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Automated high-throughput sample preparation of insulin-like growth factor 1 for targeted LC-MS/MS quantification

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PURPOSE

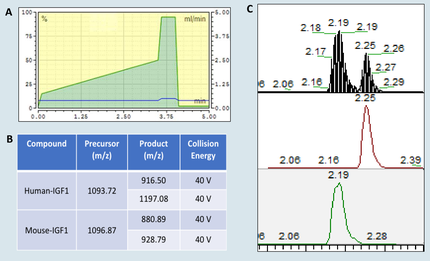
Insulin-like growth factor 1 (IGF-1) is a hormone of 70 amino acids that plays key roles in childhood growth and anabolic effects in adults. IGF1 is routinely measured in clinical laboratories to monitor for abnormal levels of the hormone which may indicate a developmental disorder or dysfunctional pituitary gland. The role of IGF1 associated with cancer, aging, neuropathy, and stroke also leads to significant interest for academic and industry research. Historically, IGF1 has been measured by immunological assays; however, recent studies have raised concerns over inter-assay variability of commercially available immunoassays. These studies have demonstrated the use of affinity enrichment of IGF1 coupled with various mass spectrometry detectors. Here, we show an alternative sample preparative method to the affinity-based enrichment method by using traditional solid phase resin chemistries on automated liquid handling system without any offline sample process (i.e. centrifugation) coupled with low resolution LC-MS/MS for monitoring IGF-1 isoforms, variants, and analogs.

OBJECTIVES

- Achieve quantitative recovery of IGF-1 from plasma samples using INtip chemistries.
- Establish a cost-effective enrichment workflow on an automated liquid handling system.
- Eliminate centrifugation for a hands-free sample preparation process

METHODS

Pooled human plasma was purchased from Fisher Scientific. EDTA and heparin treated plasma samples were used for the initial method development. Five plasma samples from healthy males and five plasma samples from diabetic males were purchased from BioIVT. Liquichek™ tumor quality control from Bio-rad was used for precision and accuracy measurements. The automated method designed by IMCS for the Hamilton STAR Microlab Workstation uses 1 mL cation exchange (CX) and 300 µL reverse phase (RP) IMCStips. All solvents were MS grade and purchased from Fisher Scientific. We optimized selected reaction monitoring using Vanquish UHPLC and TSQ Endura triple quadrupole mass spectrometer (Thermo Scientific) to measure intact IGF-1.



Compound	Precursor (m/z)	Product (m/z)	Collision Energy
Human-IGF1	1093.72	916.50	40 V
		1197.08	40 V
Mouse-IGF1	1096.87	880.89	40 V
		928.79	40 V

Figure 1. *Optimization of LC-MS quantification of intact IGF-1, 5 minutes of LC-gradient for separation of mouse and endogenous human IGF-1* ***(A)****, SRM transition of 100 picograms of mouse and human IGF-1 on TSQ Endura mass spectrometer* ***(B)****, and extracted ion chromatograms* ***(C)***

RESULTS

Dispersive pipette extraction is a novel solid phase extraction process that leverages turbulent mixing inside a tip (**Figure 2**). The loose resin contained within the tip is dispersed as the liquid sample is aspirated (**Figure 2**). The extraction process occurs as the liquid sample is pipetted allowing faster and more efficient extraction, especially when plate movements are eliminated and no intermediate sample handlings are required.

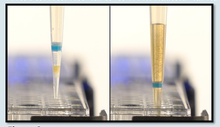
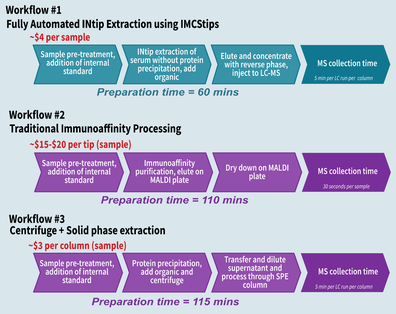


Figure 2

Here, we report INtip solid phase extraction method which is faster than previously reported methods with lower costs than the reported approaches. The entire enrichment process used conventional ion exchange and reverse phase resins, and with automation in place, the process takes less than 1 hour per 96 samples with minimal hands-on time (**Workflow 1**).

Previous workflows for IGF-1 have been published for MALDI-TOF MS or LC-MS/MS detection, where IGF-1 enrichment was performed using immunoaffinity resins or by centrifugation. The immunoaffinity enrichment study demonstrated that two 96-head robotic systems were used to process over 1,000 samples in less than 9 hours (**Workflow 2**).



However, according to the study, the sample pre-treatment and incubation time required longer preparation time and extended the batch preparation time to about two hours. Further, the antibodies specific for IGF-1 raises sample preparation costs to levels which may not be conducive for clinical operations. A traditional approach using serum protein crash with high organic solvent would be equivalent in terms of reagent costs as the INtip method, but the workflow times are double (**Workflow 3**).

There are two concerns over centrifugation. First, manual intervention is required if an automated plate gripper is not used. Manual intervention is non-continuous, where a person must physically move the samples from the operations to the centrifuge, then return the samples back to the liquid handler. This interrupted workflow can delay the process or can result in mishandling the samples (i.e. dropping plate, unbalanced plates/rotor, wrong orientation of plate back after centrifugation). However, if the plate gripper is utilized, it is possible to improperly grip and drop the plate, or not pick up the plate. Off-deck centrifuges also require more space and synchronizing the centrifuge to the robotic liquid handler's core software is an added layer of possible errors.. The INtip process negates the need for scientist intervention, ensuring positive sample identification, and thus avoiding mishandling of patient samples.

We utilized strong-cation exchange and reverse-phase INtip chemistries to enrich the polypeptide hormone from human plasma samples for robust analysis by LC-MS/MS. The sample prep workflow enables the analysis of over 5,000 samples with a single LC column.

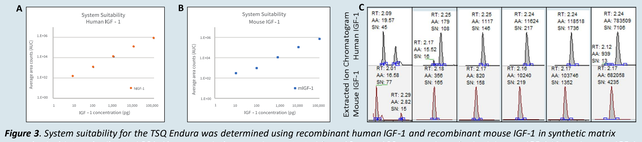


Figure 3*. System suitability for the TSQ Endura was determined using recombinant human IGF-1 and recombinant mouse IGF-1 in synthetic matrix comprised with trypsin digested BSA. Linear correlations are demonstrated from 10 pg to 100 ng on column for both human IGF-1 (**A**) and mouse IGF-1 (**B**), which is equivalent to 0.2 – 2,000 ng/mL in serum. While the average IGF-1 levels can vary significantly based on age, physiologically relevant range is 50 ng/mL to 800 ng/mL. The system is at least 2 orders of magnitude more sensitive on the lower limit and at least 1 order of magnitude sensitive for the upper limit of detection. The extracted ion chromatograms for human and mouse IGF-1 in surrogate matrix (**C**) shows LOD at 10 pg and ULOD at 100 ng.*

The extraction efficiency was assessed by spiking in varying amounts of recombinant mouse IGF-1 to human plasma samples.

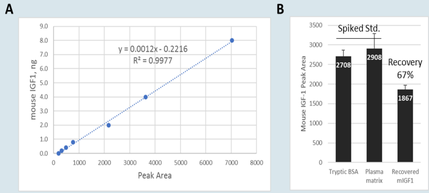


Figure 4*. Extraction efficiencies demonstrated using recombinant mouse IGF-1 spiked in human plasma samples (**A**), and comparing surrogate matrix to the extracted human plasma samples (**B**). The mouse IGF-1 spiked in human plasma was extracted using the ion exchange and reverse phase desalting. The recovery from human plasma for mouse IGF-1 was calculated at 67%.*

Linear measurement was determined for the mouse IGF-1 in human plasma from 0.2 ng/mL to 200 ng/mL (**Figure 4A**). Then, we optimized the extraction processes using the IMCStips to achieve highly efficient and reproducible enrichment (**Figure 4B**). Surrogate matrix sample using trypsin digested BSA with human IGF-1 and extracted human plasma sample as matrix matched demonstrated equivalent signals, indicating no matrix effects from the extracted sample in comparison to the surrogate matrix (**Figure 4B**). For extraction efficiency, mouse IGF-1 was spiked in human plasma samples and both human and mouse IGF-1 were enriched (**Figure 4B**). Reproducibility was demonstrated using a single human plasma sample fortified with mouse IGF-1 as the internal standard (**Figure 5**). A high and low concentration of mouse IGF-1 was assessed to determine if the addition of the recombinant mouse IGF-1 would impact quantification of the endogenous human IGF-1, and based on the results, no significant difference was observed (**Figure 5A**).

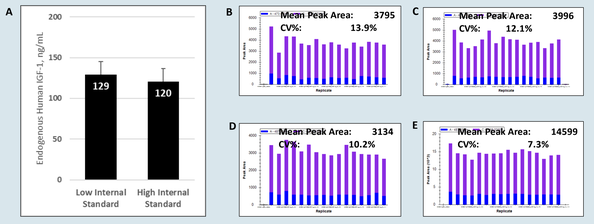


Figure 5*. Quantitative analysis of a single human plasma sample using either a high (20 ng) or low (5 ng) amount of mouse IGF-1 as a reference standard. The average calculated IGF-1 showed no significant difference between the two different amounts of mouse IGF-1 as the internal standard (**A**). The variability in the raw area counts for the endogenous human IGF-1 area counts (**B, C**) were not significantly different across the two different amounts of mouse IGF-1 (**D, E**).*

The accuracy of the INtip method was determined using 3 Liquichek™ tumor quality controls (**Figure 6A**). The relative amount of endogenous human IGF-1 levels were calculated using internal mouse IGF-1 standard. Each run represents an average of four biochemical replicates (**Figure 6B**). A commercial ELISA kit (R&D Systems) was used to also cross check against the INtip method using the three Liquichek QC vials (**Figure 6C**).

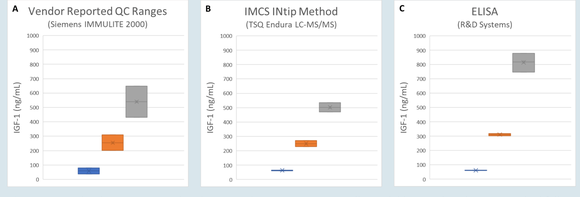
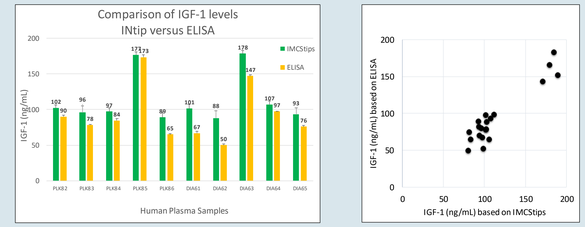


Figure 6*. IGF-1 levels were measured and compared against a commercially available ELISA kit. The INtip method falls well within the ranges reported by the vendor, and the mean values are less than 10% variance (**A, B**). The ELISA kit has increased values for QC2 and QC3 (**C**).*

Further comparison between the INtip method and ELISA kit with ten human plasma samples, five healthy male and five diabetic males, showed the opposite trend where the calculated IGF-1 levels were higher for INtip method than the ELISA results (**Figure 7**).



Additional studies will be conducted to determine the precision and accuracy of the INtip method, but its preliminary results using the commercial QC kits suggest that the method may be more accurate than ELISA. The reproducibility based on single human plasma sample with 16 replicate runs (8 each using two different mouse IGF-1 concentrations as internal standard) was less than 15% CV, which was within the system performance variability.

CONCLUSION

The results demonstrate a robust automated plasma sample preparation for high-throughput IGF-1 enrichment for targeted LC-MS/MS. This sample preparation will be useful to improve efficiency in clinical settings to quantify IGF-1 isoforms, variants, and analogs. When compared to traditional methods, this new workflow eliminates the centrifugation step and creates a fully automated hands-free sample preparation process. The INtip process ensures positive sample identification, and thus avoids mishandling of patient samples.

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