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Simultaneous determination of β-hydroxybutyrate and β-hydroxy-β-methylbutyrate in human whole blood using hydrophilic interaction liquid chromatography electrospray tandem mass spectrometry

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ABSTRACT

Objectives: For the quantification of β-hydroxybutyrate (BHB) and β-hydroxy-β-methylbutyrate (HMB) in human whole blood, a method using hydrophilic interaction liquid chromatography tandem mass spectrometry (HILIC–MS/MS) was developed, which does not require chemical modification of the analytes.

Design and methods: Samples were deproteinised by a mixture of methanol and acetonitrile, and the extracts were cleaned-up using both polymeric strong cation exchange and strong anion exchange sorbents. The analytes and their structural isomers were separated using a column with a zwitterionic stationary phase. Isotope dilution of both analytes was used for quantitative analysis.

Results: Separation of BHB from isobaric interferences was achieved through chromatography. The relative intra-laboratory reproducibility standard deviations were better than 10% for blood samples at concentration levels of 10–20 μM BHB and 1 μM HMB and better than 5% at concentration levels 10 times higher. The mean true extraction recoveries were close to 100%. The trueness expressed as the relative bias of test results was within ±5% at concentration levels of 10–1000 μM BHB and 1–20 μM HMB. The lower limits of quantification were estimated to be 3 μM for BHB and 0.4 μM for HMB.

Conclusions: A simple and highly sensitive and selective HILIC–MS/MS method was developed that is suitable for the quantification of BHB and HMB in whole blood.

Introduction

Ketone bodies (β-hydroxybutyrate (BHB), acetoacetate and acetone) are organic compounds used primarily as an alternative source of energy in the human body. In addition, ketone bodies have anticonvulsant effects in antiepileptic therapy [1] and neuroprotective properties [2]. They might also protect against oxidative stress [3]. The latter implies that ketone bodies could have an anti-ageing effect, protecting the body against age-related diseases such as Alzheimer's disease, cancer, and cardiovascular disease [3–5].

The production of ketone bodies is highly regulated and depends on physiological conditions. The primary source of ketones is the beta-oxidation of fatty acids in the liver [6,7]. In the postprandial and fed state, low plasma concentrations (<100 μM) of ketone bodies are present due to high plasma insulin levels promoting low production and utilisation of fatty acids. In contrast, high plasma concentrations (up to 6000 μM) of ketone bodies are present during fasting and starvation when plasma insulin levels are low and stress hormone levels (epinephrine, glucagon, cortisol and growth hormone) are high, promoting lipolysis, beta-oxidation of fatty acids and ultimately ketogenesis [7,8]. In pathologic conditions, such as fatal diabetic ketoacidosis, the plasma concentrations of ketone bodies reach levels higher than 20,000 μM due to insulin resistance and low insulin concentrations, leading to high lipolysis in adipose tissue and high ketogenesis in the liver [9–11].

In 1921, Russel Wilder described the "ketogenic diet", a meal with a high content of fats and low content of carbohydrates and protein [12]. It was known that fasting controlled seizures better in children with medically refractory epilepsy, and Wilder hypothesised that ketones were directly responsible for the observed anticonvulsant effects. The underlying mechanisms are still unclear, but several human and animal studies have supported a significant anticonvulsant effect of different "ketogenic diets" [4,7,13].

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The essential amino acid leucine and its metabolite β-hydroxy-β-methylbutyrate (HMB) have also received increased interest during recent years and are now widely used by sportsmen and bodybuilders as nutritional anabolic supplements. Both human and animal studies have shown effects on protein synthesis, muscle hypertrophy, decreased muscle damage and muscle soreness, modulation of the cholesterol profile and even improved emotional status [14,15].

Although ketone bodies have been investigated for decades, many of their effects are still unclear, and there is a continuing incentive to conduct mechanistic and clinical studies in the field. For such studies, sensitive analytical methods with high selectivity and specificity are needed. Head-space gas chromatography (HS-GC) has been applied in several cases for indirect determination of BHB in plasma and blood samples [16–19]. This technique requires the enzymatic conversion of BHB to acetoacetate followed by the decarboxylation of acetoacetate to acetone. An additional run without enzymatic conversion is necessary for subtraction of the endogenous content of acetoacetate and acetone. GC with mass spectrometric detection of derivatised analytes has also been reported for the determination of BHB in blood [20,21] and HMB in plasma [22]. Similarly, liquid chromatography using enzymatic oxidation followed by pre-column derivatisation of the analyte and detection by UV spectrometry has been used for the determination of BHB in plasma [23]. Direct determination of BHB by reverse phase ultra-high performance liquid chromatography tandem mass spectrometry has also been reported for quantitative analysis above a cut-off level of approximately 500 μM [24]. However, the direct determination of the analyte molecules at low concentrations using common reverse phases is generally difficult because the polar BHB and HMB show poor retention on such sorbents. Few possibilities are available for optimising the selectivity and reducing the matrix interferences by this separation technique.

The presence of endogenous γ-hydroxybutyric acid (GHB) in blood constitutes another analytical problem because of its similarity with BHB. The endogenous content of GHB in blood is normally below 40 μM [25,26]. However, higher concentrations may be observed because GHB is also abused for recreational purposes and has been found in the body fluids of suspected victims of drug-facilitated sexual assaults [27]. Published liquid chromatography mass spectrometric methods for determination of GHB in blood use either traditional reverse phase sorbents for retention of the native molecule [24,28,29] or a derivative of the molecule [30] or a hydrophilic stationary phase for retention of the native GHB [31].

The present analytical method was developed to obtain a sensitive method for the direct determination of BHB and HMB in whole blood from clinical studies, thereby helping to further define and understand the effects of these metabolites. This method achieves high selectivity and specificity by utilising hydrophilic interaction liquid chromatography tandem mass spectrometry (HILIC–MS/MS) on extracts cleaned up by solid phase extraction (SPE). The potential interference from endogenous GHB was eliminated by chromatography. The validation of the method was performed with consideration of the endogenous concentrations of BHB and HMB present in all blood samples.

Materials and methods

Chemicals

BHB sodium salt, HMB, GHB sodium salt and α-hydroxybutyric acid (AHB) sodium salt were obtained from Sigma-Aldrich (Schnelldorf, Germany). The stable isotope analogue BHB-$^{13}C_4$ sodium salt was obtained from Cambridge Isotope Laboratories (Andover, MA). HMB–D_6 was synthesised by the principle described by Krapcho and Jahngen [32] and Jacobs *et al* [33]. Formic acid, acetic acid and ammonium acetate were purchased from Merck (Darmstadt, Germany). Acetonitrile (ACN) and methanol (MeOH) of LC–MS grade were purchased from Sigma-Aldrich. Water was purified using a Direct-Q 3 apparatus (Millipore, Bedford, MA).

Samples

Samples of ante-mortem whole blood, used for the validation of the method, were obtained from the Department of Forensic Medicine, Aarhus University. The ante-mortem blood samples used in the validation study were preserved in Venosafe VF-109SFX07 tubes (Terumo Europe, Leuven, Belgium) that contained 100 mg of sodium fluoride (NaF) and 22.5 mg of potassium oxalate (FO mixture) for a 9 mL draw volume of blood. Venosafe VF-053SFC32 tubes (Terumo Europe) that contained 6.8 mg of NaF and 15.7 mg of citrate-EDTA buffer ingredients (FC mixture) for a 3 mL draw volume of blood and Venosafe VF-052SDK tubes (Terumo Europe) that contained 3.9 mg K_2EDTA for a 2 mL draw volume of blood were included in a stability study on BHB and HMB in whole blood. Whole blood samples for calibration were obtained from the Blood Bank, Aarhus University Hospital (Skejby, Denmark).

Standards

Separate 10 mM stock solutions of the active substances were prepared in MeOH and stored at −20 ± 2 °C. Combined standard solutions for the fortification of the samples and preparation of the calibrants were prepared by diluting the stock solutions with MeOH. Standard concentrations for the preparation of the calibrants were prepared at 0.2, 2, 10, 20, 30 and 40 μM HMB and at 10, 100, 500, 1000, 1500 and 2000 μM BHB. An internal standard solution (SIL–IS) containing BHB-$^{13}C_4$ and HMB–D_6 at concentrations of 500 μM and 20 μM, respectively was prepared in MeOH.

Equipment

The liquid chromatography system was a Waters Acquity UPLC system that consisted of a binary pump, an autosampler with a 10 μL sample loop thermostated at 7 ± 2 °C and a column oven set at 30 ± 2 °C (Waters, Milford, MA). The mass spectrometer was a Waters Xevo TQ MS triple–quadrupole instrument with an ESI ion source. The separation was performed using a SeQuant ZIC HILIC column (5 μm, 200 Å, 2.1 mm I.D × 100 mm) (Merck SeQuant, Umeå, Sweden). SPE was performed on a 3 mL Strata-X-C cartridge containing 60 mg of a polymeric strong cation exchange (SCX) sorbent (Phenomenex, Torrance, CA) and on a 3 mL IST Isolute cartridge containing 100 mg of a strong anion exchange (SAX) sorbent (Biotage, Uppsala, Sweden). A VacMaster-20 vacuum manifold (Biotage, Uppsala, Sweden) was used during the SPE procedure. A TurboVap LV (Caliper Life Sciences, Hopkinton, MA) was used for solvent evaporation by a stream of nitrogen. Disposable 2 mL polypropylene Safe-Lock tubes (Eppendorf, Hamburg, Germany) were used for the extractions. Autosampler vials (300 μL) made of glass (Chromacol, Welwyn Garden City, UK) were used for storage of the final extracts. Other equipment used included pipettes (Biohit, Helsinki, Finland) and a Heraeus Biofuge Pico (Thermo Scientific, Langenselbold, Germany).

Extraction and clean-up

A 200 μL volume of sample was transferred to a disposable 2 mL centrifuge tube and then 100 μL of SIL-IS solution and 100 μL of MeOH were added. The tube contents were mixed gently after each addition. Shortly thereafter, a 600 μL volume of ACN was added, and the tube was immediately closed and vigorously vortex-mixed for a few seconds. After a standing time of 5–10 min, the mixture was centrifuged at 10,000 ×*g* for 5 min. A volume of 600 μL of the supernatant was mixed with 250 μL of water and was then after a standing time of at least 5 min passed through a CX SPE cartridge with a maximum flow rate of 0.5 mL/min (gravitational force (GF) was used). The cartridge had been previously conditioned sequentially with 1 mL of MeOH followed by 1 mL of water. The eluate was collected, and the cartridge was washed with 850 μL of 60% ACN, which was collected in the same

tube. A volume of 850 μL of the combined eluate was passed through a SAX column by GF. The cartridge was previously conditioned sequentially with 1 mL of MeOH, 1 mL of 1 M ammonium acetate and 1 mL of water. The cartridge was washed with 1 mL of 80% ACN by GF and sucked dry for a few seconds. The substances were eluted from the column by 2 mL of 1% formic acid in ACN. The ACN eluate was evaporated at 30 °C under a stream of nitrogen. The residue was redissolved in 200 μL 0.1% acetic acid in ACN.

Calibration

Pure calibrants were prepared by mixing 60 μL combined standard solution with 60 μL of SIL-IS solution. After evaporation of the solvent, the residue was redissolved in 400 μL of 0.1% acetic acid in ACN. The obtained concentrations were 0.03, 0.3, 1.5, 3, 4.5 and 6 μM HMB and 50-fold higher concentrations of BHB. The calibration curves were created from weighted (1/x) linear regression analysis of the IS-normalised peak areas (analyte area/IS area).

HILIC-MS/MS analysis

The sample extracts were maintained at 7 ± 2 °C prior to analysis. A 10 μL volume was injected onto a SeQuant ZIC HILIC column running 5% mobile phase A (1 mM ammonium acetate) and 95% mobile phase B (ACN). The mobile phase was changed through a linear gradient to 45% A over 4 min. Then, the gradient was changed to 95% A over 0.2 min. Six minutes after injection, the gradient was returned to 5% A over 0.5 min, and the column was equilibrated for 4.5 min before the next injection, resulting in a total runtime of 11 min. The column flow rate was 200 μL/min, and the column temperature was maintained at 30 ± 2 °C. The eluent was diverted to waste during the time intervals of 0–2.5 and 5–11 min after injection using a post-column switch. The source and desolvation temperatures were set at 150 °C and 600 °C, respectively, and the cone and desolvation nitrogen gas flows were set at 50 L/h and 800 L/h, respectively. The mass spectrometer was operated in the negative ion mode with a capillary voltage of 2.5 kV. The dwell time was 100 ms, and at least 12 data points were obtained across the peaks. Selected reaction monitoring was applied using the conditions shown in Table 1. Argon was used for collision-induced dissociation. The data acquisition and processing were performed using MassLynx 4.1 (Waters).

Method validation

The calibration curves obtained from pure solvent based calibrants were compared with the calibration curves based on matrix matched calibrants. The matrix matched calibrants were based on donor blood and were treated using the developed procedure except that the 100 μL of MeOH was replaced by 100 μL of the mixed standard solutions containing the drug substances. Spiked sample concentrations were prepared at 0.1, 1, 5, 10, 15 and 20 μM HMB and at 50-fold higher concentrations of BHB in the original blood sample. In addition, a blank sample (a processed sample without any added analyte) and a sample spiked only with SIL–IS were also included.

Table 1
Mass spectrometric conditions in ESI(−) mode.

Substance	Transition Q1/Q3 (*m/z*)	Cone voltage (V)	Collision energy (eV)
BHB	103/59	18	10
HMB	117/59	22	11
BHB-$^{13}C_4$	107/61	18	10
HMB–D_6	123/59	22	11

The repeatability standard deviation (SD_r) (i.e., the variability of independent analytical results obtained by the same operator using the same apparatus under the same conditions on the same test sample and in a short interval of time) and the intra-laboratory reproducibility standard deviation ($SD_{R,intra\text{-}lab}$) (i.e., the variability of independent analytical results obtained on the same test sample in the same laboratory by different operators on different days) were determined on 5 different whole blood samples with endogenous contents at different levels and on 4 different blood samples spiked with 5–900 μM BHB and 0.5–18 μM HMB. Duplicate analyses were performed on 8 different days. The repeatability and intra-laboratory reproducibility parameters were calculated in accordance with ISO standard 5725-2 [34]. The method trueness (i.e., the closeness of agreement between the average value obtained from a large series of test results and the accepted reference value) was determined from the results obtained on the spiked samples in the precision study after correction for the endogenous contents. The trueness was expressed as the relative bias; relative bias = (mean test result of spiked sample– mean test result of blank sample– spiked concentration) × 100/spiked concentration.

The matrix effects (including ion-suppression and ion-enhancement effects) were investigated on 20 different samples of whole blood that were spiked after SPE at a level that was equivalent to 500 μM BHB and 10 μM HMB in the original samples. They were analysed in attenuating order along with the blank samples and pure standards at the same concentration. The matrix effect from each sample was calculated from the peak areas (A) without IS correction using the closest standards in the series; matrix effect = $(\bar{A}_{\text{pure standard}} - (A_{\text{spiked sample}} - A_{\text{blank sample}}) \times 100/\bar{A}_{\text{Pure standard}}$. The true extraction recoveries were determined from the same 20 whole blood samples that were spiked with 500 μM BHB and 10 μM HMB. The standards used for the determination of the true recoveries were the same blood samples that were spiked after SPE to concentrations equivalent to the full recovery of BHB and HMB. To compensate for changes in the matrix effects, the final extracts were spiked with SIL-IS; true extraction recovery = $(A_{\text{sample spiked before extraction}}/A_{\text{matching SIL-IS}} - A_{\text{blank sample}}/A_{\text{matching SIL-IS}}) \times 100/(A_{\text{sample spiked after SPE}}/A_{\text{matching SIL-IS}} - A_{\text{blank sample}}/A_{\text{matching SIL-IS}})$.

Results and discussion

Extraction and clean-up

The blood samples were extracted by protein precipitation using a mixture of MeOH and ACN. The MeOH was added to obtain a disperse precipitate of the protein-containing blood components. By eluting the extract through a polymeric SCX sorbent, it was possible to remove a significant portion of the dissolved matrix components before the specific SAX clean-up. Without pre-eluting the extract through a polymeric SCX sorbent, the SAX clean-up was less efficient and reproducible. The combined clean-up on SCX and SAX sorbents removed most of the blood matrix that remained after the protein precipitation.

Precursor ions and transition products

Electrospray ionisation was applied in negative ion mode (ESI(−)) for both substances, and the dominant precursor (Q1) ions were the deprotonated molecular ions ($[M\text{-}H]^-$). Both substances produced an *m/z* 59 product (Q3) ion ($[CH_3COO]^-$) from cleavage of the bond between the α- and β-positions (Table 1). Because it was not possible to obtain more than one product ion of significant abundance in ESI(−) (Fig. 1), a comprehensive clean-up of the sample extracts was applied before the HILIC–MS/MS. Both substances could also be ionised in the positive ion mode, but the sensitivities were 1–2 orders of lower magnitude.

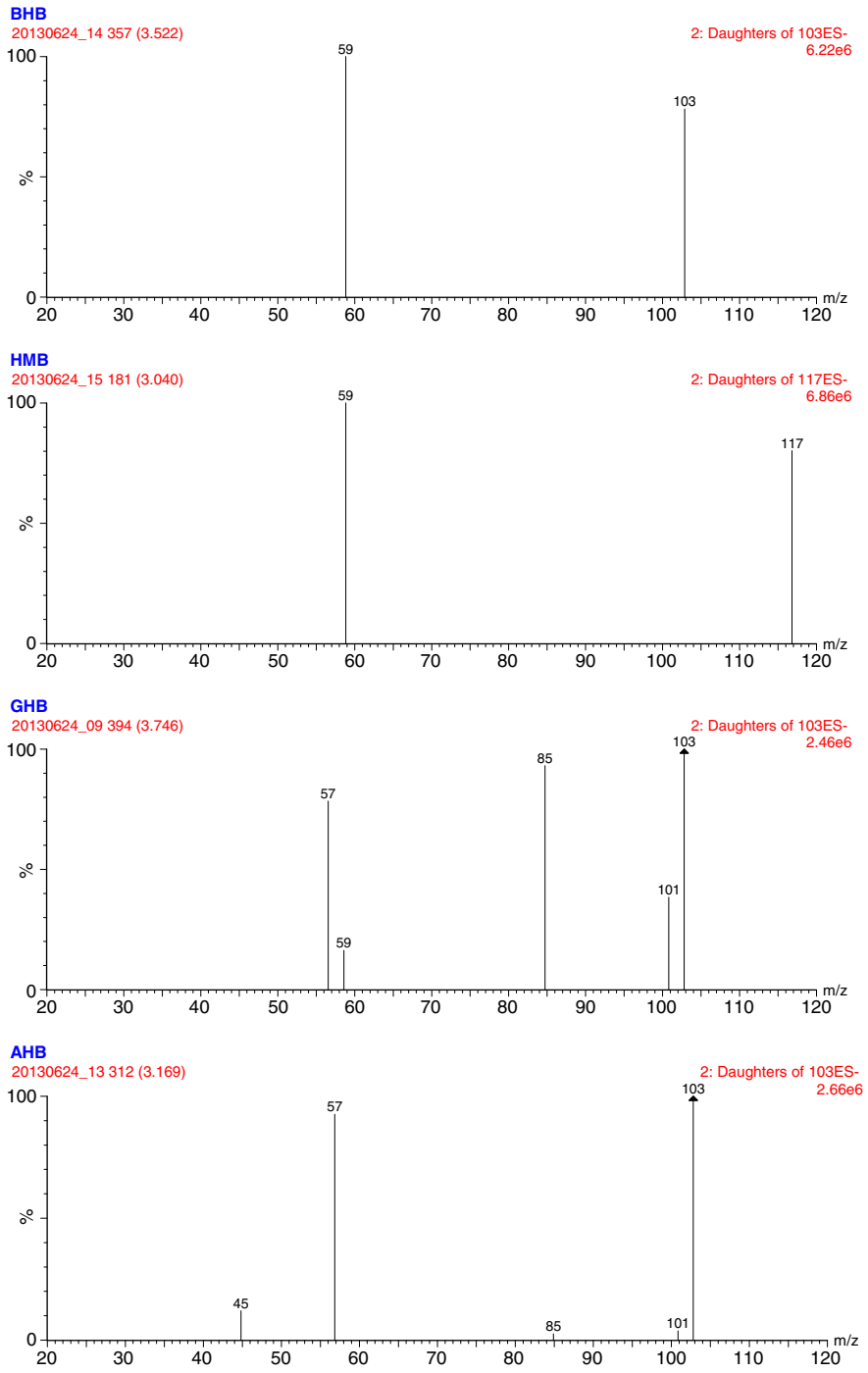

Fig. 1. Product ion scans of BHB, HMB and the structurally related substances GHB and AHB.

Chromatography

Reverse phase chromatography is a common technique used for the separation of drugs. However, by using the HILIC technique, the elution order is reversed, which makes it easier to separate very polar substances from interferences, such as matrix components and other drugs, by adjusting the composition of the mobile phase. In the present case, AHB and GHB could potentially interfere with BHB because their molecular weights are identical. The *m/z* 103/59 ion transition used for the determination of BHB also produced a signal from GHB. However, it was possible to obtain an acceptable separation between BHB and GHB within a 4 min elution period both at endogenous and at elevated concentrations of GHB (Fig. 2). The presence of GHB could also be detected by the transition products *m/z* 57 and *m/z* 85, which were not produced by BHB. AHB was almost baseline separated from BHB and did not produce a significant signal at the *m/z* 103/59 transition. No detectable carryover was observed when samples that had been spiked to the highest calibrant concentration and blank samples were analysed in attenuated order. A typical chromatogram of a blood sample is shown in Fig. 3. The gradient was changed to 95% A after the elution of the analytes to clean the column and to maintain the selectivity constant between injections.

Method performance parameters

The calibration curves were created using weighted regression analysis. The difference in the slopes of the calibration curves prepared from

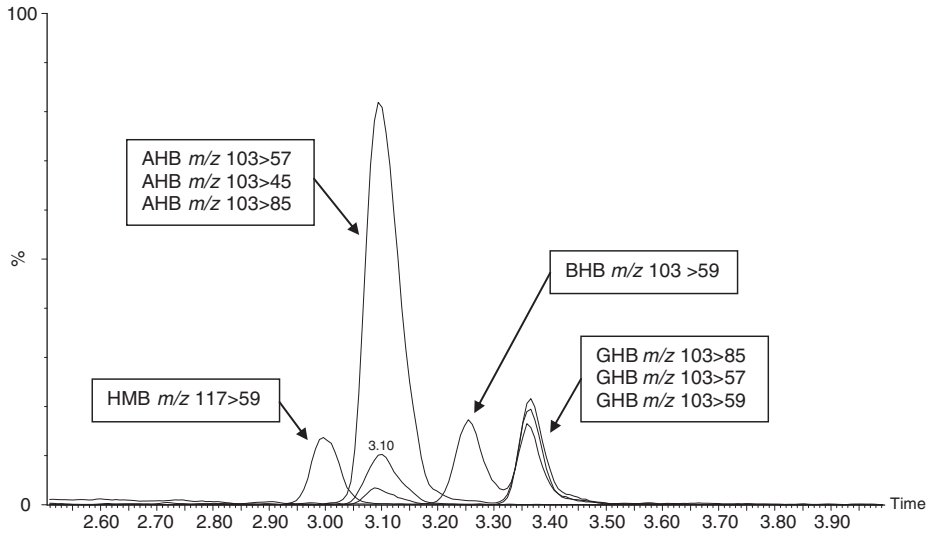

Fig. 2. Retention times of HMB, AHB, BHB and GHB from a whole blood sample that was spiked with 100 μM AHB and GHB.

the pure solvent calibrants and the matrix matched calibrants (8 independent pairs from the precision study) were less than 2%, which could not be proved as statistically significant at the 95% confidence level. The use of pure solvent calibrators is preferable when blank matrix samples cannot be obtained. The ruggedness of the calibration was not affected by the use of pure calibrants because the analytes and the corresponding SIL–IS were eluted with identical retention times. The R^2 values obtained in the precision study from 8 independent test series and analysed within a 3-week period were 0.998 ± 0.002 (mean ± SD) for BHB and 0.997 ± 0.003 for HMB for both the matrix and the pure solvent calibrants.

The average matrix suppression of the ionisation of BHB and HMB was in the range 20–30% (Table 2). Without the SAX clean-up, the matrix suppression was considerably higher, approximately 80%. The mean true extraction recoveries of BHB and HMB were greater than 95%.

The RSD_r and $RSD_{R,intra\text{-}lab}$ values obtained in the precision study were below 10% at normal endogenous concentration levels (Table 3). These RSD_R values are considered acceptable when applying the rule of Horwitz [35]. According to the Horwitz equation, RSD_R values of 20% and 11% between laboratories would be acceptable at concentration levels of 2 μM and 100 μM, respectively. The precision figures obtained from the matrix calibrants and the pure solvent calibrants were not significantly different. The relative bias determined at the low, medium and high concentration levels was within the range of ±5% (Table 3).

The LODs could not be determined exactly due to the endogenous content of BHB and HMB. However, the potential detection capability, corresponding to a peak-to-peak signal-to-noise ratio (S/N) of 3, with the noise measured just before and after the endogenous analyte peaks, was estimated at 0.5 μM BHB and 0.02 μM HMB using blood

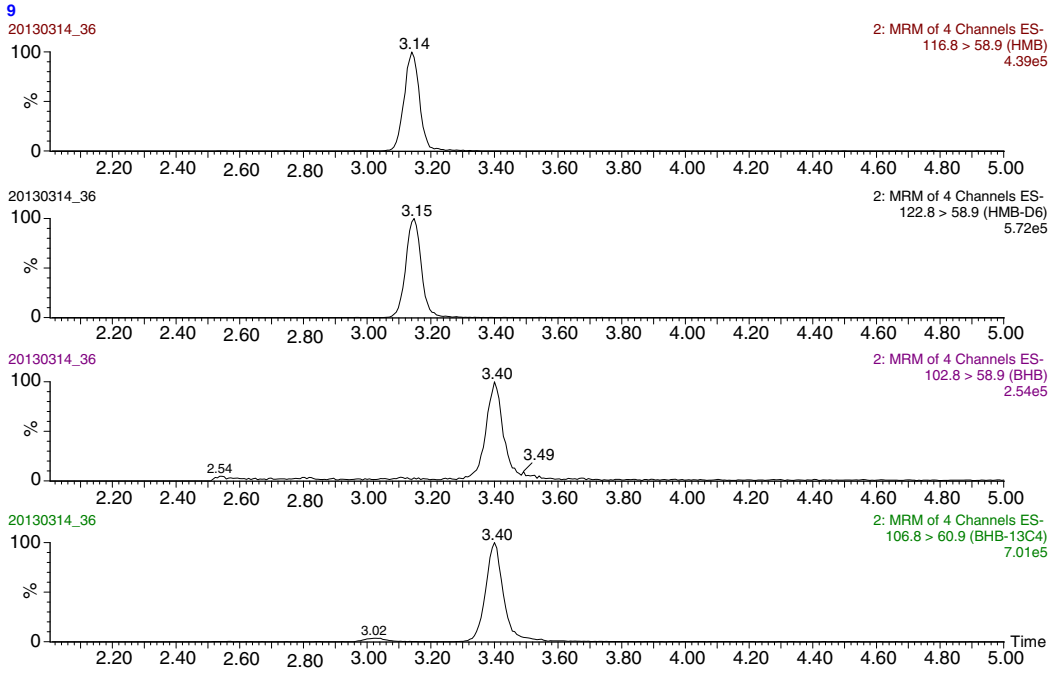

Fig. 3. Chromatograms of a whole blood sample containing low endogenous concentrations of BHB (8.0 μM) and HMB (1.1 μM).

Table 2
Matrix effects and true recoveries obtained from single determinations of spiked blood ($n = 20$). The matrix-matched standards used in the calculation of the recoveries were the same samples spiked after SPE.

Substance	Endogen conc. range μM	Spiked conc. μM	Matrix effect mean (±SD), %	True recovery mean (±SD), %
BHB	9–270	500	22 (±9)	96 (±5)
HMB	0.9–3.1	10	31 (±13)	98 (±7)

containing approximately 10 μM BHB and 1 μM HMB. The endogenous content of BHB and HMB did also complicate the determination of the lower limit of quantification (LLOQ) defined as the concentration associated with 20% $RSD_{R,intra\text{-}lab}$. However, from the precision study, absolute $SD_{R,intra\text{-}lab}$ values of 0.58 μM BHB and 0.08 μM HMB were determined for concentrations of 6.8 μM BHB and 0.9 μM HMB. If these values are considered constant at lower concentrations the LLOQ would be 3 μM for BHB and 0.4 μM for HMB. That is most likely a conservative estimate, because the absolute $SD_{R,intra\text{-}lab}$ in most cases declines with decreasing concentrations. The upper limits of quantification are 1000 μM for BHB and 20 μM for HMB, corresponding to the concentrations of the highest calibrants.

This method was compared with an HS-GC–MS method [19] on 10 blood samples containing endogenous BHB in the range of 50–1000 μM. The relationship between the methods was investigated by linear regression analysis (StatGraphics Plus version 4.0, Manugistics, Rockville, MD). The equation of the fitted model was BHB (HS-GC–MS) = −2.919 + 1.002 × BHB (HILIC–MS/MS). The SD of the slope and intercept was 0.012 and 6.12, respectively, implying that the slope and intercept of the regression line were not significantly different from 1 and 0. The SD of the residuals was 10.1, and the R^2 value was better than 0.999. The HS-GC–MS method is approximately 20 times less sensitive than the HILIC–MS/MS method.

Stability of HMB and BHB in blood and extracts

The stability of BHB and HMB in whole blood freshly preserved with FO, EDTA and FC mixtures was tested at storage temperatures of 20 ± 2 °C and 5 ± 2 °C for 8 days. The samples contained endogenous BHB and HMB at concentration levels of 15 and 1 μM, respectively.

Table 3
Method precision and trueness estimated at different concentration levels in whole blood.

Substance	Sample no.	Origin of substance	Analysed conc. μM	RSD_r[a] %	$RSD_{R,intra\text{-}lab}$[b] %	Relative bias %
BHB	1	Endogenous	6.8	7.6	8.5	
	2	Endogenous	11.5	3.4	6.3	
	3	Endogenous	14.2	6.6	8.0	
	4	Endogenous	18.1	4.1	9.4	
	5	Endogenous	135	3.2	4.8	
	6	Endogenous + 500 μM	747	1.3	1.8	
	7	Sample 2 + 25 μM	35.5	2.5	5.1	−4
	8	Sample 1 + 500 μM	514	2.5	2.8	1
	9	Sample 3 + 900 μM	922	2.8	2.9	1
	10	Sample 1 + 5 μM	11.7	5.1	7.6	−2
HMB	1	Endogenous	1.59	3.9	5.7	
	2	Endogenous	0.84	3.5	8.5	
	3	Endogenous	0.96	6.0	9.8	
	4	Endogenous	1.34	4.7	9.4	
	5	Endogenous	4.20	3.7	8.9	
	6	Endogenous + 10 μM	14.6	2.9	4.1	
	7	Sample 2 + 0.5 μM	1.35	4.1	7.8	2
	8	Sample 1 + 10 μM	11.9	3.1	3.5	3
	9	Sample 3 + 18 μM	19.2	3.5	4.5	1

[a] Relative standard deviation of repeatability.
[b] Relative standard deviation of intra-laboratory reproducibility.

The same samples were included in the stability test after fortification with the substances at concentrations of 50 μM BHB and 2 μM HMB. Both BHB and HMB were stable at 20 ± 2 °C and 5 ± 2 °C for at least 8 days when the samples were preserved with these mixtures.

The stability of the blood-based calibrants stored at −20 ± 2 °C and 5 ± 2 °C was also frequently tested over a period of 8 days. The calibrants were prepared according to the procedure, except that the internal standard was not added to the blood sample at the beginning but was instead added to the extracts on the day of the HILIC–MS/MS analysis. The slopes of the calibration curves for BHB and HMB did not change significantly during the 8 days of storage at −20 °C and 5 °C. The slopes were 0.00115 ± 0.00006 (mean ± SD) for BHB stored at 5 °C and 0.00113 ± 0.00005 for BHB stored at −20 °C. The corresponding slopes for HMB were 0.043 ± 0.0014 and 0.042 ± 0.0015. The slopes of the calibration curves of the fresh prepared calibrants were 0.00114 for BHB and 0.043 for HMB.

Application of the method

The developed method is currently being used in a clinical study performed in agreement with the local ethical guidelines. One focus of this study is different metabolic alterations with and without insulin stimulation. Using the described HILIC–MS/MS method, we currently have preliminary analytical data on four participants ($n = 4$). The mean blood concentration of HMB fell significantly after insulin stimulation from 1.79 ± 0.42 to 1.30 ± 0.33 μM ($p < 0.005$). The blood BHB concentration did not differ significantly but had a decreasing trend as the mean values fell from 211 ± 194 to 9.68 ± 2.32 μM ($p = 0.126$) with and without insulin stimulation respectively. A paired *t*-test was used for the statistical analysis. The results of this study, as well as the analytical data, will be reported in due course.

Conclusion

A sensitive and selective HILIC–MS/MS method was developed for the determination of BHB and HMB in human whole blood. By using HILIC, BHB was separated from isobaric interferences such as GHB. The ruggedness and reproducibility of the method were strengthened through the use of SIL–IS of both substances. The method was successfully tested on a set of samples from a study on insulin stimulation and metabolism.

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