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Implementation of insulin analysis by ASDTL

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Introduction

Insulin is a peptide hormone consisting of two peptide chains, chain-A and chain-B, that are cross-linked by two disulfide bridges. Five synthetic insulins were studied, Apidra (Aventis), Humalog (Eli Lilly & Company), Lantus (Aventis), Levemir and Novorapid (Novo Nordisk Pharmaceuticals Pty. Ltd.), for the analysis in serum and urine samples by liquid chromatography tandem mass spectrometry (LC/MS/MS) with electrospray ionisation. Analysis of standards for each of the insulins gave reference spectra which were compared to the amino acid sequences as identified by the manufactures of each pharmaceutical. The synthetic insulins can be differentiated from endogenous human insulin because their amino acid sequences have been modified (http://au.expasy.org/sprot/). The simplest modification is the swap of positions for the proline and lysine from human insulin to Humalog (B-chain residue 28 and 29). Humalog is therefore the hardest to differentiate by LC/MS/MS as the observed precursor molecular ions are the same. However the product ions afford distinction. The m/z 217 is observed for Humalog fragment y_2 (ProThr, B29B30) while for human insulin the m/z 226 is observed representing $y_3 - y_1$ (ProLys, B28B29).

Apidra, a rapid acting human insulin analogue, has asparagine at position B3 replaced by lysine and lysine at position B29 has been replaced by glutamic acid. Three modifications have been made for the insulin analogue Lantus: glycine replaces asparagine at A21 and two arginine amino acids are added to the COOH-terminal of the B chain. Threonine has been omitted from Levemir and a C14 fatty acid chain has been attached to the B29 amino acid, resulting in a long-acting analogue. Novorapid has the amino acid proline at B28 replaced with aspartic acid, which introduces an additional negative charge within the insulin molecule causing the rapid action of the product. Apidra, Lantus, Levemir and Novorapid are easily distinguished by LC/MS/MS analysis as each has distinct precursor ions because of the variation in molecular weight as well as distinct product ions.

Materials and Methods

The methodology for the extraction of insulin from serum or urine samples involved thawing an aliquot of 2 mL serum or 25 mL of urine, adding bovine carrier 20 µL (1 pmol/µL) to each sample, as well as 200 µL acetic acid (HOAc) to urine samples only (Thevis *et al.*; 2005, 2006). QC samples were spiked with a mixture of insulin analogues 20 µL (0.01 pmol/µL with bovine carrier 0.5 pmol/µL) and bovine carrier 10 µL (1 pmol/µL), and samples vortexed for 5 seconds. Each 2 mL serum sample was divided into 2x1 mL fractions into 1.5 mL low-bind tubes and centrifuged for 5 minutes at 14000 rpm, prior to being loaded onto immunoaffinity chromatography (IAC) columns. Urine samples are extracted by solid phase extraction (SPE) on C18 cartridges (Oasis HLB 60 mg, Waters Corporation) before IAC using the same method as after IAC extraction. The samples were transferred onto pre-conditioned IAC columns. The IAC columns were conditioned with 5 mL 2% HOAc, 2x3 mL PBS. 2 mL PBS was added to each column and vortexed for 5 seconds followed by slowly rotating the column for 30 minutes at room temperature. The IAC columns were washed with 3x1 mL PBS and eluted with 2x1 mL 2% HOAc onto a SPE cartridge (conditioned with 2 mL acetonitrile (ACN), 2 mL 2% HOAc). The cartridge was washed with 2x1 mL 2% HOAc and the insulin eluted with 1.2 mL ACN:2% HOAc mix (5:5, v/v) into a 1.5 mL low-bind tube. The sample was evaporated to dryness and resuspended in 40 µL porcine insulin (0.01 pmol/µL, volumetric internal standard) in 0.2% Formic acid in H_2O:ACN (95:5). Samples were centrifuged 12000 rpm for 5 minutes and 20 µL taken for analysis.

The samples were analysed on a 4000 Q TRAP (Applied Biosystems) with a gradient separation (Agilent 1100 LC, CapPump Binary) using a Xbridge Shield 3.5 µm RP18 150×1 mm column (Waters Corperation). Solvent A consisted of 95% H_2O 5% ACN and solvent B consisted of 90% ACN 10% H_2O both A and B contained 0.2% formic acid. The gradient began with 85% A for 2 minutes, then reduced linearly to 32.5% A by 14 minutes, solvent A increased back to 85% at 15 minutes and equilibrated the column at 85% A till 20 minutes.

Results and Discussion

The initial setup of the LC/MS/MS analysis encountered problems with available plastic ware being found unsuitable as the protein of interest bound to the surface irreversibly. A comparison study between several plastic containers and different concentration of the carrier protein was completed. In the method described by Thevis *et al.* (2005) bovine insulin

was used as a carrier. The carrier protein is added to preferentially bind to plastic ware or other surfaces thus reducing the loss of the compounds of interest. In the results for the testing of the plastic ware it was also found that a higher concentration of carrier was required to consistently reduce any losses of compound of interest. Lantus showed significant drops in peak area after 20 hours when only 0.1 pmol/µL of bovine insulin was added whereas the samples run with 1 pmol/uL were stable. The remainder of the analyses used low-bind microfuge tubes from Astral and low volume plastic inserts from Agilent Technologies.

The urine extraction described by Thevis *et al.* (2006) used 500 pmol per sample of the bovine carrier. The initial evaluation of the same protocol in our laboratory yielded very low recovery of the insulin analogues. Testing of our IAC columns concluded that the columns prepared in our laboratory as described by Thevis *et al.* (2005, 2006) had a break through capacity of about 100 pmol and thus the carrier was overloading the column. The extraction for urine samples was changed to use the same amount of carrier as for the serum samples.

A further problem was encountered with the filtration of sample extract. Once the samples have been extracted and dried they are made up in a solvent ready for injection into the LC/MS/MS instrumentation. Usually biological samples are filtered through an Ultrafree-MC centrifugal filter unit (Millipore) in our laboratory before instrumental analysis. The filters are specially designed for samples containing proteins with a low-binding Durapore polyvinylidene fluoride membrane. In the case of insulin it was found that approximately 30% of the compounds of interest were being retained on the filter membrane. The samples are now centrifuged at 12000 rpm for 5 minutes and a 20 µL aliquot is taken from the total final volume of 40 µL.

The use of techniques such as LC/MS/MS to detect synthetic insulins requires that they be extracted and concentrated prior to LC/MS/MS analysis. This requires at least one evaporation of a solution with high water content. It was found for insulin-containing solutions that a vacuum concentrator is superior to nitrogen evaporation as it is much faster and gives better recoveries (Goebel 2008).

The samples would require up to 6 hours drying time using the Turbovap nitrogen evaporator (Zymark Corporation) which resulted in the preparation taking two days before samples would be ready for analysis by LC/MS/MS. In comparison the miVac DUO vacuum concentrator (GeneVac Ltd.) dried samples in 2 hours using the alcohol method setting with heating set to 40ºC. The samples could then be extracted and ready for analysis within a

single day. Furthermore, the recoveries and recovery reproducibility of all five insulin analogues, Apidra, Humalog, Lantus, Levemire and Novorapid, were significantly improved (Table 1).

Table 1 Recovery and standard deviation for six replicate extractions by IAC of serum and urine samples (Goebel, 2008).

	Serum Turbovap		Serum miVac DUO		Urine miVac DUO	
	Average Recovery	Standard Deviation	Average Recovery	Standard Deviation	Average Recovery	Standard Deviation
Apidra	61%	8%	96%	8%	63%	7%
Humalog	60%	6%	85%	9%	41%	5%
Lantus	71%	13%	92%	5%	42%	10%
Levemir	20%	12%	87%	14%	-	-
Novorapid	109%	35%	98%	9%	57%	9%

The implementation of published protocols for the analysis of proteins and peptides is not always as simple as expected. Many factors influence the detection of low concentration levels of proteins/peptides and careful evaluation of each step needs to be done to maximise recoveries and limit unintended losses. The training of staff in the optimal protocols is crucial as well as the continued monitoring of all materials/consumables used. It is also important to re-evaluate many of these factors when analyses are developed for new proteins/peptides and not assume established protocols will work in the same way for other compounds.

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