Published in final edited form as:

Proteomics Clin Appl. 2011 August ; 5(0): 454–459. doi:10.1002/prca.201000112.

Mass Spectrometric Immunoassay of Intact Insulin and Related Variants for Population Proteomics Studies

Paul E. Oran, Jason W. Jarvis, Chad R. Borges, Nisha D. Sherma, and Randall W. Nelson^{*}
Molecular Biomarkers, The Biodesign Institute at Arizona State University, Tempe, AZ 85287

Abstract

PURPOSE—The purpose of the work presented herein was to develop a high-throughput assay for the quantification of human insulin in plasma samples while simultaneously detecting, with high mass accuracy, any additional variant forms of insulin that might be present in each sample.

EXPERIMENTAL DESIGN—A mass spectrometric immunoassay (MSIA) was designed in which anti-human insulin antibodies were immobilized to commercially available MSIA pipette tips and used to capture insulin and related protein variants from human plasma.

RESULTS—Standard curves for insulin exhibited linearity (average R^2 for three days of analysis = 0.99) and assay concentration limits of detection and limits of quantification for insulin were found to be 1 pM and 15 pM, respectively. Estimated CVs for interday experiments ($n = 3$ days) were < 8%. Simultaneously, the assay was shown to detect and identify insulin metabolites and synthetic insulin analogs (e.g. Lantus). Notably, insulin variants not known to exist in plasma were detected in diabetics.

CONCLUSIONS AND CLINICAL RELEVANCE—This introductory study sets a foundation towards the screening of large populations to investigate insulin isoforms, isoform frequencies, and their quantification.

Keywords

insulin; microheterogeneity; population proteomics

Numerous genetic polymorphisms and splice variants of human insulin have been characterized [8–11] [UniProtKB AC P01308]. Yet there remains a need for systematic methods to monitor polymorphisms, splice variants, and particularly, posttranslational modifications (e.g. degradation products) *that are not pre-defined*, in the population at large. Data arising from the application of such methods will take us one step closer to making personalized medicine in the context of diabetes a reality.

An essential and differentiating characteristic of mass spectrometric-based assays that are designed from a *top-down* perspective is that immunoreactive forms are clearly and immediately evident in the sample and can be independently quantified. Likewise, protein variants that are distinctly monitored in this way are in many circumstances the same variants that interfere with classical analytical techniques such as ELISAs and radioimmunoassays (RIA) (i.e. the variants cross-reacts with the antibody) or, conceivably, with recent *bottom-up* quantitative mass spectrometric approaches such as LC-MRM, e.g.,

^{*} Author to whom correspondence should be addressed: Randall W. Nelson, Molecular Biomarkers, The Biodesign Institute at Arizona State University, P.O. Box 876401, Tempe, AZ 85287. Tel 480-727-9929, fax 480-727-9464; randal.nelson@asu.edu.

Conflict of Interest Statement

RWN has partial ownership of Intrinsic Bioprobes

SISCAPA (Stable Isotope Standards and Capture by Anti-Peptide Antibodies) [12]. As in the case of the latter example, it is the unmodified portions of variants and native protein (in the presentation of tryptic fragments) which equally bind to the antibody and cause diminished assay specificity. These techniques (e.g. LC-MRM) result in the summation of protein variants, which may or may not have modified biological activity. Consequently, an erroneous read on the concentration of the native, bio-active form a protein may result. Indeed, this was shown with insulin twenty years ago where immunoassays were shown to cross-react with proinsulin related molecules [13]. (The same phenomenon has been documented recently with regard to human brain natriuretic peptide (BNP) [7].) Moreover, a more contemporary study showed 11 commercially available insulin assays (one RIA and 10 enzyme/chemiluminescent based) to vary by a factor of 2 indicating assay-specific cross-reactivity [14]. Consequently, several groups have responded to this challenge with the development of quantitative mass spectrometric insulin assays. With the mass spectrometer serving as the detection component of the assay, it is now possible to unambiguously quantify intact insulin out of plasma and urine distinct from preproinsulin byproducts, insulin degradation products, and synthetic insulin analogs [15–19]. While there are some relative advantages to previously reported MS techniques including the ability to monitor for unique MS² diagnostic product ion signatures resulting from insulin isoforms [19], assays such as these are not suited for large population screenings when the goal is to monitor for unanticipated forms of insulin. Herein we report a high-throughput, *full-scan mode* mass spectrometric assay for quantifying insulin which simultaneously provides accurate-mass detection of insulin-related protein variants that have not been pre-defined.

CDI (1,1'-Carbonyldiimidazole)-activated affinity pipette tips were prepared and derivatized with mouse anti-human insulin antibody (AbD Serotec, Cat No. 5329-3806), as previously described for other antibodies [20, 21]. For development of the assay, a bulk quantity (> 100 mL) of healthy human EDTA plasma from an individual female donor was used. Sixteen additional human EDTA plasma samples from patients including 8 healthy individuals and 8 type 2 diabetics (6 ID T2D (insulin dependent type 2 diabetics), and 2 non-ID T2D (non-insulin dependent type 2 diabetics)) were acquired under IRB approval. Cohorts were matched by gender. The diabetic cohort had an average age of 57 and the healthy cohort had an average age of 51. Sheep plasma was used as the matrix to generate standard curves and was acquired from Bioreclamation, Inc. All samples were stored at –80° C prior to use. Upon use, samples were centrifuged for 5 minutes at 10,500 × g and subsequently re-aliquoted into a 96 well sample tray. Porcine insulin, human insulin, and bovine serum albumin were purchased from Sigma-Aldrich. As qualitative negative controls, anti-resistin and anti-osteocalcin were purchased from R&D Systems and Novus Biological, respectively.

Five hundred microliters of human plasma was pre-treated with 250 µL of a solution containing: 4.5 % Tween 20, 150mM Octyl-β-glucopyranoside, 1.5M Ammonium Acetate, and Concentrated PBS (0.67M sodium phosphate, 1M sodium chloride), for a total analytical volume of 750 µL. Insulin and related variants were extracted with the aid of a Beckman Multimek 96 pipetting robot by repeatedly (250 repetitions) drawing and expelling (back into the sample volume) 125-µL aliquots of the sample volume through an anti-insulin affinity pipette. After extraction, the pipettes were rinsed using PBS (0.1 M sodium phosphate, 0.15 M sodium chloride, pH 7.2), HBS-P (0.01 M HEPES, 0.15 M NaCl, 0.05% v/v Tween Surfactant P20, pH 7.4), H₂O, 100mM Tris HCl (Tris(hydroxymethyl)aminomethane hydrochloride pH 4.6), and H₂O (in this order, each rinse = 10 repetitions of 150 µL), after which insulin was eluted and prepared for MALDI-TOF MS by drawing 4 µL of MALDI matrix solution (saturated aqueous solution of sinapic acid, in 33% (v/v) acetonitrile, 0.45% (v/v) trifluoroacetic acid, TFA) into the pipette and depositing onto a MALDI target.

MALDI-TOF MS was performed using a Bruker Ultraflex III MALDI-TOF-MS instrument. The instrument was used in both linear mode (for precision experiments as explained below) and with reflector engaged. In the former, positive ion, delayed-extraction mode was used with 'ion source 1' at 25.00 kV, 'ion source 2' at 23.50 kV, lens at 6.00 kV, 50 ns delayed extraction, deflection signal suppression up to m/z 2500, and 1 GS/s sample rate. When the reflector was engaged, the instrument operated in the positive ion, delayed-extraction mode with 'ion source 1' at 25.00 kV, 'ion source 2' at 21.90 kV, lens at 9.50 kV, 'reflector' at 26.30 kV, 'reflector 2' at 13.80, 190 ns delayed extraction, deflection signal suppression up to m/z 2000, and 2 GHz sampling rate. At least 10 thousand laser-shots were signal averaged for each mass spectrum to ensure good ion counting statistics. In linear mode, spectra were externally calibrated with a mixture of 4 proteins, supplied by Bruker (Cat. No. 208241), ranging from m/z 5734.52 (Insulin [M+H]⁺ to m/z 12,360.97 (Cytochrome C [M+H]⁺). When in reflector mode, MALDI-TOF-MS mass spectra were externally calibrated with a mixture of peptides and proteins supplied by Bruker including: m/z 2093.086 (ACTH_clip(1–17) [M+H]⁺; monoisotopic), m/z 3147.471 (Somatostatin [M+H]⁺; monoisotopic), m/z 5730.609 (Bovine Insulin [M+H]⁺; monoisotopic), and m/z 6179.67 (Cytochrome C [M+2H]²⁺; most abundant isotope).

All samples used for quantification were fortified with 150 pM porcine insulin (which served as the internal standard) prior to extraction of endogenous human insulin. Samples used in the generation of insulin standard curves were prepared and assayed using sheep plasma diluted 4× in 30 g/L BSA in HBS-N (0.01M HEPES and 0.15M NaCl). The curves were prepared (via serial dilution) with a human insulin concentration range of 15 pM to 1.92 nM. The curves had 9 concentration points in total, including the blank samples with only internal standard. For insulin quantification out of human samples, eight EDTA plasma samples from healthy volunteers and eight EDTA plasma samples from T2D volunteers were run in parallel with a standard curve. On each day of the interday/intraday precision experiment, curves were run in triplicate alongside samples with known concentrations of human insulin, at 50 pM (n=4), 100 pM (n=4), and 400 pM (n=4). Samples were prepared fresh daily.

Individual mass spectra were baseline subtracted (Tophat algorithm) and smoothed (SavitzkyGolay algorithm; width = 0.2 m/z ; cycles = 1) prior to peak integration using Bruker Daltonics flexAnalysis 3.0. All peaks representing porcine insulin, intact insulin, and insulin variants were integrated baseline-to-baseline (using Intrinsic Bioprobes Inc. Zebra 1.0) and tabulated in a spreadsheet for quantification.

On-target reduction of interchain disulfide bonds was employed to verify the identification of insulin in the mass spectrum: The target (96-well format MALDI-TOF-MS gold coated plate) was placed on a 40°C heating plate followed by application of a three-microliter aliquot of 100mM dithiothreitol (DTT) in 100mM tris base (pH 8.5) to an already-dried MALDI spot previously shot from a healthy human sample. Reduction was carried out for 20 minutes, during which time the aqueous environment of the samples was maintained by the addition of a 1 μ L aliquot of water at the beginning of the reduction and every 4 minutes thereafter. The reduction reaction was terminated after 20 minutes via the addition of a 3- μ L aliquot of the MALDI matrix solution which lowered the pH down to ~1–2. This effectively ended the reduction process and, upon air-drying, recrystallized the matrix spot.

In the development phase of the assay prior to the implementation of internal standards for quantification, various sample preparations and rinses were tested. Figure 1(I) shows a pre-optimized MSIA spectrum without the use of in-sample detergents, salts, and post-extraction rinses. Tween 20, Octyl- β -glucopyranoside, Ammonium Acetate, and PBS were determined, when added to the plasma sample, to minimize the non-specific binding of proteins. Next,

various rinse protocols were tested. It was determined that PBS, HBS-P, H₂O, 100 mM Tris-HCl, and finally H₂O produced the best results. Figure 1(II) shows an optimized MSIA spectrum. Overall, the optimized protocols produced spectra largely free of non-targeted proteins, high S/N ratios, and isotopic resolution for insulin. The *open m/z window* that was created after the optimization was valuable for the potential detection of additional insulin isoforms that were cross reactive with the antibody. Figure 1(III) shows an on-target-reduction spectrum of insulin. Notably, this was generated by reducing the same MALDI matrix spot used to generate Figure 1(II). Reduction of insulin breaks the two-disulfide bonds between the α -chain and β -chain and the single disulfide bond within the α -chain. Besides the specificity imparted by antibody capture and verified by the exact mass detection of intact insulin, the observation of exact masses corresponding to the α - and β -chains of insulin upon reduction confirm the qualitative specificity of the assay. Lastly, an additional measure ensuring assay specificity was achieved by generating a MALDI-TOF/TOF (MS/MS) spectrum on the insulin β -chain (Supporting Information Figure 1; methodology described elsewhere [22]). This was produced from a healthy human sample that had 50 nM recombinant human insulin spiked in pre-extraction (endogenous insulin levels did not produced adequate S/N for MS/MS).

An analog of a protein that has a different molecular weight may be used as an internal standard for quantification when using MALDI-TOF-MS [23]. In this manner, the internal standard is captured with the target protein using the same antibody (the antibody must be able to cross-react). While an internal standard can be a chemically modified form of a protein [23], the use of a protein from a different species may be used provided they differ enough in m/z [9, 10]. Considering the monoisotopic mass of human insulin at 5804.65 Da $[M+H]^+$ and a mass resolution of 15,000 ($m/\bar{m}$; FWHM), porcine insulin was determined to be an excellent internal standard which differs in mass from human insulin by -30.01 Da (the monoisotopic molecular weight is 5774.64 Da $[M+H]^+$). This change in mass results from the presence of an alanine (rather than threonine) residue at the β -30 position of porcine insulin. The anti-human insulin antibody employed in these studies was developed with human insulin as the immunogen and is known to recognize insulin and pro-insulin, but the binding epitope has not been determined. Porcine insulin proved to be, retrospectively, well chosen due to its distinct mass relative to synthetic insulin analogs and natural insulin degradation products.

Insulin MSIA curves demonstrated excellent linearity (the average r^2 value for all three days was 0.9983). The limit of detection (LOD) and limit of quantification (LOQ) were found to be 1 pM and 15 pM, respectively, provided that at least 500 μ L of plasma were employed for the assay. Three sample sets composed of four samples each with concentrations of 50 pM, 100 pM, and 400pM were used for intra/inter-assay precision experiments conducted over three days (Table 1). The intraday CVs and estimated interday CVs were $< 8\%$. A typical standard curve regression line was described by the equation $y = 2.096x - 0.034$. Using the multi-day data from the triplicate working curves run each day, the average standard error of the estimate was determined to be 24 pM. We estimate the actual captured amount of insulin to be in the range of 1 fmol at the LOQ. A typical standard curve is presented in the Supporting Information, Figure 2.

Figure 2 presents MALDI-TOF-MS spectra obtained in reflector mode from human samples using the insulin MSIA. In this figure, human insulin, des-thr³⁰-insulin (β -chain), des-arg³²⁻³¹-Lantus (β -chain), and porcine insulin (internal standard) are shown. Intact Lantus and intact Novolog were also detected in the T2D patient samples. With the exception of one outlier, mass accuracy of insulin variants detected was 15 ± 7 ppm (Supporting Information, Table 1). With this degree of mass accuracy (< 5 ppm with internal mass calibration) and knowledge of the identity and sequence of the target protein (imparted by

the specificity of the capture-antibody), mass mapping can greatly narrow a list of candidate protein modifications. Monoisotopic peak assignments were used when S/N was >3. When monoisotopic peaks did not meet this criteria (as in the case of intact Lantus), the most abundant isotope was used for assignment. Of note, we were unable to generate MS/MS information on reduced insulin isoforms due to their low concentrations. Negative control experiments were run using T2D samples known to have des-thr³⁰-insulin (β -chain), Lantus, and Novolog present using MSIA tips derivatized with anti-resistin and anti-osteocalcin. Insulin-related proteins were not detected.

The healthy and diabetic cohorts had an average intact insulin concentration and standard deviation (SD) of 82 ± 60 pM and 243 ± 359 pM, respectively. The range for the two cohorts were 15 – 176 pM and 32 – 1116 pM, respectively. Sample concentrations in the healthy cohort were not clustered near the LOQ and only one sample was found to have a concentration of 15 pM. Basal insulin concentrations in healthy humans are typically in the range of 30–90 pM, with a peak rise to 360–540 pM during meals [24]. This is consistent with our measurements of intact insulin in healthy individuals. Outlier samples below 15 pM intact insulin concentration would have to be quantified by an alternative assay, then coupled with the qualitative assay presented here to determine the presence of molecular heterogeneity.

The known urinary metabolite of insulin, des-thr³⁰-insulin (β -chain) [25], was detected in the plasma of three diabetics and the truncated form of Lantus was detected in the plasma of one diabetic. To our knowledge, these variants have previously only been detected in urine. Sample MSIA spectra for healthy and T2D patients are available as Supporting Information Fig. 3.

The research described here was designed to operate as a precursor to future, large-population based studies. In this manner, hundreds of samples (and eventually thousands) will be screened to establish the natural variation of insulin molecules in the general population. (Using the MSIA technology, a single mass spectrometer, and a single robotic pipette station, it takes 2 hours to generate data per 96-well sample plate. Accordingly, 480 data points can be acquired in each sample run resulting from the analysis of the 5 distinct insulin isoforms mentioned in this study. Using conventional methods (e.g. ELISA), five distinct assays would be required to capture the same information content and would take significantly longer.) Accordingly, due to the choice of analysis by MALDI-TOF-MS, an *open m/z window* surrounding intact insulin in every acquired spectrum may provide the opportunity for unreported variations of the molecule to reveal themselves when larger populations are scrutinized. In this framework, new isoforms and perhaps the isoforms discussed here (e.g. metabolic products, des-thr³⁰-insulin (β -chain); proinsulin byproducts) may engender clinical utility in the form of biomarkers. Such clinically applied insulin heterogeneity would be exciting considering the preexisting scope of the molecule's history including its discovery ninety years ago [26] and well-established biology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by National Institutes of Health Grants R24DK083948 and R01DK082542 to RWN.

Abbreviations

MSIA	mass spectrometric immunoassay
T2D	type 2 diabetes

References

1. Nedelkov D, Tubbs KA, Niederkofler EE, Kiernan UA, Nelson RW. High-throughput comprehensive analysis of human plasma proteins: a step toward population proteomics. *Anal Chem*. 2004; 76:1733–1737. [PubMed: 15018576]
2. Nedelkov D. Population proteomics: addressing protein diversity in humans. *Expert review of proteomics*. 2005; 2:315–324. [PubMed: 16000079]
3. Nedelkov D, Kiernan UA, Niederkofler EE, Tubbs KA, Nelson RW. Investigating diversity in human plasma proteins. *Proc Natl Acad Sci U S A*. 2005; 102:10852–10857. [PubMed: 16043703]
4. Nedelkov D, Kiernan UA, Niederkofler EE, Tubbs KA, Nelson RW. Population proteomics: the concept, attributes, and potential for cancer biomarker research. *Mol Cell Proteomics*. 2006; 5:1811–1818. [PubMed: 16735302]
5. Nedelkov D. Population proteomics: investigation of protein diversity in human populations. *Proteomics*. 2008; 8:779–786. [PubMed: 18210367]
6. Roth MJ, Parks BA, Ferguson JT, Boyne MT 2nd, Kelleher NL. "Proteotyping": population proteomics of human leukocytes using top down mass spectrometry. *Anal Chem*. 2008; 80:2857–2866. [PubMed: 18351787]
7. Niederkofler EE, Kiernan UA, O'Rear J, Menon S, et al. Detection of Endogenous B-Type Natriuretic Peptide at Very Low Concentrations in Patients with Heart Failure. *Circulation: Heart Failure*. 2008; 1:258–264. [PubMed: 19808300]
8. Chan SJ, Seino S, Gruppuso PA, Schwartz R, Steiner DF. A mutation in the B chain coding region is associated with impaired proinsulin conversion in a family with hyperproinsulinemia. *Proc Natl Acad Sci U S A*. 1987; 84:2194–2197. [PubMed: 3470784]
9. Yano H, Kitano N, Morimoto M, Polonsky KS, et al. A novel point mutation in the human insulin gene giving rise to hyperproinsulinemia (proinsulin Kyoto). *J Clin Invest*. 1992; 89:1902–1907. [PubMed: 1601997]
10. Minn AH, Kayton M, Lorang D, Hoffmann SC, et al. Insulinomas and expression of an insulin splice variant. *Lancet*. 2004; 363:363–367. [PubMed: 15070567]
11. Sakura H, Iwamoto Y, Sakamoto Y, Kuzuya T, Hirata H. Structurally abnormal insulin in a diabetic patient. Characterization of the mutant insulin A3 (Val----Leu) isolated from the pancreas. *J Clin Invest*. 1986; 78:1666–1672. [PubMed: 3537011]
12. Anderson NL, Anderson NG, Haines LR, Hardie DB, et al. Mass spectrometric quantitation of peptides and proteins using Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA). *J Proteome Res*. 2004; 3:235–244. [PubMed: 15113099]
13. Temple RC, Carrington CA, Luzio SD, Owens DR, et al. Insulin deficiency in non-insulin-dependent diabetes. *Lancet*. 1989; 1:293–295. [PubMed: 2563455]
14. Manley SE, Stratton IM, Clark PM, Luzio SD. Comparison of 11 human insulin assays: implications for clinical investigation and research. *Clin Chem*. 2007; 53:922–932. [PubMed: 17363420]
15. Kippen AD, Cerini F, Vadas L, Stocklin R, 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; 272:12513–12522. [PubMed: 9139702]
16. Stocklin R, Vu L, Vadas L, Cerini F, 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. [PubMed: 8971080]
17. 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. [PubMed: 11216004]

18. Van Uytvanghe K, Rodriguez-Cabaleiro D, Stockl 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; 21:819–821. [PubMed: 17279601]
19. Thevis M, Thomas A, Schanzer W. Mass spectrometric determination of insulins and their degradation products in sports drug testing. *Mass Spectrom Rev.* 2008; 27:35–50. [PubMed: 18000882]
20. Niederkofler EE, Tubbs KA, Kiernan UA, Nedelkov D, Nelson RW. Novel mass spectrometric immunoassays for the rapid structural characterization of plasma apolipoproteins. *J Lipid Res.* 2003; 44:630–639. [PubMed: 12562854]
21. Rehder DS, Borges CR. Possibilities and pitfalls in quantifying the extent of cysteine sulfenic acid modification of specific proteins within complex biofluids. *BMC biochemistry.* 2010; 11:25. [PubMed: 20594348]
22. Oran PE, Jarvis JW, Borges CR, Nelson RW. C-peptide Microheterogeneity in Type 2 Diabetes Populations. *Proteomics Clin Appl.* 2010; 4:1–6.
23. Nelson RW, Krone JR, Bieber AL, Williams P. Mass spectrometric immunoassay. *Anal Chem.* 1995; 67:1153–1158. [PubMed: 15134097]
24. Karam, JH. *Basic & Clinical Pharmacology.* Katzung, BG., editor. Appleton & Lange; Stamford, Connecticut: 1998.
25. Thevis M, Schanzer W. Mass spectrometry in sports drug testing: Structure characterization and analytical assays. *Mass Spectrom Rev.* 2007; 26:79–107. [PubMed: 16888758]
26. Korec R. 50th anniversary of discovery and isolation of insulin. Paulesco 1921, Banting Best and Collip 1922. *Cas Lek Cesk.* 1971; 110:416–418. [PubMed: 4930043]

Statement of Clinical Relevance

Recent studies in population proteomics have found that most proteins exist as more than one qualitatively unique form—both within an individual and across the human population [1–6]. Qualitative variations in proteins can have a dramatic effect on their biological activity and can directly impact the validity of clinical measurements [7]. Because of its high clinical profile, we felt it would be useful to develop a high-throughput-amenable quantitative assay for insulin which was simultaneously capable of detecting and tentatively (if not immediately) identifying unanticipated variant forms of insulin present in human plasma samples. Upon employing the assay, insulin variants not known to exist in plasma were identified in diabetic patient samples.

Figure 1.

I) The insulin MSIA in the development phase. Plasma from a healthy individual was used (endogenous insulin extracted) to optimize protocols involved in removing non-specific proteins. Non-specific signals include Apolipoprotein C-I and residue found within the antibody solution. II) An optimized spectrum resulting from the use of in-sample detergents, salts, and post-extraction rinses. III) The MALDI-TOF-MS spot used to generate (II) was reduced on-target to verify specificity of the antibody via the appearance of the insulin α -chain and insulin β -chain. * corresponds to sinapic acid matrix adducts. The "1" label on the y-axis indicates 100% relative abundance for the spectrum shown.

Figure 2.

Insulin MSIA spectra from diabetic samples. m/z values refer to the monoisotopic mass $[M + H]^+$: human insulin (observed 5804.57 m/z , theoretical 5804.65); des-thr³⁰-insulin (β -chain) (observed 5703.55, theoretical 5703.60); porcine insulin (internal standard) (observed 5774.66, theoretical 5774.64); and des-arg³²⁻³¹-Lantus (β -chain) (observed 5747.50, theoretical 5747.63). The “1” label on the y-axis indicates 100% relative abundance for the spectrum shown.

Table 1

Three samples (n = 4 ea.) with known concentrations of 50 pM, 100 pM, and 400 pM were tested using the insulin MSIA on three separate days (intra-assay precision). Using these data (n = 3 days), estimated inter-assay precision values were calculated. Values correspond to the average concentration $\pm$ standard deviation and to the percent coefficient of variation.

Conc (pM)	Day 1 (n = 4)	Day 2 (n = 4)	Day 3 (n = 4)	Interday (n = 3 days)
50	57.1 $\pm$ 2.8; 5.0%	48.8 $\pm$ 2.9; 5.9%	52.0 $\pm$ 3.2; 6.1%	52.6 $\pm$ 4.1; 7.9%
100	98.5 $\pm$ 0.9; 0.9%	86.6 $\pm$ 4.4; 5.1%	96.2 $\pm$ 4.3; 4.5%	93.8 $\pm$ 6.3; 6.7%
400	388.5 $\pm$ 5.3; 1.4%	347.5 $\pm$ 30.0; 8.6%	389.0 $\pm$ 10.0; 2.6%	375.0 $\pm$ 23.8; 6.4%