Short communication

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Measuring insulin in human vitreous humour using LC-MS/MS

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Besides its particular importance as a widely used therapeutic agent, insulin (and its synthetic derivatives) has been suspected, purported, and proven to be a lethal weapon in numerous cases of attempted or successful homicide and suicide. In addition to blood and urine as common matrices for clinical diagnosis and post-mortem analysis, vitreous humour has gained considerable attention in autopsy and follow-up investigations due to its ability to provide valuable information on cause and time of death. However, post-mortem insulin analyses using such specimens have been rare due to the limited penetration of peptide hormones into the vitreous body, and immunoassays were exclusively employed in those studies. In the present communication, the determination of insulin(s) from vitreous humour by means of immunopurification combined with ultrahigh performance liquid chromatography – high resolution/high accuracy (tandem) mass spectrometry is reported. Exploiting the constantly increasing sensitivity and robustness of modern mass-spectrometry-based instruments, the option to identify insulin in post-mortem vitreous samples is demonstrated with a specimen collected from a non-diabetic victim who died from an insulin overdose. This communication represents the first successful mass-spectrometry-based analysis of post-mortem material related to an insulin poisoning case.

Keywords: homicide; insulin; mass spectrometry; Orbitrap; murder

Introduction

With the discovery of insulin in 1921, its pharmaceutical production (from bovine and porcine pancreas and eventually by biotechnological methods) and introduction into medical therapy allowed numerous patients suffering from *diabetes mellitus* considerably improved conditions of living with a formerly mortal disease.[1] The facile availability of insulin proved to be both a blessing and a curse, since numerous suspected, purported, and evidenced murders by insulin have been reported.[2–4] Diagnosis of hyperinsulinemia caused by adverse insulin administration is commonly achieved by measuring blood glucose and insulin levels and, when indicated, C-peptide concentration.[5] In addition, the analysis of vitreous humour using immunoassays has been reported.[6] However, in the case of suspected and/or attempted homicide or suicide with insulin, in-depth analysis of available material by a technique beyond the non-selective ordinary immunological assays is required, and consequently mass-spectrometry-based methods were developed for insulin bioanalysis already at the turn of the twenty-first century.[7,8] Later, more sophisticated methods were established to support the differentiation and determination of human or animal insulin and their synthetic derivatives from blood and urine as employed, amongst others, for sports drug-testing purposes.[9–14] Hemolyzed post-mortem blood is, however, a very difficult material for insulin analysis by any technique due to matrix interferences and rapid degradation of insulin by the insulin-degrading enzyme; post-mortem serum can seldom be separated and frozen in a timely manner for appropriate analysis as recommended in a recent review.[15] Indeed, as early as 1972, it was concluded that quantitative assay of insulin in highly hemolyzed samples is of questionable value.[16]

Vitreous humour, the gel-like material located in the posterior segment of the eye, has been subject of toxicological analyses in early[17] and recent[18] forensic studies and the utility and limitation were outlined. The vitreous body is primarily composed of water (99.9%), collagen, hyaluronic acid, and the electrolytes Ca^{2+}, Cl^-, Na^+, and K^+, and drug penetration through the blood-ocular barrier into the vitreous is predominantly depending on diffusion rates.[19] High doses are thus required to allow for drug delivery into the eye via systemic administration routes, and dedicated membrane transport systems were identified enabling the influx and efflux of essential nutrients including, for example glucose, amino acids, and peptides.[20] Proteomic analyses identified a great number of peptidic components in the vitreous,[21] and more compound-specific analyses revealed the presence of insulin and its receptor in the vitreous,[17,22] tear film, or on the ocular surface.[23]

Consequently, it has been of interest to elucidate the option to employ vitreous humour for forensic purposes concerning insulin used as a lethal weapon. In the present study, an assay using

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immunoaffinity purification combined with liquid chromatography–(tandem) mass spectrometry (LC-(MS/)MS) was established and applied to a case of fatal insulin intoxication.

Experimental

Chemicals and reagents

Coated Dynal beads (anti-mouse IgG) were from Invitrogen (Karlsruhe, Germany). Ultrapure water, acetonitrile, trifluoroacetic acid and formic acid were purchased from Biosolve (Valkenswaard, the Netherlands). Acetic acid (glacial), acetonitrile (analytical grade), sodium dihydrogenposphate dihydrate (p.a.), disodium hydrogenposphate dodecahydrate (p.a.), and sodium chloride (p.a.) were obtained from Merck (Darmstadt, Germany). Monoclonal anti-insulin antibodies (IgG, anti-mouse) were purchased from CER-groupe (Marloie, Belgium) and solid phase extraction cartridges OASIS HLB (60 mg, 3 ml) were from Waters (Eschborn, Germany). All aqueous buffers and solutions were prepared in MilliQ water. Human insulin and its analogues Humalog Lispro, Novolog Aspart, Glulisine Apidra, and Lantus Glargine were obtained as pharmaceutical injection preparations from Sanofi-Aventis (Frankfurt am Main, Germany), Eli Lilly (Indianapolis, IN), and Novo Nordisk (Princeton, NJ, USA), respectively. Bovine insulin was purchased from Sigma (Schnelldorf, Germany).

Sample preparation

Vitreous humour analyses were adapted from recently published methods for insulin determination from plasma and urine.[13,24] The applicability of the methodology to a new matrix (i.e. vitreous humour) was tested by spiking blank specimens samples of vitreous with natural and synthetic insulin followed by routine protocol analyses. These 'blank' specimens were obtained from routine autopsies with no indication of insulin intoxication or *diabetes mellitus* and natural levels of insulin were below the method's limit of detection (LOD). In brief, a volume of 500 µl of vitreous humour was enriched with 1 pmol of bovine insulin (internal standard, ISTD), and 1 ml of acetonitrile was added prior to vortexing for 20 s. The formed precipitate was settled by centrifugation at 6000 x *g* for 5 min, the supernatant was transferred to a fresh test tube and evaporated to dryness under reduced pressure. Following reconstitution in 500 µl of phosphate-buffered saline (PBS), anti-insulin antibodies and anti-mouse IgG-coated magnetic beads were added and the mixture was incubated for 1 h at 20 °C. The beads were separated, washed with PBS (2 x 300 µl), and target analytes were eluted with 50 µl of aqueous acetic acid (2%) into low-bind polypropylene tubes for LC-MS/MS analysis.

Liquid chromatography-(tandem) mass spectrometry

Two instrumental setups were applied to obtain data with utmost information on the detected compounds. The first (routine) apparatus was composed of a nanoUPLC (WATERS Acquity, Milford, MA, USA) and a Thermo LTQ (Bremen, Germany) equipped with a nanospray source. Detailed instrument parameters are outlined elsewhere.[13] Briefly, the LC was operated with 0.1% formic acid (solvent A) and acetonitrile (solvent B) employing gradient elution from 97% A to 10% A in 21 min at a flow rate of 750 nl/min. The analytical column was a Waters BEH-130 C_{18} (75 µm x 100 mm, 1.7 µm particle size), combined with a Waters Symmetry C_{18} (180 µm x 20 mm, 5 µm particle size) trapping column. The second LC-MS system consisted of an Accela ultrahigh performance liquid chromatograph (UHPLC) and an exactive high resolution/high accuracy orbitrap mass spectrometer (Thermo, Bremen, Germany), interfaced by means of an Advion Triversa Nanomate (Ithaca, NY, USA).[24] The LC was equipped with an Agilent (Waldbronn, Germany) Zorbax SB300 analytical column (0.3 x 50 mm, 5 µm particle size) and a Zorbax SB300 guard column (1 x 17 mm, 5 µm particle size) using 0.2% formic acid and acetonitrile as solvents A and B, respectively. Also here, gradient elution was employed starting at 85% A decreasing to 25% A within 10 min. The flow of 25 µl/min was directed into the Nanomate and split to generate a nanospray at 800 nl/min. The combined information obtained by both instruments (i.e. low resolution MS/MS and high resolution/high accuracy full-scan MS data in positive mode) was used to unambiguously identify insulin in vitreous humour.

Case details

A 55-year-old non-diabetic female was hospitalized due to cardiac and hepatic issues. After five weeks of inpatient health care, the patient was transferred to the intensive care unit due to a sudden drop of blood glucose levels from normal values (89 mg/dl) to severe hypoglycemia (2 mg/dl). Corresponding blood insulin levels were 5.9 mIU/L (0.2 ng/ml, i.e. normal) and 5551 mIU/L (194 ng/ml, extremely elevated), respectively, while C-peptide concentration measurements remained within normal ranges (1.7–4.8 ng/ml) as determined with immunoassays. The patient was comatose, survived upon normalization of blood glucose levels without regaining consciousness, and deceased after four days.

Blood samples collected during intensive care treatment and vitreous humour specimens (2 x 3 ml) obtained at autopsy were stored frozen and submitted to LC-MS/MS analysis. In addition, infusion solutions, tubings, etc., being used for the patient at the day of insulin intoxication, were seized and analyzed for insulin residues.

Results and discussion

LC-MS/MS analyses of serum specimens collected 20 h before and approximately 4 and 8 h after the hypoglycaemia incidence demonstrated only the presence of human insulin at levels between 0.2 and 3.5 ng/ml (corresponding to blood glucose values between 62 and 98 mg/dl) using established methods.[4,10,13] The sample determined with the peak insulin value of 194 ng/ml (corresponding to the blood glucose concentration of 2 mg/dl) by immunological methods in the hospital was not available for LC-MS/MS re-analysis. No synthetic/modified insulin analog was detected in the provided specimens. An adenoma of the pancreatic tissue was not observed during autopsy and, although it cannot be explicitly excluded, it was considered unlikely since insulin levels stabilized after adequate treatment. Consequently, the administration of a human insulin preparation was assumed which was, however, not accomplished via the medical care bedside instruments as no traces of insulin were detected in the remnants of the infusion solution or tubings.

According to literature data,[22] immunoreactive insulin is found in vitreous humour of non-diabetic rats at average levels of approximately 0.05 ng/ml, which is about 100-fold less than corresponding serum concentrations. Considering the observation

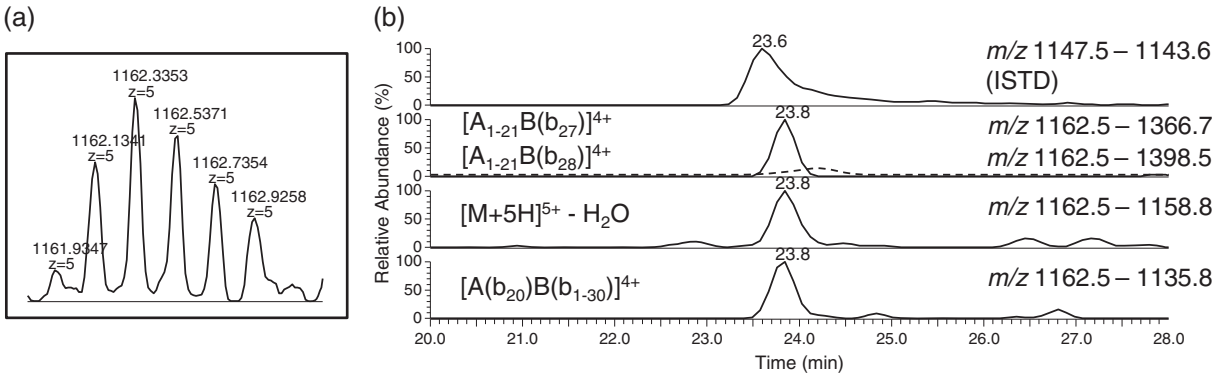

Figure 1. High resolution/high accuracy mass spectrum (a) and extracted ion chromatograms with diagnostic ion transitions (measured in low resolution MS/MS, (b)) of insulin isolated from vitreous humor after fatal intoxication.

that analyte diffusion from blood vessels via the blood-ocular barrier is concentration-dependent and blood insulin concentrations in humans are commonly found between 0.1 and 3.1 ng/ml (depending on the fasting state),[7,8] even lower vitreous humour levels of insulin are expected in humans than in rats. In a pilot study with 10 vitreous humour specimens collected from deceased insulin-dependent diabetic humans as well as non-diabetic persons, 'basal' vitreous levels of intact insulin were below the estimated limit of detection (0.1–0.2 ng/ml) of the established LC-MS/MS method (data not shown). In the present case, a vitreous humour specimen collected from a fatality allegedly caused by insulin intoxication was analyzed, and intact human insulin (approximately 1.0 ng/ml) was detected and identified by means of LC-MS/MS. The corresponding extracted ion chromatograms and full MS high resolution/high accuracy mass spectrum are illustrated in Figure 1. In contrast to most purely immunological methods, (tandem) mass spectrometry allows for the differentiation of human insulin from its synthetic derivatives such as Humalog Lispro, Novolog Aspart, Lantus Glargine, etc., all of which comprise a modified amino acid sequence and thus provide either an altered molecular mass or a distinct dissociation pattern upon collisional activation.[11] In case of human insulin, the 5-fold charged molecule is observed at *m/z* 1162 (Figure 1a), which deconvolutes to 5803.6 Da that accurately reflects the monoisotopic molecular mass and separates the analyte from most synthetic or animal analogs exhibiting higher or lower molecular masses. Humalog Lispro, however, is composed of the identical set of amino acids but differs with regard to the sequential order of the C-terminal residues B28 and B29, i.e. lysine and proline, which are reversed in case of human insulin. Consequently, diagnostic product ions at *m/z* 226 (y_3-y_1 for human insulin) and *m/z* 217 (y_2 for Humalog Lispro) or their corresponding 4-fold charged [$A_{1\text{-}21}B(b_{27})$]- and [$A_{1\text{-}21}B(b_{28})$]-ions at *m/z* 1366.7 and *m/z* 1398.5, respectively, are to monitor to provide discriminating characteristics. Due to the ion-trapping device used to generate MS/MS spectra in the present study, the low-mass cut-off hindered the use of the reporter ions at *m/z* 226 and 217, but the presence of *m/z* 1366.7 and absence of *m/z* 1398.5 illustrated in Figure 1 (extracted ion chromatogram) enabled the unambiguous identification of human insulin.

Conclusion

The considerable number of incidences where attempted or accomplished homicides or suicides with insulin have been reported has necessitated reliable analytical tools to provide (supporting) evidence to corroborate the circumstances of intoxication. Blood in particular but also urine specimens have been used for that purpose in the past, but post-mortem changes severely hinder the interpretation of blood results while urine concentrations are low and the specimen is not always available. The present communication reports, for the first time, the unequivocal mass-spectrometry-based identification of insulin in post-mortem material related to an authentic poisoning case. The study further shows that the traditional matrices can, at least in individual cases, be complemented by vitreous humour samples to differentiate the insulin species and clarify whether elevated concentrations of insulin might have contributed to the cause of death. The advantages of vitreous humour include good availability, low analytical background, and less pronounced post-mortem changes. However, further studies are required to evaluate the status of vitreous humour in post-mortem insulin diagnostics.

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