by the group (23, 37).

In summary, all aforementioned work groups (for C-peptide and insulin) agreed that a metrologically valid standardization basis was needed, i.e., comprising an accuracy-based RMP, calibrated with an international primary calibrator defined in terms of mass and used to assign values to the manufacturer's working calibrator (see SI-traceability chain in Figure I.1). However, realizing that standardization makes no sense if the assay is not sufficiently valid from the analytical point of view, the work groups also defined assay performance criteria (27, 28) to be used in a preliminary validation process with the RMP.

The outcome and conclusions from the aforementioned studies stimulated our laboratory to make work of the development of the required standardization approach. Therefore, we aimed to develop a RMP for both C-peptide and insulin and to perform a split-sample comparison using a set of native samples with values assigned by our RMP (5,9). From our expertise with standardization of other analytes, we concluded that a RMP for peptides and small proteins best be based on ID-LC/MS, because it guarantees the

specific and accurate measurement of the analyte and allows direct calibration with a primary calibrator defined in terms of mass (5,6,9).

Whereas our objectives for C-peptide would be realized entirely at the expenses of our laboratory (note that we started with C-peptide), the objectives for insulin were funded by the ADA in the form of a grant (38).

Below, the specific features of our C-peptide and insulin objectives will be detailed.

C-peptide

With regard to the development of a RMP for C-peptide in serum, we decided to start from our previously developed ID-LC/tandem MS measurement procedure for the determination of C-peptide in urine (39). With the objective in mind to move from the urine to the serum matrix, it was obvious from the beginning that we would be faced with two major challenges: the need to increase specificity (serum is a much more complex matrix) and sensitivity (the concentrations of C-peptide in serum are 50 times lower than in urine). Since the same LC/tandem MS hardware had to be used, we decided to focus in particular on the specificity of the sample pretreatment procedure in order to improve the signal-to-noise (S/N) ratio and, hence, the limits of detection (LOD) and quantification (LOQ) after processing of a reasonable volume of serum. In addition, in comparison to previously published liquid chromatography-mass spectrometry (LC-MS) procedures for C-peptide in serum (40, 41), it became our aim looking for a robust LC/tandem MS measurement procedure with a relatively short total analysis time to achieve sufficient sample throughput and column life-time. To obtain the aforementioned objectives, the following detailed strategy was applied:

- Collision-induced dissociation (CID) experiments for elucidation of the fragmentation pathway and selection of CID conditions for the final selected reaction monitoring (SRM).
- Development and optimization of a sample preparation based on a two-step solid phase extraction (SPE) procedure and comparison with the SPE-based sample preparation procedures existing in the literature (40, 41).
- Development and testing of an alternative sample preparation procedure based on immunoaffinity chromatography (IAC).
- Optimization of the LC/tandem MS parameters for superior chromatography and sensitivity.

- Validation of the ID-LC/tandem MS procedure, using the two-step SPE as the sample preparation procedure.
- Investigation of the method feasibility for the purpose of standardization by performing a method-comparison study with three representative commercial assays.

Insulin

Our objectives were to develop a sample preparation procedure that would, in combination with the good chromatographic resolution of the high performance liquid chromatography (HPLC) step prior to optimal CID tandem MS, account for the demands in specificity and S/N necessary for the quantification of insulin in physiological concentrations (LOQ). We applied also special efforts in order to achieve a RMP of the quality level as required by the performance specifications of the ADA. We based the development of our procedure on work that has been done before (40-43). However, only one method (40) had sufficient sensitivity and followed the principle of ID-MS, which is, as explained before, an essential feature for a RMP. In addition, this method employed an off-line HPLC purification with a 60-min separation time. Therefore, we aimed to improve the sample-preparation, the chromatography, and the mass spectrometric detection for the analysis of insulin in human serum. To reach these objectives, the following strategy was applied:

- Development of a suitable sample preparation procedure. Two approaches were studied, one sample preparation based on a two-step SPE procedure, attempting to improve previously published methods (40, 41) and another based on IAC being a modification of previous methods in the literature (40, 42, 43).
- Development of a sensitive LC/tandem MS procedure and comparison with previously published methods (40-43). CID experiments with different gases and instruments were performed in order to find conditions for more sensitive detection in the SRM mode.
- Establish the link between the Sigma standard and the WHO insulin 1st IRP 66/304.
- Validation of the ID-LC/tandem MS procedure using IAC as sample preparation procedure.
- Investigation of the method feasibility for the purpose of standardization by performing a method-comparison study with four representative commercial assays.

I.3. REFERENCES

1. International Federation of Clinical Chemistry, International Union of Pure and Applied Chemistry. Approved recommendations (1978) on quantities and units in clinical chemistry. Prepared for publication by R. Dybkaer. Pure Appl Chem 1979;51:2451-79.
2. International Union of Pure and Applied Chemistry (IUPAC). International Federation of Clinical Chemistry (IFCC). Properties and units in the clinical laboratory sciences: I. Syntax and semantic rules (Recommendation 1995). Prepared for publication by H. Olesen. Eur J Clin Chem Clin Biochem 1995;33:627-36.
3. BIPM, IEC, IFCC, ISO, IUPAC, IUPAP, OIML. ISO: 2007 International Vocabulary of Metrology – Basic and General Concepts and Associated Terms (VIM). 3rd ed.
4. International Federation of Clinical Chemistry, International Union of Pure and Applied Chemistry. Approved recommendations (1978) on quantities and units in clinical chemistry. Prepared for publication by R. Dybkaer. Pure Appl Chem 1979;51:2451-79.
5. Thienpont LM, Van Uytfanghe K, De Leenheer AP. Reference measurement systems in clinical chemistry. Clin Chim Acta 2002;323:73-78 [Review].
6. ISO and CEN. ISO 17511:2003 In vitro diagnostic medical devices – Measurement of quantities in biological samples – Metrological traceability of values assigned to calibrators and control materials. Available from internet: www.clsi.org or www.nccls.org (Accessed June 2007).
7. Directive 98/79/EC of the European Parliament and of the Council of 27 October 1998 on in vitro diagnostic medical devices. Official Journal of the European Communities 1998:L 331/1-37.
8. Stöckl D, Franzini C, Kratochvila J, Middle J, Ricós C, Siekmann L, et al. Analytical specifications of reference measurement procedures. Compilation and critical discussion (From the members of the European EQA-Organizers Working Group B). Eur J Clin Chem Clin Biochem 1996;34:319-37 [Review].
9. Stöckl D, Franzini C, Kratochvila J, Middle J, Ricos C, Thienpont LM. Current stage of standardization of measurements of specific polypeptides and proteins discussed in light of steps needed towards a comprehensive measurement system. Eur J Clin Chem Clin Biochem 1997;35:719-32.

10. Thienpont LM, Franzini C, Kratochvila J, Middle J, Ricos C, Siekmann L, et al. Analytical quality specifications for reference methods and operating specifications for networks of reference laboratories. Recommendations of the European EQA-Organizers Working Group B. Eur J Clin Chem Clin Biochem 1995;33:949-57.
11. Siekmann L, Doumas BT, Thienpont L, Schumann G. Reference materials and reference measurement systems in laboratory medicine. Networks of reference laboratories. Eur J Clin Chem Clin Biochem 1995;33:1013-7.
12. Lasky FD. Achieving accuracy for routine clinical chemistry methods by using patient specimen correlations to assign calibrator values: a means of managing matrix effects. Arch Pathol Lab Med 1993;117:412-9.
13. Thienpont LMR. Quality specifications for reference methods. Scand J Clin Lab Invest 1999;59:535–8.
14. ISO. ISO 15193:2002 In vitro diagnostic medical devices -- Measurement of quantities in samples of biological origin -- Presentation of reference measurement procedures.
15. ISO. ISO 15194:2002 In vitro diagnostic medical devices -- Measurement of quantities in samples of biological origin -- Description of reference materials.
16. ISO. ISO 15195:2003 Laboratory medicine -- Requirements for reference measurement laboratories.
17. JCTLM database: Laboratory medicine and in vitro diagnostics: Database of higher order reference materials and reference measurement procedures. http://www1.bipm.org/en/committees/jc/jctlm/. (Accessed June 2007).
18. Palmer JP, Fleming GA, Greenbaum CJ, Herold KC, Jansa LD, Kolb H, et al. C-peptide is the appropriate outcome measure for type I diabetes clinical trials to preserve β-cell function: report of an ADA workshop, 21–22 October 2001 [Erratum published in: Diabetes 2004;53:1934]. Diabetes 2004;53:250–64.
19. Maruyama T, Shimada A, Kanatsuka A, Kasuga A, Takei I, Yokoyama J, Kobayashi T. Multicenter prevention trial of slowly progressive type I diabetes with small dose of insulin (the Tokyo study): preliminary report. Ann N Y Acad Sci 2003;1005:362-9.
20. Greenbaum CJ, Harrison LC. Immunology of Diabetes Society. Guidelines for intervention trials in subjects with newly diagnosed type I diabetes. Diabetes 2003;52:1059-65 [Erratum in: Diabetes 2003;52:2643].

21. Service FJ, Rizza RA, Zimmerman BR, Dyck PJ, O'Brien PC, Melton LJ 3rd. The classification of diabetes by clinical and C-peptide criteria. A prospective population-based study. Diabetes Care 1997;20:198-201.
22. Clark PM. Assays for insulin, proinsulin(s) and C-peptide. Ann Clin Biochem 1999;36:541-564 [Review].
23. Nauck MS, Nauck MA, Nauck MA, Marz W, Wieland H. Six methods for the determination of C-peptide evaluated. Clin Chem Lab Med 1999;37:745–51.
24. Sacks DB, Bruns DE, Goldstein DE, Maclaren NK, McDonald JM, Parrott M. Guidelines and recommendations for laboratory analysis in the diagnosis and management of diabetes mellitus. Clin Chem 2002;48:436–72.
25. Koskinen P. Non transferability of C-peptide measurements with various commercial radioimmunoassay reagents. Clin Chem 1988;34:1575–8.
26. Robbins DC, Andersen L, Bowsher R, Chance R, Dinesen B, Frank B et al. Report of the American Diabetes Association's task force on standardization of the insulin assay. Diabetes 1996;45:242-56.
27. Marcovina S, Bowsher RR, Miller WG, Staten M, Myers G, Caudill SP, et al. Standardization of Insulin Immunoassays: Report of the American Diabetes Association work group. Clin Chem 2007;53:711-6.
28. Manley SE, Stratton IM, Clark PM, Luzio SD. Multi-comparison of human insulin assays: implications for clinical investigation and research. Clin Chem 2007;53:922-32.
29. Wiedmeyer HM, Polonsky KS, Myers GL, Little RR, Greenbaum CJ, Goldstein DE et al. International Comparison of C-Peptide Measurements. Clin Chem 2007;53:784-7. [Epub 2007 Mar 1].
30. Centers for Medicare and Medicaid Services. Medicare Coverage Database. Proposed Decision Memo for Insulin Pump: C-Peptide Levels as a Criterion for Use (CAG-00092R). http://www.cms.hhs.gov/mcd/viewdecisionmemo.asp?id=109 (Accessed June 2007).
31. Tanaka S, Endo T, Aida K, Shimura H, Yokomori N, Kaneshige M, et al. Distinct diagnostic criteria of fulminant type I diabetes based on serum C-peptide response and HbA1c levels at onset. Diabetes Care 2004;27:1936-41.
32. Bangham DR. Standardization in peptide hormone immunoassays: principle and practice. Clin Chem 1976;22:957-63.

33. Caygill CP, Gaines Das RE, Bangham DR. Use of a common standard for comparison of insulin C-peptide measurements by different laboratories. Diabetologia 1980;18:197-204.
34. Bristow AF, Das RE. WHO international reference reagents for human proinsulin and human insulin C-peptide. J Biol Stand 1988;16:179-86.
35. Heller S, Kinmonth AL: A clinical research network in diabetes for the UK. Diabetic Med 2004;21:1061-3.
36. National Institute of Diabetes & Digestive & Kidney Diseases. Type I Diabetes Special Statutory Funding Program. Research Consortia and Clinical Trials Networks. http://www.t1diabetes.nih.gov/investigator/topic.asp?topicID=17 (accessed June 2007).
37. Summary of the Expert Panel Meeting on the Special Funding Program (January 18-19, 2005). http://www.niddk.nih.gov/federal/planning/Jan-18-19-T1D-Final.pdf (accessed June 2007).
38. Insulin Assay Standardization Project. American Diabetes Association award number 10-06-Insulin (2006).
39. Fierens C, Thienpont LM, Stöckl D, Willekens E, De Leenheer AP. Quantitative analysis of urinary C-peptide by liquid chromatography-tandem mass spectrometry with a stable isotopically labeled internal standard. J Chromatogr A 2000;896:275-8.
40. Kippen AD, Cerini F, Vadas L, Stöcklin R, Vu L, Offord RE, et al. Development of an isotope dilution assay for precise determination of insulin, C-peptide, and proinsulin levels in non-diabetic and type II diabetic individuals with comparison to immunoassay. J Biol Chem 1997;272:12513-22.
41. Darby SM, Miller ML, Allen RO, LeBeau M. A mass spectrometric method for quantitation of intact insulin in blood samples. J Anal Toxicol 2001;25:8-14.
42. Stöcklin R, Vu L, Vadas L, Cerini F, Kippen AD, Offord RE, et al. A stable Isotope dilution assay for the in vivo determination of insulin levels in humans by mass spectrometry. Diabetes 1997;46:44-50.
43. Thevis M, Thomas A, Delahaut P, Bosseloir A, Schanzer W. Doping control analysis of intact rapid-acting insulin analogues in human urine by liquid chromatography-tandem mass spectrometry. Anal Chem 2006;78:1897-903.
44. Thevis M, Thomas A, Delahaut P, Bosseloir A, Schanzer W. Qualitative determination of synthetic analogues of insulin in human plasma by immunoaffinity

purification and liquid chromatography-tandem mass spectrometry for doping control purposes. Anal Chem 2005;77:3579-85.

45. Hunter SJ, Boyd AC, O'Harte FP, McKillop AM, Wiggam MI, Mooney MH, et al. Demonstration of glycated insulin in human diabetic plasma and decreased biological activity assessed by euglycemic-hyperinsulinemic clamp technique in humans. Diabetes 2003;52:492-8.

CHAPTER II.

DEVELOPMENT, VALIDATION AND APPLICATION OF A REFERENCE MEASUREMENT PROCEDURE FOR C-PEPTIDE IN SERUM

The development of the ID-LC/tandem MS RMP for serum C-peptide was published in:

- Stöckl D, Rodríguez-Cabaleiro D, Thienpont LM. Collision-induced dissociation of the $[M-2H]^{2-}$ ion of C-peptide. Rapid Commun Mass Spectrom 2004;18:3140-1.

- Rodríguez-Cabaleiro D, Stöckl D, Thienpont LM. Improvement of sample pretreatment prior to analysis of C-peptide in serum by isotope-dilution liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom 2005;19:3600-2.

The validation and application of the ID-LC/tandem MS RMP for serum C-peptide was published in:

- Rodríguez-Cabaleiro D, Stöckl D, Kaufman JM, Fiers T, Thienpont LM. Feasibility of standardization of serum C-peptide immunoassays with isotope-dilution liquid chromatography-tandem mass spectrometry. Clin Chem 2006;52:1193-6.

II.1. INTRODUCTION

II.1.1. Biosynthesis, secretion and metabolism of C-peptide and insulin in humans

Although this is a chapter dedicated to C-peptide, it will deal with the biosynthesis, secretion and metabolism of both C-peptide and insulin (mainly in sections II.1.1., II.1.2 & II.1.3). The reason for this combined description stems from their close relationship in the systemic circulation, as a result of synthesis from the same precursor in equimolar amounts.

C-peptide (31 amino acids; Mw 3020) and insulin (51 amino acids; Mw 5808) are synthesized in the rough endoplasmic reticulum of the beta-cells of the pancreatic islets of Langerhans. They are then transported to the Golgi apparatus, where they are packaged in membrane-bound granules. These granules move to the plasma membrane by a process involving microtubules, and their contents are expelled by exocytosis. C-peptide and insulin then cross the basal laminae of the beta-cell and neighboring capillary to reach the bloodstream (1).

Like other polypeptide hormones and related proteins that enter the endoplasmic reticulum, C-peptide and insulin are synthesized as part of a larger preprohormone. The gene for insulin and C-peptide is located on the short arm of chromosome 11 in humans. Preproinsulin has a 23-amino-acid signal peptide removed as it enters the endoplasmic reticulum. The remainder of the molecule is then folded, and the disulfide bonds are formed to make proinsulin (see Figure II.1). C-peptide is the “connecting” peptide between the A and B chains of insulin and facilitates the folding of the protein and then is detached in the granules before secretion.

C-peptide and insulin are synthesized from their precursors by a process of enzymatic cleavage (Figure II.2). Two endopeptidases, prohormone convertases 2 and 3 (PC2 and PC3), cleave the proinsulin molecule at two sites marked by pairs of dibasic amino acids. The type-1 endopeptidase (PC3) cleaves at the Arg^{31}-Arg^{32} site at the junction of the B/C chains of proinsulin and the second endopeptidase (PC2) cleaves at the Lys^{64}-Arg^{65} site, the proinsulin A/C junction. Both enzymes are calcium-dependent and have acidic pH optima, though their enzyme kinetics differ. Through the action of carboxypeptidase H there is a loss of basic amino acids at each site to give the ‘des’ forms of the partially processed proinsulins. When the cleavage at both sites is complete, C-peptide and insulin are produced. Gel chromatography studies have shown serum

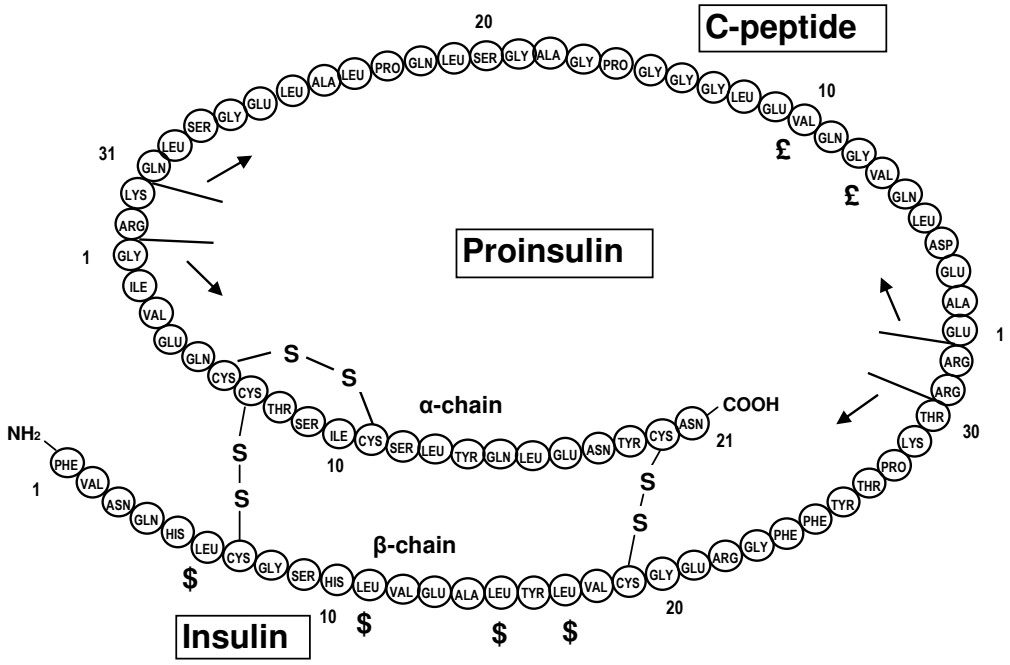

Figure II.1. Schematic structure of human proinsulin with indication of its splitting in C-peptide and insulin. **£** Indicates the positions where the internal standard (IS) of C-peptide was labeled with D8-Val. **$** Indicates the positions where the IS of insulin was labeled with D10-Leu.

C-peptide heterogeneity, which may arise either through *in vivo* metabolism and/or degradation on storage. This property has been confirmed by stable ID-MS (2). Normally, 90-97% of the product released from the beta-cells is C-peptide and insulin in equimolecular amounts. The rest is mostly proinsulin. Insulin has the major biological activity though it has been proven in the last decade that C-peptide also has biological activity as will be described later (3-7).

After secretion, C-peptide and insulin enter the portal circulation and are carried to the liver, the prime target organ. About 50% of secreted insulin is extracted and degraded in the liver; the residue is broken down by the kidneys. C-peptide is only partially extracted by the liver (and hence provides a useful index of the rate of insulin secretion), but is mainly degraded by the kidneys. The half-life of insulin in the circulation in humans is about 5 minutes. Insulin binds to insulin receptors and is internalized. It is destroyed by proteases in the endosomes formed by the endocytotic process.

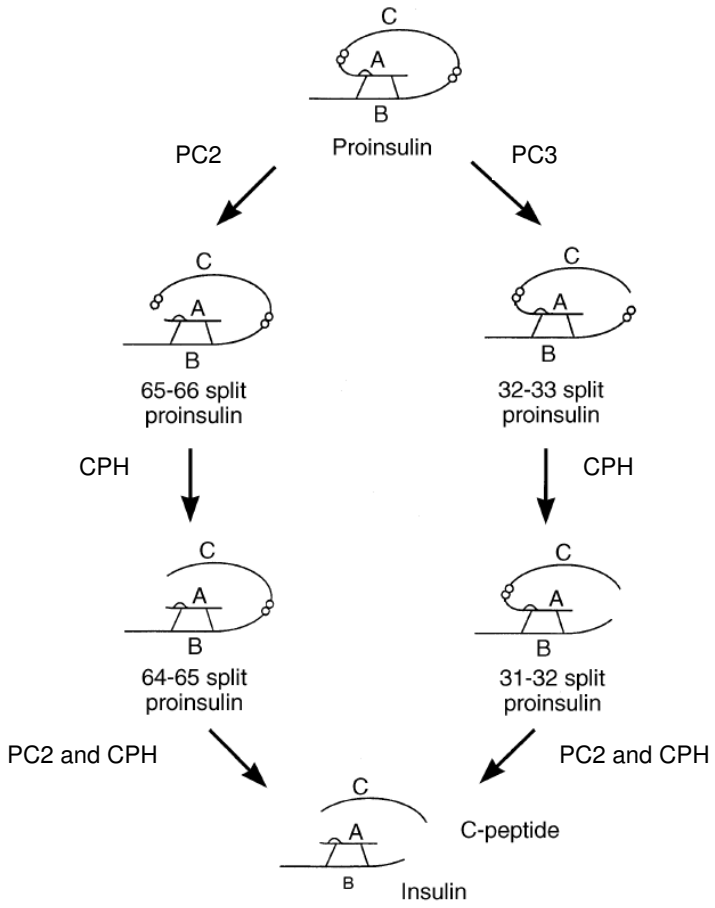

Figure II.2. Scheme showing the processing of proinsulin to major intermediates and to C-peptide and insulin. Letters identify chains, small circles represent the amino acid residues at positions 31 and 32 (Arg-Arg) and 64 and 65 (Lys-Arg). PC: proinsulin converting enzyme; CPH: carboxypeptidase H.

II.1.2. Physiological effects of C-peptide and insulin

As mentioned before, it has been suggested from experimental data and clinical studies performed during the last decade that C-peptide is a biologically active peptide (3-7). Clinical studies show that C-peptide administration in type I diabetes patients, who lack the peptide, results in improvement of diabetes-induced renal and nerve dysfunction. Molecular studies demonstrate binding to cell membranes, activation of intracellular signaling pathways, and specific end effects of importance for vascular endothelial function. These findings have prompted the hypothesis that C-peptide deficiency in type I diabetes may contribute to the development of microvascular complications, and that C-peptide replacement, together with regular insulin therapy, may be beneficial in the treatment or prevention of these complications.

Insulin is the major regulator of intermediary metabolism. The physiologic effects of insulin are far-reaching and complex (1, 8). They are conveniently divided into rapid, intermediate, and delayed actions, as listed in Table II.1.

Table II.1. Principal actions of insulin (1).

Rapid (seconds)	Increased transport of glucose, amino acids, and K^+ into insulin-sensitive cells.
Intermediate (minutes)	Stimulation of protein synthesis. Inhibition of protein degradation. Activation of glycolytic enzymes and glycogen synthetase. Inhibition of phosphorylase and gluconeogenic enzymes.
Delayed (hours)	Increase in mRNAs for lipogenic and other enzymes.

The best known is the hypoglycemic effect, but there are additional effects on amino acid and electrolyte transport, many enzymes, and growth. The net effect of the hormone is storage of carbohydrate, protein, and fat; therefore, insulin is appropriately called the “hormone of abundance”.

II.1.3. Clinical application of the measurement of C-peptide and insulin

C-peptide

Although, as explained above, insulin is the major regulatory hormone in the carbohydrate metabolism, it is generally accepted that C-peptide concentrations reflect the endogenous pancreatic insulin secretion more reliably in insulin-treated diabetics than the levels of insulin itself. C-peptide concentrations are not affected by factors limiting the utility of insulin levels, i.e., first-pass hepatic extraction, variable peripheral clearance and measurements hampered by anti-insulin antibodies or by insulin of exogenous origin in treated subjects. Therefore, to quantify the endogenous insulin secretion, C-peptide is measured basally, after fasting and after stimulation and suppression tests.

The clinical indications for C-peptide measurement are multiple:

- Direct assessment of the beta cell function via C-peptide testing is considered the most appropriate end point for clinical trials of therapies aimed at preserving or improving endogenous insulin secretion in type I diabetics (9-12).

- Detection and monitoring of the remission phase of type I diabetes.
- Adjunct in the differential diagnosis of latent autoimmune diabetes mellitus of adults (LADA) and type-2 diabetes (13-15).
- Although testing for C-peptide is never requested for the routine monitoring of diabetes, it is a valuable tool for the individual therapeutic decisions which are essential for an optimal long-term metabolic control (16,17).
- In certain countries, C-peptide testing is also part of the qualification process for medical insurance coverage of insulin pump therapy.
- Measurements of C-peptide is used as an aid in the differential diagnosis of hypoglycemia (factitious hypoglycemia and hypoglycemia caused by hyperinsulinism) to ensure an appropriate management and therapy of the patients.
- Elevated C-peptide levels may result from increased beta-cell activity observed in hyperinsulinism, but also from renal insufficiency and obesity (18).
- Contribution to the diagnosis of insulinoma (insulin-suppression test).
- Prognostic index of fetal outcome in pregnant diabetic women.
- Evaluation of insulin secretion in liver disease.
- To assess the success of surgical removal of all or part of the pancreas and islet transplantation (18).
- Correlation was also found between higher C-peptide levels and increasing hyperlipoproteinemia and hypertension (19).
- Decreased C-peptide levels are also observed in starvation and Addison's disease.

Insulin

The measurement of insulin is used in the following clinical investigations:

- Diagnosis of hypoglycemia: serum insulin determinations are mainly performed in patients with symptoms of hypoglycemia. They are used to ascertain the glucose/insulin quotients and for clarification of questions concerning insulin secretion, e.g. in the tolbutamide test and glucagon test, in the evaluation of oral glucose tolerance tests or in hunger provocation tests.
- Diagnosis of diabetes mellitus: insulin levels are normally low in patients with insulin-dependent diabetes mellitus (IDDM) and are normal or elevated in patients with non-insulin dependent diabetes mellitus (NIDDM).
- Early detection of diabetes: the insulin response to the administration of glucose may be blunted well before the onset of clinical manifestations.

- Monitoring in persons with reduced glucose tolerance: in 3% of persons with reduced glucose tolerance, the metabolic state deteriorates towards diabetes mellitus over a period of time. Reduced glucose tolerance during pregnancy always requires treatment. The clearly elevated risk of mortality for the fetus requires intensive monitoring.
- Follow-up and stabilization of insulin-treated diabetics: insulin assays can be useful at the onset of insulin therapy to evaluate the duration of action of various insulin preparations.
- Determination of the quantity of free insulin in patients with anti-insulin antibodies: although the adequacy of pancreatic insulin synthesis is frequently assessed via the determination of C-peptide, it is still generally necessary to determine insulin. For example, therapeutic administration of insulins of non-human origin can lead to the formation of anti-insulin antibodies. In this case, measurement of the concentration of serum insulin shows the quantity of free (and hence biologically active) hormone, whereas the determination of C-peptide provides a measure of the patient's total endogenous insulin secretion.
- Predicting complications of Type II diabetes: the persistent elevation of insulin is a risk factor for the development of coronary disease.
- Diagnosis of insulinoma: pancreatic beta-cell tumors may produce a state of hyperinsulinism leading to hypoglycemia. It is important for differential diagnosis of fasting hypoglycemia to discriminate between insulinoma and factitious hypoglycemia. In these applications, the glucose/insulin quotient may be more valuable than the insulin level alone
- Other uses of insulin assays have been suggested by the finding of an increase in risk factors for coronary artery disease among healthy persons with hyperinsulinemia and normal glucose tolerance (20).
- Also for forensic purposes and by the pharmaceutical industry.

II.1.4. Existing measurement procedures for serum C-peptide

Routine analysis of C-peptide in the clinical laboratory is performed with a wide variety of commercial chemiluminescence (CLIA), radio (RIA) and enzyme linked (ELISA) immunoassays (21). Most of the systems are used for the determination of C-peptide in both urine and serum. In view of the synthesis and structure of C-peptide it becomes apparent that the main analytical challenge is the measurement of low concentrations in the presence of molecules of similar structure (21). For example, the 33-residue of C-peptide (comprising two additional amino acids, lysine and arginine at the C terminus after incomplete processing of proinsulin) makes up 4-10% of the 31-residue C-peptide concentration.

Chromatographic techniques are mainly used in the pharmaceutical industry and in research studies. HPLC followed by RIA of separated fractions (22, 23) or IAC followed by HPLC and RIA (24) have also been used. Procedures based on HPLC-MS after SPE or IAC for the analysis of C-peptide in serum have also been described in the literature (2, 25, 26). Only the measurement procedures from Kippen et al. and Rogatsky E et al. (2, 26) were of the ID-LC/MS type, which is, as described previously (see chapter I), an indispensable condition for a measurement procedure to be appointed as RMP. For more details on the existing methods, Table II.2 can be consulted. Notice that the method of Rogatsky et al. (26) was published after our study on C-peptide was finished.

II.1.5. Objectives

The objective of this part of the dissertation was to develop a RMP for serum C-peptide and to apply it to the standardization of immunoassays. Previous experience gained at our laboratory on the general mass spectral and chromatographic behaviour of C-peptide and insulin (27-29) were of big utility. In particular, the previous development of a RMP for the determination of C-peptide in urine was of big use (30-32).

Table II.2. Summary of the existing LC/tandem MS procedures for serum C-peptide.

Author (reference)	IS	Sample preparation	HPLC	MS	LOD[a] (ng)
Kippen AD et al. (2)	[$^{2}H_{14}$] C-peptide	Sep-Pak® C18 SPE or IAC + Sep-Pak® C18 light SPE	Nucleosil C18 (150x1 mm) 60 min gradient of ACN[b] with 0.1% TFA	Off-line Micromass VG single quad™ ESI+[c] Short scan m/z 1003-1016	< 0.17
Darby SM et al. (25)	no	Sep-Pak® C18 SPE	Hesperia Vydac C18 (150x2.1 mm) 10 min gradient of ACN with 0.04% TFA	Finnigan MAT LCQ™ Qtrap ESI+ SIM $[M+3H]^{3+}$	< 4
Rogatsky E et al. (26)	[$^{15}N_{18}$] and [$^{15}N_{30}$] C-peptide	Sep-Pak® C8 SPE	2 dimensional HPLC Phenomenex Jupiter C5 (100x2 mm) ACN with 0.1% TFA Varian Pursuit C18 (50x2 mm) ACN/water/0.4% formic acid/1% isopropyl alcohol/2% methanol 16 min run	SCIEX API365™ API 4000™ ESI+ SIM $[M+3H]^{3+}$	$4 \cdot 10^{-4}$

[a]Note that 1 ng = 0.33 pmol

[b]Acetonitrile (ACN)

[c]Positive electrospray ionization (ESI+)

Because serum is a more complex matrix than urine, it requires a more intensive sample preparation procedure. In addition, concentrations of C-peptide in serum are 50 times lower than in urine. In consideration of the above, a selective sample preparation procedure with good recovery had to be developed and improvement of the MS sensitivity had to be achieved. Since the same LC/tandem MS hardware had to be used, we focused in particular on the specificity of the sample pretreatment procedure in order to improve the S/N ratio and hence the LOD and LOQ after processing of a reasonable volume of serum. In addition, we aimed at a robust LC/tandem MS measurement procedure with a relatively short total analysis time to achieve sufficient sample throughput and column lifetime.

Existing procedures for sample preparation, i.e., SPE with Sep-Pak® C18 cartridges, described by Kippen et al. (2), Cohen et al. (33) and Darby et al. (25), were used as a starting point combined with our LC/tandem MS conditions (31). We investigated the potential of a two-step SPE, and afterwards of IAC. As an evaluation criterion, we mainly compared the subsequent LC/tandem MS ion chromatograms with those shown for the existing methods in the literature. For improvement of the mass spectrometric conditions, i.e., in terms of more selective and sensitive SRM, we studied the CID process in greater detail.

Finally, the analytical performance characteristics of the candidate RMP based on ID-LC/tandem MS after two-step SPE were evaluated. To investigate the feasibility of this procedure for use in standardization, a split sample method comparison for parallel measurements of basal and stimulated C-peptide concentrations with three representative commercial assays was performed.

II.2. MATERIALS AND METHODS

II.2.1. Specifications and requirements for a serum C-peptide candidate RMP

There were no internationally recognized specifications for a candidate RMP for serum C-peptide. Therefore, we chose “state-of-the-art” acceptance criteria. They were a total coefficient of variation (CV_{total}) of 3.0% and a maximum deviation from target concentrations (trueness) of 5%. In view of the reference interval of C-peptide (1.1 – 3.6 µg/L), a LOQ of 0.2 µg/L was considered acceptable.

II.2.2. Calibrators, reagents, materials and sera

Calibrators

Proinsulin C-peptide fragment 33-63 and D_8-$Val^{7,10}$-C-peptide were obtained from Bachem. They were delivered in vials containing 500 µg of freeze-dried material and had a purity > 98% and a peptide content of 88.0% (C-peptide) and 86.9% (D_8-$Val^{7,10}$-C-peptide) according to the manufacturer's information. We took the peptide content into account for calculation of the C-peptide content in the calibration solutions. All the solutions were prepared gravimetrically taking into account the density of the dilution solution. Densities were measured with a Mettler Toledo DA-110M density meter (Greifensee, Switzerland) and all solutions and standard materials were weighed in a Mettler Toledo AT261 Delta Range analytical balance that allowed measurements till to 10^{-5} g. In this way, the concentration of all calibration solutions was known to four significant figures. Stock calibration solutions (250 mg/L) were prepared by adding 2 mL of 0.03 mg/L (1%) protease-free bovine serum albumin (BSA) (Sigma) solution to the 500-µg C-peptide vial. From this stock solution two consecutive 1/5 dilutions in the same BSA solution were prepared to obtain respective concentrations of 50 mg/L and 10 mg/L (note that the lowest concentration tested to be stable in storage at -20 °C is 10 mg/L in 1% BSA (31)). Immediately after preparation, the latter solutions were divided into 70-µL portions in polypropylene vials and frozen at -20 °C until the day of analysis. On that occasion, three frozen vials were thawed and diluted with ultra pure water to obtain three different final calibration working solutions with a concentration of 30 µg/L. Once thawed, the portions were never reused. The calibration mixtures used for the ID-MS

measurements were prepared by gravimetrically controlled mixing of equivalent amounts of C-peptide and D_8-Val^{7,10}-C-peptide from the final working solutions.

Reagents and materials

All chemicals used were of superquality grade and purchased from Romil (Cambridge, UK) or Fluka (Buchs, Germany). Ultra-pure water (18.2mΩ) was prepared with an ELGA Maxima system (Elga Ltd., High Wycombe, UK). For SPE, Waters Sep-Pak® C18 3 cc (200 mg) and Oasis® MCX 1cc (30 mg) cartridges (Milford, MA, USA) were mounted on a JT Baker SPE-12G Column Processor® device (Phillisburg, NJ, USA). For IAC, Trizma® hydrochloride, Trizma® base, sodium azide and ammonium acetate were purchased from Sigma-Aldrich. The IAC-gel was prepared in collaboration with the Department of Clinical Medicine, Division of Clinical Chemistry, Biomedicum Helsinki, University of Helsinki (Finland). A mouse monoclonal antibody with an affinity constant of $1x10^8$ M^{-1} (Biodesign International, Saco, ME, USA) was coupled to cyanogen-bromide-activated Sepharose (GE Healthcare, Diegem) with a capacity of 2 mg IgG/mL (0.5 mL per column). The procedure used for the preparation of the IAC-gel was done exactly as described by "Affinity Chromatography. Principles and Methods" (34). SPE after IAC was done with Sep-Pak® C18 1 cc (50 mg) cartridges.

Sera

The sera used during the development and optimization experiments came from the blood transfusion center from the Flanders Red Cross in Leuven. They were handled according to the local Ethical Committee guidelines. For internal quality control during the method comparison studies two supplemented serum pools were used (concentrations 4.1 µg/L and 14.8 µg/L). For the method comparison, samples were obtained from Ghent University Hospital from 15 ambulatory patients (2 men and 13 female, aged between 18 and 64 years) who had been subjected to an oral glucose (75 g) tolerance test after overnight fasting. Blood was drawn before the glucose load and after 30, 60, 120 and 180 min. The test was ordered by the endocrinologist for assessment of glucose tolerance, glucose handling and beta-cell function in subjects with morbid obesity and/or other risk factors for glucose resistance and intolerance. Handling of patient serum samples was performed according to the local Ethical Committee guidelines. Blood was drawn into Venosafe VF-106SAS tubes (Terumo) and allowed to clot at room temperature for at least

30 min. Serum was obtained by centrifugation at 1500 *g* for 10 min in a Jouan C412 centrifuge (Thermo Electron Corporation).

II.2.3. Instrumentation

The LC/tandem MS instrument used was a Micromass VG Quattro II™ triple quadrupole MS (Altrincham, UK) equipped with a megaflow electrospray probe. It was coupled to a Kontron Instruments Model 325 HPLC system (Milan, Italy), equipped with an autosampler 465. Chromatography was performed on a Hamilton PRP-3 column (50 x 2.1 mm, 3 µm particle size, 300 Å pore size) (Reno, NV, USA). Nitrogen was used as drying and nebulising gas at a flow rate of 450 and 14 L/h, respectively.

II.2.4. Analytical procedure for serum C-peptide

Overview

The developed sample preparation procedure for the determination of C-peptide in serum consisted of dilution of the serum and immediate purification by two-step SPE. The first step was based on a reverse phase interaction and the second step on a mixed reverse phase-cation exchange interaction. The final eluate was dried under nitrogen and the dried residue was redissolved and quantified via LC/tandem MS. A schematic representation of the procedure can be seen in Figure II.4.A.

Sampling and calibration procedure

The analysis started with gravimetric sampling of the serum (between 0.3 mL (minimum) and 2.5 mL (maximum)) and addition of D_8-Val^{7,10}-C-peptide in an equivalent amount to the endogenous C-peptide to obtain a 1:1 isotope ratio. At the same time three different standard working solutions were prepared having an approximate concentration of 30 µg/L. Subsequently, three calibration mixtures (C-peptide/ D_8-Val^{7,10}-C-peptide) with each of the standard working solutions were prepared. Immediately after preparation, the calibration mixtures were evaporated to dryness at 50 °C under nitrogen.

The calibration procedure was based on the principle of one point calibration (35). This calibration procedure, in combination with isotope dilution, provides the best results when it is done with a 1:1 isotopic ratio. The measurement procedure consisted of the analysis of three calibrators followed by the analysis of four samples, three calibrators and so on. This alternation of calibrators and samples was done to compensate any eventual drift in the response of the instrument.

Two-step SPE sample preparation procedure

After adding the IS and equilibration of the mixture during one hour at room temperature, a 1:1 dilution with 0.3% aqueous acetic acid (AcOH) was performed prior to SPE with Sep-Pak® C18 3 cc and Oasis® MCX 1 cc. Sep-Pak® C18 cartridges were preconditioned by consecutive wetting with 4 mL of ACN and 0.3% AcOH. After the diluted serum samples were loaded, they were washed with 2 mL 20/80/0.3 (v/v/v) ACN/water/AcOH and eluted with 1.5 mL 50/50/0.3 (v/v/v) ACN/water/AcOH. Oasis® MCX cartridges were preconditioned by consecutive wetting with 2 mL ACN and 0.1% trifluoro acetic acid (TFA) in water. In order to reduce the organic concentration, the extracts were diluted with 1.5 mL of 0.1% TFA in water prior to loading onto the Oasis® MCX cartridges. Elution was

done with 1.5 mL of 50/50/0.3 (v/v/v) ACN/water/TFA. Finally, the extracts were evaporated to dryness at 50 °C under nitrogen and stored at -20 °C until analysis. Note that the calibration mixtures were not submitted to the sample pretreatment procedure.

LC/tandem MS measurement procedure

The evaporated extraction residues were reconstituted with 50 µL of 15/85/0.02 (v/v/v) ACN/water/TFA and centrifuged during 5 min at 5000 rpm before injection of 40 µL into the LC/tandem MS system. Chromatography was performed with a Hamilton PRP-3 column (50 x 2.1 mm, 3 µm particle size, 300 Å pore size), in a total run time of 12 min, i.e., 5 min gradient elution from 15 to 35% ACN 0.02% TFA, followed by a fast increase (1 min) of the ACN concentration to 70%. These conditions were maintained during 2 min for column cleaning. Finally, a 3 min re-equilibration with the starting eluent was performed. At a flow rate of 0.3 mL/min, the elution time of C-peptide and its isotopically labelled analogue from the PRP-3 column was ~4.5 minutes.

ID-MS measurements were performed in the negative electrospray tandem MS mode monitoring the transitions from m/z 1509.2 to 183.8 (C-peptide) and m/z 1517.1 to 183.8 (labelled analogue). The tandem MS conditions were: collision gas argon $3x10^{-3}$ mbar, CID energy 80 eV; cone 75 V, capillary -4 kV, and source temperature 175 °C. The scan times were adapted in order to have at least 15 cycles per peak. Dwell times were set at 0.55 sec with an interchannel delay of 0.06 sec.

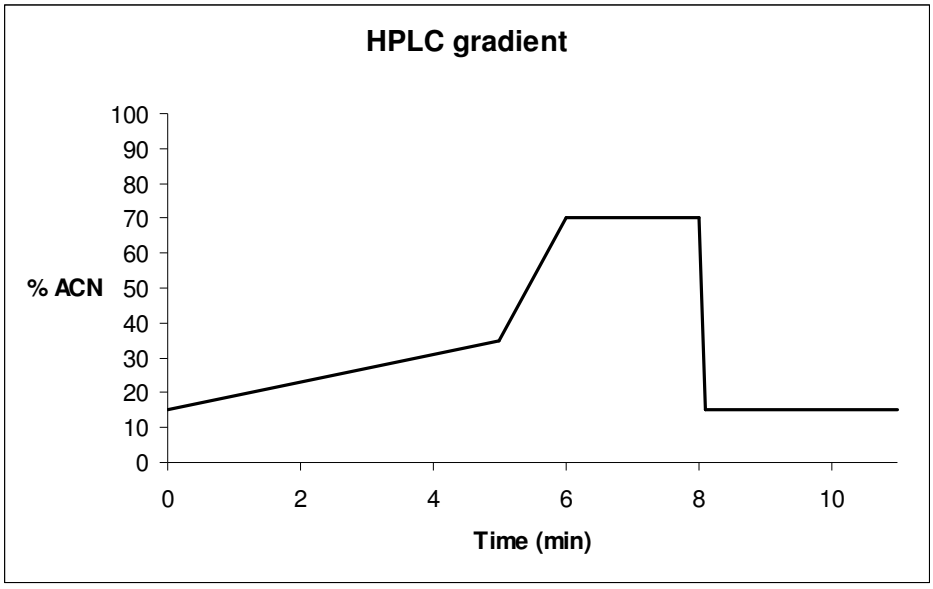

Figure II.3. Schematic representation of the HPLC-gradient

II.2.5. Comparison with sample preparation procedures in the literature

The procedures of Kippen et al. (2) and Darby et al (25) were reproduced together with our two-step SPE procedure using the same serum pool and the extracts obtained were measured under identical HPLC-MS conditions. For a schematic representation of our sample preparation procedure and the procedures previously described in the literature see Figure II.4.A and Figure II.4.B and C, respectively. Our procedure was already described in detail in Chapter II.2.4, the other procedures are described in detail below.

Procedure according to Kippen et al.

A schematic representation of this procedure can be seen in Figure II.4.B. After being diluted with 5 ml of 0.1% TFA in water, the serum samples (1 ml) were filtered through a 0.45-µm sterile filter (Millipore Corp., Billerica, MA, USA). Sep-Pak® C18 cartridges were washed with ACN and then equilibrated in 0.1% TFA in water. The serum was passed back and forth through the cartridge three times at ~1 ml/min using polypropylene syringes fixed at either end. Cartridges were washed with 4 mL of 20/80/0.1 (v/v/v) ACN/water/TFA and 80/20 (v/v) ACN/dichloromethane. Elution was done with 50/50/0.1 (v/v/v) ACN/water/TFA, and the solutions were freeze-dried.

Samples were redissolved in 120 µL of 0.1% TFA. HPLC was performed with a microbore Nucleosil 300-Å C18 column (1 x 150 mm) (Machery-Nagel, Düren, Germany). A gradient from 22 to 36% ACN with 0.1% TFA over 60 min at 0.04 mL/min was performed. The aliquots collected were freeze-dried. Aliquots were reconstituted in 10 µl of 50/49/1 (v/v/v) methanol/water/AcOH and injected via infusion into the electrospray ionization-mass spectrometer (ESI-MS) system. The samples were then analyzed by performing small scans in positive ionization mode using either: (*a*) a Fisons Instruments VG-Trio 2000™ single quadrupole instrument (Altrincham, UK) at a flow of 2 µl/min and a scan cycle of 10 s or (*b*) a Micromass VG-Platform II™ single quadrupole instrument at a flow of 10 µl/min and a scan cycle of one second. The spectrum was scanned to cover the most intense signals of the target analyte and its IS, whereas the large carrier signal was designed to be out of this scan range.

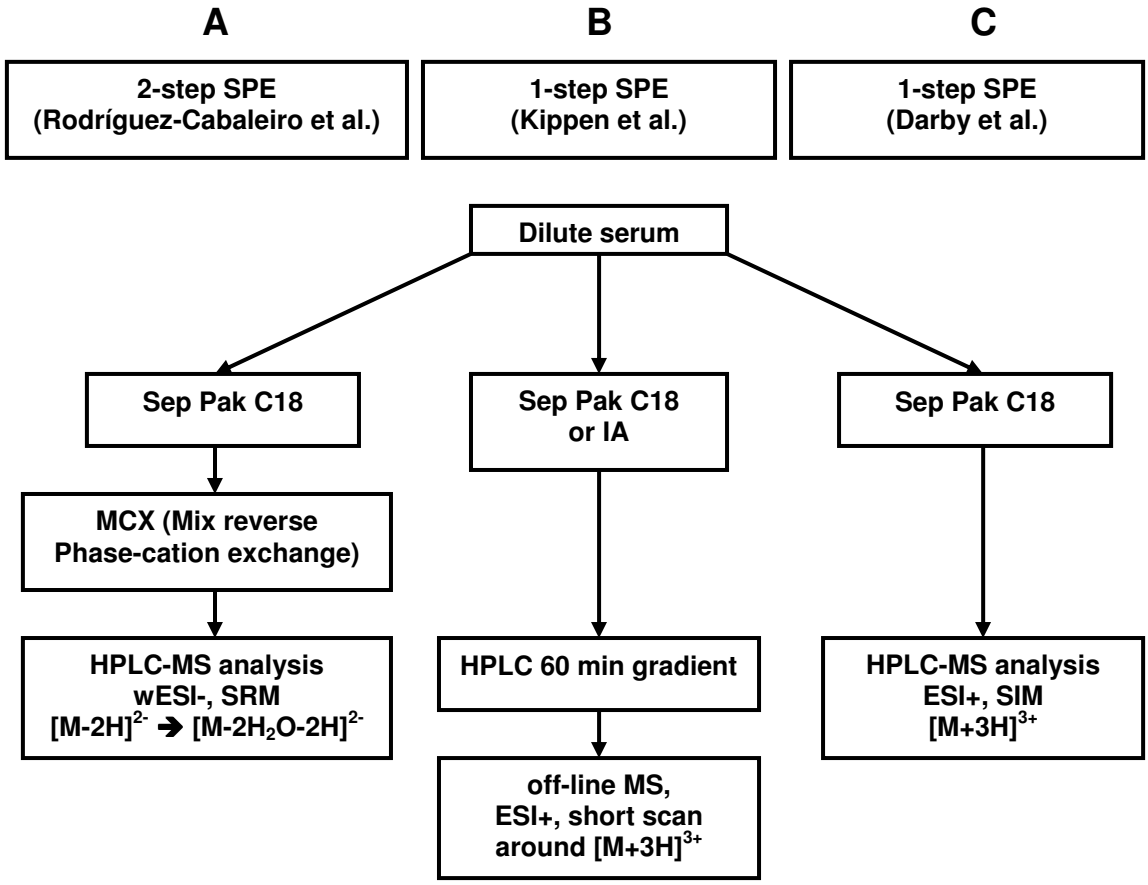

Figure II.4. Schematic representation of the developed sample preparation procedure in comparison with the others in the literature.

Procedure according to Darby et al.

A schematic representation of this procedure is presented in Figure II.4.C. Darby et al. published the following method for analysis of C-peptide in serum samples. Five milliliters of plasma were diluted 1:1 with 0.3% AcOH in water. Sep-Pak® C18 cartridges were preconditioned with 5 mL of ACN and 5 mL of 0.3% AcOH in water. The cartridges were washed with 2 mL of 20/80 (v/v) ACN/0.3% AcOH in water. Elution was performed with 2 mL of 40/60 (v/v) ACN/0.3% AcOH in water, and the solution was freeze dried.

HPLC was performed with a 2.1x50 mm Grace Vydac C18 column (Hesperia, CA, USA). A gradient from 20 to 40% ACN with 0.04% TFA over 10 min at 0.3 mL/min was performed. Measurements were done on a Finnigan MAT LCQ quadrupole ion trap (San Jose, CA, USA). The MS was set to measure in SIM the $[M+3H]^{3+}$ ion.

Protocol for the comparison between procedures

For practical reasons, the sample preparation procedure of Kippen et al. was adapted (2, 33). Sep-Pak® C18 3 cc cartridges were preconditioned with 5 mL of pure ACN and then with 0.1% TFA in water. The serum samples (2.5 mL) were diluted 1:1 with 0.1% TFA in water, and the resulting solution was applied to the cartridge. The eluate was collected and passed through the cartridge a second time (both times at a rate of ~1 mL/min). Then, a three-step washing procedure was performed, i.e., first with 2 mL of 20/80/0.1 (v/v/v) ACN/water/TFA, followed by 2 mL of 1% TFA in water, and then with 2 mL of 80/20 (v/v) ACN/dichloromethane. After the last washing step, an additional 2 mL of 20/80/0.1 (v/v/v) ACN/water/TFA were applied to remove residual dichloromethane (33). For elution, 2 mL of 50/50/0.1 (v/v/v) ACN/water/TFA were used.

Aliquots of 2.5 mL from a serum pool were processed according to the three sample preparation procedures. The serum pool had an approximate concentration of 0.6 µg/L. After sample preparation, the final SPE extracts were evaporated to dryness at 50°C under nitrogen. Afterwards, the residues were reconstituted with 50 µL of 15/85/0.02 (v/v/v) ACN/water/TFA and centrifuged before injection of 40 µL of the resulting solution into the LC/tandem MS system. The HPLC conditions were already described in chapter II.2.4.

For the MS monitoring a SRM different to the one described in the definitive method was used. C-peptide and D8-Val^{7,10}-C-peptide were monitored in the negative wrong way round electrospray ionization mode (wESI-), measuring the SRM transitions at m/z 1509 and 1517 (precursor ions $[M–2H]^{2-}$ of C-peptide and D8-C-peptide) to m/z 1500 and 1508, respectively. This SRM transition provides the same selectivity than the SRM transition used in the final method (from m/z 1509 and 1517 to 183.8) but is less sensitive. The reason for using the first transition was that at this stage of the research it was the best SRM known (30). For CID, argon gas at $3x10^{-3}$ mbar and a CID energy of 35 eV were used. The cone voltage was set to 70 V, the capillary voltage to 4.5 kV, and the source temperature to 175°C.

II.2.6. Immunoaffinity chromatography purification

In a later stage of the research immunoaffinity gel was available. The technique was qualitatively tested as an alternative to the two-step SPE sample preparation procedure described in chapter II.2.4.

Sample preparation was carried out by a combined IAC - SPE procedure (see a schematic representation in Figure II.5). The IAC-gel was stored in a buffer at pH 7.4 containing 50 mM Tris and 0.05% of sodium azide. Conditioning of the IAC columns was done by washing the columns with 3 mL of binding buffer (BiB; 50 mM Tris, pH 7.8 in water). Then, the samples were diluted 1:1 with BiB and applied to the column. Afterwards, the gel was washed with 5 mL of BiB, 4 mL of 10 mM ammonium acetate (pH 4.5) and 0.2 mL of 0.1% TFA. C-peptide was subsequently eluted with 1.5 mL of 0.05% TFA. The IAC columns were re-equilibrated with 5 mL of BiB.

The IAC eluate was loaded into a Sep-Pak® C18 1 cc cartridge, mounted upside down (see Figure III.2 in Chapter III) and previously preconditioned with 2 mL of ACN and 2 mL of 0.1% TFA. After being loaded, the cartridge was washed with 2 mL of 0.1% TFA and 2 mL of 10/90/0.1 (v/v/v) ACN/water/TFA. Finally, it was mounted in the normal flow direction for elution with 0.25 mL of 40/60/0.1 (v/v/v) ACN/water/TFA. The extracts were evaporated to dryness at 50 ºC with nitrogen and stored at -20 ºC until analysis. Residues were reconstituted in 50 µL of 15/85/0.02 (v/v/v) ACN/water/TFA and 45 µL of this mixture were injected in the LC/tandem MS system.

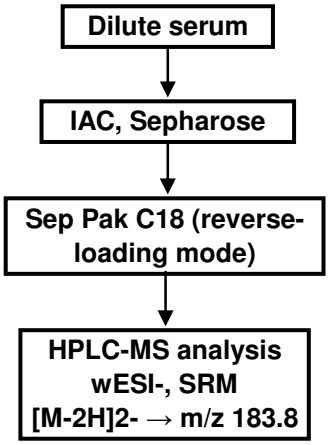

Figure II.5. Schematic representation of the IAC sample preparation procedure.

II.2.7. Optimization of the MS conditions

Based on previous experience at our laboratory (27,30), TFA was selected as modifier for chromatography and all measurements were performed in wESI- operation mode. The CID experiments were carried out in the first place to find the most sensitive (yet selective) SRM transition and in the second place to identify the fragments formed after CID.

For the realization of these experiments, chromatography was performed with a PRP-3 column (50x2.1mm) under isocratic conditions with 22/78/0.02 (v/v/v) ACN/water/TFA at a flow rate of 0.2 mL/min.

Selection of the most sensitive SRM transition

In order to find the best SRM conditions for quantification, spectra of the $[M\text{-}2H]^{2-}$ parent ion were generated at different collision energies and collision gas pressures. Additionally, helium instead of argon as a collision gas was tested. Once the most sensitive SRM transitions were selected, all MS parameters were optimized to obtain maximum sensitivity. The S/N ratios of the different SRM transitions were calculated after injection of 1 ng of C-peptide.

CID fragmentation elucidation

In a previous study at our laboratory a provisional fragmentation pathway for the CID of the doubly charged ion $[M–2H]^{2-}$ of C-peptide was assigned (37 and Fierens doctoral thesis). However, as soon as access to the labelled IS was possible, it was realized that this assignment was erroneous. Thus, the fragmentation mechanism was investigated in more depth by measuring unlabelled, labelled, Val-C-peptide and C-peptide-Val standards. The latter were C-peptide analogues elongated at the N-terminus and C-terminus with valine (Val-C-peptide/C-peptide-Val; Mw 3119.4) (both from American Peptide Company).

CID-spectra of the doubly charged ion $[M–2H]^{2-}$ of C-peptide (m/z 1509.1), labelled C-peptide (m/z 1517.1), Val-C-peptide and C-peptide-Val (m/z 1558.7) were recorded. The scans were divided in the ranges from m/z 100–1600 and m/z 1600–3150. The conditions were: 3s/scan; collision gas argon at $3x10^{-3}$ mbar; CID energy 40 eV; cone 60 V, capillary -3.5 kV, and source temperature 175 °C. Mass spectrometric resolution was 1.5 mass units, expressed as full width at half height. Note that a better resolution could not be obtained without major loss of signal intensity.

II.2.8. Stability of standards and serum

The stability of stock standard solutions was already studied (31). It was concluded that solutions down to 10 mg/L, containing 1% BSA and stored at -20°C were stable. As explained before, the concentrations of C-peptide in serum are 50 times lower than in urine. For this reason the working-day solutions for the preparation of calibrators have to be lower in concentration. Experiments were done to ensure the stability of these working solutions before preparing the calibrators with equivalent amount of IS. At the same time, the process of submitting the calibrators through analysis was tested in order to determine whether or not a difference would be observed. Finally, the stability of C-peptide in the serum samples was also addressed.

Stability of the D_8-$Val^{7,10}$-C-peptide and validity of isotopic dilution approach

Although the H/D exchange in D_8-$Val^{7,10}$-C-peptide during the extraction procedure was considered very improbable in view of the position of the deuterium atoms (present on the valine carbons and not adjacent to a functional group which may impart reactivity), it was investigated indirectly from a preliminary precision experiment. This experiment was done by analysis on six consecutive days of an aliquot of a serum pool (with a C-peptide concentration of ~4.0 µg/L) to which an equivalent amount of D_8-$Val^{7,10}$-C-peptide was added. After the measurements were performed the isotope ratios were calculated and their variation evaluated.

Stability of standard working solutions and verification of calibrators through analysis

The stability of C-peptide in the working solutions made in 0.09 µg/L (0.003%) of BSA was verified by comparing the isotope ratios of six calibration mixtures (containing unlabelled and labelled C-peptide) with another 6 calibration mixtures prepared from a solution containing 30 µg/L (1%) of BSA. Since the latter could not be directly injected into the LC/MS system they were taken through the complete sample pretreatment procedure. At the same time, this experiment validated whether it was justified to measure the calibration mixtures directly instead of after processing. The difference between the mean isotope ratios of each series was assessed for significance with a two-sided Student *t*-test (95% probability level). Note that before performing the Student *t*-test a F-test (95% probability level) has to be done to investigate if there is a significant difference between

the variances of the different data sets. Depending on the result, it would be opted for a Student *t*-test that supposes equal or different variances. This working method was followed for all Student *t*-tests described in the rest of this dissertation and will not be repeated again.

Stability of C-peptide in serum

The stability of C-peptide was tested in 9 randomly chosen samples from the stimulation curves. Their concentration range was from 3 µg/L to 12 µg/L. They were analyzed 6 times in a week with the Roche Modular Analytics E170 after different storage conditions, i.e., at room temperature (20 to 25 °C), between 2 and 8 °C and at -20 °C. The values from day 2 to 6 were tested for significance of difference with those on day one in a two-sided paired *t*-test (95% probability).

II.2.9. Method validation

The two-step SPE-LC/tandem MS measurement procedure was validated. The following experiments and materials were employed.

Specificity

Interference by other than C-peptide analytes during LC/tandem MS measurement was assessed by analysis of several serum samples selected from the method comparison study without addition of isotopically labelled C-peptide. Any peak at the m/z values used for monitoring the transitions typical for D_8-$Val^{7,10}$-C-peptide would be interpreted as an interference. Interferences in the transition corresponding to C-peptide were evaluated from the chromatography and results of precision and accuracy at different concentrations. The presence of any interference would affect the precision and accuracy to a different extent at different concentrations.

Limit of detection and quantification

The LOD and LOQ from the ID-LC/tandem MS procedure were determined from analysis of 2.5 mL of serum samples of low concentration (<0.5 µg/L) and subsequent dilution until a S/N of 3 (LOD) and 10 (LOQ).

Recovery of the sample preparation procedure

The total recovery of our combined SPE procedure was tested with a serum pool from which eight aliquots were sampled on a gravimetric basis. To four of the aliquots an amount of non-labelled and isotopically labelled C-peptide (to obtain a 1:1 isotope ratio) was added (also on a gravimetric basis) before performing the SPE; to the next four aliquots, only non-labelled C-peptide was added before processing. Collection of the extracts from the Oasis® MCX cartridges was done in tubes containing an equivalent amount of D8-C-peptide. The recovery (in %) was calculated from the isotope ratios obtained for both series of four aliquots.

Ion suppression

Ion suppression was investigated by comparing the responses for the injection of a IS solution and the injection of a processed serum sample whose extract was redissolved with the same IS solution.

Precision

For the estimation of the imprecision data from different experiments were used. First, the data from the dedicated experiments with two sera containing endogenous C-peptide concentrations (1.6 and 4.0 µg/L) was used. The sera were obtained from a voluntary blood donation after overnight fasting and 60 min after glucose stimulation. Aliquots were stored at -20 °C and analyzed in duplicate during 11 analytical runs. Secondly, the data from the two supplemented serum pools utilized for internal quality control in the method comparison study (concentrations 4.1 µg/L and 14.8 µg/L) was employed. Thirdly, we used the data from the recovery experiments (see below) performed for the estimation of accuracy and trueness was used.

The within-run and between-run CVs (%) ($CV_{between}$, CV_{within}) were calculated with Model II ANOVA (analysis of variance). The CV_{total} was obtained from the combined $CV_{between}$ and CV_{within} (square root of the sum of the squared $CV_{between}$ and CV_{within}). The CVs were calculated following the Clinical and Laboratory Standards Institute (CLSI) EP5-A2 protocol (37).

Accuracy and trueness

Accuracy and trueness were estimated from recovery studies. In these experiments, C-peptide at three concentrations (1.9 µg/L, 4.8 µg/L and 7.8 µg/L) was added to a protease-free BSA solution (70 g/L in 0.9% NaCl) and to a serum pool containing an endogenous amount of C-peptide of 1.16 µg/L. Before supplementing the BSA solution, it was verified first that this solution was C-peptide free. In a similar way, before supplementing the serum pool the base line value of C-peptide was quantified in duplicate during four analytical runs using the ID-LC/tandem MS methodology. The % recovery in each analytical run was calculated by comparing the mean value (BSA solutions) or mean difference between the observed and the baseline value (serum) with the amount of C-peptide added. The range of the calculated % recoveries and the mean of that range (± 95% confidence interval (CI)) were used as a measure of respectively the accuracy and the trueness**.**

II.2.10. Method comparison

The two-step SPE ID-LC/tandem MS measurement procedure was applied for the quality assesment of three routine measurement procedures. The following strategy and materials were employed.

Measurement strategy

The fasting blood samples were transported to the laboratory within 30 min of withdrawal. The remaining samples (after glucose stimulation) were received in the laboratory within 30 min of the last collection, after which the analysis (in duplicate) was immediately started with the three immunoassays. After successful measurement with the Roche Modular Analytics E170 (note that this immunoassay is the test system routinely used in the Laboratory for Hormonology of the Department of Clinical Chemistry of Ghent University Hospital), the remaining serum was transported ice-cooled to the MS laboratory. There, the samples were immediately processed. After evaporation, the residues were stored between 2 and 8 °C until LC/tandem MS measurement the next day.

Three routine measurement procedures were compared with the two-step SPE-ID-LC/tandem MS via split-sample measurement of 75 serum samples. All the measurements done under the routine measurement procedures were performed in the Laboratory for Hormonology of the Department of Clinical Chemistry at Ghent University Hospital. Measurement of the 15 stimulation curves by both the ID-LC/tandem MS and routine laboratory was done in seven analytical runs spread over four weeks, meaning that all curves were measured with the same batch of the immunoassays.

Each sample was measured in singlicate with the routine measurement procedures. The measurements in the ID-LC/tandem MS laboratory were done once according to a measurement protocol with bracketing of the patient and control samples (maximum six) between two times three calibration mixtures, which allowed analysis of samples of two stimulated patients per working day. As described above, the quality of each analytical run was controlled from the data of two supplemented serum pools at C-peptide concentrations of 4.1 µg/L and 14.8 µg/L.

Routine measurement procedures

The selection of the C-peptide immunoassays was done on the basis of the availability of the automated immunosystems in the Laboratory for Hormonology except for one RIA

system. The routine measurement procedures evaluated in this study were the Roche Diagnostics GmbH electrochemiluminescence immunoassay (ECLIA) for use on the Modular Analytics E170 (Basel, Switzerland); the Diagnostic Products Corporation (DPC) Immulite® 2000 C-peptide solid phase competitive chemiluminescent enzyme immunoassay (Los Angeles, CA, USA); and the Cis bio international IRMA-C-PEP ^{125}I-radioimmunometric assay (Santa Clara, CA, USA).

All assays are calibrated against the WHO 1st IRP, code 84/510 (1986) from NIBSC (38-40). The assays have measurement and/or calibration ranges from 0.01 to 40 µg/L (Roche), 0.5 to 7.0 µg/L (DPC) and 0.24 to 16.5 µg/L (Cis bio). The reference intervals quoted in the instruction sheets are: 1.1 to 4.4 µg/L (Roche; n = 96), 1.1 to 5.0 µg/L (DPC; n = 71) and 1.1 to 3.5 µg/L (Cis bio; n = 40). All measurements were performed in accordance with the respective manufacturer's instructions.

Each assay's performance was controlled either with assay specific control samples (for DPC the C-peptide Control Module with assigned target values of 1.0, 3.0 and 5.1 µg/L; for Cis bio the C-peptide MYRIA-S with targets of 0.6 and 1.4 µg/L) or with in-house prepared serum pools targeted before at 0.5 and 5.5 µg/L.

Statistical methods and graphical presentation

The method comparison data were investigated by Deming regression analysis (CBstat software version 5.1 from K. Linnet, Risskov, Denmark) and by difference plots with integrated analytical quality specifications. Analytical quality specifications derived from the total expected imprecision of each method pair (CV_{mp}; see Formula II.1) and a total error (TE) specification derived from the biological variation of C-peptide were used. The total biological error ($TE_{specification}$) was corrected for the imprecision of the RMP (formula II.2).

$$CV_{mp} = \sqrt{\frac{CV_{MS}{}^2}{n} + \frac{CV_{assay}{}^2}{n}}$$

Formula II.1. CV of the method pair. Note that n is the number of repeated measurements of the same sample with each method.

$$TE_{new} = 2 \times \sqrt{\left(\frac{TE_{specification}}{2}\right)^2 + CV_{RMP}{}^2}$$

Formula II.2. Calculation of a new specification that accounts for the imprecision of the RMP (CV_{RMP}) in the method comparison.

II.3. RESULTS AND DISCUSSION

II.3.1. Sample preparation procedure

Existing procedures for sample preparation, i.e., SPE with Sep-Pak® C18 cartridges, described by Kippen et al. and Darby et al. (2, 25), were used as a starting point for our sample preparation procedure. A procedure based on IAC purification was also developed in a later stage of this dissertation.

Comparison with other procedures described in the literature

As documented in Figures II.6.C to F, the combination of the sample pretreatment procedures from Kippen et al. and Darby et al. (adapted for practical reasons, see paragraph II.2.5) with our LC/tandem MS measurement conditions did not result in sufficient purification for a sensitive and specific LC/tandem MS measurement in a reasonable time and without damage of the chromatographic column (measured in terms of increase of column pressure). Therefore, a new procedure consisting on the combination of two SPE procedures was developed, i.e., an equivalent of the Darby C18 method (25) followed by a mixed reverse phase-cation exchange sorbent (Oasis® MCX). Our C18 SPE procedure differs from the original one in that the cartridges are loaded twice with the diluted serum sample and eluted with 1.5 mL of 50% instead of 40% ACN in order to not compromise the recovery.

Note that different deproteinization techniques prior to SPE with Sep-Pak® C18 were also tested, more specifically deproteinization was tested with: hydrochloric acid, acetonitrile, ammonium sulfate, and ultrafiltration. However, none of them gave an advantage over the “in situ deproteinization” during Sep-Pak® C18 clean up (data not shown).

The developed sample preparation procedure is superior in several aspects. The reconstructed ion chromatograms shown in Figure II.6.A and B give evidence of sufficient sample purification. Interference-free ion chromatograms with a stable baseline are obtained. It must also be noted that, after injection of reconstituted serum extracts obtained by the method of Darby et al. (25), the column back-pressure tended to increase, even after centrifugation at 5000 rpm for 5 min. We suppose that this was due to an incomplete dissolution of the extraction residue upon reconstitution. Also in terms of sample throughput, our procedure with a gradient of only 5 min and a total run time of 11

min was superior to the other methods. For example, in the method of Kippen et al. (2), purification step was needed with a Nucleosil C18 microbore column (1x150 mm) eluted with a 60 min gradient program before direct infusion into the ESI-MS system. In the method of Darby et al., (25) the elution from the Vydac C18 column (2.1x150 mm) was done with a 10 min gradient. Note that for the alternative methods only the gradient elution time can be reported, since the total HPLC run time is not mentioned in any of the references.

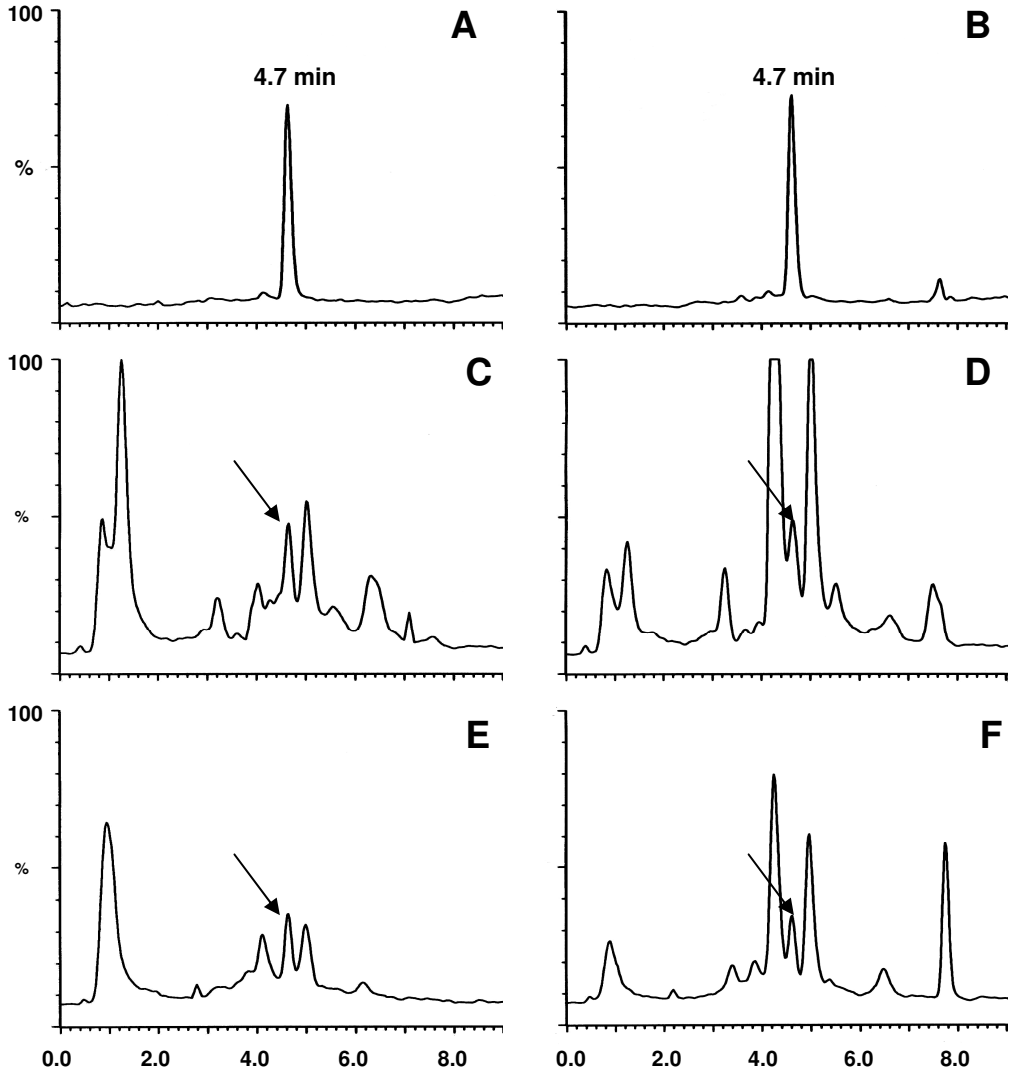

Figure II.6. Ion chromatograms of C-peptide (A, C, E) and its deuterated analogue (B, D, F) obtained by LC/tandem MS analysis (wESI-; SRM from m/z 1509 to 1500 and from m/z 1517 to 1508, respectively) of 2.5 mL of serum processed according to three sample pretreatment procedures. The serum pool had an approximate C-peptide concentration of 0.6 µg/L. The ion chromatograms shown in (A) and (B) were obtained after processing of the serum sample with our two-step SPE sample purification procedure; those shown in

(C) and (D) with the method of Kippen et al. (2) and those shown in (E) and (F) with the procedure of Darby et al. (25). The arrows in (C) to (F) indicate the position of the signals of C-peptide and the deuterated analogue. Note that all chromatograms have the same scale and respect the ratio of the absolute abundances of the peaks.

Immunoaffinity chromatography

In a later phase of the research, a sample preparation procedure based on IAC was tested. Also IAC resulted in interference free chromatograms (Figure II.7). This method is an interesting alternative to our previously described two-step SPE procedure. A major disadvantage of IAC is that the columns have to be reused due to their high cost. This increases the risk of cross contamination and loss of purification efficiency. Long term experience is needed to investigate whether these potential problems occur with the gel used.

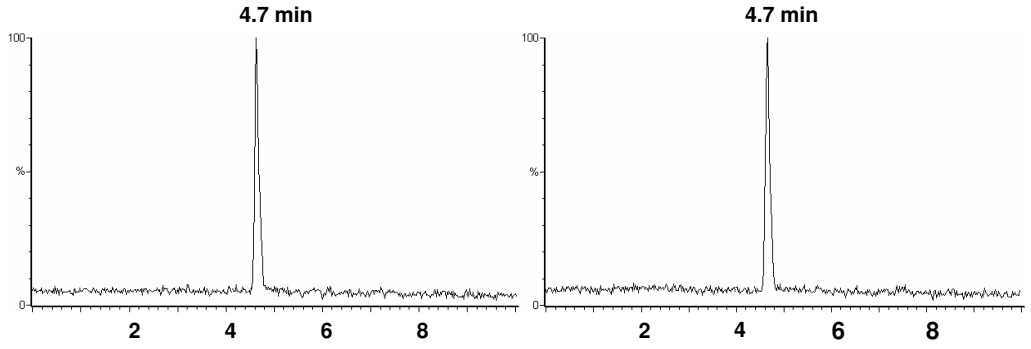

Figure II.7. Reconstructed chromatograms for a sample of 1 mL containing 2.5 ng of C-peptide (left) and deuterated analog (right) purified with IAC and measured by LC/tandem MS analysis (wESI-; SRM from m/z 1509 to 183.8 and m/z 1517 to 183.8, respectively).

II.3.2. Optimization of the MS conditions

The experience at our laboratory with C-peptide was that HPLC with TFA and MS in the wESI- mode gave the best results in terms of combining chromatography and MS performance. In this way the advantages of TFA for peptide chromatography with exceptional MS sensitivity are combined. The MS1 spectrum of C-peptide in the wESI- mode is shown in Figure II.8. The spectrum is dominated by the doubly negatively charged ion, i.e., the $[M-2H]^{2-}$ which carries almost the complete ionization. This is exceptional MS behavior for quantitative purposes. In consideration of the above, the efforts were focused on optimizing the CID conditions of this ion in order to obtain the most sensitive SRM transition.

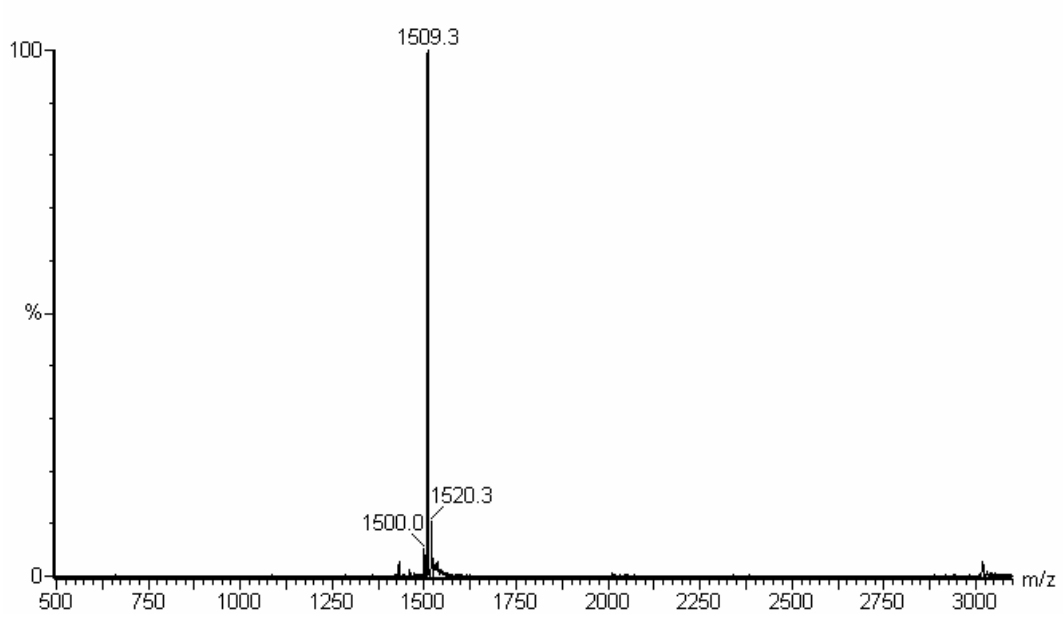

Figure II.8. wESI- spectrum of C-peptide.

Selection of the most sensitive SRM transition

Fierens et al. (37) used high mass range fragments for quantification of C-peptide. Those are most intense at low CID energies (Figure II.9). Our investigations were focused on the low-mass fragment ions generated with high CID energies (see Figure II.10). The most promising ions for SRM were m/z 183.8, 345.4, and 483.5.

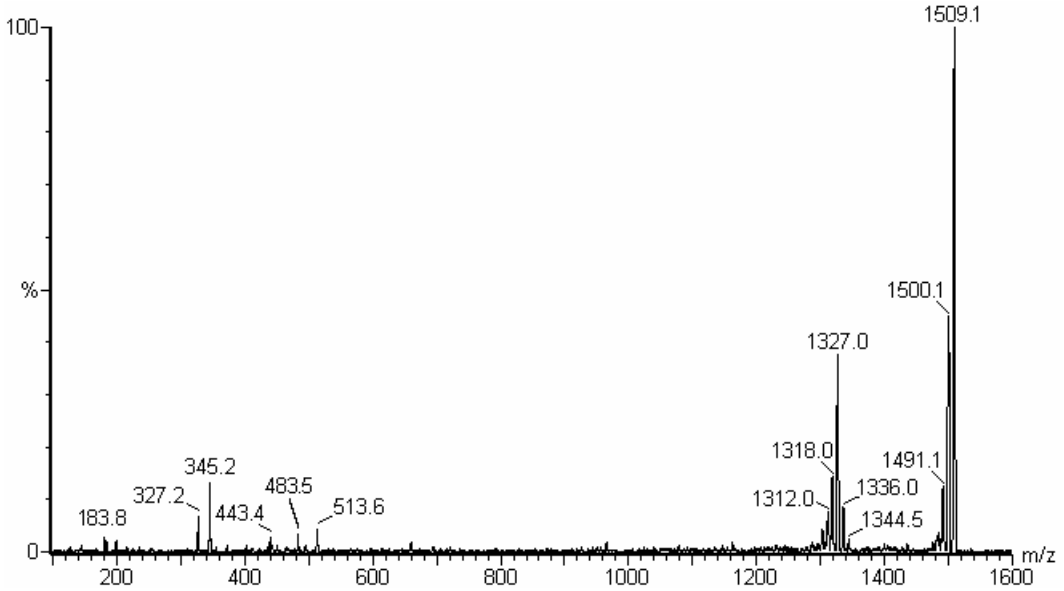

Figure II.9. CID spectrum of the $[M–2H]^{2-}$ ion of C-peptide obtained at a CID energy of 40 eV. Only the range from m/z 100–1600 is shown because no significant ions were observed at m/z values higher than 1600.

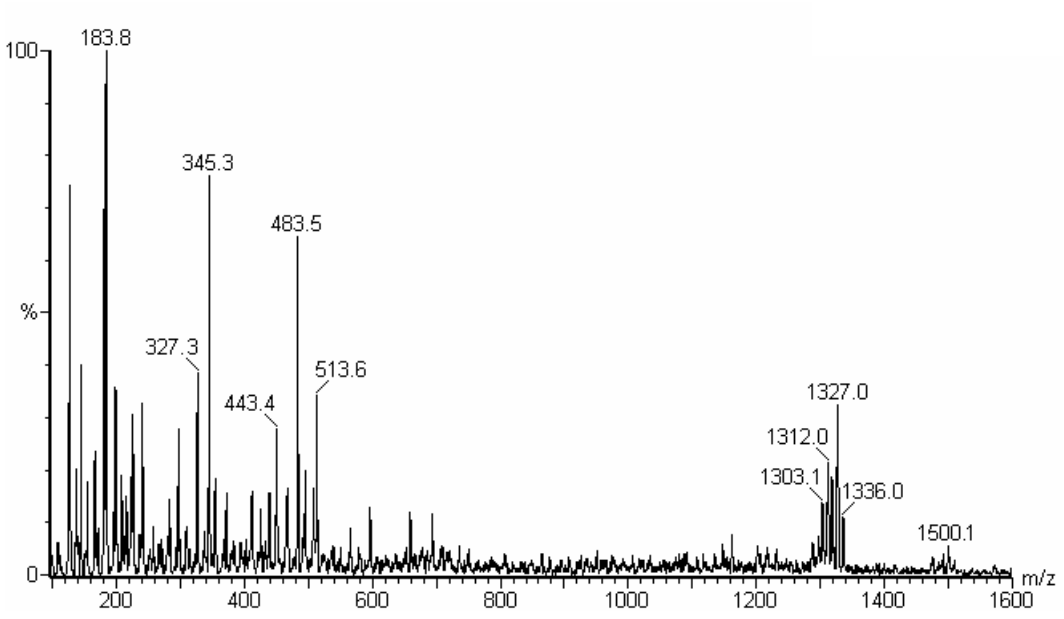

Figure II.10. CID spectrum of the $[M–2H]^{2-}$ ion of C-peptide obtained at a CID energy of 80 eV.

From those, the fragment ion with m/z 183.8 gave the best S/N ratios. Table II.3 compares the S/N ratios obtained by the low- and high-mass SRM transitions for a typical serum sample. The S/N ratio of SRM transition from m/z 1509 to 183.8 was a factor two better than those of the high-mass transitions. All three transitions had similar selectivity.

Table II.3. MS optimized conditions and S/N values for different SRM. See corresponding chromatograms in Figure II.11.

m/z SRM	Cone (eV)	Collision energy (eV)	Skimmer lens offset	S/N
1509 → 1500	82	40	13	25
1509 → 1327	80	40	14	20
1509 → 183.8	85	78	12	45

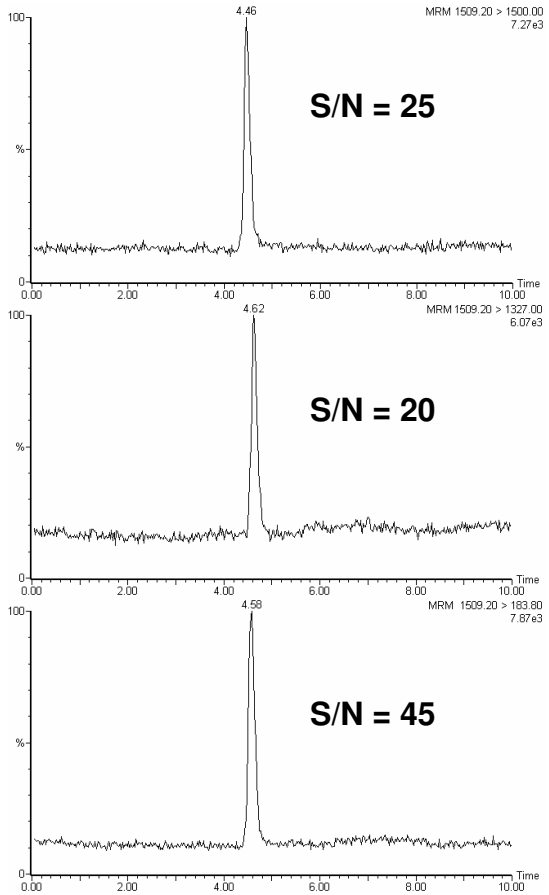

Figure II.11. Chromatograms corresponding to the measurement of 1 mL of serum with a concentration of 1.4 µg/L (the peak represents an absolute amount of 1 ng on column) obtained by monitoring of different SRM: (A) from m/z 1509 to 1500; (B) from m/z 1509 to 1327; (C) from m/z 1509 to 183.8.

CID fragmentation elucidation

The backbone fragmentation nomenclature for positive ions (41) was not used (see Figure II.12), instead the one proposed for negative ions by Bowie (42) and schematically presented in Figure II.13. The reason for that choice is that many backbone cleavage anions have no corresponding positive analogues and consequently the nomenclature for positive ions is not appropriate. Note that the scheme from Figure II.13 shows only where the molecule splits, given that the final fragment ion is often formed by complex mechanisms that include neutral loss of hydrogen as well as proton loss. As in the positive ion mode, there are many different backbone cleavages in negative ion spectra. Although two of these, α and β, provide data equivalent to those of B and Y+2 positive ion cleavages, the mechanisms of formation of the negative ions are quite different from those of their positive ion counterparts. There are also other fragmentations possible in the negative mode (ε, β', …), however, they will not be mentioned because they do not apply to the C-peptide molecule. A detailed explanation of the negative ion cleavages that take place is beyond the scope of this dissertation. For further information, the reader is referred to the review by Bowie's group (42).

$$H_2N\text{-}CHR_1\text{-}CO\text{-}NH\text{-}CHR_2\text{-}_A|^X\text{-}CO\text{-}_B|^Y\text{-}NH\text{-}_C|^Z\text{-}CHR_3\text{-}CO_2H$$

Figure II.12. Backbone fragmentation nomenclature for positive ions.

$$H_2N\text{-}CHR_1\text{-}CO\text{-}NH\text{-}CHR_2\text{-}_{(-)}|^{(-)}\text{-}CO\text{-}_\beta|^\alpha\text{-}NH\text{-}_\delta|^\gamma\text{-}CHR_3\text{-}CO_2H$$

Figure II.13. Backbone fragmentation for negative ions. Note that the fragmentation "-" is absent in the negative mode.

In Figures II.9 and II.10 the main CID fragment ions of the $[M\text{-}2H]^{2-}$ ion of C-peptide can be seen. A summary of the m/z values and fragment assignment for the four molecules studied (C-peptide, (D_8-$Val^{7,10}$)-C-peptide, Val-C-peptide and C-peptide-Val) is presented in Table II.4. The CID spectrum of C-peptide is characterized by two abundant ion series at m/z below 1509.1 (m/z 1500.1, 1491.1, etc.) and around m/z 1327.0 (m/z 1344.5, 1336.0, 1327.0, 1318.0, 1312.0) and some minor fragment ions in the mass range m/z 320-520 (327.3, 345.3, 443.4, 483.5, 513.6). At higher CID energies the low mass ions m/z 183.8, 345.4, and 483.5 are most prominent. Comparison with the data from Val-

C-peptide and C-peptide-Val (Table II.4) shows that the higher mass series are doubly charged ions (expected difference 49.6 Th), while the lower mass fragments are singly charged ones (expected difference 99.1 Th). From the spectra of the homologues it could be inferred that the ion series at m/z below 1509.1 do not correspond with a backbone cleavage but with multiple loss of water.

In addition, the spectra show that the ion series around m/z 1327.0 and at m/z 513.6 and 483.5 are attributable to the C-terminus (α or γ type; no mass difference for Val-C-peptide), whereas the ions at m/z 443.4, 345.3 and 327.3 come from the N-terminus (β or δ type; no mass difference for C-peptide-Val). The data for the deuterated C-peptide confirm these findings. The Asp residue in position 4 (D^4) dominates the backbone cleavages (42), leading to the series of doubly charged fragment ions around m/z 1327.0 and to the singly charged fragment ions at m/z 345.3 and 327.3 (see Table 1 for possible mechanisms). The other backbone cleavages occur between D^4/L^5 and L^{26}/E^{27} (see Table II.4 for details). Major side-chain cleavages are observed due to the presence of 1 D/4 E (loss of H_2O), 4 Q (loss of NH_3), and 2 S (loss of CH_2O) (42).

The transitions from m/z 1509 and 1517 to m/z 1500 and 1508 corresponding to a loss of H_2O from side-chain cleavages, are typical in peptides containing Asp and Glu residues and produce some of the most abundant ions in the negative ion spectra. In an initial stage of the method development these transitions were selected for measurement because they provided a good specificity and S/N ratio for quantification of C-peptide in serum samples.

The generation of the fragment ion at m/z 183.8 was also investigated by measurement of unlabelled, labeled, Val-C-peptide and C-peptide-Val. All four compounds gave very similar spectra indicating that the ion at m/z 183.8 is not generated by the known fragmentation mechanisms involving the cleavage of a single backbone bond.

Formalistically, the ion m/z 183.8 corresponds to a $\{[\text{-NH-CH}_2\text{-CO-NH-CH}(C_3H_6NO)\text{-CO}]\text{-[H]}\}^-$ fragment of the amino acids G and Q (G and Q are adjacent to each other in positions 8 and 9). This ion could be generated by cleavage of two backbone bonds. However, to the best of our knowledge, fragmentation mechanisms for negative peptide fragment ions under "high" collision energies (for quadrupole mass spectrometers) have not been investigated yet. Therefore, the assignment of m/z 183.8 must remain a speculation. Further investigation (e.g. high resolution measurements) was beyond the scope of the thesis.

Table II.4. Negative fragment ions obtained from the $[M-2H]^{2-}$ precursor ion of the different C-peptide molecules studied (the backbone cleavages are indicated below).

C-peptide	V-C-peptide	C-peptide-V	$(D_8\text{-}V^{7,10})$-C-peptide	Fragment assignment [a]	Charge
1509.1[b]	1558.7	1558.7	1517.1	$[M-2H]^{2-}$ precursor ion	-2
1500.1	1549.7	1549.7	1508.1	$-H_2O$	-2
1491.1	1540.7	1540.7	1499.1	$-2H_2O$	-2
1344.5	1344.5	1394.1	1352.5	α between E^3 and D^4	-2
1336.0	1336.0	1385.5	1344.0	γ at D^4 or α between E^3 and D^4-NH_3	-2
1327.0	1327.0	1376.5	1335.0	γ at D^4-H_2O or α between E^3 and D^4-NH_3-H_2O	-2
1318.0	1318.0	1367.5	1326.0	γ at D^4-$2H_2O$ or α between E^3 and D^4-NH_3-$2H_2O$	-2
1312.0	1312.0	1361.5	1320.0	γ at D^4-H_2O-CH_2O or α between E^3 and D^4-NH_3-H_2O-CH_2O	-2
513.6	513.6	612.7	513.6	α between L^{26} and E^{27}-H_2O	-1
483.5	483.5	582.7	483.5	α between L^{26} and E^{27}-H_2O-CH_2O	-1
443.4	542.5	443.4	443.4	β between D^4 and L^5	-1
345.3	444.5	345.3	345.3	δ at D^4	-1
327.3	426.4	327.3	327.3	δ at D^4-H_2O	-1
183.8	183.8	183.8	183.8	{[-NH-CH_2-CO-NH-CH(C_3H_6NO)-CO] - [H]}$^-$ fragment of G^8 and Q^9	-1

[a] E1AE3|D^4|L^5QV$G^8$$Q^9$VELGGGPGAGSLQPLAL^{26}|$E^{27}$GSLQ^{31}

[b] For better comparison, the theoretical m/z values are listed. All observed masses were within ±0.3 u of the theoretical values.

II.3.3. Stability of standards and serum

Stability of D_8-$Val^{7,10}$-C-peptide and validity of isotopic dilution approach

The coefficient of variation of 4.3% obtained for the isotope ratios during the preliminary precision experiment was able to confirm that there was no significant H/D exchange, if any. This proves the identical behavior (stability) of C-peptide and the deuterated analog and, as a consequence, the validity of the isotope dilution approach. In addition, the internal validation data in terms of precision, accuracy and trueness (see Chapter II.3.5 and Table II.7 below) and the agreement between isotope ratios of calibration mixtures measured directly and after passing through analysis (see next paragraph) document the advantages of the ID principle. The ID approach ensures, throughout the entire procedure, compensation for eventual losses, degradation or incomplete recovery of C-peptide. This is a condition *sine qua non* to claim that a measurement procedure is of a higher hierarchical order.

Stability of standard working solutions and verification of calibrators through analysis

The data of the calibrators prepared from standard solutions containing 1% BSA and taken through the entire sample preparation procedure and the standards prepared in 0.003% BSA and directly measured are shown in Table II.5. Statistical testing of the variances and means of the isotope ratios obtained for the two series of calibration mixtures did not reveal a significant difference (P 0.63 in the F-test and P 0.92 in the t-test). It can be concluded that the working standard solutions prepared on the day of analysis are stable and that there is no need for taking the standards through analysis.

Stability of C-peptide in serum

The data of the samples stored at room temperature (20 to 25 °C), between 2 and 8 °C and at -20 °C and analyzed in six consecutive days is presented in Table II.6. In Figure II.12 the average concentration (%) for each day is plotted. The paired t-test on the data for the stability testing upon storage revealed a significant difference versus the reference from day two on for samples stored at room temperature. The concentrations decreased in average by 5% (day two) to 20% (day six). For the other storage conditions the differences were not significant ($P > 0.05$). The results from the stability testing prove that the storage conditions in our study were satisfactory to preserve the stability of C-peptide

Table II.5. Results of the experiment to determine the stability of the working standards and calibration solutions.

	Standards in 1% BSA taken through analysis					
Weight ratio (C-peptide/IS)	1.0333	1.0246	0.9828	0.9998	0.9465	0.9580
Area ratio (C-peptide/IS)	1.0272	0.9758	0.9740	0.9817	0.9114	1.0409
Normalized (Area ratio/weigh ratio)	0.9941	0.9524	0.9911	0.9818	0.9629	1.0866
Mean ± 95% CI	0.9948 ± 0.0550					
	Standards in 0.003% BSA directly injected					
Weight ratio (C-peptide/IS)	0.9915	0.9859	0.9879	1.0195	1.0472	1.0276
Area ratio (C-peptide/IS)	1.0006	0.9839	1.0067	0.9930	1.0775	0.9503
Normalized (Area ratio/weigh ratio)	1.0092	0.9980	1.0190	0.9740	1.0289	0.9248
Mean ± 95% CI	0.9923 ± 0.0438					

in serum. In addition, they evidence that storage of the samples at -20 ºC, necessary for logistic reasons during split-sample comparison will not jeopardize the standardization process. This is a comforting observation since the susceptibility of C-peptide to cleavage by proteolytic enzymes has always been considered a potential pitfall in standardization programs (9, 21).

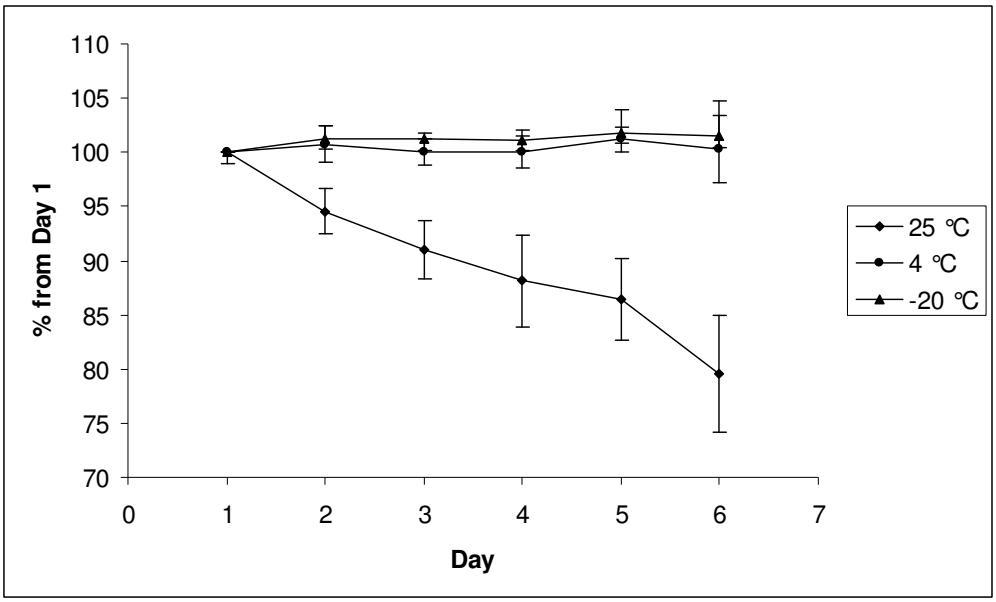

Figure II.12. Average concentration (%) for samples kept at different temperatures during 6 days with the correspondent 95% CI.

Table II.6. Stability results (µg/L) for selected serum samples kept at different temperatures during 6 days.

Sample	Conditions	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
1	20-25 ℃	3.43	3.31	3.20	3.16	3.06	2.95
	2-8 ℃	3.43	3.44	3.45	3.47	3.49	3.57
	-20 ℃	3.43	3.47	3.46	3.44	3.51	3.56
2	20-25 ℃	6.79	6.33	6.07	5.86	5.69	5.25
	2-8 ℃	6.79	6.92	6.77	6.78	6.92	6.66
	-20 ℃	6.79	6.84	6.89	6.89	6.88	6.85
3	20-25 ℃	7.50	7.01	6.71	6.51	6.39	5.84
	2-8 ℃	7.50	7.42	7.4	7.38	7.6	7.51
	-20 ℃	7.50	7.52	7.57	7.53	7.47	7.35
4	20-25 ℃	9.17	8.78	8.53	8.31	8.20	7.34
	2-8 ℃	9.17	9.24	9.21	9.23	9.29	9.15
	-20 ℃	9.17	9.37	9.25	9.35	9.33	9.50
5	20-25 ℃	11.34	10.65	10.20	9.63	9.56	8.66
	2-8 ℃	11.34	11.56	11.44	11.35	11.32	11.27
	-20 ℃	11.34	11.59	11.54	11.50	11.78	11.46

II.3.4. Method validation

Specificity

There was no indication of ion suppression or interference by analytes other than C-peptide or D_8-$Val^{7,10}$-C-peptide during LC/tandem MS measurements. The serum extracts without added D_8-$Val^{7,10}$-C-peptide and monitored at the m/z value typical for the transition of the isotopically labeled analogue did not show any peak between 3 and 6 min (see Figure II.13), except from the overlap around 5% in the SRM from m/z 1509 to 183.8. This overlap is taken into account in the calculation of sample concentrations. Note that this overlap was also present in the injections of standard solution. From the very clean chromatograms and the good results of precision at different concentrations, it can be concluded that the chances for interference are very low.

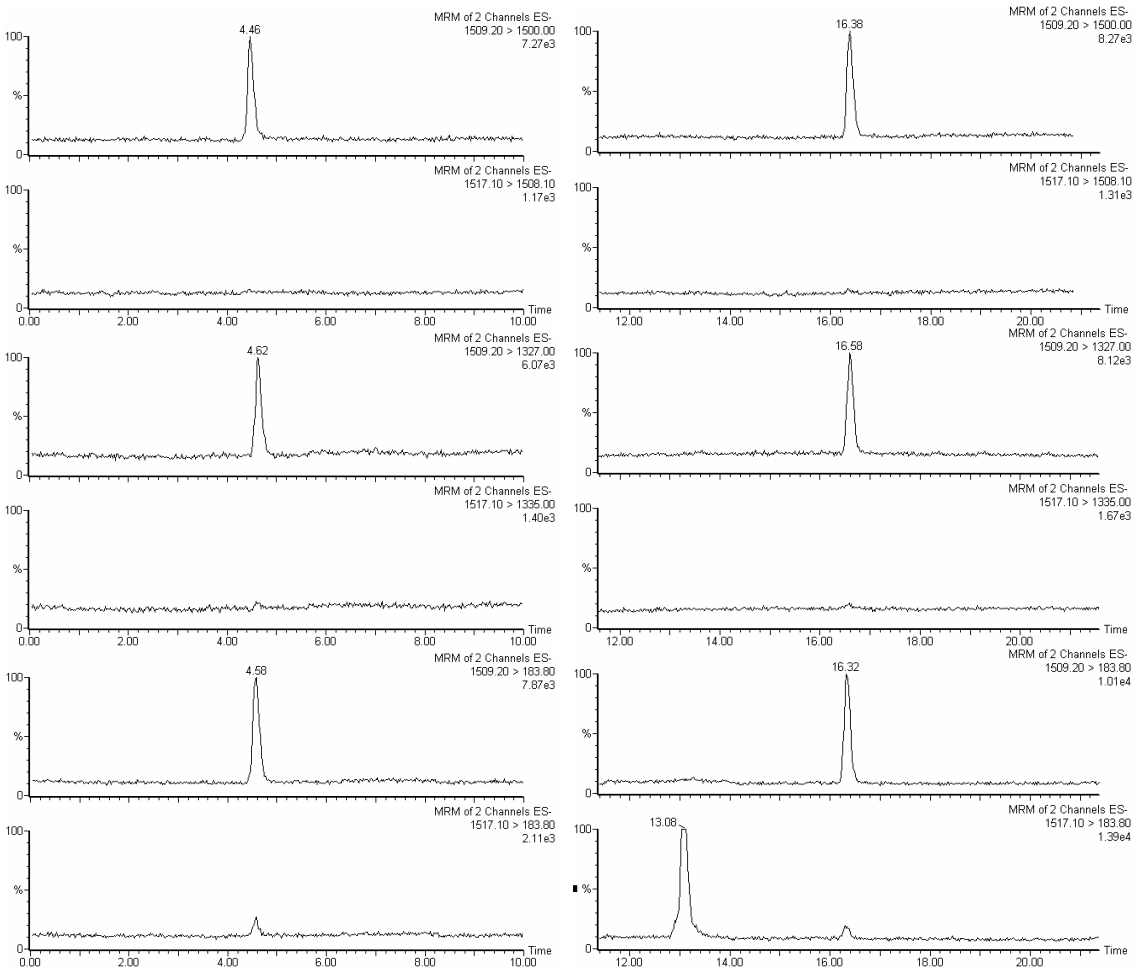

Figure II.13. Chromatograms for the standard solution (on the left) and samples (on the right) measured at different SRM, note that no IS was added.

Limit of detection and quantification

The analysis of 2.5 mL of a sample with a concentration of 0.4 µg/L resulted in a chromatogram with a S/N ratio of 32 (see Figure II.14). Extracts of the same sample were diluted before injection until S/N ratios of 10 and 3 were obtained (data not shown).

The LOD and LOQ of the ID-LC/tandem MS procedure were estimated to be 0.03 µg/L (S/N ratio of 3) and 0.15 µg/L (S/N ratio of 10), respectively when processing 2.5 mL of serum. This was considered sufficient for reliable quantification of physiological C-peptide concentrations in serum (typically between ~1 and 4 µg/L).

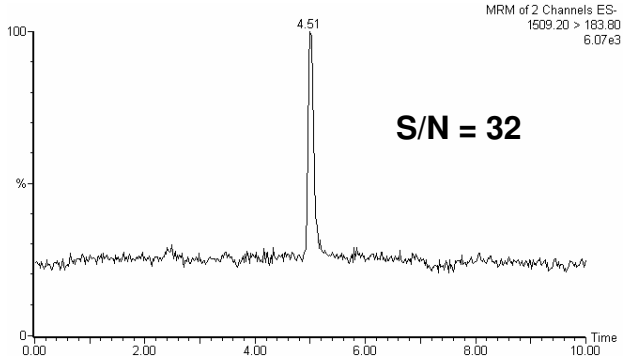

Figure II.14. Chromatogram of a processed sample of 2.5 mL containing 0.4 µg/L.

Recovery of the sample preparation procedure

With regard to the total recovery, no significant decrease in our sample pretreatment procedure as opposed to that of the Kippen et al. was observed, i.e., 77±0.8% (SEM; n=4) vs. 80% (2). Note that Darby et al.(25) do not specify the recovery.

Ion suppression

The response for the injection of a IS solution and the injection of a processed serum sample whose extract was redissolved with the same IS solution were compared. Both gave similar intensities (see Figure II.15) (n=3), indicating the absence of ion suppression.

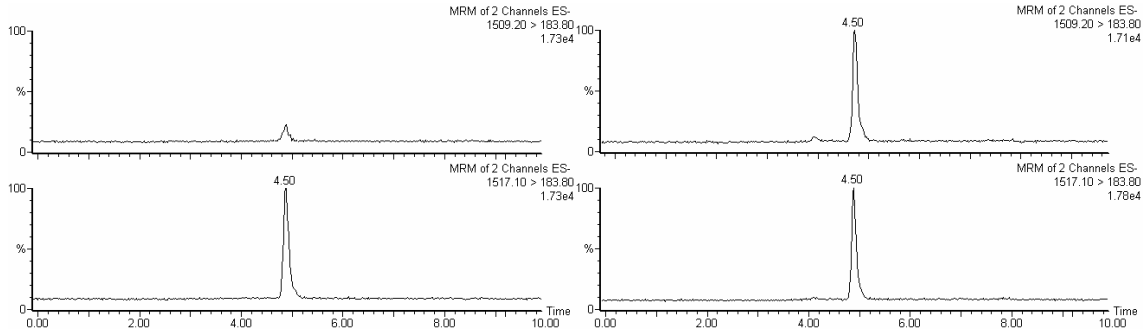

Figure II.15. The left chromatogram represents 3 ng of an IS solution. The right chromatogram represents a serum extract reconstituted with an IS solution containing 3 ng. Note that the small peak at the m/z value of C-peptide after injection of the IS is due to the spectral overlap of the IS on the ion of the standard.

Precision

The data for the precision of the ID-LC/tandem MS procedure is summarized in Table II.7. The CV_{within} ranged from 1 to 3.5%, the $CV_{between}$ from 0.1 to 2.5%, and the CV_{total} from 1.8 to 3.8%. In general, the data demonstrates that the method is capable of reaching the predefined imprecision goal of a CV_{total} of 3%.

Accuracy and trueness

The data for the accuracy and trueness of the ID-LC/tandem MS is summarized in Table II.7. The accuracy ranged between 94.6% and 104.1%, and the minimum trueness was 98.1% (95% CI, 96.2%–100.0%). These data indicate that the method fulfills the predefined trueness goal of 5%.

Note, however, that the trueness of the method relies on the manufacturer certificate for what concerns the purity of the standard and the filling weight. If an international standardization of C-peptide measurements is considered, a certified primary reference material needs to be developed. Unfortunately, the WHO standard IRP 84/150 cannot be considered as such a material because the ampoule content and the purity of that material are insufficiently characterized.

Table II.7. Precision, accuracy and trueness of the ID-LC/tandem MS procedure.

	CV_{within}, %	$CV_{between}$, %	CV_{total}, %	Accuracy, %	Trueness (95% CI), %
Dedicated sera[a]					
1.6 µg/L (n = 11)[b]	1.6	2.5	2.9	-	-
4.0 µg/L (n = 11)	2.1	1.7	2.7	-	-
Internal quality control materials[c]					
4.1 µg/L (n = 16)	3.5	1.3	3.7	-	-
14.8 µg/L (n = 12)	3.5	1.3	3.8	-	-
Albumin solutions (n = 5)					
1.9 µg/L	1.6	0.8	1.8	96.7 - 99.8	98.1 (96.2-100.0)
4.8 µg/L	1.9	0.1	1.9	97.6 - 101.0	99.8 (98.1-101.5)
7.8 µg/L	1.0	1.6	1.8	97.6 - 102.8	100.0 (97.2-102.8)
Supplemented sera[d] (n = 5)					
1.9 µg/L	1.3	1.5	2.0	94.6 - 104.1	98.1 (95.3-100.9)
4.9 µg/L	2.1	0.6	2.2	98.3 - 103.8	100.2 (97.6-102.8)
7.8 µg/L	1.7	1.4	2.3	97.1 - 103.6	99.5 (96.1-102.9)

[a]Mean endogenous serum C-peptide concentration.

[b]Number of analytical runs in which a sample was assayed in duplicate.

[c]Mean C-peptide concentration in a supplemented serum pool.

[d]The basal C-peptide concentration was 1.16 (95% CI, 1.11-1.21 µg/L) µg/L (n = 4).

II.3.5. Method comparison

The two-step SPE-ID-LC/tandem MS measurement procedure was applied to the evaluation of three routine immunoassays. The results of the method comparison study are presented in the form of i) scatter plots with Deming regression (Figure II.16), ii) absolute and % difference plots before and after recalibration (Figure II.16 to 19).

Data before recalibration

With ID-LC/tandem MS, the basal C-peptide concentrations in the 15 patient samples ranged from 0.5 to 3.7 µg/L; the stimulated concentrations ranged from 1.2 to 9.5 µg/L. The Deming regression analysis (see also Table II.8) indicated that the commercial assays considerably overestimated the C-peptide concentrations in a proportional way (slopes significantly >1) and to a different extent (slopes: Cis bio = 1.90; Roche = 1.74; DPC = 1.53). In addition, a statistically significant negative intercept for the Cis bio assay was documented. This interassay discrepancy documents the fact that calibration with a common calibrator (IRP 84/510) did not provide comparability of the measurements. On the other hand, all immunoassays showed excellent correlation coefficients (Roche, r = 0.9914; Cis bio, r = 0.9889; DPC, r = 0.9815) and low standard errors of the estimates (*Sy/x* = 0.46–0.59 µg/L). The magnitude of *Sy/x* is nearly entirely the result of the combined imprecision of the immunoassay and the ID-LC/tandem MS measurement procedure. This is in contrast to the suggested differences in specificity of different antisera (21). The high correlation coefficients and low *Sy/x* values are a good basis for recalibration of the investigated immunoassays by use of the regression equations of the method comparison. The outcome of a recalibration based on the method comparison data is outlined below.

Table II.8. Deming regression data of the different immunoassays compared with ID-LC/tandem MS.

	Slope (95% CI)	Intercept (95% CI) (µg/L)	Sy/x (µg/L)	Correlation coefficient (*r*)
Roche	1.74 (1.695 - 1.786)	-0.15 (-0.348 - 0.056)	0.461	0.9914
DPC	1.53 (1.468 - 1.600)	-0.016 (-0.313 - 0.281)	0.585	0.9815
Cis bio	1.90 (1.837 - 1.959)	-0.90 (-1.18 - -0.626)	0.570	0.9889

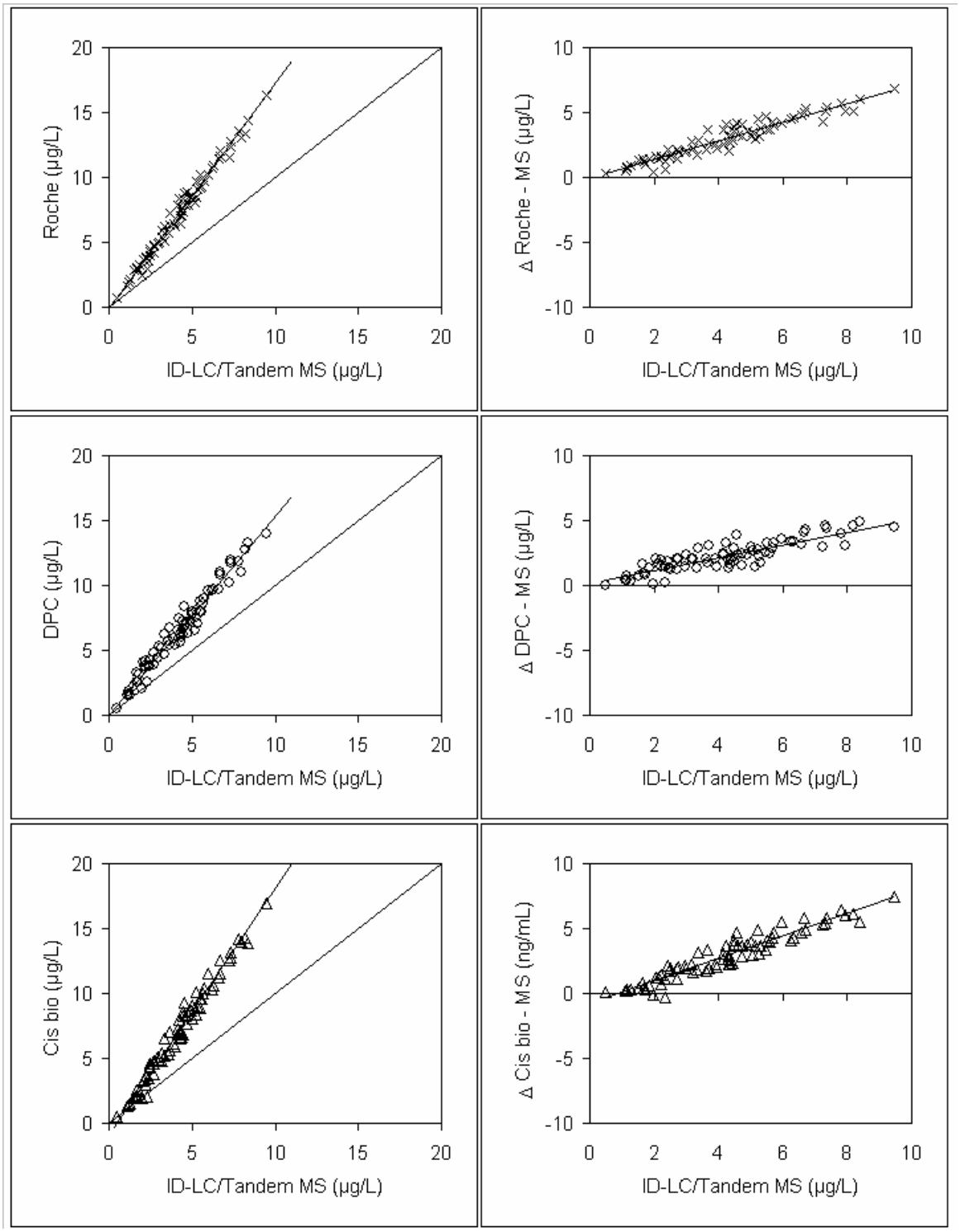

Figure II.16. Method comparison between three immunoassays and the ID-LC/tandem MS measurement procedure. For each immunoassay the scatter and absolute difference plot is shown. Scatter plots show also the curve from Deming regression analysis and the $x = y$ line.

Data after recalibration

Comparing Figure II.17 A and B with C and D shows that the immunoassays can successfully be recalibrated by the outcome of the method comparison study. The residual spread of the data is only an effect of the within-assay deviations from the ID-LC/tandem MS method.

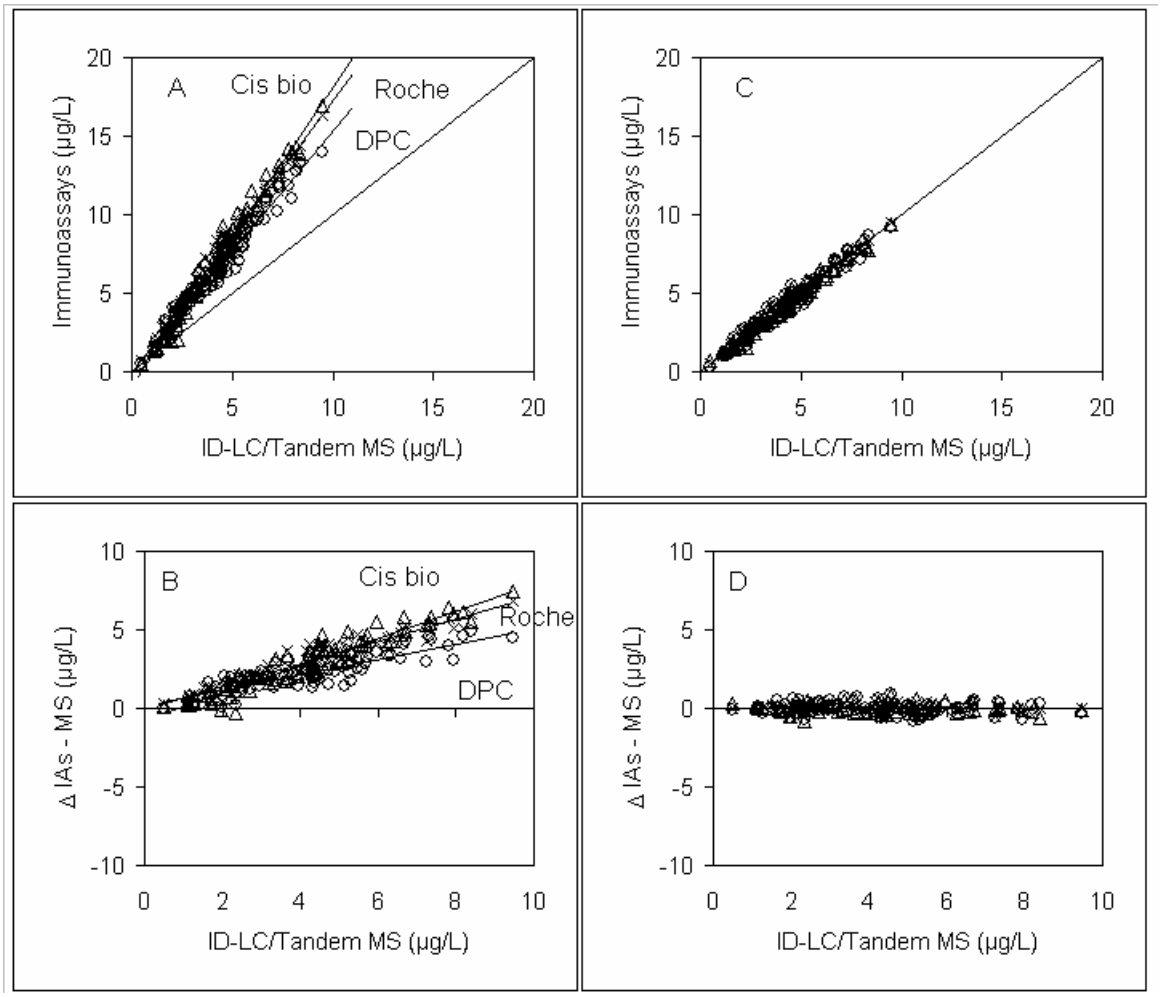

Figure II.17. Combined scatter and absolute difference plots before (A and B) and after (C and D) recalibration with the respective weighted Deming equations.

Recalibrated data and expected total variation

For a more detailed investigation of the residual spread of the data after recalibration, the % difference plots after recalibration shall be used (Figure II. 18). This Figure also includes the expected spread of the data if only the imprecision of the respective method pairs is considered (see also Table II.9).

Table II.9. Total imprecision corresponding to each method, to the method pair and 95% expected interval for the method comparison results.

	CV_{total}	CV_{mp}	1.96 x CV_{mp}
MS	3.8	-	-
Roche	2.3	4.5	8.8
DPC	8.8	9.6	18.8
Cis Bio	6.6	7.6	14.9

The investigation of the plots shows that somewhat more than the expected 5% of the data fall out of limits that represent 1.96 X CV_{mp}. However, it requires only a slight expansion of the limits to observe only 5% of the values outside the limits. This confirms the prior expressed statement that the main contribution to the variability around the regression line comes from the combined imprecision of both the field immunoassay and ID-LC/tandem MS. Sample related effects play only a minor role.

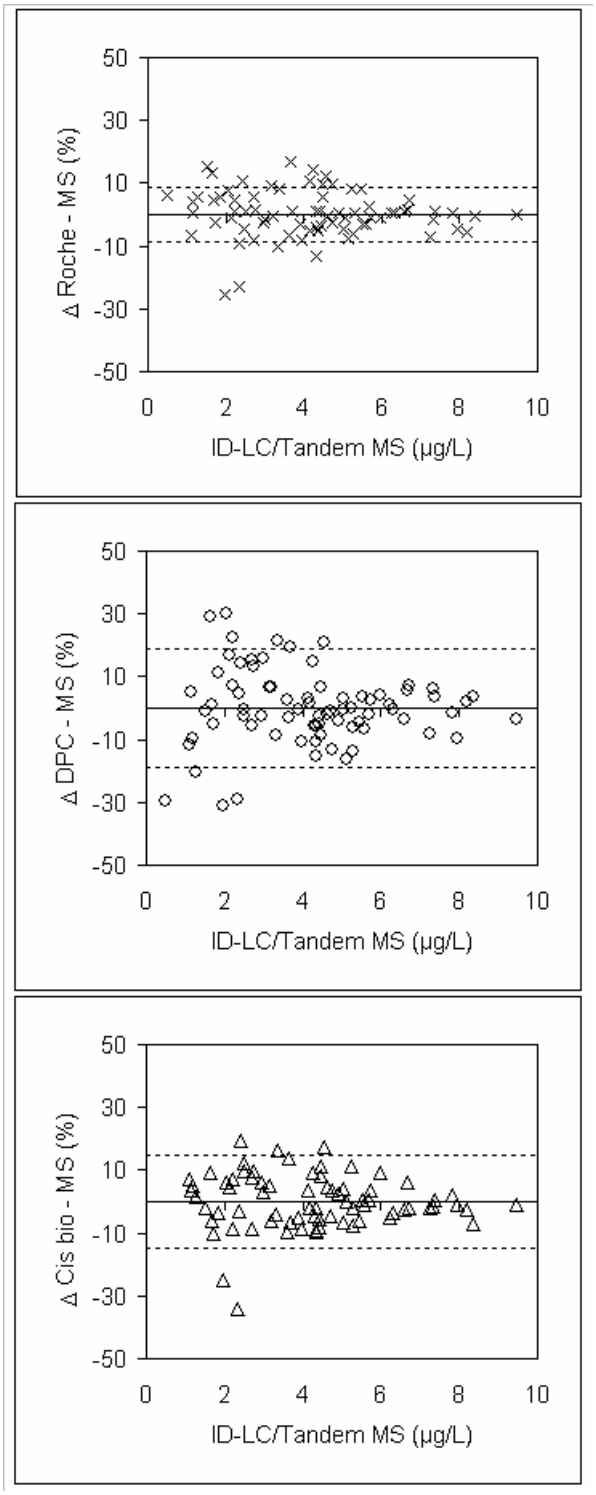

Figure II.18. Percentage difference plots after recalibration for each individual immunoassay. The dotted lines represent the 95% expected interval ($1.96 \times CV_{mp}$).

Recalibrated data and total error limits derived from biology

Additionally, the outcome of recalibration shall be investigated in light of an objective quality goal for total error. Such a goal can be derived from the biological variation of C-peptide: TE = 0.25 $CV_{Between}$ + 1.65 x [0.5 x CV_{Within}] = 11.7%. However, this goal applies only to a situation where the RMP is error-free. In our application, we have to adjust the TE by the imprecision of the RMP as calculated in Materials and Methods, which results in a TE value of 14%. This value is shown in the % difference plots after recalibration in Figure II. 19. Inspection of the figure indicates that the Roche and the CIS bio methods may be able to achieve the total error goal of 14 % (roughly 5% of the data out of the limit), while the DPC method may not fulfill the limit (considerably more than 5% of the data outside the limit).

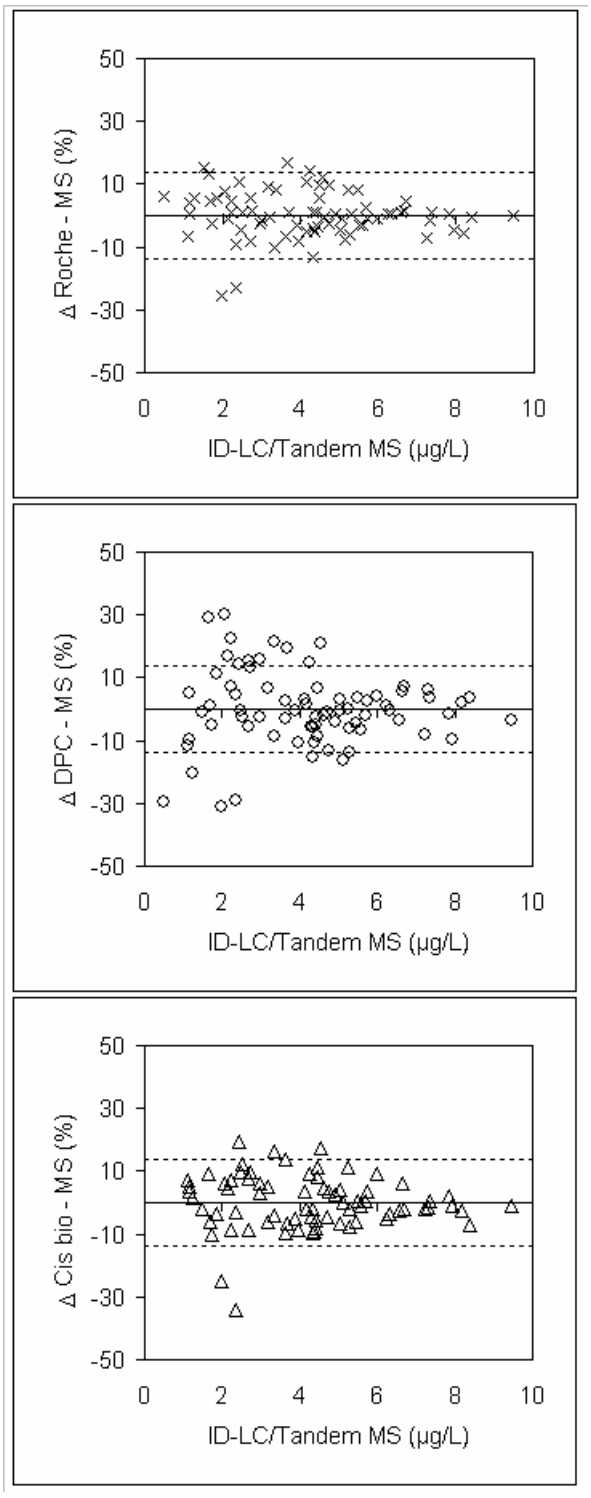

Figure II.19. Percentage difference plots after recalibration for each individual immunoassay. The dotted lines represent the corrected TE specification of 14% that results from the combined biological variation (11.7%) and the imprecision of the ID-LC/tandem MS method.

II.4. CONCLUSION

We developed and validated an ID-LC/tandem MS procedure with a sufficient LOD/LOQ and specificity for interference-free measurement of basal and stimulated serum C-peptide concentrations. The internal validation data documented the compensating effects of ID for eventual losses, degradation, or incomplete recovery of C-peptide, which is necessary for a RMP. The procedure had reasonable recovery and short run time.

We reached this by combining ID-MS with a two-steps SPE procedure of serum (43) and applying negative electrospray ionization and high energy CID of the doubly charged deprotonated C-peptide/D_8-$Val^{7,10}$-C-peptide molecule $[M-2H]^{2-}$ from m/z 1509.2 to 183.8 and 1517.1 to 183.8, respectively. The existing sample pretreatment procedures prior to analysis of C-peptide in serum with ID-LC/tandem MS were improved.

We were able to elucidate the fragmentation pathway of C-peptide with ESI- and CID monitoring of the doubly charged deprotonated molecule $[M–2H]^{2-}$. For example, backbone fragmentation is dominated at the Asp site and losses of water from side-chain cleavages of Asp and Glu residues occur.

The method was successfully applied to a method comparison study with three immunoassays. The comparison showed the need of standardization of C-peptide measurements. It was demonstrated that standardization could be achieved on the basis of a method comparison study such as has been described in this dissertation.

It should be noted, however, that the presented ID-LC/tandem MS measurement procedure does not yet have the status of a SI-traceable RMP. To this end, it will be necessary to develop an internationally accepted primary calibrator that is certified in terms of mass units. Note that the WHO IRP 84/510 does not meet this criterion, since the ampoule content was only nominally assigned the value of 10 µg by comparison with several commercial C-peptide preparations. Additionally, the ID-LC/tandem MS measurement procedure should, as part of an external validation process, be challenged in round-robin trials with other laboratories performing the same or a variant of the ID-MS measurement procedure in order to assess a sufficient level of interlaboratory agreement. Finally, it should be nominated for identification by an authoritative organization such as the recently established JCTLM (44).

II.5. REFERENCES

1. Review of medical physiology. Willian F Ganong. New York (N.Y.): Lange medical books/McGraw-Hill, 21st edition, 2003.
2. Kippen AD, Cerini F, Vadas L, Stocklin R, Vu L, Offord RE et al. Development of an isotope dilution assay for precise determination of insulin, C-peptide, and proinsulin levels in non-diabetic and type II diabetic individuals with comparison to immunoassay. J Biol Chem 1997;272:12513-22.
3. Wahren J, Ekberg K, Jornvall H. C-peptide is a bioactive peptide. Diabetologia 2007:50:503-9 [Epub 2007 Jan 18].
4. Henriksson M, Nordling E, Melles E, Shafqat J, Stahlberg M, Ekberg K et al. Separate functional features of proinsulin C-peptide. Cell Mol Life Sci 2005;62:1772-8.
5. Steiner DF, Rubenstein AH. Proinsulin C-peptide-Biological Activity?. Science 1997;277:531-2.
6. Steiner DF. The proinsulin C-peptide--a multirole model. Exp Diabesity Res 2004;5:7-14. Review.
7. Marques RG, Fontaine MJ, Rogers J. C-peptide: much more than a byproduct of insulin biosynthesis. Pancreas 2004;29:231-8 [Review].
8. Parveen Kumar, Michael Clark. Clinical medicine. 6th edition. Elsevier Saunders.
9. Palmer JP, Fleming GA, Greenbaum CJ, Herold KC, Jansa LD, Kolb H, et al. C-peptide is the appropriate outcome measure for type I diabetes clinical trials to preserve beta-cell function: report of an ADA workshop, 21-22 October 2001. Diabetes 2004;53:250-64. Erratum in: Diabetes 2004;53:1934.
10. Maruyama T, Shimada A, Kanatsuka A, Kasuga A, Takei I, Yokoyama J, et al. Multicenter prevention trial of slowly progressive type I diabetes with small dose of insulin (the Tokyo study): preliminary report. Ann N Y Acad Sci 2003;1005:362-9.
11. Greenbaum CJ, Harrison LC. Immunology of Diabetes Society. Guidelines for intervention trials in subjects with newly diagnosed type I diabetes. Diabetes 2003;52:1059-65. Erratum in: Diabetes 2003;52:2643.
12. TrialNet Study. Further research to prevent and treat type I [No authors listed]. Diabetes Forecast 2004;57:77-9. htpp://clinicaltrials.gov/ct/show/NCT00105352 (Accessed June 2007).
13. Pourmotabbed G, Kitabchi AE. Hypoglycemia. Obst Gynecol Clin North Am 2001;28:383-400.

14. Batstra MR, Aanstoot H-J, Herbrink P. Prediction and Diagnosis of Type I Diabetes Using B-cell Autoantibodies. Clin Lab 2001;47;497-507.
15. Törn C. C-peptide and Autoimmune Markers in Diabetes. Clin Lab 2003;49:1-10.
16. Haupt E, Benecke A, Haupt A, Herrmann R, Vogel H, Walter C. The KID Study VI: diabetic complications and associated diseases in younger type II diabetics still performing a profession. Prevalence and correlation with duration of diabetic state, BMI and C-peptide. Exp Clin Endocrinol Diabetes 1999;107:435-41.
17. Sacks DB, Bruns DE, Goldstein DE, Maclaren NK, McDonald JM, Parrott M. Guidelines and recommendations for laboratory analysis in the diagnosis and management of diabetes mellitus. Clin Chem 2002;48:436-72.
18. Sacks DB. Carbohydrates. In: Burtis CA, Ashwood ER, eds. Tietz Textbook of Clinical Chemistry, 3rd ed. Philadelphia: WB Saunders 1999:750-808.
19. Haupt E, Haupt A, Herrmann R, Benecke-Timp A, Vogel H, Walter C. The KID Study V: the natural history of type II diabetes in younger patients still practising a profession. Heterogeneity of basal and reactive C-peptide levels in relation to BMI, duration of disease, age and HbA1. Exp Clin Endocrinol Diabetes 1999;107:236-43.
20. Zavaroni I, Bonora E, Pagliara M, Dall'Aglio E, Luchetti L, Buonanno G et al. Risk factors for coronary artery disease in healthy persons with hyperinsulinemia and normal glucose tolerance. N Engl J Med 1989;320:702-6.
21. Clark PM. Assays for insulin, proinsulin(s) and C-peptide. Ann Clin Biochem 1999;36:541-64 [Review].
22. Knip M. Analysis of Pancreatic Peptide Hormones by Reversed-Phase High-Performance Liquid Chromatography. Horm Metabol Res 1984:487-491.
23. Komjati M, Nowotny P, Waldhäusl W. Radioimmunologic Determination of Human C-Peptide and Proinsulin in Plasma with Anti-C-Peptide Serum after Prepurification by High-Performance Liquid Chromatography. Anal Chem 1987:2010-2.
24. Kuzuya M, Blix PM, Horwitz DL, Steiner DF, Rubenstein AH. Determination of free and total insulin and C-peptide of insulin-treated diabetics. Diabetes 1977;36:22-9.
25. Darby SM, Miller ML, Allen RO, LeBeau M. A mass spectrometric method for quantitation of intact insulin in blood samples. J Anal Toxicol 2001;25:8-14.
26. Rogatsky E, Balent B, Goswami G, Tomuta V, Jayatillake H, Cruikshank G et al. Sensitive quantitative analysis of C-peptide in human plasma by two-dimensional

liquid chromatography-mass spectrometry isotope-dilution assay. Clin Chem 2006;52:872-9 [Epub 2006 Mar 23].

27. Fierens C, Stockl D, Thienpont LM, De Leenheer AP. Strategies for determination of insulin with tandem electrospray mass spectrometry: implications for other analyte proteins?. Rapid Commun Mass Spectrom 2001;15:1433-41.
28. Fierens C, Stockl D, Thienpont LM, De Leenheer AP. A convenient method for the generation of negative and positive electrospray ionization mass spectra of proteins by gas-phase admission of volatile bases and acids via the nebulizing gas. Rapid Commun Mass Spectrom 2001;15:451-3.
29. Fierens C, Thienpont LM, Stöckl D, De Leenheer AP. Matrix effect in the quantitative analysis of urinary C-peptide by liquid chromatography tandem mass spectrometry. Rapid Commun Mass Spectrom 2000;14:936-937.
30. Fierens C, Thienpont LM, Stöckl D, Willekens E, De Leenheer AP. Quantitative analysis of urinary C-peptide by liquid chromatography-tandem mass spectrometry with a stable isotopically labelled internal standard. J Chromatogr A 2000;896:275-8.
31. Fierens C, Stöckl D, Baetens D, De Leenheer AP, Thienpont LM. Application of a C-peptide electrospray ionization-isotope dilution-liquid chromatography-tandem mass spectrometry measurement procedure for the evaluation of five C-peptide immunoassays for urine. J Chromatogr B Analyt Technol Biomed Life Sci 2003;792:249-59.
32. Fierens C, Stöckl D, Baetens D, De Leenheer AP, Thienpont LM. Standardization of C-peptide measurements in urine by method comparison with isotope-dilution mass spectrometry. Clin Chem 2003;49:992-4.
33. Cohen RM, Given BD, Licinio-Paixao J, Provow SA, Rue PA, Frank BH et al. Proinsulin radioimmunoassay in the evaluation of insulinomas and familial hyperproinsulinemia. Metabolism 1986;35:1137-46.
34. Amersham Biosciences. Affinity Chromatography. Principles and Methods. Amersham Biosciences AB 2002.
35. Thienpont LM, Van Nieuwenhove B, Stöckl D, De Leenheer A. Calibration for isotope dilution mass spectrometry – Description of an alternative to the bracketing procedure. J Mass Spectrom 1996;31:1119-25.
36. Fierens C, Thienpont LM, Stockl D, De Leenheer AP. Overcoming practical limitations for the application of ultrafiltration in sample preparation for liquid

chromatography/ mass spectrometry of small proteins. Anal Biochem 2000;285:168-9.

37. Clinical and Laboratory Standards Institute (CLSI). Evaluation of precision performance of clinical chemistry device; approved guideline. CLSI document EP5-A2 Wayne: CLSI, 2004.
38. Roche. Instruction sheet of C-peptide Elecsys® 1010/2010/Modular Analytics E170 (2004 version).
39. DPC. Instruction sheet of C-peptide Immulite® (2004 version).
40. Technogenetics (distributed by Cis bio international). Instruction sheet of IRMA-C-PEP (2004 version).
41. Biemann K, Martin SA. Mass spectrometric determination of the amino acid sequence of peptides and proteins. Mass Spectrom Rev 1987;6:1-75.
42. Bowie JH, Brinkworth CS, Dua S. Collision-induced fragmentations of the (M-H)- parent anions of underivatized peptides: an aid to structure determination and some unusual negative ion cleavages. Mass Spectrom Rev 2002;21:87-107 [Review].
43. Rodríguez-Cabaleiro D, Stöckl D, Thienpont LM. Improvement of Sample Pretreatment Prior to Analysis of C-peptide in serum by Isotope Dilution-Liquid Chromatography-Tandem Mass Spectrometry. Rapid Commun Mass Spectrom 2005;19:3600-2.
44. Bureau International des Poids et Mesures. Joint Committee for Traceability in Laboratory Medicine. Declaration of cooperation between the CIPM, IFCC and ILAC, for the establishment of a Joint Committee for Traceability in Laboratory Medicine. http://www.bipm.org/utils/en/pdf/establish-jctlm.pdf. JCTLM Datatbase of Reference Materials and Reference Measurement Procedures. http://www.bipm.org/jctlm/ (Accessed June 2007).

CHAPTER III.

DEVELOPMENT AND APPLICATION OF A REFERENCE MEASUREMENT PROCEDURE FOR INSULIN IN SERUM

The development of the ID-LC/tandem MS measurement procedure for serum insulin was published in:

- Van Uytfanghe K, Rodríguez-Cabaleiro D, Stöckl D, Thienpont LM. New liquid chromatography/electrospray ionization tandem mass spectrometry measurement procedure for quantitative analysis of human insulin in serum. Rapid Commun Mass Spectrom 2007;21:819-21.

The validation of the ID-LC/tandem MS measurement procedure for serum insulin and investigation of the method feasibility for the purpose of standardization by performing a method-comparison study with four immunoassays was published in:

- Rodríguez-Cabaleiro D, Van Uytfanghe K, Stove V, Fiers T, Thienpont LM. Pilot study for the standardization of insulin immunoassays with isotope-dilution liquid chromatography-tandem mass spectrometry. Clin Chem 2007;53 (in press).

III.1. INTRODUCTION

III.1.1. Insulin measurements in the clinical laboratory

In the clinical laboratory, measurements of insulin are performed with a great variety of immunoassays (1). However, the quality of these assays has long been a matter of concern. The problems encountered are lack of standardization, non-specificity of the used antibodies and/or the presence of anti-insulin antibodies and insulin auto-antibodies in the patients samples (1, 2). For example, the ADA task force on standardization of insulin assays reported widely disparate results from immunoassays (2). Problems with insulin immunoassays have been confirmed recently (3, 4). These problems hinder efforts towards achieving consistent measures for treatment guidelines. To address this issue, the ADA in conjunction with the NIDDK and the Centers for Disease Control and Prevention (CDC) convened an international work group in 2004 to evaluate the specificity of different assays, to establish guidelines for assay acceptability, and to develop a standardization program to achieve uniform, accuracy-based values. A recent report on the progress made by the work group concluded that "a common insulin reference preparation did not change the among-assay CV and failed to improve harmonization of results among assays" and that the "investigation of a RMP for insulin should be a priority to provide a metrologically appropriate basis to evaluate the accuracy of routine methods" (3). Similarly, a European group stated in a recent report that "a reference method for measurement of insulin is required with an international standard defined in terms of mass" (4). This publication evoked an editorial with the title “Insulin immunoassays: fast approaching 50 years of existence and still calling for standardization” (5). Altogether, this shows the urgency and timeliness for the development of a RMP for serum insulin.

III.1.2. Instrumental analytical measurement procedures for insulin

A variety of HPLC-based measurement procedures for insulin have been described for pharmaceutical or research applications (6-15) and for the analysis of insulin in biological matrices (16-23). However, only a few methods for the analysis of insulin in serum have been described that are based on LC-MS (24-27). An overview of these methods can be found in Table III.1. Note that only the procedure from Kippen et al. (24) has enough sensitivity to measure endogenous low-level insulin (reference interval of 0.07-0.87 µg/L

(= 12-150 pmol/L or 1.7-22 mIU/L)) (28) and makes use of an isotopically labeled IS. However, a disadvantage of the procedure from Kippen et al. is the off-line sample preparation with a 60 min gradient and MS sample introduction via infusion.

Table III.1. Overview of the LC/MS procedures for insulin in serum described in the literature.

Author (reference)	IS	Sample preparation	HPLC	MS	LOD[a] (fmol)
Kippen AD et al. (24)	[$^2H_{16}$] insulin	Sep-Pak® C18 SPE or IAC + Sep-Pak® C18 light SPE	Nucleosil C18 (150x1 mm) 60 min gradient with ACN/water/0.1% TFA	Off-line Micromass VG single quad™ ESI+ Short scan m/z 1159-1168 $[M+4H]^{4+}$	< 3
Darby SM et al. (25)	no	Sep-Pak® C18 SPE	Hesperia Vydac C18 (150x2.1 mm) 10 min gradient with ACN/water/0.04% TFA	Finnigan MAT LCQ™ Qtrap ESI+ SIM m/z 1453 and 1936	172
Hunter SJ et al. (26)	no	Sep-Pak® C18 SPE	Vydac C8 (250x4.6 mm) 35 min gradient ACN /water/0.1% TFA	Off-line Finnigan MAT LCQ™ Qtrap ESI+ Scan m/z 300 – 2000 $[M+3H]^{3+}$	-
Thevis M et al. (2005) (27)	Bovine insulin	IAC + Oasis® HLB SPE	Agilent Zorbax 300SB-C18 (50x1 mm), 5 µm Gradient with ACN /water/0.5% acetic acid/0.01% TFA	Applied Biosystems QTrap™ ESI+ Product ion scan m/z 1163 to 100-1200	155

[a] Absolute amount of insulin injected in the system.

III.1.3. Objectives

The objective was the development of an ID-LC/tandem MS measurement procedure for serum insulin and its application for the evaluation and standardization of insulin immunoassays. The method development focused on the following topics:

- Standardization
The stabilization effects of glycerol, albumin, glargine and insulin-depleted serum were investigated. The link between our standard and the WHO insulin 1st IRP 66/304 was investigated.

- Sensitive mass spectrometric detection
CID experiments were performed with different gases on two different LC-tandem MS configurations.

- Effective sample purification
Two approaches were studied: a double stage SPE procedure and an IAC method.

- Validation of the finally selected ID-LC/tandem MS procedure
Finally, the feasibility of the method for the purpose of standardization was tested by performing a method comparison study with four representative commercial assays.

Note that the method development has been done in cooperation with K. Van Uytfanghe.

III.2. MATERIALS AND METHODS

III.2.1. Specifications and requirements for a serum insulin candidate RMP

The ADA work group proposed the following performance specifications for such a procedure: a LOQ of 0.07 µg/L (= 12 pmol/L or 1.7 mIU/L), a CV_{total} of maximum 3% (6 – 7% at the LOQ), and a bias limit of ±5% (29).

III.2.2. Calibrators, reagents, materials and sera

Calibrators

Recombinant human insulin expressed in yeast (Sigma-Aldrich) was used as standard material. It was delivered in vials containing 50 mg of freeze-dried material and had a purity and potency of respectively >98% and 27.5 kIU/g (according to the manufacturer's information). 4-[D10]Leu-insulin, labeled in positions 6, 11, 15, and 17 of the beta-chain (see Figure II.1), used as IS, was bought from Peptide Institute (Osaka, Japan). The producer stated a purity of 98.5%, determined by HPLC analysis.

For the preparation of the calibration solutions, two different procedures were applied during the method comparison study (Procedure 1) and method validation (Procedure 2).

Procedure 1: the standard stock solution was prepared by weighing and dissolving a minimum of 5 mg of standard material in 1 mL of a 60/40 (v/v) mixture of 50 mM hydrochloric acid in water and glycerol. The stock solution was diluted in two consecutive steps with the same solvent to obtain a final concentration of 25 mg/L (0.62 kIU/L). Immediately after preparation, the latter solution was divided into 350-µL portions and stored at -20 °C until the day of analysis. These portions were never used more than four times. Each day of analysis fresh working solutions with a concentration of 20 µg/L (= 0.5 IU/L) were prepared. This was done by diluting a portion of the stock solution of 25 mg/L with a 60/40 (v/v) mixture of water/glycerol containing 0.1 g/L of protease-free BSA. Note that two individually weighed stock solutions were always diluted to prepare a total of four different working solutions (2 from each stock). The stock and working solutions of the IS were prepared in an identical way. Thirty µL of a 60/40 (v/v) mixture water/glycerol containing 0.1 g/L of BSA and 10 mg/L of glargine (Lantus® from Sanofi-Aventis) were pipetted into the empty vial, which was vortexed before adding the standard working

solutions. Glargine was used in this and other steps (see also Procedure 2) because it proved to have a carrier effect.

Procedure 2: the standard stock solution was prepared exactly as described in Procedure 1. Subsequently, it was diluted in two consecutive steps with insulin-depleted serum (Scipac) to obtain a final concentration of 25 mg/L (= 0.62 kIU/L). The pH of the insulin-depleted serum was adjusted to 7.4 by adding 100 µL of HEPES buffer (185 g/L HEPES acid and 8.8 g/L NaOH, both from Sigma) per mL of serum. Immediately after preparation, the latter solution was divided into 50-µL portions and frozen at -20 °C until the day of analysis. On that occasion, a frozen vial of the stock solution of 25 mg/L was thawed and diluted with a 60/40 (v/v) mixture of water/glycerol containing 0.1 g/L of BSA and 10 mg/L glargine to obtain working solutions of 20 µg/L (= 0.5 IU/L). This dilution was never reused. Note that two individually weighed stock solutions were always diluted to prepare a total of four different working solutions (2 from each stock). The stock and working solutions of the IS were prepared as described in Procedure 1. One hundred µL of a 60/40 (v/v) mixture of water/glycerol containing 0.1 g/L of BSA and 10 mg/L of glargine were pipetted into the empty vial and vortexed before adding the standard working solutions.

In both procedures low protein-binding Sorensen® vials (BioScience Inc, West Salt Lake City, Ut, USA) were used. Also all volumetric steps involved in the preparation/sampling of the calibration solutions were gravimetrically controlled, using a Mettler Toledo analytical balance, type AT261 Delta Range that allowed mass measurements till 10^{-5} g. Finally, the calibrators were prepared by mixing of equivalent amounts of the insulin and IS working solutions. The calibration mixtures were stored at -20°C until the time of analysis. The analysis of calibrators taken through the sample preparation procedure analysis verified that they could be directly injected in the LC/tandem MS system. A one-point calibration procedure was used (30).

Reagents and materials

Glycerol (Sigma-Aldrich), HPLC-water (Biosolve, Valkenswaard, Holland), ACN (Romil, Cambridge, UK), TFA (Fluka, Buchs, Switzerland) and cyclohexane (Merck, NJ, USA) with pro-analyse, gradient grade, LC/MS grade and for protein use qualities, respectively. For IAC, Trizma® hydrochloride, Trizma® base, NaN_3 and ammonium acetate were purchased from Sigma-Aldrich. Experiments were started with an IAC-gel made in collaboration with the Department of Clinical Medicine, Division of Clinical Chemistry,

Biomedicum Helsinki, University of Helsinki (Finland). A mouse monoclonal antibody (Biodesign International, Saco, ME, USA) coupled to cyanobromide sepharose (GE Healthcare, Diegem, Belgium) was used. The procedure used to make de IAC columns was taken from 'Affinity Chromatography. Principles and Methods' (31) and will not be describe in more detail. Afterwards, further optimization and final application of the IAC procedure was done with a commercial immunoaffinity gel from "Laboratoire d'Hormonologie" (C.E.R. Groupe, Marloie, Belgium). Empty Poly-Prep columns from Bio-Rad (Hercules, CA, USA) were filled with 0.25 mL of IAC-gel. The properties of these two different IAC-gels are summarized in Table III.2. The gel was stored in a buffer at pH 7.4 containing 50 mM Tris and 0.05% of sodium azide in water. Sep-Pak® C18 cartridges (1 cc, 50 mg) from Waters (Milford, MA, USA) were used for SPE after IAC.

Table III.2. Properties of the IAC used columns.

Self made	**Commercially available**
Mouse monoclonal antibody (IgG1,k)	Mouse monoclonal antibody (IgG1,k)
Affinity constant: 6 x10^{8} M^{-1}	-
CNBr sepharose matrix	CNBr sepharose matrix
2 mg IgG/mL capacity	10 mg IgG/mL capacity
0.5 mL gel/column	0.25 mL gel/column

Sera

The sera employed during the method development and optimization experiments came from the blood transfusion center of the Flanders Red Cross at Leuven. They were handled according to the local Ethical Committee guidelines.

III.2.3. Instrumentation

For LC/tandem MS two different instruments were used. Experiments were started with a Micromass VG Quattro II™ triple stage MS (Altrincham, UK) equipped with a megaflow electrospray probe. It was coupled to a Kontron HPLC system Model 325 from (Milan, Italy) equipped with an autosampler 465 from the same brand. Nitrogen was used as the drying and nebulizing gas at a flow rate of respectively 450 and 14 L/h. Later on, a newer instrument with better performance characteristics was used. This was an Applied Biosystems API 4000™ triple stage MS from (Foster City, CA, USA) equipped with an electrospray probe and coupled to an Agilent 1100 Series HPLC and autosampler (Palo Alto, CA, USA). In Table III.3, the settings of the two instruments are summarized.

Table III.3. Main settings of the MS configurations used for the measurement of human insulin (transition monitored m/z 1453 to 226).

Setting	**VG Quattro II™**	**API 4000™**
Cone voltage / Declustering potential	60 V	50 V
Capillary / Ion spray voltage	4 kV	5 kV
Drying / nebulising gas	450 / 14 L/h (nitrogen)	-
Curtain gas / Gas 1 and 2	-	35 (nitrogen) / 65
EP / CXP	-	11 / 12
Source temperature	175 °C	500 °C

III.2.4. Optimization experiments

III.2.4.1. Stability of standard solutions

The stabilization effects of glycerol, albumin, glargine and insulin-depleted serum were investigated.

III.2.4.2. Optimization of HPLC conditions

Experiments were done with a Hamilton PRP-3 column (50x2.1, 3 µm particle size) (Reno, NV, USA), with a Waters XTerra column (2.1x10 mm, 5 µm) and with Waters XBridge columns (Milford, MA, USA). From the latter, different lengths (10 mm and 150 mm) and particle sizes (3.5µm and 5µm) were tested.

III.2.4.3. CID experiments with different instruments and collision gases

CID experiments were performed for the 4-times protonated molecular ion (m/z 1453) with the Micromass VG Quattro II^{TM} and the API 4000^{TM} MS configurations. The collision gases tested were helium, argon, and nitrogen in the VG Quattro II^{TM}. The API 4000^{TM} was only operated with nitrogen, the standard installed gas for CID. Parameters that were optimized comprised the gas pressure and the CID energy. All the other MS settings are summarized in paragraph III.2.3 (see Table III.3).

Samples were introduced by an online HPLC system, under the same conditions as described in paragraph III.2.6. Chromatography was performed with a XBridge C18 column (10x2.1 mm, 5 µm particle size) connected to a prefilter from Alltech (Deerfield, II, USA).

All the measurements were done in the positive wrong way round electrospray ionization (wESI+) mode and monitoring the CID spectra of the four times protonated molecular ion $[M+4H]^{4+}$ (m/z 1453) of human insulin. CID-scans in the range from m/z 50 to 400 were acquired at one scan per second in both instruments.

III.2.5. Sample preparation procedure development

During the development of the sample preparation procedure two different methods were created, one consisted in a combined IAC-SPE (see Figure III.1.A) and the other in a two-step SPE procedure (see Figure III.1.B). The first was already presented and described in detail in the doctoral thesis of Dr. K. Van Uytfanghe. Below the two-step SPE procedure is described in detail together with the way both procedures were evaluated and compared. For a detailed description of the combined IAC-SPE method see paragraph III.2.7.

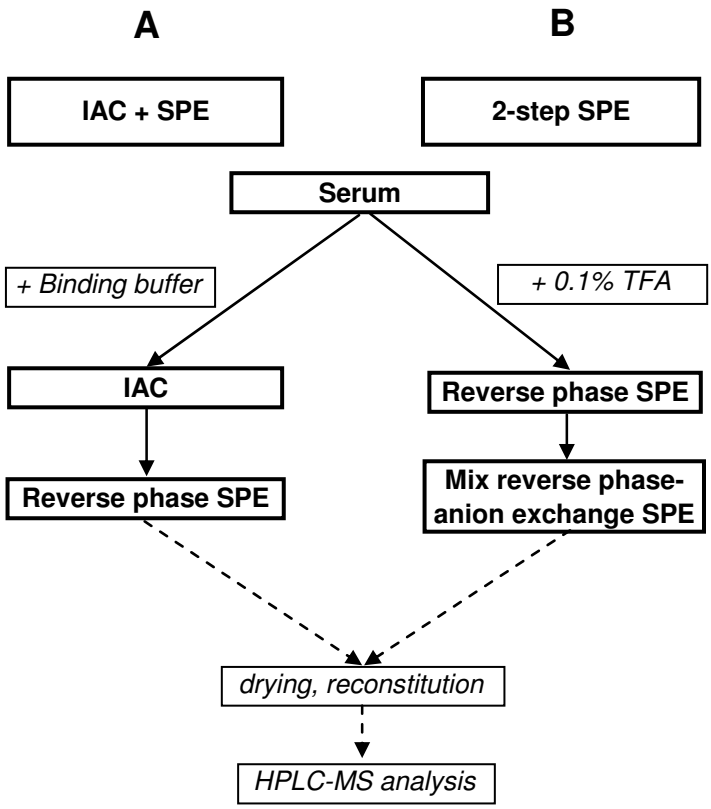

Figure III.1. Schematic representation of both developed methods.

Two-step SPE procedure

This procedure is schematically presented in Figure III.1.B. Samples were diluted 1:2 with 0.1% TFA prior to SPE with Sep-Pak® C18 3 cc (200 mg). Sep-Pak® C18 cartridges were preconditioned by consecutive wetting with 2 mL of ACN and 2 mL of 0.1% TFA. After loading the diluted serum samples twice, cartridges were washed consecutively with 2 mL 20/80/0.3 (v/v/v) ACN/water/TFA, 2 mL 30/70 v/v ACN with 0.1% TFA/dichloromethane

and 0.7 mL 20/80/0.1 ACN/water/TFA. Elution was performed with 2 mL 35/65/0.6 ACN/water/TFA in a test tube containing 0.1 mL HPLC-water and 300 ng glargine. Oasis® MAX 1 cc (30 mg) cartridges were preconditioned by consecutive wetting with 1 mL ACN and 1 mL of HPLC-water. Prior to loading of the extracts onto the Oasis® MAX cartridges, 7 µL of 25% ammonia were added to the Sep-Pak® eluate. The cartridges were washed consecutively with 1.5 mL 23.33/76.67/0.4/0.42 ACN/water/TFA/NH_4OH (i.e., 2 mL of the eluent for elution from Sep-Pak® with 1 mL of water and 50 µL of 25% ammonia) and 1 mL of 5/95/0.5 ACN/water/acetic acid. Elution was done with 1.2 mL 80/20/0.5 ACN/water/acetic acid in a vial containing 100 µL of ACN. Finally, the extracts were evaporated to dryness at 50 °C under nitrogen (~30 min) and stored at -20 °C until analysis. Before LC/tandem MS analysis, 0.25 mL of cyclohexane were added, vortexed and removed. The remaining residue was dried and reconstituted in 50 µL of a 10/90 (v/v) mixture of ACN/water for injection into the chromatographic system (5 to 45 µL, depending on the concentration).

The two-step SPE procedure was qualitatively compared with the combined IAC/SPE procedure. An optimized version of the combined IAC/SPE procedure is described in detail in the final method presented in paragraph III.2.7, but for this experiment (performed in an early stage of the method development), the procedure was slightly different. The IAC columns contained 0.5 mL of the self made gel (paragraph III.2.2). The last washing step of the IAC was done with 0.5 mL of 0.05% TFA. Elution was performed by twice adding 0.4 mL of 0.05% TFA.

The two techniques were evaluated by comparing representative ion chromatograms of an extracted serum pool with a concentration of 5 µg/L (124 mIU/L), from which 2 mL were taken through analysis.

Optimization of the C18 SPE procedure used in the IAC method

Reverse phase cartridges (C8 or C18) from different brands and with different packing size were tested, more specifically: Waters Sep-Pak® C18 1 cc (50 mg) (Milford, MA, USA), Oasis® HLB 1 cc (30 mg) also from Waters, Varian SPEC® C8 and C18 1 cc (15 mg) (Palo Alto, CA, USA) and 3M Empore® C18 1 cc (4mg) with high (12 µm particle size) and standard (55 µm particle size) densities (St. Paul, MN, USA). Additionally, the different cartridges were tested in the normal flow direction and in the “reverse-loading mode” (see Figure III.2). The upside down mounting was done by connecting (with tubing) the loading side of a cartridge containing the solid phase to the vacuum manifold.

Subsequently, the cartridge's elution side was connected to the same side of an empty cartridge, serving as the solvent container.

The cartridges were compared by loading 50 µL of a mixture 40/60 (v/v) glycerol/water with 0.4 g/L BSA containing 1 ng of insulin. Cartridges were then washed with 1 mL of 10/90/0.1 (v/v/v) ACN/water/TFA and eluted with 100 – 150 µL of 70/30/0.1 (v/v/v) ACN/water/TFA for the 4 and 15 mg packing cartridges respectively (following manufacturer instructions), while with 250 µL for the cartridges with 30 and 50 mg.

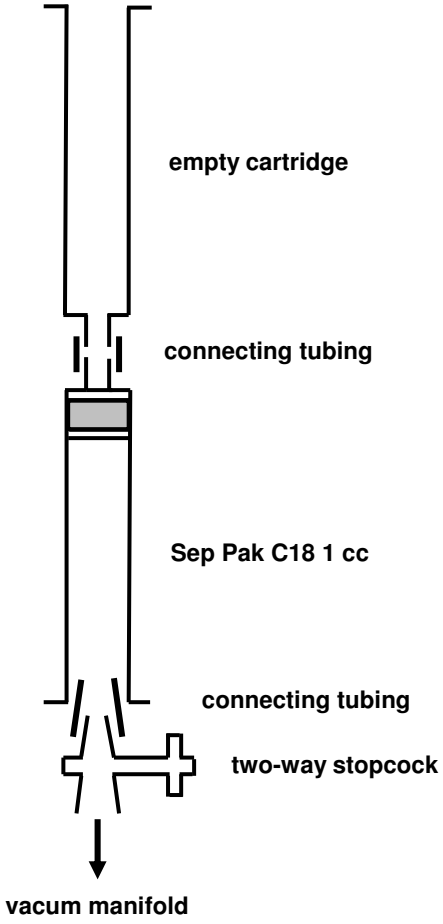

Figure III.2. Schematic representation of the technique used for loading the cartridge in the opposite direction of normal flow.

III.2.6. Establishment of the relationship between the WHO and the Sigma insulin standard

For the comparison of the WHO 1st IRP (66/304) and the Sigma recombinant insulin standard material the following procedure was applied. Two ampoules of the WHO standard were diluted in 60/40 (v/v) glycerol/50 mM HCl in water until a concentration of 130 µg/g. At the same time, two stock solutions of around five µg/mg of the Sigma standard were prepared by weighing five mg of insulin and dissolving them in 1 mL of 40/60 (v/v) glycerol/50 mM HCl in water. The Sigma stock solutions were diluted a second time with 40/60 (v/v) glycerol/50 mM HCl in water also until a concentration of 130 µg/g. At continuation, the Sigma and WHO intermediate solutions were diluted with insulin-depleted serum to a final concentration of 0.2 µg/g. This dilution took place in two steps, first some insulin-depleted serum was pipetted in the empty vial, then supplemented with the appropriate amount of standard solution and finally insulin-depleted serum was added until the desired total volume. Note that the pH of the insulin-depleted serum was adapted to 7.4 with HEPES buffer by adding 100 µL of this buffer (185 g/L HEPES (acid); 8.8 g/L NaOH) per each mL of serum. The IS was diluted in 60/40 (v/v) glycerol/water with 0.1 g/L BSA also to a final solution of approximately 0.2 mg/L. From each of the final solutions three calibration mixtures were prepared, i.e., standard/IS mixtures. First, 200 µL of the standard solution were pipetted, followed by the appropriate volume of IS solution in order to approximately achieve the isotope ratio of one. Afterwards, the sample preparation as described below in paragraph III.2.7 was performed on these mixtures. Analysis of these mixtures was carried out by alternating analysis of Sigma and WHO standards. The results of isotope ratios standard/IS were calculated and normalized with the mass ratios from standard/IS. The mean ratios of isotope ratio/mass ratio from the Sigma and WHO calibrators were compared. The difference between the ratios of each series was assessed for significance with a two-sided Student *t*-test (95% probability level).

III.2.7. Analytical procedure for serum insulin

The sample preparation procedure for the determination of insulin in serum consisted in a combined IAC – SPE procedure. A schematic representation of this procedure can be seen in Figure III.1.A. This procedure was validated and applied during the method comparison with the routine insulin immunoassays. The procedure was already described in detail in the doctoral thesis of Dr. K. Van Uytfanghe but will be mentioned here again for completeness of information.

Sampling and calibration procedure

The procedure started with gravimetric sampling of the serum, between 0.3 mL (minimum) and 4 mL (maximum) depending on the concentration (based on data available from the Roche immunoassay) and available volume. Ideally, an absolute amount of insulin of 2 ng was processed. A known amount of IS was added to obtain an isotope ratio of 1:1 within ±15%, and the samples were equilibrated for at least 30 min. Samples with a total amount of insulin lower than 0.6 ng were analyzed at an isotope ratio of 0.5:1, together with a set of calibration mixtures prepared at the same ratio.

The calibration procedure was based in the principle of one point calibration (30). The measurement procedure consisted of the analysis of three calibrators followed by the analysis of four samples, three calibrators and so on. This alternation of calibrators and samples was done to compensate any eventual drift in the response of the instrument.

Immunoaffinity chromatography sample preparation procedure

IAC was performed with 0.25 mL of the commercially available gel (see paragraph III.2.2). The gel was stored in a buffer at pH 7.4 containing 50mM Tris/0.05% sodium azide in water. The immunoaffinity gel containing columns were used in the gravity feed mode. Conditioning was done by adding 3 mL of binding buffer (50 mM Tris, pH 7.8 in water) for immediate application of up to 4 mL of serum (depending on the concentration) diluted 1:1 with binding buffer. Then, the gel was rinsed with 5 mL of binding buffer, 4 mL of 10 mM ammonium acetate (pH 4.5) and 0.2 mL of 0.1% TFA in water (this first rinse with TFA did not cause elution since the volume corresponds with the dead volume of the column). Human insulin and 4-[D10]Leu-human insulin were subsequently eluted with two times 0.4 mL 0.1 % TFA in water, taking care that a time interval of 5 min elapsed between

application and elution of the TFA solutions. Finally, the IAC columns were re-equilibrated with 5 mL of binding buffer.

The eluate from IAC was loaded into a Sep-Pak® C18 cartridge, mounted upside down in order to perform a reverse loading mode (see Figure III.2). The cartridge was conditioned with 2 mL of ACN and 2 mL of 0.1% TFA in water. After being loaded, the cartridge was washed with 2 mL of 10/90/0.1 (v/v/v) ACN/water/TFA. Finally, it was mounted in the normal flow direction for The extracts were evaporated to dryness at 50 ºC with nitrogen and the residues stored at -20 ºC until analysis. Before processing by LC/tandem MS, 0.25 mL of cyclohexane were added, vortexed and removed. The remaining residue was dried and reconstituted in 50 µL of a 10/90 (v/v) mixture of ACN/water for injection into the chromatographic system of 15 to 45 µL (depending on the concentration).

LC/tandem MS measurement procedure

MS was done with an API 4000™ system, operated in wESI+ mode and connected with an HP 1100 HPLC system. The latter was equipped with a XBridge C18 column (2.1x10 mm, 5 µm) maintained at room temperature. Gradient elution was done with eluents A and B containing ACN and water (in a v/v ratio of 10/90 and 70/30, respectively) and 0.0025% ammonium hydroxide. The percentage of ACN was increased from 10% to 40% in 3 min at a flow rate of 0.25 mL/min and kept constant during 3 min. Afterwards, it was increased to 70% in the next 0.1 min and kept constant again for 3 min to clean the column. Finally, the column was re-equilibrated with eluent A for 5 min. For a schematic representation of the HPLC gradient see Figure III.3 below.

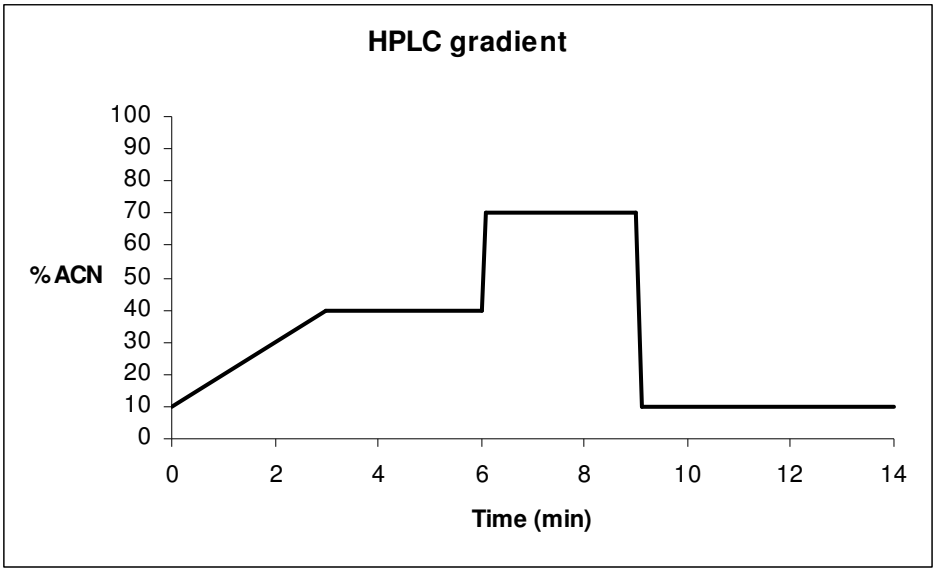

Figure III.3. Schematic representation of the HPLC-gradient.

SRM transitions of the four times-protonated molecular ions $[M+4H]^{4+}$ from m/z 1453 to 226 (insulin) and 1463 to 226 (4-[D10]Leu-insulin) with dwell time of 0.5 s were monitored. This resulted in at least 15 scan cycles under the peak. The declustering potential was 50 V, the collision energy was 85 eV and the N_2 collision gas setting was 12.

III.2.8. Method validation

The IAC-LC/tandem MS measurement procedure was validated. The following experiments and materials were employed.

Specificity

Specificity was assessed indirectly by visual inspection of chromatograms obtained in the method comparison study. Indirect evidence was also gained from the accuracy and precision studies. The presence of any interference would affect the precision and accuracy to a different extent at different concentrations. In order to study the interferences at the transition typical for the 4-[D10]-Leu-insulin, analysis of several serum samples selected from the method comparison study without the addition of IS were measured.

Limit of detection and quantification

The determination of the LOD was done by injection of calibration solutions. The LOD was calculated as the quantity of insulin on column necessary to obtain a S/N of 3.

The LOQ was estimated from the analysis of a pool of insulin-depleted serum supplemented with insulin to a final concentration of 0.0796 µg/L.

Recovery of sample preparation procedure

A preliminary estimation of the absolute recovery of the insulin extracted from the serum was determined with a serum pool that was divided in eight portions of 2 mL. Two portions were supplemented with 344 fmol (2 ng) of IS. The other six portions were purified and 344 fmol of IS were added at three different stages (n=2) of the sample preparation procedure, more exactly, after IAC, after SPE and after drying of the SPE-eluates. The recovery (in %) was calculated from the comparison of the isotopic ratios of the different portions.

Ion suppression

The suppression in the ionization process was qualitatively studied through the comparison of the absolute signal of IS in a calibration solution and in a purified sample were the IS was added after the drying step (n=2).

Precision, accuracy and trueness

The performance of the ID-LC/tandem MS procedure (imprecision, accuracy and trueness) was validated with insulin-depleted serum supplemented with human insulin to obtain three pools, a low pool at 0.0796 µg/L (= 1.97 mIU/L) (LOQ), a medium and high level pool at 0.769 µg/L (= 19.0 mIU/L) and 5.56 µg/L (= 137.8 mIU/L), respectively. Aliquots were stored at -20 °C and analyzed in duplicate on 6 different days (n = 12). The insulin content (±95% CI) of the insulin-depleted serum was determined beforehand.

For the recovery data, two-sided 95% CI were calculated and a 1-sample *t*-test was performed. Statistical data analysis was done with CBstat, Version 5.1 (CBstat software) and MedCalc, Version 9.2.0.0 (MedCalc software). Model II ANOVA was used to calculate the CV_{within}, $CV_{between}$ and CV_{total} (%). The CV_{total} was obtained from the combined $CV_{between}$ and CV_{within} (square root of the sum of the squared $CV_{between}$ and CV_{within}).

III.2.9. Method comparison study

The study was purposely undertaken in an early developmental stage of the ID-MS procedure in order to have a fast answer on questions about the feasibility of the project, to know the expected impact on immunoassay results after recalibration and to be able to do a focused refinement of our ID-MS procedure. That is the reason why the protocol for preparation of calibrators is slightly different between the method comparison studies and the method validation.

Measurement strategy

For the method comparison, 80 serum samples from 16 ambulatory patients (four males and 12 females, aged between 21 and 74 years) from Ghent University Hospital were used. The patients had been subjected to an oral glucose (75 g) tolerance test after overnight fasting. The insulin tests were ordered by the endocrinologist for assessment of glucose tolerance, glucose handling, beta cell function and degree of insulin sensitivity in subjects with morbid obesity and/or other risk factors for insulin resistance and glucose intolerance. Handling of patient samples was done according to the local Ethical Committee guidelines. Blood was drawn into Venosafe VF-106SAS tubes (Terumo) before the glucose load and also after 30, 60, 120 and 180 min. The collected blood was allowed to clot for at least 30 min and centrifuged at 1500 g for 10 min. The fasting blood samples were transported to the laboratory within 30 min of withdrawal, whereas the remaining samples were received within 30 min of the last collection. Then, the analysis was immediately started with the four immunoassays. The remaining serum was transported ice-cooled to the MS laboratory. There, the samples were immediately processed by combined IAC/SPE as described above. The evaporated extracts were stored at -20 °C until LC/tandem MS analysis the next day.

Each sample was measured singly with each of the routine immunoassays. The measurements in the ID-LC/tandem MS laboratory were done once according to a measurement protocol with bracketing of the patient and control samples (maximum six) between two times three calibrators, which allowed analysis of samples of two stimulated patients per working day. The values of all calibrators were used for the calculation of the sample concentration. Measurement of the 16 stimulation curves by both the ID-LC/tandem MS and routine laboratory was done in eight different analytical runs (batches of 10 samples and one control sample) spread over three weeks. The quality of each

analytical run was controlled by the use of a high level lyophilized control material (estimated mean = 4.426 µg/L; CV_{total} = 2.6%; n=8) from INSTAND e.v. (Düsseldorf, Germany).

Routine measurement procedures

The method comparison was done with the four following assays: electrochemiluminescence immunoassay for use on the Diagnostic Products Corporation (DPC) Immulite® 2000 Insulin from (Los Angeles, CA, USA), the Roche Diagnostics GmbH Modular Analytics E170 (Basel, Switzerland) immunoassay analyzer, the Abbott AxSYM insulin microparticle enzyme immunoassay (Abbott Park, Illinois, USA) and the Beckman Coulter Access Ultrasensitive insulin chemiluminescent immunoassay (Fullerton, CA, Los Angeles). All assays are calibrated against the WHO insulin 1st IRP 66/304 (NIBSC). All measurements with the commercial insulin immunoassays were performed with the same batch and in accordance with the respective manufacturer's instructions for use. Each assay's performance was controlled either with assay specific control samples (for DPC the insulin Controls LINC1 and LINC2 with assigned target values of 9.2 and 48 mIU/L; for Abbott the AxSYM insulin L, M and H with targets of 8, 40 and 120 mIU/L), with commercial control materials (for Beckman the Lyphochek Immunoassay Plus Control Level 1, 2 and 3 from Bio-Rad with assigned values of 7.1, 36 and 95 mIU/L) or with in-house prepared serum pools (for Roche) targeted before at 11.1 and 60.0 mIU/L.

Statistical methods and graphical presentation

The results from the method comparison were investigated by scatter-plots and correlation and Deming regression analysis (CBstat software version 5.1 from K. Linnet, Risskov, Denmark). The two highest concentration values were not taken into account for the regression and correlation analysis because they are outliers with respect to the distribution of the insulin concentrations. In the graphs, however, the regression line is extrapolated to show the performance of the assays in the high concentration range. Further, the results were investigated by difference plots containing total error limits derived from the combined imprecision of both measurement procedures (CV_{mp}) (calculated with Formulas III.1 and III.2) and total error limits specified by the ADA (32%). The latter limits are derived from the biological variation of serum insulin (3).

$$CV_{mp} = \sqrt{\frac{CV_{MS}^{2}}{n} + \frac{CV_{assay}^{2}}{n}}$$

Formula III.1. CV of the method pair. Note that n is the number of repeated measurements of the same sample with each method.

$$TE = 1.96 \times CV_{mp}$$

Formula III.2. Total error due to the combined imprecision of both methods.

III.3. RESULTS AND DISCUSSION

III.3.1. Method development and optimization

Note, that since the method development was published (32), the method has been refined and further applied to the measurement of 45 serum samples to be used in an international project for the evaluation and standardization of insulin immunoassays. This work has been performed under an ADA grant (29). Unfortunately, at this point, the ADA did not consent to the use of the data in the current thesis. Therefore, only some general statements can be made about the progress of the method since.

III.3.1.1. Stability of standard solutions

Many factors can cause the degradation or loss of proteins in a solution (33), like oxidation, contamination of proteases from our skin, polymerization, loss due to adsorption and damage during the freezing process. In the case of insulin, the desamidation at acidic pH (Asn in position A21) and at neutral pH (Asn in position B3) may also take place. Indeed, in the preparation of standards without any additives, differences of up to 20% were observed in isotope ratios between calibrators prepared from different working solutions. This was attributed to decomposition or loss due to absorption.

Many techniques have been described to stabilize proteins and to avoid their adsorption: addition of trehalose to decrease polymerization; maintaining the pH at a higher value than the pKa (34) of the protein to prevent desamidation at Asn residues; use of EDTA to complex any metals which can produce aggregation or oxidation of –SH groups; storing solutions at -20 °C without freezing by adding 30 to 50% glycerol (or 10 to 50% isopropanol) (35); adding albumin; rapid freezing with liquid nitrogen; use of antioxidants; use of gloves or protease inhibitors; avoiding aeration or foaming by mixing gently; use of LBP or coated vials and tips.

We stabilized insulin by making solutions in 40/60 (v/v) glycerol/water with 0.1 g/L BSA (32). This allowed the direct injection of the standards in the HPLC system. Unfortunately, longer-term experience demonstrated that this was not sufficient to guarantee stability within the 5% limit for systematic error set by the ADA. Therefore, we

had to make stock and standard solutions in insulin-depleted serum. This required taking the standards through the complete analysis.

III.3.1.2. Optimization of the HPLC conditions

All current LC/MS measurement procedures for quantitative analysis of human insulin in serum work under acidic LC conditions and ESI+. The HPLC-MS procedures mainly utilized TFA (25-27), while one of the off-line procedures made use of acetic acid (24). Triggered by the development of base-stable C18 reversed phase columns, we investigated LC under alkaline conditions (with ammonia) and operating the mass spectrometer in the wESI+ mode. First chromatograms of insulin standards were encouraging: insulin eluted as a single peak with good symmetry and sensitivity (see Figure below corresponding to an XBridge column).

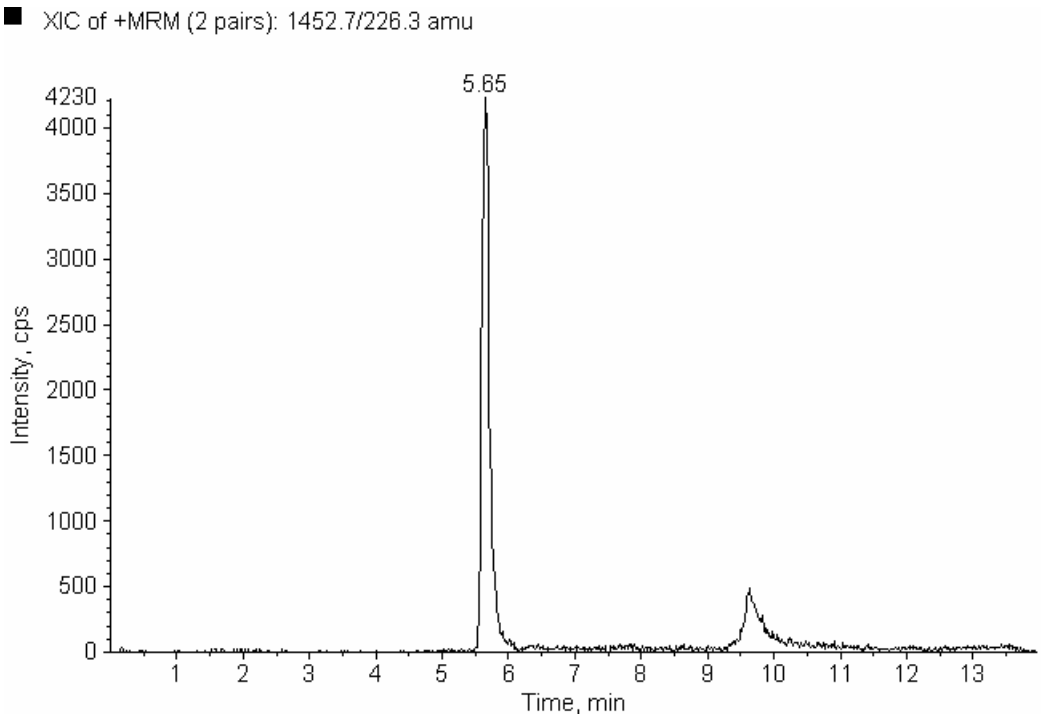

Figure III.4. Chromatogram corresponding to a sample of 0.75 µg/L (19 mIU/L).

Therefore, we further evaluated a PRP-3 column (50x2.1, 3 µm particle size), an XTerra C18 column (2.1x10 mm, 5 µm), and various XBridge C18 columns (2.1x10 mm, 5 µm;

2.1x10 mm, 3.5 µm; 2.1x150 mm, 5 µm). The best results were obtained with the short XBridge columns (see below for chromatograms of processed samples). The advantage of the short columns is their price, which allows treating them as “disposables”. While the method comparison study has been done with the 5 µm columns, experiments are being performed to investigate the long-term robustness of the 3.5 µm column. The latter is somewhat superior in terms of resolution and S/N ratio.

Unfortunately, it was not possible to directly compare the sensitivity of our procedure with those of the previously published procedures, because they applied different instruments, different scan conditions (e.g. short scan over the five-times protonated molecular ion (24), bigger scan and measuring the three times protonated molecular ion (26), product ion scan (27), or SRM (25)) or off-line HPLC (24, 26). However, with our MS configuration and conditions, the new procedure was ~25 times more sensitive than a procedure optimized with 0.04% TFA.

III.3.1.3. Optimization of MS conditions: CID experiments

Parent spectrum

The parent spectrum of insulin in the wESI+ mode (Figure III.5) is dominated by the three-, four- and five-times protonated molecular ions (m/z 1937, 1453 and 1162.6, respectively). The four-times protonated molecular ion, being the most abundant ion of insulin in the wESI+ mode, was selected for CID.

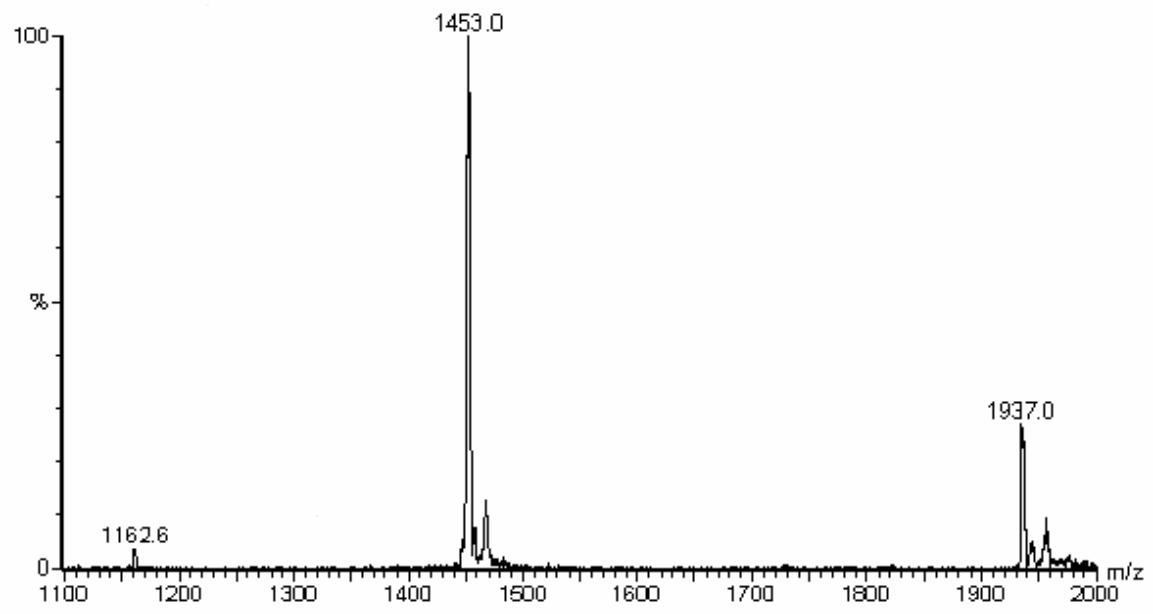

Figure III.5. Mass spectrum of human insulin obtained in positive mode on the VG Quattro II^{TM}. m/z 1162.6 corresponds to $[M+5H]^{5+}$, 1453 to $[M+4H]^{4+}$ and 1937 to $[M+3H]^{3+}$.

CID spectra of m/z 1453

Figures III.6.A and III.6.B show the product ion mass spectra of m/z 1453 under CID conditions optimized for the ions at m/z 110 and m/z 226, respectively.

The optimized spectrum for m/z 110 (Figure III.6.A) is dominated by fragments of insulin amino acids like valine (m/z 72), leucine or isoleucine (m/z 86), histidine (m/z 110), phenylalanine (m/z 120), arginine (m/z 129) and tyrosine (m/z 136). Some product ions can correspond to amino acid side chain fragments (m/z 69, 72 and 83) (38) (for reviews about the low mass (m/z < 200) region of the CID spectra of proteins see: 36-38).

The spectrum optimized for m/z 226 (Figure III.6.B) is dominated by the m/z 226 fragment, which corresponds to the y3-y1 fragment of the insulin β-chain (lysine-proline). The fragment at m/z 345 corresponds to the complete y3 fragment (threonine-lysine-proline) (39).

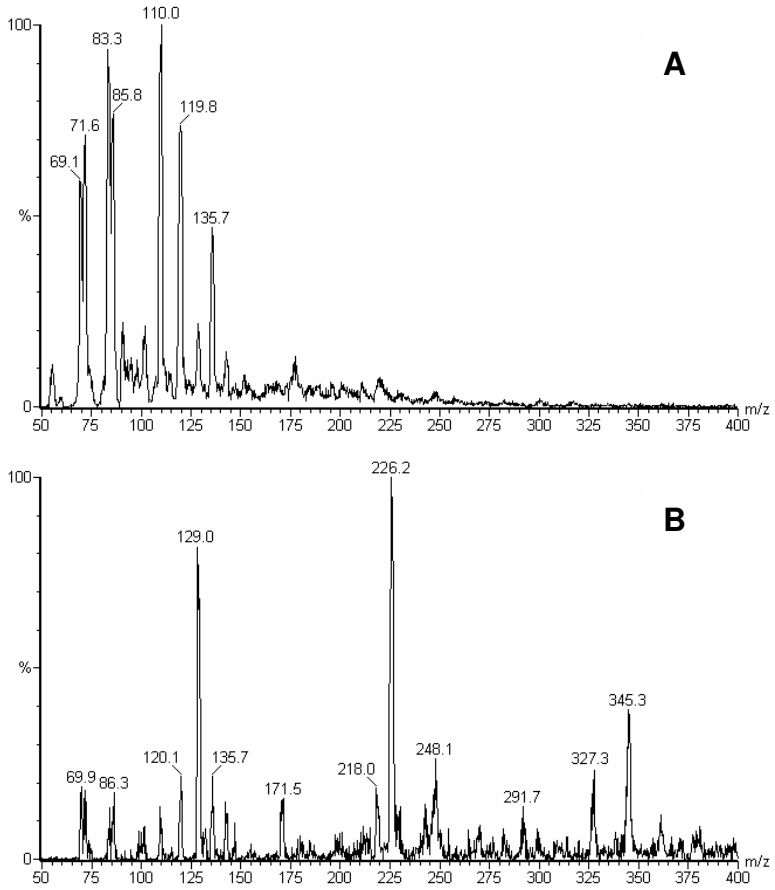

Figure III.6. CID-spectra of m/z 1453 obtained with the Quattro II, optimized for m/z 110 (A) and m/z 226 (B).

Comparison of various collision gases in the VG Quattro II^{TM} configuration

Table III.4 shows the optimal conditions for the generation of m/z 226 and m/z 110 CID fragment ions of m/z 1453 precursor ion from insulin using different collision gases. In general, it can be seen that the collision energies for the formation of the fragment ion m/z 110 were higher than for the formation of the m/z 226 fragment ion.

For argon, the collision energy and the gas pressure was lower for both transitions than for nitrogen. Helium requires even higher gas pressures and collision energies. Therefore, it was impossible to optimize the conditions for the formation of the fragment m/z 110. On the other hand the spectra were quite similar to the various gases under optimized conditions.

For our application, argon and nitrogen gave the same sensitivity. However, the advantage of nitrogen is its lower cost.

Table III.4. Optimal MS conditions for the generation of the most sensitive CID fragment ions of m/z 1453 precursor ion in the Micromass VG Quattro II^{TM} MS using different CID gases.

	Argon		Nitrogen		Helium	
m/z	eV	mBar	eV	mBar	eV	mBar
226	25	3.3E-3	75	1.1E-2	130	4.0E-2
110	145	3.3E-3	190	1.1E-2	Could not be optimized	

Comparison between the VG Quattro II™ and API 4000™ configurations

Figure III.7 shows the CID-spectra of m/z 1453 obtained with the API 4000™, optimized for m/z 110 (A) and m/z 226 (B).

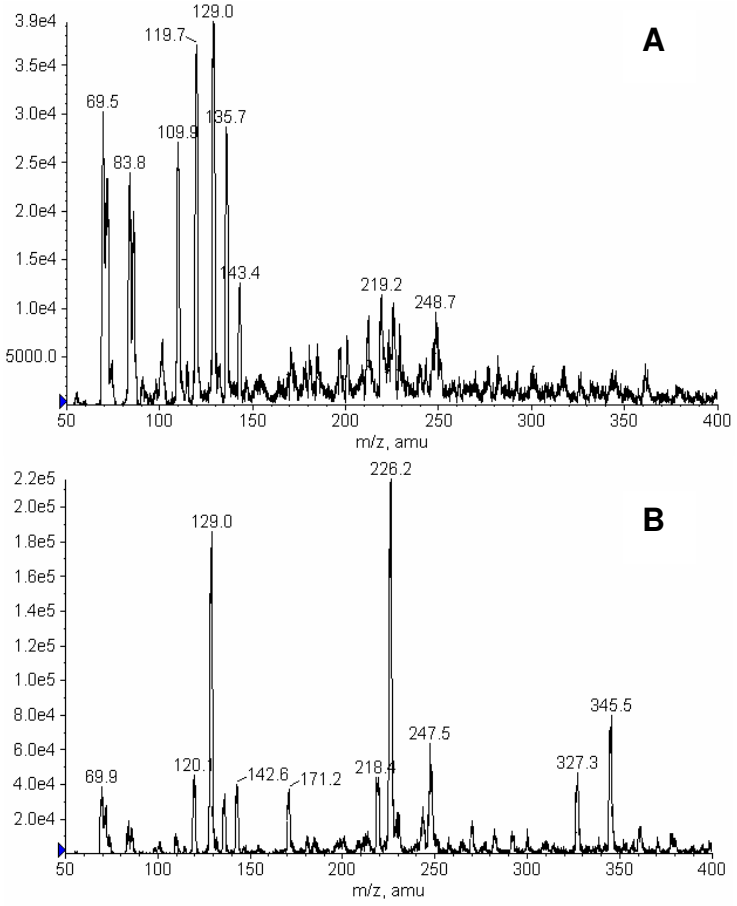

Figure III.7. CID-spectra of m/z 1453 obtained with the API 4000™, optimized for m/z 110 (A) and m/z 226 (B).

First, it should be noted that it was not possible to find conditions in the API 4000™ to obtain m/z 110 as the most intense ion in the spectrum (see Figure III.7 A). This is mainly attributed to the pressure limitation of the API 4000™ collision cell. Unfortunately, we were unable to install argon as collision gas on the API 4000™. According to contacts with the company we had to "do this at our own risk". A further limitation is the maximum usable collision energy of about 190 eV (this voltage depends also on other voltages used in the CID mode).

On the other hand, the spectra optimized for m/z 226 were quite similar with the VG Quattro II™ and the API 4000™ (compare Figures III.6 B and III.7 B).

Selection of the most selective and sensitive SRM transition

On the VG Quattro II™, m/z 110 was the most sensitive transition, five times more than m/z 226. On the API 4000™, the transition to m/z 129 was the most sensitive one. However, the transition to m/z 226 was the most selective one on both instruments. The API 4000™ was approximately 10 times more sensitive for that transition than the VG Quattro II™.

III.3.1.4. Development of sample preparation

Figure III.8 shows the chromatograms obtained with the two-step SPE procedure (A) and the IAC procedure (B) for a sample containing 5 µg/L (860 pmol/L) of insulin. Note that the less selective transition to m/z 110 was monitored in order to better visualize the differences in the sample preparation procedures. The visual inspection of the chromatograms shows that the IAC procedure gives clearer chromatograms. Also, pressure increases were observed when injecting samples purified by the two-step SPE procedure. Therefore, the IAC procedure was finally chosen for sample preparation.

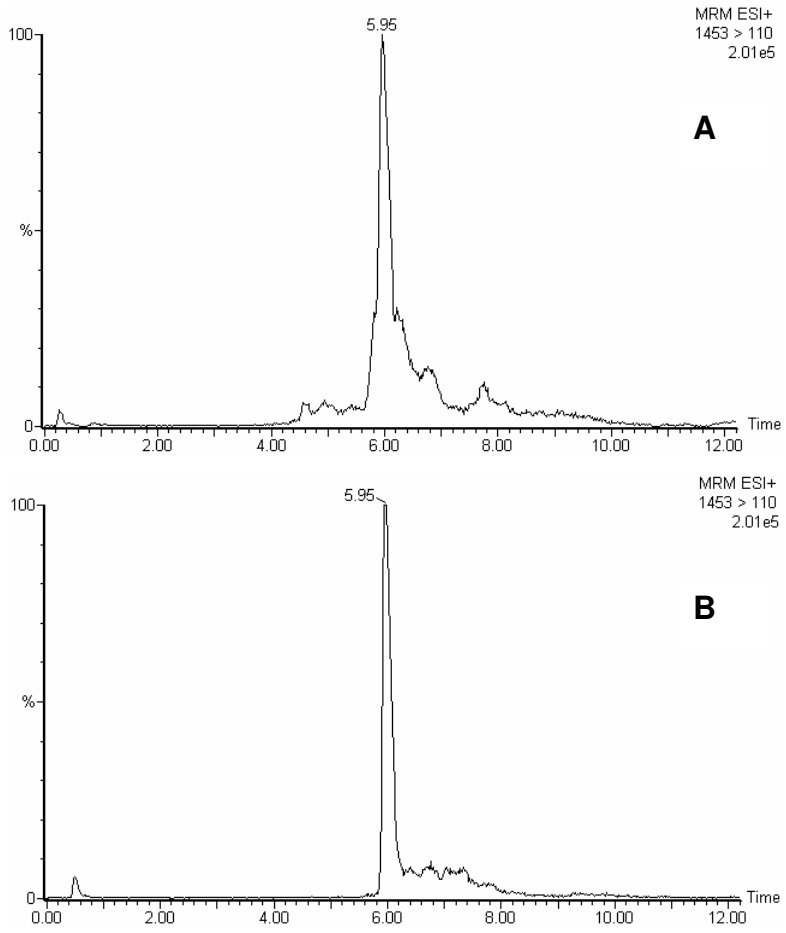

Figure III.8. Chromatograms obtained after purification of a sample with 5 µg/L (860 pmol/L) of insulin with two-step SPE (A) and IAC (B).

Note, however, that the IAC columns are relatively expensive. This requires using the columns several times. The experience gained up to now did not give an unequivocal

indication of how many times the columns could be used. Depending on the samples purified and the columns prepared, the columns could be used 6 to 20 times. Future investigation is needed to increase the robustness of the IAC purification step.

Optimization of the C18 SPE procedure used in the IAC method

Cartridges with lower packing size were tested in order to reduce elution volumes and hence increase recoveries in the preconcentration step. Reducing elution volumes to approximately 100 µL was possible, unfortunately, worse recovery results were obtained than by using Sep-Pak® C18 1cc (50 mg) in the "reverse-loading mode" (see Figure III.2). The idea behind the reverse loading of the SPE cartridges was restricting the adsorption of insulin to a small part of the solid phase, hence, to minimize losses and reduce the elution volume.

III.3.1.5. Relationship between the WHO and the Sigma insulin standard

All development, validation and application experiments described in this dissertation were done with commercially available recombinant human insulin from Sigma with a purity >98%. The quality of this material was good enough for the purpose of the research but to be able to evaluate the calibration status of the routine immunoassays, the link between this standard and the first IRP for insulin (66/304) must be determined. Note that all commercial immunoassays are calibrated with this WHO material (1). This international standard is prepared from human pancreas extracts and is distributed in ampoules of 1 mL that contain approximately 130 µg of insulin and 4 mg of sucrose. Per definition, one ampoule contains three IU of insulin, with a filling variation of 1.08%. A great disadvantage of this international standard is that it is not defined in terms of mass. That has the consequence that the link between the international unit (IU) and the effective content of insulin in this standard (expressed in g or mol) is not yet established. In addition, several conversion factors are use, varying from 6 to 7.5 (conversion from IU to pmol/L) (1). This is also the reason why this standard was not used for our candidate RMP as primary calibrator. It is also important to mention that in the international standardization of insulin immunoassays organized by ADA and IFCC it will be necessary the use of a state-of-the-art primary calibrator. This can be a commercially available recombinant insulin preparation like the one used in our experiments but it should preferably be certified by a metrological institute such as NIST (National Institute of Standards and Technology) or IRMM (Institute for Reference Materials and Measurements).

Table III.5 lists the results obtained for the measurements of the WHO and the Sigma standard. The results indicate that the insulin content of the WHO standard is approximately 9% lower than that of the Sigma standard (P = 0.0005 in the t-test).

Table III.5. Results from the comparison between the WHO and Sigma standard

Stock	Weight ratio (WR)	Isotope ratio (IR)	Ratio IR/WR	Mean ratio	Overall mean	CI*
Sigma 1	1.0063	1.2282	1.2205	1.2356	1.2198	2.27%
	0.994	1.2204	1.2278			
	1.0042	1.2638	1.2585			
Sigma 2	1.0241	1.2522	1.2227	1.2040		
	1.0179	1.2346	1.2129			
	0.9999	1.1763	1.1764			
WHO 1	0.9857	1.117	1.1332	1.1196	1.1074	1.85%
	0.9886	1.1141	1.1269			
	0.994	1.0921	1.0987			
WHO 2	0.9898	1.0766	1.0877	1.0951		
	0.9918	1.0789	1.0878			
	1.02	1.132	1.1098			
Difference Sigma versus WHO					**9.2%**	

* 95% CI for overall mean

III.3.2. Method validation

Specificity

Figure III.9 shows representative chromatograms for processed serum samples with respectively concentrations of 0.174 µg/L (30 pmol/L), 0.918 µg/L (158 pmol/L) and 2.904 µg/L (500 pmol/L). They show a nearly symmetric insulin peak with a base width of ~0.3 min and a baseline that is stable and interference-free over the entire chromatographic run.

Dedicated interference studies, for example with proinsulin or split-products, have not been performed yet. Since the mass spectrometric point of view, these substances are unlikely to interfere, but, as those may interfere with immunoassays they are usually tested also in a mass spectrometric procedure.

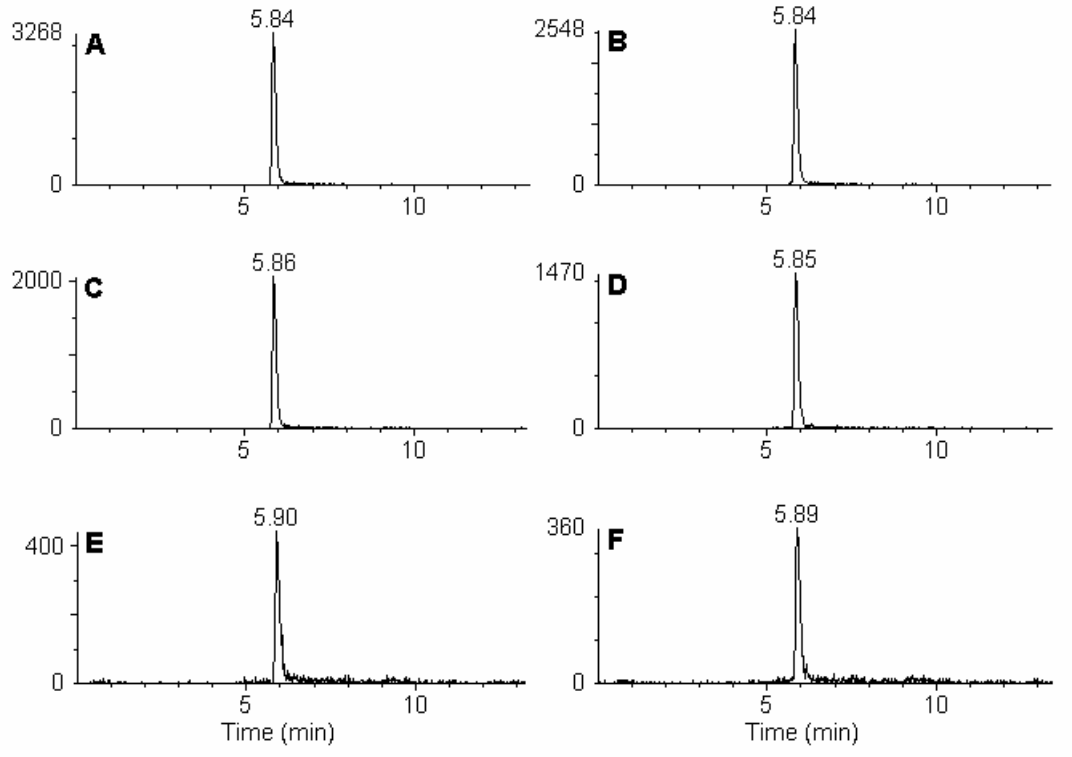

Figure III.9. Chromatograms obtained for processed serum samples containing 2.904 (A, B), 0.918 (C, D) and 0.174 (E, F) µg/L of human insulin (500, 158 and 30 pmol/L respectively). A, C, E: from m/z 1453 to 226; B, D, F: from m/z 1463 to 226.

Indirect evidence for the specificity of the method was also gained from the accuracy and precision studies. The presence of any interference would affect the precision and accuracy to a different extent at different concentrations. These data (see Table III.6) did not indicate interference problems.

The absence of interference at the transition of the IS could be demonstrated by analysis of several samples from the method comparison study without the addition of IS.

Limit of detection and quantification

The LOD (defined as S/N = 3), estimated from the injection of consecutively diluted standard solutions, was 3.5 pg (0.6 fmol or 86 nIU). The LOQ (defined as S/N = 10) was 0.07 µg/L (12 pmol/L or 1.83 mIU/L). This LOQ and the described application on patient samples (see Figure below) document that the measurement procedure is suitable for quantification of insulin in human sera at concentrations typical for the reference range 0.070-0.87 µg/L (12–150 pmol/L or 1.7-21.6 mIU/L) and after glucose stimulation (up to 11.6 µg/L (2000 pmol/L or 288 mIU/L)).

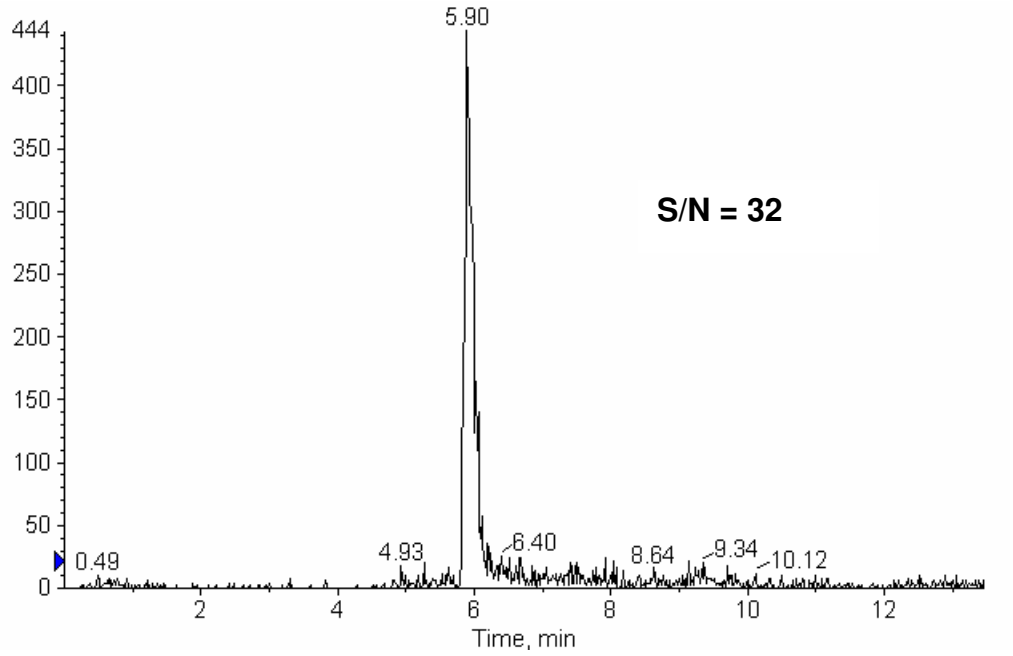

Figure III.9. Chromatogram corresponding to 138 pg (3.4 µIU) of insulin on column from a sample analyzed during the method comparison studies.

Recovery of sample preparation procedure

Preliminary validation of the procedure showed a total recovery of 30–40%, as estimated from the peak areas obtained for processed serum samples in comparison to those of the calibrators. From further experiments a loss of 10–15% was observed in each of the sample pretreatment steps, i.e., IAC, SPE, drying and sample transfer.

The sample transfer step was investigated in somewhat more detail. Often, the dried extract residue after processing of the sample is redisolved and transferred to an insert in order to be able to inject the maximum of this solution. This transfer from the original vial to an insert can result in losses due to adsorption to the pipette tip, the glass insert as well as due to the fact that is impossible to transfer 100 % of the solution. Therefore, it was our objective to inject the sample directly from the evaporation vial. Different sort of vials from different brands and with different shapes were tested. Finally, the best recovery was found with glass vials having a conic bottom. For the injection of calibrators, PBP vials were used because they gave a somewhat better sensitivity than the conical glass vials. This may be attributed to adsorption to the glass or formation of adduct ions with ions coming from the glass. PBP vials, however, could not be used as samples because of considerably longer drying times.

Ion suppression

The absolute signal of IS in a calibration solution and in a purified sample were the IS was added after the drying step were of the same order in both instruments. This indicates the absence of ion suppression on both, the VG Quattro II™ and the API 4000™ instruments.

Precision, accuracy and trueness

The preliminary performance characteristics of the ID-LC/tandem MS procedure are presented in Table III.6. The insulin content (95% CI) of the insulin-depleted serum used to prepare the three pools was estimated to be 8 ng/L (1-15 ng/L) (0.02-0.37 mIU/L) (n = 6). The within-run imprecision ranged from 3.2 to 6.3%, the total imprecision from 4.9 to 12.1% (listing sequence from the high to the low pool). In all cases the between-run precision was greater than the within-run precision. Analysis of the low pool was used to confirm that our measurement procedure met the LOQ demand of the ADA Workgroup. The average S/N ratio obtained at the LOQ was 25. This LOQ was confirmed by the analysis of a real patient sample (lowest concentration 0.073 µg/L or 1.83 mIU/L). The

recovery of insulin added to insulin-depleted sera ranged from 101.8 to 104.1% (identical listing sequence as before).

Table III.6. Precision and trueness data of the ID-LC/tandem MS procedure.

Precision data (design: duplicates on 6 days (n = 12))			
	Sample		
	Low (= LOQ)	**Middle**	**High**
Mean (µg/L)	0.0829	0.792	5.656
CV_{within} (%)	6.3	3.5	3.2
$CV_{between}$ (%)	10.3	5.0	3.8
CV_{total} (%)	12.1	6.1	4.9
Trueness data (design: duplicates on 6 days (n = 12))			
	Sample		
	Low (= LOQ)	**Middle**	**High**
Target# (µg/L)	0.0796	0.769	5.556
Mean (µg/L)	0.0829	0.792	5.656
95% CI (%)	11.8	5.8	4.6
Recovery (%)	104.1	102.9	101.8
LCL$ (%)	92.4	97.1	97.2
UCL$ (%)	115.9	108.8	106.4
P§	0.424	0.258	0.369
#Spike plus content of insulin-depleted serum			
$LCL = lower confidence limit; UCL = upper confidence limit (both two-sided with 95% P)			
§Probability of the 1-sample *t*-test			

III.3.3. Method comparison study

The results of the method comparison study are summarized in Figures III.10-13. Figure III.10 shows the scatter and absolute difference plot before recalibration for each immunoassay. In Figure III.11, the combined scatter and absolute difference plots before (A and B) and after recalibration with the respective weighted Deming equation (C and D) are given. Figures III.12 and 13 document the percentage difference plots after recalibration for the individual assays. They include the total error limit expected from the combined imprecision of the method pairs (Figure III.12) and the 32% total error limit (Figure III.13) as proposed by the ADA work group (3). The results are reported in mIU/L because the 4 manufacturers use different conversion factors for the calculation of results in mass units (µg/L or pmol/L). To convert the ID-LC/tandem MS results into IU, a relative molecular mass of 5807.6 and a factor of 6.945 for the conversion of mIU/L to pmol/L were used.

Data before recalibration

With ID-LC/tandem MS, the basal insulin concentrations ranged from 3.74 to 14.7 mIU/L, the stimulated concentrations from 1.83 to 247.6 mIU/L. The weighted Deming regression data for the method comparison are Roche = 1.21 (± 0.02, SE) x MS – 0.33 (±0.31) mIU/L (Sy/x 0.12 mIU/L), DPC = 1.27 (± 0.07) x MS + 0.74 (± 1.28) mIU/L (Sy/x 0.34 mIU/L), Beckman = 0.84 (± 0.01) x MS – 0.20 (± 0.12) mIU/L (Sy/x 0.09 mIU/L), and Abbott = 1.08 (± 0.02) x MS – 0.33 (± 0.21) mIU/L (Sy/x 0.11 mIU/L). The observed bias is +20% for Roche, +42% for DPC, -17% for Beckman, and +6% for Abbott. The standard deviation of the differences of all assay results from ID-LC/tandem MS is 9.6 mIU/L (8.9 mIU/L without the DPC assay). The correlation data are $r = 0.991$ for Roche, $r = 0.920$ for DPC, $r = 0.993$ for Beckman, and $r = 0.992$ for Abbott.

Weighted Deming regression and correlation analysis of the method comparison data showed considerable between-assay variation, however, the correlation of those with the ID-LC/tandem MS is excellent with exception of the DPC assay. These data provide a reliable basis for the recalibration of the immunoassays based on the Deming regression equations (see below).

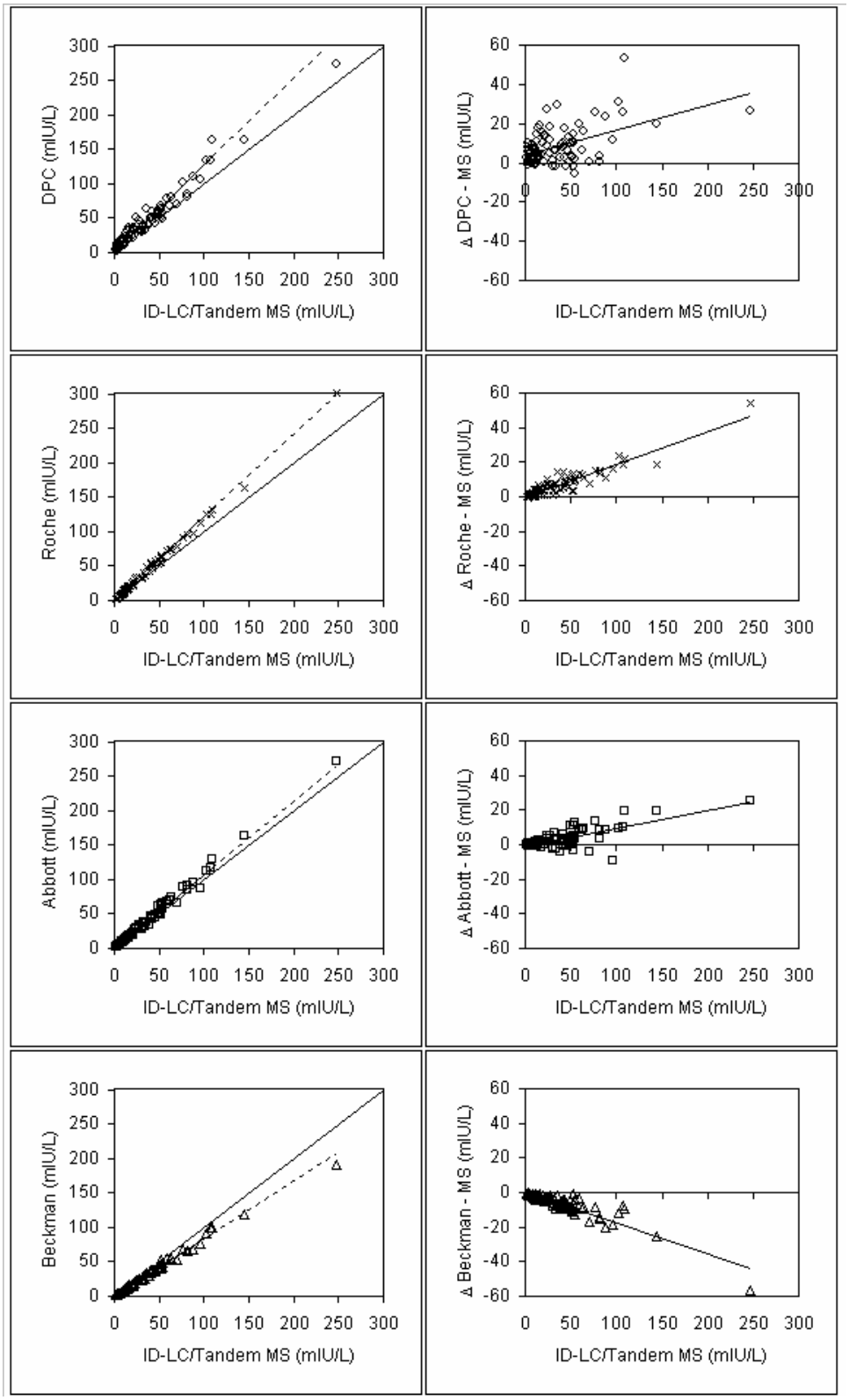

Figure III.10. Method comparison study between four immunoassays and ID-LC/tandem MS: for each immunoassay the scatter and absolute difference plot before recalibration is shown.

Data after recalibration

Recalibration of the immunoassay results considerably reduced the between-assay variation. It reduced the standard deviation of the differences of all assay results from ID-LC/tandem MS from 9.6 µIU/mL to 5 µIU/mL (from 8.9 µIU/mL to 3.4 µIU/mL without the DPC assay) (compare also panels A and B with panels C and D). Note that the variation in the recalibrated data is dominated by the DPC assay.

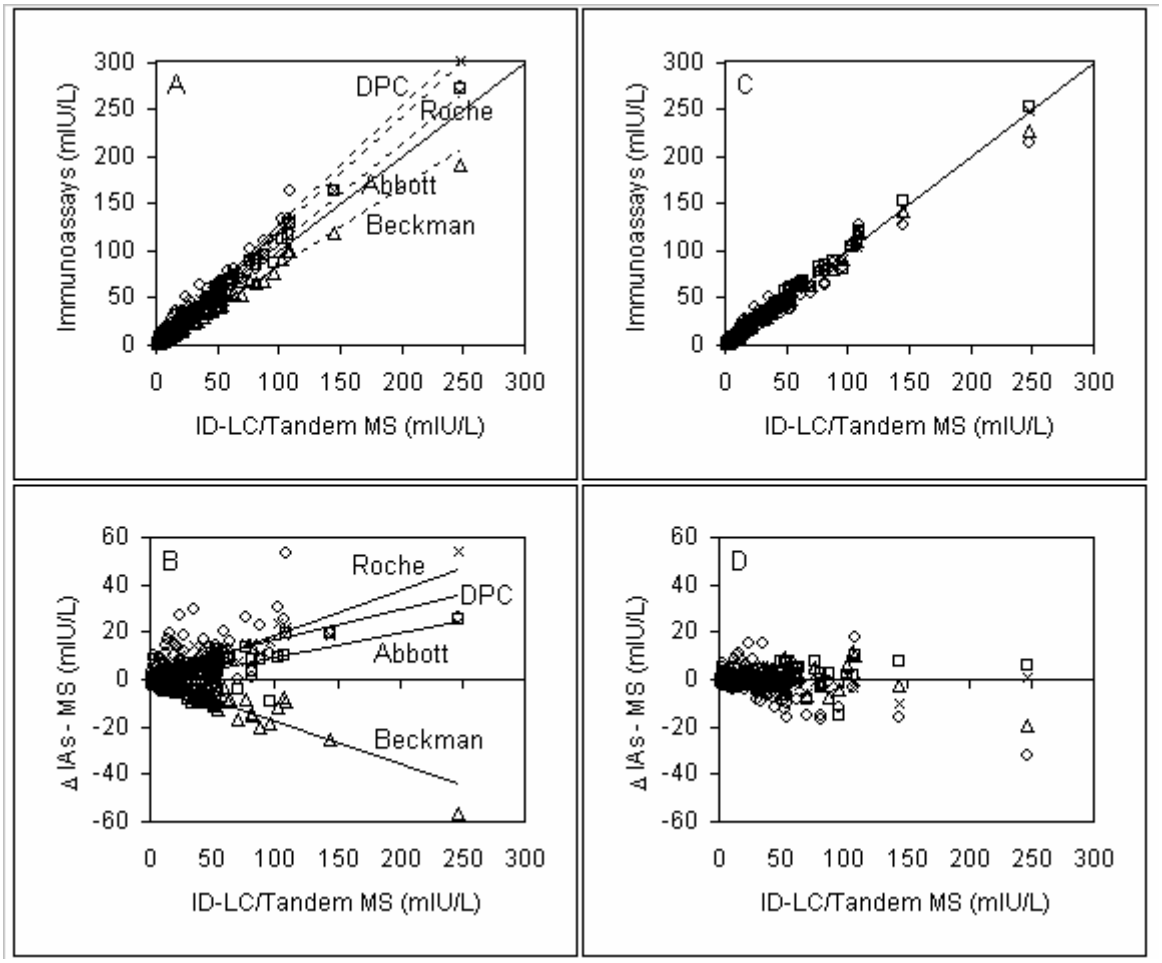

Figure III.11. Combined scatter and absolute difference plots before (A and B) and after (C and D) recalibration with the respective weighted Deming equations.

Recalibrated data and expected total variation

For a more detailed investigation of the residual spread of the data after recalibration, the % difference plots after recalibration shall be used (Figure III.12). This Figure includes also the expected spread of the data if only the imprecision of the respective method pairs is considered (see also Table III.7).

Except for the DPC assay, 95% of the differences between the recalibrated data and the ID-LC/Tandem MS method lie within the expected total variation of the method comparison. This indicates sample related effects for the DPC only. Most probably, this assay suffers from specificity problems.

Table III.7. Total imprecision corresponding to each method, to the method pair and 95% expected interval for the method comparison results.

	CV_{total}	CV_{mp}	1.96 x CV_{mp}
MS	12.1	-	-
DPC	7.3	14.1	27.7
Roche	4.9	13.1	25.6
Abbott	5.3	13.2	25.9
Beckman	5.6	13.3	26.1

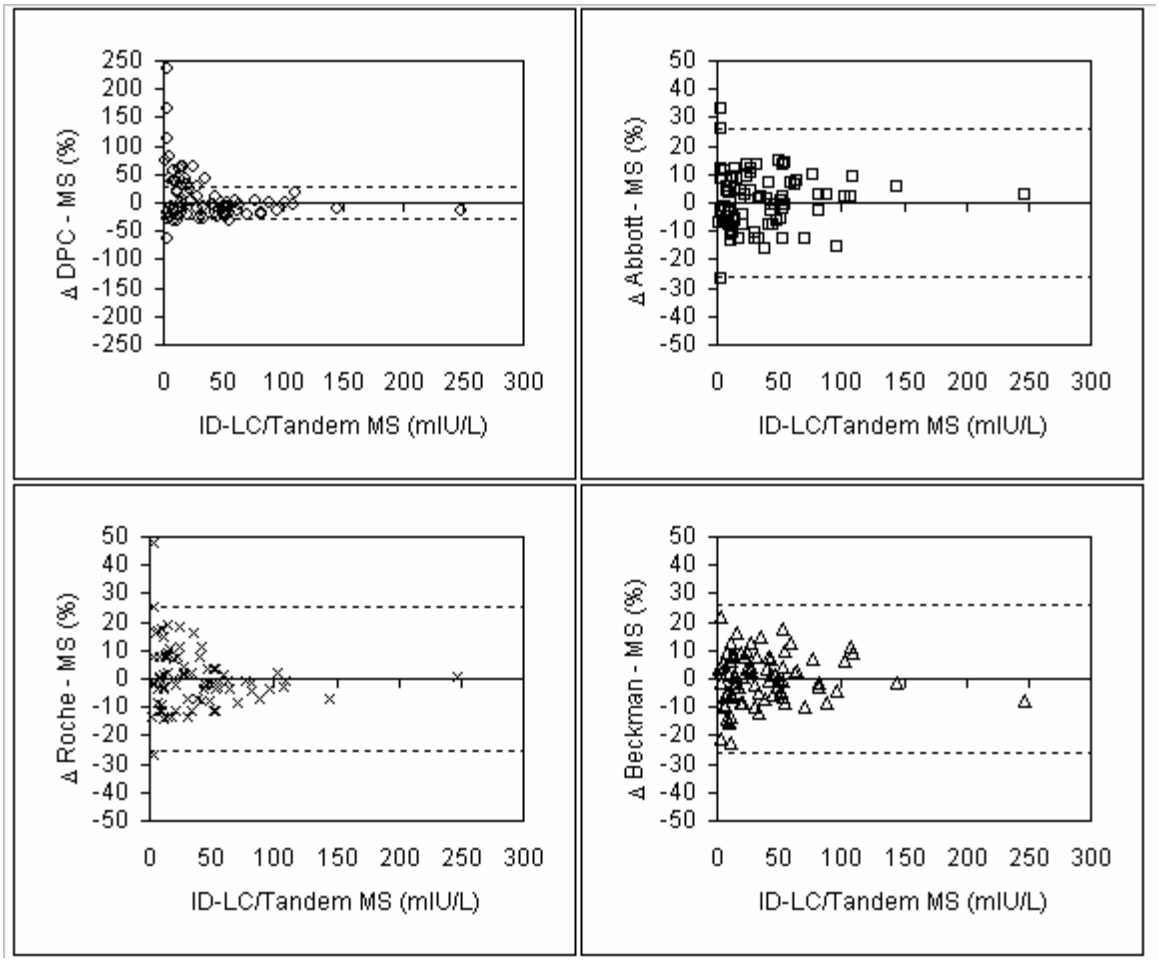

Figure III.12. Percentage difference plots after recalibration for each individual immunoassay. The dotted lines represent the 95% expected interval ($1.96 \times CV_{mp}$).

Recalibrated data and ADA total error limit

Figure III.13 shows that, after recalibration, all assays, with the exception of DPC, fulfill the total error specification of 32% set by the ADA work group (3). This demonstrates that the state-of-the-art of insulin immunoassays is quite good and that their standardization by ID-LC/Tandem MS may provide a major step forward to accurate measurement of serum insulin by the immunoassay technique. It should be noted further that the MS measurements have been done only once. An even better outcome may be expected for the ADA panel where the MS measurements are done in four-fold (four independent measurements on four different days).

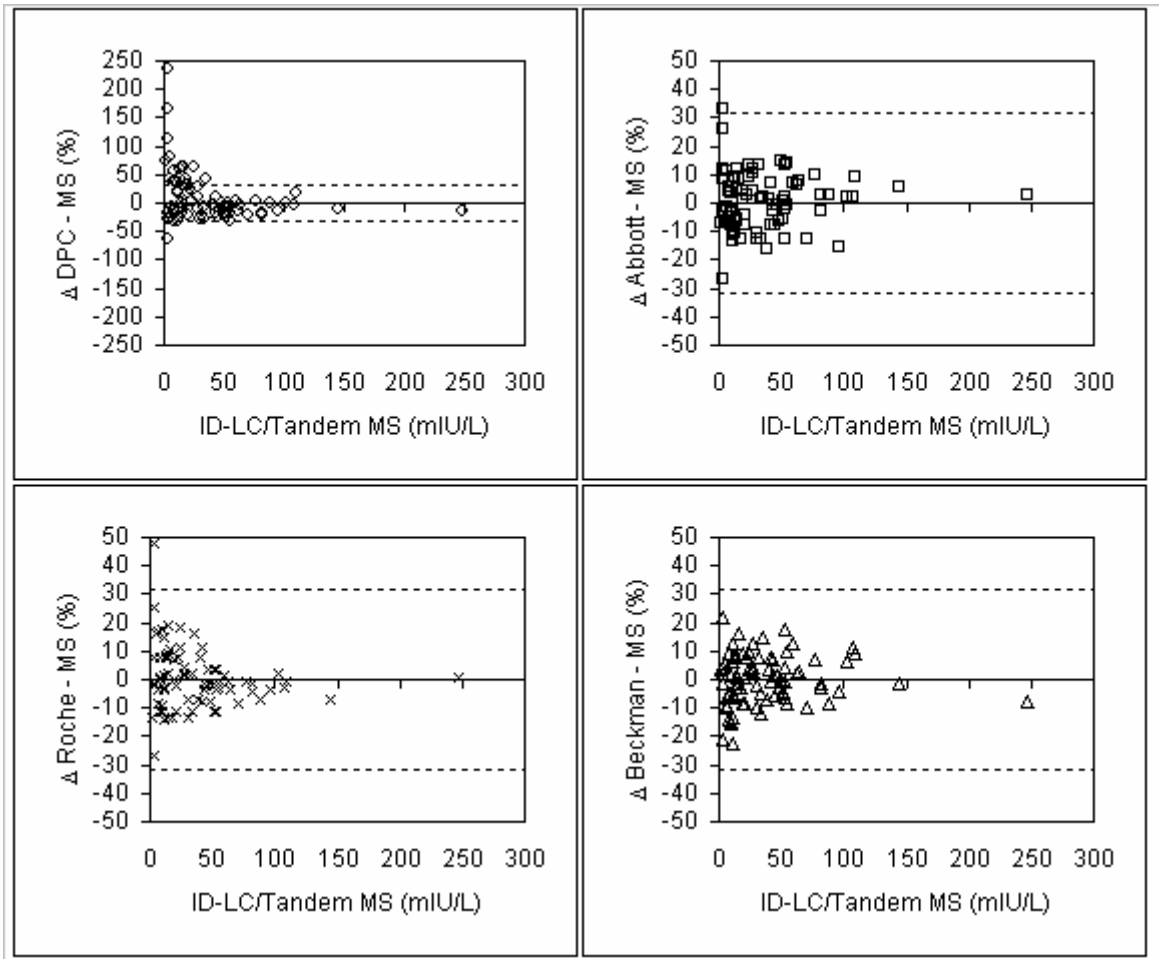

Figure III.13. Percentage difference plots after recalibration for each individual immunoassay. The dotted lines at ±32% represent the total error specification proposed by the ADA work group.

III.4. CONCLUSION

In conclusion, we were able to develop an LC/tandem MS measurement procedure for human insulin in serum that was superior in terms of sensitivity in comparison to previous methods described in the literature.

Our pilot study demonstrated that recalibration of insulin assays by regression equations established from method comparison with ID-LC/tandem MS can result in successful standardization and fulfillment of the total error criterion proposed by the ADA work group.

III.5. CURRENT WORK

Currently, the method is used for the certification of 40 single donation and 5 serum pools to be used for standardization of insulin immunoassays. This work is done under an ADA grant (29). Unfortunately, at this point, the ADA did not give consent to use the data in the current thesis. Therefore, only some general statements can be made about the progress of the method since.

The accuracy and precision study demonstrated that an improvement of the CV_{total} was desirable (range of 5 – 12%). This was achieved during the current study. The typical CV_{total} (four independent measurements on four different days) for the measurement of the ADA panel was 2 - 3%, with somewhat increased values in the low concentration range (4 - 6%; 100 ng/L range). This was reached by preparing the insulin standards in insulin free serum. However, this required to taking the standards through analysis.

Also, the method had a relatively low total recovery (approximately 30 – 40 %). We could increase the recovery to 60 – 70% by performing the IAC at room temperature instead of at 4 °C.

III.6. REFERENCES

1. Clark PM. Assays for insulin, proinsulin(s) and C-peptide. Ann Clin Biochem 1999;36:541-64 [Review].
2. Robbins DC, Andersen L, Bowsher R, Chance R, Dinesen B, Frank B et al. Report of the American Diabetes Association's task force on standardization of the insulin assay. Diabetes 1996;45:242-56.
3. Marcovina S, Bowsher RR, Miller WG, Staten M, Myers G, Caudill SP, et al. Standardization of Insulin Immunoassays: Report of the American Diabetes Association work group. Clin Chem 2007;53:711-6 [Epub 2007 Feb].
4. Manley SE, Stratton IM, Clark PM, Luzio SD. Multi-comparison of human insulin assays: implications for clinical investigation and research. Clin Chem 2007;53:922-32 [Epub 2007 Mar 15].
5. Sapin R. Insulin immunoassays: fast approaching 50 years. Clin Chem 2007;53:810-2.
6. Knip M. Analysis of pancreatic peptide hormones by reversed-phase high-performance liquid chromatography. Horm Metab Res 1984;16:487-91.
7. Halban PA, Rhodes CJ, Shoelson SE. High-performance liquid chromatography (HPLC): a rapid, flexible and sensitive method for separating islet proinsulin and insulin. Diabetologia 1986;29:893-6.
8. Farid NA, Atkins LM, Becker GW, Dinner A, Heiney RE, Miner DJ, Riggin RM. Liquid chromatographic control of the identity, purity and "potency" of biomolecules used as drugs. J Pharm Biomed Anal 1989;7:185-8. Review.
9. Ohta M, Tokunaga H, Kimura T, Satoh H, Kawamura J. Analysis of insulins by high-performance liquid chromatography. III. Determination of insulin in various preparations. Chem Pharm Bull 1984;32:4641-9.
10. Sato H, Tsuji A, Hirai K, Kang YS. Application of HPLC in disposition study of A14-125I-labeled insulin in mice. Diabetes 1990;39:563-9.
11. Lookabaugh M, Biswas M, Krull IS. Quantitation of insulin injection by high-performance liquid chromatography and high-performance capillary electrophoresis. J Chromatogr 1991;549:357-66.
12. Sergeev NV, Gloukhova NS, Nazimov IV, Gulyaev VA, Shvets SV, Donetsky IA et al. Monitoring of recombinant human insulin production by narrow-bore reversed-phase high-performance liquid chromatography, high-performance capillary

electrophoresis and matrix-assisted laser desorption ionization time-of-flight mass spectrometry. J Chromatogr A 2001;907:131-44.

13. Brix R, Spliid H, Hansen SH, Sorensen E. From experimental design to uncertainty estimation for the European Pharmacopoeia HPLC analysis of human insulin. Analyst 2002;127:1676-81.
14. Moslemi P, Najafabadi AR, Tajerzadeh H. A rapid and sensitive method for simultaneous determination of insulin and A21-desamido insulin by high performance liquid chromatography. J Pharm Biomed Anal 2003;33:45-51.
15. Sawicka K, Sahota T, Taylor MJ, Tanna S. Development of a reversed-phase high-performance liquid chromatography method for the analysis of components from a closed-loop insulin delivery system. J Chromatogr A 2006;1132:117-23 [Epub 2006 Aug 9].
16. Komjati M, Nowotny P, Waldhausl W. Radioimmunologic determination of human C-peptide and proinsulin in plasma with anti-C-peptide serum after prepurification by high-performance liquid chromatography. Anal Chem 1987;59:2010-2.
17. Murozono S, Sato K, Picazo J, Ishii H. Measurement of proinsulin in human pancreatic juice. Horm Metab Res 1989;21:619-22.
18. Given BD, Ostrega DM, Polonsky KS, Baldwin D Jr, Kelley RI, Rubenstein AH. Hypoglycemia due to surreptitious injection of insulin. Identification of insulin species by high-performance liquid chromatography. Diabetes Care 1991;14:544-7.
19. Linde S, Welinder BS, Nielsen JH. Analysis of proinsulin and its conversion products by reversed-phase high-performance liquid chromatography. J Chromatogr 1993;614:185-204 [Review].
20. Muronetz VI, Sholukh M, Korpela T. Use of protein-protein interactions in affinity chromatography. J Biochem Biophys Methods 2001;49:29-47 [Review].
21. Subramanian A. Immunoaffinity chromatography. Mol Biotechnol 2002;20:41-7 [Review].
22. Miyazaki S, Morisato K, Ishizuka N, Minakuchi H, Shintani Y, Furuno M et al. Development of a monolithic silica extraction tip for the analysis of proteins. J Chromatogr A 2004;1043:19-25.
23. Chen WH, Lee SC, Sabu S, Fang HC, Chung SC, Han CC et al. Solid-phase extraction and elution on diamond (SPEED): a fast and general platform for proteome analysis with mass spectrometry. Anal Chem 2006;78:4228-34.

24. Kippen AD, Cerini F, Vadas L, Stocklin R, Vu L, Offord RE et al. Development of an isotope dilution assay for precise determination of insulin, C-peptide, and proinsulin levels in non-diabetic and type II diabetic individuals with comparison to immunoassay. J Biol Chem 1997;272:12513-22.

25. Darby SM, Miller ML, Allen RO, LeBeau M. A mass spectrometric method for quantitation of intact insulin in blood samples. J Anal Toxicol 2001;25:8-14.

26. Hunter SJ, Boyd AC, O'Harte FP, McKillop AM, Wiggam MI, Mooney MH, et al. Demonstration of glycated insulin in human diabetic plasma and decreased biological activity assessed by euglycemic-hyperinsulinemic clamp technique in humans. Diabetes 2003;52:492-8.

27. Thevis M, Thomas A, Delahaut P, Bosseloir A, Schanzer W. Qualitative determination of synthetic analogues of insulin in human plasma by immunoaffinity purification and liquid chromatography-tandem mass spectrometry for doping control purposes. Anal Chem 2005;77:3579-85.

28. Sacks DB. Carbohydrates. In: Burtis CA, Ashwood ER, eds. Tietz textbook of clinical chemistry, 2nd ed. Philadelphia: WB Saunders Company;1994:945.

29. Insulin Assay Standardization Project. American Diabetes Association award number 10-06-Insulin (2006).

30. Thienpont LM, Van Nieuwenhove B, Stöckl D, De Leenheer A. Calibration for Isotope Dilution Mass Spectrometry - Description of an Alternative to the Bracketing Procedure. J Mass Spectrom 1996;31:1119-25.

31. Amersham Biosciences. Affinity Chromatography. Principles and Methods. Amersham Biosciences AB 2002.

32. Van Uytfanghe K, Rodríguez-Cabaleiro D, Stöckl D, Thienpont LM. New liquid chromatography/electrospray ionisation tandem mass spectrometry measurement procedure for quantitative analysis of human insulin in serum. Rapid Commun Mass Spectrom 2007;21:819-21.

33. Manning MC, Patel K, Borchardt RT. Stability of protein pharmaceuticals. Pharm Res 1989;6:903-18 [Review].

34. Strickley RG, Anderson BD. Solid-state stability of human insulin. II. Effect of water on reactive intermediate partitioning in lyophiles from pH 2-5 solutions: stabilization against covalent dimer formation. J Pharm Sci 1997;86:645-53.

35. Lougheed WD, Albisser AM, Martindale HM, Chow JC, Clement JR. Physical stability of insulin formulations. Diabetes 1983;32:424-32.

36. Wells JM, McLuckey SA. Collision-induced dissociation (CID) of peptides and proteins. Methods Enzymol 2005;402:148-85 [Review].
37. Medzihradszky KF. Peptide sequence analysis. Methods Enzymol 2005;402:209-44 [Review].
38. Papayannopoulos IA. The interpretation of collision-induced dissociation tandem mass spectra of peptides. Mass Spectrometry Reviews 1995;14:49-73 [Review].
39. Thevis M, Thomas A, Delahaut P, Bosseloir A, Schanzer W. Doping control analysis of intact rapid-acting insulin analogues in human urine by liquid chromatography-tandem mass spectrometry. Anal Chem. 2006;78:1897-903.

Summary

As explained in Chapter I, this thesis entirely fits into establishing the 'metrological traceability' of in-vitro diagnostic systems. Starting with a number of metrological considerations on measurements and measurement results in the clinical laboratory, the first chapter immediately addresses the problem of poor inter-laboratory and inter-method comparability of measurement results and continues with the generally accepted concept to solve this problem. This concept comprises the establishment of SI-traceability of commercial in-vitro diagnostic systems based on the use of a 'reference measurement system' as part of a traceability chain. The structure and elements of such a traceability chain are discussed in detail, as is its application. In this regard, it is clarified that traceability is the key to trueness/accuracy of a measurement result. Because reference measurement procedures are essential parts of a reference measurement system, this chapter also deals with the requirements and analytical specifications such measurement procedures have to meet in more detail, as well as with the internationally accepted mechanisms ("Joint Committee for Traceability in Laboratory Medicine" and dedicated ISO standards) to identify adequate reference measurement procedures and competent reference laboratories. At this point a first reference is made to the suitability of the technique of isotope dilution-mass spectrometry (ID-MS) to serve as a reference measurement procedure for quantification of organic compounds. It is also emphasized that doing a method comparison with a reference measurement procedure on native sera is the best way to establish traceability of a routine diagnostic system. As described in a later passage in this chapter, the development and application of ID-MS reference measurement procedures are two of the major objectives of this dissertation. The context in which these objectives are described is the so-called 'diabetes epidemic' that gradually affects humans worldwide and consequently increases the interest for routine C-peptide and insulin determinations. After addressing briefly the importance of laboratory investigations, this chapter immediately repeats the problem of non-comparability of measurement results. Although the clinical laboratory community found an intermediate solution to the problem, i.e., using method-dependent reference intervals, the need for establishing better comparability remained great. This need has even increased since authoritative organizations such as the American Diabetes Association (ADA) have aimed at publishing general guidelines and decision criteria with regard to the diagnosis of diabetes and therapeutic monitoring. Recently, the pharmaceutical industry increased the

necessity as a result of the development of new drugs (the so-called insulin sensitizers), which are recommended for therapy on the basis of the homeostasis model assessment (HOMA) insulin-resistance indices. In view of the considerations and explanation given in Chapter I, it is obvious that the need can be satisfied through the development of a reference measurement system, in order to make serum C-peptide and insulin measurements SI-traceable. Since at the start of this dissertation, to the best of our knowledge, the necessary reference measurement systems and traceability chain did not exist and the development of a reference measurement procedure for C-peptide and insulin became our first objective. A second objective consisted on demonstrating the utility of the respective reference measurement procedures in a method comparison with representative routine diagnostic systems. To be able to apply this method comparison on authentic patient samples, cooperation with the Department for Hormonology of the Ghent University Hospital was established. The major objectives of this dissertation fit excellently with those of the ADA Task Force and the National Institute of Diabetes & Digestive & Kidney Diseases (NIDDK). Indeed, because the activities of the latter groups did not succeed in terms of metrological traceability, they recently called upon trueness-based standardization of C-peptide and insulin measurements. As a result an award was granted to our laboratory for the research towards an insulin reference measurement procedure. Finally, this chapter describes in a detailed way the focus of our research to reach the aforementioned objectives. The distinct steps are discussed in detail in the Chapters II and III.

Chapter II deals with the development, validation and application of a reference measurement procedure for C-peptide in serum. By way of introduction the biosynthesis, secretion and metabolism of C-peptide in humans is described in short. In view of the close relationship between C-peptide and insulin, both of which originate from the common precursor proinsulin, this introduction in the C-peptide chapter is extended also to insulin. The same applies for the discussion of the physiological effects and the clinical applications of laboratory measurements. With regard to the latter, specifically for C-peptide, an overview of the existing measurement procedures is given. Besides those for routine use, also chromatographic and mass spectrometric procedures, some of them in the combined mode, are discussed, in particular because the latter will served as a basis for the development of the aimed reference measurement procedure. These developments are systematically described. They focus in particular on the specificity for interference-free analysis of C-peptide in the serum matrix and on the achievement of

sufficient sensitivity for measurement of concentrations typical within the physio(patho)logy of C-peptide. Also the analysis time and robustness of the measurement procedure played an important role. Specifications for total precision, trueness and limit of quantification were set by ourselves. In the C-peptide study special attention was paid to the collision induced dissociation (CID) of the doubly charged deprotonated molecular ion $[M–2H]^{2-}$ of C-peptide. On the basis of the experiments and their evaluation, we finally opted for an isotope dilution-liquid chromatography/tandem mass spectrometry (ID-LC/tandem MS) procedure after serum purification with solid phase extraction in two steps. For MS detection we worked under conditions of negative electrospray ionization (ESI) and high-energy CID of the $[M\text{-}2H]^{2-}$ C-peptide/D_8-$Val^{7,10}$-C-peptide molecule (transitions from m/z 1509.2 to 183.8 and 1517.1 to 183.8, respectively). Validation of the most important analytical characteristics of the selected ID-LC/tandem MS procedure proved that it met the preset requirements and specifications. With regard to the second objective, our study confirmed the need for standardization of C-peptide routine measurement procedures. It also documented that standardization via a method comparison between the developed ID-LC/tandem MS measurement procedure and the routine test systems on authentic patient samples was feasible. The performance of the tested routine systems after recalibration was estimated against specifications. This chapter is finalized by giving perspectives concerning further activities and/or requirements for state-of-the-art standardization at the international level.

In Chapter III the development of a reference measurement procedure for insulin on the basis of ID-LC/MS is discussed. The research is conducted in the same way as described for C-peptide, i.e., by starting from existing LC/MS measurement procedures and seeking for a sensitive and specific reference measurement procedure, that satisfies the specifications for total precision, trueness and limit of quantification defined by the ADA. Since this research was conducted together with a colleague, it must be mentioned that the outcome of the experiments done and described as part of this dissertation, is not necessarily withheld in the final measurement procedure (e.g., the two-step solid phase extraction was finally substituted with a combined immuno affinity-solid phase extraction). During the method optimization experiments with various collision gasses in two different MS configurations were done in order to increase sensitivity. Special attention was paid to the stability of the standard solutions. The relationship between the commercial standard material (used for the calibration of our measurement procedure) and the WHO standard (used by the in-vitro diagnostic companies as calibration basis) was investigated. On the

basis of the combined results of all experiments presented in this dissertation and the one of a colleague, we finally opted for an ID-LC/tandem MS procedure with a sample preparation procedure based on a combined immunoaffinity chromatography-solid phase extraction. For the LC procedure we opted for alkaline gradient elution with ammonia on an basic-pH resistant stationary phase in combination with wrong way round positive ESI mode and monitoring of the transition of the four-times protonated molecular ions $[M+4H]^{4+}$ from m/z 1453 to 226 (insulin) and from m/z 1463 to 226 (4-[D10]Leu-insulin). As a result of these LC/tandem MS conditions a remarkable increase in the sensitivity was observed in comparison with the existing measurement procedures operated under conventional conditions (acidic mobile phase and positive ESI). The validation of the analytical characteristics of the selected measurement procedure showed that they met the ADA specifications, apart from the imprecision component. Like in the C-peptide study, we showed the utility of the ID-LC/tandem MS procedure for method comparison. After recalibration of the tested routine systems it was possible on the basis of this method comparison, to investigate their performance against the ADA specifications for total error derived from the biological variation of insulin in human. For the tested insulin systems the impact of the recalibration from the method comparison was considered rather moderate. This chapter is concluded with the perspective that our ID-LC/tandem MS procedure will be used by the ADA for standardization of commercial insulin routine procedures. Unfortunately, at this point, the ADA did not give consent to use the data in the current dissertation. Therefore, only general statements have been made about the progress of the measurement procedure since.

Samenvatting

Zoals in Hoofdstuk I uitgelegd, kadert deze thesis integraal in de verwezenlijking van 'metrologische herleidbaarheid' van in-vitro diagnostische systemen. Vanuit een aantal algemene metrologische beschouwingen rond metingen en meetresultaten in het klinisch laboratorium, wordt in dit hoofdstuk onmiddellijk de problematiek van onvoldoende vergelijkbaarheid van meetresultaten tussen methoden en laboratoria aangesproken, om vervolgens het algemeen aanvaarde concept tot oplossen van deze problematiek voor te stellen. Dit concept bestaat in het verwezenlijken van SI-herleidbaarheid van commerciële in-vitro diagnostische systemen via aanwenden van een 'referentiemeetsysteem' als onderdeel van een herleidbaarheidsketen. De structuur en de elementen van zo'n herleidbaarheidsketen worden in detail besproken, evenals de aanwending ervan. In dit verband wordt duidelijk gemaakt dat herleidbaarheid de enige sleutel tot juistheid/nauwkeurigheid van een meetresultaat is. Aangezien referentiemeetprocedures essentiële onderdelen van een referentiemeetsysteem zijn, gaat dit hoofdstuk ook dieper in op de vereisten en analytische specificaties waaraan dergelijke meetprocedures moeten voldoen, evenals op de internationaal aanvaarde mechanismen ("Joint Committee for Traceability in Laboratory Medicine" en specifieke ISO standaarden) om adequate referentiemeetprocedures en competente referentielaboratoria te identificeren. Hier wordt reeds een eerste keer verwezen naar de geschiktheid van de techniek van isotoop dilutie-massaspectrometrie (ID-MS) als basis voor een referentiemeetprocedure van organische componenten. Tevens wordt benadrukt dat voor het realiseren van herleidbaarheid van een routine diagnostisch systeem, deze laatste best aan een methodevergelijking met de referentiemeetprocedure en gebruik van natieve sera wordt onderworpen. Zoals verder in dit hoofdstuk beschreven, behoort de ontwikkeling en toepassing van ID-MS referentiemeetprocedures trouwens tot de hoofdobjectieven van deze thesis. De context van deze objectieven is de zogenaamde 'diabetes epidemie' die wereldwijd toeslaat en maakt dat diagnostisch gezien de interesse voor routine C-peptide en insulinebepalingen in humaan serum gestaag toeneemt. Na een korte uiteenzetting van het belang van deze laboratoriumbepalingen, komt dit hoofdstuk onmiddellijk terug op de problematiek van niet-vergelijkbaarheid van meetresultaten. Ofschoon de klinische laboratoriumgemeenschap zich aan dit probleem heeft aangepast door o.a. het gebruik van methodeafhankelijke referentie-intervallen, is de nood tot verwezenlijken van betere vergelijkbaarheid zeer groot, des te meer aangezien gezaghebbende organisaties zoals

de "American Diabetes Association" (ADA) willen overgaan tot het uitvaardigen van algemene richtlijnen en beslissingscriteria in verband met de diagnose van diabetes en/of opvolgen van de therapie. Recent heeft ook de farmaceutische industrie zich bij deze nood geschaard, meer bepaald sedert de ontwikkeling van nieuwe geneesmiddelen (de zogenaamde insuline "sensitizers"), die therapeutisch aanbevolen worden op basis van indexen voor insulineresistentie, zelf afgeleid uit "homeostasis model assessment" (HOMA). Vanuit de overwegingen en uitleg in Hoofdstuk I is het duidelijk dat deze nood kan gelenigd worden door de ontwikkeling van een referentiemeetsysteem om zowel serum C-peptide als insulinemetingen SI-herleidbaar te maken. Aangezien bij het aanvatten van deze thesis, voor zover wij er kennis van hadden, de noodzakelijke referentiemeetsystemen en herleidbaarheidsketens onbestaande waren, werd de ontwikkeling van een referentiemeetprocedure voor C-peptide en insuline ons eerste objectief. Een tweede objectief bestond erin de bruikbaarheid van de respectievelijke referentiemeetprocedures in een methodevergelijking met representatieve routine diagnostische systemen aan te tonen. Om deze methodevergelijking op authentieke patiëntenstalen te kunnen doen, werd een samenwerking met het Departement voor Hormonologie van het Universitair Ziekenhuis te Gent tot stand gebracht. De voornoemde objectieven sloten zeer goed aan bij de objectieven van o.a. de ADA "Task Force" en de "National Institute of Diabetes & Digestive & Kidney Diseases" (NIDDK). Inderdaad, aangezien de activiteiten van laatstgenoemde groepen tot nog toe geen succes in termen van metrologische herleidbaarheid hadden opgeleverd, hadden zij recent nog een oproep gedaan tot juistheidgebaseerde standaardisatie van C-peptide en insulinemetingen. Op die manier kwam zelfs voor ons laboratorium een financiële ondersteuning van het wetenschappelijk onderzoek naar een insulinereferentiemeetprocedure tot stand. Tot slot wordt in dit hoofdstuk gedetailleerd uiteengezet op wat het wetenschappelijk onderzoek zich zou toespitsen om voornoemde objectieven te bereiken. De verschillende stappen in dit onderzoek komen in de Hoofdstukken II en III uitgebreid aan bod.

In Hoofdstuk II wordt de ontwikkeling, validatie en toepassing van een referentiemeetprocedure voor C-peptide in serum behandeld. Bij wijze van inleiding wordt de biosynthese, secretie en het metabolisme van C-peptide bij de mens kort besproken. Gezien de verwantschap van C-peptide en insuline vanuit de gemeenschappelijke precursor, het proinsuline, wordt de inleiding van het C-peptidehoofdstuk met insuline uitgebreid. Hetzelfde geldt voor de bespreking van de fysiologische effecten en het klinisch toepassen van laboratoriummetingen. In dit laatste verband wordt vervolgens

specifiek voor C-peptide een overzicht van de bestaande meetprocedures gegeven. Naast deze voor routinegebruik worden ook chromatografische en massaspectrometrische procedures, al dan niet gecombineerd, besproken, vooral omdat deze laatste als basis zullen dienen bij de ontwikkeling van de beoogde referentiemeetprocedure. Deze ontwikkelingen worden stapsgewijs besproken. Zij spitsten zich vooral toe op de specificiteit voor interferentievrije analyse van C-peptide in de serummatrix, op het bereiken van voldoende gevoeligheid voor meting van concentraties typisch voor de fysio(patho)logie van C-peptide. Ook de analysetijd en de robuustheid van de meetprocedure speelden een belangrijke rol. Specificaties voor totale precisie, juistheid en bepaalbaarheidsgrens werden door onszelf vooropgesteld. Een speciaal aandachtspunt in de C-peptidestudie was de collisie-geïndiceerde dissociatie (CID) van het tweevoudig geladen gedeprotoneerde moleculair ion $[M–2H]^{2-}$ van C-peptide. Op basis van de doorgevoerde experimenten en hun beoordeling werd finaal geopteerd voor een ID-LC/tandem MS procedure, na serumopzuivering via vaste fase-extractie in twee stappen. Voor MS-detectie werd gewerkt onder voorwaarden van negatieve elektrospray ionisatie (ESI) en hoge-energie CID van de $[M-2H]^{2-}$ C-peptide/D_8-$Val^{7,10}$-C-peptide molecule (transities van m/z 1509.2 tot 183.8 en 1517.1 tot 183.8, respectievelijk). De validatie van de voornaamste analytische karakteristieken van de geselecteerde ID-LC/tandem MS procedure bewees dat zij voldeed aan de door ons vooropgestelde eisen en specificaties. In verband met het tweede objectief, bevestigde onze studie de nood aan standaardisatie van C-peptide routinemeetprocedures. Zij toonde tevens aan dat deze standaardisatie via een methodevergelijking tussen de ontwikkelde ID-LC/tandem MS meetprocedure en de routine testsystemen op authentieke patiëntenstalen mogelijk is. De performantie van de geteste routinesystemen na recalibratie werd tegen specificaties beoordeeld. Dit hoofdstuk wordt afgesloten met de formulering van een aantal perspectieven over wat er nog te doen staat om de feitelijke standaardisatie op internationaal niveau volgens de regels van de kunst uit te voeren.

In Hoofdstuk III wordt de ontwikkeling van een referentiemeetprocedure voor insuline op basis van ID-LC/MS besproken. Het wetenschappelijk onderzoek wordt grotendeels volgens hetzelfde stramien als beschreven voor C-peptide uitgevoerd, meer bepaald door uitgaande van de bestaande LC/MS meetprocedures te zoeken naar een gevoelige en specifieke referentiemeetprocedure, die voldoet aan specificaties voor totale precisie, juistheid en bepaalbaarheidsgrens vooropgesteld door de ADA. Aangezien dit onderzoek in samenwerking met een collega gebeurde, werd het resultaat van de

experimenten in deze thesis uitgevoerd en beschreven, niet noodzakelijkerwijze in de finale meetprocedure weerhouden (bv. de tweestaps vaste fase-extractie werd in de finale procedure gesubstitueerd met een gecombineerde immunoaffiniteits-vaste fase-extractie). Speciale aandachtspunten in de insulinestudie waren de CI dissociatie met verschillende collisiegassen in twee verschillende MS configuraties, de stabiliteit van de standaardoplossingen en het verband tussen het commerciële standaardmateriaal, dat wij ter calibratie van onze meetprocedure gebruikten, en de WHO-standaard, die door de in-vitro diagnostica firma's als calibratiebasis wordt gebruikt. Op basis van de gecombineerde resultaten van alle experimenten (in deze thesis en die van een collega) werd finaal geopteerd voor een ID-LC/tandem MS procedure, na serumopzuivering via een gecombineerde immunoaffiniteitschromatografie-vaste fase-extractieprocedure. Voor de LC procedure werd geopteerd voor een alkalische gradiëntelutie met ammonia op een hoge pH-bestendige stationaire fase in combinatie met "wrong way round" positieve ESI, waarbij de transitie van de viervoudig geprotoneerde moleculaire ionen $[M+4H]^{4+}$ van m/z 1453 tot 226 (insuline) en 1463 tot 226 (4-[D10]Leu-insuline) werden gevolgd. Het resultaat van deze LC/tandem MS condities was een opmerkelijke toename van de gevoeligheid in vergelijking met de bestaande meetprocedures op basis van klassieke zure positieve ESI LC/MS. Na validatie van de analytische karakteristieken konden wij bewijzen dat de geselecteerde meetprocedure voldeed aan de ADA specificaties, op vooral de imprecisiecomponente na. Net zoals bij de C-peptidestudie werd de bruikbaarheid van de ID-LC/tandem MS procedure voor methodevergelijking in een toepassing gedemonstreerd. Na recalibratie van de geteste routinesystemen op basis van deze methodevergelijking was het mogelijk hun performantie tegen de ADA specificaties voor totale fout op basis van de biologische variabiliteit voor insuline te beoordelen. Opmerkelijk was dat voor de geteste insulinesystemen de impact van de recalibratie op basis van de methodevergelijking niet zo ingrijpend was als voor de C-peptidesystemen. Dit hoofdstuk wordt afgesloten met het perspectief dat de ID-LC/tandem MS procedure door de ADA als basis voor de standaardisatie van de commerciële insuline routineprocedures zal gebruikt worden, waarvoor de ADA echter tot op heden geen toestemming tot gebruik in deze thesis gaf. Daarom kunnen slechts een aantal algemene gegevens over de verdere vorderingsstaat van de studie worden gegeven.