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ORIGINAL ARTICLE

Preliminary results on the postmortem measurement of 3-beta-hydroxybutyrate in liver homogenates

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Abstract The concentrations of 3-beta-hydroxybutyrate (3HB) in blood and two liver samples were retrospectively examined in a series of medicolegal autopsies. These cases included diabetic ketoacidosis, nondiabetic individuals presenting moderate to severe decompositional changes and nondiabetic medicolegal cases privy of decompositional changes. 3HB concentrations in liver sample homogenates correlate well with blood values in all examined groups. Additionally, decompositional changes were not associated with increases in blood and liver 3HB levels. These results suggest that 3HB can be reliably measured in liver homogenates when blood is not available at autopsy. Furthermore, they suggest that metabolic disturbances potentially leading or contributing to death may be objectified through liver 3HB determination even in decomposed bodies.

Keywords Postmortem biochemistry · 3-Beta-hydroxybutyrate · Liver · Diabetes · Ketoacidosis

Introduction

Specific molecules may prove much more difficult to identify in biological samples collected during autopsy compared to clinically derived specimens. Indeed, the presence of compounds generated by putrefaction and the often-altered (decomposed) nature of the samples limit the direct applicability of clinically validated assays in a postmortem setting [1]. While peripheral blood, urine, and gastric contents represent the ideal biological fluids for toxicological purposes, they may be partly or completely unavailable in infant autopsies and severely decomposed corpses. Furthermore, should body damage in cases such as traffic accidents, fire fatalities, and explosions be extensive, the collection of sufficient amounts of blood, urine, and other samples for toxicology and biochemistry would be consequently limited.

Significant effort has therefore been directed towards identifying alternative, liquid or solid, biological specimens for toxicological analyses. Alternatives considered include liver, kidney, skeletal muscle, brain, lung, adipose tissue, and bone marrow samples as well as possible larvae of insects feeding on the host [1, 2].

Postmortem biochemical analyses are routinely performed on vitreous humor (glucose and electrolytes), whole blood (glycated hemoglobin and ketones) and postmortem serum obtained from peripheral or cardiac blood (markers of hepatic, cardiac and renal functions, various hormones, tryptase, markers of inflammation, and sepsis). Urine, pericardial, cerebrospinal, and synovial fluids are occasionally used for chemical and biochemical investigations. However, blood and urine samples collected during autopsy are usually reserved for toxicological analyses. Vitreous humor, pericardial, synovial, and cerebrospinal fluids are not systematically sampled. Additionally, their use is rather episodic and usually limited to situations in which blood and urine are unavailable.

As far as toxicology is concerned, the nature of the biological fluids collected from putrefied bodies may significantly reduce their application possibilities, rendering the direct application of clinically validated methods to decomposed samples for biochemical purposes impracticable.

Over the last 20 years, there has been a growing interest in the development of methods for qualitative and quantitative analysis of several compounds in postmortem matrices alternative to blood, despite the existence of objective limits related to sample handling and processing.

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These difficulties are mainly due to decompositional changes, postmortem autolysis, bacterial contamination, tissue homogenization, challenges in obtaining representative samples, time consuming techniques, as well as analytical and chromatographic obstacles. The analytical methods must therefore be able to minimize the role of potentially interfering substances such as lipids, proteins, and numerous other molecules which are usually present in high concentrations in such matrices [3–6].

Tissue samples obtained from the liver and kidney are often used in postmortem toxicology analysis, especially when blood is unavailable. Kidney and liver tissues are suitable to prepare homogenates; yet, as already mentioned, they also contain high concentrations of lipids, which may interfere with the analytical procedures' performance.

As a specimen, liver has the advantage of being relatively unaffected by postmortem redistribution compared to blood, although drug concentrations in its left side, which is proximal to the stomach and small intestine, may be affected by the postmortem diffusion of some compounds. Since most drugs are metabolized in the liver, both the parent substance and its metabolites may be present in high concentrations in the hepatic tissue. Additionally, one of the main problems in using liver samples for forensic purposes is the lack of a database concerning the hepatic concentrations of most analyzed molecules. The results obtained from liver analysis are therefore more complicated to interpret [7].

In the realm of postmortem biochemistry, C-reactive protein (CRP) has mainly been determined in postmortem serum from femoral blood. It has, however, been experimentally measured in other biological matrices, such as pericardial fluid, vitreous humor, cerebrospinal fluid, and liver homogenates, at times with promising results [8]. Astrup and Thomsen [9] investigated CRP levels in 50 forensic autopsy cases in different specimens, including liver samples, and concluded that liver CRP levels correlate well with postmortem serum concentrations, suggesting the possibility of using liver samples for CRP determination should blood or postmortem serum prove unavailable. Apart from this example, liver homogenates have not been used further for postmortem biochemical purposes.

The aim of this study was to investigate 3-beta-hydroxybutyrate (3HB) levels in blood and liver samples in a series of medicolegal autopsies that included cases of diabetic ketoacidosis and bodies presenting decompositional changes. The intention was to characterize 3HB concentrations in liver homogenates in comparison to blood levels as well as evaluate the usefulness of 3HB determination in liver samples in order to assess the metabolic disturbances potentially leading or contributing to death.

Material and methods

Forensic autopsy cases

A total of 48 subjects (28 males and 20 females) were included in this study and retrospectively selected from 2008 to 2012. Subject age range was between 16 and 78 years old, with a mean age of 54.6 years. All cases included in the study underwent complete autopsies preceded by unenhanced CT scans. Autopsies were ordered by the public prosecutor due to unclear circumstances of death and the bodies were transferred to the medicolegal center. Toxicology and histology were performed in all cases included in this study despite the presence of decompositional changes in most organs in a portion of the selected cases. Medical records, social histories of the deceased when available, as well as police reports were reviewed in all cases before conclusions were made.

The availability of both blood (peripheral or cardiac) and liver samples at autopsy was the inclusion criterion for the cases chosen.

The selected study population consisted of 7 diabetic ketoacidosis cases without decompositional changes, 1 insulin-dependent diabetic individual presenting severe decompositional changes, 20 nondiabetic cases (according to medical records) presenting moderate to severe decompositional changes, and 20 nondiabetic cases (according to medical records) without decompositional changes.

Vitreous glucose concentrations were measured in all cases where vitreous humor was collected during autopsy.

According to the literature [10], diabetic ketoacidosis was determined to be the cause of death when vitreous glucose concentrations were above 10 mmol/l (corresponding to 104 mg/ml) and blood 3HB levels were higher than 2.5 mmol/l (corresponding to 26 mg/ml), as well as when other causes of death were excluded based on all postmortem investigations. The insulin-dependent diabetic individual with severe decompositional changes showed a cardiac blood 3HB concentration consistent with the hypothesis of ketoacidosis as the cause of death (9.6 mmol/l, corresponding to 100 mg/ml). Vitreous humor was unavailable in this case.

The causes of death in the 20 individuals presenting moderate to severe decompositional changes were hangings (two cases), gunshot wounds (two cases), drug intoxication (three cases), drowning (two cases), natural deaths (six cases), and unknown (five cases).

The causes of death in the 20 individuals without decompositional changes were determined to be drug intoxication (four cases), hangings (six cases), coronary thrombosis (three cases), and ischemic heart disease (seven cases).

Blood samples

Peripheral blood samples were collected by aspiration with a sterile needle and a syringe from the left or right femoral vein(s) during autopsy. Blood samples were drawn after clamping the vein(s) at the proximal end and lifting the lower limb(s) for several minutes. Peripheral blood was not available in 11 out of 20 cases with moderate to severe decompositional changes as well as the insulin-dependent diabetic individual presenting a high degree of decomposition. In these cases, blood was sampled from the right and left cardiac cavities. Both peripheral and cardiac blood samples were stored in tubes containing sodium fluoride and immediately frozen at −20 °C.

Vitreous samples

Undiluted vitreous humor samples were obtained by aspiration using a sterile needle and syringe as soon as possible upon arrival of the bodies at the morgue. Right and left vitreous samples were collected through a scleral puncture at the lateral canthus, aspirated from the center of each eye, pooled in the same syringe, and mixed together. After collection, vitreous samples were immediately centrifuged at 3,000×*g* for 15 min. The separated supernatant was collected and stored in preservative-free tubes. Vitreous samples were transferred to the laboratory immediately after having performed the autopsies. When glucose determination was delayed, vitreous samples were stored at −20 °C.

Liver samples

Two samples (A sample and B sample) were collected in each case from the central part of the right lobe of the liver during autopsy. The first set of samples (A samples) was immediately homogenized using a laboratory blender. The tissue specimens were cut into small pieces with a disposable scalpel and 4 g was weighed out. Four milliliters of sterile, sodium chloride solution 0.9 % was added to the tissue (dilution 1:1). The second set of samples (B samples) was stored in preservative-free tubes and frozen at −20 °C. After 30 days, samples were thawed overnight at 4 ° C and homogenized using the same protocol as was used with the A samples. Complete homogenization of the tissue samples was not always possible due to specimen quality. However, approximately 90 % tissue homogenization was achieved by using the laboratory blender for 4 min.

Laboratory assays

3HB concentrations were determined on a Cobas Mira Plus (Roche Diagnostics, Switzerland) by an enzymatic photometric method adapted in house from the technique described by Ruell and Gass [11]. Frozen femoral and cardiac blood samples were thawed overnight at 4 ° C and deproteinized with perchloric acid. Supernatant was used for analysis. Similarly, liver homogenates (samples A and B) were deproteinized with perchloric acid and supernatant was used for analysis. 3HB concentrations were expressed in millimole per liter.

Glucose was determined on vitreous samples by enzymatic assays on a Dimension® Xpand® Plus Integrated Chemistry System (Siemens Healthcare Diagnostics Inc., Deerfield, IL, USA). Glucose concentrations were expressed in millimole per liter.

Statistical analysis

In diabetic ketoacidosis cases, in nondiabetic cases presenting moderate to severe decompositional changes and in nondiabetic cases privy of decompositional changes, 3HB concentrations in blood and liver samples were compared by matched pair test. The comparisons between patient groups were carried out by using the Wilcoxon test.

The linear relationship between 3HB in blood and liver samples was explored by Pearson's product–moment test. Correlation coefficient value ≥0.8 and between 0.7 and 0.79 was considered to define a very strong and strong correlation between variables, respectively, while coefficient ranging between 0.69 and 0.5 and 0.49 to 0.3 was considered to define moderate and low correlation, respectively.

Ethical aspects

Ethical aspects were discussed with the local ethics committee. Since the medicolegal autopsies had been ordered by the judicial authorities, analyses were performed as part of the investigations and no further ethical permission was required.

Results

3HB concentrations in blood and liver samples as well as the relative differences between values observed in A sample versus B sample and A sample versus blood in the three studied groups are summarized in Table 1.

In cases of diabetic ketoacidosis, 3HB levels ranged from 6.70 to 21.50 mmol/l (corresponding to 69.80 and 223.95 mg/ml, respectively), from 6.60 to 20.50 mmol/l (corresponding to 68.75 and 213.55 mg/ml, respectively), and from 5.30 to 18.50 mmol/l (corresponding to 55.20 and 192.70 mg/ml, respectively) in blood, A samples and B samples, respectively. Concentrations were statistically higher in blood than A samples (mean difference 1.46; 95 % CI 0.84, 2.09; p value <0.001) and in A samples than B samples (mean difference 0.78; 95 % CI −0.27, 1.28; p

Table 1 3-Beta-hydroxybutyrate concentrations in blood and liver samples and relative difference between values observed in A samples versus B samples and A samples versus blood in the three studied groups

		Diabetic ketoacidosis	Nondiabetic cases presenting moderate to severe decompositional changes	Nondiabetic cases privy of decompositional changes
Liver A	Median	9.95 (103.65)	0.16 (1.67)	0.12 (1.25)
	Range	6.60–20.50	0.10–0.17	0.10–0.16
		(1.04–1.67)	(68.75–213.55)	(1.04–1.77)
Liver B	Median	9.60 (100.00)	0.15 (1.56)	0.12 (1.25)
	Range	5.30–18.50	0.10–0.16	0.10–0.16
		(55.20–192.70)	(1.04–1.67)	(1.04–1.67)
Blood	Median	11.90 (123.96)	0.17 (1.77)	0.13 (1.35)
	Range	6.70–21.50	0.10–0.18	0.10–0.16
		(69.80–223.95)	(1.04–1.87)	(1.04–1.67)
(liver A − liver B)/liver A	Range	2.91 to 19.7 %	0 to 12.5 %	0 to 9.09 %
(liver A − blood)/liver A	Range	−21.05, −1.51 %	−15.38 %, 0	−10 %, 0

3HB concentrations are expressed in millimole per liter and (milligram per deciliter)

value=0.008). The relative differences between 3HB levels in A samples versus B samples and A samples versus blood were ≤21 % of A sample concentration in all cases of diabetic ketoacidosis.

Similarly, in nondiabetic cases presenting moderate to severe decompositional changes and in nondiabetic cases privy of decompositional changes, 3HB concentrations were statistically higher in blood than A samples (mean difference 0.01; 95 % CI 0.008, 0.01; p value <0.001 and mean difference 0.0045; 95 % CI 0.0021, 0.0089; p value <0.001, respectively) and in A samples than in B samples (mean difference 0.009; 95 % CI 0.005, 0.012; p value <0.001 and mean difference 0.002; 95 % CI 0.0001, 0.0039; p value= 0.042, respectively) (Table 1).

The relative differences between 3HB levels in A samples versus B samples and A samples versus blood were ≤15 and ≤10 % of A sample concentration in all the nondiabetic cases presenting decompositional changes and in all the nondiabetic cases without decompositional changes, respectively.

3HB concentrations were significantly higher in cases of diabetic ketoacidosis than in the other studied groups in both blood and liver samples ($p<0.001$ for every comparison). 3HB values were higher in nondiabetic cases presenting moderate to severe decompositional changes than in nondiabetic cases privy of decompositional changes in both blood and liver samples ($p<0.001$ for every comparisons). However, in all tested samples of both groups, 3HB concentrations were lower than the blood clinical reference value (0.17 mmol/l, corresponding to 1.77 mg/ml).

Linear dependence between the three variables was shown by Pearson's product–moment coefficient in the three groups (Table 2). Pearson's product–moment coefficient was ≥0.9, p value<0.001, and R-square ≥0.84 in every correlation between variables and in every group.

The linear function of 3HB levels in A samples versus B samples and in A samples versus blood is reported in Table 3. Slopes and intercept of the regression line equations were very close to 1 and 0, respectively, and their values ±2 standard error included 1 and 0 for every correlation (Table 3).

Table 2 Correlation between 3HB in blood and liver samples in the three studied groups assessed by Pearson's product–moment coefficient test

Group	Variable by variable	Pearson's product–moment coefficient test	
		Correlation	p value
DB	Liver A vs blood	0.99	<0.001
	Liver B vs blood	1	<0.001
	Liver B vs liver A	0.99	<0.001
DECO	Liver A vs blood	0.99	<0.001
	Liver B vs blood	0.9	<0.001
	Liver B vs liver A	0.92	<0.001
Control	Liver A vs blood	0.96	<0.001
	Liver B vs blood	0.96	<0.001
	Liver B vs liver A	0.97	<0.001

DB diabetic ketoacidosis, *DECO* nondiabetic cases presenting moderate to severe decompositional changes, *Control* nondiabetic cases without decompositional changes

Table 3 The linear function of 3HB levels in A samples versus B samples and A samples versus blood in the three studied groups (in parentheses standard error of intercept and slope)

Group	Linear function	*R*-square
DB	liver A=−0.099 (0.565)+1.084 (0.051)×liver B	0.99
	liver A=−1.127 (0.93)+0.983 (0.07)×blood	0.97
DECO	liver A=−0.006 (0.016)+1.1 (0.112)×liver B	0.84
	liver A=0.001 (0.005)+0.931 (0.036)×blood	0.97
Control	liver A=0.0059 (0.006)+0.968 (0.053)×liver B	0.95
	liver A=−0.0025 (0.009)+0.984 (0.0568)×blood	0.91

DB diabetic ketoacidosis, *DECO* nondiabetic cases presenting moderate to severe decompositional changes, *Control* nondiabetic cases without decompositional changes

Equivalency emerged from the bivariate fit of 3HB levels in A samples versus blood/B samples in diabetic ketoacidosis cases is shown in Fig. 1a. Equivalency emerged from the bivariate fit of 3HB levels in A samples versus blood/B samples in nondiabetic cases presenting decompositional changes (DECO) and in nondiabetic cases without decompositional changes (control) is shown in Fig. 1b. The regression line for every correlation described angle bisector between the axes.

Discussion

The postmortem diagnosis of diabetes mellitus with fatal complications, particularly ketoacidosis, requires the determination of specific biochemical markers. Vitreous glucose has been proposed by several authors as the most appropriate laboratory parameter to estimate antemortem blood glucose levels [12–16]. Other analyses involving the determination of the glycosylation level of some blood proteins [17–22] and ketone measurement in body fluids [23–31] have also been indicated to corroborate the hypotheses of constant antemortem hyperglycemia and increased blood ketone concentrations at the time of death, respectively.

Determination of ketone bodies in blood or alternative biological fluids, along with measurements of other biochemical parameters including vitreous glucose, urine adrenaline, and postmortem serum hormones, proteins, and markers of chronic alcohol consumption, has also been proposed in diagnosing alcoholic and starvation ketoacidosis as well as in supporting the hypothesis of hypothermia as the cause of death [10, 32–41].

The possibilities of establishing reliable diagnoses of diabetic ketoacidosis by using biochemical investigations can be extremely compromised when vitreous and blood are unavailable during autopsy or advanced putrefactive changes are present [42].

These limitations, however, may be drastically reduced if ketone measurements in the postmortem setting can be performed in biological fluids other than blood with equally reliable results [25, 26, 32, 35, 38, 39]. The need to identify alternative biological samples is therefore paramount. These should be collectable even when autopsies are performed on bodies presenting advanced decompositional changes and, above all, the measured compounds should normally be present and not increase with the onset of putrefaction.

Decompositional phenomena may markedly influence toxicological and biochemical results because they induce modifications in drug concentrations and may be responsible for molecule degradation. Additionally, putrefaction may lead to the production of numerous lower molecular weight compounds, drugs, or new substances by way of artefact, which may affect the way in which case results are interpreted [3, 43–46].

Iten and Meier [10] analyzed the relationship between blood 3HB concentrations and the interval after death in a series of medicolegal autopsies and did not observe any statistical increase in postmortem blood 3HB levels. These findings led to the conclusion that decompositional changes were not associated with 3HB production and that blood 3HB levels in decomposed bodies could be considered an appropriate biochemical parameter in the estimation of 3HB concentrations at the time of death. Similar results were obtained in two former studies, in which we investigated

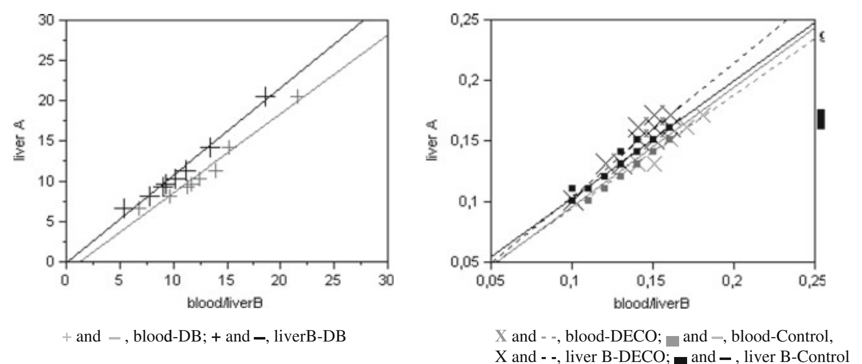

Fig. 1 Bivariate fit of 3HB levels in A samples versus blood/B samples in diabetic ketoacidosis cases (*DB*) (**a**) and in nondiabetic cases presenting decompositional changes (DECO) and in nondiabetic cases without decompositional changes (control) (**b**)

blood 3HB levels in a series of medicolegal autopsies that included bodies with decompositional changes [40, 47]. Kadiš et al. [39] had already postulated that 3HB does not increase after death but, at most, may decrease due to spontaneous molecule degradation.

Besides decompositional changes, other situations frequently encountered in the forensic setting may force the pathologist to deal with biological fluid insufficiency or unavailability during autopsy or to collect biological samples of poor quality for toxicological and biochemical purposes. Indeed, only small amounts of blood may be sampled during infant autopsy and specimens are often missing completely in severely damaged victims [48]. In these situations, the collection of alternative biological samples that can be reliably analyzed and whose results can be faithfully exploited to produce pertinent data is of utmost importance. This is especially crucial when faced with pathological processes, such as hypothermia, diabetic, alcoholic, and starvation ketoacidosis, which may be characterized exclusively by biochemical changes and limited (or absent) morphological findings [49].

The liver has been deemed the primary solid tissue of use in postmortem toxicology with the xenobiotic analysis resulting from this tissue often complementing blood toxicology data. Furthermore, the liver has the advantage of being the main metabolic organ, allowing sufficient quantities of tissue to be collected during autopsy for analyses. Liver samples are therefore the specimen of choice should blood prove unavailable due to massive blood loss, fire victims, or advanced decompositional changes. Liver samples can additionally be readily homogenized. Though the liver is relatively unaffected by postmortem redistribution or postmortem diffusion compared with blood, xenobiotic concentrations in the lobe proximal to the stomach and small intestine may artificially increase due to postmortem diffusion in cases of oral drug overdose. Therefore, use of tissue from the deep part of the right lobe is preferred for toxicological purposes [50–52].

In this study, we aimed to evaluate 3HB levels in liver samples obtained from medicolegal autopsies including cases of diabetic ketoacidosis fatalities and bodies presenting decompositional changes. Our goal was to characterize 3HB concentrations in liver homogenates and compare them with peripheral (or cardiac) blood 3HB levels in order to evaluate the usefulness of 3HB determination in liver samples to objectify the metabolic disturbances that can potentially cause or contribute to death.

The results of our study indicate that 3HB concentrations in liver homogenates correlate well with blood values in all examined groups, thus allowing diabetic ketoacidosis to be diagnosed.

Additionally, in nondiabetic decomposed bodies and nondiabetic individuals without decompositional changes, 3HB concentrations in both blood and liver samples were within clinical blood reference values, suggesting that decompositional changes are not associated with increased blood 3HB levels. This observation concurs with the conclusions of two former studies that focused on the interpretation of postmortem blood 3HB levels in decomposed bodies [10, 39].

Lastly, according to the results of our analysis, decompositional changes do not seem to be associated with increases in 3HB liver concentrations.

Though further studies are required to confirm these preliminary observations, our data suggest that 3HB can be reliably measured in liver homogenates when blood is unavailable during autopsy or when priority in using limited amounts of blood must be given to toxicological investigations. Liver homogenates can be considered appropriate, alternative, biological samples for 3HB determination when blood, vitreous, urine, pericardial, and cerebrospinal fluids cannot be collected during autopsy and biochemical investigations could prove useful in evaluating the metabolic disturbances that may have potentially led or contributed to death.

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Conflict of interest The authors have no conflict of interest to declare.

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