

# Bioanalytical Determination of Human Insulin and Insulin Detemir in Human Plasma by Immunoenrichment and LC-MS/MS

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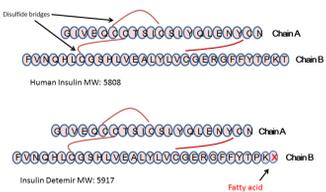


## Introduction

Insulin is a hormone produced by the pancreas then released in the blood stream where it helps to regulate levels of sugar. The American Diabetes Association recommends that bioanalytical assays, used to measure insulins, should be able to achieve an LLOQ of 0.070 ng/mL. Traditionally ELISA is used for the determination of insulin and insulin isoforms in human serum and plasma based on the inherent sensitivity of this technique. However, in some cases, antibody-based methods may suffer for poor specificity and selectivity or cross reactivity when several isoforms or metabolites are present in the biological matrix. LC-MS/MS approaches are expected to overcome such limitations and enable the simultaneous determination of insulin isoforms in the biological matrices.

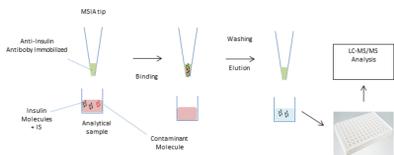
The aim of this feasibility study was the development of an LC-MS/MS approach for the determination of human insulin and insulin detemir in human plasma. Insulin detemir is a long-acting human insulin analogue used to maintain the basal level of insulin activity in the body. This small protein carries a fatty acid moiety (myristic acid) which is covalently bound to the lysine 29 of the protein beta chain (Figure 1).

**Figure 1. Human Insulin and Insulin Detemir. Insulin detemir carries fatty acid (myristic acid) that is covalently bound to the lysine 29 of the protein beta chain.**



The higher complexity of proteins and peptides are often the basis for poor sensitivity and selectivity of the mass spectrometric methods used for their analysis. Recently, the introduction of orthogonal approaches based on immunoenrichment prior to the mass spectrometric determination, alleviates some of these problems. In such methodologies antibodies immobilized on a solid support first capture the molecule of interest from the matrix, then subsequent washing cycles are used to remove contaminants and finally the analyte is eluted prior to its analysis by LC-MS/MS. Samples were immunoenriched prior to LC-MS/MS using MSIA™ technology (Thermo Scientific) (Figure 2).

**Figure 2. Schematic Representation of MSIA Technology Workflow.**



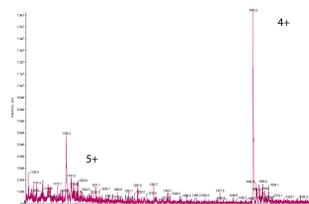
Bovine insulin was used as an internal standard. Standards were prepared in insulin immunodepleted serum while QC samples were prepared directly in human plasma.

## Method

Analyte	Human insulin and insulin detemir
Internal Standard	Bovine insulin
Matrix	Human insulin depleted serum for the calibration curve human plasma for quality control samples
Sample Volume	500 µL/250 µL
Calibration Range	0.0500 - 10.0 ng/mL
Extraction Method	Thermo Scientific MSIA
LC Method	UPLC reverse phase
Detection	SCIEX Triple Quad 6500
Run Time	7 min
LC System	Waters ACQUITY UPLC® I-Class System (SM-FTN)
Analytical Column	BEH C18, 2.1mm x 50mm, 1.7 µm particles
Mobile Phase A	Water/Acetic acid (100/0.2)
Mobile Phase B	Acetonitrile/Acetic acid (100/0.2)
Human Insulin	(6+) 969.2 → 1130.0
Insulin Detemir	(5+) 1184.3 → 1366.7
Bovine Insulin	(6+) 956.3 → 1114.8

Solutions of human insulin and insulin detemir were analysed in infusion experiments at 300 µL/min in 30/70/0.2 acetonitrile/water/formic acid. The MRM transitions selected were optimized for CE, DP, CXP and EP. Figure 3 shows a representative MS1 spectrum for insulin detemir.

**Figure 3. MS1 Spectrum of Insulin Detemir.**



### Impact of Modifier on Analyte Signal Intensities

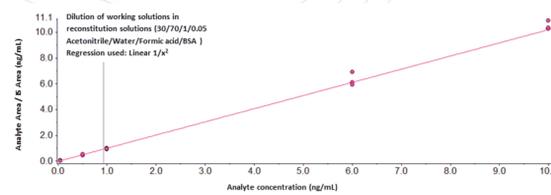
To determine the effects of the modifier on the signal intensities, human insulin and insulin detemir were infused in reconstitution solvent (30/70 acetonitrile/water) modified either with 0.2% formic acid or 0.2% acetic acid at chromatographic compatible flow rate.

Whilst no clear difference in charge state distribution was evident, an overall sensitivity difference was observed. For both human insulin and insulin detemir, acetic acid favoured ionization of the analytes and increased signal intensities of 140-170%.

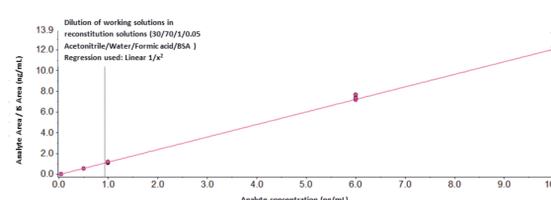
### Linearity of the Calibration Curves in Pure Solution

Working solutions were prepared and diluted to a concentration similar to that reached in the samples after elution of the analytes from the MSIA tips. Solutions were analyzed by LC-MS/MS using a linear gradient of water, acetonitrile modified with 0.2% acetic acid.

**Figure 4. Human Insulin Calibration Curve in Pure Solutions.**



**Figure 5. Insulin Detemir Calibration Curve in Pure Solutions.**

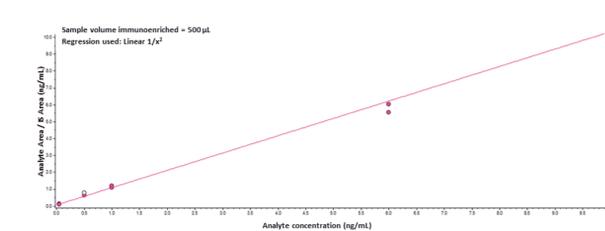


All the back-calculated standard concentrations for both human insulin and insulin detemir in pure solutions were within 95-105% accuracy. Good linearity of the calibration curves was observed.

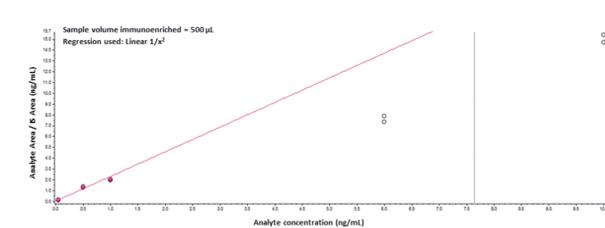
### Linearity of the Calibration Curves in Depleted Serum

To determine the linearity of the calibration curve in a biological matrix, working solutions spiked in human insulin depleted serum (Figures 7 and 8). The amount of the endogenous human insulin present in such matrix is negligible; therefore, it represents an ideal surrogate for human insulin calibration curve determination. 500 µL of sample was immunoenriched via MSIA technology, eluted and analyzed by LC-MS/MS analysis.

**Figure 6. Human Insulin Calibration Curve in Depleted Human Serum.**



**Figure 7. Insulin Detemir Calibration Curve in Depleted Human Serum.**



All the back-calculated standard concentrations for human insulin in depleted plasma were within 95-105% accuracy. Good linearity of the calibration curves was observed.

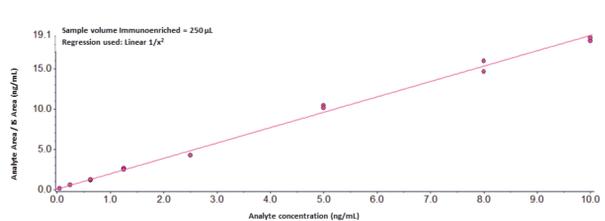
The insulin detemir standards at 6.00 and 10.0 ng/mL deviated from the linear fit. Two hypotheses that would explain the poor accuracy observed at high analyte concentrations were investigated:

- Insulin detemir adsorption/solubility phenomena at high concentration of analyte in depleted serum
- Saturation of antibody binding capacity for insulin detemir immunoenrichment

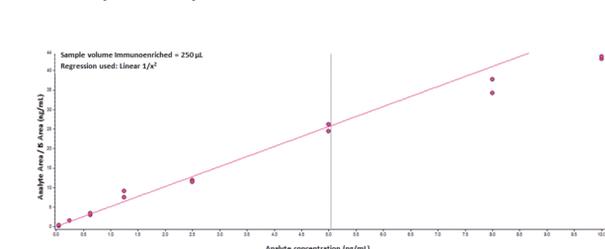
### Investigation of Non Linearity

To control the effect of possible adsorption/solubility phenomena, serial dilutions to generate samples for the calibration curve were made. To reduce possible saturation of the antibody binding capacity the sample volume processed with MSIA technology was reduced from 500 to 250 µL.

**Figure 8. Human Insulin Calibration Curve. Serial dilution of the stock solution was made to generate standards in human insulin depleted serum. 250 µL of sample was used for immunoenrichment.**



**Figure 9. Insulin Detemir Calibration Curve. Serial dilution of the stock solution was made to generate standards in human insulin depleted serum. 250 µL of sample was used for immunoenrichment.**



**Table 1. Precision and Accuracy of Human Insulin and Insulin Detemir Calibration Curve.**

Analyte	Concentration (ng/mL)	% CV	Accuracy	Analyte	Concentration (ng/mL)	% CV	Accuracy
Human Insulin	0.0500	1.23	98.2	Insulin Detemir	0.0500	4.89	96.3
	0.250	1.80	112		0.250	2.22	115
	0.625	2.46	93.8		0.625	9.02	98.7
	1.25	4.18	104		1.25	13.6	128
	2.50	0.518	88.2		2.50	2.14	90.9
	5.00	1.82	107		5.00	4.90	98.8
	8.00	6.04	99.7	8.00	6.90	87.8	
	10.0	1.18	97.3	10.0	1.08	84.4	

### Human Insulin

- All the calibration standards were within 85-115% accuracy and were measured with a precision better than 7%
- Good linearity of the calibration curves was observed

### Insulin Detemir

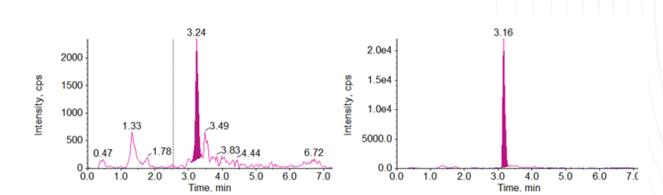
- Deviation from a linear fit of the calibration curve was observed. Although linearity was overall slightly improved

The poor linearity for the insulin detemir calibration curve cannot be attributed only to possible adsorption or antibody saturation phenomena. Sufficient sensitivity could be achieved using 250 µL samples; therefore, this value was applied to the method.

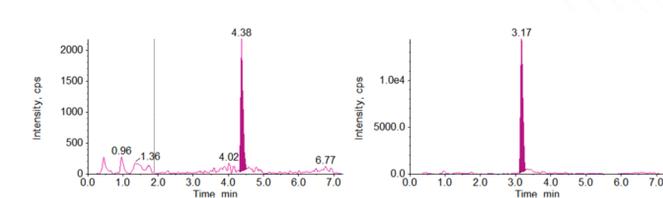
## Method Evaluation

250 µL of human depleted serum at concentration of 50 pg/mL was immunoenriched using MSIA technology and analyzed by LC-MS/MS.

**Figure 10. Standard Sample of Human Insulin at 0.0500 ng/mL was Immunoenriched via MSIA Technology and Analyzed by LC-MS/MS.**



**Figure 11. Standard Sample of Insulin Detemir Spiked at 0.0500 ng/mL was Immunoenriched via MSIA Technology and Analyzed by LC-MS/MS.**



Good sensitivity was achieved for both human insulin and insulin detemir using 250 µL samples.

Quality control samples were prepared in human plasma. The endogenous concentration of the human insulin in a control sample was determined by a validated ELISA method developed in house (DAKO). The concentration was determined to be 0.527 ng/mL. QC control samples spiked at 0.050 and 0.150 ng/mL.

**Table 2. Precision and Accuracy of Human Insulin and Insulin Detemir Quality Control Samples.**

Analyte	QC Concentration (ng/mL)	% CV	Accuracy	Analyte	QC Concentration (ng/mL)	% CV	Accuracy
Human Insulin	0.500	0.587	106	Insulin Detemir	0.0500	8.38	71.9
	0.600	2.86	96.2		0.150	0.332	104

### Human Insulin

- The endogenous concentration of human insulin measured in control plasma was 0.450 ng/mL. Good correlation between the ELISA and LC-MS/MS estimation was measured (15.8% from the mean concentration value)
- Good accuracy and precision was measured for the QC samples

### Insulin Detemir

- Insulin detemir spiked in human plasma at 0.0500 and 0.150 ng/mL. Poor accuracy was measured for Insulin Detemir at the LLOQ

## Conclusions

In this feasibility study a protocol for the immunoenrichment approach prior to mass spectrometric was applied for the determination of the human insulin and insulin detemir. A good linearity over the quantitation range was demonstrated for the human insulin. The data showed that accuracy at high concentration of insulin detemir decreased considerably. The experiments performed excluded analyte adsorption/solubility or saturation of antibody binding capacity as the main sources of the problem. One possible explanation of the phenomenon could be related to the physicochemical characteristics of insulin detemir different from human insulin. This insulin analogue carries a fatty acid chain attached on the lysine 29 of the protein beta chain. This could influence the binding to the antibody used for its immunopurification when insulin detemir is present at high concentration in the sample. Experiments are continuing to further optimize this method.