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Application Note

Reducing Sample Volume and Increasing Sensitivity for the Quantification of Human Insulin and 5 Analogs in Human Plasma Using ionKey/MS

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Abstract

In this current work, we undertook to a) transfer the original analytical scale method to the ionKey/MS System, and b) to decrease sample volume and further increase sensitivity through the inherent characteristics of ionKey/MS. IonKey Technology integrates the UPLC analytical separation directly into the source of the mass spectrometer.

Benefits

- High sensitivity assay with LLOQ of <25–50 pg/mL in human plasma
- Reduced solvent consumption (50X) compared to 2.1 mm scale = significant cost savings
- Use of mixed-mode SPE reduces matrix interferences and enhances selectivity of the extraction for insulins in plasma
- 96-well µElution plate format enables concentration of the sample while maintaining solubility and minimizes peptide losses due to adsorption
- Selective, fast SPE extraction (<30 minutes) without time-consuming immuno-affinity purification
- Versus 2.1 mm scale, proof of concept studies yield greater signal-to-noise from 2.5X less sample and 1/3 injection volume, allowing for greater confidence in results, more tests per sample, and more injections

Introduction

Recombinant human insulin and its analogs (Figure 1) are perhaps the best known and most widely sold biotherapeutics. Historically, such biologics have been quantified using ligand binding assays (LBAs). However, specifically in the case of insulin and analogs, these affinity-based assays lack standardization, are subject to matrix effects, and in some cases lack adequate specificity. Furthermore, multiplexing is desirable as diabetes treatment typically consists of combination dosing with both long and fast acting versions. LBAs do not allow for simultaneous quantification of human insulin and its important analogs. In spite of these short comings, LBAs are incredibly sensitive and consume only minimal sample. Over the past few years, there has been a trend toward the analysis of large molecules by LC-MS/MS. LC-MS/MS has the advantage of shorter development times, greater accuracy and precision, the ability to multiplex, and can readily distinguish between closely related analogues, metabolites, or endogenous interferences. However LC-MS

has struggled to achieve the sensitivity of LBAs and often requires significantly more sample. The need for robust and sensitive analysis of peptide species challenges both the chromatographic separation and mass spectrometry.

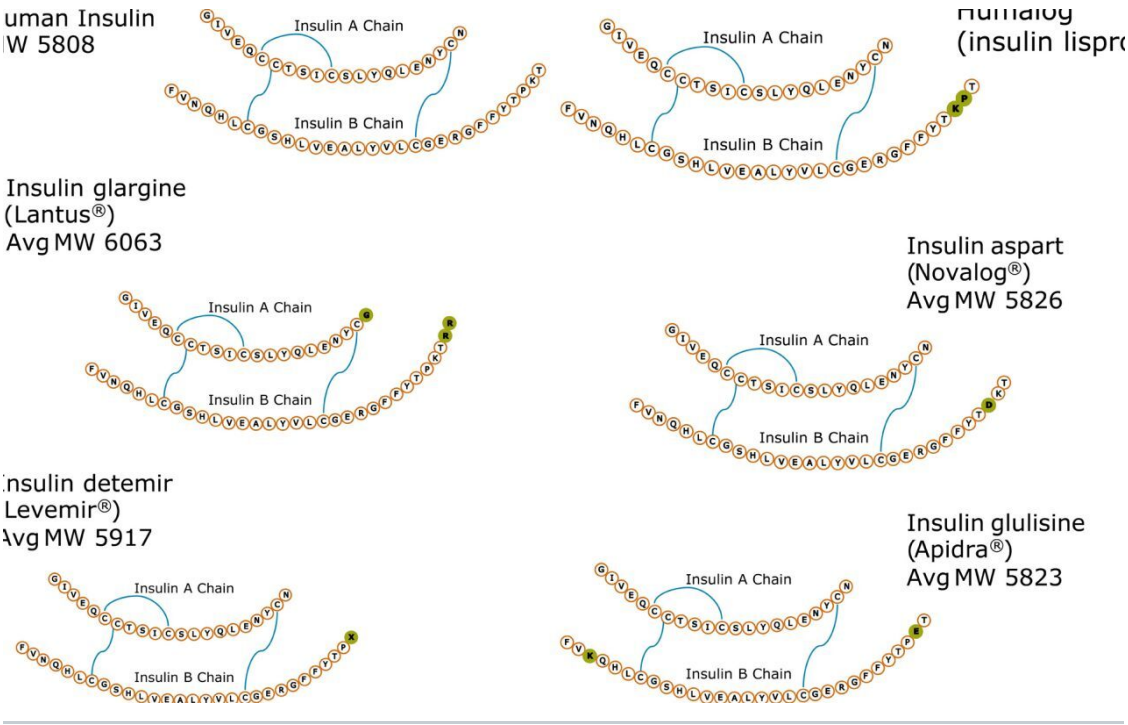

Figure 1. Structures for human insulin and analogs quantified in this application.

Experimental

Sample prepration

Samples were pretreated using protein precipitation (PPT) and extracted on an Oasis MAX 96-well µElution Plate according to a previously published method. [1] For this work, either 100 or 50 µL of human plasma were extracted.

Method conditions

UPLC conditions

LC system:	ACQUITY UPLC M-Class, configured with

	optional trap and back flush elution
Separation device:	iKey Peptide BEH C_{18}, 130Å, 1.7 µm, 150 µm x 100 mm (p/n 186006766)
Trap column:	ACQUITY UPLC M-Class Symmetry C_{18} Trap Column, 100Å, 5 µm, 300 µm x 50 mm (p/n 186007498)
Mobile phase A:	0.1% formic acid in water
Mobile phase B:	0.1% formic acid in acetonitrile
Loading solvent:	85:15 mobile phase A:B, 25 µL/min for first two minutes, reverse valve
Valve position:	Initial position one (forward loading of trap), switch to position two at two minutes (back flush elute of trap onto the analytical column)
Gradient:	25–55% B over 5 minutes
Elution flow rate:	2.5 µL/min
iKey temp.:	75°C
Sample temp.:	15°C
Final injection volume:	10 µL
Total run time:	13.5 minutes
Collection plates:	Waters 1 mL Collection Plates

MS conditions

MS system:	ionKey/MS
Ionization mode:	ESI positive
Capillary voltage:	3.6 kV
Source temp.:	120 °C
Cone gas flow:	50 L/hr
Collision cell pressure:	3.83 X $10^{(-3)}$ mbar
Collision energy:	Optimized by component, see Table 1
Cone voltage:	Optimized by component, see Table 1

Data management

Chromatography software:	MassLynx 4.1
Quantification software:	TargetLynx

In an earlier publication,[1] we described an ultra-high sensitivity quantitative assay for human insulin and 5 analogs. The method was carefully optimized to maximize sensitivity for the insulins in the following manner: a multi-dimensional LC system was used to enable at-column-dilution and a trap/back elute strategy to increase loading volume and then refocus the analyte band. Mixed-mode SPE and a high-efficiency chromatographic system using a solid-core column with a positively-charged particle surface improved specificity and facilitated the differentiation of human insulin and insulin LisPro. In this earlier method, 250 µL of human plasma were extracted to reach detection limits between 50 and 200 pg/mL with a 30 µL injection volume.

Many of the insulins described in the earlier method have either recently come off patent or are due to shortly. This has resulted in a flurry of research activity aimed at alternate dosing regimes, pediatric extensions, and the development of replacement insulins. In many of these cases, a further decrease in detection limit and reduction in sample volume required were requested.

In this current work, we undertook to a) transfer the original analytical scale method to the ionKey/MS System, and b) to decrease sample volume and further increase sensitivity through the inherent characteristics of the ionKey/MS System. The ionKey/MS System integrates UPLC analytical separation directly into the source of the mass spectrometer. The iKey Separation Device (150 µm internal diameter), shown in Figure 2, contains the fluidic channel, electronics, ESI interface, heater, eCord, and the chemistry to perform UPLC separations. Perhaps most importantly, The ionKey/MS System can provide increased sensitivity compared to 2.1 mm chromatography with the same injection volume, or equivalent or greater sensitivity with reduced sample consumption, making it ideal for insulin analyses. As previously mentioned, it is common for bioanalytical LC-MS assays to consume high volumes of both solvent and sample, thus increasing the cost of the assay and limiting the number of replicates that can be analyzed. This study combines µElution solid-phase extraction (SPE) and the novel and highly efficient ionKey/MS System to improve a quantitative assay for insulins in human plasma.

Figure 2. iKey Separation Device.

We investigated the potential for increasing sensitivity whilst simultaneously reducing sample volume using the ionKey/MS System. This study demonstrates a cumulative ~15X benefit over a 2.1 mm I.D. scale method for all 6 insulins studied. We were able to reduce sample size by 2.5X, reduce injection volume 3X, and increase sensitivity by a minimum of 2X through effective adaptation of the method to the ionKey/MS System. Specifically, sample volume was decreased to 100 µL and an LLOQ of 25 pg/mL was achieved for most insulins. A human plasma starting volume of 50 µL yielded a 50 pg/mL LLOQ.

Results and Discussion

Mass Spectrometry

A stock solution of all insulins was infused via an infusion iKey Separation Device to confirm mass spectrometry conditions previously described for an analytical scale method.[1] Methods in the literature have demonstrated that it is possible to see a shift in relative abundance of multiply-charged peptide precursors at different flow rates.[2] It becomes important, therefore, to evaluate this when adapting a method from analytical LC flow to microflow. In this instance, MS conditions remained the same and are summarized in Table 1.

Specific insulin	MRM transition	Cone voltage (V)	Collision energy (eV)
Glargine	1011.0 → 1179.0	60	25
	867.0 → 984.0	60	18
Lispro	1162.0 → 217.0	50	40
	968.5 → 217.0	50	40
Detemir	1184.0 → 454.4	60	20
	1184.0 → 1366.3	60	20
Aspart	971.8 → 660.8	60	18
	971.8 → 1139.4	12	18
Glulisine	1165.0 → 1370.0	14	22
	1165.0 → 346.2.0	14	22
Bovine (IS)	956.6 → 1121.2	60	18
Human insulin	1162.0 → 226.0	50	40
	968.5 → 217.0	50	40

Table 1. MRM transitions and MS conditions for human insulin, 5 insulin analogs, and the internal standard bovine insulin; transitions highlighted in blue are the primary quantitative MRMs.

Chromatography

Chromatographic separation of human insulin, 5 key analogs, and the IS was achieved using a novel microfluidic chromatographic iKey Separation Device (shown in Figure 2). The iKey Separation Device has a channel with UPLC grade sub-2-µm particles that permits operation at high pressure and results in highly efficient LC separations. By integrating microscale LC components into a single platform design, problems associated with capillary connections, including manual variability, leaks, and excessive dead volume are avoided. Insulin peak widths are 3 to 4.2 seconds wide at base, as shown in chromatograms from extracted human plasma, Figure 3. Interestingly, at analytical scale human insulin and insulin lispro completely co-elute. Normally, one would expect to have to resort to nano-flow, very shallow gradients, and unduly long run times to affect separation. However, the two peaks begin to separate under the microflow conditions shown

here, within a run time that is compatible with routine bioanalytical assays.

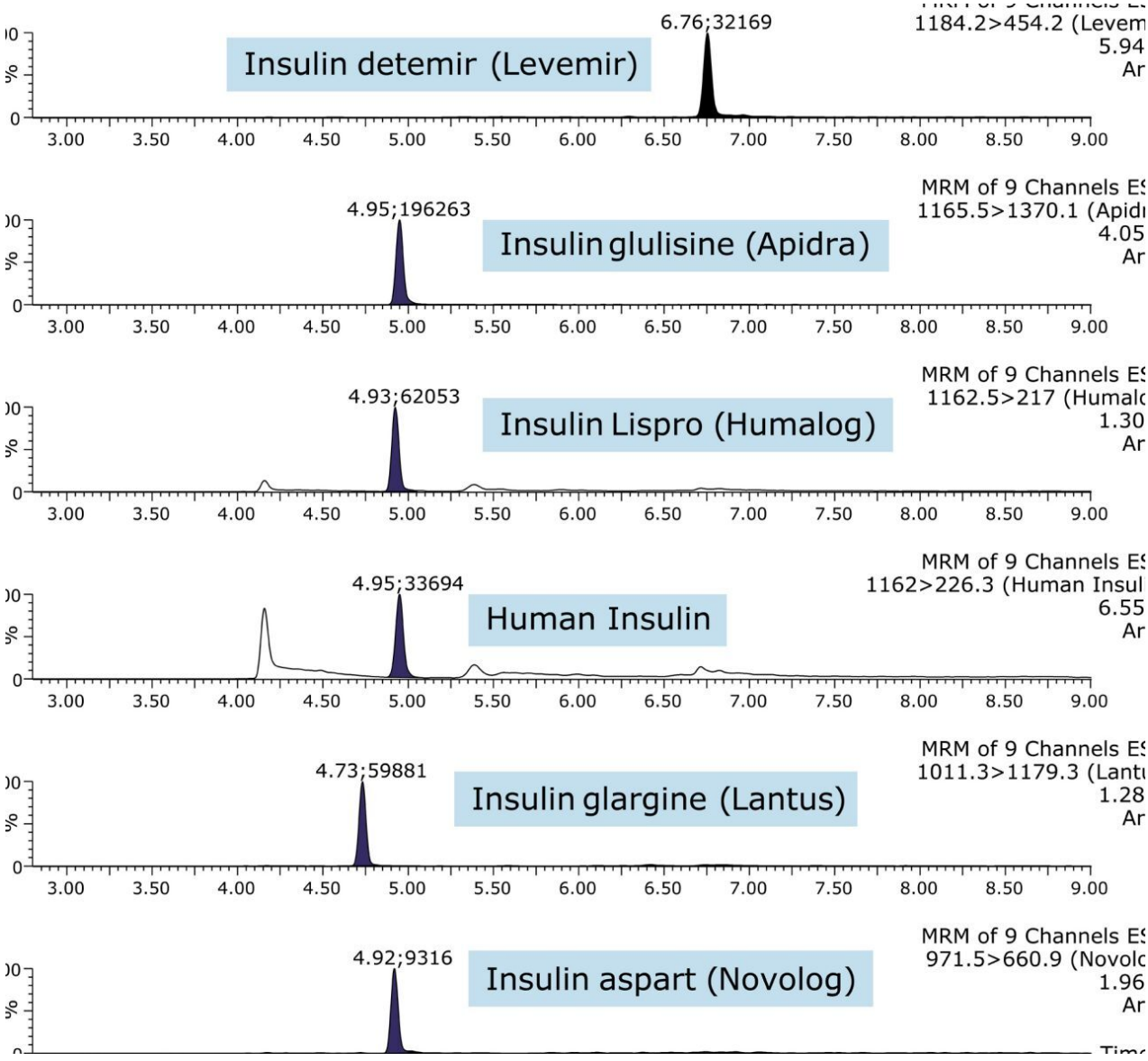

Figure 3. Representative chromatograms of human insulin and analogs extracted from 100 µL human plasma.

The separation was performed using the optional Trap Valve Manager (TVM) configured for single-pump trapping in trap and back elute mode, Figure 4. This configuration facilitates the injection of larger volumes of plasma extracts to improve sensitivity. In this application, the injection volume is 10 µL, which, if properly scaled to a 2.1 mm I.D. column, would equate to approximately a 2 mL injection. Even at analytical scale, this would normally require some type of trapping to focus the analyte band. Furthermore, the final injection solvent (after SPE of the plasma and dilution of the eluate with water) is 30% methanol and 5% acetic acid.

This composition is necessary in order to keep the insulins soluble throughout the chromatographic process. Direct injection without the TVM would result in severe breakthrough due to the organic content which cannot be reduced further without resulting in adsorptive losses and poor solubility. The composition of the injection solvent also plays a key role in eliminating non-column related carry-over. Representative chromatograms of human insulin and analogs extracted from 100 µL human plasma are shown in Figure 3.

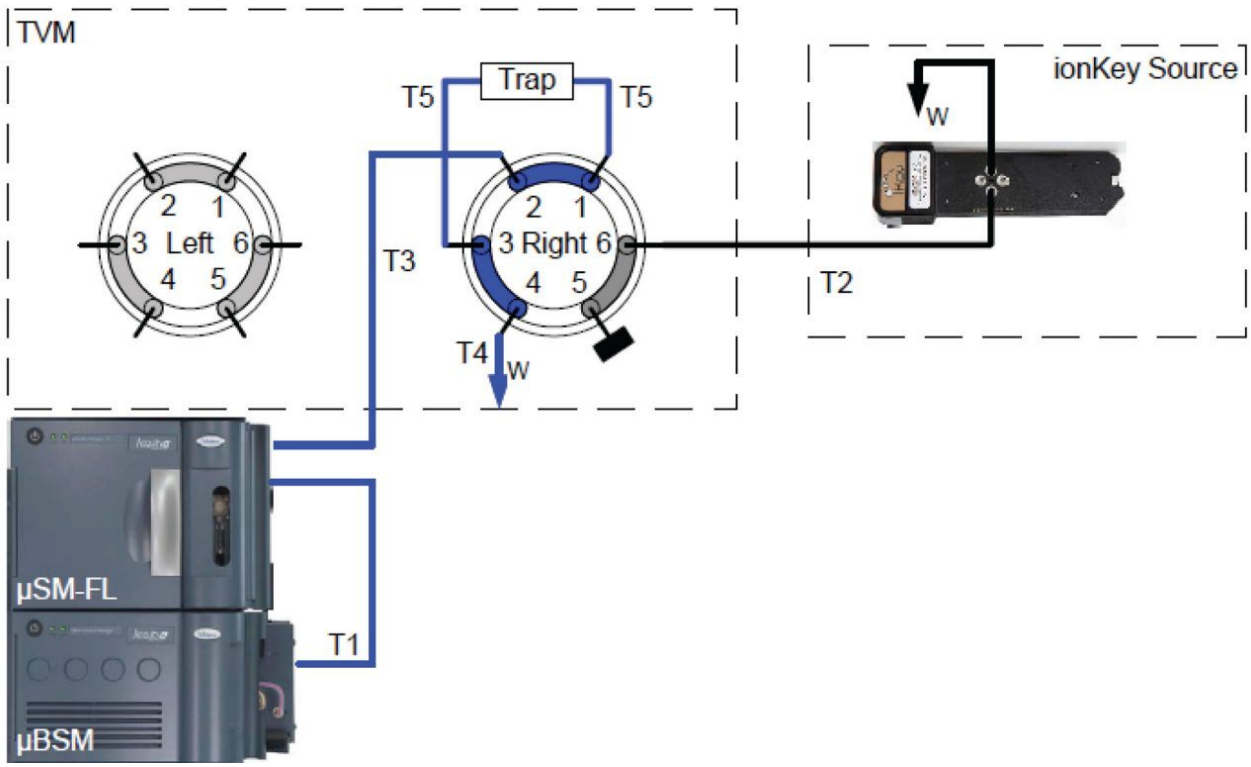

Figure 4. Configuration for single pump trapping on the ionKey/MS System.

Linearity and sensitivity

To demonstrate proof of principle, standard curve points were prepared by fortifying human plasma with human insulin and the 5 analogs at the following final concentrations: 25, 50, 100, 200, 500, 1,000, 2,000, 5,000, and 10,000 pg/mL. Quality control (QC) samples were prepared separately at 150, 750, 2,500, and 7,500 pg/mL. Bovine insulin was used as the internal standard (IS). Peak area ratios (PARs) of the analyte to the IS peak were calculated. The calibration curve was constructed using PARs of the calibration samples by applying a one/concentration (1/x) weighted linear regression model. All QC sample concentrations were then calculated from their PARS against the calibration curve. For human insulin, the standard addition method was used for quantification. Basal insulin level was determined by calculating the x intercept from the slope of the calibration line. Using 1/x regression, standard curves for all insulins were linear, with all R^2

values greater than 0.99. Representative standard curves are shown in Figure 5. A summary of standard curve performance for all insulins (25–10,000 pg/mL) is shown in Table 2.

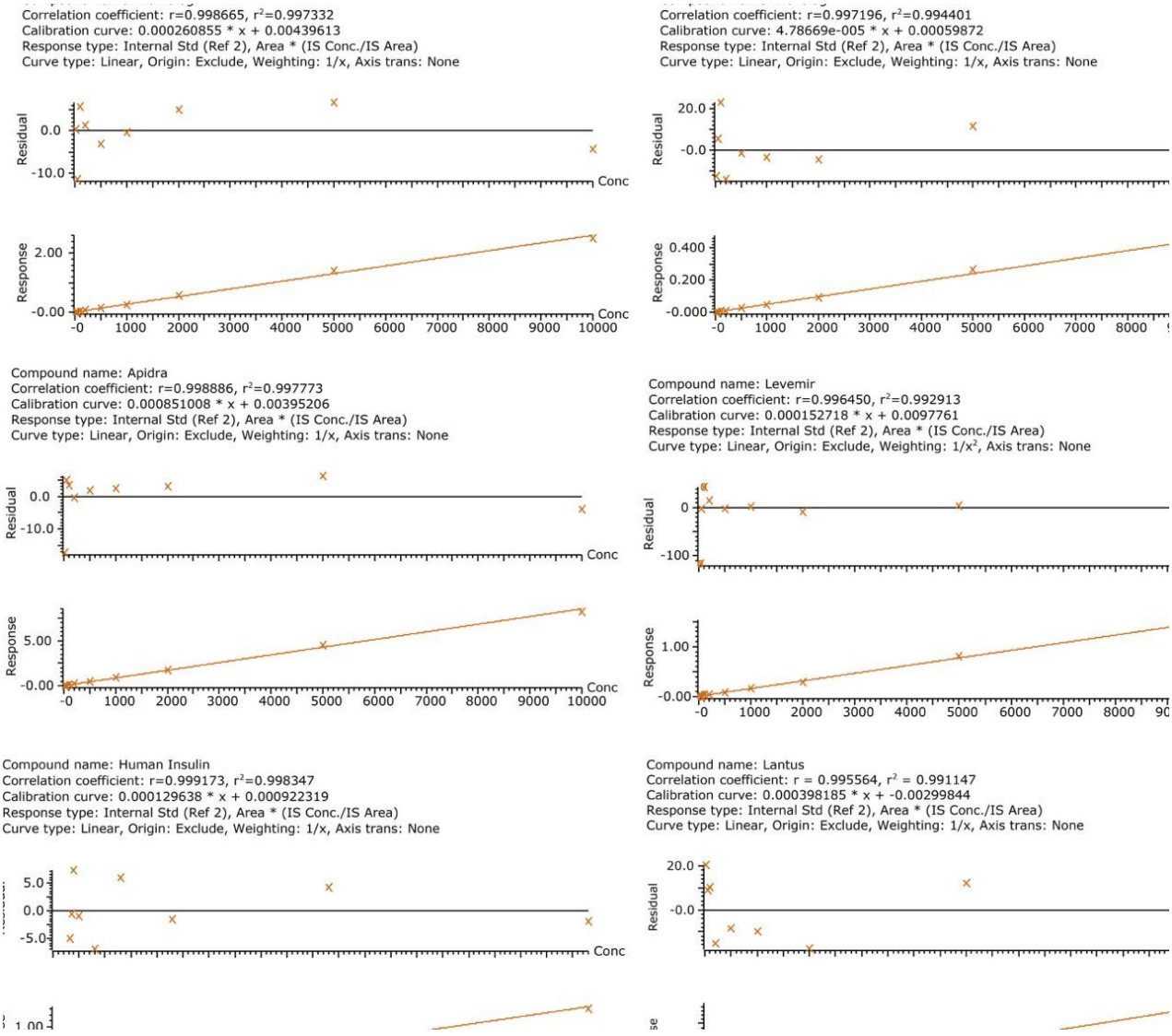

Figure 5. Representative standard curves for human insulin and analogs.

Figure 6 and Figure 7 contain representative chromatograms for insulin glargine and insulin glulisine (respectively) at 25, 50, and 100 pg/mL extracted from 100 µL human plasma, as compared to blank extracted plasma. All QC samples demonstrated very good accuracy and precision, with mean accuracies ranging from 94–109% and mean %CV's of 3.4–8.8%. A summary of QC statistics is shown in Table 3. These results easily meet the recommended FDA acceptance criteria outlined in the white papers describing best practices in bioanalytical method validation for LC-MS/MS assays.[3,4]

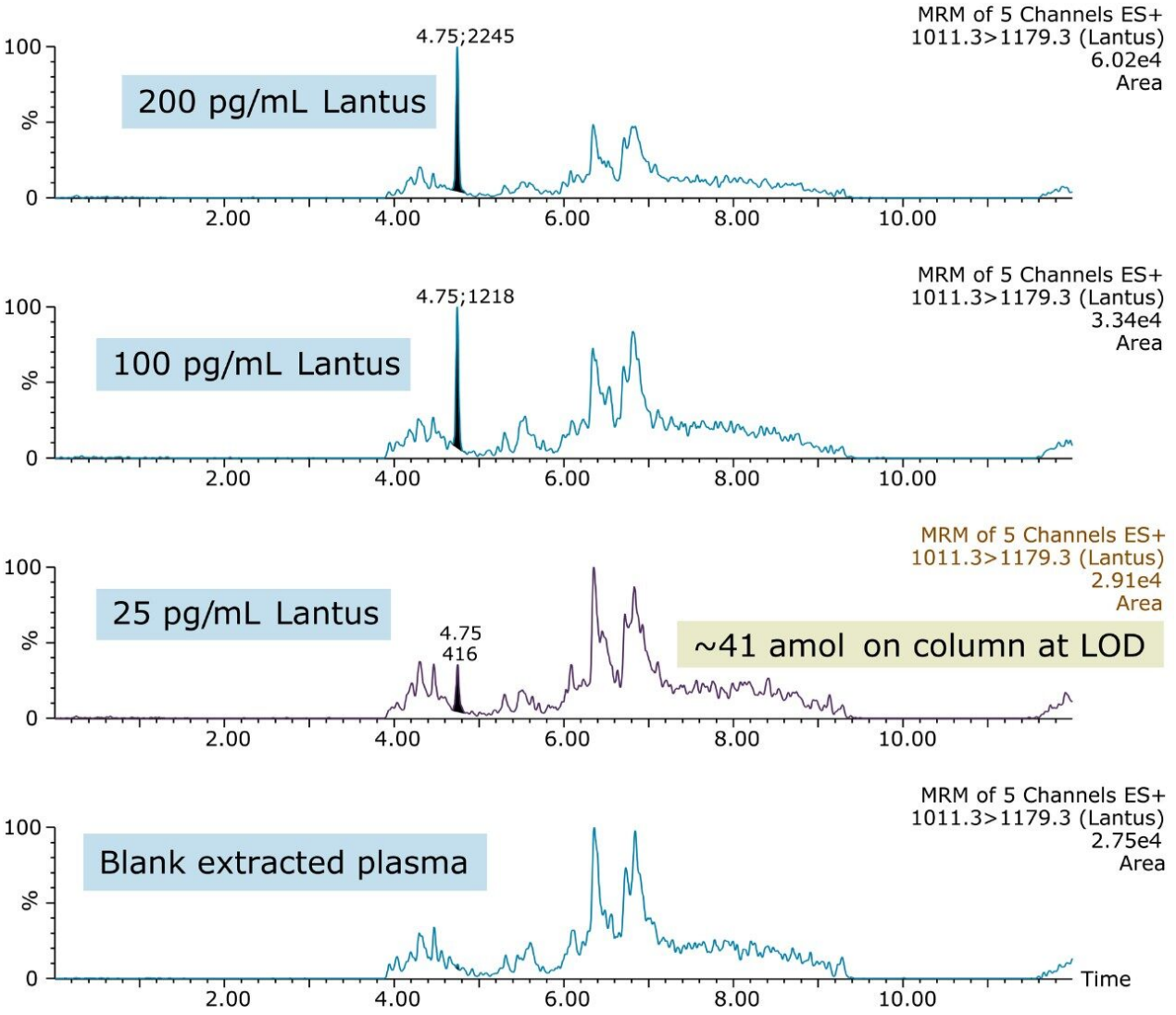

Figure 6. The ionKey/MS System's analysis of insulin glargine (Lantus) from 100 µL human plasma sample, 10 µL injection.

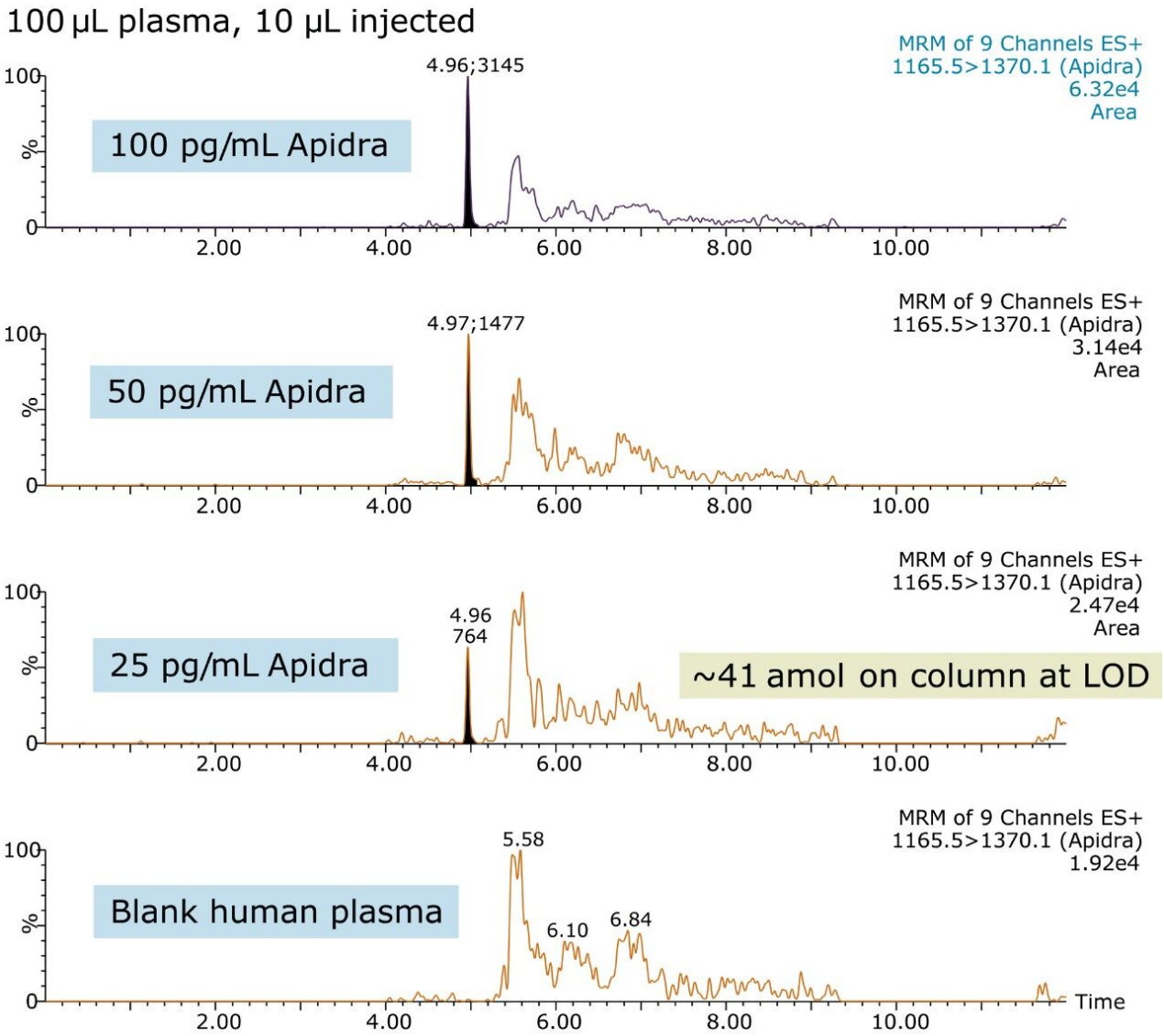

Figure 7. The ionKey/MS System's analysis of insulin glulisine (Apidra) from 100 µL human plasma sample, 10 µL injection.

Insulin variant	Std curve range (pg/mL)	R^2	Avg % accuracy	Avg. % CV
Human	25–10,000	0.998	97	2.0
Glargine	25–10,000	0.991	97	9.5
Aspart	25–10,000	0.994	98	8.6
Glulisine	25–10,000	0.998	98	3.5
Detemir	50–10,000	0.993	101	9.7
Lispro	25–10,000	0.997	99	4.0

Table 2. Summary statistics for standard curve performance in a proof of principle study for human insulin and 5 analogs.

Insulin Variant	Avg accuracy QC 1 (150 pg/mL)	Avg accuracy QC 2 (750 pg/mL)	Avg accuracy QC 3 (2,500 pg/mL)	Avg accuracy QC 4 (7,500 pg/mL)
Human	-1.6	4.1	0.8	-2.9
Glargine	10.1	-0.9	5.1	2.0
Aspart	0.8	6.4	14.4	0.8
Glulisine	6.4	2.3	4.7	-3.5
Detemir	14.6	-10.3	-4.0	0.3
Lispro	11.5	-2.5	2.7	-0.8

Table 3. Summary QC statistics for human insulin and 5 analogs, extracted from human plasma.

When comparing sensitivity, the current ionKey/MS System method provides a cumulative benefit of approximately 15X over earlier work[1] from our labs. The ionKey/MS System used 2.5X less sample, injects 3X less, and achieves at least a 2X improvement in detection limit. While the original method had an LOD of ~618 amol on column, the ionKey/MS System has an LLOQ of only 41 amol on column (Figures 6 and 7).

If a further reduction in sample volume is desired, comparable performance (with a slightly higher LLOQ) can be achieved extracting only 50 µL of human plasma. Chromatograms of insulin glargine and insulin glulisine are used as representatives and are shown in Figures 8 and 9.

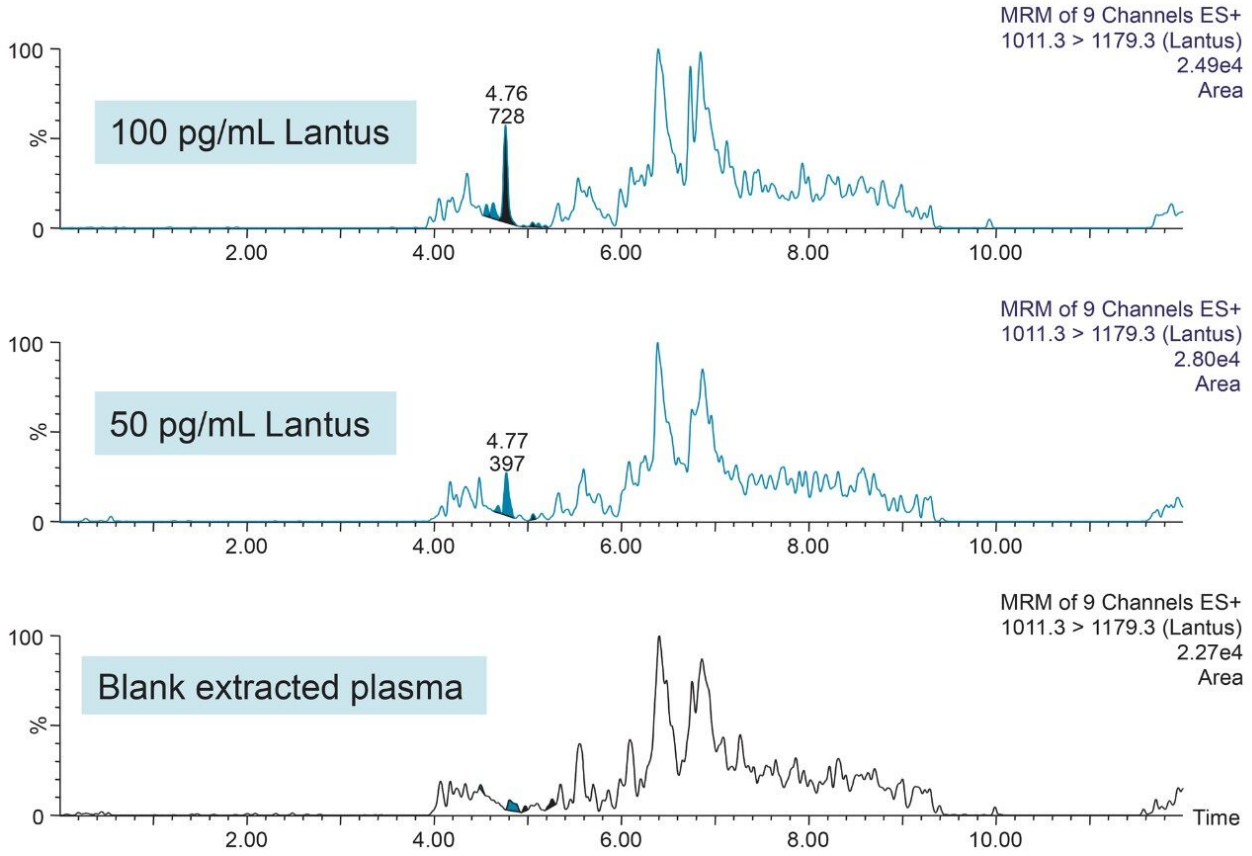

Figure 8. The ionKey/MS System's analysis of insulin glargine (Lantus) from 50 µL human plasma sample, 10 µL injection.

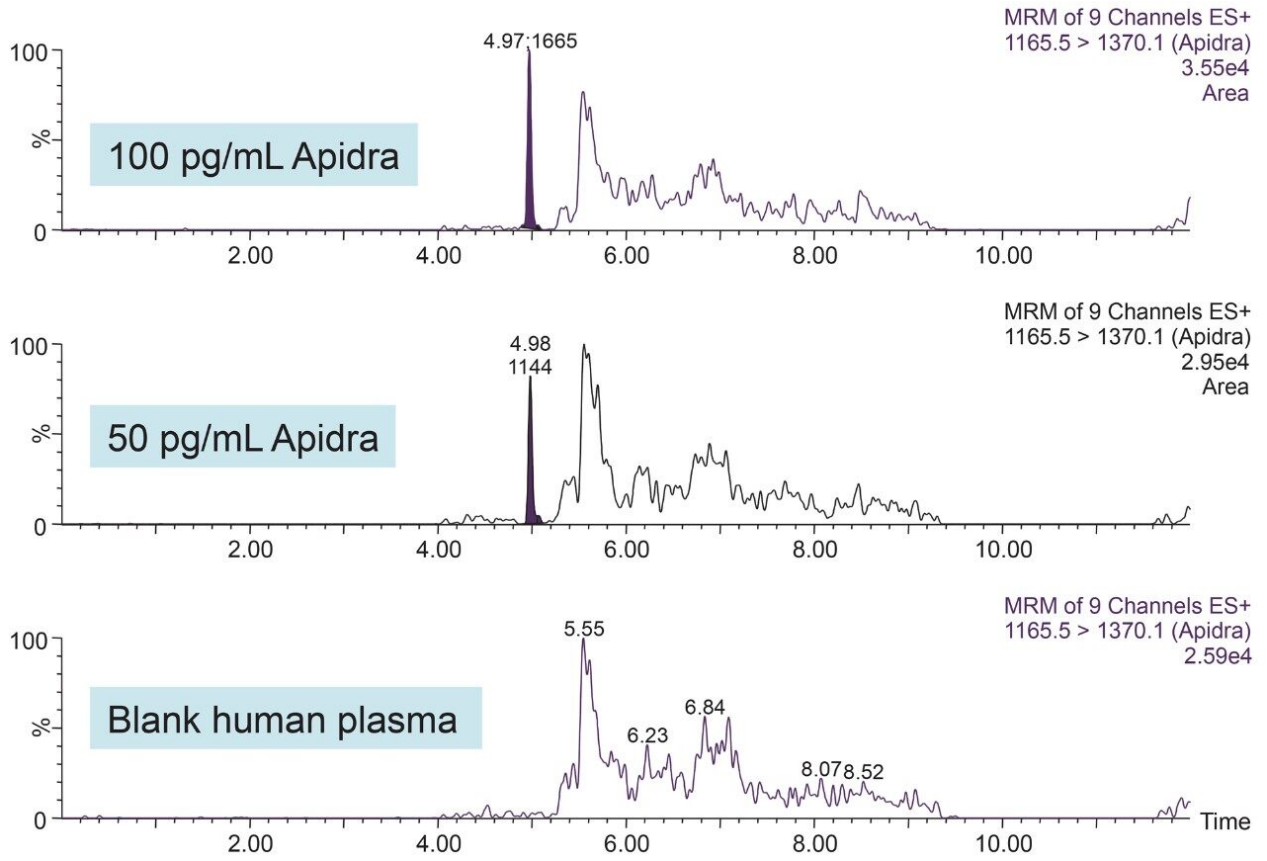

Figure 9. The ionKey/MS System's analysis of insulin glulisine (Apidra) from 50 µL human plasma sample, 10 µL injection.

Conclusion

The use of the ionKey/MS System facilitated a reduction in sample volume and concomitant, increase in sensitivity for the quantification of human insulin, and 5 important analogs. Extraction of 100 µL human plasma yields an LLOQ of 25 pg/mL for insulin glargine, lispro, glulisine, aspart, and endogenous insulin, with a 10 µL injection. Extraction of 50 µL of human plasma yields quantification limits of 50 pg/mL for most insulins. Standard curves were accurate and precise from 25–10,000 pg/mL. QC samples at all levels easily met recommended FDA regulatory criteria with mean accuracies ranging from 94–109% and mean %CVs of 3.4–8.8%, indicating an accurate, precise, and reproducible method. The ionKey/MS System provided a cumulative 15X benefit over an existing analytical scale method by reducing sample required by 2.5 to 5X, reducing injection volume 3X, all whilst increasing sensitivity >2X. In addition, the ionKey/MS System also

reduces solvent consumption by approximately 60X, thereby reducing cost. The reduction in sample volume required for this analysis allows for multiple injections of samples for improved accuracy, more tests per sample, or to meet the guidelines for ISR. This method shows great promise for high sensitivity quantification of intact insulins in patient samples from PK studies using the ionKey/MS System if further validation was performed.

References

1. Chambers, E.E., et al., Multidimensional LC-MS/MS Enables Simultaneous Quantification of Intact Human Insulin and 5 Recombinant Analogs in Human Plasma. *Analytical Chemistry*, 2014. 86(1): p. 694–702.
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