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REVIEW ARTICLE

Disorders of glucose metabolism–post mortem analyses in forensic cases: part I

Cornelius Hess · Frank Musshoff · Burkhard Madea

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Abstract In developed countries, diabetes is one of the ten most common causes of death. Post mortem diagnosis of glucose metabolism disorders can be difficult and vague because of the lack of characteristic morphological findings. Reviews of the literature are presented concerning biochemical problems in cases of unclear hyper- or hypoglycemia. After repetition of causes, frequency, and mortality of diabetic metabolism disorders, we give hints for the detection of diabetic ketoacidosis, hyperosmolar coma, insulinoma, and insulin- or oral diabetic-induced hypoglycemia. The first part discusses the analytes glucose and lactate, glycated proteins and oral antidiabetics, with special regard to their matrices post mortem, to reference concentrations, stability data and to analytic procedures that should be used in clinical or toxicological laboratories to detect diabetic metabolism disorders after death.

Keywords Diabetes mellitus · Glucose metabolism · Post mortem · Hypoglycemia · Hyperglycemia · Oral antidiabetics

Cornelius Hess and Frank Musshoff contributed equally to this work.

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C. Hess (✉) · F. Musshoff · B. Madea
Institute of Forensic Medicine, University of Bonn,
Stiftsplatz 12,
53111 Bonn, Germany
e-mail: corny.hess@web.de

Introduction

Among chronic diseases, diabetes mellitus is frequent, and it is one of the ten most common causes of death in developed countries. A common problem is the case of sudden deaths of diabetics followed by a negative autopsy [1]. Post mortem analytes and forensic analyses in cases of deaths due to disorders of glucose metabolism will be reviewed. The first part discusses analytes that are rather relevant in clinical laboratories, but with the need for further forensic interpretation. The second part [2] deals with sophisticated chromatographic methods for the determination of human and synthetic insulins, C-peptide, proinsulin, and ketone bodies in specialized (forensic) laboratories.

Diabetes and acute complications of glucose metabolism

Despite widely variable intervals between meals or the occasional consumption of substantial carbohydrates, human blood glucose levels remain within a narrow range, usually (3.3–7.7 mmol/l even after a meal [3]) because of several pathophysiological mechanisms. Those mechanisms are deranged in diabetics and may lead to the following consequences.

Constant hyperglycemia

Constant hyperglycemia (Online resource 1) in diabetics is due to a lack of insulin. Human insulin, a protein hormone secreted by the pancreas, has a molecular weight of 5,808 Da. It is made out of two peptide chains connected by two disulfide bridges and contains 51 amino acids, the A chain having 21 amino acids and the B chain having 30 amino acids. Proinsulin, one of the precursor molecules, is

produced by enzymatic division of preproinsulin. It is made out of one single chain containing 86 amino acids and is transported into the Golgi apparatus. There, the maturation of secretory granules is associated with conversion of proinsulin into major intermediates and into insulin and C-peptide (Online resource 2). C-peptide is a polypeptide (containing 31 amino acids) with a molecular mass of 3,017 Da and has an isoelectric point of 2.8 [4]. Mature secretory granules in the pancreas contain equimolar amounts of insulin and C-peptide plus 2–6% of intact and split proinsulin. They release their content into the bloodstream when β-cells are stimulated by high blood glucose concentration [5]. Proinsulin has 5–10% of the bioactivity of insulin [6], but both of them can lower the blood glucose level. The physiological function of C-peptide is still widely unknown.

Complications: diabetic ketoacidosis and hyperosmolar coma

Diabetic coma is the most severe form of hyperglycemic metabolic disorder. It can be differentiated between diabetic ketoacidosis and non-ketoacidotic hyperosmolar crisis. The pathogenesis of both types is described in Online resource 3. High blood glucose levels and ketone bodies in blood may lead to medical complications including disorientation, dehydration, coma, and death. Annual incidence and mortality rates of glucose metabolism disorders are summarized in Online resource 4.

Hypoglycemia

The common definition of hypoglycemia is related with low plasma or low blood glucose concentration (Online material 5). Common reasons for a hypoglycemic attack are insufficient nutrition, abnormal physical encumbrance, and chronic alcoholism. Other reasons are non-islet cell tumor hypoglycemia, reactive hypoglycemia after the intake of sugar, and autoimmune hypoglycemia, but there are many other rare reasons summarized in literature [7, 8]. However, the most important reason for hypoglycemia is the drug-induced hypoglycemia as side effect of antidiabetic therapy, especially if insulin is used (9–26% of the cases [9]). Type I diabetics and many of the type II diabetics have to rely on the injection of insulin or its synthetic analogs to replace the hormone in a manner which reproduces physiological insulin secretion as closely as possible and to rebuild normal glucose levels. Hypoglycemia is the main and the most dreaded complication of insulin therapy [10] in diabetics and may be associated with permanent brain damage and significant mortality if not treated with fast absorbable carbohydrates [11]. The incidence of hypoglycemic episodes is higher in patients with type I diabetes mellitus than in patients with type II diabetes (1.70 vs. 0.73 periods per patient and year [11]). However, absolute numbers depend on the definition of hypoglycemia. In the majority of cases, hypoglycemic situations are defined as situations with no way of self-escape for the sufferer. Unintentional overdoses caused by insulin application occur quite often (0.16% [12] and 0.70% [13] of the reported substance exposures involving insulin in 2005 and 2007, respectively). The use of insulin analogs in diabetes therapy reduced the rate of hypoglycemia to nearly 25% (insulin lispro vs. human insulin [14]); other studies only showed that there is a tendency to reduce the frequency of hypoglycemia [15]. The knowledge of possible fatal complications of insulin overdosages occasionally leads to incidents when insulin is used as a weapon either against oneself (suicide or factitious hypoglycemia), against a dependent (Munchausen by proxy), or against another person (homicide) [16]. Arem [17] found 81 cases of factitious hypoinsulinemia in the world literature written until 1985, and more suicides committed by insulin application are documented [18–27]. After human insulin was replaced by synthetic insulin, rapid-acting as well as long-acting insulin formulations (57.8% vs. 42.8%) [28, 29] were used. Murder committed by insulin application is rare. Marks [30, 31] collected data of all 66 detected cases of murder or attempted murder committed by insulin application.

After insulin injection, it takes at least 20 min to develop hypoglycemia. Doses of insulin and insulin types are found to be closely related to duration of hypoglycemia, but not to its severity [28]. Patients who had insulin doses up to 3,000 U fully recovered (1 U=45 μg human insulin), but there are cases of insulin application of 100 U (range: 100–3,000 U) [32] and 300–550 U [31] that ended fatally. Insulin doses used to commit suicide range from 100 to 2,000 U [33].

In general, insulin poisoning is ineffective because it takes a long period until death occurs. Furthermore, it is simple to diagnose and to treat hypoglycemia which might save victims from dying [31].

Drugs for the therapy of patients with type II diabetes also include oral antidiabetics (Online resource 6). Treatment with insulinotropic substances leads to hypoglycemia as a side effect, but sulfonylureas are associated with significantly lower rates of severe hypoglycemia than insulin [34, 35]. Sulfonylureas are the most commonly used oral antidiabetic drug overdose, especially when treatment is with first- and second-generation sulfonylureas. Newer compounds like glimepirid or glinides rarely show such disadvantages [36, 37]. Hypoglycemia can occur with even the smallest doses of any sulfonylureas or glinides; there is no constant dose–effect relationship [36–38]. In literature, there are 93 cases of factitious hypoglycemia due

to sulfonylurea overdose [39] and only one suicidal attempt committed by the application of a glinide [40]. Other oral antidiabetic substances show hypoglycemia less frequently, and there was no case found in literature.

Analytes for post mortem diagnosis of glucose metabolism disorders

Glucose and lactate

If there is a need to investigate glycemic disorder situations in forensic cases, unfortunately, glucose might be an unreliable factor. Besides food intake, glucose infusion, loss of islet cell control in some cases of acute pancreatitis or head trauma [41, 42], or glucose increase as an "agonal alarm reaction" in association with increased catecholamines [43], there might occur glycogen mobilization caused by epinephrine administration, and glycogen and glucose can circulate through the victim's body during cardiopulmonary resuscitation. Blood glucose–at its maximum at the moment of death–is rapidly metabolized into lactate by glycolysis. Hence, it is not possible to draw important conclusions concerning primary blood glucose levels [44, 45]. Hourly rates of glucose decomposition were shown to be about 0.7 mmol/L h^{-1}, and blood sugar is metabolized to lactate within 8 h [46]. Until 10 h after death, lactate levels rise to about 10–15 mg/dl h^{-1} and can reach levels up to 450–680 mg/dl. Besides blood, vitreous fluid (VF) and cerebrospinal fluid (CSF) are often used for analysis in post mortem cases. Glucose levels in VF correlate with glucose ante mortem serum levels [47] and they amount to 50% of ante mortem serum concentrations [47] and 85% of post mortem serum concentrations [48]. The vitreous is less exposed to bacterial contamination, to autolysis, to putrefaction, and to post mortem biochemical changes, e.g., glycolysis, but its defined content might be a disadvantage. However, the considerable influence of environmental temperature to glycolysis (hourly decrease of 35% and 70% within 6 h at room temperature) in vitreous fluid (VF) [49–51] and CSF (0.28 mmol/l and 2.5 mmol/l h^{-1} [52] in diabetic coma after death) was demonstrated. Therefore, post mortem biochemical evaluation of glucose metabolism has to be based on combination of glucose and lactate levels in VF [44, 48] or CSF by addition of both concentrations, estimated in milligrams per deciliter [53]. This "formula of Traub", especially concerning VF, should be used to diagnose hyperglycemia thresholds (see Online resource 7). However, thresholds described in literature could not always be complied [61]. Several studies showed an increase of the sum value until 30 h post mortem [33] or until the first 2 days after death [60, 61]. After this interval, it stays almost unmodified for about 1 week [33]. It should also be kept in mind that in some circumstances, lactic acid is built in vivo like in malignant tumors, chronically inflammable illnesses like uremia, respiratory insufficiency, inflammations of the CNS, alcohol-induced lactatacidosis, or fasting [62, 63]. High glucose values in a few non-diabetics have been reported in asphyxial deaths, cerebral hemorrhage, congestive heart failure, and electrocutions [54].

In contrast to blood glucose, urinary glucose is less reliable. Glucose is reabsorbed through the renal tubuli. Although glucose is detectable in urine of patients with high increased blood glucose concentration (about 1.67 mmol/l in diabetics and <0.84 mmol/l in non-diabetics), it gives no information about blood glucose concentrations below the variable renal glucose threshold, which is very variable between individuals (about 10 mmol/l) [3]. That means that a negative urine glucose test does not distinguish between hypoglycemia, euglycemia, and mild or moderate hyperglycemia. During diabetic coma, values higher than 27.75 mmol/l were observed (mean value 160.67 mmol/l) [3]. Post mortem urine glucose [55] was measured in six cases of hyperglycemia with concentrations between 4.77 and 191.4 mmol/l (mean value 85.97 mmol/l). Therefore, urinary glucose should exclusively be used to assure hyperglycemia as a plausibility control.

Post mortem analyses

If blood is used to measure the glucose level, it could be demonstrated that there are significant differences between specimens if they are taken from diverse parts of the heart. Samples taken from the right side of the heart or the inferior vena cava in general show a high glucose content [64] as a result of glyconeogenesis in the liver which is followed by glucose diffusion into surrounding blood vessels. Instead, it was recommended to take blood from a peripheral side [39]. Wark [39] recommended to avoid taking blood from the right atrium, the right ventricle, the inferior vena cava, or the vena hepatica.

The stability of samples is an important knowledge. In blood samples prepared without preservatives (glycolysis-inhibiting sodium fluoride or additions of maleinimid, of lithium iodacetate, or mannose), a degradation of glucose to lactate can be observed [59]. When combined with Ca oxalate as an anticoagulant [65], and in concentration of 2.5 g/l or 4.3 g/l, blood sodium fluoride is recommended as a stabilizer for blood glucose and lactate [59]. Mannose inhibits glucose metabolism and can be added in concentrations of about 2 g/l blood. Mannose functions only about 4 h hence, NaF should be added, too [59, 66]. Such containers allow separate determination of glucose values and lactate values. Glucose concentration is constant at room temperature in those containers for about 12 h. If

stored at 4°C, the concentration keeps constant for 2 [67] to 6 days [68] and for several months [68] if samples are deep frozen (−21°C). If urinary glucose is measured, samples should be analyzed within 2 h because of low stability [59].

Glycated proteins

Different studies demonstrated that serum proteins are glycated posttranslationally caused by a slow nonenzymatic reaction taking place between glucose and amino groups of proteins [69].

HbA1c

HbA1c is defined as D-glucose added to one or both N-terminal valines belonging to the β-chain of hemoglobin. The rate of synthesis of HbA1c is a function of the concentration of glucose to which the erythrocytes are exposed [70, 71]. Diverse methods have been described, but each of them is validated only for the method they have been designed for (i.e., [71]). Recently, it was recommended to change the unit of HbA1c (% of total hemoglobin) to millimoles HbA1c per mole of hemoglobin. In addition, another value was introduced: A1C-derived average glucose (values are explained in literature [71–73]). The measurement of glycated hemoglobin (HbA1c) is an approved method to ensure glycemic control in routine management of patients with diabetes mellitus [74]. Once formed, glycated hemoglobin is stable and accumulates the life span of the red blood cell which makes it an adequate index during the preceding 120 days and to assess stable glycemia [70, 74]. But not before a 12-h hyperglycemia the HbA1c concentration is influenced [75], therefore, it is less suited to monitor rapid changes in glucose levels in a diabetic death.

Measurement of glycated hemoglobin is expedient when death was due to undiagnosed diabetes mellitus to distinguish between diabetic and non-diabetic patients. In cases of increased ketone bodies, the increased HbA1c allows to differentiate ketoacidosis caused by alcohol or starvation. Glycated hemoglobin is stable after death for at least 36 h [76], and it remains unaffected by hemolysis [55, 77]. Furthermore, it shows the smallest deviation from healthy subjects, and there is no difference due to the etiology of death [78, 79]. Winecker et al. [80] showed that HbA1c may increase after post mortem collection. Increased HbA1c (>86 mmol/mol) correlated positively to the sum value of Traub in CSF [77]. However, conversely, this is not always the case. Post mortem HbA1c values of diabetics were demonstrated to be 72–145 mmol/mol [64], 58–191 mmol/mol [80] or "elevated with values that may be seen in patients with poorly controlled diabetes" [76] (reference values 15–44 mmol/mol and <48 mmol/mol in well-adjusted diabetics). Extremely low glycated hemoglobin in treated diabetics as a consequence of periodically repeating hypoglycemic states can give a hint to hypoglycemia.

Post mortem analyses

HbA1c is measured in whole blood. There was little or no difference of HbA1c values when different sampling sites were tested [78]. Winecker et al. [80] found no difference in HbA1c values if samples were stored in tubes with EDTA or with NaF. Another study showed that conservation of HbA1c at 4°C was possible for about 40 days [64], for about 3 months in whole blood samples collected with fluoride and for 6 months in samples collected in a dry or heparinized tube [79]. When temperature was varied, values increased 4–7% above the initial values.

There are many different HbA1c assay methods currently in use. In most of them, HbA1c is quantified, others quantify "total glycated hemoglobin" which includes both HbA1c and other hemoglobin–glucose adducts. Therefore, immunological procedures showing cross-reactivity are no longer appropriate. A specific reference method for HbA1c has been developed to achieve uniform standardization of HbA1c measurement [81, 82]. In the first step, hemoglobin is cleaved into peptides by the enzyme endoproteinase Glu-C. In the second step, the glycated and non-glycated N-terminal hexapeptides of the obtained beta-chain are separated and quantified by HPLC and electrospray ionization mass spectrometry. Other mass spectrometrical methods are summarized by Lapolla et al. [83].

Fructosamin

Most of the plasma proteins show a biological half-life of 5–15 days. Thus, measurement of glycated serum protein levels provide a more rapidly responding parameter than HbA1c and serves as an index of intermediate glucose control (1–3 weeks) [84]. Fructosamin ("blood-based glycated proteins") concentration largely reflects glycated albumin concentration although 20% of the reducing activity is derived from other serum proteins [85].

Reference ranges in non-diabetics are 205–285 μmol/l and >370 μmol/l in less well-adjusted diabetics [56, 57]. After death, blood fructosamin was demonstrated to be 325.3±147.1 μmol/l in non-diabetics [78] and 500 μmol/l [56, 57] or up to 1,300 μmol/l (mean value of 200 μmol/l) [58] in diabetics. Fructosamin values did not change significantly until 72 h after death. The usefulness of determining fructosamin in VF during autopsy has also been demonstrated (up to 1,500 μmol/l in autopsied cases in VF) [56, 57].

Post mortem analyses

The "fructosamin test" is based on the ability of ketoamines to reduce nitroblue tetrazolium to formazan at pH 10.35 ([85] and improvements in [86–88]). The fructosamin test results should always be corrected for either total protein or albumin concentrations, and correction for serum proteins can be done by multiplication with 72/total protein (g/l) [59]. In addition, chromatography and immunoassays are used to assay glycated total serum protein [89, 90]. Little or no difference was observed when different sampling sites were tested [78]. Serum should be measured because plasma gives different values due to the presence of fibrinogen and coagulation factors [59]. In the original method used by Johnson, heparin (80,000 IU/l) and EDTA (5.5 g/l) decrease fructosamin activity to a small but statistically significant amount [91]. Stored at ambient temperatures, fructosamin activity is unaffected for up to 24 h [92]. Patients' samples may be stored at −20°C for several months [85] and for 2 weeks at 4°C [92]. However, Koskinen et al. [93] reported considerable changes in specimens stored at −20°C. This is why it is recommended to separate samples within 24 h, to store them at 4°C, and to analyze them within 2 weeks [87].

α1-Antitrypsin and haptoglobin, both glycosylating considerably more rapidly, appear to allow reliable ante mortem and post mortem discrimination between normoglycemic and hyperglycemic metabolic states, but further investigations are indicated [94].

Oral antidiabetics

Oral antidiabetics on the market are summarized in Online resource 6. Peak plasma levels occur within a range of 1–8 h after ingestion, and the onset of hypoglycemia in acute overdose occurs in less than 8 h [95] or may be delayed for several days [96]. Doses, plasma levels [97, 98], and half-lives [99] of oral antidiabetics are described in literature. As the third-generation sulfonylureas are almost completely excreted in the urine in a metabolized form, samples of oral antidiabetics in the urine always have to include the main metabolites of the agents.

To our knowledge, the inclusion of all oral antidiabetics in one analytical procedure was only described twice, meaning LC-MS/MS methods for the determination of sulfonylureas, glinides, and glitazones in equine plasma [100] and in human urine [101]. Recently, such a method to determine all of the oral antidiabetics in human serum has been submitted by us also [102].

Integrity of research The authors declare that they have no conflict of interest.

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