The Determination of Insulin Overdose in Postmortem Investigations

Laura M. Labay, Casey P. Bitting, Kevin M. Legg, Barry K. Logan

ABSTRACT

The analysis of biological specimens for the presence of exogenous insulin is of special interest in select postmortem investigations. Insulin analogues are primarily used to mediate the regulation of blood glucose concentrations; however, their use has also been implicated or suspected as a cause of death in suicides, accidents, and homicides. Toxicological analysis for these compounds is challenging due to the large molecular weight, the limited stability of insulin in whole blood, and complexities associated with sample preparation and instrumental testing. As a consequence, determination of insulin in postmortem specimens is not routinely offered by most forensic toxicology laboratories. Forensic death investigation is further complicated by interpretative difficulties such as the frequent absence of anatomical findings, concentration interpretation in known insulin users, and addressing the impact of chemical instability and postmortem redistribution. There are ongoing efforts, however, to develop and validate robust methods that may be used for this analysis on these challenging samples and that are capable of withstanding scientific and legal scrutiny for forensic use. In this regard, in recent years, methods for the detection of exogenous insulin in postmortem samples have been reported and results of this testing has been published in a handful of cases. The purpose of this article is to review the primary functions of insulin, the disease states associated with the therapeutic use of exogenous insulin, the current state of laboratory testing, and to provide case summaries that summarize the timeline of advancements and underscore the importance of this work. *Acad Forensic Pathol.* 2016 6(2): 174-183

AUTHORS

Laura M. Labay PhD F-ABFT, NMS Labs - Toxicology

Roles: Data acquisition, analysis and/or interpretation, manuscript creation and/or revision, approved final version for publication, accountable for all aspects of the work, general administrative support, writing assistance and/or technical editing.

Casey P. Bitting DO, University of New Mexico School of Medicine - Pathology

Roles: Project conception and/or design, data acquisition, analysis and/or interpretation, manuscript creation and/or revision, approved final version for publication, accountable for all aspects of the work, general administrative support, writing assistance and/or technical editing.

Kevin M. Legg PhD, Center for Forensic Science Research and Education

Roles: Data acquisition, analysis and/or interpretation, manuscript creation and/or revision, approved final version for publication, accountable for all aspects of the work, writing assistance and/or technical editing.

Barry K. Logan PhD F-ABFT, NMS Labs - Toxicology

Roles: Project conception and/or design, manuscript creation and/or revision, approved final version for publication, accountable for all aspects of the work, principal investigator of the current study, general supervision, writing assistance and/or technical editing.

CORRESPONDENCE

Barry K. Logan MD, 3701 Welsh Rd, Willow Grove PA 19090, Barry.Logan@nmslabs.com

ETHICAL APPROVAL

As per Journal Policies, ethical approval was not required for this manuscript

STATEMENT OF HUMAN AND ANIMAL RIGHTS

This article does not contain any studies conducted with animals or on living human subjects

STATEMENT OF INFORMED CONSENT

No identifiable personal data were presented in this manuscript

DISCLOSURES & DECLARATION OF CONFLICTS OF INTEREST

The authors, reviewers, editors, and publication staff do not report any relevant conflicts of interest

FINANCIAL DISCLOSURE

The authors have indicated that they do not have financial relationships to disclose that are relevant to this manuscript

KEYWORDS

Forensic pathology, Insulin analogues, C-peptide, Toxicology, Overdose, Analytical testing, Cause and manner of death, Vitreous humor, Postmortem, Suicide, Homicide, Mass spectrometry, Liquid chromatography, Immunoassay

INFORMATION

ACADEMIC FORENSIC PATHOLOGY: THE OFFICIAL PUBLICATION OF THE NATIONAL ASSOCIATION OF MEDICAL EXAMINERS

©2017 Academic Forensic Pathology International • (ISSN: 1925-3621) • <https://doi.org/10.23907/2016.019>

Submitted for consideration on 19 Apr 2016. Accepted for publication on 24 May 2016

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 often coupled with a history of insulin abuse and/or misuse, and chemical confirmation of the presence of the exogenous compound. Considering these requirements, it stands to reason that a proportion of these deaths likely go unrecognized or misclassified. From a toxicological perspective, a key contribution to the investigations would be the ability to determine whether exogenous insulin is present and if possible, to determine the concentration and its significance. The major obstacle to this has been that traditional methods such as immunoassays used in clinical settings are typically not amenable for postmortem specimens due to interferences with hemolyzed samples in immunoassay methods. In the last decade, improvements in sample preparation and instrumentation have made progress in the detection and differentiation of insulin analogues in both insulin preparations and medical equipment (intravenous lines, and bags) and

using alternative biological matrices, most notably vitreous fluid (1-3). It is likely that these analytical advancements, 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 review article will summarize advances in technology that support the investigation of forensic cases where insulin use is suspected to have contributed to death.

DISCUSSION

Insulin Structure and Functions

Proinsulin, the precursor molecule of insulin, is a peptide that is synthesized in the β cells of the pancreas. It is cleaved by the action of a series of proteolytic enzymes to form insulin. This step removes the 31-amino acid residue termed C-peptide between the amino- and hydroxy-terminal ends. The final insulin peptide 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**) (4). In response to rising blood glucose concentrations, insulin is excreted with C-peptide in equimolar quantities into the portal circulation (5).

Figure 1: Primary peptide structure of human insulin adapted from USNLo. EXUBERA - insulin human drug label information, 2008 (4).

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 (6-8).

Insulin Therapy

Problems with insulin production and utilization characterize types 1 and 2 diabetes mellitus, respectively. Type 1 DM (T1DM) is caused by autoimmune destruction of the insulin-producing pancreatic β cells, resulting in low or absent blood insulin concentrations, and presents in childhood or early adulthood. Type 2 DM (T2DM) typically presents later in life and is characterized by insulin resistance in peripheral tissues (i.e., muscle, fat, and liver), diminishing insulin excretion and increased hepatic glucose production. Treatment for T1DM and for advanced T2DM is insulin injection (9).

While the symptoms of T1DM have been described for centuries, the pancreatic histological findings of T1DM were first described in 1902 by the German pathologist, Martin Schmidt, in an autopsy specimen of a 10-year-old child with diabetes (10). The Nobel Prize was later awarded to Banting and Best who first

isolated insulin from the pancreas in 1922, allowing for purification of animal insulin for treatment (9, 10). While this therapy was life-saving, the administration of animal insulin is associated with the development of antibodies to the exogenous insulin molecule (10). Since then, the development and use of recombinant human insulin has resulted in a dramatic decrease in symptomatic immune responses to insulin (11).

Like purified animal insulin, recombinant human insulin, or insulin analogues, are exogenous types of insulin because these are made outside of the human body. Insulin analogues are available in several forms with molecular structures similar to endogenous human insulin, some differing by as little as one amino acid. Synthetic human insulin (Humulin) is produced in bacteria or yeast and is structurally identical to endogenous human insulin. Exogenous insulins are classified as rapid-acting, intermediate-acting or long-acting depending on the speed of onset and duration of activity (**Table 1**). Chemical and pharmacological properties of the insulins are detailed in **Table 2**.

Specimen Handling

In a postmortem assessment, the most common matrix collected and then submitted for toxicological evaluation is blood. Unfortunately, due to the variations associated with postmortem blood such as putrefaction, hemolysis, and clotting, this specimen type is not amenable to insulin analysis by immunoassay platforms. The use of serum or plasma samples that have been saved by the hospital, especially if drawn within minutes or hours of the suspected time of insulin intoxication, and if refrigerated or frozen, may be viable for immunoassay testing. Studies show that 80% to 95% of insulin is recoverable from serum or plasma

Table 1: Insulin Classification by Onset, Peak, and Duration

Classification	Onset	Peak	Duration
Rapid-Acting	10-30 min	30 min-3 hours	3-5 hours
Short-Acting	30-60 min	2-5 hours	Up to 12 hours
Intermediate-Acting	90 min-4 hours	4-12 hours	Up to 24 hours
Long-Acting	45 min-4 hours	Minimal	Up to 24 hours

after six days of refrigeration at 4°C. However, insulin is nearly completely degraded by four to five days at room temperature (12). Thus, any biological specimen collected for insulin analogue testing should be collected in as timely a manner as possible relative to the initiation of the investigation and immediately stored frozen (no warmer than -20°C and ideally at -80°C).

Analytical Methods

Several methods capable of detecting and quantifying insulin have been reported. The insulin radioimmunoassay (RIA) was published by Berson and Yalow in 1960 (13). The sensitivity and reproducibility of RIA soon established it as the method of choice for most insulin investigations. The technique may also be employed for C-peptide determinations. Disadvantages to RIA, however, is that the test is time-consuming, subject to interferences, and requires laboratory personnel to work with radiolabeled agents. Today, commercially available enzyme-linked immunosorbent assay (ELISA) kits have been designed and developed to quantitatively measure human insulin and C-peptide in serum and/or plasma samples. The advent of these assays has improved sensitivity and reproducibility and, from a laboratory perspective, has lowered cost and reduced turnaround time.

From a forensic perspective however, these tests may not suffice, especially in the absence of supportive anamnestic case information. This is principally because current ligand-binding tests are unable to deliver discriminatory quantitative results from the laboratory to

the death investigator. Immunoassay cross-reactivity with nontarget compounds, dynamic range limitations and, most importantly, the ability to unambiguously differentiate endogenous insulin from recombinant pharmaceutical analogues remains likely unsolvable with traditional antibody-based immunoassays (14-16). Because of this, the identification of the insulin analogue should be made by a technique such as liquid chromatography tandem mass spectrometry (LC-MS/MS) that provides the ability to discriminate between the various synthetic analogues. Becoming increasingly popular for clinical endocrinology and sports medicine testing, LC-MS/MS assays are not subject cross-reactivity, provide higher specificity if not sensitivity, and are amenable to multiplexing multiple targets in a single test (17, 18). From a reporting standpoint, a well-designed LC-MS/MS assay should eliminate the concern that structurally similar compounds cross react, and therefore enable distinct identification and quantification of either endogenous human insulin or the analogous recombinant varieties.

Proteins can be simply defined as biological molecules that perform cellular functions. Ultimately, protein function is dictated by amino acid sequence leading to highly specialized tertiary and/or quaternary structure. While all common pharmaceutical analogues have been developed with slight structural differences (Table 3) to achieve various desired pharmacokinetic and pharmacodynamic properties, the ultimate biological function of these insulin analogues is the regulation of blood glucose concentrations (19, 20). However, because of their very similar molecular characteristics, including mass and structure, these

Table 2: Chemical and Pharmacological Properties

Compound Name	Trade Name	Empirical Formula	Molecular Weight (Da)	Onset of Action	Peak Action	Effective Duration	Maximum Duration
Insulin (human)	Humulin	C ₂₅₇ H ₃₈₃ N ₆₅ O ₇₇ S ₆	5808.0	0.5-1 hr	2-4 hr	6-8 hr	---
Lispro	Humalog	C ₂₅₇ H ₃₈₃ N ₆₅ O ₇₇ S ₆	5808.0	15-30 min	30 min-1.25 hr	3-4 hr	4-6 hr
Aspart	NovoLog	C ₂₅₆ H ₃₈₁ N ₆₅ O ₇₉ S ₆	5825.8	15-30 min	30 min-1.25 hr	3-4 hr	4-6 hr
Glulisine	Apidra	C ₂₅₈ H ₃₈₄ N ₆₄ O ₇₈ S ₆	5823.0	15-30 min	30 min-1.25 hr	3-4 hr	4-6 hr
Glargine	Lantus	C ₂₆₇ H ₄₀₄ N ₇₂ O ₇₈ S ₆	6063.0	8-16 hr	18-20 hr	18-20 hr	20-24 hr
Detemir	Levemir	C ₂₆₇ H ₄₀₂ O ₇₆ N ₆₄ S ₆	5916.9	6-8 hr	14 hr	14 hr	~2 hr

compounds also have similar analytical characteristics, specifically, chromatographic retention time and fragmentation spectra. Taken together, clear differentiation remains a challenge even for modern LC-MS/MS based approaches, requiring careful method optimization, thorough method validation, and strict quality control.

In recent years, several LC-MS/MS based methods have been published. The most commonly employed technique combines low flow chromatography (microbore or nanobore) with anti-insulin antibody immunopurification (3, 21-23). While these approaches have seen some success, complex sample preparation steps associated with immunocapture purification, as well as lengthy run times, lack of robustness, and reproducibility associated with low flow separations make these approaches largely unsuitable for forensic analysis (24, 25). More recent applications have omitted immunopurification in lieu of multidimensional chromatographic purification as well as introducing MS-level separation via ion mobility mass spectrometry (26, 27). However, no single approach has successfully integrated a straightforward, production-compatible, preparation method with unequivocal, unambiguous, discrimination between insulin and the recombinant analogues. Combining additional

approaches and advances such as immunopurification automation, novel column chemistries and preparation techniques (e.g., enzyme digestion) are likely to provide a solution in the near future (28).

Interpretation of Findings

There are two main patient populations to consider: those that are not prescribed insulin and those that are prescribed insulin. In the former group, if exogenous insulin is identified – regardless of concentration – this is substantive evidence that insulin may have at least contributed to death, especially in the absence of a more competent cause. In the latter population, it might be expected for the analytical method to detect an exogenous insulin. Quantitative analysis to determine concentration would be warranted in this circumstance and should be compared to concentrations corresponding to compliant therapeutic use when available for that particular specimen type.

Another interpretive avenue is the consideration of the insulin to C-peptide (I:C) ratio. This ratio has been used to indicate the use or administration of exogenous insulin (12, 29). In living individuals, the plasma half-life of insulin is between five to eight minutes, while the plasma half-life of C-peptide is between ten

Table 3: Amino Acid Sequence of Human and Insulin Analogues

Compound	Chain	Sequence
Insulin (Human)	α -chain	GIVEQCCTSICSLYQLENYCN
	β -chain	FVNQHLCGSHLVEALYLVCGERGFFYTPKT
Lispro (Humalog)	α -chain	GIVEQCCTSICSLYQLENYCN
	β -chain	FVNQHLCGSHLVEALYLVCGERGFFYTKPT
Aspart (Novalog)	α -chain	GIVEQCCTSICSLYQLENYCN
	β -chain	FVNQHLCGSHLVEALYLVCGERGFFYTDKT
Glulisine (Apidra)	α -chain	GIVEQCCTSICSLYQLENYCN
	β -chain	FVKQHLCGSHLVEALYLVCGERGFFYTPET
Glargine (Lantus)	α -chain	GIVEQCCTSICSLYQLENYCG
	β -chain	FVNQHLCGSHLVEALYLVCGERGFFYTPKTRR
Detemir (Levemir)	α -chain	GIVEQCCTSICSLYQLENYCN
	β -chain	FVNQHLCGSHLVEALYLVCGERGFFYTPK + Myristic acid

to 20 minutes (30, 31). The liver breaks down insulin during first metabolism (a lesser amount of insulin is subsequently eliminated by the kidneys), whereas C-peptide is removed from circulation exclusively via the kidneys (32, 33). Most importantly, because both insulin and C-peptide are secreted from the pancreas in equimolar concentrations, the ratio of insulin to C-peptide should be near 1.0 or even slightly lower due to the somewhat longer half-life of C-peptide (34). For example, the I:C ratio in a normal healthy living person is 0.12 - 0.47 and never exceeds 1.0. In contrast, for people who use exogenous insulin, the I:C ratio would be expected to be greater than 1.0 (29). Taking this information into account, calculation of I:C ratio using total insulin (endogenous and exogenous) and C-peptide values may be beneficial, particularly when the analytical method (e.g., immunoassay) does not differentiate endogenous insulin from a suspected exogenous insulin.

It is important to consider that, like insulin, C-peptide concentrations may be influenced by certain disease states or through the use of certain drugs and/or medications. For example, insulin and C-peptide concentrations are both elevated in insulinoma, renal failure, and Cushing syndrome. Therefore, even though the patient is experiencing a medical event, the insulin to C-peptide molar ratio is preserved at less than or equal to 1. In contrast, in patients with insulin autoantibodies, as occurs in T1DM, the insulin to C-peptide ratio may be reversed to greater than 1, due to the prolonged half-life of autoantibody-bound insulin. Lastly, failing insulin secretion, as occurs in T1DM and longstanding T2DM, is associated with corresponding reductions in serum C-peptide concentrations (35).

In addition to directly testing for insulin and C-peptide, alternate analytes such as glucose, lactic acid, and ketone bodies may be considered. A postmortem glucose concentration of less than 200 mg/dL is considered normal. Because glucose concentrations decline rapidly following death, a postmortem vitreous glucose concentration of greater than or equal to 200 mg/dL is diagnostic of uncontrolled diabetes (36). The finding of either a normal or elevated vitreous glucose concentration suggests that exogenous insulin

is not present at a concentration consistent with a lethal outcome. The same rationale may be applied to the evaluation of ketone bodies. When the pancreas produces insufficient amounts of insulin or no insulin, cells receive inadequate quantities of glucose. As a consequence, the body begins to break adipose (and, depending on the concentration of insulin, possibly muscle) stores, and ketone bodies mainly consisting of β -hydroxybutyrate, acetoacetate, and acetone are produced. β -hydroxybutyrate is the major ketone produced in alcoholic and diabetic ketoacidosis (DKA). Ketones may also be detected in cases of malnutrition/starvation and isopropanol ingestion (37). Similar to all toxicological interpretation, it is relevant to consider the impact of any medical intervention prior to death that would affect the presence and/or amounts of these analytes.

Autopsy Studies

The discovery of the insulin molecule in 1922 (9, 10), while life-saving for so many diagnosed with T1DM, has been tainted by unintentional therapeutic mismanagement, sport doping-related abuse (38), and even malicious use (3, 6, 12, 29, 37, 39, 40) of the molecule in ensuing years. Hospital emergency rooms are familiar with hypoglycemia in the setting of insulin misuse, and the number of such presentations representing suicidal attempts or gestures is unmistakably underestimated (41). Of a total of 2 424 180 calls to poison control centers in the United States in 2005, 3934 (0.16%) were related to insulin exposure. While this number is comparatively low compared to other calls for toxic exposures, the majority of calls for insulin toxicity seem to be related to deliberate self-poisoning. In a retrospective study of 160 telephone inquiries to one regional poison unit, 89.4% were suicidal or parasuicidal, 5.0% were accidental, 1.9% were due to criminal overdose, and 3.7% were classified as another reason (42). Indeed, both T1DM and T2DM are associated with a two-fold higher rate of depression as compared to the general population (43). While the majority of self-administered insulin toxicities are by patients with diabetes, a portion is by nondiabetics using insulin not prescribed to them (5, 44).

From a forensic standpoint, establishing insulin toxicity as a cause of death remains challenging. A thorough investigation noting and collecting insulin syringes and vials, a suicide note, or even an eyewitness account, is particularly valuable. In the case of investigating hospital-associated overdose, whether accidental or intentional, catheter-associated lines and fluid bags should be collected and examined for insulin residue (3). When available, antemortem serum and urine samples, especially those closely temporally related to the toxic administration, should be stored frozen for later insulin detection studies (3, 40). Additionally, anamnestic information such as a history of depression, prior suicide attempts, substance abuse, diabetic status, occupation, and relationship to someone with a diagnosis of diabetes is also useful (6). Unfortunately, such helpful information is often not present and the diagnosis of insulin toxicity may rely solely on autopsy findings, which may be unremarkable.

From a morphological perspective, external examination of insulin toxicity cases may reveal little or no insight. Because insulin is most often administered using high-gauge needles, puncture sites may be difficult or impossible to find. When identified, however, photodocumentation is warranted, and some authors have reported the utility of excising the injection site for insulin detection (6, 45-47), as well as examination of the tissue by immunohistochemistry (48, 49). The importance of ruling out other causes of hyperinsulinemia merits careful inspection of the pancreas for an insulinoma. Morphological evidence of central nervous system dysregulation, though nonspecific, may be apparent in the form of pulmonary edema and cerebral edema (44). Depending on post-injection survival interval, as well as on hospitalization or postmortem imaging modalities, magnetic resonance imaging findings of hypoglycemia-related encephalopathy may be evident as disseminated hypersignals of the cerebral gray matter (50).

The diagnosis of hypoglycemia secondary to hyperinsulinemia as a cause of death is a great deal more difficult than the diagnosis of such in the living. In the setting of an acute presentation to the emergency

room, a patient with suspected insulin toxicity might be thoroughly evaluated through blood glucose, insulin, and C-peptide concentrations. In fact, autopsy workup of insulin toxicity has historically proceeded in the same manner. However, postmortem sampling is complicated by an endless number of factors. For example, death following insulin overdose is not necessarily immediate and, depending on the type of insulin used (onset and duration of action), the time to hypoglycemic coma can be 20 minutes or much longer (39). Such a length of time allows for metabolism and elimination of the insulin molecule prior to death. Furthermore, decedents may be found hours, days, or longer after death, in which case the insulin molecule is additionally subjected to insulinase, an enzyme present in several tissues and released by red blood cells during hemolysis (51, 52). Insulinase activity, like all enzymes, is highly dependent on temperature (12), explaining the importance of rapid sample recovery and of freezing samples for insulin analysis as described above (6, 12). Similar to insulin concentrations, blood glucose concentrations (53) and C-peptide concentrations have also been shown to decrease after death (29, 54, 55).

Iwase and Kobayashi studied 18 control cadaver cases to determine postmortem I:C ratio in order to substantiate a case of homicidal insulin intoxication (29). The authors quantified insulin and C-peptide in the victim and controls using commercial radioimmunoassay kits on blood samples, which have known limitations. Control cases in which hemolysis of the sample had occurred were excluded. The authors found a mean insulin value in control cadavers of $8.1 \pm 5.1 \mu\text{U/mL}$ ($0.058 \pm 0.037 \text{ nmol/L}$) (CV=156%) and C-peptide concentrations ranging 55 to 390 pmol/L ($0.2\text{-}1.4 \text{ ng/mL}$). The mean I:C ratio was 0.478 ± 0.247 (range 0.081-1.03) (CV=193%). Interestingly, cadavers examined less than 24 hours after death had a significantly lower I:C ratio (0.387 ± 0.195 ; $n=12$) than cadavers examined more than 24 hours after death (0.660 ± 0.255 ; $n=6$) ($p < 0.01$). The authors concluded that as time after death increases, the C-peptide concentration decreases and the I:C increases (29). This finding is somewhat counterintuitive to the known half-lives of insulin and C-peptide, which would predict a de-

crease in I:C over time as insulin is preferentially broken down, and C-peptide is one of the products. This phenomenon deserves further investigation once more robust analytical methods are available.

Another study found that insulin concentrations in nondiabetic postmortem blood ranged from 0-69 $\mu\text{U/mL}$ (0-0.50 nmol/L) (6). Kernbach-Wighton and Puschel performed a retrospective review of 12 cases of suicide by insulin, with a postmortem time frame of one day to six weeks, also utilizing RIA for insulin quantification in blood and cerebrospinal fluid (CSF) (44). The authors found that peripheral blood insulin concentrations were elevated (range 1-155 $\mu\text{U/mL}$; mean 49 $\mu\text{U/mL}$), and that the mean CSF insulin values were about 30% greater than the peripheral blood values (44). Therefore, the authors indicated a preference for CSF (and vitreous humor) over blood for postmortem analysis of glucose, lactate and insulin (44). Furthermore, the authors found that the Traub score (calculated by adding the glucose and lactate concentrations in the CSF or vitreous in mg/dL), used to make conclusions about the antemortem status of blood glucose concentrations and metabolism (56, 57), could not be used to diagnose lethal hypoglycemia in the case of longer postmortem intervals (44). The value of the Traub score has been further questioned by other workers who demonstrated limited effectiveness in the estimation of antemortem glucose and the diagnosis of hyperglycemia due to increases in lactate after death, noting instead that vitreous glucose concentration itself appears to be the most reliable marker along with other candidate biochemical markers such as acetone, 3- β -hydroxybutyrate, urine glucose, and glycated hemoglobin, to confirm diabetic ketoacidosis as the cause of death (58).

While immunoassays, especially radioimmunoassays, may be able to provide the necessary sensitivity for use in quantifying insulin concentrations in the forensic setting (59), immunopurification combined with ultrahigh performance liquid chromatography-high resolution/high accuracy (tandem) mass spectrometry provides the specificity to differentiate between the various insulin analogues (3). Indeed, the latter method has been successfully employed in the identifica-

tion of insulin in postmortem material (vitreous humor) in a case of insulin poisoning (3). In that case, a nondiabetic female was admitted, comatose, to intensive care following a severe drop in her blood glucose concentration from a normal concentration of 89 mg/dL to severe hypoglycemia (2 mg/dL). She died from complications four days later. The corresponding change in human insulin concentrations determined by the authors LC-MS/MS, before and after admission to the intensive care unit went from normal (5.9 mIU/L; 0.2 ng/mL), to extremely elevated (5551 mIU/L; 194 ng/mL). The C-peptide concentration measurements determined by immunoassay were within the normal range (1.7-4.8 ng/mL) on both draws. The death was ultimately determined to be a poisoning by nonmedically administered human insulin (3).

CONCLUSION

Investigation of purported insulin deaths must include several facets including thorough scene and postmortem investigations, a comprehensive medical record review, and appropriate toxicological testing. The latter, based upon current analytical approaches, would ideally utilize mass spectrometry so that the identification of any insulin analogue is verified. If C-peptide is included in the scope of analysis and a quantitative method used, the I:C ratio can be calculated. This latter technique could be accomplished by mass spectrometry and immunoassay platforms. It is recommended that specimen handling practices and submission guidelines be reviewed with the testing laboratory prior to actual need to mitigate as many pre-analytical variables as possible.

REFERENCES

- 1) Blackburn M. Advances in the quantitation of therapeutic insulin analogues by LC-MS/MS. *Bioanalysis*. 2013 Dec; 5(23):2933-46. PMID: 24295119. <http://dx.doi.org/10.4155/bio.13.257>.
- 2) Hess C, Madea B, Daldrup T, Musshoff F. Determination of hypoglycaemia induced by insulin or its synthetic analogues post mortem. *Drug Test Anal*. 2013 Sep-Oct; 5(9-10):802-7. PMID: 23847093. <http://dx.doi.org/10.1002/dta.1500>.
- 3) Thevis M, Thomas A, Schänzer W, et al. Measuring insulin in human vitreous humour using LC-MS/MS. *Drug Test Anal*. 2012 Jan; 4(1): 53-6. PMID: 22102262. <http://dx.doi.org/10.1002/dta.368>.
- 4) Medicine. USNLo. EXUBERA - insulin human drug label information. 2008.

- 5) Winston DC. Suicide via insulin overdose in nondiabetics: the New Mexico experience. *Am J Forensic Med Pathol.* 2000 Sep; 21(3): 237-40. PMID: 10990283. <http://dx.doi.org/10.1097/00000433-200009000-00010>.
- 6) Fletcher SM. Insulin. A forensic primer. *J Forensic Sci Soc.* 1983 Jan; 23(1):5-17. PMID: 6339673. [http://dx.doi.org/10.1016/s0015-7368\(83\)71537-9](http://dx.doi.org/10.1016/s0015-7368(83)71537-9).
- 7) Grossman SP. The role of glucose, insulin and glucagon in the regulation of food intake and body weight. *Neurosci Biobehav Rev.* 1986 Fall; 10(3):295-315. PMID: 3095717. [http://dx.doi.org/10.1016/0149-7634\(86\)90015-1](http://dx.doi.org/10.1016/0149-7634(86)90015-1).
- 8) Sonksen P, Sonksen J. Insulin: understanding its action in health and disease. *Br J Anaesth.* 2000 Jul; 85(1):69-79. PMID: 10927996. <http://dx.doi.org/10.1093/bja/85.1.69>.
- 9) Hall JE, Guyton AC. Guyton and Hall textbook of medical physiology. 12th ed. Philadelphia: Saunders/Elsevier; 2010. 1120 p.
- 10) Williams RH, Larsen PR. Williams textbook of endocrinology. 10th ed. Philadelphia: Saunders; 2003. 1927 p.
- 11) Scherthner G, Borkenstein A, Fink M, et al. Immunogenicity of human insulin (Novo) or pork monocomponent insulin in HLA-DR-typed insulin-dependent diabetic individuals. *Diabetes Care.* 1983 Mar-Apr; 6 Suppl 1:43-8. PMID: 6343038.
- 12) Haibach H, Dix JD, Shah JH. Homicide by insulin administration. *J Forensic Sci.* 1987 Jan; 32(1):208-16. PMID: 3546589. <http://dx.doi.org/10.1520/jfs12344j>.
- 13) Yalow RS, Berson SA. Immunoassay of endogenous plasma insulin in man. *J Clin Invest.* 1960 Jul; 39(7):1157-75. PMID: 13846364. PMCID: PMC441860. <http://dx.doi.org/10.1172/jci104130>.
- 14) Parfitt C, Church D, Armston A, et al. Commercial insulin immunoassays fail to detect commonly prescribed insulin analogues. *Clin Biochem.* 2015 Dec; 48(18):1354-7. PMID: 26171976. <http://dx.doi.org/10.1016/j.clinbiochem.2015.07.017>.
- 15) Heurtault B, Reix N, Meyer N, et al. Extensive study of human insulin immunoassays: promises and pitfalls for insulin analogue detection and quantification. *Clin Chem Lab Med.* 2014 Mar; 52(3): 355-62. PMID: 24150210. <http://dx.doi.org/10.1515/ccclm-2013-0427>.
- 16) Glenn C, Armston A. Cross-reactivity of 12 recombinant insulin preparations in the Beckman Unicel Dxi 800 insulin assay. *Ann Clin Biochem.* 2010 May; 47(Pt 3):264-6. PMID: 20392753. <http://dx.doi.org/10.1258/acb.2010.010002>.
- 17) van den Ouweland JM, Kema IP. The role of liquid chromatography-tandem mass spectrometry in the clinical laboratory. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2012 Feb 1; 883-884:18-32. PMID: 22197607. <http://dx.doi.org/10.1016/j.jchromb.2011.11.044>.
- 18) Carvalho VM. The coming of age of liquid chromatography coupled to tandem mass spectrometry in the endocrinology laboratory. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2012 Feb 1; 883-884:50-8. PMID: 21907642. <http://dx.doi.org/10.1016/j.jchromb.2011.08.027>.
- 19) Sheldon B, Russell-Jones D, Wright J. Insulin analogues: an example of applied medical science. *Diabetes Obes Metab.* 2009 Jan; 11(1): 5-19. PMID: 19120431. <http://dx.doi.org/10.1111/j.1463-1326.2008.01015.x>.
- 20) Vajo Z, Fawcett J, Duckworth WC. Recombinant DNA technology in the treatment of diabetes: insulin analogs. *Endocr Rev.* 2001 Oct; 22(5):706-17. PMID: 11588149. <http://dx.doi.org/10.1210/er.22.5.706>.
- 21) Hess C, Thomas A, Thevis M, et al. Simultaneous determination and validated quantification of human insulin and its synthetic analogues in human blood serum by immunoaffinity purification and liquid chromatography-mass spectrometry. *Anal Bioanal Chem.* 2012 Oct; 404(6-7):1813-22. PMID: 22865008. <http://dx.doi.org/10.1007/s00216-012-6271-5>.
- 22) Ojanperä I, Sajantila A, Vinogradova L, et al. Post-mortem vitreous humour as potential specimen for detection of insulin analogues by LC-MS/MS. *Forensic Sci Int.* 2013 Dec 10; 233(1-3):328-32. PMID: 24314537. <http://dx.doi.org/10.1016/j.forsciint.2013.10.009>.
- 23) Thomas A, Schänzer W, Delahaut P, Thevis M. Immunoaffinity purification of peptide hormones prior to liquid chromatography-mass spectrometry in doping controls. *Methods.* 2012 Feb; 56(2):230-5. PMID: 21871962. <http://dx.doi.org/10.1016/j.ymeth.2011.08.009>.
- 24) Percy AJ, Chambers AG, Yang J, et al. Comparison of standard- and nano-flow liquid chromatography platforms for MRM-based quantitation of putative plasma biomarker proteins. *Anal Bioanal Chem.* 2012 Sep; 404(4):1089-101. PMID: 22547352. <http://dx.doi.org/10.1007/s00216-012-6010-y>.
- 25) Gaspari M, Cuda G. Nano LC-MS/MS: a robust setup for proteomic analysis. *Methods Mol Biol.* 2011; 790:115-26. PMID: 21948410. http://dx.doi.org/10.1007/978-1-61779-319-6_9.
- 26) Chambers EE, Fountain KJ, Smith N, et al. Multidimensional LC-MS/MS enables simultaneous quantification of intact human insulin and five recombinant analogs in human plasma. *Anal Chem.* 2014 Jan 7; 86(1):694-702. PMID: 24345052. <http://dx.doi.org/10.1021/ac403055d>.
- 27) Thomas A, Schänzer W, Thevis M. Determination of human insulin and its analogues in human blood using liquid chromatography coupled to ion mobility mass spectrometry (LC-IM-MS). *Drug Test Anal.* 2014 Nov-Dec; 6(11-12):1125-32. PMID: 25219675. <http://dx.doi.org/10.1002/dta.1710>.
- 28) Schoenherr RM, Saul RG, Whiteaker JR, et al. Anti-peptide monoclonal antibodies generated for immuno-multiple reaction monitoring-mass spectrometry assays have a high probability of supporting Western blot and ELISA. *Mol Cell Proteomics.* 2015 Feb; 14(2): 382-98. PMID: 25512614. PMCID: PMC4350033. <http://dx.doi.org/10.1074/mcp.o114.043133>.
- 29) Iwase H, Kobayashi M, Nakajima M, Takatori T. The ratio of insulin to C-peptide can be used to make a forensic diagnosis of exogenous insulin overdose. *Forensic Sci Int.* 2001 Jan 1; 115(1-2): 123-7. PMID: 11056282. [http://dx.doi.org/10.1016/s0379-0738\(00\)00298-x](http://dx.doi.org/10.1016/s0379-0738(00)00298-x).
- 30) Berne R, Levy M, Koeppen B, Stanton BA Physiology. 4th ed. St. Louis: CV Mosby; 1998. 1131 p.
- 31) Matthews DR, Rudenski AS, Burnett MA, et al. The half-life of endogenous insulin and C-peptide in man assessed by somatostatin suppression. *Clin Endocrinol (Oxf).* 1985 Jul; 23(1):71-9. PMID: 2863015. <http://dx.doi.org/10.1111/j.1365-2265.1985.tb00185.x>.
- 32) Johnson DG, Alberti KG, Faber OK, Binder C. Hyperinsulinism of hepatic cirrhosis: Diminished degradation or hypersecretion? *Lancet.* 1977 Jan 1; 309(8001):10-3. PMID: 63654. [http://dx.doi.org/10.1016/s0140-6736\(77\)91652-x](http://dx.doi.org/10.1016/s0140-6736(77)91652-x).
- 33) Kuzuya T, Matsuda A, Sakamoto Y, et al. C-peptide immunoreactivity (CPR) in urine. *Diabetes.* 1978; 27 Suppl 1:210-215. PMID: 564800. <http://dx.doi.org/10.2337/diab.27.1.s210>.
- 34) Lebowitz MR, Blumenthal SA. The molar ratio of insulin to C-peptide. An aid to the diagnosis of hypoglycemia due to surreptitious (or inadvertent) insulin administration. *Arch Intern Med.* 1993 Mar 8; 153(5):650-5. PMID: 8439228. <http://dx.doi.org/10.1001/archinte.1993.00410050082011>.

- 35) Merck manual: professional version [Internet]. Kenilworth (NJ): Merck Sharp & Dohme Corp.; c2016. Blood tests: normal values; [cited 2016 May 27]. Available from: <http://www.merckmanuals.com/professional/appendixes/normal-laboratory-values/blood-tests-normal-values>.
- 36) Rose KL, Collins KA. NewsPath: Vitreous postmortem chemical analysis [Internet]. Washington: College of American Pathologists; 2008 Dec [cited 2016 Apr 19]. 4 p. Available from: http://www.cap.org/apps/docs/newspath/0812/vitreous_postmortem_chemical_analysis.pdf.
- 37) Green RP, Hollander AS, Thevis M, et al. Detection of surreptitious administration of analog insulin to an 8-week-old infant. *Pediatrics*. 2010 May; 125(5):e1236-40. PMID: 20385635. <http://dx.doi.org/10.1542/peds.2009-2273>.
- 38) Holt RI, Sönksen PH. Growth hormone, IGF-I and insulin and their abuse in sport. *Br J Pharmacol*. 2008 Jun; 154(3):542-56. PMID: 18376417. PMCID: PMC2439509. <http://dx.doi.org/10.1038/bjp.2008.99>.
- 39) Marks V. Murder by insulin: suspected, purported and proven-a review. *Drug Test Anal*. 2009 Apr; 1(4):162-76. PMID: 20355194. <http://dx.doi.org/10.1002/dta.38>.
- 40) Koskinen PJ, Nuutinen HM, Laaksonen H, et al. Importance of storing emergency serum samples for uncovering murder with insulin. *Forensic Sci Int*. 1999 Oct 25; 105(1):61-6. PMID: 10605076. [http://dx.doi.org/10.1016/s0379-0738\(99\)00111-5](http://dx.doi.org/10.1016/s0379-0738(99)00111-5).
- 41) Russell KS, Stevens JR, Stern TA. Insulin overdose among patients with diabetes: a readily available means of suicide. *Prim Care Companion J Clin Psychiatry*. 2009 Oct; 11(5):258-62. PMID: 19956464. PMCID: PMC2781038. <http://dx.doi.org/10.4088/pcc.09r00802>.
- 42) von Mach MA, Meyer S, Omogbehin B, et al. Epidemiological assessment of 160 cases of insulin overdose recorded in a regional poisons unit. *Int J Clin Pharmacol Ther*. 2004 May; 42(5):277-80. <http://dx.doi.org/10.5414/cpp42277>.
- 43) Rush WA, Whitebird RR, Rush MR, et al. Depression in patients with diabetes: does it impact clinical goals? *J Am Board Fam Med*. 2008 Sep-Oct; 21(5):392-7. PMID: 18772293. <http://dx.doi.org/10.3122/jabfm.2008.05.070101>.
- 44) Kernbach-Wighton G, Püschel K. On the phenomenology of lethal applications of insulin. *Forensic Sci Int*. 1998 Apr 22; 93(1):61-73. PMID: 9618911. [http://dx.doi.org/10.1016/s0379-0738\(98\)00032-2](http://dx.doi.org/10.1016/s0379-0738(98)00032-2).
- 45) Dickson SJ, Cairns ER, Blazey ND. The isolation and quantitation of insulin in post-mortem specimens--a case report. *Forensic Sci*. 1977 Jan-Feb; 9(1):37-42. PMID: 838414. [http://dx.doi.org/10.1016/0300-9432\(77\)90063-2](http://dx.doi.org/10.1016/0300-9432(77)90063-2).
- 46) Phillips AP, Webb B, Curry AS. The detection of insulin in postmortem tissues. *J Forensic Sci*. 1972 Jul; 17(3):460-3. PMID: 4669593. <http://dx.doi.org/10.1520/jfs10134j>.
- 47) Campbell IW, Ratcliffe JG. Suicidal insulin overdose managed by excision of insulin injection site. *Br Med J (Clin Res Ed)*. 1982 Aug 7; 285(6339):408-9. PMID: 6809104. PMCID: PMC1499212. <http://dx.doi.org/10.1136/bmj.285.6339.408>.
- 48) Hood I, Mirchandani H, Monforte J, Stacer W. Immunohistochemical demonstration of homicidal insulin injection site. *Arch Pathol Lab Med*. 1986 Oct; 110(10):973-4. PMID: 3533005.
- 49) Lutz R, Pedal I, Wetzel C, Mattern R. Insulin injection sites: morphology and immunohistochemistry. *Forensic Sci Int*. 1997 Nov 10; 90(1-2):93-101. PMID: 9438369. [http://dx.doi.org/10.1016/s0379-0738\(97\)00153-9](http://dx.doi.org/10.1016/s0379-0738(97)00153-9).
- 50) Mégarbane B, Deye N, Bloch V, et al. Intentional overdose with insulin: prognostic factors and toxicokinetic/toxicodynamic profiles. *Crit Care*. 2007; 11(5):R115. PMID: 17963523. PMCID: PMC2556768. <http://dx.doi.org/10.1186/cc6168>.
- 51) Roth RA, Mesirow ML, Cassell DJ, et al. Characterization of an insulin degrading enzyme from cultured human lymphocytes. *Diabetes Res Clin Pract*. 1985 Mar; 1(1):31-9. PMID: 3915257. [http://dx.doi.org/10.1016/s0168-8227\(85\)80026-7](http://dx.doi.org/10.1016/s0168-8227(85)80026-7).
- 52) Sapin R, Ongagna JC, Gasser F, Grucker D. Insulin measurements in haemolysed serum: influence of insulinase inhibitors. *Clin Chim Acta*. 1998 Jun 8; 274(1):111-7. PMID: 9681602. [http://dx.doi.org/10.1016/s0009-8981\(98\)00051-5](http://dx.doi.org/10.1016/s0009-8981(98)00051-5).
- 53) Gormsen H, Lund A. The diagnostic value of postmortem blood glucose determinations in cases of diabetes mellitus. *Forensic Sci Int*. 1985 Jun-Jul; 28(2):103-7. PMID: 4043894. [http://dx.doi.org/10.1016/0379-0738\(85\)90066-0](http://dx.doi.org/10.1016/0379-0738(85)90066-0).
- 54) Logemann E, Pollak S, Khalaf AN, Petersen KG. [Postmortem diagnosis of exogenous insulin administration]. *Arch Kriminol*. 1993 Jan-Feb; 191(1-2):28-36. German. PMID: 8452472.
- 55) Given BD, Ostrega DM, Polonsky KS, et al. Hypoglycemia due to surreptitious injection of insulin. Identification of insulin species by high-performance liquid chromatography. *Diabetes Care*. 1991 Jul; 14(7):544-7. PMID: 1914793. <http://dx.doi.org/10.2337/diacare.14.7.544>.
- 56) Naumann HN. Studies on postmortem chemistry. *Am J Clin Pathol*. 1950 Apr; 20(4):314-24. PMID: 15432319. <http://dx.doi.org/10.1093/ajcp/20.4.314>.
- 57) Naumann HN. Postmortem chemistry of the vitreous body in man. *Arch Ophthalmol*. 1959 Sep; 62:356-63. PMID: 14426195. <http://dx.doi.org/10.1001/archophth.1959.04220030012003>.
- 58) Palmiere C, Sporkert F, Vaucher P, et al. Is the formula of Traub still up to date in antemortem blood glucose level estimation? *Int J Legal Med*. 2012 May; 126(3):407-13. PMID: 22327538. <http://dx.doi.org/10.1007/s00414-011-0659-1>.
- 59) Nowicka J, Skowronek R, Czech E, et al. Comments on 'Measuring insulin in human vitreous humour using LC-MS/MS' by Thevis et al. *Drug Test Anal*. 2013 Feb; 5(2):133-4. PMID: 22887877. <http://dx.doi.org/10.1002/dta.1392>.