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REVIEW ARTICLE

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

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Abstract In continuation to part I, a literature review is presented concerning biochemical problems of forensic post mortem cases of unclear hyperglycaemia or hypoglycaemia. Clinical parameters for this purpose were recently reviewed. Particular attention was paid to the detection of diabetic ketoacidosis, of hyperosmolar coma, insulinoma, insulin-induced or oral diabetic-induced hypoglycaemia. The second part of the review discusses the analytes ketone bodies, synthetic insulins, human insulin, C-peptide, proinsulin and insulin antibodies. Special interest is given to post mortem matrices for those analytes to reference concentrations, stability data, analytic interferences and analytical procedures which should be used in toxicological laboratories willing to detect diabetic metabolism disorders after death.

Keywords Synthetic insulin · C-peptide · Hyperosmolar coma · Diabetic ketoacidosis

Introduction

The diagnosis of glucose metabolism disorders post mortem is often complicated and imprecise caused by the missing of characteristic morphological findings. In many cases, the forensic pathologist may not be able to demonstrate unequivocal biochemical or histological proof for hyperglycaemia or hypoglycaemia [1]. The present part of the review continues source [2] and describes chromatographic methods for the determination of human and synthetic insulins, C-peptide, proinsulin and ketone bodies in specialized (forensic) laboratories.

Ketone bodies

Diabetic ketoacidosis (DKA) is initially characterized by an increased production of the ketone bodies acetoacetate, acetone and β-hydroxybutyrate (β-OHB) and determination of ketones in urine and blood are widely used for diagnosis of DKA. In hyperosmolar nonketotic hyperglycaemia, significant blood levels of ketone bodies are lacking [3, 4]. The principal ketone bodies β-OHB and acetoacetate usually present 78% and 20% of all ketone bodies. Acetone which usually appears in small quantities derives from spontaneous decarboxylation of acetoacetate and does not differ appreciably between diabetic and healthy subjects. The presence of ketone bodies in urine may indicate impending or even established ketoacidosis. Elimination in urine depends on the glomerular filtration rate [5]. In persons with normal kidney function, the urinary ketone level is proportional to the blood level but it is affected by urine volume and concentration [6]. Therefore for measuring, blood should be prefered. β-OHB reference concentrations in urine were described as <50 mg/l.

Determination of ketone bodies in corpse fluid and its application to forensic practice has been analysed by several authors [7–13]. β-OHB serum concentrations provide more information about severity of ketoacidosis whether it is related to diabetes [14], alcohol [9] or starvation [15] than other ketone bodies. In most cases,

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hypoglycaemia associated with plasma β-OHB levels of less than 600 μmol/l is due to increased plasma insulin or insulin-like activity, no matter if endogenous or exogenous insulin. In most cases of spontaneous hypoglycaemia, e.g. in cases caused by alcohol, hypoglycaemia is associated with modest ketonemia >600 μmol/l [16]. Alcohol can cause hypoglycaemia and alcoholic ketoacidosis. In a fasting person, hypoglycaemia occurs 6–36 h after alcohol consumption. In such a situation, hypoglycaemia goes along with a hypoinsulinaemia and an elevated concentration of β-hydroxybutyrate and lactate. Blood concentration of β-OHB in fatal alcoholic ketoacidosis amount to 3,000 to 47,000 μmol/l (mean value, 13,000 μmol/l) [17] which is higher than in diabetic ketoacidosis (Electronic supplementary material, Online resource 1).

β-OHB can be reliably measured in post mortem samples of vitreous fluid (VF) in blood and in urine. Pounder [13] found a strong relation between blood and vitreous ketone body levels in the diagnosis of alcoholic ketoacidosis in autopsies. VF was less affected by agonal or post mortem increase in ketone body levels [18].

Analytics

In ketone body assays, central venous blood and arterial blood can both be used [6]. Whole blood samples for ketone measurement are stable at 4°C for up to 24 h, at −20°C for 'prolonged periods' [6]. β-OHB in whole blood is a stable analyte for up to 2 days. Acetoacetate is an unstable analyte, because it undergoes nonenzymatic decarboxylation to acetone and enzymatic transformation to β-OHB [19]. In forensic practice, the headspace gas chromatographic procedure is the preferred method [10] based on enzymatical and thermal (decarboxylation) conversion of acetoacetate and β-hydroxybutyrate to acetone. The Electronic supplementary material (Online resource 2) shows a chromatogramm of the three ketone bodies which are transformed into acetone. Further methods that satisfy forensic requirements are electrospray mass spectrometry techniques with single-ion monitoring after liquid/liquid extraction and methoxim/tert-butyldimethylsilyl [20] or tert-butyldimethylsilyl-N-methyltrifluoroacetamide derivatization [21].

Human insulin, synthetic insulin analogues, C-peptide and proinsulin

To achieve therapeutic benefit, the insulin molecule was modified. The therapy of diabetes mellitus includes regular insulin (insulin without any addition which delays resorption), neutral protamine Hagedorn (human insulin bound to protamine to lower solubility) and insulin analogues (Electronic supplementary material, Online resource 3). Rapid-acting insulin analogues (insulin lispro (Humalog®); insulin aspart (Novomix®); insulin glulisine (Apidra®)) mimic postprandial insulin secretion by possessing considerably reduced self-association and are therefore faster in bioavailablity and shorter in duration after subcutaneous injection. The advantage of the long-acting analogues (insulin glargine (Lantus®), insulin detemir (Levemir®)) is their peak-free and constant action, which mimics basal or background insulin secretion.

In the Electronic supplementary material (Online resource 4), the onset of action, peak of action and duration of action of the different types of insulin on the market are summarized. In cases of acute poisoning, insulin levels reflect delays of insulin activity, including delayed absorption from the injection site and possibly prolonged clearance of absorbed insulin. In insulin (self)-overdose exogenous insulin follows first-order kinetics, resulting in a linear decrease in concentrations using a semi-logarithmic scale [22]. Using non-compartmental analysis in a case of human insulin intoxication in a type 1 diabetic, Shibutani and Ogawa [23] found an elimination half-life of 6.2 h. In another case of insulin poisoning in a type 1 diabetic patient, Fasching [24] identified a slow biphasic decrease with half-lives of 4 and 10 h for the two successive phases, respectively. Forensic scientists reported that serum insulin concentration in corpses varies significantly amongst individuals [25]. Iwase et al. [26] said that insulin level may decrease after death. Powner and Thicoipe [27, 28] reported that serum insulin levels either stay at the same level as antemortem levels or are higher.

Relevance

Reference levels of human insulin, C-peptide, the molar I/C-ratio (insulin/C-peptide) and proinsulin are summarized in Table 1. Reference values should be based on corresponding glucose levels [29]. Analysis of those peptides are of forensic interest in the investigation of unclear hypoglycaemia [30, 31]. In cases of human insulin injection, it is impossible to distinguish between insulin secreted by β-cells and exogenous insulin. The relationship human insulin/C-peptide is very complex. Due to different half-lives (insulin, 5 min [32, 33]; C-peptide, 30 min [34]) and clearance rates (insulin, 11–34 ml/min; C-peptide, 4.4 ml/min), the peptides reach their maximum concentration at different times after a meal and are not always present in equimolar amounts. Concentrations of circulating C-peptide in venous blood are five to ten times larger than insulin concentrations. Degradation of insulin is mainly the result of glutathione insulin transhydrogenase in the liver acting on A and B chains that have been separated by reductive cleavage of disulfide bonds [35], but insulin is

Table 1 Reference levels of relevant peptides in blood (I/C-ratio=molar ratio of insulin to C-peptide; insulin: μU/ml×6=pmol/l, C-peptide: μg/l×0.28=nmol/l, proinsulin: ng/l×0.11=pmol/l)

	Human insulin	C-peptide	I/C-ratio	Proinsulin
Reference range for non-diabetics	5–30 μU/ml [26, 95, 96, 136]	0.5–4 μg/L [39, 137, 138]	0.13 [139]	4.0 pmol/l [140]
			0.1 [39]	6.7±1.7 pmol/l [39]
				2.2-6.2 pmol/l [82]
				<3.0 pmol/l [5]
After glucose intake	Up to 120 μU/ml [141]	Up to 10 μg/L [95, 137, 142, 143]	0.19 [139]	8.5-11.3 pmol/l [6]
	Up to 200 μU/ml [6]	Up to 5.7 μg/ml [6]	0.12±0.47 [39]	
After overnight fasting	<6 μU/ml [6]	<0.7 μg/l [6]		<3 pmol/l [6]
For a hypoglycaemia diagnosis in the living	<4.14 μU/ml [144]	<1.07 μg/l [144]		<20 pmol/l [144]
	<4.97 μU/ml [45]	<0.71 μg/l [45, 145, 146]		<5 pmol/l [145]
Reference range in urine for non-diabetics	2.76–6.9 μU/ml [101, 102, 147, 148]	40–150 μg/l, 32–165 μg/l, 10–120 μg/l [101, 102, 147, 148]		

degraded in kidney, placenta, muscles and to a lesser extent, in plasma too [32]. Approximately 50% of insulin is removed by the liver in a single pass. C-peptide is cleared primarily by the kidney, transverses the liver without being extracted from hepatocytes. I/C in a living person is thought to reliably distinguish between hypoglycaemia due to exogenous insulin (I/C>1 meaning high insulin, low C-peptide which is under the detection limit most of the time [36]) and hypoglycaemia due to an insulinoma or a sulfonylurea overdose [37]. The suppression of C-peptide level is due to a negative feedback system that stops the cleavage of the proinsulin molecule after the detection of the active insulin molecule in bloodstream [38]. Hypoglycaemia caused by oral substances is biochemically indistinguishable from hypoglycaemia caused by an insulinoma. In both cases, C-peptide levels and insulin levels are increased, the ratio of insulin to C-peptide is <1 (mean value 0.16 [37, 39]) and their concentration is 31.66± 17.25 μU/ml [38] (human insulin), 3.44±1.45 μg/l and 7.85±3.21 μg/l (C-peptide) and 15.7±2.3 pmol/l (proinsulin) [39, 40]. The diagnosis of insulin-secreting tumours relies mainly on biochemical profile because of their often small size. In case of an insulinoma, the ratio of insulin to C-peptide in patients is equivalent to the one in healthy individuals (0.2 [41, 42]). Glucose levels >3.0 mmol/l exclude organic hyperinsulinism [41]. Even if several authors give the following thresholds of insulin (>3 μU/ml [43], >5 μU/ml [44], >6 μU/ml [45], >10 μU/ml [46]), of C-peptide (>0.6 μg/l [45], >0.71 μg/l [47], >0.9 μg/l [44]) and proinsulin (>5 pmol/l [45, 47], >20 pmol/l [30], >22 pmol/l [48]) at the time of symptomatic hypoglycaemia lying under 2.5 mmol/l there are reports of insulinoma patients that showed insulin levels below [49–51]. Clearly elevated serum insulin levels might be caused by intentional insulin overdose because levels of 1.000 μU/ml are rarely seen in patients with insulin-secreting tumours.

To interpret correctly the ratio of insulin to C-peptide their absolute concentration, blood glucose concentration, sampling conditions and sampling time must be brought into relation [52, 53]. Iwase et al. [26] demonstrated that the ratio of insulin to C-peptide in corpses that were examined less than 24 h after death was significantly lower than in those who were examined later.

Urinary insulin and C-peptide

Insulin is filtered at the kidney's glomerulus and is almost completely reabsorbed. Thus, a small but variable amount of insulin enters the urine (Table 1). Less than 1% of intact human insulin is excreted into urine [54]. Instead, human insulin [42, 54–57]—like its synthetic analogues Lantus and Levemir which are not observed intactly in urine specimens [58]—undergoes extensive metabolic reactions [59]. The enzymes 'insulin-degrading enzyme' (IDE) and 'endosomal-acidic insulinase' have been identified as the main insulin-degrading enzymes [60–62]. Several cleavage sites located at different A- and B-chain positions (A13/14, A14,15, B9/10, B10/11, B13/14, B16/17, B23/24, B24/25, B25/26, B29/30) were determined [54, 60, 61, 63]. Degradation products of Lantus are desB30-32, desB31-32 and desB24-32. The major degradation pathway of Detemir includes deacylation of the myristinic acid residue which yields desB30 human insulin [63]. About 6% of the C-peptide produced by the pancreas is excreted intact into urine [64].

Insulin in other matrices

Bile as specimen was used in a case of a patient who had been admitted to hospital for 9 days after insulin injection [65]. Not recommended to measure the insulin level are tissue samples of liver, brain or kidney [66]. Coe reported

that insulin and C-peptide rarely penetrate the blood-vitreous barrier [67]. In one case of an insulin intoxication, Winston et al. [68] measured vitreous insulin in a concentration of 31 μU/ml but there are no reference data or validated methods for VF and cerebrospinal fluid. If an injection site exists, the excision of the surrounding dermal area should be taken into consideration [69, 70] and a forensic toxicologic analysis of insulin in the injection site can be done.

Proinsulin

Proinsulin usually is released into blood circulation in sparse amounts. As proinsulin is not removed by the liver, its half-life is longer than that of insulin (90 min) and it accounts for 15–20% of the total amount of insulin+ proinsulin in the basal state [71]. There are two major proinsulins in plasma, called proinsulin-like material: the intact proinsulin and the des31,32 proinsulin, both usually appearing in equimolar amounts [72, 73]. Proinsulin-like material is increased in clinical conditions such as in the case of insulinoma [39, 74, 75], familial hyperproinsulinaemia [76, 77] and non-insulin dependent diabetes mellitus (non-IDDM) [78–81]. Fasting proinsulin levels are elevated in recent-onset IDDM patients [82]. The ratio of insulin to proinsulin amounts 3.4:1 in non-insulin-dependent diabetic patients and 6:1 in non-diabetic patients [39]. Proinsulin secretion is suppressed by hypoglycaemia, and it is not part of pharmaceutical insulin preparations thus its presence in plasma of a hypoglycaemic subject is evidence that any insulin that is present is of endogenous origin, too [47].

Analytics of the peptides

The methods routinely used to measure insulin are almost invariably different types of immunoassays (summarized in [30, 83]). However, the subsequent discovery of proinsulin [84], the demonstration that proinsulin-like molecules which account for 10–20% of the immunogenicity and even for more in diabetic patients [78] exist in human plasma [85, 86] and that human proinsulin, anti-insulin antibodies or synthetic analogues react like human insulin in many human insulin radioimmunoassay methods has put in question the specificity of human plasma insulin radioimmunoassays. Cross-reactivity varies from 40–100% depending on the polyclonal antibodies that are used, on the sample dilution, the assay incubation time and the temperature [79]. Furthermore, commercially available insulin (immuno)assays which are designed to measure human insulin do variably detect synthetic insulin or lead to false results due to partial reaction [87–89] although improved immunoassays for human insulin [90–92] and its analogues [93, 94] have been developed. Mass spectrometral methods would serve forensic purposes, however, a lot of methods do not cover synthetic insulins [95–97]. In sports drug testing, there was recently published a procedure to allow the unequivocal identification of synthetic insulin analogues and metabolic products derived from human insulin and its artifical counterparts in urine and plasma specimens [63, 98]. Figure 1 shows a chromatogramm of human insulin measured in mass spectrometry-enhanced product ion (MS-EPI) mode (molecule ion $(M+5H)^{5+}$=1,162.5 Da) and the appropriate product ion spectra determined by a modified procedure described by Thomas et al. [63].

Immunoassays to analyse plasma and urinary C-peptide are well established (summarized in [83, 99]). However, C-peptide is a small molecule with low antigenicity. thus the specificity of immunoassays is questionable due to differences in antibody specificities or matrix effects. If compared immunoassays gave consistently higher results than mass spectrometric assays [95, 100]. C-Peptide catabolic products with an increased half-life relative to intact C-peptide could contribute to C-peptide immunoreactivity, but more data are required to elucidate C-peptide metabolism and the relationship between immunoreactive species and intact C-peptide detected by immunoassays. Alternatives to immunoassays in forensic laboratories are LC-MS/MS after ultrafiltration of urine [101, 102] or solid-phase extraction [95, 100, 103]. The use of positive [100] and negative [102, 104] MS mode is possible, however, in all methods stable isotope-labeled analogue of C-peptide as internal standard was used ($[^2H_{14}]$C-peptide [102] or d8-Val7,10-C-peptide [104]). Figure 2 shows a chromatogramm in multiple reaction monitoring mode of the specific ion transitions 1,007→86, 1,007→70 and 1,007→130 for human C-peptide.

Proinsulin is mostly identified by immunochemical assays (summarized in [83]) which had to face critics because they could not distinguish between intact proinsulin and its intermediate forms. The problem was that they showed cross-reactivity with Des64,65-proinsulin and split 65–66-proinsulin. There is no mass spectrometric method for proinsulin described in the literature.

Analytical interferences and sample storage for the peptides

The main sources of interference with insulin determination are haemolysis, insulin degradation and different types of circulating antibodies [105–107]. It is recommended that the serum should be separated from red blood cells as soon as possible. The IDE, a peroxisomal protease [108], is widely distributed in various tissues including red blood cells especially in haemolysate [109, 110]. Plasma C-peptide measurement is unaffected by haemolysis [107]. The activity of IDE is decreased at 4°C or -20°C and insulin in blood and plasma samples is relatively stable

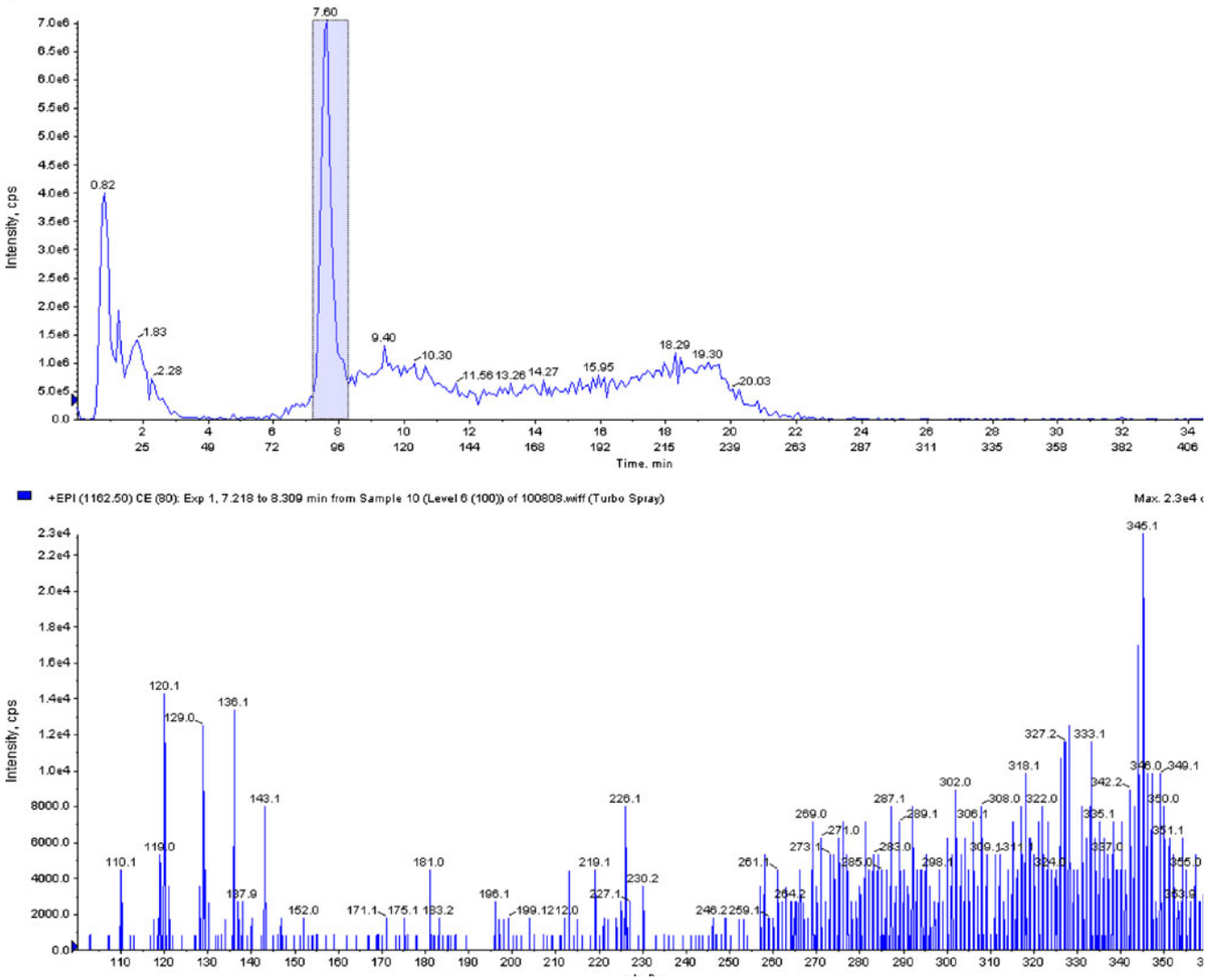

Fig. 1 Chromatogramm of a blank plasma sample spiked with 40 µU/ml human insulin (retention time, 7.60 min) measured in MS-EPI mode (molecule ion $(M+5H)^{5+}$=1,162.5 Da) and the appropriate product ion spectra determined after a modified procedure according to Thomas et al. [63]

(for at least 5 days [111] at 4°C). Serum collected at autopsy must be separated from cellular elements immediately and stored at −20°C [25, 66, 112–115] at which insulin is stable for several months, C-peptide for at least 40 days. However, a stability at 4°C for C-peptide is not given [63]. The effect of repeated freeze/thaw cycles on measured C-peptide appears to be variable: some authors reported no effect [116] although instability on storage at −20°C was found [115]. For human insulin and proinsulin there is no such effect [116–119]. Insulin degradation can also be prevented by adding IDE inhibitors into the sample tube before blood collection. The most effective inhibitors are sulfhydryl modifying reagents such as diamide, *p*-hydroxymercuribenzoate (1 mmol/l) [120] and *p*-chloromercuriphenylsulphonic acid (100 mg/l) [29, 107, 121]. *N*-ethylmaleimide (1 mmol/l) has been reported as an effective inhibitor [122], but it lacked stability in aqueous solution [121]. Appropriate preservatives for blood specimens of insulin and C-peptide are sodium fluoride serum separator tubes [123], heparinized samples are acceptable and the use of EDTA has been discussed controversially [103, 124–126]. All plastic and glassware contacting with C-peptide should be washed with a 1% bovine serum albumin solution for example [127] because of an enormous loss of peptide due to adsorption to glass surfaces. Another possibility to avoid losing the peptide during purification and measurement is to use low protein-bind polypropylen tubes. In post mortem insulin assays the insulin values measured in right heart blood were about ten times higher probably due to post-mortal diffusion of insulin via the portal vein [67, 128]. The measurements should therefore be made on peripheral venous (femoral or iliac) blood [25].

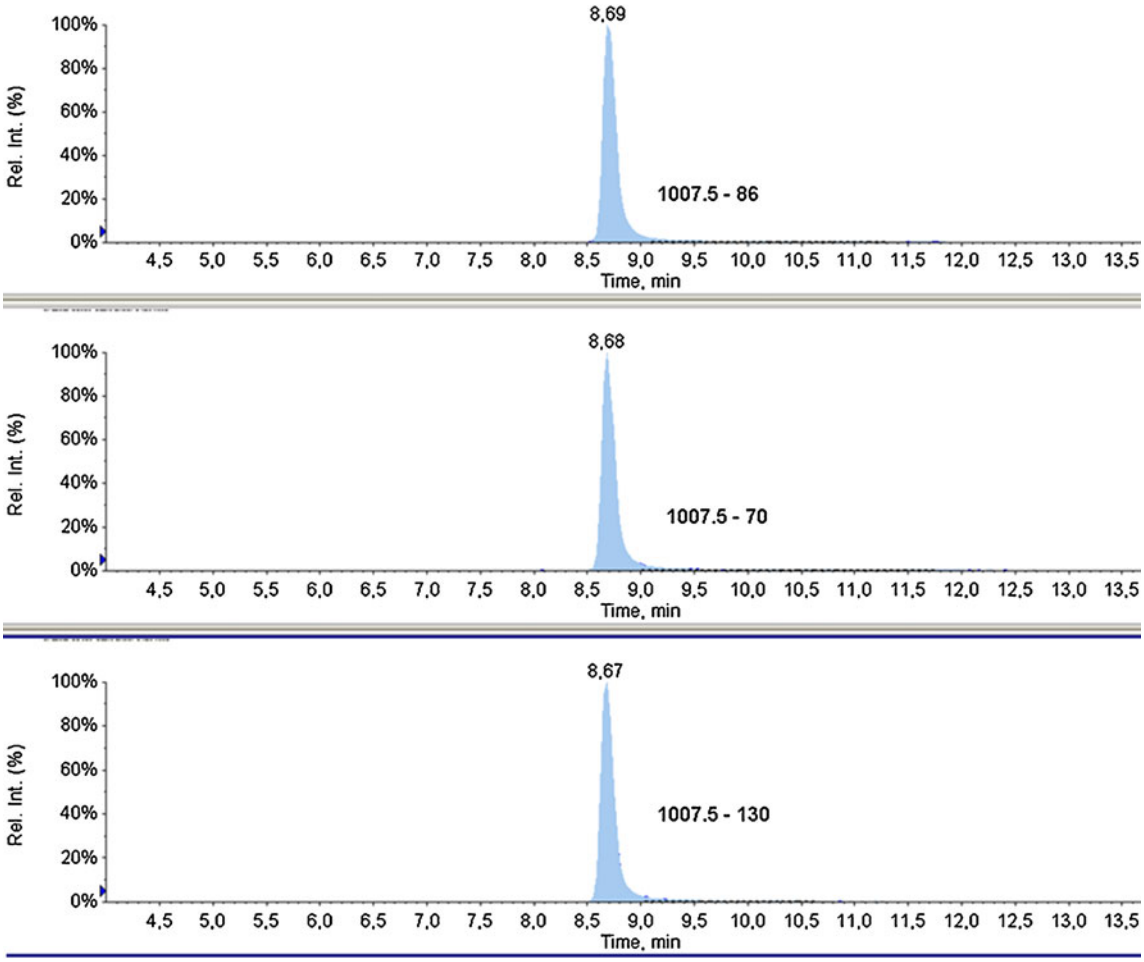

Fig. 2 Chromatograms in multiple reaction monitoring mode of a blank plasma spiked with 10 ng/ml C-peptide. C-peptide is isolated from human plasma by immunoaffinity precipitation with C-peptide-antibody-coated magnetic beads. After a 60 min binding step C-peptide is eluted from the beads by lowering the pH with acetic acid. After the sample preparation and the chromatographic separation over a C18 column the threefold protonated molecule ion $(M+3H)^{3+}=$ 1,007.5 is isolated in Q1. The three chromatograms show the specific ion transitions Q1→Q3 m/z 1,007.5→86, 1,007.5→70 and 1,007.5→130

Insulin antibodies

Patients with factitious hypoglycaemia usually have no detectable insulin antibodies [129, 130] as they used to have when animal insulin was the only type commercially available [131, 132]. Human and synthetic insulins are less antigenic. The antibodies can arise in development of type 1-diabetes, in insulin autoimmune hypoglycaemia [133] or following therapy with certain thiol drugs [83]. However, antibodies may be detectable in persons without hypoglycaemia [134] and in rare cases in people with insulinomas [135]. Anti-insulin antibodies can be present in a person who never received exogenous insulin before thus the cause and effect-relation between the magnitude of the increase in insulin antibodies and the degree of insulin resistance is quite unclear.

Summary

The diagnosis of glucose metabolism complications is often problematical in forensic practice in living people as well as in autopsy cases. Anamnesis and morphology as well as histology should be investigated with attention. However, the main evidence can be derived by biochemical parameters. A forensic laboratory engaged to detect a diabetic metabolism disorder should at least be able to detect glucose, lactate, HbA1c (and fructosamine) and ketone bodies by the methods described above. More important is the doubtless detection of human and synthetic insulins, C-peptide, (proinsulin) and oral antidiabetics which can cause hypoglycaemias: This doubtless evidence is only given by mass spectrometral methods which have to be inserted in forensic practice. In the Electronic supplemen-

tary material (Online resource 5), biochemical parameters characteristic for the diagnosis of insulinoma, factitious hypoglycaemia by human insulin or by oral antidiabetics are summarized.

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

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