Document Title:	**Development of a Comprehensive Workflow for Postmortem Insulin Testing in Cases of Suspected Insulin Homicide**
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Development of a Comprehensive Workflow for Postmortem Insulin Testing in Cases of Suspected Insulin Homicide

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INTRODUCTION

The investigation of death due to exogenous insulin administration is complicated by the lack of tests that have been validated on autopsy specimens. This is due to a combination of factors including cost, pre-analytical issues regarding specimen handling, and pronounced analytical challenges. Considering insulin toxicity as a cause of death and then accurately determining manner of death requires a systematic investigation that is often complex and lengthy. For this reason, it follows that a proportion of these deaths go unrecognized, misclassified as to the cause and manner of death, or are suspicious but unconfirmed for an insulin overdose. From a toxicological perspective, a key contribution to these investigations would be the ability to determine whether exogenous insulin is present, its concentration, and significance. The major obstacle to this is that methods used in clinical settings are typically not amenable for postmortem specimens such as hemolyzed serum or whole blood. In the last decade, improvements in sample preparation and instrumentation have made notable progress in the detection and differentiation of insulin analogues in both insulin preparations and medical equipment (e.g., intravenous lines, and bags) and using alternative biological matrices (e.g., vitreous fluid) that are frequently collected during autopsy. Further analytical advancements with the development of interpretation resources, in conjunction with the knowledge base that already exists for insulin biochemistry and physiology, will allow for more complete forensic investigations of suspected insulin-related deaths. This work contributes to the scientific data that supports the insulin death investigations.

INSULIN STRUCTURE AND FUNCTION

Proinsulin, the precursor molecule of insulin, is a peptide that is synthesized in the β-cells of the pancreas. Proteolytic enzymes cleave the 31-amino acid residue (C-peptide) between the amino- and hydroxy-terminal ends away from proinsulin to form insulin. The insulin structure consists of a 21-amino acid α-chain (carboxy terminal end) and a 30-amino acid β-chain (the amino terminal end) held together by two interchain disulfide bonds. The α-chain contains an additional, single, intrachain disulfide bond (**Figure 1**). In response to rising blood glucose concentrations, insulin is excreted with C-peptide in equimolar quantities into the portal circulation.

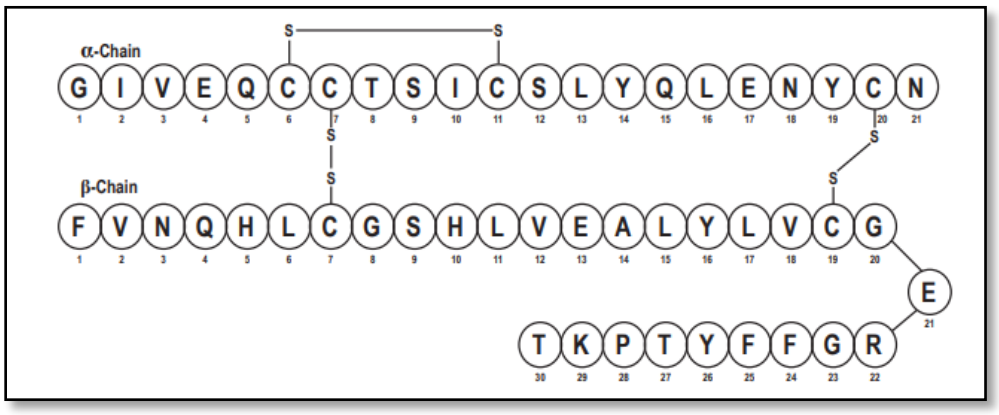

Figure 1

Insulin is a vital hormone that has numerous anabolic physiological functions including stimulating DNA and RNA synthesis, oxidative phosphorylation, intracellular transport, lipogenesis, and glucose utilization. Foremost in allowing these actions to occur, insulin allows glucose to enter cells where it can be metabolized and used to drive cellular activity. Insulin also promotes anabolic processes by inhibiting protein catabolism and decreasing the release of glucagon, a catabolic hormone, from pancreatic α-cells. In contrast to insulin, glucagon promotes the breakdown of glycogen, the storage form of glucose. The two hormones, working in opposition, serve to stabilize glucose blood concentrations so that they are maintained within appropriate limits.

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EXOGENOUS INSULIN

Physiological problems with insulin production and utilization characterize types 1 and 2 diabetes mellitus, respectively. Treatment for T1DM and for advanced T2DM is insulin injection. Insulin was first isolated from the pancreas in 1922, which allowed for the purification of animal insulin for treatment purposes. While this therapy was lifesaving, the administration of animal insulin can lead to the development of antibodies to the exogenous insulin. Since then, the development and use of recombinant human insulin has resulted in a dramatic decrease in symptomatic immune responses to insulin. Exogenous insulin analogues are available in several forms with molecular structures like endogenous human insulin. The following insulin analogues were included in this study: Glargine, Glulisine, Aspart, Lispro, Human, Detemir, and Deludec.

RESEARCH OBJECTIVES

There are four research objectives for this study. These are as follows:

1. Analytical Method Development
    - Development of a quantitative multiple reaction monitoring (MRM) method on a triple quadrupole mass spectrometer.
2. Automated Sample Processing
    - Development of an automated method that can be used to extract insulin and C-peptide from postmortem blood and vitreous humor.
3. Method Validation
    - Validation of the developed method in concordance with SWGTOX (Scientific Working Group for Forensic Toxicology) guidelines.
4. Population Assessment
    - Paired postmortem blood and vitreous humor samples will be collected and tested to establish therapeutic and overdose concentrations.
    - Acetone and β-hydroxybutyrate testing will be performed on the same blood and vitreous humor samples, and glucose testing will be performed on vitreous fluid samples.

RESEARCH QUESTIONS

There are five research questions for this study. These are as follows:

1. Can a robust automated immunopurification technique be applied to the extraction of exogenous insulins from vitreous fluid and postmortem blood?
2. Do method validation outcomes show that the developed method is appropriate for use in forensic settings?
3. What percentage of vitreous humor samples submitted for death investigation purposes were positive using the developed method?

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4. How will sample collection and storage conditions affect exogenous insulin concentrations in vitreous humor?
5. Is it possible to develop reference intervals for each exogenous insulin that show the therapeutic concentration range and concentrations associated with overdose?

RESEARCH DESIGN, METHODS, ANALYTICAL AND DATA ANALYSIS

RESEARCH DESIGN

Compared to standard "small molecule" mass spectrometry, large molecule quantitation remains a challenge even when employing modern analytical techniques. This is true regarding sample preparation and instrumentation requirements. Purification frequently employs several front-end approaches (e.g., protein precipitation, ultrafiltration), and instrumentation necessitates multi-stage chromatographic separation (e.g., size exclusion, ion exchange, and/or reverse phase fractionation) with mass spectrometry quantification. Difficulties arise because of multiple charge states, dispersed isotope distributions, variable post-translational modifications while also exhibiting poor fragmentation spectra depending on molecular weight and amino acid composition. The totality of these challenges adversely means that testing for insulin analogues and C-peptide in samples collected postmortem is not feasible for routine use. At the core of the research are three novel technical approaches that will be developed and validated to minimize the complexity of sample preparation. These are 1) fully automated "hands free" robotic sample preparation, 2) antibody-based immunoaffinity extraction of target compounds, and 3) insulin β-chain mass spectral analysis.

METHODS

1. Automation
    - Sample preparation robotics have become an increasingly essential technology for laboratories facing heavy caseloads with insufficient manpower. Robotic liquid handlers are routinely used in the biopharmaceutical industry to characterize and quantitate protein pharmaceuticals. These systems expedite complex analyses, reducing turnaround times while simultaneously improving precision, decreasing errors, and minimizing sample processing costs.
2. Antibody-based Immunoaffinity Extraction
    - This purification process has become a popular technique in the proteomic and drug discovery workspaces for multiplex assays requiring the absolute maximal sensitivity. High affinity antibodies are used to "pull out" target antigens from biological matrices, resulting in highly pure final extracts.

3. β-chain mass spectral analysis
    - By analyzing the insulin β-Chain in lieu of the intact molecule, analytical challenges regarding chromatographic separation and mass analysis can be minimized or eliminated entirely. Chromatographic separation of insulin(s) has been a challenge due to the nearly identical amino acid composition and structure. Therefore, baseline chromatographic resolution of these molecules has not been possible by any analytical approach to date. This has been particularly challenging regarding human insulin and Lispro, which are identical in molecular weight, and only differ by 2 amino acids (lysine and proline rotation) at the C-terminal of the β-chain.

ANALYTICAL AND DATA ANALYSIS

- LC/MS/MS analysis of the insulin β-chain was performed on a 1290 series UHPLC coupled with an Agilent 6495 triple quadrupole mass spectrometer.
- Data analysis was completed using Agilent's Masshunter Quantitative Analysis software.

EXPECTED APPLICABILITY OF THE RESEARCH

Investigation of purported insulin deaths ideally should be held to the same standard as all other deaths referred to the coroner or a medical examiner. This includes thorough scene investigation, a comprehensive medical record review, and appropriate toxicological testing. Therefore, it is expected that the successful completion of this project will provide an analytical method that forensic laboratories can use to test samples for exogenous insulin and C-peptide. Method validation outcomes (e.g., stability evaluation) can be used to inform forensic pathologists and morgue technicians about best sample handling practices to mitigate as many pre-analytical variables as possible.

PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

When this project was originally submitted for consideration, ten collaborating organizations had committed to either providing samples with case history for testing, application support, or other consultation as needed. At the time of project completion, over 75 additional agencies and organizations (e.g., medical examiners and law enforcement) had submitted samples for insulin testing. The latter is of special relevance because testing was requested for death investigation purposes.

CHANGES IN APPROACH FROM ORIGINAL DESIGN AND REASON FOR CHANGE, IF APPLICABLE

1. C-peptide was not included in final analytical scope of the method.
    - Reason(s): A suitable antibody for the immunopurification of C-peptide could not be consistently sourced.

2. Deludec was added to the final analytical scope of the method.
    - Reason(s): This insulin was approved by the FDA in 2015. We believed this was relevant addition to the test panel.
3. Method validation for blood was not completed.
    - Reason(s): The required reagent (HemogloBind™ from Biotech Support Group) was not obtainable for the final validation experiments. As such, the method was not used to test blood samples for reference interval determinations.
4. Delay of project completion.
    - Reason(s): Every effort was made to keep pace with the proposed project schedule. However, pandemic related issues dramatically slowed progress during the award period. Extended supply-chain disruptions meant several essential pieces of labware such as low retention plates and microcentrifuge tubes were incredibly difficult to obtain. Also, because CFSRE and NMS Labs are classified as "essential businesses", an executive-level decision was made to limit in-person access to facilities to keep laboratory personnel healthy so that the timely testing of patient samples would remain unaffected. With these factors in place, project completion was delayed until 2022.

OUTCOMES

ACTIVITIES/ACCOMPLISHMENTS

1. Analytical Method Development
    - Development of a quantitative multiple reaction monitoring (MRM) method on a triple quadrupole mass spectrometer.

    This project goal was completed. A comprehensive analytical workflow was developed and validated to determine exogenous analyte concentrations in vitreous fluid.

2. Automated Sample Processing
    - Development of an automated method that can be used to extract insulin and C-peptide from postmortem blood and vitreous humor.

    This project goal was partially completed. A comprehensive analytical workflow was developed and validated to determine exogenous insulin concentrations in vitreous fluid.

3. Method Validation
    - Validation of the developed method in concordance with SWGTOX (Scientific Working Group for Forensic Toxicology) guidelines.

This project goal was completed. An analytical quantitative method was developed and validated for the determination of exogenous insulins in vitreous fluid. This work was published in the Journal of Analytical Toxicology in 2019.

4. Population Assessment
    - Paired postmortem blood and vitreous humor samples will be collected and tested to establish therapeutic and overdose concentrations.
    - Acetone and β-hydroxybutyrate testing will be performed on the same blood and vitreous humor samples, and glucose testing will be performed on vitreous fluid samples.

This project goal was partially completed. Reference intervals were unable to be determined due to variables such as not receiving enough samples that were positive for each insulin included in the study. It should be noted, however, that over 200 case samples were tested during this project. The acetone, β-hydroxybutyrate, and glucose testing as described above was completed.

RESULTS AND FINDINGS

LINEARITY

Linearity was evaluated over 5 days for all analytes. The calibration parameters are shown in **Table 1**.

Table 1

	Glargine	Glulisine	Aspart	Lispro	Human	Detemir	Deludec
Slope	1.018	1.026	1.017	1.011	1.02	1.012	0.99
R2	0.99	0.99	0.99	0.99	0.99	0.99	0.99
Model	Linear	Linear	Linear	Linear	Linear	Linear	Linear
Curve Weighting	1/x	1/x	1/x	1/x	1/x	1/x	1/x

LIMIT OF DETECTION (LOD)

The LOD was defined as the lowest concentration which yielded an average signal of the negative samples plus 3.3 times the standard deviation. The LOD was assessed over 3 days in triplicate. The LODs for Glargine, Glulisine, Aspart, Lispro and human insulin was 0.125 ng/mL. The LOD for Detemir was 0.5 ng/mL. Because Deludec was a later addition to the test panel, the LOD for Deludec was not determined.

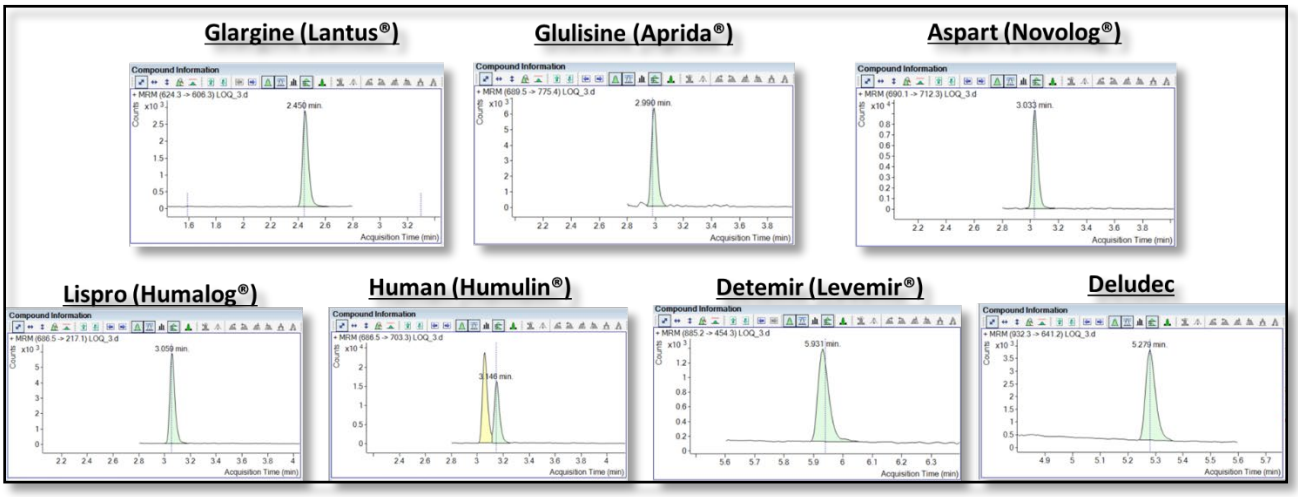
LIMIT OF QUANTIFICATION (LOQ)

Precision and accuracy of the LOQ (0.5 ng/mL) was evaluated with three replicates over three analytical batches. All targets performed acceptably with %CV values < 10% and all values within ± 20% of the target concentration. The mean and %CV for each insulin evaluated are shown in **Table 2** and representative chromatography is shown in **Figure 2**.

Table 2

	LOQ TARGET CONCENTRATION: 0.5 ng/mL						
	Glargine	**Glulisine**	**Aspart**	**Lispro**	**Human**	**Detemir**	**Deludec**
Mean	0.551	0.529	0.516	0.503	0.523	0.496	0.503
%CV	5.1	7.8	4.2	5.2	4.1	14.6	7.9

Figure 2: Chromatography at the LOQ (0.5 ng/mL)



PRECISION AND BIAS

Assessment of precision and bias occurred over three separate runs over a period of five days, with five replicates per day, at the LowQC (2.0 ng/mL), MidQC (4.0 ng/mL), and HighQC (8.0 ng/mL). Between and within run precision is %CV ≤ 20% at every level. Bias was <20% for all analytes. Results are shown in **Table 3** and **Table 4**.

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Table 3

	Precision (%CV)			Precision (%CV)		
	Within			Between		
	LowQC	MidQC	HighQC	LowQC	MidQC	HighQC
	(2.0 ng/mL)	(4.0 ng/mL)	(8.0 ng/mL)	(2.0 ng/mL)	(4.0 ng/mL)	(8.0 ng/mL)
Glargine	2.5	3.5	4.6	2.8	3.8	4.9
Glulisine	2.1	3.5	3.5	2.6	4.3	5.8
Aspart	2.8	1.9	2.2	2.9	2.6	3.5
Lispro	1.5	2.1	3.4	2.2	2.9	4
Human	0.9	5.9	4.8	2	9.6	4.7
Detemir	10.8	5.5	4.7	11.3	8.3	8.6
Deludec	4.6	5.7	2.2	4.9	6.9	4.2

Table 4

	Bias (%Difference)			Bias (%Difference)		
	Within			Between		
	LowQC	MidQC	HighQC	LowQC	MidQC	HighQC
	(2.0 ng/mL)	(4.0 ng/mL)	(8.0 ng/mL)	(2.0 ng/mL)	(4.0 ng/mL)	(8.0 ng/mL)
Glargine	-10.5	-4.9	-1.1	-10.1	-5.1	-1.2
Glulisine	-6.7	-0.7	2.8	-6.5	-2.3	1.7
Aspart	-4.1	-1.7	-1.2	-4.6	-2.1	-1.8
Lispro	-4.6	-1.6	-2.4	-4.9	-1.6	-2.3
Human	-5.6	-6.1	-0.8	-5.7	-6.1	-0.5
Detemir	-17.7	-9.8	11.9	-18.56	-10.5	8.6
Deludec	-3.4	1.4	9.1	-5.2	-0.7	6.9

CARRYOVER

Carryover was assessed by analyzing blank vitreous humor that was injected immediately following the highest calibration standard (25 ng/mL). Carryover was observed for Detemir. As such, blank injections consisting of 2% acetic acid in 70% acetonitrile were tested between all samples, calibration standards and quality controls.

INTERFERENCES

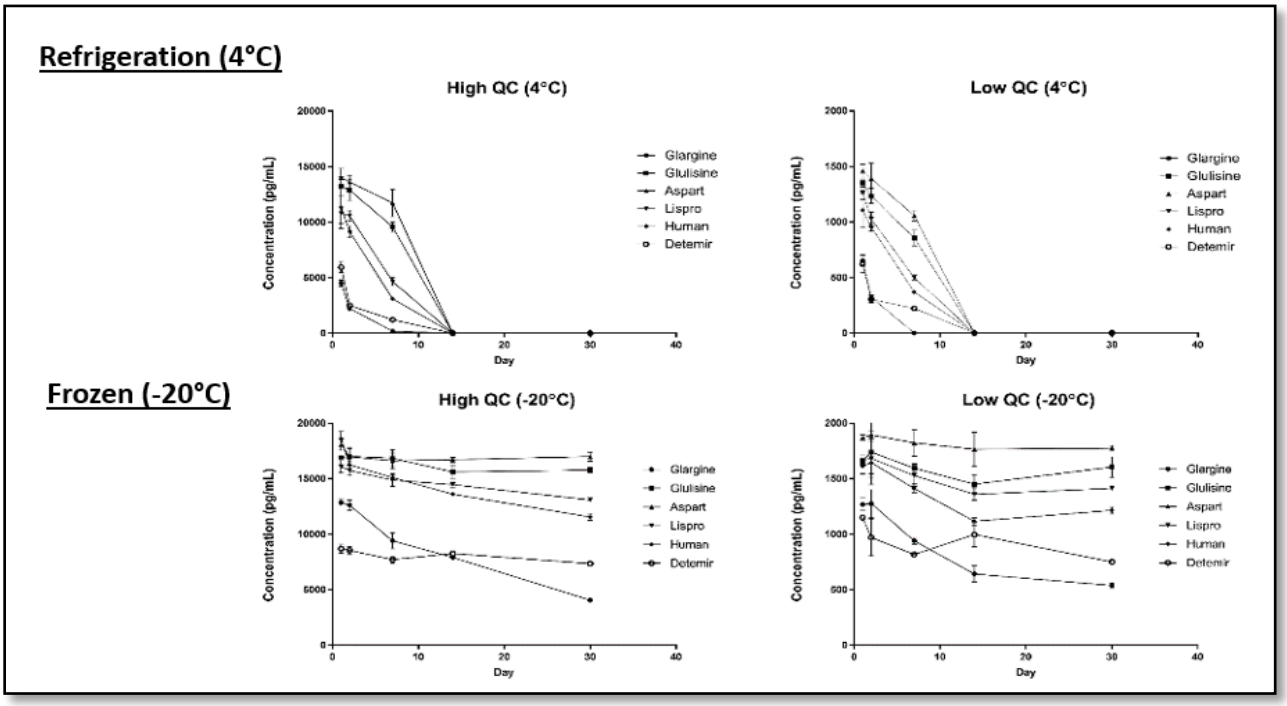
The interference assessment consisted of testing a series of commonly encountered drugs at concentrations consistent with therapeutic, toxic, and fatal levels. In addition, commercially available mammalian insulins including bovine (*Bos Taurus*) and equine (*Gallus equus*) were assayed. No interferences were observed from any low

molecular weight “small molecule” drugs. Bovine insulin did interfere (contribute signal) to the porcine internal standard, as expected since the β-chain sequences for these two insulins are identical.

STABILITY

Long-term stability was assessed using the LowQC and HighQC at room temperature, 4°C, and −20°C on Days 1, 2, 3, 7, 14 and 30. To ensure a realistic assessment of analyte stability, all samples were prepared in red-top glass vials. All insulin analogs were undetectable after one day when stored at room temperature. A complete loss of all insulin analogs was observed by Day 14 at 4°C. Results for all insulins except for Deludec are shown in **Figure 3**.

Figure 3



POPULATION ASSESSMENTS

Part 1: Vitreous Humor samples collected and submitted by project collaborators were tested to determine if reference intervals or other interpretive insight could be obtained. These groups with qualitative result outcomes shown in **Table 5**.

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Table 5

Group #	Sample Size	Group Description with Results
1	5	Description Insulin and non-insulin users where insulin use is believed be unrelated to their death.
		Results No insulin detected.
2	186	Description Insulin users where insulin is suspected to be related to their death.
		Results 48 = Positive – 17 = Aspart – 19 = Lispro – 12 = Human Insulin
3	44	Description Non-insulin users where insulin is suspected to be related to their death.
		Results 43 = None Detected – 1 = Positive for Detemir

Part 2: Acetone, vitreous glucose (VG), and to a lesser extent, beta-hydroxybutyric acid (BHB) are three target substances that are relied upon in postmortem toxicology as diagnostic markers of ketoacidosis. In this study (n=11), acetone, BHB, and VG concentrations were measured to evaluate their probative value in cases of suspected insulin overdose (**Table 6**). From this work, no correlations between ketone body, VG, and insulin concentrations were identified. Overall, this study substantiates that routine postmortem toxicology tests cannot be used to identify and/or confirm insulin overdose.

Table 6

ACETONE (mg/dL)	BHB (mcg/mL)	GLUCOSE (mg/dL)	INSULIN RESULT (ng/mL)
ND	170	46.8	**Aspart 7.6**
ND	ND	30.7	**Aspart < 0.5**
ND	ND	12.9	**Aspart < 0.5**
ND	44	19.2	**Lispro < 0.5**
ND	ND	12.6	**Aspart < 0.5**
ND	44	ND	**Aspart 1.6**
ND	ND	47.7	**Aspart 5.2**
ND	54	ND	**Human 0.62**
ND	120	380	**Human 0.98**
ND	50	ND	**Lispro 4.4**
ND	52	ND	**Aspart < 0.5**

LIMITATIONS

Two critical limitations that compromise the detection of exogenous insulin in any given sample and the development and practical use of reference intervals were identified. The first limitation is analyte stability. The stability assessment showed that all insulin analogues were undetectable after 1 day at room temperature and after 14 days at refrigerated temperature (4°C). This is relevant in practice because biological samples collected at autopsy are most often stored refrigerated. The second limitation pertains to concentration decreases observed when standard glassware is used as the collection container. This work shows that all target insulins exhibited concentration loses between 10 to 50% when samples were not collected and stored in low bind plastic tubes. This is relevant because the gray and red top collection containers are likely the most stocked and used for postmortem sample collections. Low bind plastic tubes need to be specially ordered and set aside for suspected insulin-involved deaths. These pre-analytical limitations are important because without an immediate case history and/or scene evidence that is suggestive of an exogenous insulin overdose, by the time the need for testing is identified, any insulin that was present may no longer be detectable. Furthermore, even if enough samples could be collected under carefully controlled conditions for reference interval determination, their usefulness especially in the prescribed-user population is expected to be minimal.

REFERENCE AND RESEARCH MATERIALS

List of products (e.g., publications, conference papers, technologies, websites, databases), including locations of these products on the Internet or in other archives or databases.

Publications:

Kevin M Legg, Laura M Labay, Sally S Aiken, Barry K Logan, Validation of a Fully Automated Immunoaffinity Workflow for the Detection and Quantification of Insulin Analogs by LC–MS-MS in Postmortem Vitreous Humor, *Journal of Analytical Toxicology*, Volume 43, Issue 7, September 2019, Pages 505–511, https://doi.org/10.1093/jat/bkz014

Dissemination Activities:

- West Virginia University College of Law "The Forensic Perspective", A Medicolegal Symposium on the Serial Murder Case of Reta Mays, 2021 Morgantown, WV.
- American Academy of Forensic Sciences, "Healthcare Serial Killers: Analytical Proof", 2020 72nd annual scientific meeting, Anaheim, CA.
- National Association of Medical Examiners "Identification and Quantification of Exogenous Insulin Analogs in Postmortem Specimens, 2019 NAME Annual Meeting, Kansas City, MO.
- Society of Forensic Toxicologists, "Identifications and Quantification of Exogenous Insulin Analogs in Postmortem Specimens" 2019 SOFT Annual Meeting, San Antonio, TX.
- American Academy of Forensic Sciences, "Robotic Extraction and LC/MS/MS Forensic Analysis of Insulin Analogs in Post-Mortem Samples", 2018 70th annual scientific meeting, Seattle, WA.
- American Academy of Forensic Sciences, "Automated Detection and Quantification of Insulin Analogs by LC/MS/MS in Post-Mortem Vitreous Humor", 2017 69th annual scientific meeting, New Orleans, LA.

Data Sets Generated:

Method development, validation, and case sample analytical data. Data exists as .d files on secure server.