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The (non)sense of routinely analysing beta-hydroxybutyric acid in forensic toxicology casework

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ABSTRACT

Beta-hydroxybutyric acid (BHB) is a ketone body which is generated from fatty acids as an alternative energy source when glucose is not available. Determination of this compound may be relevant in the forensic laboratory as ketoacidosis – an elevated level of ketone bodies – may contribute to the cause of death. In this study, we aimed at determining the relevance of routinely implementing BHB analysis in the forensic toxicological laboratory, as BHB analysis typically requires an additional workload. We therefore performed an unbiased retrospective analysis of BHB in 599 cases, comprising 553 blood, 232 urine and 62 vitreous humour samples. Cases with BHB concentrations above 100 mg/L (in blood, urine and/or vitreous humour) were invariably associated with elevated levels of acetone, another ketone body, the detection of which is already implemented in most forensic laboratories using the gas chromatographic procedure for ethanol quantification. Our retrospective analysis did not reveal any positive case that had been missed initially and confirms that BHB analysis can be limited to acetone positive cases.

1. Introduction

Ketoacidosis is a metabolic disturbance caused by an elevated blood level of the ketone bodies acetoacetate, acetone and beta-hydroxybutyric acid (BHB). These compounds are generated by the liver when there is insufficient glucose available. Under these circumstances, lipids – stored as triglycerides in fatty tissue – are an alternative energy source. During lipolysis, triglycerides are degraded into fatty acids, resulting in the generation of acetyl-CoA. This co-enzyme is converted into acetoacetate, a precursor molecule of BHB and acetone. The latter compound is generated by decarboxylation of acetoacetate and is eliminated by urine and breath, resulting in a typical sweet odour of breath and urine. Since acetone may be reduced to isopropanol, also the latter may be found in case of ketoacidosis. On the other hand, acetoacetate may also be converted enzymatically into BHB. This small low molecular weight compound is readily water-soluble and can easily cross the blood brain barrier. There, BHB is converted into acetyl-CoA, which is on its turn via the Krebs cycle converted to ATP, an energy-carrying molecule. Consequently, high levels of ketone bodies indicate deficient sugar metabolism which may be caused by uncontrolled diabetes. Also the use of atypical antipsychotics may induce ketoacidosis. Other sources of ketoacidosis are chronic alcoholism, starvation, hypothermia or infection [1]. Conclusively, determination of ketone bodies is relevant in a forensic context as they may elucidate pathological findings or the cause of death. Yet, there remains a lot of uncertainty about the interpretation of (concentrations of) ketone bodies. E.g. it is unclear if ketone bodies (i.e. acetone, acetoacetate and/or BHB) should routinely be measured in all cases or if measurement can be limited to some specific cases. It is also unclear if all ketone bodies should be measured or if the determination of one ketone body suffices. Up till now, acetone is already co-detected routinely in blood and/or urine using a standard ethanol quantification procedure via headspace sampling–gas chromatography–flame ionization detection (HS–GC–FID). Even a simple dipstick test readily sheds some light on the extent of positivity of acetone and acetoacetate in urine. On the other hand, BHB analysis is not performed routinely. Mostly, BHB is determined when there is a suspicion of ketoacidosis, based on background information. However, sometimes there is no access to background information. Additionally, the presence of alcoholism and/or diabetes – two important factors causing ketoacidosis – is not always known.

Abbreviations: BHB, beta-hydroxybutyric acid; DBS, dried blood spots; FID, flame ionization detection; GC, gas chromatography; HS, headspace; IS, internal standard; LC, liquid chromatography; MS, mass spectrometry; QC, quality control.

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There is also some controversy about the preferred biological matrix. E.g. can vitreous humour be considered as an alternative for blood?

In this manuscript, we evaluate the relevance of routine implementation of BHB analysis in a forensic toxicological laboratory, taking into account that a dedicated analysis is required for the quantification of this ketone body, as this analyte is typically not covered by other routinely applied liquid chromatography (LC) or GC-based general screening procedures. To this end, we applied a recently developed dried spot-based method to perform retrospective BHB analysis of a large number of samples which were received for forensic toxicological analysis. In this retrospective analysis, we worked unbiased, i.e. we did not select samples based upon background information (e.g. known diabetic or alcoholic) and earlier obtained toxicological results.

2. Materials and methods

2.1. Chemicals and stock solutions

Heptafluorobutanol, acetic anhydride, pyridine and the sodium salt of BHB were provided by Sigma-Aldrich (Diegem, Belgium). Suprasolve ethyl acetate and methanol were obtained from Merck (Darmstadt, Germany). Deuterated GHB (GHB-d6) was used as internal standard (IS) and provided by Lipomed (Arlesheim, Switzerland).

A BHB stock solution was prepared by dissolving 50 mg BHB in one mL of methanol. This stock solution was diluted and used to prepare calibration standards of 5, 15, 25, 50, 100 and 300 mg/L. Quality control samples (QCs) of 65, 125 and 250 mg/L were prepared from an independent stock solution. For the IS, we made a 50 mg/L GHB-d6 solution in methanol. All solutions were stored at −20 °C.

2.2. Samples

We performed retrospective BHB analysis of routine samples – mostly post-mortem samples – which were received for forensic toxicological analysis in the Laboratory of Toxicology of Ghent University and stored at −20 °C. Since BHB has been found to be stable in post-mortem samples [2], the age of the samples was not an issue. All available samples were analysed, irrespective of background information and earlier obtained (toxicological) results. Finally, we had access to 599 cases, comprising 553 blood samples, 232 urine samples and 62 vitreous humour samples. Of these samples, dried matrix spots were prepared by pipetting 15 μL of biofluid onto a 7-mm pre-punched disc of Whatman 903 filter paper. These spots were dried for at least 2 h and stored at room temperature in a zip-closure plastic bag with desiccant before analysis. Acetone and isopropanol were qualitatively assessed in the procedure for routine ethanol quantification (ISO/IEC 17025 accredited), applied at the day of arrival using HS–GC–FID.

2.3. BHB analysis

For the quantification of BHB, a previously developed and validated GC coupled to mass spectrometry (GC–MS) method was slightly adapted [3]. This adaptation consisted of using 7-mm pre-punched discs, onto which a fixed volume of 15 μL biofluid was applied, rather than using 6-mm partial punches from a dried blood spot (DBS). The protocol used can be summarized as follows: first, a fixed volume of 15 μL of blood, urine or vitreous humour is spotted onto a 7-mm pre-punched disc of Whatman 903 filter paper. After adding 5 μL IS (50 mg/L GHB-d6 in methanol) to the dried spots, the punches are subjected to "microwave-assisted on-spot derivatization", as described elsewhere [3]. Briefly, derivatization is achieved by direct application of 25 μL acetic anhydride and 25 μL pyridine onto the spots and microwave heating for 90 s at maximum power, followed by a second derivatization for 90 s at maximum power with 25 μL heptafluorobutanol. Following evaporation under nitrogen, 100 μL of ethyl acetate was added to the samples. Finally, one μL of the derivatized extract is injected into an Agilent 6890 GC coupled to a 5973 MS system. Chromatographic separation is achieved on a 30 m × 0.25 mm i.d. × 0.25 μm Agilent HP-5MS column. Helium is used as carrier gas with a flow rate of 1.1 mL/min. A splitless injection is chosen. The injection temperature is set at 250 °C. The initial oven temperature is 60 °C, which is held for 2 min. Then, the temperature ramped at 8 °C/min to 110 °C, raised 30 °C/min until 230 °C, followed by an increase of 50 °C/min to 300 °C, which is held for 2 min. The transfer line temperature, MS ion source temperature and MS quadrupole temperature are set at 300 °C, 230 °C and 150 °C, respectively. Quantification of BHB and GHB-d6 is performed in SIM mode using *m/z* 227, 268 and 285 for BHB and 231, 245, 273 and 291 for GHB-d6. Quantifier ions are underscored.

Validation of the procedure starting from a 6-mm disc of a 25-μL DBS prepared from fresh whole blood [3] encompassed the following: calibration lines were linear over the 5–300 mg/L concentration range, applying a 1/x weighted linear regression. Within-day and between-day precision criteria (<20% at LLOQ, <15% at 3 other QC levels) were fulfilled for all QC levels, whereas accuracy was slightly above the acceptance criterion for two QC levels, i.e. >15% but still below 20%. No carry-over was observed following injection of the highest concentration of the calibration curve. Samples could be diluted 4-fold without influencing precision and accuracy. Stability studies revealed no significant alteration ofBHB concentration in DBS which were stored in a zip-closure plastic bag with desiccant for 84 days at room temperature [3].

3. Results and discussion

BHB analysis was based on a previously developed GC–MS method for the detection of several polar low molecular weight compounds with relevance in forensic and clinical toxicology [3]. This validated procedure (with punching a 6-mm disc from a 25-μL DBS) was slightly modified in our application as we wished to apply the method on a divergent set of matrices (urine, vitreous humour, post-mortem blood, . . .) which have a different spreading on the filter paper. To this end, a whole-cut approach with application of a fixed volume of 15 μL of biofluid onto a 7-mm pre-punched disc was utilized instead of using partial punches. To ensure the validity of the slightly adapted method we applied, a calibration line and QC samples were run every day of analysis. %CV was below 20% and %bias below 13% for 3 QC levels (65, 125 and 250 mg/L). These QC concentrations were based on proposed cut-off levels (see below).

The BHB concentrations obtained in blood, vitreous humour and urine were arbitrarily grouped in different classes (Fig. 1): BHB concentrations below 50 mg/L were considered as 'low', concentrations between 50 and 100 mg/L as 'slightly elevated', concentrations between 100 and 250 mg/L as 'moderately elevated', and concentrations above 250 mg/L as 'high and pathologically significant'. Although this classification is rather based on decision levels for BHB in blood and vitreous humour, we also applied these ranges for urine, as suggested by Elliott et al. [4].

As can be seen in Fig. 1a, a 'slightly or moderately elevated' BHB blood level was found in 28 cases (5%) and a 'high' BHB level in 5 (0.9%) blood samples. Also in vitreous humour (Fig. 1b), most samples (89%) had a 'low' BHB concentration. There were no vitreous humour samples with a BHB concentration higher than