Development of an SPE-LC/MS/MS Clinical Research Method for Quantification of Four Synthetic Insulins in Human Plasma: Challenges of working with Large Peptides

Waters
THE SCIENCE OF WHAT'S POSSIBLE.™

Erin E. Chambers and Kenneth J. Fountain
Waters Corporation 34 Maple Street Milford MA, 01757 USA

INTRODUCTION

Insulin is perhaps one of the best known and earliest peptide therapeutics. For quantification of biologics, LC-MS/MS has the advantage of short development times, high accuracy and precision, the ability to multiplex, no cross-reactivity, and can readily distinguish between closely related insulins. Intact insulins are particularly difficult to analyze by LC-MS/MS, as MS sensitivity is low due to poor transfer into the gas phase and poor fragmentation due to the presence of multiple stabilizing disulfide bonds. In addition, insulin and its analogs suffer from non-specific binding and poor solubility, making LC and sample preparation method development difficult. A few LC-MS/MS methods exist, however most involve time-consuming and laborious immunoaffinity purification and/or multidimensional or nano-flow LC. This work provides a single, simple method for the simultaneous quantification of multiple intact insulin analogs (Figure 1) in human plasma, achieving LODs of 0.2 to 0.5 ng/mL.

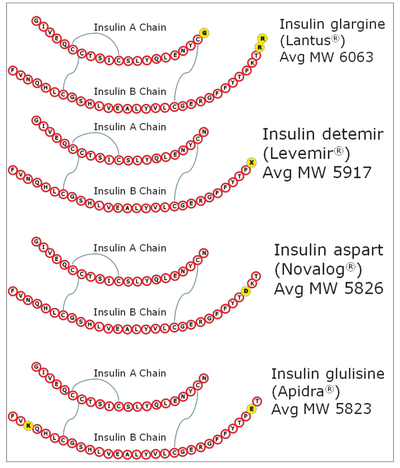

Figure 1: Representative structures and molecular weights of the insulin analogs used in this study

METHODS

ACQUITY UPLC IClass Conditions

Column: ACQUITY UPLC® CSH C_{18}, 2.1 x 50 mm, 1.7 µm
Mobile Phase A: 0.1% HCOOH in H_2O
Mobile Phase B: 0.1% HCOOH Acetonitrile
Flow Rate: 0.25 mL/min
Gradient:

Time (min)	Profile %A	%B	Curve
0.0	80	20	6
2.0	35	65	6
2.1	2	98	6
2.6	2	98	6
2.7	80	20	6

Injection Volume: 15.0 µL
Column Temperature: 60 °C
Sample Temperature: 15 °C

Waters Xevo™ TQ-S Conditions, ESI+

Capillary Voltage: 3.0 kV
Desolvation Temp: 550 °C
Cone Gas Flow: 150 L/Hr
Desolvation Gas Flow: 1000 L/Hr
Collision Cell Pressure: 2.6 x $10^{(-3)}$ mbar
MRM transition monitored, ESI+: See Table 1

Specific Insulin	MRM Transition	Cone Voltage (V)	Collision Energy (eV)
Glargine	867->984	60	18
	1011->1179	60	25
Detemir	1184-> 454.4	60	20
	1184-> 1366.3	60	20
Aspart	971.8 -> 660.8	60	18
	971.8 -> 1139.4	12	18
Glulisine	1165 -> 346.2	14	22
	1165 -> 1370	14	22
Bovine (IS)	1147.5 -> 315	50	35

Table 1. MRM transitions, collision energies, and cone voltages for insulin analogs and bovine insulin, the internal standard (IS)

Sample Preparation Protocol

Oasis® HLB µElution 96-well plate

Condition: 200 µL methanol
Equilibrate: 200 µL water
Load sample: 300 µL human plasma diluted with 300 µL 10mM TRIS Base
Wash: 200 µL 5% methanol, 1% acetic acid in water
Elute: 2 X 25 µL 60/30/10 methanol/water/acetic acid
Dilute: 50 µL water
Inject 15 µL

RESULTS/DISCUSSION

Several multiply charged precursors were observed for each of the analogs; spectra are shown in Figure 2. MSMS spectra obtained at the optimal collision energy are shown in Figure 3. Although immonium ion fragments are readily produced and are present at high intensity, they do not provide adequate specificity for the assay (Figure 4.) Use of higher m/z fragments yields significantly improved specificity, facilitating the use of simpler LC and SPE methodologies than what has previously been reported.

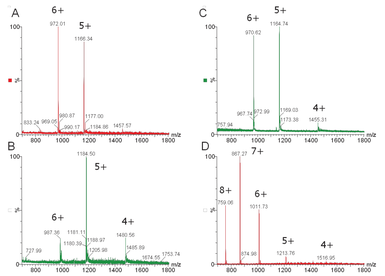

Figure 2. MS spectra of insulin analog precursors; A= aspart, B= detemir, C= glulisine, and D= glargine

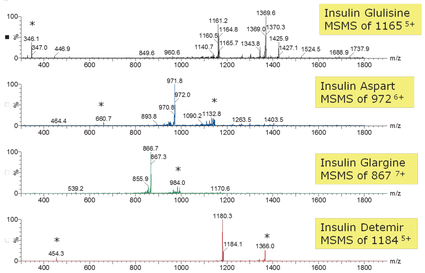

Figure 3. MSMS spectra of insulin analogs

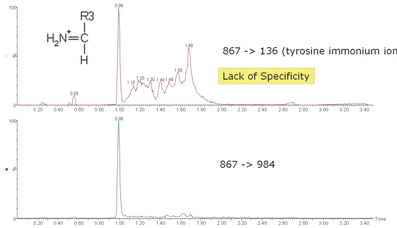

Figure 4. LC-MS/MS analysis of insulin glargine extracted from human plasma, analyzed monitoring an immonium ion fragment (top) and a sequence ion fragment at m/z 984 (bottom)

Significantly narrower peak widths were obtained for the analogs using a novel charged surface Hybrid (CSH) column than a traditional C18 column. The resultant separation is shown in Figure 5. Peak widths at base are <3 seconds wide for all analytes.

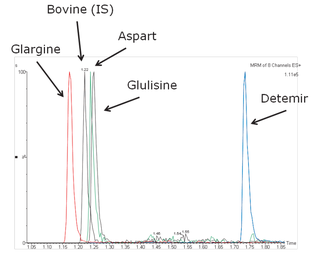

Figure 5. UPLC separation of insulin analogs and internal standard using a 2.1 X 50mm ACQUITY UPLC CSH column

Sample pretreatment prior to SPE proved to be critical in improving specificity. A typical plasma pretreatment is often dilution with acid. In this case, dilution with TFA resulted in a major peak at 5.66 minutes in the final SPE eluates (Figure 6B, top.) MS spectra under the peak were summed and yielded a distinct protein envelope from about m/z 600 to m/z 1000 (Figure 6B, bottom.) Deconvolution of the protein envelope produced an intact mass of 66,400. This provided putative identification as human serum albumin. When plasma samples were pretreated with TRIS base, this peak was absent as shown in Figure 6A.

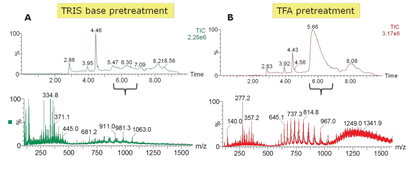

Figure 6. MS scan data from final SPE eluates from samples that were initially pretreated with either TRIS base (A) or TFA (B.) Summed spectra from 5.5 to 6.25 minutes from each eluate is shown in the bottom panels.

The combination of proper MS fragment choice, selective SPE clean-up and optimal LC column enabled us to achieve quantification and detection limits in the 0.2 to 0.5 ng/mL range for all 4 insulin analogs. Figure 7 contains representative spectra for 0.2 and 0.5 ng/mL glulisine extracted from human plasma as compared to blank extracted plasma. Figures 8 and 9 are representative extracted standard curves, from 0.2 or 0.5 to 25 ng/mL for glulisine and detemir, respectively, in human plasma. Finally, the standard curve and QC statistics for the same analogs are summarized in Tables 2 and 3.

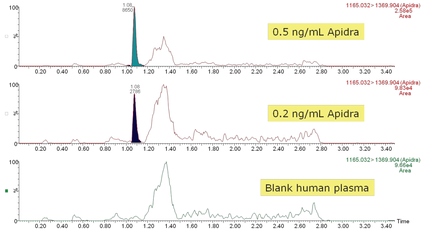

Figure 7. Representative chromatograms from insulin glulisine extracted from human plasma at 0.2 and 0.5 ng/mL, compared to extracted blank plasma.

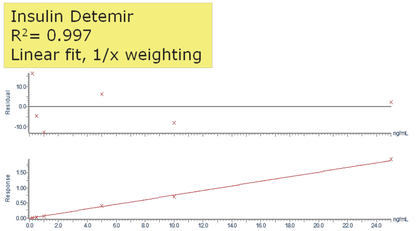

Figure 8. Representative standard curve from insulin detemir in human plasma, from 0.5 to 25 ng/mL.

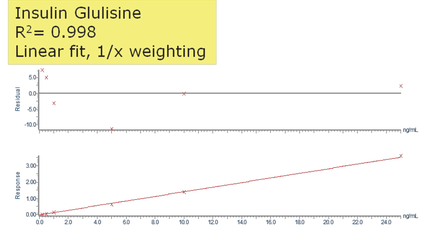

Figure 9. Representative standard curve from insulin glulisine in human plasma, from 0.2 to 25 ng/mL.

Name	Conc ng/mL	Area	Response	IS Area	%Dev	Calc Conc ng/mL
Blank plasma		31.5				
200 pg/mL plasma	0.2	153.3	0.012	13239.5	16.6	0.23
500 pg/mL plasma	0.5	375.6	0.03	12437.3	-4.6	0.48
1 ng/mL plasma	1	750.2	0.06	12419.1	-12.7	0.87
5 ng/mL plasma	5	4714.5	0.399	11801.8	6.3	5.31
10 ng/mL plasma	10	8984.8	0.696	12907.3	-8	9.20
25 ng/mL plasma	25	25007.1	1.948	12836.3	2.4	25.60
QC 350 pg/mL plasma	0.35	241.6	0.02	12200.7	-2.6	0.34
QC 750 pg/mL plasma	0.75	733.6	0.058	12560.6	12.9	0.85
QC 2 ng/mL plasma	2	1969.4	0.141	13954.4	-3.5	1.93
QC 8 ng/mL plasma	8	7486.7	0.615	12168.7	1.8	8.14
QC 20 ng/mL plasma	20	20549.6	1.616	12712.9	6.3	21.26

Table 2. Representative standard curve and QC statistics from insulin detemir extracted from human plasma.

Name	Conc ng/mL	Area	Response	IS Area	%Dev	Calc Conc ng/mL
Blank plasma		36.9				
200 pg/mL plasma	0.2	230.3	0.013	17486.8	7.4	0.22
500 pg/mL plasma	0.5	1017.4	0.057	17807.0	5	0.53
1 ng/mL plasma	1	2068.0	0.12	17249.8	-3.2	0.97
5 ng/mL plasma	5	11330.0	0.61	18563.8	-11.4	4.43
10 ng/mL plasma	10	23803.2	1.396	17051.6	-0.2	9.98
25 ng/mL plasma	25	57342.0	3.606	15903.8	2.3	25.58
QC 350 pg/mL plasma	0.35	626.1	0.035	17654.8	6.3	0.37
QC 750 pg/mL plasma	0.75	1700.4	0.101	16856.5	11.2	0.83
QC 2 ng/mL plasma	2	4744.9	0.31	15317.0	15.5	2.31
QC 8 ng/mL plasma	8	18609.8	1.013	18366.4	-9	7.28
QC 20 ng/mL plasma	20	47779.3	2.828	16894.6	0.5	20.09

Table 3. Representative standard curve and QC statistics from insulin glulisine extracted from human plasma.

CONCLUSIONS

- One extraction method was developed for 4 insulin analogs from human plasma. µElution format SPE plates eliminate the need for evaporation, reducing losses due to adsorption.
- A single fast, simple, analytical scale LC method was developed for separation of 4 synthetic insulin analogs. Total LC cycle time was 3.5 minutes.
- Detection or quantification limits of 0.2 to 0.5 ng/mL were achieved for all 4 insulin analogs extracted from 250-300 µL human plasma.
- This work demonstrates the importance of: column chemistry, sample pretreatment, addressing NSB, concentration without evaporation, and proper fragment choice.
- The method shows promise for application to doping studies, forensic cases involving insulin over dose, and perhaps future use in diabetes treatment and monitoring.

For Clinical Research Use Only, Not for Use in Diagnostic Procedures

TO DOWNLOAD A COPY OF THIS POSTER, VISIT WWW.WATERS.COM/POSTERS