THE CENTER FOR FORENSIC SCIENCE RESEARCH & EDUCATION

Identification and Quantification of Exogenous Insulin Analogs in Postmortem Specimens

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INSULIN DEATHS

NEWS

Germany: Care worker charged with 6 murders by insulin injection

The alleged murders took place across Germany between April 2017 and February 2018. Police accuse the man of murdering his diabetic victims by injecting them with lethal doses of insulin.

NATIONAL

Second Death At West Virginia VA Hospital Ruled As Homicide

August 28, 2019 · 11:15 PM ET

ITV REPORT 23 November 2018 at 3:46pm

Deleted 'insulin overdose ' search found on Middlesbrough murder accused's phone, court told

Mitesh Patel. Credit: Gazette Media Syndication

Inquiry: Nurse's colleagues called her 'angel of death'

By Jonathan Sher
More from By Jonathan Sher

Published on: June 13, 2018 | Last Updated: June 13, 2018 2:17 PM EDT

INSULIN ANALYSIS CHALLENGES

- Current techniques
  - Antibody-based (ELISA)
    - Cross reactivity & analog differentiation
- Analytical issues via LC/MS
  - Laborious sample prep
    - SPE/SPE + 2D-LC + nLC
    - Manual immunoaffinity purification
  - Analog co-elution via LC/MS
    - Isotope distribution
  - Poor fragmentation
  - Low-specificity transitions

Review — Drug Testing and Analysis

Received: 25 March 2009 Revised: 29 April 2009 Accepted: 6 May 2009 Published online in Wiley Interscience:

Murder by insulin: suspected, purported and proven – a review

Vincent Marks*

Murder by insulin – whether attempted, suspected or proven – is rare. Only 66 cases worldwide could be found for this review. A conviction was secured in 31 cases and additional weapon was employed in 11. Differentiation of attempted homicide from Munchausen syndrome by proxy in the young and from 'mercy killing' in the elderly was not attempted. Most perpetrators were close relatives and most victims were alive when discovered and responded to treatment. Hypoglycaemia is the first clue to homicidal insulin use in living subjects and requires the demonstration of a plasma insulin concentration of generally more than 1000 pmol/L and undetectable plasma C-peptide concentration to establish the diagnosis. Serum glucose measurements are valueless in victims found dead. The presence near the body of insulin vials, syringes or needles, loose talk by the suspected perpetrator or their ready access to insulin may be the only clue. The demonstration of insulin in tissue around an injection site by immunohistopathology or by measuring it in an extract clinches the diagnosis. Immunoassays suitable for clinical use to detect and measure insulin and C-peptide are subject to random errors and cannot be relied upon unless special precautions including separation by gel filtration or HPLC are undertaken prior to analysis. They do not detect or measure accurately a new generation of synthetic insulin analogues. Mass spectrometry will be required to do this and to validate clinical immunoassays, upon which convictions have always had to rely in the past.

"Immunoassays suitable for clinical use to detect and measure insulin and C-peptide are subject to random errors and cannot be relied upon [....] They do not detect or measure accurately a new generation of **synthetic insulin analogues**. **Mass spectrometry** will be required to do this [....]"

HUMAN & SYNTHETIC SEQUENCES

Most variability occurs at the c-terminal of the beta-chain

Human & Humulin® — **Native**

GIVEQCCTSICSLYQLENYCN
FVNQHLCGSHLVEALYLVCGERGFFYTPKT

Lispro (Humalog®) — **Fast Acting**

GIVEQCCTSICSLYQLENYCN
FVNQHLCGSHLVEALYLVCGERGFFYTKPT

Glargine (Lantus®) — **Long Acting**

GIVEQCCTSICSLYQLENYCG
FVNQHLCGSHLVEALYLVCGERGFFYTPKTRR

Aspart (Novolog®)

GIVEQCCTSICSLYQLENYCN
FVNQHLCGSHLVEALYLVCGERGFFYTDKT

Detemir (Levemir®)

GIVEQCCTSICSLYQLENYCN
FVNQHLCGSHLVEALYLVCGERGFFYTPK
Myristic acid

Glulisine (Aprida®)

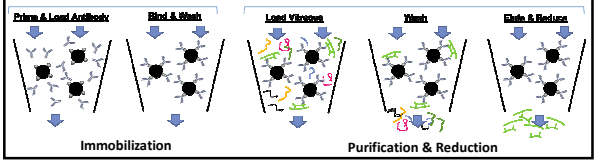
GIVEQCCTSICSLYQLENYCN
FVKQHLCGSHLVEALYLVCGERGFFYTPET

SAMPLE PREPARATION: IMMUNOAFFINITY MASS SPECTROMETRY

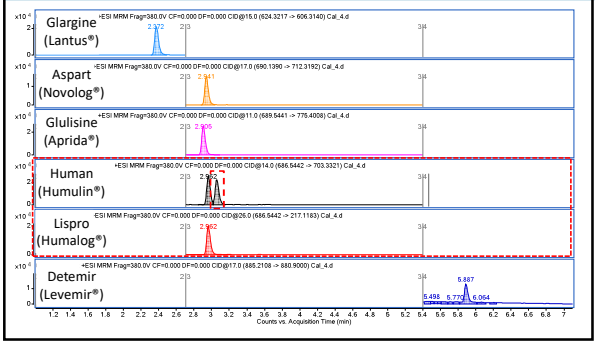
- Hands-free Immunoaffinity Purification
  - Agilent AssayMap Bravo
    - Reproducibility
    - Accuracy
    - Throughput
- Initial Preparations
  - 250 µL vitreous humor. Centrifuge 12,000 x g 5 min.
  - Dilute 200 µL vitreous with 100 µL Phosphate Buffer
  - Add ITSD (Porcine Insulin)
  - **Corning Non Binding Surface/NBS microplate**

SAMPLE PREPARATION: IMMUNOAFFINITY MASS SPECTROMETRY

- Immobilization
  - Prime Protein G cartridges with 50 µl PBS buffer
  - Load 1 µg each antibody
  - Wash 50 µl PBS buffer with 0.02% sodium azide. Store 4°C until use
- Purification
  - Equilibrate 50 µl PBS buffer
  - Load 250 µL dilute vitreous at 3 µL/min. Wash with 4x PBS followed by 20% ACN in 50 mM ABC
  - Elute with 15 µL 2% Acetic Acid into existing volume of 40 mM TCEP-HCL in 30% ACN
  - Eppendorf LoBind PCR plate. Seal, incubate 45°C 15 min, run on 6495 QQQ

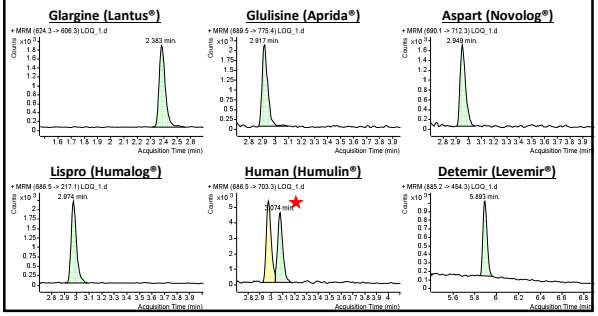


BETA-CHAIN CHROMATOGRAPHY



METHOD VALIDATION: LIMIT OF QUANTITATION (500 PG/ML)

Response and concentration (%CV) assessed with 10 replicates/day over 3 days



CASE SAMPLES 2017-2019

- Tested 58 vitreous humor samples where insulin was suspected to be involved with death
  - 19 (32%) yielded positive results
  - Aspart (Novolog®) & Lispro (Humalog®) most commonly found
  - No Glargine Identified
  - Sensitivity remains a challenge
    - <LOQ results
  - Positive results are corroborating with case history

STABILITY

- Standard glass vials vs. Protein low bind plastics
  - 10 to 50% loss in target concentration in standard glassware

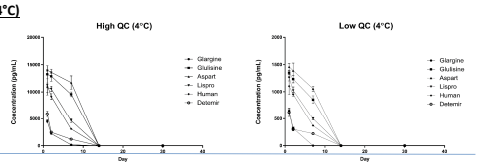
	Glargine (Lantus®)	Glulisine (Aprida®)	Aspart (Novolog®)	Lispro (Humalog®)	Human (Humulin®)	Detemir (Levemir®)
Glass HQC (pg/mL)	14,470	16,590	17,834	16,339	17,064	9,620
Plastic HQC (pg/mL)	19,696	20,133	19,387	19,443	20,004	18,406

20,000 pg/mL target concentration

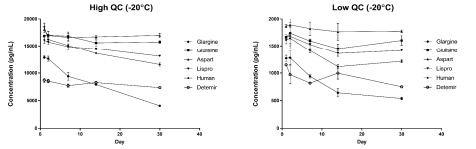
LONG-TERM STABILITY

Loss of all insulin analogs by Day 14 at 4°C

Refrigeration (4°C)

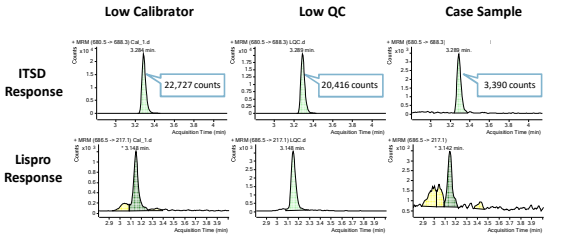


Frozen (-20°C)

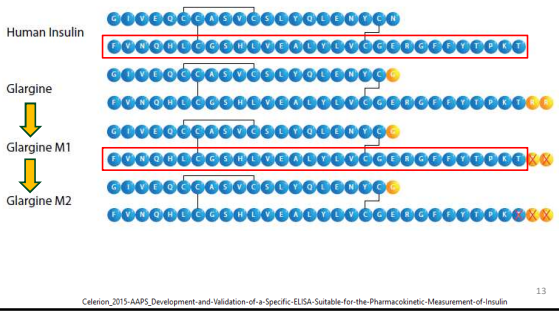


SAMPLE CONDITION IS CRITICAL!
HEMOLYZED VITREOUS

- Hemolyzed case sample
  - Bench notes state "Debris in sample. Mild hemolysis, light pink color"
  - ≈10x loss in internal standard response.
  - Lispro response ratios out of bounds



GLARGINE M1 & M2 METABOLITES



Celerion_2015-AAPS_Development-and-Validation-of-a-Specific-ELISA-Suitable-for-the-Pharmacokinetic-Measurement-of-Insulin

CASE - PRESENTATION

- 38 year old female was found deceased in bed
  - No obvious signs of trauma
  - Needle with bottle of insulin next to bed
- One of her primary physicians stated that the decedent had been mildly suicidal
- Comment from husband (insulin user)
  - "An insulin overdose would be a peaceful way to die."

CASE – TOXICOLOGY FINDINGS

- Toxicological Findings
  - Bupropion: 140 ng/mL
  - Fluoxetine: 1500 ng/mL
  - Norfluoxetine: 1300 ng/mL
  - Insulin Lispro (Humalog®): 2.37 ng/mL
  - Human insulin was not detected
- Husband verified as Humalog® user
- Cause of death documented as "Hypoglycemia due to intentional injection of insulin" and the manner of death was suicide

INSULIN 2.0: R & D for 2019/2020

- Scope Additions
  - Postmortem Blood
  - Deludec (Tresiba®), C-Peptide, Glargine metabolites
- Tissues
  - Injection sites as well as Brain/Liver
  - 1 gram tissue + 5 mL DI water, 1:10 dilution in blank vitreous

	2 ng/mL Target		20 ng/mL Target	
	Brain	Liver	Brain	Liver
Glargine	1.68	1.83	19.82	19.20
Glulisine	2.22	1.68	23.70	18.79
Aspart	2.04	1.68	21.00	19.41
Lispro	2.26	2.14	22.61	20.07
Human	2.29	1.80	24.10	20.41
Detemir	0.78	0.65	8.04	5.47

CONCLUSIONS

- Therapeutic insulin (vitreous) levels unknown
- Stability is a major concern!
  - Freeze following sample collection
- Hemolyzed samples have lowered analytical response
- Injection sites, blood, tissues