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Research Article

Development and validation of a multiplexed LC-MS/MS ketone body assay for clinical diagnostics

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ABSTRACT

Objectives: Ketone bodies (KBs) serve as important energy sources that spare glucose, providing the primary energy for cardiac muscle, skeletal muscle during aerobic exercise, and the brain during periods of catabolism. The levels and relationships between the KBs are critical indicators of metabolic health and disease. However, challenges in separating isomeric KBs and concerns about sample stability have previously limited their clinical measurement.

Methods: A novel 6.5-minute liquid chromatography-mass spectrometry-based assay was developed, enabling the precise measurement of alpha-, beta- and gamma-hydroxybutyrate, beta-hydroxyisobutyrate, and acetoacetate. This method was fully validated for human serum and plasma samples by investigating extraction efficiency, matrix effects, accuracy, recovery, intra- and inter-precision, linearity, lower limit of quantitation (LLOQ), carryover, specificity, stability, and more. From 107 normal samples, reference ranges were established for all analytes and the beta-hydroxybutyrate/acetoacetate ratio.

Results: All five analytes were adequately separated chromatographically. An extraction efficiency between 80 and 120 % was observed for all KBs. Accuracy was evaluated through spike and recovery using 10 random patient samples, with an average recovery of 85–115 % for all KBs and a coefficient of variation of $\leq$ 3 %. Coefficients of variation for intra- and inter-day imprecision were $<$ 5 %, and the total imprecision was $<$ 10 %. No significant interferences were observed. Specimens remained stable for up to 6 h on ice or 2 h at room temperature.

Conclusions: The developed method is highly sensitive and robust. It has been validated for use with human serum and plasma, overcoming stability concerns and providing a reliable and efficient quantitative estimation of ketone bodies.

Introduction

Ketone bodies (KBs) are crucial energy sources that spare glucose. They primarily serve as the main energy source for cardiac muscle, skeletal muscle during aerobic exercise, and the brain during periods of catabolism [1–3]. Ketones are synthesized from fatty acids and ketogenic amino acids in the liver and are oxidized in cardiac muscle, skeletal muscle, and brain during catabolism to conserve glucose. The measurement of serum or plasma beta-hydroxybutyrate (BHB) and acetoacetate (AcAc) concentrations provides insights into fatty acid β-oxidation and ketogenic amino acid catabolism [4–6]. Atypical ketones, such as alpha-hydroxybutyrate (AHB) and beta-hydroxyisobutyrate (BHIB), possess diagnostic significance in certain pathophysiological conditions. For example, elevated levels of AHB have been observed in individuals with increased intracellular reduced nicotinamide adenine dinucleotide (NADH) and decreased oxidized nicotinamide adenine dinucleotide (NAD^+). These elevated levels indicate conditions such as mitochondrial disease or hypoxia [7,8], as well

Abbreviations: AcAc, Acetoacetate; AHB, Alpha-Hydroxybutyrate; AMR, Analytical Measurement Range; BHB, Beta-Hydroxybutyrate; BHIB, Beta-Hydroxyisobutyrate; CHOP, Children's Hospital of Philadelphia; ESI, Electrospray Ionization; GHB, Gamma-Hydroxybutyrate; HIBCH, 3-Hydroxyisobutyral-CoA Hydrolase; IS, Internal Standard; KB, Ketone Body; LC-MS/MS, Liquid Chromatography-Tandem Mass Spectrometry; LLOQ, Lower Limit of Quantitation; MRM, Multiple Reaction Monitoring; NADH, Reduced Nicotinamide Adenine Dinucleotide; NAD, Oxidized Nicotinamide Adenine Dinucleotide; QC, Quality Control; RT, Room Temperature; SSADH, Succinate Semialdehyde Dehydrogenase Deficiency; SST, Serum Separator Tube.

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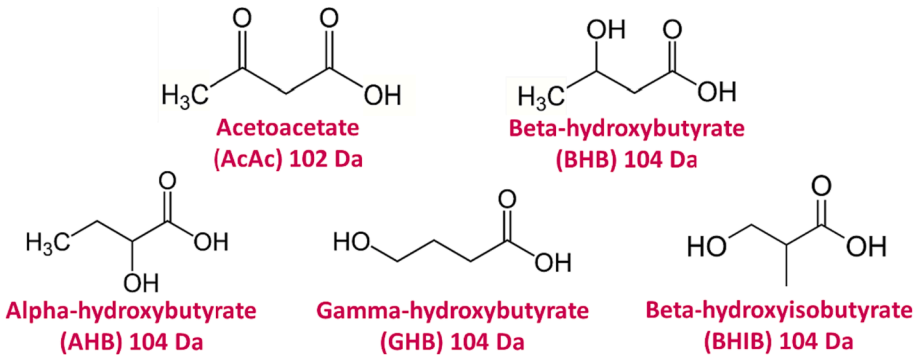

Fig. 1. Molecular structures of the five targets that comprise the ketone body panel assay including their molecular weight showing the inclusion of four isomers.

Table 1
MS parameters and MS/MS transitions of the ketone bodies and their respective IS.

Analyte	RT	Description	Q1 (*m/z*)	Q3 (*m/z*)	Cone (V)	Collision energy (V)	Dwell time (ms)
AcAc	1.39	Quantifier	101.02	57.03	25	9	80
		Qualifier	101.02	57.08	25	6	80
AcAc-C4	1.39	Quantifier	105.04	60.04	25	9	80
		Qualifier	105.04	60.09	25	6	80
BHB	1.86	Quantifier	103.04	59.01	25	9	20
		Qualifier	103.04	59.06	25	6	20
BHB-D4	1.82	Quantifier	107.06	59.01	25	9	20
		Qualifier	107.06	59.06	25	6	20
AHB	1.77	Quantifier	103.04	57.03	25	9	20
		Qualifier	103.04	57.08	25	6	20
GHB	1.67	Quantifier	103.04	57.03	25	9	20
		Qualifier	103.04	57.08	25	6	20
BHIB	2.01	Quantifier	103.04	73.03	25	9	20
		Qualifier	103.04	73.08	25	6	20

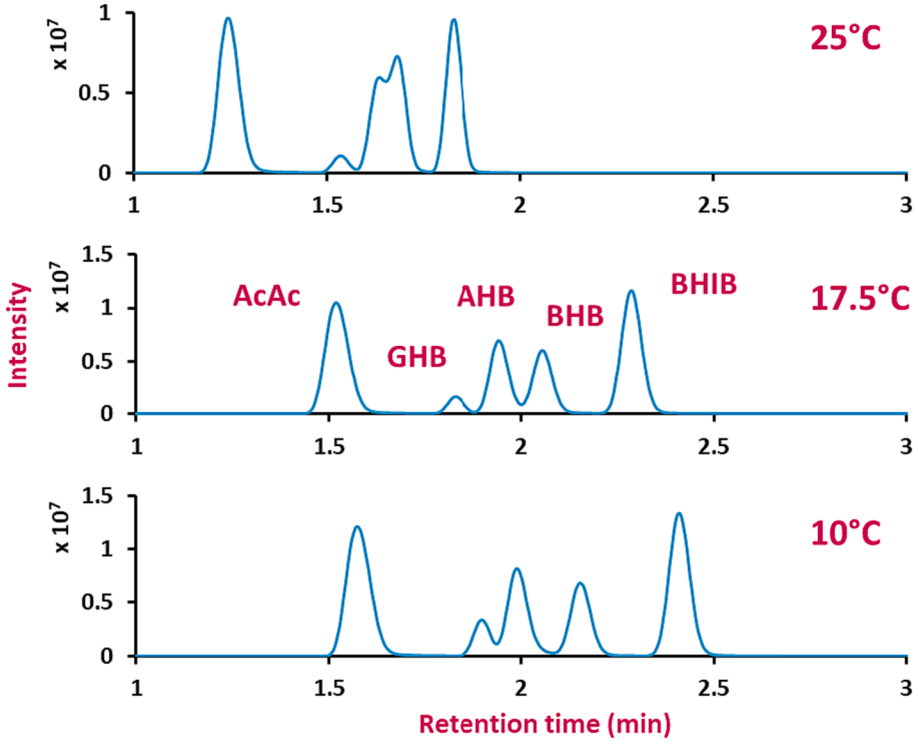

Fig. 2. TICs show that reducing the column temperature increased the separation power of the KB isomers; however, the viscosity and system pressure also increased. At 17.5 °C optimum separation and flow rate were established. KB mix at 0.025 mM.

Table 2
Spike and recovery results from random serum samples (n = 10) spiked with 0.05 mM of each analyte.

Analyte	Recoveryrange (%)	Average recovery (%)	%CV
AcAc	96.2–104.5	99.9	2.2
BHB	98.7–107.9	102.7	3.0
AHB	93.0–98.0	95.3	2.1
GHB	81.5–88.1	85.7	2.1
BHIB	84.6–91.4	87.5	2.0

as early-stage insulin resistance [9,10]. Likewise, BHIB, a biomarker of valine catabolism in skeletal muscle [11], is elevated in patients with 3-hydroxyisobutyral-CoA Hydrolase (HIBCH) deficiency, a specific type of mitochondrial disease. Gamma-hydroxybutyrate (GHB) is a marker for succinate semialdehyde dehydrogenase deficiency (SSADH) [12], although it can also originate from exogenous intake [13,14].

BHB and AcAc have been analyzed in biological matrices in a variety of ways, including global metabolomics studies using nuclear magnetic resonance spectroscopy [15–17], liquid chromatography-mass spectrometry [18–20], and—most commonly—by gas chromatography paired with mass spectrometry [3,21,22]. However, accurately measuring the ratio between BHB and AcAc in a clinical setting has been challenging due to the absence of an appropriate internal standard, stability, and sensitivity limitations of AcAc [2,23–25]. In order to overcome these limitations, a rapid liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based assay has been developed. This new method does not require derivatization and has a low sample volume requirement. The assay facilitates precise measurement of BHB, AcAc, and their ratio, as well as other KB isomers, including AHB, GHB, and BHIB (Fig. 1). The method developed is highly sensitive, robust, and has been validated with just 10 μL of human serum or plasma. It effectively overcomes stability concerns and offers a reliable and efficient quantitative estimation of KBs.

Overall, understanding the dynamics of KBs, including AcAc, BHB, AHB, GHB, and BHIB, is crucial for comprehending energy metabolism and its implications in various physiological and pathological conditions [26]. The development of this LC-MS/MS assay for directly measuring the BHB/AcAc ratio represents a significant advancement, providing a reliable and efficient method for quantifying KBs [27]. This assay aids not only in indirectly assessing mitochondrial function and redox state, but also in quantitatively measuring AHB, GHB, and BHIB, potentially expanding the diagnostic capabilities for mitochondrial-related disorders. The availability of this novel clinical assay offers promise for improving the diagnosis, monitoring, and understanding of

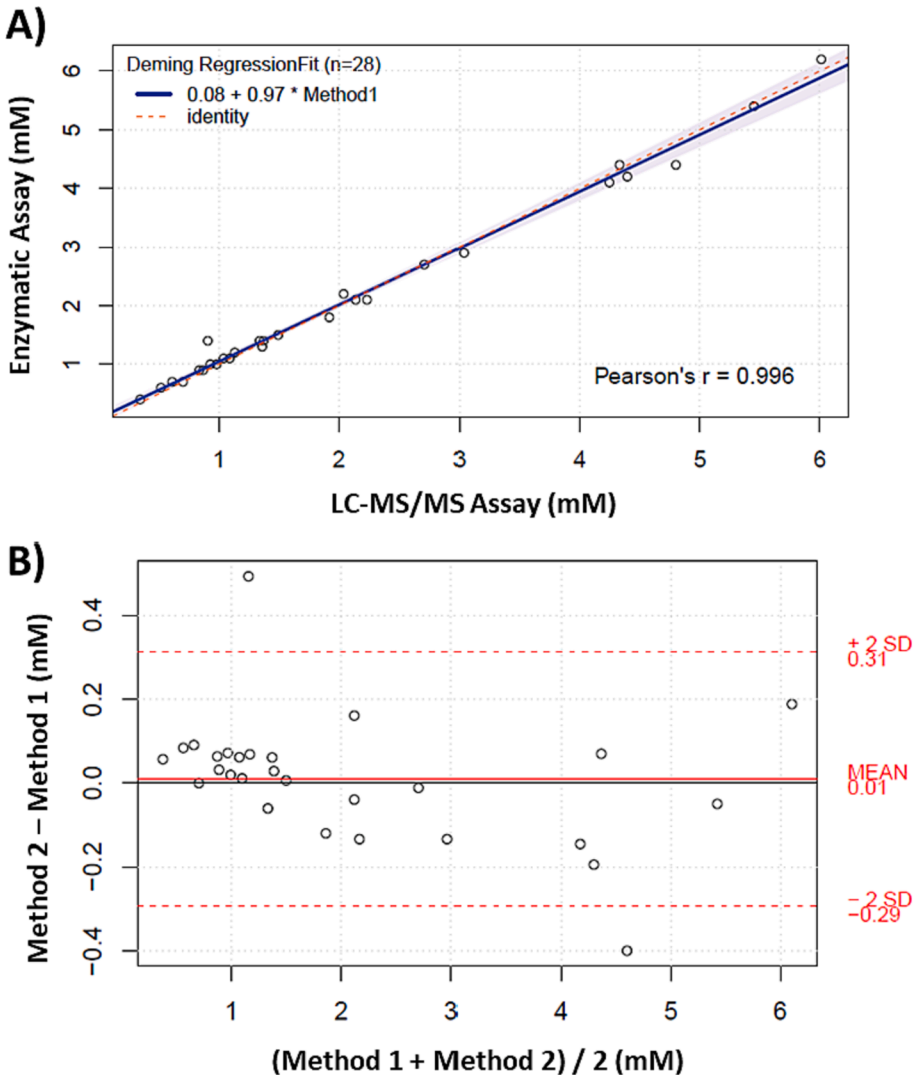

Fig. 3. Method comparison of BHB between the newly developed LC-MS/MS ketone body panel (method 1) and CHOP's enzymatic assay (method 2). A) Deming regression plot with 95% interval in purple with Jackknife method, and B) Difference plot showing estimated bias. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Precision study results of 20 days, 2 replicates sample preparations, and 2 injections in dialyzed serum at three levels.

Level	Spiked Concentration (mM)	Total (%CV)	Inter-assay Day (% CV)	Intra-assay	
				Replicate (%CV)	Injection (%CV)
AcAc Low	0.005	5.1	2.5	0.0	4.4
AcAc Med	0.05	3.8	2.8	1.8	1.7
AcAc High	0.5	2.8	1.5	2.0	1.3
BHB Low	0.005	4.2	2.3	1.8	3.1
BHB Med	0.05	3.3	1.3	2.3	2.0
BHB High	0.5	4.2	2.3	1.8	3.1
AHB Low	0.005	6.3	3.9	3.5	3.4
AHB Med	0.05	5.3	2.6	4.5	1.1
AHB High	0.5	3.7	1.2	3.3	1.3
GHB Low	0.005	6.3	4.2	1.6	4.4
GHB Med	0.05	4.8	2.5	3.7	1.8
GHB High	0.5	4.1	3.1	1.8	1.8
BHIB Low	0.005	4.2	3.5	1.2	2.0
BHIB Med	0.05	4.0	1.5	3.6	1.0
BHIB High	0.5	3.0	1.9	2.0	1.1

Table 4
Established analytical measurement range (AMR), clinically reportable range (CRR), and clinical reference range.

Analyte	AMR (mM)	CRR (mM)	Reference Range (mM)
AcAc	0.0025 – 1.5	0.0025 – 75.0	0.0072–0.2409
BHB	0.0050 – 1.5	0.0050 – 75.0	0.0078–0.4101
BHB/AcAc	–	–	0.4757–2.5654
AHB	0.0025 – 0.5	0.0025 – 25.0	0.0106–0.1031
GHB	0.0025 – 0.5	0.0025 – 25.0	<0.0025
BHIB	0.0050 – 0.5	0.0050 – 25.0	<0.0050–0.0439

mitochondrial dysfunction and related pathophysiological conditions. Furthermore, the availability of this novel assay opens new possibilities for research and clinical applications in the field of energy metabolism.

Materials and Methods

Chemicals and reagents

LCMS grade water and methanol were purchased from Honeywell, while LCMS grade acetonitrile, sodium hydroxide solution (10 N), and hydrochloric acid solution (6 N) were obtained from Fisher Scientific. Acetic acid (ACS grade), lithium acetoacetate (90 %), DL-Beta-Hydroxybutyric acid sodium salt (98 %), and DL-2- Hydroxybutyric acid sodium salt (97 %), DL-sodium-B-Hydroxyisobutyrate (96 %) were sourced from Sigma Aldrich. Santa Cruz Biotech provided the 4-hydroxybutyric acid methyl ester, and Cambridge Isotope supplied the sodium DL-3-hydroxybutyrate [3,4,4,4-D4, 98 %] at 1 mg/mL in water (95 %), ethyl acetoacetate [1,2,3,4–13C4, 99 %] (98 %), and sodium borodeuteride [D4, 99 %] (95 %). Dialyzed serum FBS (one shot) was obtained from Gibco.

Stock solutions, working solutions, calibrators, and quality controls

Individual stock solutions of AcAc, BHB, AHB, GHB, and BHIB were prepared at 50 mM in water. All five KB stock solutions were mixed equally to create a 10 mM KB-Mix, which was used to prepare dilutions for working solutions, calibrators, and quality controls (QCs). Stock solutions of the internal standards (ISs) (i.e., AcAc-IS and BHB-IS) were prepared at 5 mM in water and a mixture of these two ISs was created at 1 mM by combining the stock solutions. Due to unavailability of commercially produced GHB and AcAc-IS during the method development and validation, both compounds were synthesized in-house by hydrolyzing 4-hydroxybutyric acid methyl ester and ethyl acetoacetate [13C4], respectively [28]. Further details are provided in the Supporting Information.

Nine calibrators were prepared in water using the KB-Mix, with concentrations equivalent to serum samples: 0.0025, 0.005, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, and 1.5 mM. Due to the lack of KB-free matrices, three levels of QC materials were prepared in pooled dialyzed fetal bovine serum, serving as a surrogate matrix. The same materials were used for the precision study. Blank dialyzed serum was tested to ensure that KB levels were below the lowest calibrator and QC. All stock solutions, mixtures, dilutions, and QC materials were stored at −80 °C in small aliquots to minimize free-thaw cycles.

Specimens

All plasma and serum specimens included in this study were received at the Children's Hospital of Philadelphia for Metabolic Lab testing, BHB testing, or routine well-visits. Upon receipt, specimens were spun down, aliquots were created, and stored at −80 °C prior to analysis. One aliquot was used for routine clinical testing, other aliquots were utilized for LC-MS/MS KB comparison testing and validation studies. Additional whole blood samples were drawn via venipuncture from consenting healthy donors to perform stability testing. The Children's Hospital of Philadelphia Institutional Review Board (IRB)/Ethics Committee provided a determination of exemption for this study (IRB 23–021290).

Sample preparation

A straightforward protein precipitation was performed, adapted from Puchalska, et al. [25] 10 μL of serum or plasma was transferred into a microcentrifuge tube, 10 μL of 25 μM IS mix was added, followed by 30 μL of water. All samples were thoroughly homogenized prior to adding 200 μL of ice-cold extraction solution (50 % methanol in water, v/v). A pellet was created by centrifugation at 15,000 x g, chilled at 4 °C for 10 min; 200 μL of the supernatant was transferred into a new tube and dried down under nitrogen flow at room temperature. Finally, the samples were reconstituted with 160 μL 0.0125 % acetic acid in water (v/v) and injected onto the column. Throughout the sample preparation, samples, QCs, and reagents remained on ice unless otherwise noted.

LC-MS/MS method

Analyses were performed on a Waters Acquity Classic UPLC system equipped with a binary pump, coupled to a Waters Xevo TQS triple quadrupole MS utilizing electrospray ionization (ESI). Separation of the analytes was carried out on a Waters Cortecs UPLC T3 (100x2.1 mm, 1.6 μm) column at 17.5 °C. 0.0125 % acetic acid in water was selected as mobile phase A and 0.0125 % acetic acid in water:methanol (60:40) as mobile phase B (v/v). The solvent gradient started at 0 % B, increasing to 10 % B and then 20 % B at 0.5 and 1.9 min, respectively. At 2.0 min mobile phase B was increased to 30 % and at 3.0 min to 90 % and held constant for one minute. Re-equilibration of the column was performed by reducing B back to 0 %; total run time was 6.5 min. The flow rate was 0.34 mL/min; however, from 2 to 4.5 min this was reduced to 0.22 mL/min. 3 μL of sample material was injected onto the column; following

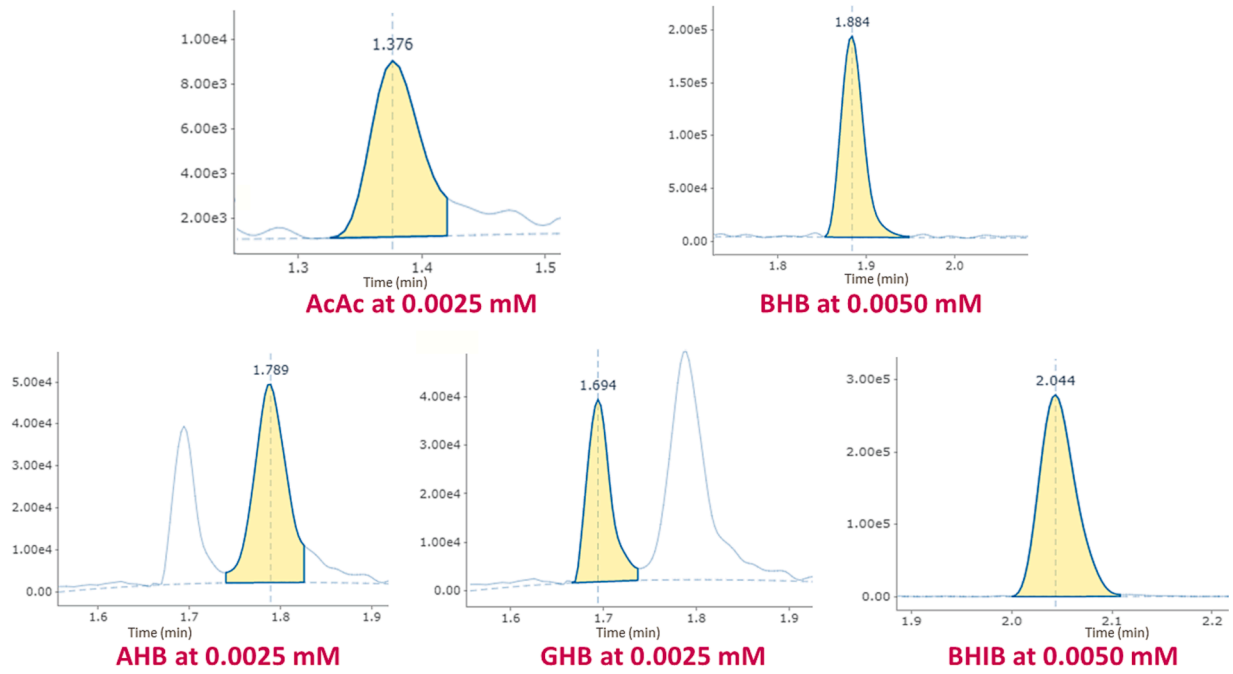

Fig. 4. Integrated chromatograms at LLOQ levels for each analyte.

each injection a needle wash was performed using 80 % methanol in water (strong wash) followed by 5 % methanol in water (weak wash) (v/v).

Ionization was carried out in negative mode using 0.8 kV; other optimized MS parameters were: nebulizer pressure at 7 bar, source gas flow of 165 L/H at 130 °C, and desolvation gas flow of 700 L/H at 425 °C. Experiments were performed using multiple reaction monitoring (MRM), and optimized MS parameters and ion transitions, including applied collision energies, are listed in Table 1.

Method validation

The LC-MS/MS method was fully validated and tested by examining extraction efficiency, matrix effects, accuracy, recovery, intra- and inter-precision, linearity, lower limit of quantitation (LLOQ), diluent selection, dilution, carryover, specificity, stability, specimen tube selection, and assay robustness, in accordance with the Clinical and Laboratory Standards Institute (CLSI) C62-A guidelines [29].

Extraction efficiency or recovery was determined by comparing spiked dialyzed serum pre- and post-extraction at three levels in triplicate. Matrix effects were investigated by comparing spiked dialyzed serum post-extraction versus a neat standard at the expected concentration post-extraction. A similar experiment was conducted with pooled serum. Two pooled serum samples were analyzed - spiked and non-spiked. The difference between the two samples was compared against the neat standard; both experiments were performed in triplicate.

Accuracy and recovery were evaluated by analyzing 10 random serum patient samples pre- and post-spike with a 0.05 mM KB mixture. For BHB comparison, forty-three random serum patient samples were measured using an FDA-approved enzymatic assay (Stanbio LiquiColor, performed on Ortho Diagnostics Vitros 4600), and the results were compared with those from the new LC-MS/MS KB assay.

Intra- and inter-assay precision studies were performed over 20 days, with each day involving a duplicate extraction, and each replicate was injected twice. Three levels (low, medium, and high) of spiked dialyzed serum were evaluated.

Linearity and LLOQ were determined by analyzing 11 mixtures of the lowest and highest spiked dialyzed serum (0.00125–1.5 mM) in triplicate. Subsequently, at the anticipated LLOQ level and slightly above (i.e., 0.00125 and 0.0025 mM), five replicates of each concentration were prepared in dialyzed serum and analyzed for five days to measure the imprecision and bias.

Diluent selection, dilution maximum, and carryover were established by spiking pooled patient serum at 0.1 mM and performing five dilutions between 2 and 100x in triplicate using water and dialyzed serum. Additionally, a 10 mM KB mix was spiked into pooled serum. First, a blank was measured, followed by the spiked sample, and then three blanks. The presence of analytes in the blanks was assessed. The same strategy was carried out for the system check sample at the LLOQ level. The system check was analyzed prior to and post the 10 mM spiked sample, and the percent deviation of the reported peak area was measured.

The specificity of the assay was established by measuring dialyzed serum to ensure the absence of the targets, in addition to conducting a comprehensive interference study. This study included 16 common therapeutic drugs as well as unconjugated bilirubin (icterus), conjugated bilirubin, hemoglobin (hemolysis), triglycerides (lipemic), and protein levels using the Assurance Interference Kits from Sun Diagnostics. Pooled patient serum was spiked with all KBs at two levels, 0.1 mM (low) and 1.0 mM (high), and exposed to each potential interferent. Bias compared with the control was then measured.

Specimen stability was determined through the full testing process, including pre-spin down, post-spin down, and freeze–thaw cycles, as well as extract stability. For the pre-spin down study, four serum separator tube (SST) samples were drawn from six donors and were each kept under different conditions (2 or 6 h on ice, and 2 or 6 h at room temperature). Following an immediate refrigerated spin down, the serum was stored at −80 °C for analysis. In the post-spin down study, two sample pools of newly drawn and spun down serum (SST) and plasma (NaHeparin) were spiked with GHB, since it is absent in most healthy subjects. Aliquots were stored in duplicate for each time point at various temperatures. Serum and plasma aliquots at time zero (control) were prepared for immediate analysis after aliquoting. Other samples were moved from their storage temperature to a −80 °C freezer at designated time points for analysis upon completion of the study. In addition to time zero, the study evaluated time points of 6 h, 18 h, and 24 h at room temperature, and 1, 2, 3, 4, 7, 17, and 90 days at 4 °C, −20 °C, and −80 °C. Freeze-thaw stability was assessed using the same pooled serum

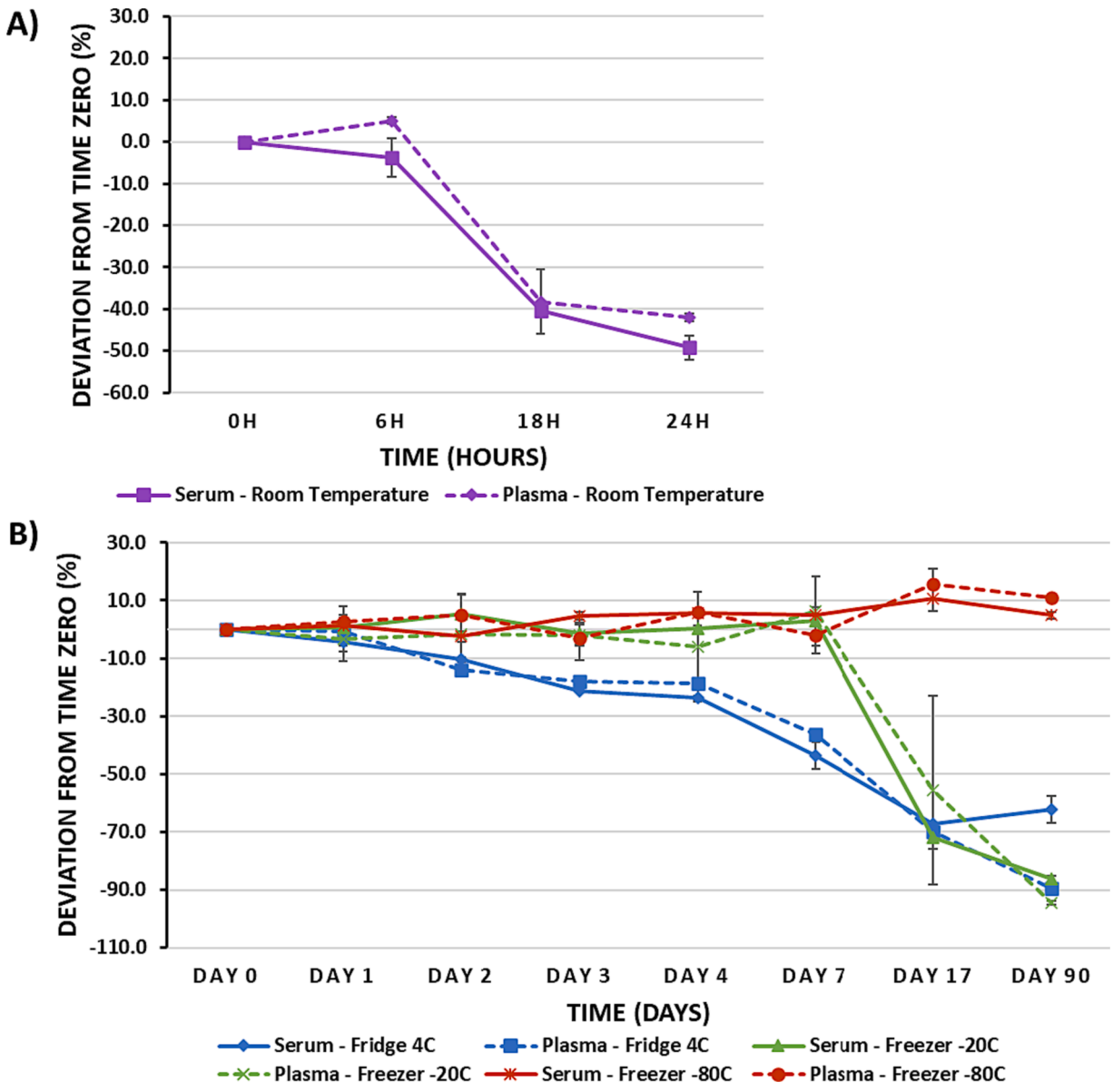

Fig. 5. Stability plot of AcAc in serum and plasma at (A) room temperature, (B) 4 °C, −20 °C, and −80 °C, error bars are 1xSD, n = 2.

and plasma used for the post-spin down stability study, with aliquots subjected to up to 5 cycles of thawing and refreezing. Extract stability was evaluated by keeping a batch of 10 random patient samples in the autosampler at 4 °C after the initial analysis and re-running them over the next four days. This experiment was replicated with another batch where three aliquots of each extract, post-initial analysis, were stored at −20 °C and reanalyzed after 1, 2, and 5 days.

The performance of the matrix and specimen collection tube was examined by measuring pooled serum (SST) and plasma (NaHeparin). Each sample pool was spiked with GHB at different concentrations due to its absence in these pooled samples. Mixtures at different ratios in serum and plasma (i.e., 3:1, 1:1, and 1:3) were prepared in triplicate. The percent deviation from the expected concentration was calculated to assess differences in performance. In addition, blood was drawn from two donors using four different collection tubes (SST or Gold Top, Red Top, NaHeparin or Green Top, and EDTA or Purple Top) to compare the percent deviation of reported concentrations.

Throughout the validation process, the robustness of the assay was evaluated by testing three different column lots, two different LC-MS/MS platforms of the same model, and several brands of pipettes and tips.

Data analysis

Throughout the method development phase, MassLynx and TargetLynx V4.2 software from Waters were utilized to acquire data, optimize parameters, and batch process. All validation data collected using the final LC-MS/MS method were analyzed and curated with Ascent V4 from Indigo BioAutomation. Validated customized rules were applied to reduce processing times and human errors. Statistical data analysis was performed with R 4.2.3 [30]. Deming regression was performed with the assumption of equal variance using the "mcr" package [31]. Assessment of inter- and intra-day precision was performed using the "VCA" package [32].

Results and discussion

Method development

A 6.5-minute LC-MS/MS method was developed and validated for the quantification of AcAc, BHB, AHB, GHB, and BHIB in serum and plasma using protein precipitation. A previously developed method was

Table 5
Overview KB stability, bias < 20 % was set as cutoff.

Analyte	From draw tospin-down		From spin-down to storage				Freeze-thaw cycles
	RT	4 °C	RT	4 °C	−20 °C	−80 °C	
AcAc	6H	2H	6H	2 days	7 days	At least 90 days	At least 5
BHB	At least 6H	At least 6H	At least 24H	At least 90 days	At least 90 days	At least 90 days	At least 5
AHB	At least 6H	At least 6H	At least 24H	At least 90 days	At least 90 days	At least 90 days	At least 5
GHB	At least 6H	At least 6H	At least 24H	At least 90 days	At least 90 days	At least 90 days	At least 5
BHIB	At least 6H	At least 6H	At least 24H	At least 90 days	At least 90 days	At least 90 days	At least 5

Table 6
Matrix comparison of spiked serum SST and plasma NaHeparin collected samples; calculated %bias towards the expected KB concentrations after mixing the sample pools.

Serum: Plasma Ratio	3: 1	1: 1	1: 3
AcAc	3.9	4.7	5.1
BHB	4.6	7.9	7.3
AHB	1.9	3.1	5.7
GHB	1.6	5.8	8.3
BHIB	4.7	4.1	8.5

Table 7
Comparison of freshly drawn blood in four different collection tubes, %bias is calculated towards the serum SST tube as control. D1 and D2 are donor 1 and 2.

Analyte	Plasma (NaHeparin)		Plasma (EDTA)		Serum (Red Top)	
	D1	D2	D1	D2	D1	D2
AcAc	−13.7	6.5	−4.8	13.6	1.0	−5.8
BHB	−5.5	7.7	15.8	17.9	3.0	−2.5
AHB	−2.5	3.7	−5.2	4.0	−2.6	4.0
GHB	ND	ND	ND	ND	ND	ND
BHIB	1.9	0.0	18.5	13.7	−1.5	1.0

adapted to improve separation and peak shape and reduce the analysis time [25]. All five analyte peaks were adequately separated chromatographically. For example, AHB and GHB are isomers and share the same MS/MS transition; however, their LC peaks were baseline resolved at 1.77 and 1.67 min, respectively. Lowering the column temperature was an important step in the chromatographic development to separate all isomers. As shown in Fig. 2, at 25 °C, minimal separation between GHB and BHB was observed; however, at a lower temperature, increased separation was observed. In the final method, the column temperature was set at 17.5 °C using a column chiller (+/−0.5 °C) to achieve appropriate separation while maintaining a sufficiently low viscosity of the mobile phase to avoid pressure issues, which were observed at lower temperatures. Additionally, the flow rate was reduced to 0.22 mL/min between 2 and 4.5 min to mitigate the pressure increase due to increased viscosity. Chromatography performance was measured over 472 injections across 20 batches, including calibrators, QCs, and unknowns, reporting a percent coefficient of variation (%CV) of <1 % for all analytes. Details are listed in the Supporting Information.

KBs are small molecules that easily fragment, and due to their small size, only one or two suitable fragments were recorded with the application of minimal collision energy. As shown in Table 1, unique fragments were selected for the four KB isomers whenever possible. Selecting adequate qualifier ions with sufficient abundance and a similar background was difficult, so different collision energies were applied to produce a qualifier ion. For example, hydroxybutyrate ions may produce fragments with *m/z* 57 and *m/z* 85; however, due to the inadequate intensity of *m/z* 85 and the presence of background noise, only *m/z* 57 was used. To avoid instrument errors, a slight mass offset was applied, which is negligible on a unit resolution MS [33]. In this scenario ion ratios can still be monitored to ensure fragmentation is consistent. Unfortunately, this approach may provide less analytical specificity than a true qualifier ion if the ratio of quantifier transition to pseudo-qualifier transition does not change significantly in the presence of an interferant.

Extraction efficiency and matrix effects were constantly monitored during the method development phase. An extraction efficiency between 95 and 105 % was observed for all KBs, except AcAc. The latter is the least stable, but still reported an extraction efficiency between 80 and 95 %, which was within our aim of 80–120 %. The optimized protein precipitation workflow, using an extra reconstitution step, demonstrated acceptable matrix effect for all analytes, averaging between 1.5 and 13.9 % in dialyzed serum and pooled serum. See Supporting Information for a full overview.

Accuracy and precision

Accuracy was evaluated through spike and recovery using 10 random patient samples. Table 2 presents the data, demonstrating an average recovery between 85 and 115 % for all compounds being studied, with a tight %CV of ≤ 3 %. AHB, GHB, and BHIB exhibited slightly lower recoveries compared to the other KBs, which may be due to the absence of a suitable IS. Additionally, accuracy was assessed by analyzing three levels of QCs across 20 batches. The mean values of each level for all KBs demonstrated accuracy between 85 and 115 %. Further, the comparison between BHB measurements using an existing FDA-approved enzymatic assay (Stanbio LiquiColor, performed on Ortho Vitros 4600) in the Core Laboratory of the Children's Hospital of Philadelphia and the new LC-MS/MS method showed a strong correlation. Out of the 43 samples compared, 13 had values below 0.3 mM (LLOQ) in the enzymatic assay, while all these samples were still within the analytical measurement range (AMR) in the LC-MS/MS method, with reported values below 0.3 mM. Moreover, samples with assigned values in both methods were compared using the Deming regression fit, resulting in a Pearson's R of 0.996 and a slope of 0.97 (Fig. 3A). The variance was equally distributed across the measured concentrations, and a mean bias of 0.01 was observed (Fig. 3B).

The precision study was carried out at low, medium, and high QC levels. Intra-precision was determined by assessing the injection precision and the replicate extraction precision within-day. Inter-precision was determined by analyzing the same QC materials over a span of 20 days to calculate between-day precision. The reported %CV values for intra- and inter-imprecision were < 5 %, and the total imprecision was < 10 %, which was within our target acceptance criteria (Table 3).

Linearity and LLOQ

Eleven-point curves were created using a mix of 0.0125 mM and 1.5 mM samples in triplicate, representing equal intervals. Nominal values were plotted against the measured concentrations for each analyte. AcAc and BHB demonstrated linearity across the tested range, while AHB, GHB, and BHIB exhibited linearity up to 1.35 mM, likely due to the absence of an IS. The correlation coefficients were > 0.9993, and the calculated residuals reported a bias of < 15 % for all analytes.

The LLOQ was established by measuring five replicate extractions over five days at two levels. At 0.00125 mM, all analytes reported a CV

of < 15 %, but a calculated bias of > 15 %. At 0.0025 mM, AcAc, AHB, and GHB reported a CV and bias of < 15 %, while BHB and BHIB performed well at 0.0050 mM. Given the assigned nominal values to the calibrators, appropriate analytical measurement ranges (AMRs) were set and listed in Table 4. Integrated chromatograms at LLOQ level are displayed in Fig. 4.

Diluent, dilution maximum, and carryover

Dilutions were performed in triplicate using both dialyzed serum and water at 2, 5, 10, 50, and 100x. All targets demonstrated an imprecision and bias of < 15 % when using either diluent up to 50x dilution. Based on the established maximum dilution of 50x, a clinically reportable range (CRR) was determined and listed in Table 4, using the upper limit of the AMR and the maximum dilution.

Throughout the validation process, no carryover was observed in the blank sample following the highest calibrator. Moreover, blank samples analyzed after a spiked patient sample with 10 mM KB mix, values were reported < LLOQ. Furthermore, when comparing peak areas of a system check sample at the LLOQ level measured before and after analysis of the 10 mM spiked sample, the deviation was <20 %.

Specificity

Twenty-one interferences were tested using pooled serum that was spiked at both low and high concentrations of KBs. No significant interferences were observed, and all five analytes reported a bias of <10 % for the individually surveyed interferences. The only exceptions were high levels of triglycerides and protein, which resulted in a bias of <15 %.

Stability

Due to the known limited stability of acetoacetate, it was important to conduct a comprehensive stability study to ensure the accuracy of the assay. The stability of the KBs was assessed from the time of sample collection until centrifugation at the laboratory, with the aim of evaluating any potential biases in the measurements. When comparing the stability of specimens kept on ice for 6 h or at room temperature (RT) for 2 h to those kept on ice for 2 h (which represents the fastest typical time from blood draw to receipt in our clinical setting), the average bias for all KBs remained below 15 %. However, when samples were kept at RT for a longer period after collection, the bias increased from 14.5 % (2 h at RT) to 25.9 % (6 h at RT) for AcAc. Additionally, the bias for the BHB/AcAc ratio increased from 16.6 % (2 h at RT) to 37.1 % (6 h at RT). Nevertheless, the remaining analytes demonstrated biases of less than 10 % across all studied time points. Notably, GHB was excluded from this experiment due to its absence in most healthy subjects and the challenges associated with accurately spiking freshly drawn blood without knowledge of the exact volume. Detailed results are provided in the Supporting Information.

Additionally, the stability of all KBs, including GHB, was evaluated after spinning down the serum and plasma samples at various temperatures: RT, 4 °C, −20 °C, and −80 °C. The results showed that all analytes, except AcAc, remained stable for up to 24 h in both serum and plasma when stored at RT. However, although AcAc displayed a bias of less than 10 % for up to 6 h, but this bias significantly increased to over 35 % after this time point in both plasma and serum. Conversely, at 4 °C, −20 °C, and −80 °C, all analytes, except AcAc, demonstrated stability for at least 90 days in both serum and plasma, with biases below 17 %. AcAc maintained stability for 2 days at 4 °C, 7 days at −20 °C, and at least 90 days at −80 °C. The stability profile of AcAc is depicted in Fig. 5.

Additionally, the impact of freeze–thaw cycles on analyte stability was examined. The results indicated that all analytes remained stable for at least 5 cycles in both serum and plasma, with biases below 20 %. However, AHB exhibited an upward trend immediately after the first cycle in both matrices, and after two cycles, a steady bias between 15 and 20 % was observed, particularly in serum. A comprehensive overview of the stability results can be found in Table 5. Finally, the stability of a fully extracted batch was assessed at 4 °C for four days and at −20 °C for five days. BHB demonstrated stability under both storage conditions, whereas AcAc remained stable for one day at 4 °C, but achieved full stability at −20 °C. AHB and BHIB displayed stability for one day at 4 °C, but were not stable at −20 °C. The post-extraction stability of GHB was not determined as none of the tested batches contained GHB in patient samples. Further details regarding the stability experiments, including stability plots, can be found in the Supporting Information.

Matrix and specimen collection tubes

To evaluate the commutability of different matrices, pooled serum and plasma samples were spiked and mixed in various ratios. The objective was to investigate any potential bias between the two matrices. The results showed that all analytes exhibited a bias of less than 10 %. In general, absolute bias was slightly less in serum (Tables 6 and 7).

In addition to matrix mixing, blood samples were freshly drawn into different specimen collection tubes, namely SST, Red Top, NaHeparin, and EDTA tubes, from two donors. Since most of the validation was performed using serum from SSTs, it was considered as the control. For AcAc and AHB, the bias was below 15 % across all four tested tubes. BHB and BHIB showed good agreement between the NaHeparin and Red Top tubes. However, a bias greater than 15 % was observed for the EDTA tube (Table 7). GHB was not detected in any of the tubes from either donor. Based on these findings, the EDTA tubes were deemed unsuitable and were excluded from the list of acceptable collection tubes.

Reference range

New reference ranges for all analytes and the BHB/AcAc ratio have been established for patient diagnosis. Initially, 156 specimens were analyzed; after a chart review, ultimately 107 samples were included in the reference range. Samples were excluded due to factors such as recent vomiting, extreme fasting, eating disorders, or the presence of interfering metabolic diseases. The newly determined reference ranges, representing the central 95 % of included samples, are presented in Table 4. Additional details regarding the distribution of each analyte among the reference samples can be found in the Supporting Information.

Conclusions

A rapid and robust LC-MS/MS-based clinical assay was developed for the quantification of KBs, including AcAc and BHB, as well as three additional isomers of hydroxybutyrate (i.e., AHB, GHB, and BHIB). The method exhibited minimal imprecision, acceptable bias in accuracy testing, and high specificity with respect to common interferences. Notably, the instability of AcAc was overcome with strict guidelines for sample handling. Despite limited stability, the validation study identified three acceptable sample collection tubes (i.e., SST, Red Top, and NaHeparin). This flexibility in collection tubes allows for minimizing collection volumes when blood is collected for multiple simultaneous tests. Finally, a reference range was established for all five KBs, in addition to the BHB/AcAc ratio. The reference range of the BHB/AcAc ratio and AHB are expected to be valuable in diagnosing and monitoring mitochondrial dysfunction, aiding in the accurate assessment and diagnosis of these associated conditions. This assay also provides highly sensitive measurement of gamma-hydroxybutyrate which will be useful in diagnosing and monitoring patients with succinate semialdehyde dehydrogenase deficiency.

Full disclosures

Dr. Kemperman has nothing to disclose.

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Ethics statement

The Children's Hospital of Philadelphia Institutional Review Board (IRB)/Ethics Committee provided a determination of exemption for this study (IRB 23–021290).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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