

Simultaneous Analysis of Intact Human Insulin and Five Analogs in Human Plasma Using μ Elution SPE and a CORTECS UPLC Column

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Abstract

This work provides a single, simple method for the simultaneous, direct quantification of intact human insulin and multiple insulin analogs in human plasma.

Benefits

- CORTECS UPLC Column for highest sensitivity and narrowest peaks widths
- Selective, fast extraction without timeconsuming affinity purification
- Quantitative accuracy; more accurate and precise than traditional LBA methods
- High sensitivity
- Specificity, including differentiation between human insulin and insulin lispro
- Simultaneous quantification of human insulin, glargine, lispro, aspart, glulisine, and detemir
- Quantification without digestion
- Excellent asset utilization; allows the use of current LC-MS instrumentation

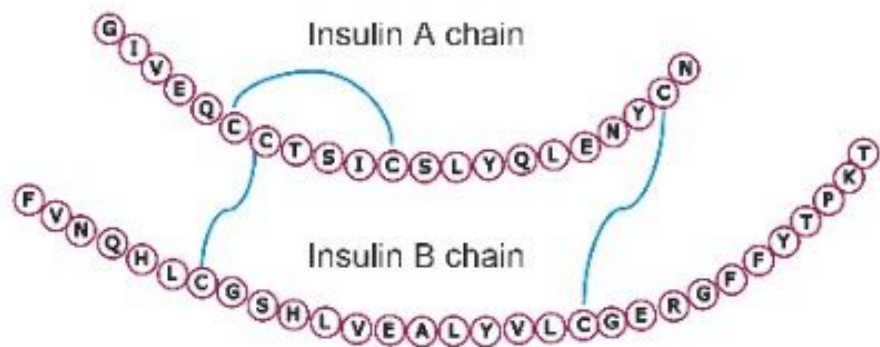
Introduction

Insulin is perhaps one of the best known and earliest peptide therapeutics. Multiple long and fast-acting analogs have also been developed, and a patient may often be prescribed one of each for diabetes control. Quantification of biologics, such as insulins, has historically been performed using ligand binding assays (LBAs) such as ELISAs. LC-MS/MS, however, has certain advantages over LBAs, such as shorter development times, higher accuracy and precision, the ability to multiplex, no cross-reactivity, and the ability to 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 patterns exist 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 do exist; however, most of those methods involve time-consuming and laborious immunoaffinity purification and/or nano-flow LC. Distinguishing between human insulin and insulin lispro (Humalog) is a very specific challenge for quantifying insulins, as they differ by a simple reversal in the

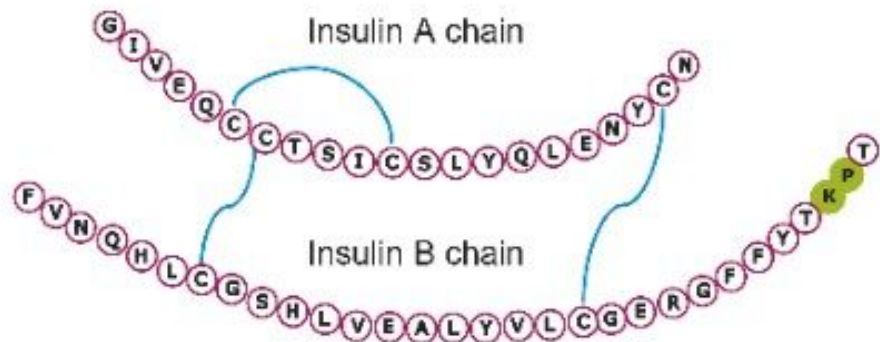
position of two amino acids. Only a single low-molecular weight fragment differentiates the two, making selective sample preparation and chromatography critical.

This study takes advantage of mixed-mode solid-phase extraction (SPE) and a high-efficiency, solid-core particle column that contains a low-level positive surface charge to eliminate interferences while facilitating high sensitivity quantification. Furthermore, selectivity studies show that the presence of high levels of human insulin, such as one might encounter in type II diabetic patients, does not interfere with quantification of lispro or any of the other analogs. This work provides a single, simple method for the simultaneous, direct quantification of intact human insulin and multiple insulin analogs in human plasma (Figure 1), achieving LODs of 50 to 200 pg/mL for each. Average accuracy for standard curve points was 99% to 100%. Average inter- and intra-day accuracies for QC samples were 98% and 94%, respectively. Average inter- and intra-day precisions for QC samples were 7.5% and 5.3%, respectively. Matrix factors for all analogs were calculated in six sources of human plasma and CVs of matrix factors were <15% in all cases, further supporting the selectivity of the method.

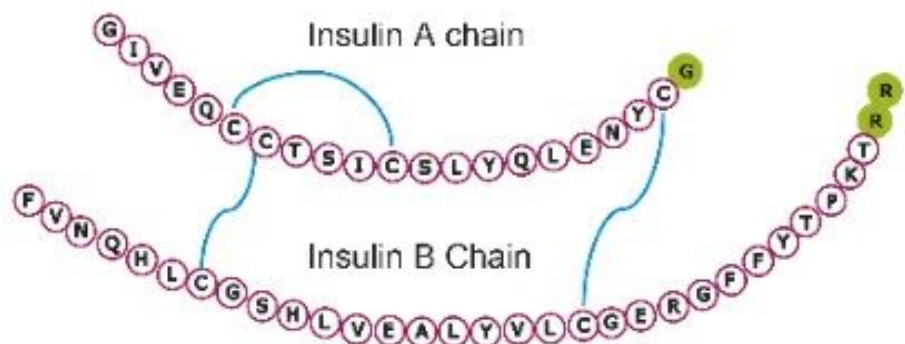
Human insulin



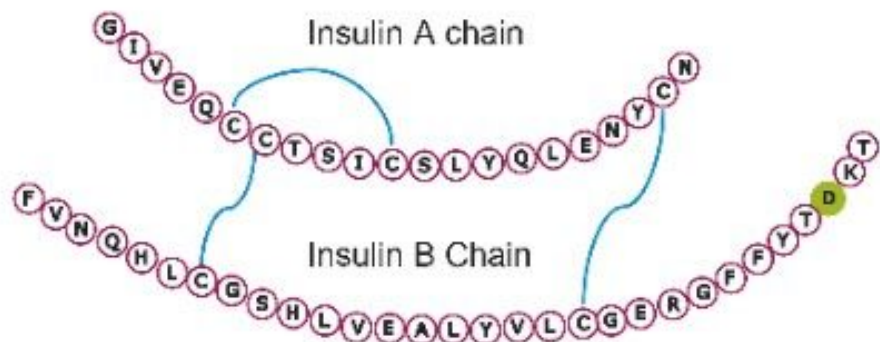
Humalog (insulin lispro)



Lantus (insulin glargine)



Novalog (insulin aspart)



Levemir (insulin detemir)

25 µL of bovine insulin (internal standard, final concentration 10 ng/mL) was added to 250 µL human plasma and mixed.

Samples were precipitated with 250 µL 1:1 methanol/acetonitrile containing 1% acetic acid, and centrifuged for 10 minutes at 13,000 rcf.

The supernatant was transferred to a 2-mL 96-well plate containing 900 µL of 5% concentrated NH₄OH in water (v:v) and mixed.

Step 2: SPE using an Oasis MAX µElution 96-well Plate

Condition:	200 µL methanol
Equilibrate:	200 µL water
Load sample:	Entire diluted PPT supernatant was loaded onto the extraction plate in two steps of approximately 700 µL each
Wash:	200 µL 5% NH ₄ OH in water
Wash:	200 µL 5% methanol + 1% acetic acid
Elute:	2 × 25 µL 60:30:10 methanol/water/ acetic acid
Dilute:	50 µL water
Inject:	30 µL

UPLC conditions

System:	ACQUITY UPLC I-Class with 2D Technology, configured for at-column dilution with trap and back elution
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Analytical column:	CORTECS UPLC C ₁₈ + 1.6 µm, 2.1 × 50 mm (p/n 186007114)*
Trap column:	XBridge C ₁₈ IS 3.5 µm, 2.1 × 20 mm (p/n 186003019)
Elution mobile phase A:	0.1% HCOOH in water
Elution mobile phase B:	0.1% HCOOH in acetonitrile
Loading solvent:	85:15 mobile-phase A:B; 0.1 mL/min for first two minutes, reverse valve, then clean trap column with ramp from 40% to 90% B
Dilution solvent:	100% mobile-phase A, 0.3 mL/min for first two minutes
Valve position:	Initial position one (forward loading of trap), switch to position two at two minutes (back elute of trap onto analytical column), back to position one at 6.5 minutes
Gradient:	Load for two minutes; switch valve and back elute from trap column onto analytical column with a linear gradient from 15% to 40% B over four minutes
Elution flow rate:	0.25 mL/min
Column temp.:	60 °C
Sample temp.:	15 °C
Injection volume:	30 µL

Run time: 8.5 minutes

Collection plates: Waters 1-mL ACQUITY collection plates

*Column conditioning is required, refer to "Column conditioning" section

MS conditions

Mass spectrometer: Xevo TQ-S

Ionization mode: ESI positive

Capillary voltage: 3.0 kV

Desolvation temp.: 600 °C

Cone gas flow: 150 L/h

Desolvation gas flow: 1000 L/h

Collision cell pressure: 3.8×10^{-3} mbar

Collision energy: Optimized by component, see Table 1

Cone voltage: Optimized by component, see Table 1

Specific insulin	MRM transition	Cone voltage (V)	Collision energy (eV)	Ion type
Glargine	1011→1179	60	25	
Lispro	1162→217	50	40	
Detemir	1184→454.4	60	20	y2
Aspart	971.8→660.8	60	18	y11
Glulisine	1165→1370	14	22	
Bovine (IS)	956.6→1121.2	60	18	
Human insulin	1162→226	50	40	

Table 1. MS conditions for human insulin, insulin analogs, and the internal standard bovine insulin.

Data management

Chromatography software: MassLynx

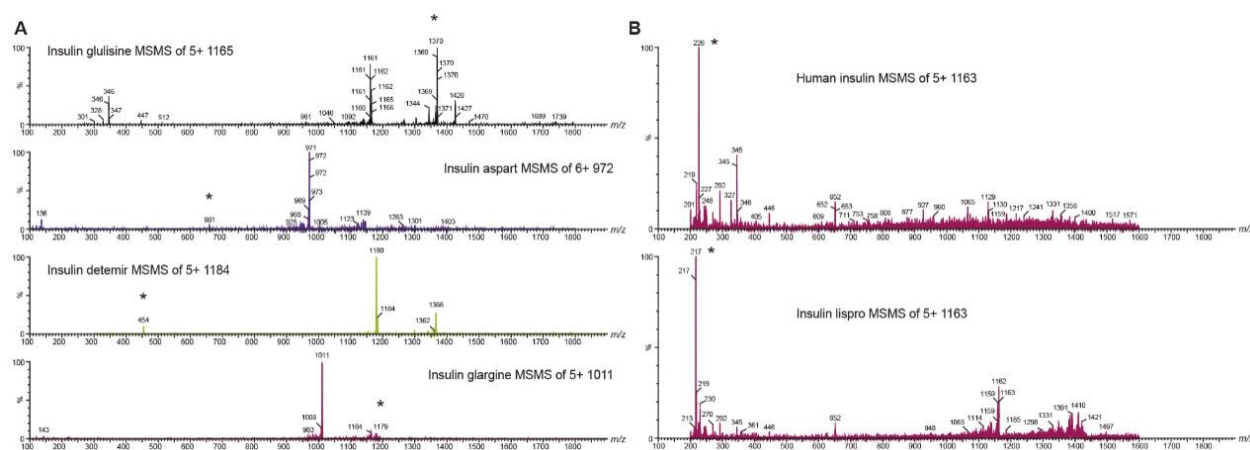
Quantification software: TargetLynx

Results and Discussion

Mass Spectrometry

Several multiple charged precursors were observed for each of the analogs, typically the two most abundant were selected for CID, and either one or two diagnostic fragments monitored during method development. Representative MS/MS spectra at the optimal collision energies for the primary transitions chosen for quantification are shown in Figures 2A and 2B. In several cases, higher intensity MRM transitions existed

than the one that was ultimately chosen. Specifically, most insulins will produce very intense immonium ion fragments. Glargine, for example, produces a tyrosine immonium ion fragment at m/z 136. However, tests in extracted plasma demonstrated that the use of higher m/z precursor and fragment pairs (Table 1) yielded significantly improved specificity in matrix, outweighing any apparent sensitivity difference (Figure 3). This facilitates the use of analytical scale LC and traditional SPE methodologies versus nano-scale chromatography and affinity-based purification schemes previously reported. Human insulin and lispro, however, share almost complete overlap in their fragmentation patterns due to a simple reversal in the positions of amino acids 28 and 29 in the B chain (Figure 1). A single low-molecular weight fragment differentiates the two, with human insulin yielding a fragment at m/z 226 and lispro producing a fragment at m/z 217 (Figure 2B), both arising from the last two amino acids in the B chain. Without baseline chromatographic separation, the MS must rely on these non-specific fragments for quantification, making both selective sample preparation and high efficiency chromatography crucial.



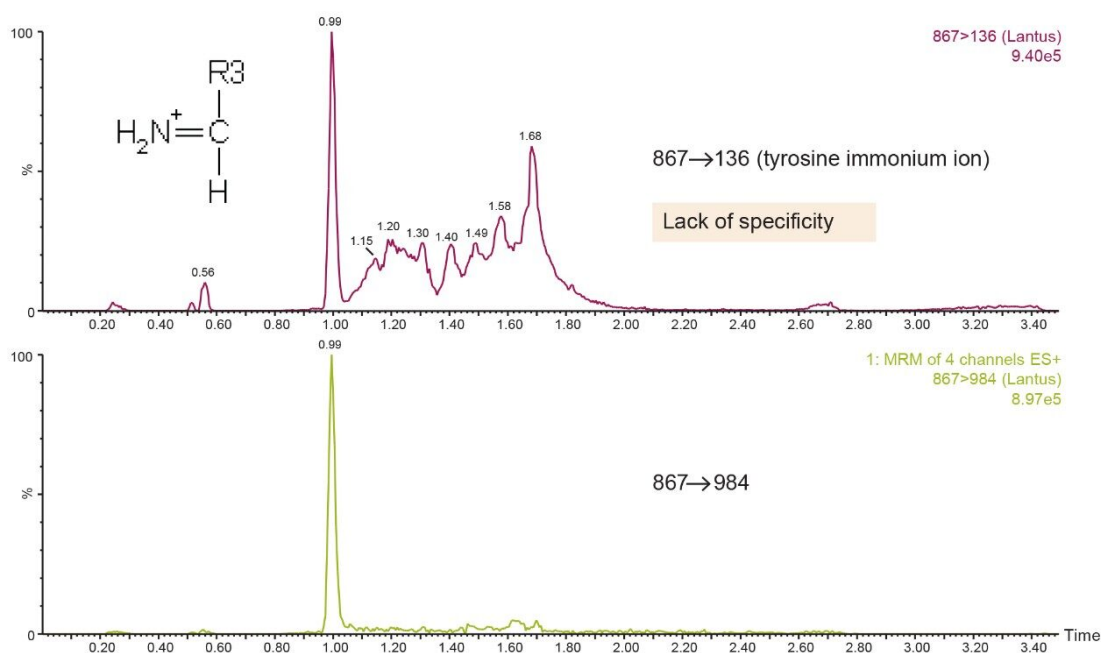


Figure 3. Extracted ion chromatograms from two different transitions for insulin glargine. Use of the immonium ion fragment (top panel) shows a lack of specificity relative to the use of a higher m/z precursor and fragment pair (bottom panel).

Column conditioning

This section details the methodology for column conditioning and optimization which uses a simple 1D gradient method. For best insulin peak widths and area counts, the CORTECS C₁₈+ Column must be conditioned prior to use. Injections of the supernatant from precipitated rat plasma have been shown to effectively condition the CORTECS C₁₈+ Column for the analysis of insulin and its analogues under reverse-phase conditions.

Injections are made using a 2 minutes linear gradient from 20%B to 65%B with a ramp to 98%B in 0.1 minutes and a hold time of 0.5 minutes. Re-equilibrate back to 20%B in 0.2 minutes and hold for 1.8 minutes before the next injection. Mobile-phase A is 0.1% formic acid in water. Mobile-phase B is 0.1% formic acid in acetonitrile. The flow rate is maintained at 0.25 mL/min using a column temperature of 60 °C. The conditioning injections are best made without the mass spectrometer in-line. The effectiveness of the conditioning should be confirmed as shown in Figure 4. The mass spectrometer settings and conditions are the same as those stated on page 3.

1D Linear gradient used for conditioning and obtaining chromatograms in Table 2.

Time (min)	Flow rate (mL/min)	A	B
Initial	0.250	80	20
2	0.250	35	65
2.1	0.250	2	98
2.6	0.250	2	98
2.7	0.250	80	20
4.6	0.250	80	20

Table 2. 1D linear gradient used for conditioning and obtaining chromatograms.

The following describes the supernatant preparation method:

Combine equal volumes of rat plasma and a solution containing 1:1 acetonitrile/methanol with 1% acetic acid. Mix to precipitate, and centrifuge for 10 minutes at 13,000 rcf. Transfer supernatant to a fresh tube. Vortex/mix the tube containing the supernatant, and centrifuge a second time to further clarify the supernatant. Transfer this supernatant to a sample vial and use to condition the column.

Perform multiple injections totaling 250 μ L of the prepared rat plasma supernatant to fully condition the column. Figure 4A shows typical UPLC-MS/MS of total ion chromatograms (TIC) for human insulin and five analogs both before and after conditioning a CORTECS C₁₈+ Column under current 1D methods and conditions.

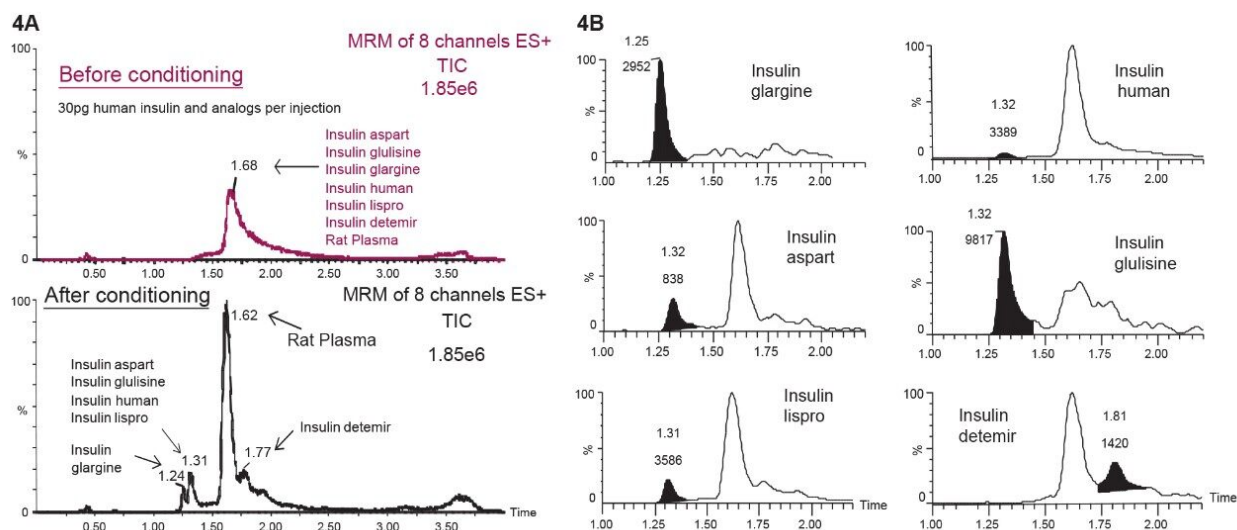


Figure 4A and 4B. Typical UPLC-MS/MS chromatograms for human insulin and five analogs. Figure 4A (left). UPLC-MS/MS TIC for human insulin and five-insulin analogs before (top) and after (bottom) column conditioning. Figure 4B (right) is the extracted ion chromatogram (shaded areas) for human insulin and five-insulin analogs after conditioning column.

The insulin mixture used in the chromatograms of figure 4A and 4B was prepared from formulations of insulin and analogs present at 100 IU/mL for Lantus, Apidra, Novolog, Humalog, and Levemir which are equivalent to 3.64, 3.49, 3.5, 3.5, and 14.2 mg/mL of insulin glargine, insulin glulisine, insulin aspart, insulin lispro, and insulin detemir, respectively (values as stated in product inserts). Lyophilized human insulin was dissolved in 0.01 M HCl to generate a concentrated stock at 1 mg/mL.¹ The sample diluent was 65/30/5 Milli-Q water/methanol/acetic acid (v/v/v) containing 0.05% rat plasma, added to prevent non-specific binding of the insulin and its analogs.

A 2 μ L injection of human insulin and the five analogs at 15 ng/mL (total in diluent) was made on the unconditioned and conditioned column shown in Figure 4A. Upon conditioning, the human insulin and its five analogs could be quantitatively identified exhibiting peak widths of <4 seconds with increased area counts as shown in Figure 4B.

Liquid Chromatography

Unlike small molecules, larger peptides and small proteins, i.e., insulins, suffer from poor mass transfer in and out of fully-porous particles. Thus, using a column packed with solid-core particles allows for sharper peak shapes at the higher flow rates typically needed for bioanalytical studies.^{1,2} Specifically for insulins, it is documented that using a column packed with particles containing a low-level positive surface charge gives

superior peak shape and resolution to other columns.³ CORTECS C₁₈+ Columns combine the benefits of solid-core particle technology and a low-level positive surface charge to obtain a new level of separation performance for this application. With CORTECS C₁₈+, insulin peaks are typically 4.0 to 4.5 seconds wide with 2X higher area counts than state-of-the-art, fully-porous particle columns. Representative chromatograms are shown in Figure 5.

The use of multidimensional chromatography facilitated higher injection volumes, through the use of at-column dilution (ACD), while providing additional sample cleanup by employing a trap and back-elute strategy. The combination of these chromatographic system elements allowed us to inject up to 40 µL without experiencing insulin breakthrough. Chromatographic breakthrough was evident on a 1D system with injection volumes >10 µL (data not shown).

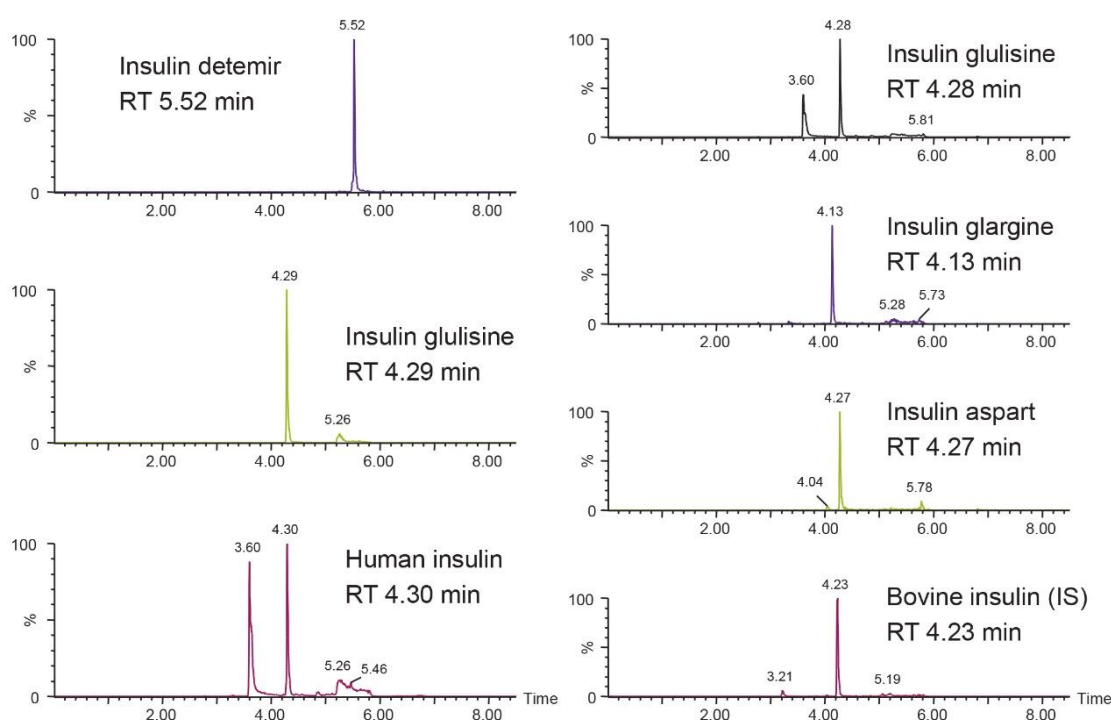


Figure 5. UPLC-MS/MS chromatogram for human insulin, five-insulin analogs, and bovine insulin (IS).

Sample preparation

A proof-of-concept study published earlier in 2013³ used reversed-phase only SPE to extract four insulin analogs. Although the method clearly demonstrated feasibility, endogenous background was high in the transitions for human insulin and lispro, with average LLOQs of 200 to 500 pg/mL reported for the four

insulins, making accurate low-level quantification and distinction between the lispro and human insulin impossible. This application presents a significantly more selective two-step extraction utilizing a protein precipitation (PPT) pre-treatment step and a strong anion-exchange mixed-mode SPE device to effectively reduce endogenous background, as well as improve specificity and detection limits.

During optimization of protein precipitation conditions for insulin, various ratios of organic solvent to plasma, as well as different precipitation solvents were tested. Protein precipitation (PPT) with a 1:1 ratio of various solvents (ACN or ACN modified with one of the following: 5% NH_4OH , 1% acetic acid, or 2% formic acid) resulted in >90% recovery of the six insulins without precipitating the peptides themselves. Protein precipitation with higher ratios of organic resulted in peptide loss due to undesired precipitation of the insulins. The PPT pre-treatment effectively eliminated protein binding and reduced endogenous interferences from large proteins such as albumin.

The PPT supernatants were then diluted with 5% concentrated NH_4OH to bring the pH to ~11. At this pH, insulin and its analogs will carry a net negative charge, putting them in the proper charge state to bind to Oasis MAX by ion exchange. A 2007 study⁴ determined that mixed-mode SPE (especially in its ion-exchange elution) was significantly more selective than reversed-phase only SPE. For this assay specifically, where the use of low m/z fragments for human insulin and lispro was necessary, it was imperative to have the most selective sample preparation possible.

The diluted supernatant was then applied to conditioned SPE plates, and analytes were well retained on the SPE sorbent during the basic pH load step. Optimization of the elution solution was critical to fully elute the insulins, maintain solubility, and minimize interferences from the plasma matrix. The optimum elution solution was 60% organic with 10% acetic acid. The final eluate was diluted with an equal volume of water.

Linearity, accuracy, and precision

To generate standard curves, human plasma was fortified with a mixture of the insulins at the following final concentrations: 50, 100, 200, 500, 1000, 2000, 5000, and 10,000 pg/mL. Quality control (QC) samples were prepared in human plasma at the following concentrations: 150, 750, 2500, and 7500 pg/mL.

Bovine insulin (final concentration 10 ng/mL) was used as the internal standard (IS) for all insulins. Peak area ratios (PARs) of the analyte peak area to the IS peak were calculated. Calibration curves, prepared in human plasma, were constructed using PARs of the calibration samples by applying a one/concentration weighted linear regression model. All QC sample concentrations were then calculated from their PARs against their respective calibration lines. All curves were linear using the $1/x$ regression. A summary of standard curve performance for all insulins is shown in Table 3.

Analyte	Std. curve range pg/mL	Std. curve range fmol/mL	r^2 , linear fit, 1/x weighting	Mean % accuracy of all points
Insulin lispro	50 to 10,000	8.6 to 1720	0.998	99.99
Insulin glargine	50 to 10,000	8.3 to 1650	0.996	99.98
Human insulin	50 to 10,000	8.6 to 1720	0.996	100.00
Insulin detemir	200 to 10,000	33.8 to 1690	0.998	96.40
Insulin glulisine	50 to 10,000	8.6 to 1720	0.995	100.00
Insulin aspart	100 to 10,000	17.2 to 1716	0.995	100.00

Table 3. Standard curve ranges, r^2 values, and mean accuracy for curve points for all compounds.

For human insulin, the basal concentration in pooled or individual control plasma was determined by calculating the x-intercept. The basal level of human insulin (average = 1937 pg/mL) was then added to the spiked concentration for all standard curve and QC samples to enable accurate quantification. A representative standard curve for insulin lispro is shown in Figure 6.

Compound name: Humalog

Correlation coefficient: $r = 0.998354$, $r^2 = 0.996710$

Calibration curve: $0.00024951 * x + 0.000753141$

Response type: Internal std (Ref 2), Area * (IS conc./IS area)

Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None

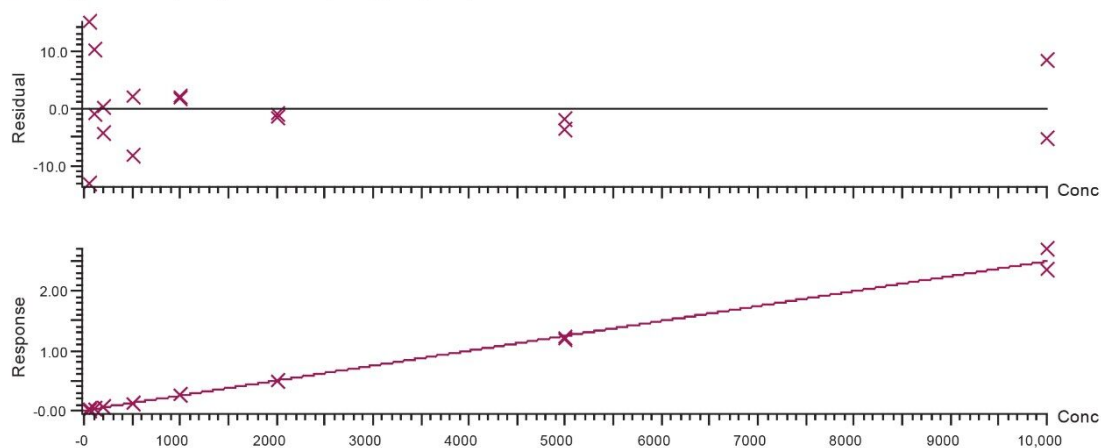


Figure 6. Representative standard curve for insulin lispro.

Inter- and intra-day accuracy and precision were calculated for all QC samples. Representative summary statistics for insulin glargine, lispro, and human insulin can be found in Tables 4, 5, and 6. All other analogs

exhibited similar performance.

Insulin Glargine

Inter-day n=9

Inter-day n=9

QC conc. (pg/mL)	Mean calc. conc.	Std. dev.	%CV	Mean accuracy
150	150.1	18.7	12.4	102.7
750	718.4	47.3	6.6	95.8
2500	2369.3	131.2	5.5	94.8
7500	7648.5	511.3	6.7	102.0

Intra-day n=3

QC conc. (pg/mL)	Mean calc. conc.	Std. dev.	%CV	Mean accuracy
150	167.4	16.6	9.9	111.6
750	757.7	62.4	8.2	101.1
2500	2378.0	184.9	7.8	95.1
7500	7949.5	257.9	3.2	106.0

Table 4. Inter- and intra-day accuracy and precision for QC samples for insulin glargine.

Insulin Lispro

Inter-day n=9

Inter-day n=9

QC conc. (pg/mL)	Mean calc. conc.	Std dev	% CV	Mean accuracy
150	144.0	17.5	12.2	96.0
750	721.8	32.3	4.5	96.2
2500	2447.1	202.9	8.3	97.9
7500	7697.5	634.8	8.2	102.6

Intra-day n=3

QC conc. (pg/mL)	Mean calc. conc.	Std dev	% CV	Mean accuracy
150	164.6	14.9	9.1	109.8
750	748.2	19.8	2.6	99.8
2500	2417.6	230.4	9.5	96.7
7500	8215.4	243.1	3.0	109.5

Table 5. Inter- and intra-day accuracy and precision for QC samples for insulin lispro.

Human Insulin

Inter-day n=9 (Avg basal level was 1937 pg/mL)

Inter-day n=9 (Avg basal level was 1937 pg/mL)				
QC conc. (pg/mL)	Mean calc. conc.	Std dev	% CV	Mean accuracy
150	1915.1	125.4	6.5	92.0
750	2542.5	141.0	5.5	94.8
2500	4326.0	146.7	3.4	97.6
7500	9819.0	960.3	9.8	104.0
Intra-day n=3 (Basal level was 1872 pg/mL)				
QC conc. (pg/mL)	Mean calc. conc.	Std dev	% CV	Mean accuracy
150	2056.5	16.7	0.8	90.2
750	2506.3	46.6	1.9	99.3
2500	4269.8	206.4	4.8	101.3
7500	10,233.2	265.2	2.6	100.3

Table 6. Inter- and intra-day accuracy and precision for QC samples for human insulin.

Specificity

Matrix factors and CVs of matrix factors for all analogs were calculated in six individual sources of human plasma, as outlined in the 2007 AAPS white paper.⁵ The % CV of the matrix factors were 12.3, 11.6, 11.8, 9.0, and 7.7 for insulin detemir, glargine, aspart, glulisine, and lispro, respectively. All easily met the recommended criteria of <15% CV.

In addition, a study was performed to assess the impact of high levels of human insulin, expected to be found in type II diabetic patients, on the assay specificity. This is particularly important for two reasons. First, not all analytes are chromatographically resolved from one and another. Second, due to the use of a nominal mass instrument (triple quadrupole) and the high degree of sequence homology between the analogs themselves and with human insulin, the possibility of MS overlap in some of the transitions exists. For this test, plasma samples were fortified to a final concentration of 5 ng/mL with all of the analogs. A subset of these samples was also spiked with human insulin at 200X higher concentration, to a final concentration of 1 µg/mL human insulin. All samples were then pre-treated, extracted, and quantified. There was no significant change in area counts for any of the analogs when human insulin was present in high concentration.

Conclusion

Using analytical scale chromatography and simple 96-well SPE sample preparation, this method reaches

detection limits for intact insulins, comparable to those previously achieved using immuno-precipitation and nano-scale chromatography.⁶⁻¹¹

Development of this assay was challenging due to a high degree of non-specific binding (NSB), protein binding, and difficulty maintaining peptide solubility throughout the SPE extraction and elution process. Sample pre-treatment prior to SPE proved to be critical in improving recovery and specificity. Protein precipitation with 1% acetic acid 1:1 acetonitrile/methanol resulted in 80% to 100% recovery without precipitating the peptides themselves. Mixed-mode strong anion SPE provided an additional layer of selectivity, and facilitated the use of the low m/z MS fragments that were necessary to distinguish human insulin from lispro.

The use of the CORTECS UPLC C₁₈+ Column provided significantly improved sensitivity and peak shape for insulin and its analogs versus charged-surface fully-porous columns and traditional C₁₈ solid-core columns. This led to an improvement in detection limits of approximately 2X, when combined with proper MS fragment choice, and selective SPE cleanup enabled quantification limits of 50 to 200 pg/mL for the six insulins.

All FDA criteria¹² for accuracy and precision of the method were easily met. Average accuracies for standard curve points and QC samples were >92%, with most close to 99%. Inter- and intra-day precision for all QC samples was greater than 7.5%. CVs of matrix factors across six lots of human plasma were <15%, further supporting the selectivity of the method.

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