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 LOQ of 0.070 ng/mL. Traditionally ELISA is the determination of insulin and insulin isoforms in human plasma and based on the inherent sensitivity of this technique. However, in some cases, antibody-based methods may suffer by poor specificity and selectivity or cross-reactivity when several isomers 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 MISA® technology (Thermo Scientific) (Figure 2).

Figure 2. Schematic Representation of MISA Technology Workflow.

Method

Insulin Detemir (5+) 1184.3
Mobile Phase B  Acetonitrile/Acetic acid (100/0.2)
Mobile Phase A  Water/Acetic acid (100/0.2)
Analytical Column  BEH C18, 2.1mm x 50mm, 1.7 μm particles
Run Time 7 min
Extraction Method Thermo Scientific MSIA
Calibration Range 0.0500 - 10.0 ng/mL
Sample Volume 500 μL/250 μL

Analyte Human insulin and insulin detemir
Internal Standard Bovine insulin
Matrix Human insulin depleted serum while QC samples were prepared directly in human plasma.
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 SCIEC 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 (s) 9691.2 ± 113.0
Insulin Detemir (s) 1914.3 ± 1366.7
Bovine Insulin (s) 986.3 ± 1174.8

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, CID 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 at 0.50/70, 0.50/20 and 0.62/50 acetonitrile/water/formic acid at chromatographic compatible flow rate.

The influence of modifier concentration on signal intensity was assessed. Unmodified solutions were used as controls. The solutions were analysed by LC-MS/MS using a linear gradient of water, acetonitrile modified with 0.2% acetic acid.

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/saturation or inhibition 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.

Table 1. Precision and Accuracy of Human Insulin and Insulin Detemir Calibration Curves.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>LOQ</th>
<th>CV %</th>
<th>Accuracy</th>
<th>Analyte</th>
<th>LOQ</th>
<th>CV %</th>
<th>Accuracy</th>
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</thead>
<tbody>
<tr>
<td>Human Insulin</td>
<td>0.050</td>
<td>6.57</td>
<td>98.3</td>
<td>Insulin Detemir</td>
<td>0.050</td>
<td>7.99</td>
<td>98.3</td>
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<tr>
<td>Human Insulin</td>
<td>1.000</td>
<td>8.16</td>
<td>97.4</td>
<td>Insulin Detemir</td>
<td>1.000</td>
<td>7.86</td>
<td>97.7</td>
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</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Analyte</th>
<th>LOQ</th>
<th>CV %</th>
<th>Accuracy</th>
<th>Analyte</th>
<th>LOQ</th>
<th>CV %</th>
<th>Accuracy</th>
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<tr>
<td>Human Insulin</td>
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<td>5.78</td>
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<td>Insulin Detemir</td>
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<tr>
<td>Human Insulin</td>
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<td>5.87</td>
<td>97.4</td>
<td>Insulin Detemir</td>
<td>0.150</td>
<td>5.93</td>
<td>97.5</td>
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