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Quantitative Mass Spectrometric Immunoassay of Insulin Like Growth Factor 1

Randall W. Nelson,* Dobrin Nedelkov, Kemmons A. Tubbs, and Urban A. Kiernan

Intrinsic Bioprobes, Inc., 625 S. Smith Road, Suite 22, Tempe, Arizona 85281

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Reported in this work are the development of mass spectrometric immunoassay (MSIA) devices and methods for the qualitative analysis of IGF-1 and -2, and the rigorous quantification of IGF-1 from human plasma. A method involving addition of SDS in moderate concentration to unfractionated plasma for disrupting IGF/IGFBP complexes was initially developed. The method is suitable for the direct extraction of the IGFs and subsequent mass spectrometric analysis. Rat plasma, containing IGF-1 that is mass shifted from human IGF-1, was used as an internal reference standard (IRS) for the quantification of IGF-1 directly from human plasma. A standard curve with linear dynamic range of at least 2 orders of magnitude was constructed from serially diluted IGF-1 standards containing equal amounts of rat plasma. Using the standard curve, IGF-1 levels in plasma samples from eight individuals were determined. The limit of detection for the IGF-1 MSIA was also evaluated and established to be ~15 pM. The assay is rapid and can be performed in parallel via high-throughput robotics processing. Furthermore, the mass spectrometry aspect of the developed IGF-1 immunoassay offers a new dimension in the ongoing study of IGF-1 and related diseases.

Keywords: mass spectrometry • immunoassay • IGF • quantification

Introduction

Insulin like growth factors, which include IGF-1 and IGF-2, are circulating peptides involved in the regulation of cell proliferation, differentiation, and apoptosis. Small in size, the IGFs share significant sequence (structural) homology with pro-insulin and are known to interact with two transmembrane receptors (IGF-1R and IGF-2R) and a family of at least six circulating binding proteins (IGFBP-1 thru -6), which regulate the availability of IGFs to interact with the IGF receptors.[1] Normal IGF-1 levels in plasma range between 20 and 600 μg/L, dependent on the age and sex of the individual.[2,3] Plasma levels are known to increase from the normal age- and sex-stratified levels in response to acromegaly and decrease in growth hormone deficient children.[4] Moreover increased levels of IGF-1 have been implicated as prognostic/diagnostic markers for several cancers, most notably, prostate cancer.[5−7]

IGF-1 levels in plasma are typically measured by radio-immunoassay (RIA) or immuno-radiometric assay (IRMA).[8,9] Recently, these assays have been expanded upon to include chemiluminescence and mass spectrometry-based detection.[3,10,11] Regarding the use of mass spectrometry for the quantification of IGF-1, de Kock et al. have reported on a method in which electrospray ionization (ESI) ion-trap−MS is used to detect IGF-1 and R^3IGF-1 (human IGF-1 with Glu^3 substituted by Arg^3) after on-line liquid chromatography (LC); the R^3IGF-1 serving as a mass-resolved internal standard for the subsequent quantification of IGF-1.[11] Likewise, Bobin et al. used the same internal standard in the preliminary development of a strikingly similar LC−ESI/MS based approach.[10] Both authors made note of the presence of variably oxidized versions of the internal standard, which required judicious treatment of the mass spectrometric data in order to achieve quantitative linearity − with de Kock et al. and Bobin et al. using the non-oxidized and di-oxidized versions of R^3IGF-1 as internal standards, respectively. Additionally, at the time of the reports, both studies were focused on the demonstration of useable working curves using bottled standards and neither study was applied in the analysis of plasma-borne IGF-1, which requires the disruption of in vivo IGF/IGFBP (see below). These idiosyncrasies notwithstanding, these reports represent some of the first examples of applying proteomics approaches, mass spectrometry in particular, to the quantification of IGF-1 in various organisms.

In past works, we have demonstrated the use of a hybrid proteomics approach, mass spectrometric immunoassay (MSIA), in quantifying proteins present in biological fluids.[12−14] Briefly, the approach is that of using affinity isolation to simultaneously extract a target protein and a suitable internal standard from the fluid in preparation for MALDI-TOF mass spectrometry.[14] Given appropriate experimental design, the target protein and internal standard register as distinct signals in the mass spectrum, and their relative ion signals are used in the rigorous quantification of the target protein. The approach takes on a practical aspect by using devices specifically tailored to the task of extracting the proteins for mass spectrometric detection.

* To whom correspondence should be addressed. Tel: (480) 804−1778. Fax: (480) 804−0778. E-mail: rnelson@intrinsicbio.com.

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Accordingly, we have devoted much effort in perfecting such devices, in the form of affinity pipet tips, and demonstrated their use in the analysis of several plasma and urine-borne human proteins.[13−20] As part of ongoing studies, we report here the development of devices and methods for the qualitative analysis of IGF-1 and -2, and the rigorous quantification of IGF-1, from human plasma. Areas of investigation included: (1) Determining a method for disrupting IGF/IGFBP complexes that is suitable for the direct extraction of the IGFs (for subsequent mass spectrometric analysis), (2) Choice of an internal reference standard (IRS) that is cross-reactive with the extraction antibody and is sufficiently resolved from the human IGF-1, (3) Constructing usable working curves, (4) The application of the approach to a small sample population, and (5) Determining the limit of detection of the approach.

Experimental Section

Materials. CDI (1,1′-Carbonyldiimidazole)-activated affinity pipet tips were prepared and derivatized with rabbit anti-human IGF-1 antibody (Cell Sciences; Canton, MA), or in combination with rabbit anti-human IGF-2 polyclonal antibody (Cell Sciences; Canton, MA), as previously described.[13] For the development of the assay, human blood was obtained from a single subject recruited from within Intrinsic Bioprobes Inc. (IBI), following a procedure approved by the IBI's Institutional Review Board (IRB), only after reading and signing an Informed Consent form. In short, 50 μL human blood was drawn under sterile conditions from a lancet-punctured finger with a heparinized microcolumn (Drummond Scientific Co., Broomall, PA), mixed with 50 μL of HEPES buffered saline (HBS-EP) buffer (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 0.005% (v/v) polysorbate 20, 3 mM EDTA) and centrifuged for 30 s (at 7000 rpm, 2500 × *g*) to pellet the red blood cells. The supernatant (2× diluted plasma) was used for a single assay as described below. Additional human plasma samples that were used in the population experiments were purchased from ProMedDX (Norton, MA).

Mass Spectrometric Immunoassay. Forty μL of human plasma was mixed with 60 μL of HEPES buffered saline (HBS-EP) and 60 μL of 0.5% (w/v) sodium dodecyl sulfate, agitated with a vortexer and allowed to stand at room temperature. After 10-min, an additional 840 μL of HBS-EP was added (for a total analytical volume of 1 mL) and the IGFs were extracted by repeatedly (50 repetitions) drawing and expelling (back into the analytical volume) 200 μL aliquots of the analytical volume through an anti-IGF affinity pipet. After extraction, the pipets were rinsed using HPS-EP, H_2O, acetonitrile:water (20:80; v/v), and H_2O (in this order, each rinse = 5 repetitions of 200 μL), after which the IGFs were eluted and prepared for MALDI-TOF MS by drawing 4 μL of MALDI matrix (saturated aqueous solution of α-cyano-4-hydroxycinnamic acid (ACCA), in 33% (v/v) acetonitrile, 0.4% (v/v) trifluoroacetic acid, TFA) into the pipet and depositing onto a mass spectrometer target. MALDI-TOF MS was performed using a Bruker autoflex instrument operating in the positive ion, delayed-extraction mode with a 1.85 kV draw out pulse, 200 ns after the laser pulse delay and a full accelerating potential of 20 kV. One hundred laser-shots were signal averaged for each mass spectrum.

Generation of Working Curve. Standards for the generation of the IGF-1 working curve were prepared and assayed as described above, with the following exceptions: 1) The 40 μL aliquot of human plasma was replaced by a 40 μL aliquot of human IGF-1 standard (in HBS-EP) at a known concentration, 2) A 20 μL aliquot of rat plasma was added to the mixture (the rat IGF-1 present in the plasma served as an IRS), and 3) A 40 μL aliquot, instead of 60 μL aliquot, of HBS-EP was used to make volume during the dissociation of the complexes. Eight standard samples, with IGF-1 concentrations ranging from 7.8 μg/L to 1000 μg/L were prepared and assayed in parallel using an octa-pipet fitted with anti-IGF-1 affinity pipets. Five 100 laser-shot mass spectra were taken for each sample. Signals (peak heights) for the rat and human IGF-1 were integrated to baseline and the hIGF-1 signal normalized to the rIGF-1—these operations were performed using IBI software (IBI Proteome Analyzer; produced by Beavis Informatics Ltd., Winnipeg, Canada). The normalized values for the five spectra were averaged and plotted against the hIGF-1 concentration. The resulting response versus hIGF-1 plot was correlated using linear least-squares fit, with error bars reflecting the standard error of each point.

Application to Sample Population. Samples from eight individuals were prepared as described in the previous section, with the exception of replacing the IGF-1 standard with a 40 μL aliquot of undiluted plasma. All other steps remained the same. Five spectra (100 laser shot each) were taken from each sample and the data treated as described in the previous section.

Determination of the Limit of Detection. Samples were prepared for limit of detection studies by serially diluting the plasma of one of the individuals and treating 40 μL aliquots as described in the MSIA protocol described above. Additionally, IGF-1 stock (US Biological, Swampscott, MA) was serially diluted with HBS-EP buffer containing 1.0 g/L human serum albumin carrier, and assayed at low concentrations.

Results and Discussion

Disruption of IGF/IGFBP Complexes. Approximately 99% of plasma IGFs circulate in complex with IGFBPs and acid labile subunit (ALS),[21] creating the need to free the IGF prior to analysis. The most common methods employed in releasing IGF-1 for conventional immunological assays use acid disruption of the complex followed by treatment with ethanol or hydrophobic interaction chromatography to, respectively, precipitate or scavenge IGFBPs from solution.[22,23] Oftentimes, the removal of IGFBPs is incomplete and these procedures are augmented by the addition of exogenous IGF-2 in order to complex with residual (free) IGFBPs prior to analysis of IGF-1—essentially precluding the re-binding of IGFBP to IGF-1 by saturating binding sites with IGF-2. These procedures are not compatible to the MSIA approach for two reasons. First, the procedures require that any quantitative measurements be corrected with respect to extraction efficiency, which must be determined for each approach and can vary dependent on ambient conditions. (From a fundamental analytical point of view, if at all possible, it is preferred to perform quantitative analyses using a procedure that extracts the analyte directly from the plasma, rather than one that fortifies the plasma with analyte by indirectly extracting interferences.) And second, the procedures preclude the analysis of IGF-2 from the processed plasma sample. Thus, alternate methods for the disruption of the IGF/IGFBP complex were investigated.

Figure 1 shows mass spectra comparing two sample preparations. In the first sample, no attempts were made to disrupt the IGF/IGFBP complexes (i.e., plasma was simply diluted in HBS-EP). In the second sample, complexes were disrupted by adding SDS to the plasma sample to a concentration of ~0.02%.

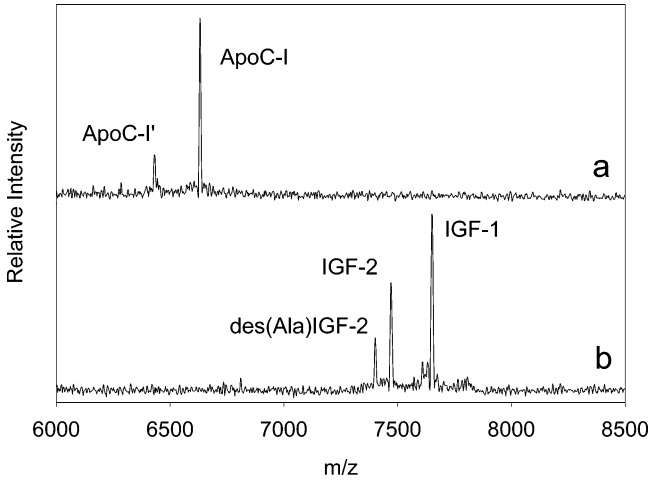

Figure 1. Results of anti-IGF-1 & -2 multi-affinity mass spectrometric immunoassay (MSIA). (a) MSIA analysis of diluted (in HBS-EP) plasma. Only signals from apolipoprotein C−I and C−I′ are observed (nonspecifically retained); signals from IGFs are absent from the spectrum. (b) MSIA analysis of plasma pretreated with SDS. Only signals from the two IGFs (and a truncated IGF-2 variant) are observed, indicating the successful liberation of IGF-1 and IGF-2 from their protein complexes.

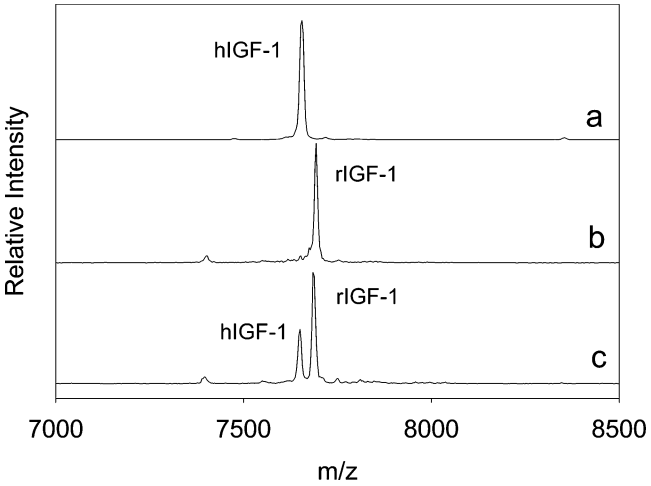

Figure 2. IGF-1 MSIA spectra of: (a) Human plasma, (b) Rat plasma, and (c) Mixture of human and rat plasma. Signals at m/z = 7648 and 7691 are observed, corresponding to the human IGF-1 (hIGF-1) and rat IGF-1 (rIGF-1), respectively.

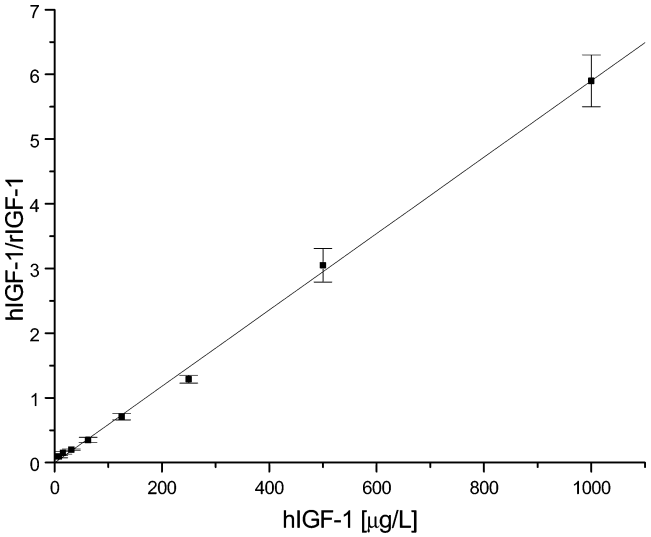

Figure 3. Quantitative MSIA working curve, constructed from serially diluted IGF-1 standards, and covering an IGF-1 plasma concentration range of 7.8 μg/L to 1000 μg/L (0.3−40 μg/L analytical sample concentration range).

Briefly, the approach was that of disrupting the IGF/IGFBP complexes using a moderate concentration of SDS, which was then diluted to a lower concentration (so not to interfere with the extraction of the IGFs) prior to MSIA. To gauge the ability to extract both (endogenous) IGF-1 and IGF-2, MSIA-Tips derivatized with both anti-IGF-1 and anti-IGF-2 polyclonal antibodies were used in the investigations. The nontreated sample shows signals at m/z = 6629.4 and 6431.1 due to nonspecifically retained apolipoproteins C−I and C−I′, and no discernible signals from either of the IGFs. The treated sample, however, shows strong signals at m/z = 7400.2, 7469.6, and 7650.3, corresponding to des(Ala)-IGF-2, IGF-2, and IGF-1 ($MW_{(calc)}$ = 7398.3, 7469.4, and 7648.7, respectively). In other studies along these lines (data not shown), it was found that the limits of SDS treatment were ∼0.002% to ∼0.2%, at which point the treatment either did not disrupt the complexes or interfered with the MSIA process, respectively. These results are in agreement with findings utilizing BIA/MS technology in the study of bound and free IGF-1 and -2.[24]

Selection of Internal Reference Standard. Internal reference standards (IRS) are required for the quantitative MALDI-TOF MS analysis of polypeptides.[25] Preferably, the internal standards share properties similar to those of the analyte, which, in the case of MSIA, is accomplished through the use of mass-shifted analogues or homologues of the target protein.[12] Importantly, the internal reference is present in all sample preparation steps, i.e., it is retrieved, rinsed, eluted and MALDI-analyzed simultaneously with the target protein. Although chemically modified versions of the target protein serve well as IRS,[12] there are occasions when the same protein from a different animal species can be used.[13,14] Considering a molecular weight of 7650 and a mass resolution of ∼900 (m/dm fwhm), the preferred internal reference should be mass shifted by at least 30 Da from the human IGF-1. Additionally, the IRS needs to cross-react with the immobilized polyclonal anti-IGF-1 antibody. Accordingly, plasmas from several animal species were evaluated—by database searching to eliminate inter-species IGF-1 isoforms that have molecular weights not easily resolved from human IGF-1, and empirical screening to determine cross-reactivity using a given antibody and experimental conditions—to determine their suitability for use as IRS. Figure 2 shows the results of performing IGF-1 MSIA on human and rat plasma and a mixture of both plasmas. All spectra show little nonspecific binding and strong signal from both the human and rat IGF-1s (labeled hIGF-1 and rIGF-1, respectively). Important to the development of the quantitative human IGF-1 MSIA, the signals for the human and rat IGF-1 are fully resolved from each other.

Standard Protocol and Generation of Working Curve. A quantitative working curve was generated by replacing the human plasma sample with a 40 μL aliquot of hIGF-1 at a known concentration. A serial dilution series (factor of 2) of eight standard samples was used in the construction of the working curve. Results of the working curve standards are shown in Figure 3. The curve, covering an IGF-1 concentration range of 7.8 μg/L to 1000 μg/L shows good linearity (linear least-

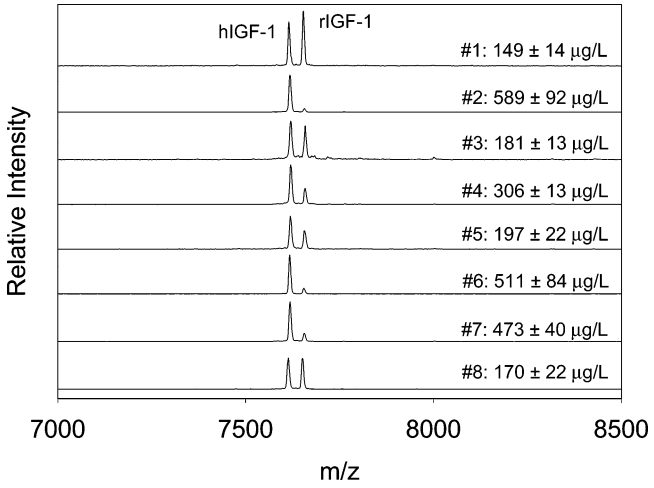

Figure 4. Quantitative MSIA analysis of eight plasma samples. The concentrations of IGF-1 in each sample were determined with the help of the standard curve shown in Figure 3.

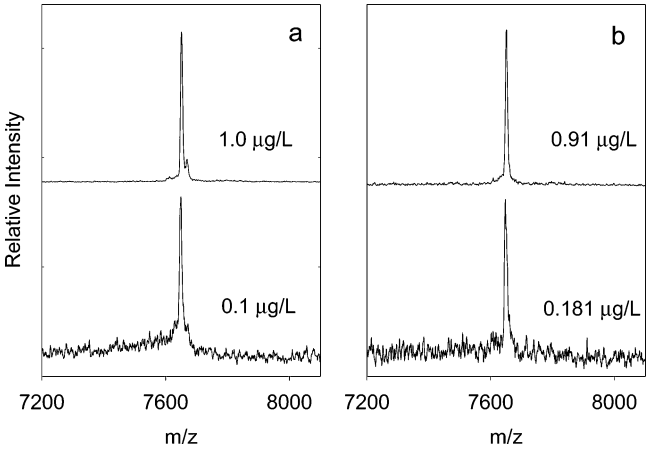

Figure 5. Exploring the limit of IGF-1 MSIA detection. (a) MSIA analyses of 1.0 and 0.1 μg/L (analytical volume concentrations) standard IGF-1 solutions, (b) MSIA-analyses of human plasma (sample #3 in Figure 4), diluted 8-fold (0.91 μg/L analytical concentration) and 40-fold (0.181 μg/L).

squares fit $R^2 = 0.998$; SEE = 0.080) and an (essentially) zero intercept (hIGF-1/rIGF-1 = 0.0059 [hIGF-1 (in μg/L)] − 0.0022) indicating nonsaturating extraction/analysis conditions and adequate resolution between hIGF-1 and rIGF-1 (and lack of molecular interferences), respectively. The inter-point error (i.e., standard error of the line equation) was determined to be ~8%, which is approximately equivalent to the average intra-point error (i.e., error of the five-replicate analyses for each point (as reflected in the error bars)) of 9.6%. These values suggest that the MSIA approach can be used to determine IGF-1 levels in plasma with a errors on the order of <10%, which is comparable with values obtained using commercial IGF-1 ELISAs.[26] Note: For convenience, the working curve is plotted as relative intensity versus IGF-1 concentration in plasma. However, the concentration of the IGF-1 exposed to the MSIA-tip is actually 1/25th of the plotted concentration, or 0.3−40 μg/L, due to the dilutions made during analysis.

Quantification of IGF-1 in Human Plasmas. Plasma samples from eight healthy individuals were analyzed to evaluate any unforeseen difficulties that may be encountered in the analysis of multiple individuals. All sample preparation, extraction, rinsing and depositions steps were performed in parallel with the aid of an octa-pipet. The total time required for analysis of the eight samples was approximately 1-hour. Figure 4 shows the results of the screening. With the help of the working curve shown in Figure 3, the plasma IGF-1 concentration was determined to range between 149 and 589 μg/L for the eight individuals, which is consistent with literature values for healthy individuals.[3] Although only a small study, these results show much promise in the application of the IGF-1 MSIA to larger study populations using, for instance, robotic workstations and automated MALDI-TOF MS.[14−16]

Investigation of the Limit of Detection. In keeping with the development of an IGF-1 MSIA, a final study was undertaken to determine the nonquantitative lower limit of detection of the MSIA approach. Dilution series ranging to a concentration low of 0.1 μg/L were prepared from (commercially available) IGF-1 standard, and plasma sample #3 (which was determined to have an IGF-1 concentration of 181 μg/L, see Figure 4). Figure 5 shows the results of the investigation. Strong signal (signal-to-noise ratio (S/N) better than 20) is observed for the standard and plasma dilution series at a concentration of ~ 1 μg/L. Signal is still observed (with a S/N of at least 5), at concentrations of 0.1 μg/L and 0.18 μg/L for the standard solution and diluted plasma, respectively. These results indicate a lower limit of detection on the order of ~15 pM for the MSIA assay, which is comparable to the limit of detection of a commercially available sandwich ELISA (R&D Systems, Minneapolis, MN).

Conclusion

It is becoming readily apparent that the field of proteomics, which for the past decade has been used mainly to identify/discover proteins in biological systems (largely with mass spectrometry), is rapidly crossing over into the arena of human clinical biology. In doing so, there emerges a pressing need for translating results derived from discoveries (whether they be past, present or future) into targeted, high information content analyses. Ideally, such analyses are able to structurally characterize and quantify a given protein, and can be applied to lower-level proteins when in the presence of other proteins. Presented here has been the development of a mass spectrometric immunoassay, for insulin-like growth factor 1, that is in keeping with these requirements. Issues key to its development, such as the design of affinity-extraction devices, the determination of methods suitable for the disruption of IGF-protein complexes, and the choice of appropriate internal reference standard, were investigated and optimized to ultimately produce a rigorously quantitative IGF-1 MSIA with a linear dynamic range of at least 2 orders of magnitude with relatively low standard error. Additional studies indicate a limit of detection of ~15 pM and the ability to apply the approach to multiple individuals in parallel; the former issue illustrating the potential of using affinity isolation in combination with mass spectrometry to characterize low-level plasma proteins; the latter issue is of particular importance when considering the use of high throughput robotic processing in e.g., population screening and clinical studies. Given these abilities, we believe the IGF-1 MSIA demonstrated in this preliminary study is able to assist in the ongoing investigation of IGF-1 related disease, which to date have not benefited from the use of mass spectrometry.

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References

(1) Zapf, J.; Froesch, E. R.; Schmid, C. Metabolic Effects of IGFs. In. *Contemporary Endocrinology17: The IGF System−Molecular Biology, Physiology and Clinical Applications*; Conn, M., Ed.; Humana Press: New Jersey, 1999.

(2) Strasburger, C. J.; Bidlingmaier, M.; Wu, Z.; Morrison, K. M. Normal values of insulin-like growth factor I and their clinical utility in adults. *Horm. Res.* **2001**, 55 Suppl. 2, 100−105.

(3) Brabant, G.; von zur Muhlen, A.; Wuster, C., et al. Serum insulin-like growth factor I reference values for an automated chemiluminescence immunoassay system: results from a multicenter study. *Horm. Res.* **2003**, *60*, 53−60.

(4) Monzavi, R.; Cohen, P. IGFs and IGFBPs: role in health and disease. *Best Pract. Res. Clin. Endocrinol. Metab.* **2002**, *16*, 433−447.

(5) Wolk, A.; Mantzoros, C. S.; Andersson, S. O., et al. Insulin-like growth factor 1 and prostate cancer risk: a population- based, case-control study. *J. Natl. Cancer Inst.* **1998**, *90*, 911−915.

(6) Chan, J. M.; Stampfer, M. J.; Giovannucci, E., et al. Plasma insulin-like growth factor-I and prostate cancer risk: a prospective study. *Science* **1998**, *279*, 563−566.

(7) Hankinson, S. E.; Willett, W. C.; Colditz, G. A., et al. Circulating concentrations of insulin-like growth factor-I and risk of breast cancer. *Lancet.* **1998**, *351*, 1393−1396.

(8) Chestnut, R. E.; Quarmby, V. Evaluation of total IGF-I assay methods using samples from Type I and Type II diabetic patients. *J. Immunol. Methods* **2002**, *259*, 11−24.

(9) Clemmons, D. R. Commercial assays available for insulin-like growth factor I and their use in diagnosing growth hormone deficiency. *Horm. Res.* **2001**, *55*, 73−79.

(10) Bobin, S.; Popot, M. A.; Bonnaire, Y.; Tabet, J. C. Approach to the determination of insulin-like-growth-factor-I (IGF-I) concentration in plasma by high-performance liquid chromatography-ion trap mass spectrometry: use of a deconvolution algorithm for the quantification of multiprotonated molecules in electrospray ionization. *Analyst* **2001**, *126*, 1996−2001.

(11) de Kock, S. S.; Rodgers, J. P.; Swanepoel, B. C. Growth hormone abuse in the horse: preliminary assessment of a mass spectrometric procedure for IGF-1 identification and quantitation. *Rapid Commun Mass Spectrom* **2001**, *15*, 1191−1197.

(12) Nelson, R. W.; Krone, J. R.; Bieber, A. L.; Williams, P. Mass-Spectrometric Immunoassay. *Anal. Chem.* **1995**, *67*, 1153−1158.

(13) Tubbs, K. A.; Nedelkov, D.; Nelson, R. W. Detection and quantification of beta-2-microglobulin using mass spectrometric immunoassay. *Anal. Biochem.* **2001**, *289*, 26−35.

(14) Niederkofler, E. E.; Tubbs, K. A.; Gruber, K. et al. Determination of beta-2 microglobulin levels in plasma using a high-throughput mass spectrometric immunoassay system. *Anal. Chem.* **2001**, *73*, 3294−3299.

(15) Kiernan, U. A.; Tubbs, K. A.; Gruber, K., et al. High-Throughput Protein Characterization Using Mass Spectrometric Immunoassay. *Anal. Biochem.* **2002**, *301*, 49−56.

(16) Kiernan, U. A.; Nedelkov, D.; Tubbs, K. A.; Niederkofler, E. E.; Nelson, R. W. High-throughput analysis of human plasma proteins. *Am. Biotech. Lab* **2002**, *20*, 26−28.

(17) Kiernan, U. A.; Tubbs, K. A.; Nedelkov, D.; Niederkofler, E. E.; Nelson, R. W. Comparative phenotypic analyses of human plasma and urinary retinol binding protein using mass spectrometric immunoassay. *Biochem. Biophys. Res. Commun.* **2002**, *297*, 401−405.

(18) Niederkofler, E. E.; Tubbs, K. A.; Kiernan, U. A.; Nedelkov, D.; Nelson, R. W. Novel mass spectrometric immunoassays for the rapid structural characterization of plasma apolipoproteins. *J. Lipid Res.* **2003**, *44*, 630−639.

(19) Kiernan, U. A.; Tubbs, K. A.; Nedelkov, D.; Niederkofler, E. E.; Nelson, R. W. Detection of novel truncated forms of human serum amyloid A protein in human plasma. *FEBS Lett.* **2003**, *537*, 166−170.

(20) Kiernan, U. A.; Tubbs, K. A.; Nedelkov, D.; Niederkofler, E. E.; McConnell, E.; Nelson, R. W. Comparative urine protein phenotyping using mass spectrometric immunoassay. *J. Proteome Res.* **2003**, *2*, 191−197.

(21) Baxter, R. C. Insulin-like growth factor binding proteins in the human circulation: a review. *Horm. Res.* **1994**, *42*, 140−144.

(22) Blum, W. F.; Breier, B. H. 1994 Radioimmunoassays for IGFs and IGFBPs. Growth Regul. 4 Suppl 1: 11−119.

(23) Bang, P.; Baxter, R. C.; Blum, W. F., et al. Valid measurements of total IGF concentrations in biological fluids. Recommendations from the 3rd International Symposium on Insulin-like Growth Factors. *J. Endocrinol.* **1994**, *143*, C1−2.

(24) Nedelkov, D.; Nelson, R. W.; Kiernan, U. A.; Niederkofler, E. E.; Tubbs, K. A. Detection of bound and free IGF-1 and IGF-2 in human plasma via biomolecular interaction analysis mass spectrometry. *FEBS Lett.* **2003**, *536*, 130−134.

(25) Nelson, R. W.; McLean, M. A.; Hutchens, T. W. Quantitative-Determination of Proteins By Matrix-Assisted Laser-Desorption Ionization Time-of-Flight Mass-Spectrometry. *Anal. Chem.* **1994**, *66*, 1408−1415.

(26) Renehan, A. G.; Jones, J.; O'Dwyer, S. T.; Shalet, S. M. Determination of IGF−I, IGF−II, IGFBP-2, and IGFBP-3 levels in serum and plasma: comparisons using the Bland-Altman method. *Growth Horm. IGF Res.* **2003**, *13*, 341−346.

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