A rapid HILIC-MS/MS method for the determination of β-hydroxybutyrate in post-mortem blood

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Introduction

In ketoacidosis, the concentration of the ketone body β-hydroxybutyrate (BHB) (Fig. 1) reaches a level that impacts the natural pH of the blood. Because ketoacidosis can be fatal, it is relevant to determine the concentration of BHB in autopsy cases where the cause of death may have been affected by this metabolic state. Interpretation of BHB concentrations in post-mortem blood is often performed by using the following ranges: normal, <500 µM; raised, 500-2,500 µM; and high and pathologically significant, >2,500 µM [1].

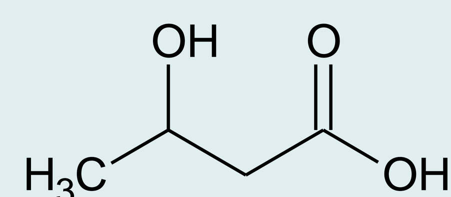

Fig. 1
Molecular structure of BHB.

The quantitative determination of BHB in post-mortem blood is mostly performed using headspace gas chromatography mass spectrometry (HS-GC-MS) after the enzymatic conversion of BHB to acetoacetate, followed by decarboxylation of the acetoacetate to acetone. The analysis must be repeated without the enzymatic reaction to identify the BHB. In this study, a simple hydrophilic interaction liquid chromatography tandem mass spectrometric (HILIC-MS/MS) method was developed for the direct determination of BHB in post-mortem blood.

Experimental

Standards and reagents

Standard solutions: BHB (Sigma-Aldrich, Schnelldorf, Germany) in methanol (MeOH) at concentrations of 100; 1,000; 10,000; 20,000; 30,000 and 40,000 µM. Internal standard solution (IS): BHB-$^{13}C_4$ (Cambridge Isotope Laboratories, Andover, MA) in MeOH at 500 mg/L. Mobile phase A, 1 mM ammonium acetate; mobile phase B, acetonitrile (MeCN).

Equipment

Mass spectrometer: Xevo TQ MS triple-quadrupole instrument with an ESI ion source (Waters, Milford, MA). LC system: Acquity UPLC system (Waters). Solid phase extraction (SPE) cartridges: polymeric strong cation exchange sorbent, 60 mg Strata-X-C (Phenomenex, Torrance, CA). HPLC column: SeQuant ZIC HILIC (5 µm, 200 Å, 2.1 mm I.D. × 100 mm) column (Merck SeQuant, Umeå, Sweden).

Extraction and clean-up

Post-mortem blood (100 µL) was mixed with 100 µL of water, 100 µL of methanol (MeOH) and 100 µL of the IS. The proteins were precipitated by adding 600 µL of MeCN, followed by centrifugation at 10,000×g. A 600 µL volume of the supernatant was mixed with 250 µL of water and filtered through an SPE cartridge, previously conditioned with 1 mL of MeOH followed by 1 mL of water. A 100-µL volume of the effluent was mixed with 900 µL of 0.1% acetic acid (HAc) in MeCN.

Calibration

Pure standard solutions were used for calibration. Volumes of 60 µL of the BHB standard solution and 60 µL of the IS were mixed and diluted with 730 µL of 50% MeCN. Then, 100 µL of this solution was mixed with 900 µL of 0.1% HAc in MeCN. The calibrant concentrations were equivalent to 100, 1,000, 10,000, 20,000, 30,000 and 40,000 µM BHB in the blood samples.

Chromatography and mass spectrometry

A volume of 10 µL of the extract was injected onto the column, which was maintained at 30°C, and elution was performed with a gradient (Table 1). The BHB concentration was measured in both the negative and positive ESI ion modes (Table 2).

Table 1
Chromatographic conditions.

Time (min)	Mobile phase A (%)	Flow (µL/min)	MS bypass (min)
0 - 4	5 > 45 linear	200	0 – 2.5
4 - 4.2	45 > 95 linear	200	
4.2 - 5	95	200	4.5 - 10
5 - 5.5	95 > 5 linear	200	
5.5 - 10	5	200	

Table 2
Mass spectrometric conditions. The source and desolvation temps. were 150 and 600°C, respectively.

ESI mode	Substance	Q1 *m/z*	Q3 *m/z*	Capillary voltage (kV)	Cone voltage (V)	Collision energy (eV)
-	BHB	103	59	2.4	18	10
-	BHB-$^{13}C_4$	107	61	2.4	18	10
+	BHB	105	87	3.0	10	5
+	BHB-$^{13}C_4$	109	91	3.0	10	5

Results and discussion

BHB was separated from γ-hydroxybutyrate (GHB) by chromatography (Fig. 2), and one abundant transition product was obtained by both ESI(-) and ESI(+) (Table 2). However, ESI(-) was more sensitive, and GHB did not produce a significant transition product at the ESI(-) *m/z* 59. No significant differences were observed between the slopes of calibration curves based on the pure calibrants and the matrix-matched calibrants; additionally, the retention times of BHB and BHB-$^{13}C_4$ were equal. Consequently, pure calibrants were selected for calibration, which eliminated the uncertainty of the correction for the endogenous content of BHB in the calibrants.

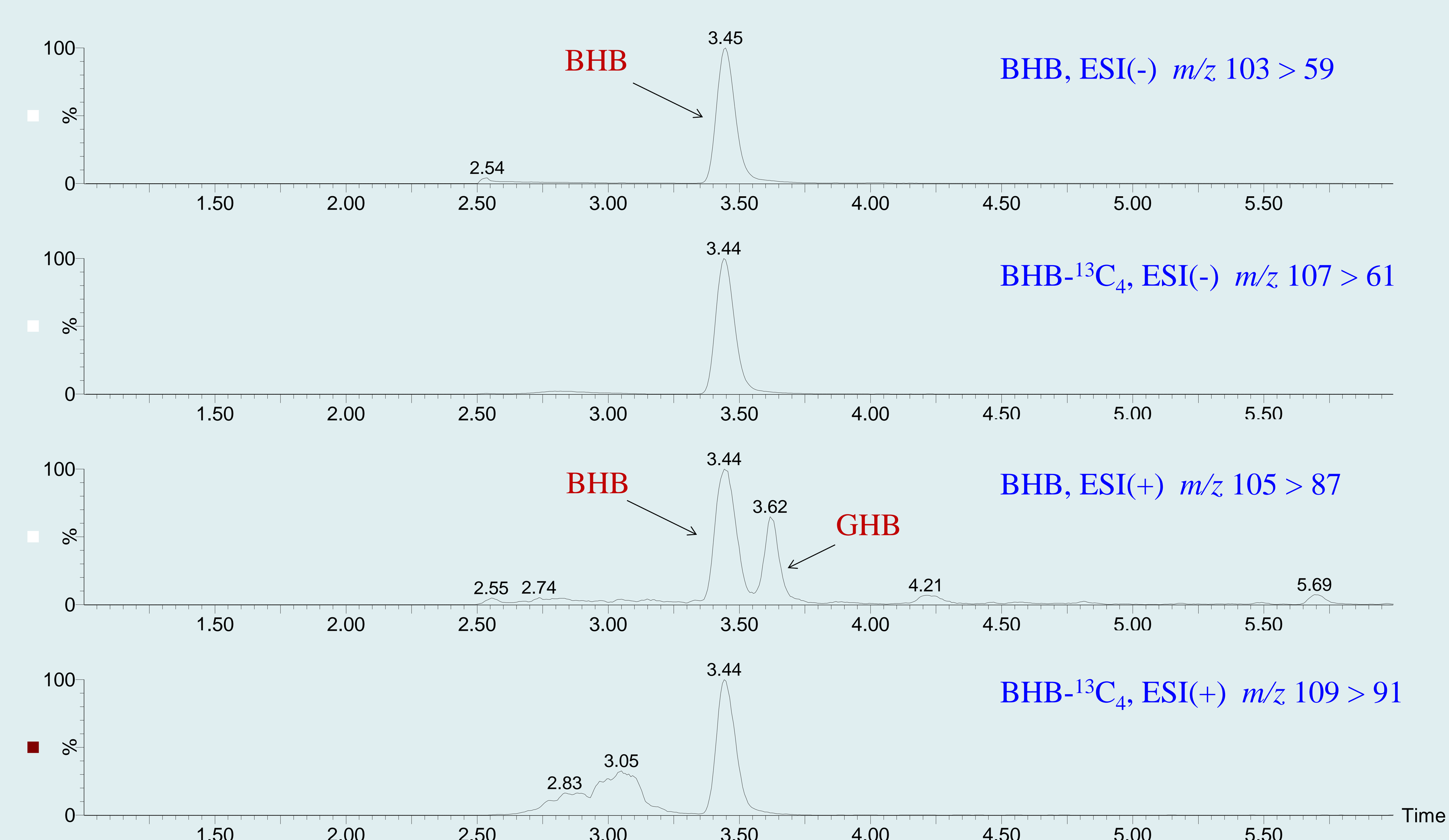

Fig. 2
Chromatograms of post-mortem blood containing 1.200 µM BHB.

The relative repeatability standard deviation (RSD_r) and the relative intra-laboratory reproducibility standard deviation ($RSD_{R,intra\text{-}lab}$) were less than 10% at raised and high concentrations (Table 3). The mean true extraction recovery was greater than 95% at levels of 1,000 and 20,000 µM BHB. The matrix effect (ion suppression) was 38±4% in ESI(-) and 3±19% in ESI(+). The lower limit of quantification (LLOQ), corresponding to an $RSD_{R,intra\text{-}lab}$ of 20%, could not be determined exactly because of the endogenous content of BHB. However, if the absolute $SD_{R,intra\text{-}lab}$ determined at a level of 150 µM is considered constant in the range of LLOQ-150 µM, LLOQ values of 20 and 100 µM BHB were obtained for ESI(-) and ESI(+), respectively. That is most likely a conservative estimate, because the absolute $SD_{R,intra\text{-}lab}$ in most cases declines with decreasing concentrations

Table 3
Precision values obtained from determination of the endogenous content of BHB in post-mortem blood (n = 4). Duplicate analyses were performed on eight different days.

Transition	Conc. level (µM)	RSD_r (%)	$RSD_{R,\ intra\text{-}lab}$ (%)
ESI (-) *m/z* 103 > 59	150/400/3,700/16,000	3/3/2/3	3/4/3/5
ESI(+) *m/z* 105 > 87	150/400/3,700/16,000	8/8/4/4	14/8/5/5

This method was compared with an HS-GC-MS method [2] on 10 post-mortem blood samples containing endogenous BHB in the range of 100-16,000 µM (Fig. 3). The slope and intercept of the regression line was not significantly different from 1 and 0, and the R^2 value was greater than 0.98 for the range 100-1000 µM and greater than 0.99 for the range 100-16,000 µM.

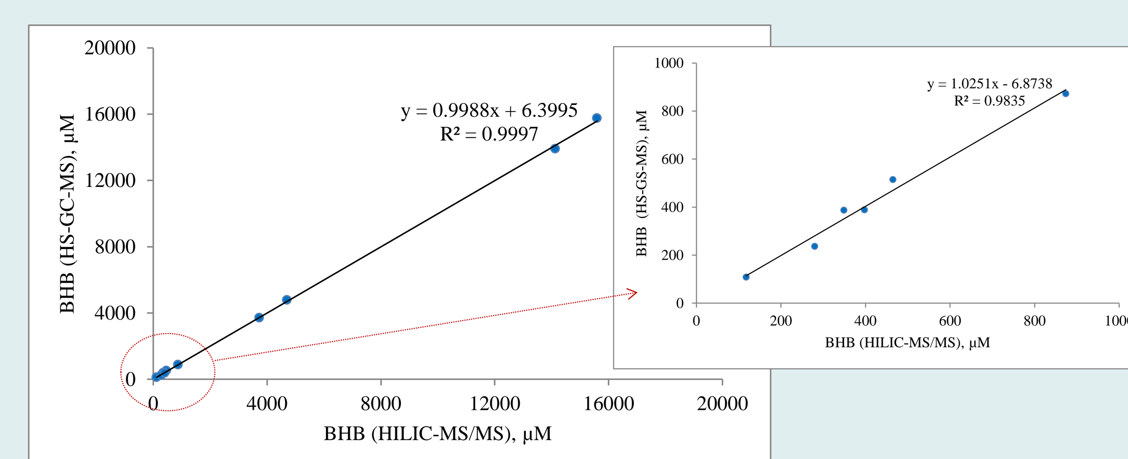

Fig. 3
Comparison of HILIC-MS/MS operating in ESI(-) mode with HS-GC-MS on post-mortem blood.

Conclusion

A rapid and selective HILIC-MS/MS method was developed to determine BHB in post-mortem blood samples. The procedure does not require the conversion of BHB to acetone or derivatisation of the native molecule.

References

[1] S. Elliott, C. Smith, D. Cassidy, Forensic Sci. Int. 198 (2010) 53-57.
[2] K.M.D. Holm, K. Linnet, B.S. Rasmussen, A.J. Pedersen, J. Anal. Toxicol. 34 (2010) 549-554.

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