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Determination of ketone bodies in human plasma

A LC/MS method for quantification and tracer analytics of β-Hydroxybutyrate

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Table of contents

Abstract

Ketone bodies, consisting of β-hydroxybutyrate (BHB), acetoacetate (ACAC) and acetone, are part of several mammalian metabolic pathways with a broad concentration range. Ketone bodies are recently found to have a protective role for heart and liver and are discussed for therapeutic use. The aim of this master thesis was to implement a method to measure BHB and its isotope labelled equivalent with liquid chromatography coupled to mass spectrometry

Sample preparation was performed with protein precipitation. Chromatography was performed by a 1290 Infinity II Liquid Chromatography (LC) System of Agilent with a Waters Acquity UPLC BEH C_{18} column. The analytes were detected with an Agilent 6495 Triple Quadrupole mass spectrometer (MS) in multiple reaction mode (MRM) with internal standardization using $^{13}C_2$-BHB. The quantification of BHB was evaluated according to the guidelines of the US Food and Drug Association (FDA).

ACAC can also be measured with the implemented LC/MS method. The chromatography is able to separate the keto and enol form of ACAC. Further experiments are needed to clearly define the tautomeric forms and to accurately quantify ACAC.

$^{13}C_4$-BHB was implemented into the LC/MS method for prospective tracer enrichment studies. The tracer enrichment showed a linear course over the expected concentration range of BHB.

With the implemented LC/MS method it was possible to measure samples from a clinical trial as well as to establish an enzymatic assay so far.

1. Introduction and aim of this work

Ketone bodies are an energy source in organisms and part of different mammalian metabolic pathways. Humans exhibit a daylong variation of the total ketone body concentration with a broad range from 50 µM up to 20 mM depending on the metabolic state (Puchalska and Crawford, 2017). Commercial ketone tests for urine and blood are based on the so called Legal reaction, in which acetoacetate (ACAC) reacts with the chemicals to produce a purple color on the test strip. This method is semiquantitative and does not react to β-hydroxybutyrate (BHB). Furthermore, the Legal reaction is associated with a significant risk for false-positive or false-negative results. These test strips are mainly used for self-control of Diabetes Mellitus (DM) (Laffel, 1999). For clinical predictions, quantification of BHB is therefore performed with rapid enzymatic methods based on the method of Williamson et al., the spectrophotometric measurement of the reduction of diphosphopyridine nucleotide in the presence of BHB using β-hydroxybutyrate dehydrogenase (BDH) (Williamson et al., 1962).

Most of the assays are designed for high concentrations of ketone bodies to monitor possible development of ketosis and therefore are unreliable in physiological range (Laun et al., 2001). Furthermore, ketone bodies are not measured directly and ACAC is a rather unstable and therefore difficult analyte. As mentioned above, ketone bodies are part of different metabolic pathways and are currently found to have a protective role in heart and liver. Moreover, they are discussed as therapeutic options for diseases of the nervous system and cardiovascular or obesity-related diseases, respectively. Thus, there is interest in a more sensitive and specific quantification of ketone bodies for further examination of their different roles in the metabolism. Aim of this master thesis is to implement a method for the direct quantification of ketone bodies. This is managed with the combination of liquid chromatography coupled to mass spectrometry (LC/MS).

We settled on quantification of BHB because there is a strong correlation between BHB and ACAC levels. Therefore, BHB alone can serve as an analyte for the overall process of ketogenesis (Laun et al., 2001). Over the course of this thesis, BHB was measured for a

clinical trial to get an insight of the effects of special diabetic drugs. The validation of the method was carried out based on the guidelines of the US Food and Drug Association (FDA).

Further insights on the metabolic ways of ketone bodies can be achieved by in vivo kinetics of BHB using tracer dilution in clinical trials. Here, an isotopic tracer, a labeled equivalent to BHB, is infused into the blood. The samples are then analyzed for the enrichment of the labeled BHB versus the endogenous produced one as well as for the concentration of BHB. For this purpose, the distinction between labeled BHB and endogenous BHB is necessary, which can be accomplished by the measurement with LC/MS.

While working on the main aim, the LC/MS method was applied to a clinical trial about the effects of diabetic drugs. An enzyme activity assay based on the LC/MS method was also realized to directly determine the turnover of BHB to ACAC. A poster about this experiment was presented at HPLC 2017 in Prague. The poster is attached to this thesis in the appendix.

2. Theoretical background information

2.1. Chromatography and Mass Spectrometry

2.1.1. Liquid Chromatography

LC is a method in analytical chemistry to separate molecules or ions in a solvent due to differences in size, adsorption, ion-exchange or partitioning. In general, LC contains a stationary and a mobile phase. The mobile phase is introduced with a constant flow to the stationary phase. The sample is injected into the constant flow and then gets separated by interaction with both phases. The different compounds are then eluted with different retention times and introduced to the detector, for instance a mass spectrometer. Improved separation power is achieved with smaller particle sizes, which result in higher pressures for the desired solvent flow rate. Advances in instrumentation and column technology lead to the nowadays commonly used ultra high performance liquid chromatography (UHPLC) systems (Waters).

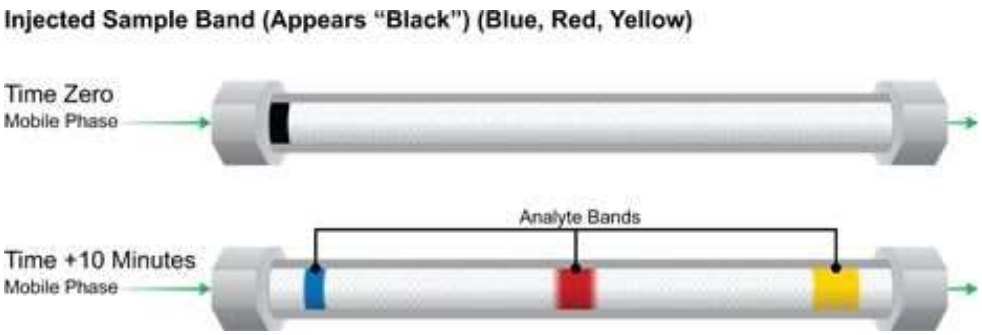


Figure 1: Schematic separation in a chromatographic column (Waters)

2.1.2. Mass Spectrometry

A MS detects ions based on their movement in vacuum. A quadrupole consists of four parallel rods with specific voltages applied. In triple quadrupoles, the first quadrupole only transmits the precursor ion of interest with a specific m/z, which then is fragmented in the collision cell, the second quadrupole. A specific product ion with defined m/z is then transmitted to and subsequently quantified at the third. This technique enables isolation and examination of multiple precursor to product ion transitions, called multiple reaction monitoring (MRM) (Agilent Technologies, 2014). Dynamic MRM mode allows the instrument to acquire the MRM data only during the retention time window of the analyte. MRM mode is particularly useful for analysis in complex matrices such as the blood (Stone, Glauner, Kuhlmann, Schlabach, & Miller, 2009).

The Agilent 6495 Triple Quad combines an enhanced electrospray ionization (ESI) and an Agilent Jet Stream with a hexabore capillary sampling array. The Agilent Jet Stream utilizes a thermal gradient focusing technology with super-heated nitrogen sheath gas to confine the nebulizer spray and to dry and concentrate the ions more effectively. After ionization, the analyte enters the hexabore capillary sampling array. Consequently, an increasing number of ions can be sampled but also a large volume of gas is transmitted. An ion optics element - the iFunnel - is needed to separate the ions from the gas. This element consists of two funnels, the first one at high and the second at low pressure, displayed in figure 2 (Momoh, et al., 2010).

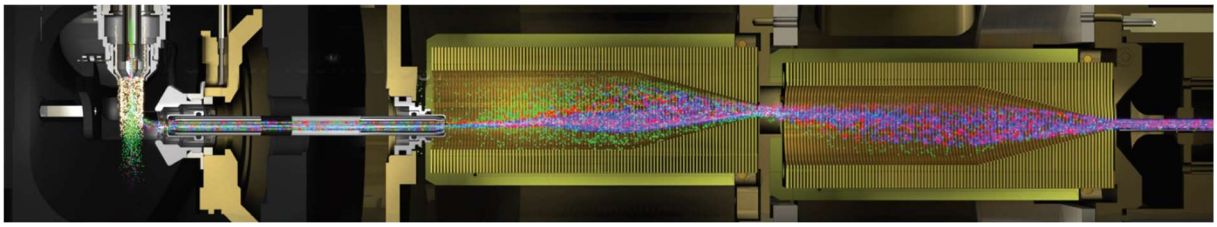


Figure 2: Display of iFunnel technology. The analyte is ionized by a ESI with Jet Stream and collected with a hexabore capillary sampling. The ions are focused further with the iFunnel system (Agilent Technologies, 2017).

2.2. Ketone bodies

Ketone body refers to the sum of BHB, ACAC and acetone as energy source in the organism. They are part of different mammalian metabolic pathways like β-oxidation, tricarboxylic acid cycle, gluconeogenesis, biosynthesis of sterols or de novo lipogenesis (Puchalska and Crawford, 2017).

Specific concentrations of ketone bodies in healthy humans are not yet defined due to the broad concentration range. A typical postprandial concentration of BHB is about 50 µM (Laeger, Metges, & Kuhla, 2010). Therefore, basal blood levels are about a few µM but rise to a few hundred µM after fasting, reaching 1-2 mM after an overnight fast or intense exercise, 5-6 mM with prolonged fasting and up to 20 mM in pathological states like diabetic ketoacidosis (DKA) or alcohol abuse (Newman and Verdin, 2014; Puchalska and Crawford, 2017). ACAC concentration levels range from 0 to 70 µM under non-fasting conditions. There is a strong correlation between BHB and ACAC levels, whenever measured (Laun et al., 2001).

ACAC and BHB provide acetoacetyl-CoA and acetyl-CoA for synthesis of cholesterol, fatty acids and complex lipids (HMDB, 2017b). During early postnatal periods these compounds are preferred over glucose as substrates for synthesis of phospholipids and sphingolipids and supply adequate surfactant lipids to maintain lung function and therefore are important for brain growth and maintenance of lung function during early days of life (Yeh and Sheehan, 1985).

Ketone bodies, mainly BHB and ACAC, are clinically used as markers for diagnosing type 1 diabetes mellitus (T1DM) and ketoacidosis as a result of diabetes or extensive alcohol abuse (Yamato et al., 2009; Dahl et al., 2012). In addition, their measurement has a prognostic value in acute heart failure or hepatitis (Laun et al., 2001). Moreover, a ketogenic diet exert neuroprotection in different models of diseases, including ischemic stroke, Parkinson´s disease, epileptic spasms or Alzheimer´s disease (Puchalska and Crawford, 2017).

2.2.1. Ketone body metabolism

During fasting, stores of glycogen are depleted first, then fatty acids are mobilized and transported to the liver. During β-oxidation, these fatty acids are broken down to acetyl-CoA which then is utilized to generate energy via the citric cycle (figure 3).

In hepatic mitochondrial matrix accumulating acetyl-CoA is used for ketogenesis. Catalyzed by 3-hydroxymethylgutaryl-CoA synthase 2 (HMGCS2), Acetyl-CoA and acetoacetyl-CoA are condensated to hydroxymethylgutaryl (HMG)-CoA. HMG-CoA is then cleaved by a lyase to form ACAC and acetyl-CoA. BDH**Fehler! Textmarke nicht definiert.** catalyses the reduction of ACAC to BHB in a NAD^+/NADH-coupled near-equilibrium reaction. Ketone bodies are then transported to metabolically active tissues, such as muscles or brain, for terminal oxidation as glucose-sparing energy source. Excess ACAC can spontaneously decarboxylate to acetone.(Newman and Verdin, 2014; Puchalska and Crawford, 2017).

The interconversion of BHB and ACAC is regulated primarily by the ratios of substrates and cofactors (Newman and Verdin, 2014).

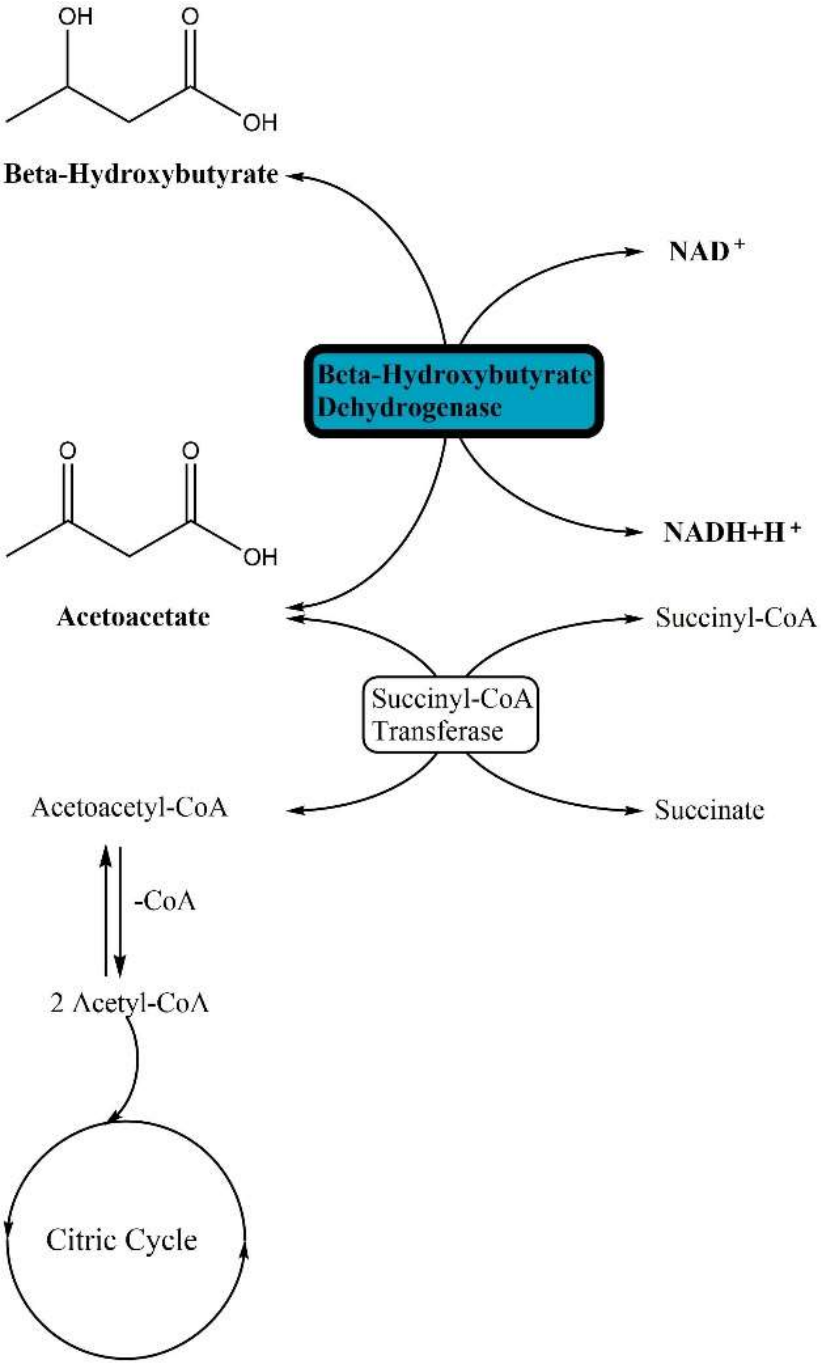


Figure 3: Schematic degradation of ketone bodies

2.2.2. β-Hydroxybutyrate

BHB is the most abundant circulating ketone body and less likely degrades spontaneously into acetone than ACAC (Newman and Verdin, 2014). BHB has the chemical formula $C_4H_8O_3$ and a molecular weight of 104.1 (HMDB, 2017a).

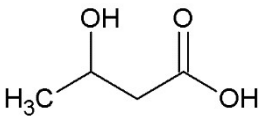


Figure 4: Structure of β-hydroxybutyrate (Chemspider, 2015c)

Under starving conditions, the brain can also utilize BHB as the primary fuel covering about 60% of its energy requirement. Moreover, the immature brain depends on BHB as important precursor for amino acids as well as for lipid biosynthesis necessary for cellgrowth and restructuring of cell connections. Utilization of BHB is not homogenously distributed in the brain due to the permeability of the blood-brain-barrier, which declines with age and is modulated by dietary changes, i.e. high fat diet (Laeger et al., 2010).

BHB also has signaling functions. It is a ligand for at least two G-protein-coupled receptors (GPR). Inhibition of GPR41 reduces total energy expenditure and heart rate. In addition, it functions as a feedback mechanism by activation of hydroxycarboxylic acid receptor 2 (expressed in immune cells and adipose tissue) and resulting inhibition of lipolysis in adipocytes. It also may reduce sympathetic tone and lower the metabolic rate (Newman and Verdin, 2014; Puchalska and Crawford, 2017).

2.2.3. Acetoacetate

ACAC is a weak organic acid with a molecular weight of 102.09 and the chemical formula $C_4H_6O_3$. Excessive fatty acid breakdown leads to accumulation of ACAC and its partially convertion to acetone. In newborns a persistent mild hyperketonemia is common since these compound serves as an energy source for extrahepatic tissue, especially the lung and brain in the early stages of life (HMDB, 2017b).

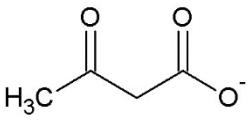


Figure 5: Structure of acetoacetate (Chemspider, 2015a)

2.2.4. Acetone

Acetone has the chemical formula C_3H_6O and a molecular weight of 58.08. The physiological role of acetone is not yet clear, but it is not regarded as a waste product. It is excreted either in urine or through respiration (HMDB, 2017c).

In DKA ketone body production exceeds the degrading capacity presenting a challenge to the pH regulatory system. Acetone is produced by decarboxylation of ACAC and is further degraded to C3 fragments. Therefore acetone keeps maintenance of pH buffering capacity and provision of fuel for peripheral tissues (HMDB, 2017c).

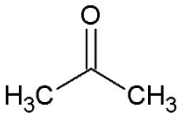


Figure 6: Structure of acetone (Chemspider, 2015b)

2.3. Diabetes mellitus

In response to rise in blood glucose the hormone insulin is secreted rapidly from the β-cells in the pancreas to transport glucose into cells. Reduced sensitivity of the cells for insulin, also called insulin resistance, or the inability to produce enough of the hormone leads to diabetes mellitus (DM). Elevated levels of blood glucose lead to hyperglycaemia, which can damage various tissues, leading to life-threatening health complications like cardiovascular disease, neuropathy or nephropathy. DKA is another consequence of sustaining hyperglycaemia, leading to osmotically driven dehydration in combination with increased ketone body concentration and decreased pH. According to the WHO, diabetes is a major cause of blindness, kidney failure, heart attacks, stroke and lower limb amputation. The global prevalence among adults over 18 years was 8.5% in 2014. In 2017, 8.8% of Europeans of age 20-79 are living with diabetes. There are three main types of diabetes (European Medicines Agency, 2015; IDF, 2017; WHO, 2017).

2.3.1. Type 1 Diabetes Mellitus

T1DM**Fehler! Textmarke nicht definiert.** is caused by autoimmune reactions against the β-cells, which lead to impaired insulin production. Risk factors include certain HLA-antigens (HLA-DQ8 and HLA-DR4) and environmental triggers like viral infections (Böcker et al., 2012; IDF, 2017).

2.3.2. Type 2 Diabetes Mellitus

Type 2 DM (T2DM) accounts for around 90% of all cases of diabetes. Cause of the disease is insulin resistance and impaired production of insulin. Obesity, physical inactivity and a poor diet account as risk factors for T2DM (IDF, 2017).

2.3.3. Gestational diabetes mellitus

Gestational diabetes mellitus (GDM) is hyperglycaemia first diagnosed during pregnancy. GDM arises because of diminished action of insulin due to hormone production by the placenta. GDM usually resolves once the pregnancy ends (IDF, 2017).

2.3.4. Diagnosis and therapies

DM is diagnosed with elevated glucose levels in blood plasma (126 mg/dl in empty stomach) or with HbA1c > 6.5% (Böcker et al., 2012). T1DM is treated with a daily insulin injection to maintain a physiological blood glucose level (IDF, 2017).

Hyperglycaemia of T2DM is controlled with oral medication, most commonly metformin. If metformin is not adequate, add-on therapy with antidiabetic medication like sodium-glucose cotransporter-2 (SGLT-2) inhibitors, dipeptidyl peptidase 4 (DPP-4) inhibitors, or glucagon-like peptide 1 (GLP-1) agonists is possible (IDF, 2017). SGLT-2 are the main transporter of glucose in the proximal tubule in the kidney. SGLT-2 is a high-capacity cotransporter with a 1:1 sodium to glucose molecule transport ratio. Inhibition of this reabsorption of glucose reduces blood glucose levels (Devenny et al., 2012). GLP-1 is a hormone that increases insulin secretion of β-cells and inhibits glucagon release. Additionally, it enhances lipogenesis in adipose tissue. GLP-1 is degraded by DPP-4 (Waller and Sampson, 2018).

Recent studies indicate a risk of euglycaemic DKA in patients treated with SGLT-2 inhibitors. These cases are concerning because the absence of typical symptoms of hyperglycaemia complicates the detection of DKA, a delayed detection leads to delayed treatment and graver complications, like coma or death (European Medicines Agency, 2015).

A healthy lifestyle, including a healthy diet, physical activity and smoking cessation, is part of the therapy for both types of diabetes (IDF, 2017).

3. Materials and methods

3.1. Chemicals

3.1.1. Standards

BHB	(*R*)-3-Hydroxybutyric acid	1 g (Sigma Aldrich, St. Louis, USA)
$^{13}C_4$-BHB Tracer	Sodium D-3-Hydroxybutyrate ($^{13}C_4$-BHB)	500 mg (Cambridge Isotope Laboratories, Tewksbury, MA, USA)
AHB	2-Hydroxybutyric acid sodium salt	5 g (Sigma Aldrich, St. Louis, USA)
ACAC	Sodium aceto-acetate	500 mg (Shanghai iChemical Technology, Shanghai, China)
$^{13}C_2$-BHB; ISTD	Sodium DL-3-hydroxybutyrate-1,3-$^{13}C_2$	500 mg (Sigma Aldrich, St. Louis, USA)

3.1.2. Reagents

Antimycin A	from Streptomyces sp (Sigma Aldrich, St. Louis, USA)
Bovine Serum Albumine	lyopholized (Sigma Aldrich, St. Louis, USA)
Elomel	isoton (Fresenius Kabi, Graz, Austria)
Ethylacetate	LC-MS CHROMASOLV® (Sigma Aldrich, St. Louis, USA)

Formic Acid	Formic acid, reagent grade, 5 ml (Agilent, Waldbronn, DEU)
L-Cystein	BioUltra (Sigma Aldrich, St. Louis, USA)
Magnesium Chloride	Anhydrous powder, ≥98%(Sigma Aldrich, St. Louis, USA)
Methanol	Chromasolv®, for HPLC, ≥99.9 % (Sigma Aldrich, St. Louis, USA)
Milli-Q Water	18.2 MΩ*cm, Milli Q® Academic water purification system (Millipore GmbH, Vienna, AUT)
Nicotinamide adenine dinucleotide (NAD^+)	Grade I, free acid (Sigma Aldrich, St. Louis, USA)
Nicotinamide	HPLC grade (Sigma Aldrich, St. Louis, USA)
Potassium Chloride	Cell culture tested (Sigma Aldrich, St. Louis, USA)
Potassium Cyanide Bioultra	BioUltra (Sigma Aldrich, St. Louis, USA)

3.2. Equipment

AJS-MS/MS	Agilent 6495 Triple Quadrupole LC/MS	Agilent, Waldbronn, GER
Centrifuge Large	Heraeus Multifuge X3R Centrifuge	Thermo Scientific, Rockford, USA
Evaluation Software	MassHunter	Agilent, Waldbronn, GER
Evaporator Large	Evaporator	Liebisch Labortechnik, Bielefeld, GER
Evaporator Small	MiniVap, 96 needle head	Porvair Sciences, Wales, UK
Pipettes	Reference, Research plus/pro, Mulitpette M4	Eppendorf, Hamburg, GER
Pre Column	VanGuardTM Precolumn	Waters, Massachusetts, USA
SPE	*Oasis HLB 96-well µElution*	Waters, Massachusetts,

	plate 2 mg, 30 µm	USA
U-HPLC	1290 Infinity II LC System	Agilent, Waldbronn, DEU
U-HPLC-Column	Waters Acquity UPLC® BEH C18 (2.1x150 mm; 1.7 µm)	Waters, Massachusetts, USA
Ultrasonic Bath	Sonorex Super	Bandelin, Berlin, DEU
Vacuum Pump System	DOA-V517-BN	GAST Manufactoring Inc., Benton Harbor, USA

3.3. Consumables

HPLC vials	Verex Vials, 9mm Screw, 2 ml Amber	Phenomenex, California, USA
PCR tubes	PCR tubes, 0.2 ml	Agygen Scientific, California USA
Pipette Tips	epT.I.P.S., Combitips advanced	Eppendorf, Hamburg, GER
Safe Lock Tubes	Safe Lock tubes 0.5 ml, 1.5 ml, 2 ml, 5 ml	Eppendorf, Hamburg, GER
Reagent Reservoir	Reagent Reservoir 25 ml	VWR, Vienna, AUT
Sealing mats	Deepwell Mat 96, 1.2 ml	Eppendorf, Hamburg, GER
Vial Inserts	Vial insert, 250 µl pulled point glass	Agilent, Waldbronn, GER
Vial Caps	Blue Screw Cap, PT FE/SIL Banded Septa	Agilent, Waldbronn, GER

3.4. Methods-Overview

Standard solutions of the analytes were used to develop the method. Purpose of this method is to quantitate the ketone body concentration and to separate the labeled BHB ($^{13}C_4$-BHB), which is used as tracer in later studies, from the endogenous BHB. MRM is used as the scan

mode for MS to ensure the separation of co-eluting substances. In this mode, the analyte molecules are fragmented in a collision cell and the analytes can be identified with their precursor mass and their specific fragments.

3.5. Preparation of Standards

The standards are provided as dry chemicals. For the pretests, a set of A-stocks are prepared and stored at -80°C. Fresh B-stocks are prepared on the respective test day. All stocks are freshly prepared for the method validation and stored at -80°C until the date of utilization.

3.5.1. A-Stocks

The standards BHB, $^{13}C_2$-BHB, $^{13}C_4$-BHB, ACAC and α-hydroxybutyrate (AHB) are delivered as dry chemicals and have to be dissolved in methanol. For the A-stocks 100 mg of each standard is dissolved in 10 ml of methanol individually, except for ACAC which was dissolved in methanol/MilliQ (50/50, v/v).

The dilutions are aliquoted into safe lock tubes and stored at -80°C.

3.5.2. B-Stocks

B-stocks are prepared by dilution of the A-Stocks with methanol/MilliQ (20/80, v/v) to a final concentration of 1 mM. The stocks were used for the preparation of the calibration standard solutions.

For the B-stock of $^{13}C_2$-BHB a final concentration of 200 µM is prepared and used as internal standard (ISTD).

3.5.3. Calibration standard solutions

For quantification of the analytes a calibration curve is established. The concentrations of the calibration solutions range from 1 to 800 µM with 8 calibration levels for the pretests and from 3 to 800 µM for the method validation and the clinical trial. The LC/MS based enzyme assay was evaluated with calibration standards from 1 µM to 10 mM in five levels. Further

information about the preparation of the calibration solutions can be found in table 16 in the appendix.

3.6. Sample collection

Human plasma is used for the analysis of the ketone bodies. The blood is drawn and prepared by the Clinical Research Center and passed on to the analytical laboratory as sodium fluoride/potassium oxalate plasma on ice.

3.7. Sample preparation

3.7.1. Protein precipitation

100 µl of plasma are used for the analysis. 25 µl of the B-stock of $^{13}C_2$-BHB - the ISTD - are added to the samples so that the final concentration of $^{13}C_2$-BHB is 40 µM. For protein precipitation 400 µl of cold methanol (-20°C) are added. Mixing is followed by an incubation at 4°C for 10 minutes. The mixture is centrifuged at 3000 g at 4°C for 10 minutes to separate the protein. 200 µl of the supernatant are evaporated with a stream of nitrogen at ambient temperature. The samples are redissolved in 100 µl of methanol/MilliQ (20/80, v/v) and stored until quantification by LC/MS at 5°C.

3.7.2. Solid phase extraction

A solid phase extraction (SPE) was additionally performed after the protein precipitation to achieve a higher purity and concentration of the ketone bodies. 200 µl of the obtained supernatant are diluted with 500 µl of MilliQ/formic acid (FA) (100/0.02, v/v) and used for the SPE. The used material for the SPE is the hydrophilic-lipophilic balance (HLB) copolymer, which is a reversed phase sorbent used for acids, bases and neutrals. Prior to sample loading, conditioning is performed with 1 ml of methanol, followed by equilibration with 1 ml of MilliQ/FA (100/0.02, v/v). The samples are loaded with the help of a vacuum manifold and a vacuum pump. The washing step is performed with 1 ml of methanol/MilliQ (5/95, v/v). The elution of the analytes is performed with 200 µl of methanol followed by 200 µl of ethylacetate. The eluate is collected into a 96-wellplate and then evaporated with nitrogen and redissolved in 100 µl of methanol/MilliQ (20/80, v/v).

3.8. Preliminary mass spectrometric tests

3% bovine serum albumin (BSA) in Elomel was spiked with BHB, ACAC or AHB to a concentration of 100 µM to perform the pretests. MRM transitions of the analytes are selected based on literature. The optimizer tool of Agilent is used to get the collision energy with the best abundances. The optimizer tool runs the method with different collision energies and compares the responses.

3.9. Tracer enrichment

For an evaluation of the measurement of the tracer enrichment later on in the clinical trial a calibration curve with different enrichments of the tracer is measured. The concentration levels of BHB, the tracee, ranges from 25 µM to 10 mM whereas the concentration of $^{13}C_4$-BHB, the tracer, is 5 µM for each BHB concentration level. Each calibration level for the enrichment curve, eight in total, is measured five times.

3.10. Worklist Design

After equilibration of the HPLC-column the run is started by injecting methanol/MilliQ (20/80, v/v), which was also analyzed regularly in between the runs to detect a possible carry over.

At the beginning and the end of the analyses of batches measurements of the eight calibration levels were performed.

3.11. Chromatographic separation

Samples were kept in the Agilent autosampler at 5°C. A chromatography is performed prior to mass spectrometry to separate the components. An Agilent 1290 Infinity II LC System, which was used for this purpose, is a single dimension UHPLC system and is able to work up to a pressure of 1300 bar.

An Acquity UPLC® BEH C_{18} column from Waters is used. It has 1.7 µm sized BEH (ethylene bridged Hybrid) particles, a length of 150 mm and a diameter of 2.1 mm. It is used in combination with a precolumn with the same specifications except for the length.

For the separation, a constant flow of 0.2 ml/min was adjusted and the column temperature was set to 35°C. A gradient elution was chosen for the chromatographic separation. Therefore, two solvents were prepared, called solvent A and solvent B. Solvent A consists of MilliQ and formic acid (100/0.02, v/v) and solvent B was absolute methanol. The time table for the gradient can be found in table 1.

Table 1: Time table of the gradient elution for the chromatographic separation. Solvent A was MilliQ/FA (100/0.02, v/v) and solvent B was methanol. Flow and maximum pressure were kept constant at 0.2 ml/min and 1300 bar, respectively.

Time	Solvent A	Solvent B
[min]	[%]	[%]
0.00	98	2
1.50	45	55
1.51	10	90
3.51	10	90
3.52	98	2
7.00	98	2

3.12. Determination by Mass Spectrometry

Ketone bodies were determined with an Agilent 6495 Triple Quadrupole System with iFunnel technology. Dynamic MRM mode was used for quantification. This mode allows the instrument to acquire the MRM data only during the retention time window of the analyte. The chosen transitions for the MRM are shown in table 2. Instrumental parameters of the method are shown in table 3.

Table 2: Transitions of the compounds. Collision Energy and cell accelerator Voltage were kept constant at 9 and 4, respectively.

Compound	Precursor Ion > Product Ion	Retention Time [min]
BHB	103 > 59.1	3.3
$^{13}C_2$-BHB	105 > 60.2	3.1
$^{13}C_4$-BHB	107 > 61.2	3
AHB	103 > 57.3	3.6
ACAC	101 > 57.3	2.9 and 3.2

Table 3: Method parameters for MS determination

Parameter	
Delta EMV (-)	300
Polarity	negative
Source	
Gas Temperature	290°C
Gas Flow	11 l/min
Nebulizer	20 psi
Sheath Gas Temp	250 °C
Sheath Gas Flow	11 l/min
Negative Capillary	3000 V
Nozzle Voltage	1500 V
iFunnel Parameters	
High Pressure RF	90 V
Low Pressure RF	60 V

3.13. Data Processing

Qualitative and Quantitative MassHunter Software from Agilent was used for the data processing.

All calibration levels are analyzed at least twice to construct a calibration curve. There are 8 points for the pretests, method validation as well as for the samples from the clinical trial. For the enzyme kinetics a 5-point calibration was used. The levels of the calibration range from 3 µM to 800 µM, so that the physiological concentration range should be included. A weighting factor of 1/x was applied. $^{13}C_2$-BHB is used as the ISTD at a concentration of 40 µM. The ratio of analyte to ISTD is utilized for the calculations of the concentrations of the samples.

3.14. Method validation

Method validation is performed according to the guidelines of the FDA on bioanalytical method validation. The validation should prove the reliability and reproducibility of the quantification of the ketone bodies in blood samples (FDA, 2001). Included are accuracy, precision, reproducibility and stability of the analyte BHB.

Three concentration levels are chosen as quality controls of the method and are referred to as QC-L, QC-M and QC-H. A plasma pool with a BHB concentration of 13.69 µM was used as the lowest concentration level QC-L. The medium (QC-M) and high (QC-H) concentration plasma pools are spiked with the BHB standard substance.

3.14.1. Accuracy and precision

The accuracy of a method describes the closeness of the mean test results to the true concentration of the analyte. The determination is performed by replicate analysis of samples with a known amount of the analyte (FDA, 2001). Accuracy is calculated with the mean measured concentration and the nominal concentration according to equation 1.

Equation 1: Accuracy [%Diff]

$$\%Diff = \frac{(mean_{conc_{measured}} - conc_{nominal})}{conc_{nominal}} * 100$$

Precision is obtained by repeated performance of the method on multiple aliquots of a homogenous volume of the appropriate matrix and describes the closeness of these individual measures to each other (FDA, 2001). Precision is determined by using equation 2. Standard deviation and mean concentration of the measured samples are needed for the calculation.

Equation 2: Precision [%]

$$\%\ RSD = \frac{SD(conc_{measured})}{mean(conc_{measured})} * 100$$

The data of this experiment was also used to evaluate the LLOQ and linearity of the calibration curve.

3.14.1.1. Intra-batch

Three aliquots of each concentration level were processed individually and measured two times to calculate intra-batch precision. Hence, each concentration was measured six times in one batch.

3.14.1.2. Inter-batch

Each quality control level was processed for each batch separately. The data of three consecutive days are compared to each other regarding accuracy and precision.

3.14.2. Linearity of the calibration curve and LLOQ

The levels of the calibration curve should be selected to adequately define the relationship of concentration to response of the analyte. It should consist of a blank and a zero sample and six to eight spiked samples. The calibration standards should be prepared in the same biological matrix as the samples and the accuracy should be ±15% for all levels except for the lower limit of quantification (LLOQ), where ±20% are valid (FDA, 2001). It is nearly impossible to filter small endogenous molecules like BHB out of blood samples completely to gain blank matrix. Therefore 3 % BSA dissolved in isotonic Elomel is used as the surrogate matrix. Besides the blank and zero sample, the calibration curve consists of eight levels with the concentration-range of 3 to 800 µM, the detailed list is shown in table 16 in the appendix. Each level is measured five times to calculate the linearity.

The LLOQ is the lowest standard of the calibration curve. The response should have a precision of 20% and an accuracy of 80-120% (FDA, 2001). For LLOQ a concentration of 3 µM is measured five times for the calculation of accuracy and precision.

3.14.3. Stability

The stability of the analyte during sample collection and handling, long-term and short-term storage, after going through freeze-thaw cycles and during the analytical process should be documented (FDA, 2001).

3.14.3.1. Short-term stability

The stability of the analyte over the run time of a batch was validated by storage of the processed samples in the autosampler at 5°C and analysis after 0, 25 and 69 hours.

3.14.3.2. Long-term stability

Long-term stability is validated with storage of unprocessed and processed samples of all three QCs at -80°C. Analysis was performed after 4 and 7 weeks.

3.14.3.3. Stock Solution stability

The A-stocks of BHB from the pretests are diluted to a concentration of 100µM. The measured concentration is compared to the nominal concentration of 100 µM.

3.14.3.4. Freeze-Thaw stability

Stability after freezing of the samples is validated by freezing three aliquots of each concentration level. After 25 hours all three aliquots are thawed unassisted at room temperature. One aliquot of each concentration level is then processed and analyzed whereas the remaining aliquots are stored again at -80°C. This approach is repeated the following day (21 hours later) and the last aliquots of QC-L, QC-M and QC-H are frozen again. After another 23 hours of storage these are also processed and analyzed.

The stability of processed samples was tested after two freeze-thaw cycles with QC-L, QC-M and QC-H. The processed samples were frozen at -80°C and thawed for analysis after 4 weeks. Then the QC set was frozen again and thawed after another 3 weeks of storage at -80°C.

Precision and accuracy are calculated with the measured concentrations.

3.14.4. Selectivity

Selectivity of the method is given if the differentiation and quantification of the analyte is possible in the presence of other components. For the determination of the selectivity at least six different blanks of the appropriate biological matrix should be measured. Endogenous matrix components, metabolites or medication should be considered as possible interferences (FDA, 2001). 3% BSA as matrix blank and a zero sample is part of the calibration of every analyzed batch.

4. Results and Discussion

4.1. Stock preparation

The resulting concentrations of the A-stocks are 97 mM for BHB, 78 mM for $^{13}C_2$-BHB, 6 mM for $^{13}C_4$-BHB, 9 mM for ACAC and 79 mM for AHB.

4.2. Sample Collection

Sodium fluoride/potassium oxalate plasma is used. These additives are mainly used for glucose quantifications. Sodium fluoride limits the ex vivo consumption of glucose in blood specimen. Since sodium fluoride has a poor anticoagulation effect it is combined with potassium oxalate, a calcium-chelating anticoagulant. Oxalate also inhibits several other enzymes (Bowen and Remaley, 2014). Therefore, these additives were chosen to limit the ex vivo conversion of BHB and ACAC by enzyme inhibition.

4.3. Sample preparation

For sample preparation protein precipitation and additional SPE were tested. According to Dahl et al. the analytes do not show sufficient retention on reversed phase SPE material to follow a standard protocol (Dahl, Olsen, & Strand, 2012). Additionally, there was no significant signal after an SPE even with spiked samples. Hence the elution of the analytes during the sample loading is also tested. The elute is collected in a 96-wellplate and then evaporated with nitrogen to a volume of about 100 µl. Full drying within a reasonable time was not possible due to the large sample volume of 750 µl. The samples were measured with LC/MS. Although a significant signal was found in this elutes, SPE was not performed in further experiments. This was due to reasons of efficiency. As mentioned above, drying was time intensive and a lot of nitrogen was needed in the process. Furthermore, protein precipitation showed satisfying accuracy, was not as time consuming as SPE and was cheaper. Therefore, for reasons of practicality protein precipitation was the method of choice for the sample preparation.

4.4. Chromatographic separation and detection by MS

The transitions of the analytes were chosen based on the abundance determined by the optimizer tool and are shown in table 2. Thus, the identity of each compound was determined by retention time and the MS/MS transition.

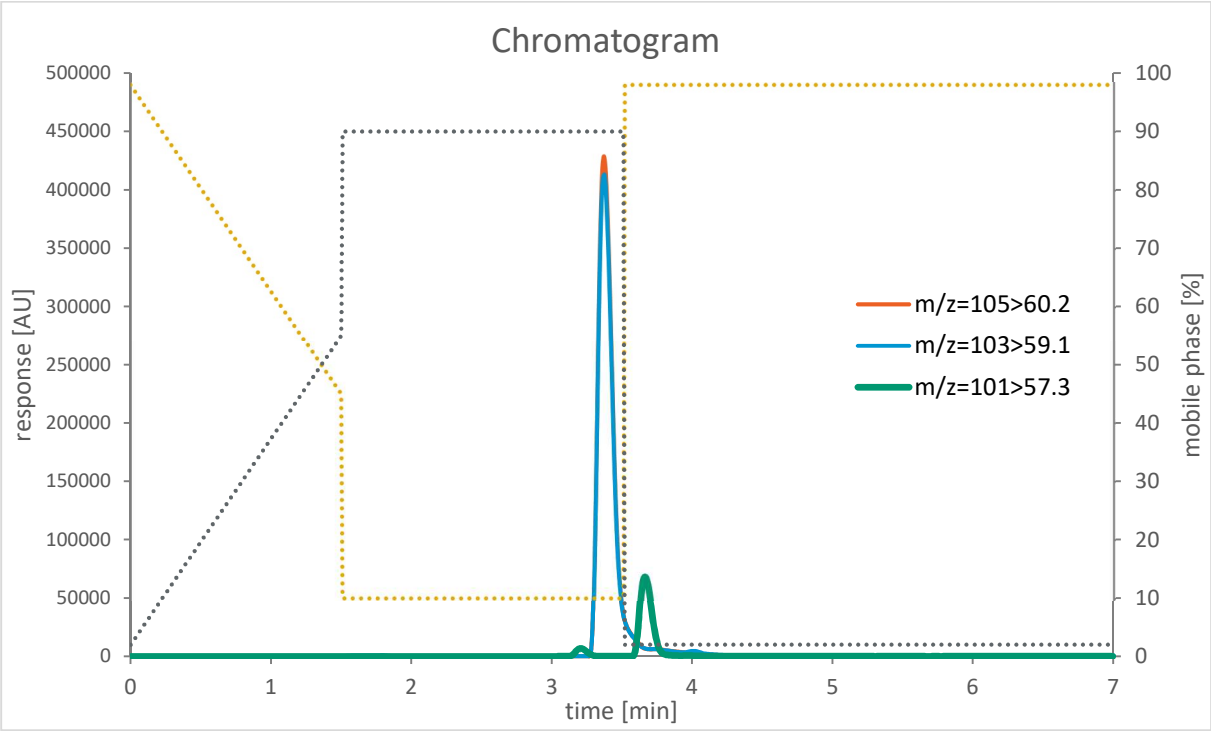


Figure 7: Chromatographic seperation of ketone bodies. BHB (blue), ACAC (green), ISTD (orange); The gradient flow consistent of mobile phase A (yellow) and mobile phase B (grey) is shown as dotted line.

ACAC showed two peaks in the chromatogram, which reproducibly occurred. The first assumption was an instability of the standard and so three different standard solutions were analyzed to check the stability. All three solutions showed two separable peaks with the same retention time but different ratio of the peak intensities, shown in figure 8.

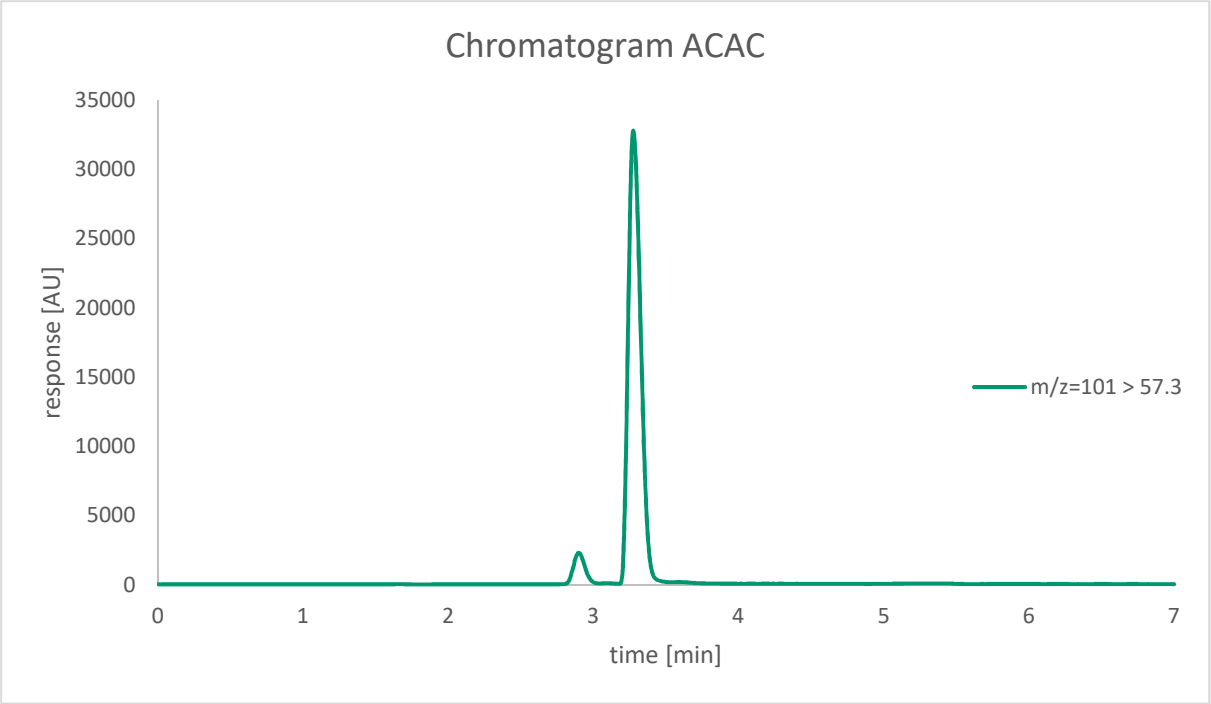


Figure 8: Chromatogram of ACAC. The peaks appear at 2.9 and 3.2 minutes and show the keto-enol-tautomerism of the analyte.

The two separable peaks which can be seen in the chromatogram of ACAC indicate a tautomerism of ACAC. Keto-enol tautomerization occurs by proton transfer of the solvent water. According to Chiang et al ACAC is a stable tautomer in an aqueous solution with 25°C and the enol tautomer is unstable (Chiang et al., 1999). ^{1}H NMR spectra showed that ACAC favors the keto form in the solid state when crystallized from carbon tetrachloride and that the keto form is the more polar tautomer. In general, the enol concentration is decreasing with increasing of the solvent (Grande and Rosenfeld, 1980). Belova et al expect a mixture of both tautomeres with the preference of the enol form for non-symmetrically substituted β-diketones (Belova et al., 2010).

Further experiments are needed to define the tautomerism more specifically. At this point it is not defined which peak defines which tautomer. Another column may not be able to separate the ketone from the enol form. It is also unclear, which form shows a longer retention time and which one is mainly found in human plasma. Sample preparation and storage hold a high potential for the influencing factors of the equilibrium of the tautomerism, like solvent, temperature, or the presence of other hydrogen bonding species (Belova et al., 2010). The

main object of the method was the quantification of BHB and therefore not enough time was devoted to ACAC. Hence, circumstances regarding the keto-enol-tautomerism have still to be clarified.

AHB is isobaric to BHB and therefore a priori not securely distinguishable by MS. A preceding chromatographic separation is needed. In contrast to these expectations, preliminary tests showed not only chromatographic separation but also different product ions of BHB and AHB (figure 9). While BHB shows a transition from 103 to 59.1, AHB shows the transition from 103 to 57.3. Therefore, AHB is clearly separable from BHB and no interference in the quantification of BHB.

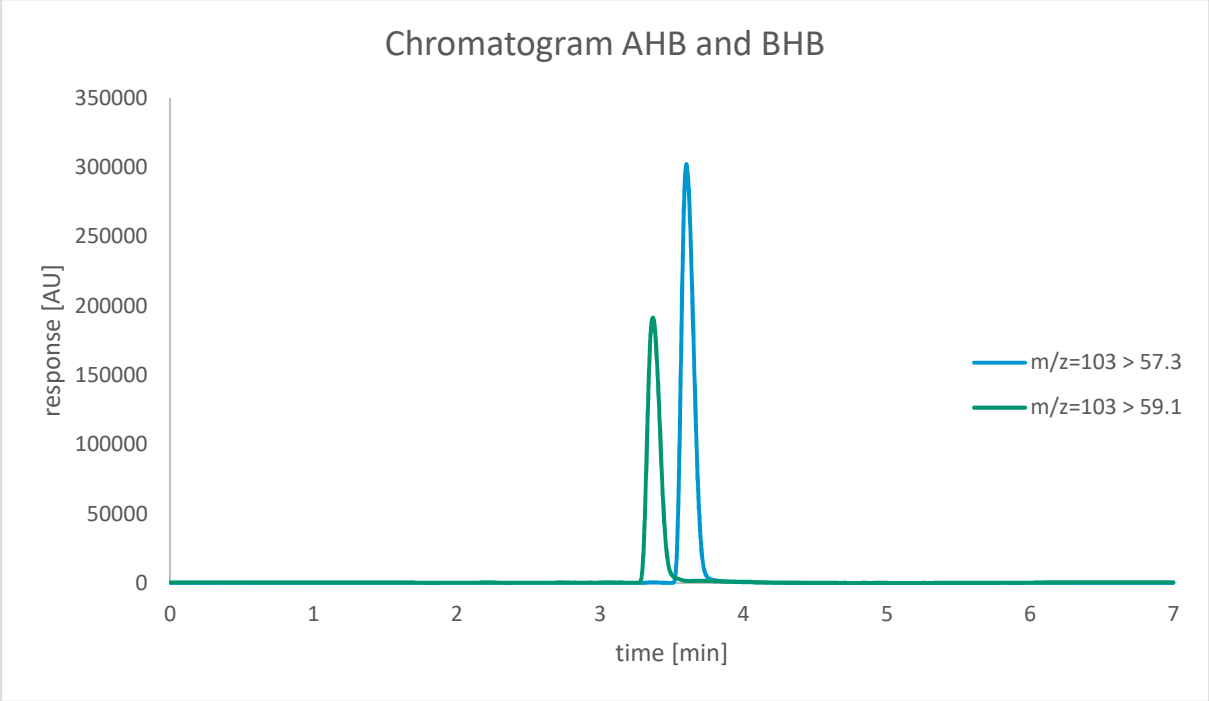


Figure 9: Chromatogram of BHB (green) and AHB (blue). The two analytes are separated by retention time and transitions in SRM-Mode.

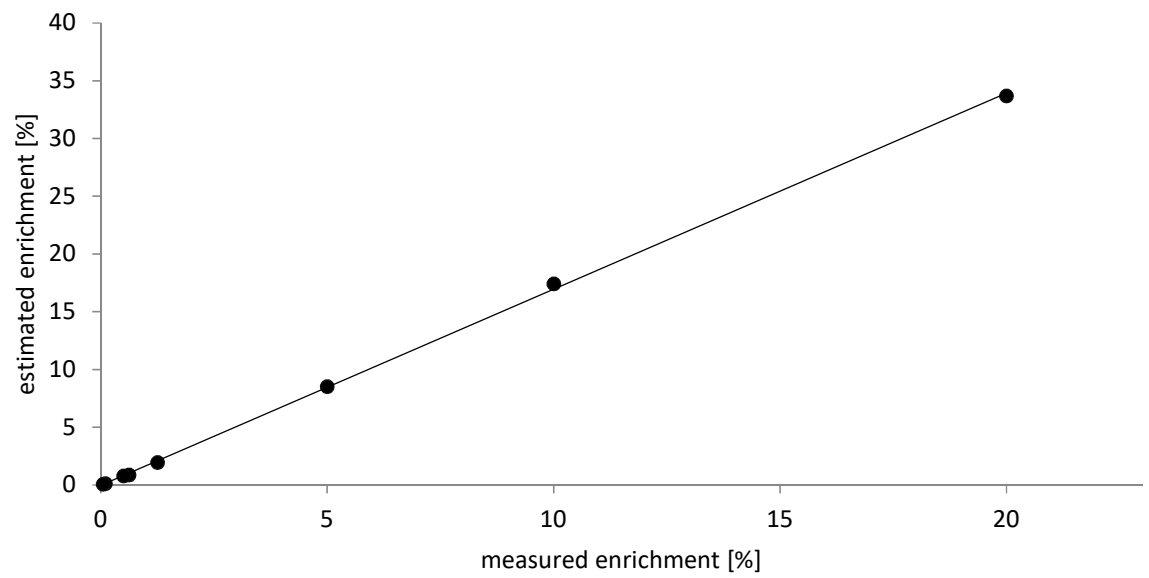
4.5. Tracer enrichment

The concentration range of BHB starts with 25µM and ends with 10 mM, lower concentration levels are disregarded. This is due to the future clinical studies. Ketone bodies will be tested at fasting conditions. It is known that serum concentration increases to 400µM after an

overnight fast (Laeger et al., 2010) and therefore, concentration under 25µm are not expected.

The results of the analysis are shown in table 4. While the enrichment curve and the calculated concentrations show a good correlation shown in figure 10, comparison of the areas over increasing BHB concentrations showed a non-constant area of the ISTD and $^{13}C_4$-BHB. This can have several reasons, concluding impure samples due to the protein precipitation, saturation or binding of the analyte to proteins or the tube surface. Another plausible reason may be ion suppression.

A



B

$$y = 1.698x - 0.0319$$

$$R^2 = 0.9997$$

Figure 10: Tracer enrichment. A shows the graph of the enrichment of the tracer with different BHB concentrations. In **B** the linear equation and the coefficient of determination of the enrichment curve are shown.

Ion suppression is a matrix effect, which influences the ionization of an analyte and is often observed as a loss in response. ESI often loses the linearity of response at high concentrations (>10^{-5} M). With ESI, the characteristics as well as the concentration of an

analyte determines the efficiency of its ionization. Ion suppression can lead to false negative results, or even to false positive if the ISTD is suppressed. Ion suppression is highly affected by sample preparation and chromatographic selectivity. Protein precipitation is known to cause a loss of analyte and a lower sensitivity resulting from dilution. (Volmer and Jessome, 2006). To reduce this matrix effect, different approaches have to be tested. It could help to reduce the volume of sample injection or to dilute the sample further. Reducing the ESI flow rate to the nanoliter-per-minute range, so that smaller and more highly charged droplets are generated, could also reduce the ion suppression. Compared to the highly concentrated BHB, the low concentration of the tracer might not produce a clear peak and may result in problems with the detection.

Table 4: Calibration curve of the tracer enrichment

	ISTD	Concentration BHB	Tracer	Enrichment	
	40 µM	[µM]	5 µM	Nominal [%]	Measured [%]
C1	✓	800	✓	20	32.90
C2	✓	400	✓	10	17.26
C3	✓	100	✓	5	8.36
C4	✓	50	✓	1.25	1.89
C5	✓	25	✓	0.625	0.89
C9	✓	1 000	✓	0.5	0.75
C10	✓	5 000	✓	0.1	0.13
C11	✓	10 000	✓	0.05	0.07
C0	✓	✗	✗		
C00	✗	✗	✓		
C000	✓	✗	✓		

The slope coefficient for estimated and measured enrichment with 1.67 deviates from unity, which is most probably due to ion suppression. The reproducibility of the slope coefficient was not tested. A separate calibration curve for the enrichment is needed to get verified results for any further enrichment studies.

4.6. Method Validation

Protein precipitation is a frequently used step in sample preparation due to its simplicity. It is also used successfully in this study to eliminate the proteins in the plasma samples. However, the main type of interferences that is removed are proteins, thus, apart from the analytes other components are present in the measured probes. Method validation is performed to ensure a reproducible result. Validation was performed with spiked plasma pool samples, called QC. The spiked samples had a concentration of 13.6 µM for QC-L, 84.13 µM for QC-M and 500.82 µM for QC-H.

4.6.1. Linearity and LLOQ

A calibration curve is processed with every batch and also with each day of method validation. The linearity and reproducibility of the calibration curve was tested by measuring each calibration level for five times. The results concluding the accuracy of the measurement can be found in table 5. The range of variation was between 0% and 8% for the calibration levels and an average of 15% for the LLOQ. The 40 data points from the spiked surrogate matrix combine to the calibration curve, shown in figure 11. The linear equation for BHB can also be found in figure 11 and the coefficient of determination is R^2= 0,9991. All criteria for LLOQ, linearity and the calibration curve according to the FDA guidelines are met.

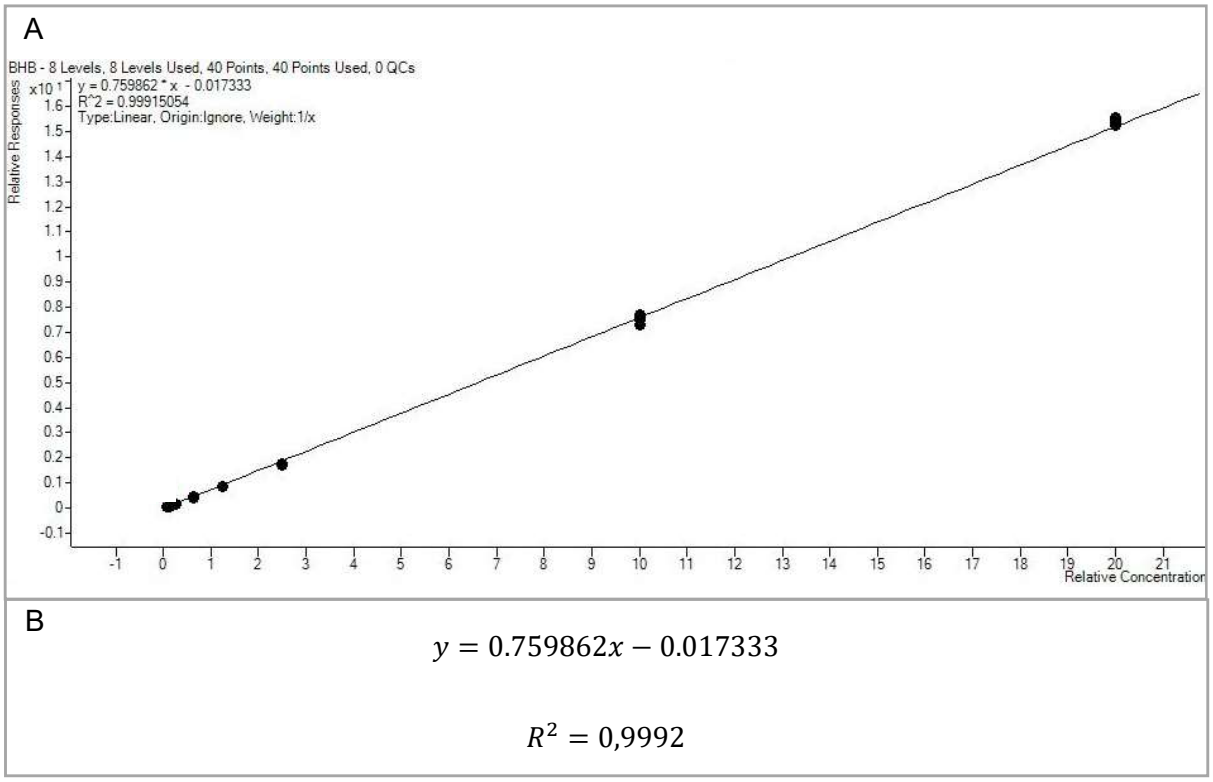


Figure 11: Calibration curve of the validation. The linear curve is shown in **A**, weighting was 1/x. Each concentration level was measured 5 times. **B** shows the equation and coefficient of determination of the calibration curve.

Table 5: Results of the validation of linearity and LLOQ. Calibration consists of 8 concentration levels reaching from 3 µM to 800 µM. Each concentration was measured 5 times. The lowest concentration level represents the LLOQ.

Calibration level	Nominal concentration	Measured concentration	Average concentration	Accuracy
	[µM]	[µM]	[µM]	[%Diff]
C1_1		811.98		1
C1_2		809.75		1
C1_3		807.65		1
C1_4		819.87		2
C1_5	800.00	803.81	810.61	0
C2_1		404.81		1
C2_2		396.59		-1
C2_3		404.50		1
C2_4		386.21		-3
C2_5	400.00	407.03	399.83	2
C3_1		92.30		-8
C3_2		93.32		-7
C3_3	100.00	93.48	93.69	-7

C3_4		95.11		-5
C3_5		94.22		-6
C4_1		47.39		-5
C4_2		46.37		-7
C4_3		47.42		-5
C4_4		46.98		-6
C4_5	50.00	47.00	47.03	-6
C5_1		23.76		-5
C5_2		23.93		-4
C5_3		23.36		-7
C5_4		23.20		-7
C5_5	25.00	23.15	23.48	-7
C6_1		9.75		-3
C6_2		9.62		-4
C6_3		9.84		-2
C6_4		9.54		-5
C6_5	10.00	9.52	9.65	-5
C7_1		5.23		5
C7_2		5.29		6
C7_3		5.19		4
C7_4		5.20		4
C7_5	5.00	5.21	5.22	4
C8_1		3.47		16
C8_2		3.44		15
C8_3		3.54		18
C8_4		3.46		15
C8_5	3.00	3.49	3.48	16

4.6.2. Accuracy and precision

Three concentration levels of BHB were examined within three consecutive days regarding intra-batch and inter-batch precision and accuracy. The results are shown in table 6.

Table 6: Results for the validation of accuracy and precision. The three QC levels were measured on three consecutive days**.** Only the average measured concentration per batch is shown, specific concentrations can be found in the appendix.

Sample ID		Nominal concentration	Average concentration	Intra-batch precision	Inter-batch precision	Accuracy
		[µM]	[µM]	[%]	[%]	[%Diff]
QC-L	Batch 1	13.66	13.60	5.85	7.9	-8.6
	Batch 2		11.76	3.19		
	Batch 3		11.94	1.13		
QC-M	Batch 1	84.13	84.13	2.34	6.2	-7.5
	Batch 2		73.95	1.00		
	Batch 3		75.50	2.41		
QC-H	Batch 1	500.82	500.82	1.47	5.2	-6
	Batch 2		445.79	1.19		
	Batch 3		464.78	2.01		

In one batch each concentration level was measured six times for the calculations. QC-L with a nominal concentration of 13.69 µM showed an accuracy of 9% over all three batches. The intra-batch precision varied from 1% to 6% and the inter-batch precision was 8%.

QC-M had a nominal concentration of 84.13 µM and showed an accuracy below 8%. The intra-batch precision varied between 1% and 3%, inter-batch precision amounts to 6%.

QC-H with the nominal concentration of 500.82 µM had an accuracy of 6%, intra-batch precision was lower than 2% and inter-batch precision was 5%, respectively.

To conclude, accuracy of all three QC levels accounts to less than 9% and the highest value of precision was 8%. Hence, according to the FDA criteria, which require accuracy and precision below 15%, the method is valid for all three concentration levels.

4.6.3. Stability

The stability of an analyte is defined by the difference of measured concentration over different periods of time and is a hint for the quality of the method.

4.6.3.1. Short-term stability

The short-term stability describes the stability of processed samples at 5°C in the autosampler. This should show the stability of the samples over the analysis time. The samples were processed and directly measured. The measurement was repeated after 26 and 69 hours. In the meantime, they were stored in the autosampler at 5°C.

QC-L showed a difference of 8% and 9% over 25 and 69 hours, respectively. QC-M had a difference of 11% after 25 hours and 10% after 69 hours. For QC-H a variance of 9% over 25 and 69 hours was calculated. The results are presented in table 7 in detail.

Table 7: Results of the validation of short term stability. The three QC levels were measured after 0, 25 and 69 hours of storage in the autosampler at 5°C. Only the average measured concentration per batch is shown, specific concentrations can be found in the appendix.

Sample ID	Storage time	Nominal concentration	Average concentration	Intra-batch precision	Stability
	[h]	[µM]	[µM]	[%]	[%Diff]
QC-L	0	13.60	13.60	5.9	0.0
	25		12.35	8.4	-9.2
	69		12.37	7.1	-9.0
QC-M	0	84.13	84.13	2.3	0.0
	25		75.05	1.6	-10.8
	69		75.44	2.2	-10.3
QC-H	0	500.82	500.82	1.5	0.0
	25		453.41	2.1	-9.5
	69		453.87	1.6	-9.4

4.6.3.2. Long-term stability

The stability of the analyte in processed as well as in unprocessed samples is measured after four and seven weeks and compared to the initial measured concentrations. The samples were stored at -80°C.

Unprocessed, the results differed from the initial measurement by 5% after 4 weeks and were identical after 7 weeks of storage for QC-L samples. QC-M showed no difference after

4 weeks and a difference of 1% after 7 weeks. The greatest deviation showed QC-H samples with nearly 1% after 4 weeks but 8% after 7 weeks. The results are shown in table 8.

Table 8: Results of the validation of longterm stability of unprocessed samples. Each QC level was stored unprocessed at -80°C for 4 and 7 weeks. Only the average measured concentration per batch is shown, specific concentrations can be found in the appendix.

Sample ID	Storage time	Nominal concentration	Average concentration	Intra-batch precision	Stability
	[weeks]	[ng/ml]	[ng/ml]	[%]	[%Diff]
QC-L	0	13.60	13.60	5.9	0.0
	4		12.88	2.2	-5.3
	7		13.63	2.6	0.2
QC-M	0	84.13	84.13	2.3	0.0
	4		83.44	1.9	-0.8
	7		85.05	1.4	1.1
QC-H	0	500.82	500.82	1.5	0.0
	4		505.26	1.2	0.9
	7		538.68	1.8	7.6

The processed samples had a deviation of 11% after 4 weeks and 2% after 7 weeks for QC-L. A deviation of 8% after 4 weeks and none after 7 weeks is shown by QC-M and QC-H showed 6% and 3% difference after 4 and 7 weeks, respectively.The results are presented in table 9.

The samples were stable for a time period of at least 7 weeks at -80°C regardless of the manner of storage (processed or unprocessed). Processed samples showed a higher deviation after storage but were still in the tolerance area of 15%.

Table 9: Results of the validation of longterm stability of processed samples. Each QC level was processed and stored at -80°C for 4 and 7 weeks. Only the average measured concentration per batch is shown, specific concentrations can be found in the appendix.

Sample ID	Storage time	Nominal concentration	Average concentration	Intra-batch precision	Accuracy
	[weeks]	[ng/ml]	[ng/ml]	[%]	[%Diff]
QC-L	0	13.60	13.60	5.9	0.0
	4		12.18	2.7	-10.5

	7		13.32	2.1	-2.0
QC-M	0	84.13	84.13	2.3	0.0
	4		77.77	2.8	-7.6
	7		83.79	0.7	-0.4
QC-H	0	500.82	500.82	1.5	0.0
	4		472.63	1.2	-5.6
	7		515.11	1.1	2.9

4.6.3.3. Stock solution stability

The processed A-stocks of the pretests show an accuracy of 13.7% compared to the nominal concentration. Therefore, the FDA criteria for stability are met.

Table 10: Results of the validation of stock stability. A-stocks from the pretest phase were spiked into surrogate matrix and processed to compare them to freshly prepared A-stock regarding the stability. Only the average measured concentration per batch is shown, specific concentrations can be found in the appendix.

Sample ID		Nominal concentration	Measured concentration	Average measured concentration	Intra-batch precision	Accuracy
		[µM]	[µM]	[µM]	[%]	[%]
Pre-test stock	S1-1	100	112.54	113.68	2.7	13.7
	S1-2		110.30			
	S2-1		111.92			
	S2-2		112.36			
	S3-1		117.74			
	S3-2		117.22			

4.6.3.4. Freeze-thaw stability

Freeze-thaw stability was tested for processed and unprocessed samples of the three concentration levels L, M and H.

Unprocessed samples passed three freeze-thaw cycles and were analyzed after each cycle. The measurements were compared to directly measured samples without a freeze-thaw process, results are shown in table 11.

The deviations of QC-L were after all three cycles below 13%.

QC-M showed a decreasing deviation with consecutive freeze-thaw cycles. After the first cycle the deviation was the highest with 12%.

The same decreasing deviation can be found with QC-H, the highest deviation was 11%.

Table 11: Results of freeze-thaw stability of unprocessed samples. Each concentration level passed up to 3 freeze-thaw cycles and compared to directly measured samples. Only the average measured concentration per batch is shown, specific concentrations can be found in the appendix.

Sample ID	Freeze-thaw cycles	Nominal concentration	Average concentration	Intra-batch precision	Stability
		[µM]	[µM]	[%]	[%Diff]
QC-L	0	13.60	13.60	5.9	0.0
	1		11.81	2.7	-13.1
	2		11.88	0.6	-12.6
	3		12.12	1.7	-10.9
QC-M	0	84.13	84.13	2.3	0.0
	1		73.95	1.0	-12.1
	2		75.32	1.3	-10.5
	3		78.31	1.2	-6.9
QC-H	0	500.82	500.82	1.5	0.0
	1		445.79	1.2	-11.0
	2		461.82	1.7	-7.8
	3		470.92	2.6	-6.0

Two cycles of freeze and thaw were performed with processed samples, results are shown in detail in table 12.

All three concentration levels showed a deviation between 2% and 11%.

Table 12: Results of freeze-thaw stability of processed samples. Each concentration level passed one freeze-thaw cycle and compared to directly measured samples. Only the average measured concentration per batch is shown, specific concentrations can be found in the appendix.

Sample ID	Freeze-thaw cycles	Nominal concentration	Average concentration	Intra-batch precision	Accuracy
		[µM]	[µM]	[%]	[%Diff]
QC-L	0	13.60	13.60	5.9	0.0
	1		12.18	2.7	-10.5
	2		12.88	4.8	-5.3

QC-M	0	84.13	84.13	2.3	0.0
	1		77.77	2.8	-7.6
	2		82.81	1.3	-1.6
QC-H	0	500.82	500.82	1.5	0.0
	1		472.63	1.2	-5.6
	2		511.97	1.5	2.2

In conclusion deviation did not exceed 15%. Hence, samples can be frozen and stored for at least 7 weeks without significantly changing the results.

4.6.4. Selectivity

Selectivity is defined by the FDA as the ability to differentiate and quantify the analyte in the presence of other components in the biological matrix (FDA, 2001). In case of endogenous substances, the analyte needs to be removed from the biological matrix. This can be achieved by addition of activated carbon, followed by mixing, centrifugation and filtering. The carbon particles need to be removed completely, otherwise they would react with the spiked analyte too. This method removes more than only the analyte and a different matrix is the result. More recommended is the affinity extraction with antibodies (Hess et al., 2018). Both methods are not practical or sufficient enough for BHB. Therefore, 3% BSA in saline solution is used as a surrogate matrix. A zero sample, which is surrogate matrix with ISTD, was part of each batch to ensure the selectivity of the method. Concentrations were always < 10% of LLOQ, hence the method shows a good selectivity.

5. Applications

In the course of this master thesis the LC/MS method for the quantification of BHB was applied to a clinical trial regarding the effects of diabetic drugs. Another application was the implementation of an enzymatic assay for BDH.

5.1. Clinical trial: effects of diabetic drugs

Different glucose lowering agents are used as add-on therapy for non-insulin dependent type 2 diabetes patients, e.g. as oral agents (SGLT-2 inhibitors or DPP-4 inhibitors) or injectable ones (insulin or GLP-1 receptor agonists).

Recent studies indicate a risk of DKA for SGLT-2 inhibitor treatment. The underlying causes are not fully understood but may be due to an interplay of glucagon levels, endogenous glucose production and lipolysis (Peters et al. 2015). A study with a clamp setting after seven day treatment with Dapagliflozin, a SGLT-2 inhibitor, Saxagliptin, a DPP-4 inhibitor, or a combined treatment of both as add-on therapy to metformin was designed to get further insights into this indication (Sach-Friedl et al.).

The primary objective of this trial was to investigate the response of glucagon, endogenous glucose production and glycerol rate of appearance to treatment with these agents, which will will not be considered in this thesis. Additionally, BHB concentrations were measured with the implemented LC/MS method to investigate lipolysis in subjects with T2DM (Sach-Friedl et al.).

5.1.1. Materials and methods

The study was a randomized, double-blind, three arm, three treatment period, cross-over trial.

Plasma glucose (PG) level is regulated by a variable intra venous infusion of glucose or human soluble insulin. The subjects undergo a time course with different PG levels which are shown in figure 12. Blood samples are assessed chronologically at plateau concentrations of 5.5 mmol/l with low insulin, 11.1 mmol/l, 5.5 mmol/ with high insulin levels, 3.5 mmol/l and 2.5 mmol/l (Sach-Friedl et al.).

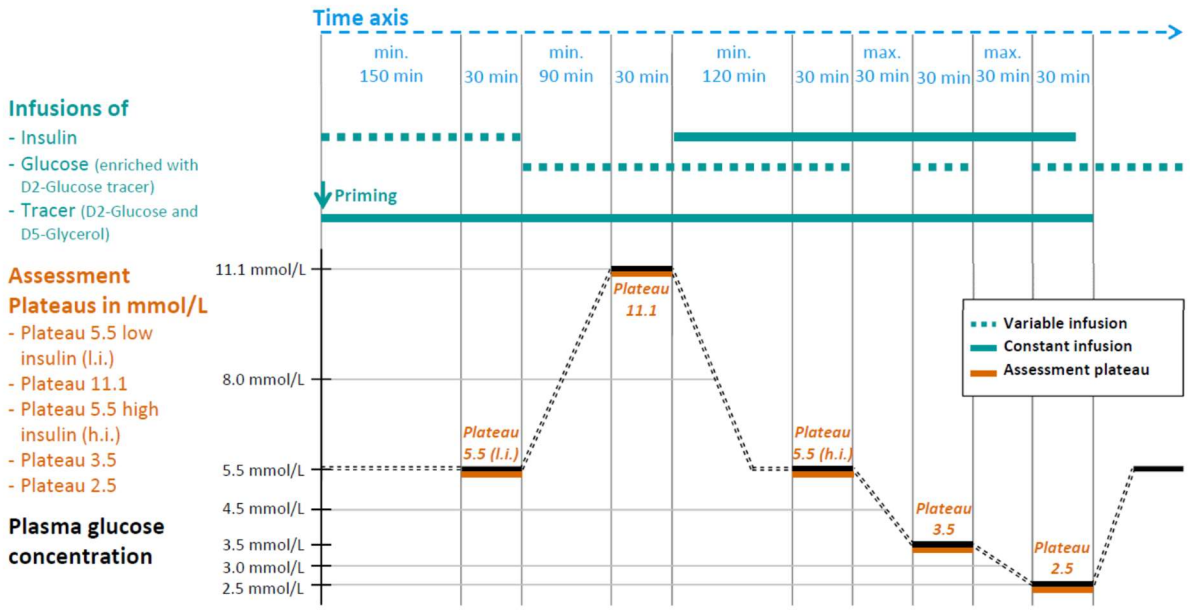


Figure 12: Clamp design of the clinical trial. (Sach-Friedl et al.)

Samples from 17 subjects were measured. Each subject passed four clamp visits with five samples each. Visit 2 is defined as the baseline, subjects continued their normal metformin therapy. Subjects were randomly assigned to one of six treatment sequences as shown in table 13 (Sach-Friedl et al.).

Samples were processed and measured as already described before in chapter 3.11 and 3.12.

Table 13: Treatment sequences for the clinical trial with allocation of the subjects (Sach-Friedl et al.).

	Visit 4	Visit 6	Visit 8	Allocations
1.	SGLT-2	DPP-4	SGLT-2 + DPP4	R102 R111 R115 R119
2.	SGLT-2	SGLT-2 + DPP4	DPP-4	R104 R118
3.	DPP-4	SGLT-2	SGLT-2 + DPP4	R105 R107 R117
4.	DPP-4	SGLT-2 + DPP4	SGLT-2	R103 R108

				R114
5.	SGLT-2 + DPP4	SGLT-2	DPP-4	R110 R113
6.	SGLT-2 + DPP4	DPP-4	SGLT-2	R101 R109 R116

5.1.2. Results and Discussion

At first, BHB was quantified with a routine assay with a LLOQ of 100 µM. A statement based on the concentration of BHB was only possible for the first and second assessment plateau, the others had concentrations below 100 µM (Sach-Friedl et al.). Therefore, the more accurate implemented LC/MS method with an LLOQ of 3 µM is needed.

Ketone bodies show a significant decrease with saxagliptin treatment (DPP-4 inhibitor) compared to no treatment during hyperglycaemia and to dapagliflozin (SGLT-2 inhibitor) and combined treatment during eu- and hypoglycaemia at high insulin levels. Dapagliflozin did significantly increase ketone body concentrations during induction of hypoglycaemia compared to no treatment or combined treatment (shown in figure 13) (Sach-Friedl et al.). Concentrations of each subject are shown in the appendix in table 24.

The study concludes that the risk of DKA during treatment with SGLT-2 inhibitor dapagliflozin is not mediated via glucagon associated fuel shift. A general shift towards ketone production during treatment with dapagliflozin over a wide glycemic range is not confirmed (Sach-Friedl et al.).

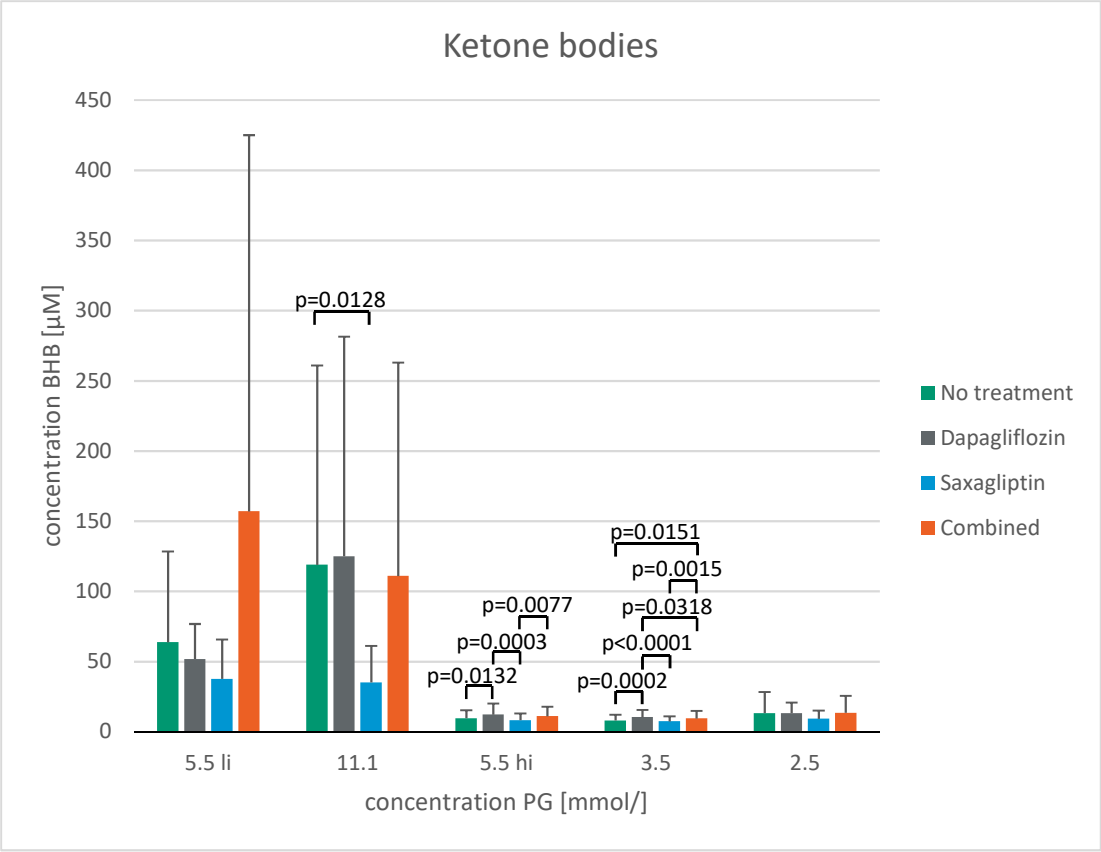


Figure 13: BHB concentration at different assessment plateaus. Concentrations of each subject can be found in the appendix (Sach-Friedl et al.).

5.2. Enzyme Assay

Enzymes are proteins with catalytic capacity and each one is unique for a given reaction (Gomes and Rocha-Santos, 2018). A key enzyme in the ketone body pathway is BDH. It catalyzes the reversible oxidation of BHB to ACAC. Aim of this experiment was to implement an enzyme activity assay based on LC/MS to directly measure the product of BHB. In this approach, the analysis of the activity of BDH is used to determine the turnover of BHB to ACAC in rat tissue.

The activity of an enzyme can be determined by the product formed or the substrate used up over a given time. For the measurement, there is the possibility of the stopped assay, which will be used for the LC/MS-based assay. A stopped assay terminates the reaction after a specific time to measure the formed product. The reaction is stopped by inhibitors that are irreversibly bound to the enzyme or by denaturing the enzyme. The LC/MS based assay is

compared to a commonly used photometric assay, which will be measured continuously. A continuous assay follows the progress of the reaction as it occurs and shows the result immediately (Scopes, 2002).

5.2.1. Methods

The assay conditions were adapted from Lehninger et al. (Lehninger et al., 1960) to be appropriate for photometric determination as well as for LC/MS-based measurements.

5.2.1.1. Mitochondrial Extraction

Whole cell homogenates would only have a low concentration of BDH, most likely lower than the detection limit for the photometric assay. Therefore, concentrating the BDH is necessary. This was carried out by extraction of the mitochondria, where the enzyme is located.

Mitochondria are isolated out of rat liver and heart. For homogenization the tissue is plunged in a Dounce homogenizer 10 or 15 times for the liver or heart, respectively. The used buffer contains sucrose (250 mM) and ammonium formate (5 mM) with a pH of 7.4 at 4°C. After plunging the tissue, it is washed by centrifugation and resuspension with buffer for four times. The washing protocol can be found in table 14. The washing steps are followed by a bicinchoninic acid assay to determine the protein concentration.

Table 14: Washing protocol for mitochondrial extraction.

	[g]	[min]
1	1 047	3
go on with the supernatant		
2	11 630	10
pour of the supernatant; resuspend pellet with buffer		
3	11 630	10
pour of the supernatant; resuspend pellet with buffer		
4	11 630	10
pour of the supernatant; resuspend pellet with small volume of buffer		

5.2.1.2. Assay

The compounds of the assay buffer are shown in table 15. The basic components of the assay were assay buffer and isolated mitochondria with a protein amount of 50 µg and lecithin as an additional activating compound. A baseline was recorded for 15 minutes. The assay was started by adding BHB as the substrate, with a final concentration of 20 mM. Possible increase of NADH can be measured at a wavelength of 340 nm. The photometric assay shows the continuous increase of NADH at 340 nm.

Table 15 Assay buffer: contained substances and their concentrations.

concentration [mM]	compound
50	Ammonium formate (pH 8.2)
12.5	$MgCl_2$
15	KCl
50	Nicotinamide
10	L-cysteine
2	NAD

For the LC/MS based enzyme assay aliquots of the reaction mixture were taken and terminated at different timepoints by protein precipitation. After the precipitation, the reaction mixtures are centrifuged at 3000g for 10 minutes and 200 µl of supernatant are evaporated with nitrogen. The samples and calibrations are then resuspended in 100 µl of 20% MeOH and analyzed with the LC/MS system. For the enzyme assay five levels of calibration standards were used.

5.2.2. Results

Experiments with whole cell homogenate showed no activity of BDH. The reason may be the relative low concentration of BDH in the cells and interferences of other proteins and enzymes in the homogenate. Concentrating BDH resolved this problem. BDH is located in the mitochondria, hence a mitochondrial extraction was the method of choice.

Lehninger et al used a buffer with antimycin A and potassium cyanide presumable for the preservation of the buffer. Using the buffer without these substances showed no

considerable changes. Since these substances are known to be toxic, further experiments were performed without using them.

The assay was performed with a great excess of all components, as described by Lehninger et al., which was common in the initial enzyme experiments. It would be possible to perform the assay with lower concentrations. Due to lack of time it was not possible to establish experiments with lower concentrations, therefore the concentrations found in the literature were used.

A baseline is needed to reduce the unspecific rise of NADH in the photometric assay due to other enzymes in the homogenate. After 15 minutes the emission was constant and the substrate was added to the reaction mix.

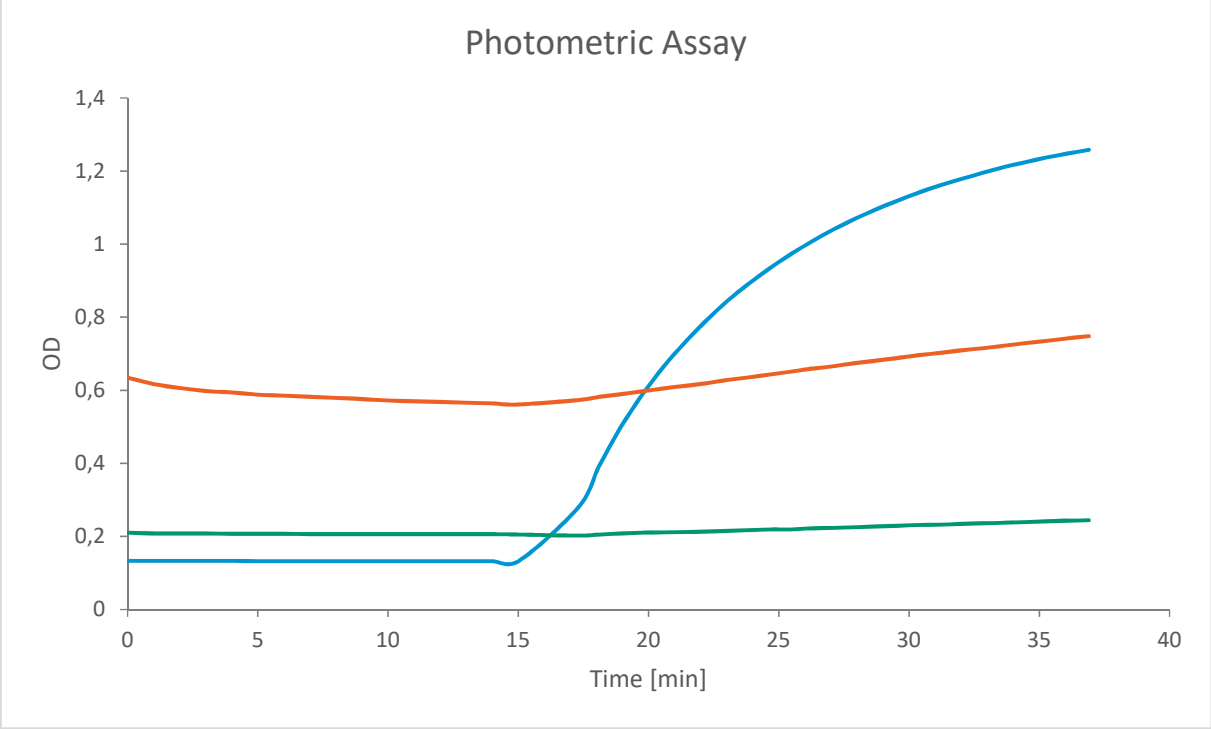


Figure 14: Photometric assay of pure BDH (blue) and of mitochondrial isolations of rat heart (green) and rat liver (orange).

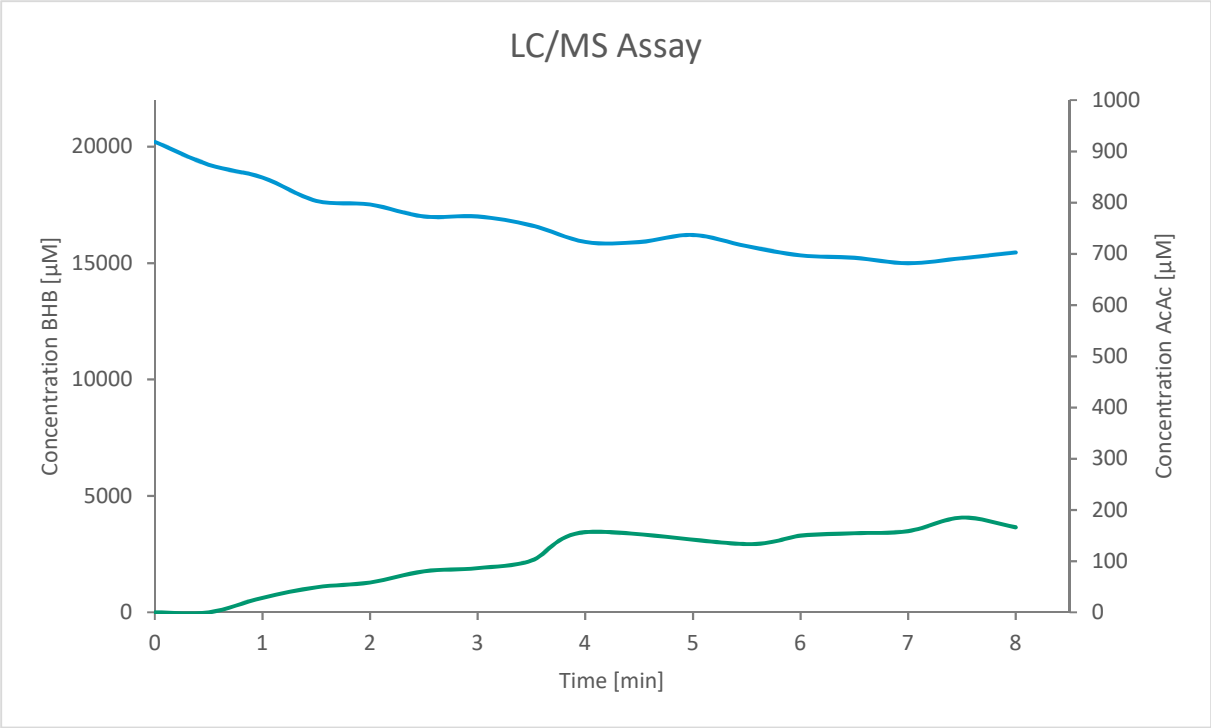


Figure 15: LC/MS based assay of pure BDH with decrease of BHB (blue) and simultaneous increase of ACAC (green).

The photometric assay shows the increase of NADH and is therefore a measure for the indirect activity of BDH (figure 14). Cell debris and other mitochondrial proteins cause turbidity of the homogenates. Therefore, higher OD of heart and liver homogenates compared to pure BDH can be observed.

In contrast to the photometric assay the LC/MS based assay determines specific substrate and product of BDH. The LC/MS method for the quantification of BHB and ACAC is described in chapters 3.11 and 3.12 in detail. ACAC shows two peaks in the chromatogram due to its keto-enol-tautomerism. The peak with a retention time of 3.2 minutes was used to quantify the increase of ACAC for the enzyme assay. An increase of the second peak was not noticeable. Decrease of BHB and increase of ACAC can be observed simultaneously with the LC/MS based assay (figure 15). Thus, it has a better selectivity and accuracy of the enzyme kinetic of BDH. A disadvantage is the limitation of the time points due to an elaborate sample preparation.

6. Conclusion and Outlook

Most existing analytical methods for the determination of ketone bodies are established to diagnose ketoacidosis and are therefore designed for much higher concentration levels than the physiological range, whereas the above described method facilitates a direct and accurate quantification of BHB with a LLOQ of 3 µM.

Protein precipitation as sample preparation is a fast and easy to handle method and the disadvantages are insignificant for this LC/MS method. BHB and its ISTD ($^{13}C_2$-BHB) are defined by their retention times and transitions, isomers like AHB can clearly be separated.

ACAC shows its keto-enol tautomerism in the chromatogram and cannot be accurately quantified by now. Further experiments are needed to clarify the tautomer positions and their influencing factors.

The determination of an isotopically labeled equivalent of BHB ($^{13}C_4$-BHB) is established as well and enables prospective tracer studies with the corresponding determination of the enrichment of the tracer $^{13}C_4$-BHB. Unfortunately, a clinical trial itself is not part of this thesis due to a time delay of the clinical trial development. The planed trial is still outstanding at this time point.

Validation of BHB quantification is a stable method with accurate and precise measurement of the analyte in a concentration range from 3 µM to 800 µM. Furthermore, stability of BHB is proved for storage for at least 69 hours at 5°C and for at least up to seven weeks at -80°C.

During this master thesis the implemented LC/MS method was used for two applications. First it was used for the quantification of BHB in a clinical trial regarding the effect of diabetic drugs. The LC/MS method had a lower LOQ than the assay used before and enabled a detailed data on metabolic shifts during treatment with dapagliflozin in metformin-treated patients with T2DM. The second application was the implementation of a LC/MS based enzymatic assay to examine the turnover of BHB and ACAC in rat tissue, which offers a better selectivity, sensitivity and accuracy of the enzyme kinetic of BDH in comparison to the

conventional photometric assay. Furthermore, it can be used for studies of drug effects on BDH.

Overall, the implemented LC/MS method offers a reliable and accurate quantification of BHB with a fast sample preparation and many future applications including in vivo tracer analytics.

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II. Abbreviations

AHB *α-hydroxybutyrate*

BDH *β-hydroxybutyrate dehydrogenase*

BSA *Bovine serum albumin*

DKA *Diabetic ketoacidosis*

DM *Diabetes Mellitus*, *Diabetes mellitus*

DPP-4 *Dipeptidyl peptidase-4*

FA *Formic acid*

FDA *US Food and Drug Association*

GDM *Gestational diabets mellitus*

GLP-1 *Glucagon-like peptide 1*

GPR *G-protein-couples receptor*

HLB *Hydrophilic-lipophilic balance*

HMG-CoA *Hydroxymethylgutaryl-CoA*

HMGCS2 *3-hydroxymethylgutaryl-Coa synthase*

ISTD *Internal standard*

LC *Liquid chromatography*

LC/MS *Liquid chromatography coupled to mass spectrometry*

LLOQ *Lower limit of quantification*

MRM *Multiple reaction mode*

MS *Mass spectrometer*

NAD^+ *Nicotinamide adenine dinucleotide*

PG *Plasma glucose*

SGLT-2 *Sodium-glucos cotransporter-2*

SPE *Solid phase extraction*

T1DM *Type 1 diabetes mellitus*

T2DM *Type 2 Diabetes Mellitus*

UHPLC *Ultra high performancce liquid chromatography*

III. Table of figures

III.I Figures of the Appendix

IV. Table of equations

V. List of tables

V.I. Tables of the Appendix

VI. Appendix

Table 16: Calibration levels and their concentration

Sample ID	Concentration
	[µM]
C1	800
C2	400
C3	100
C4	50
C5	25
C6	10
C7	5
C8	3
C0	Zero sample, processed with ISTD
MB	Matrix blank processed without analyte or ISTD

Table 17: Results for the validation of accuracy and precision. The QC levels were processed and measured on three consecutive days. Each QC level had three aliquots per batch and each aliquot was measured two times.

Sample ID			Nominal concentration	Measured concentration	Average concentration	Intra-run precision	Inter-batch precision	Accuracy
			[µg/ml]	[µg/ml]	[µg/ml]	[%]	[%]	[%Diff]
QC-L	Batch 1	11-1		14.68				
		11-2		14.57				
		12-1		13.11				
		12-2		12.97				
		13-1		13.12				
		13-2	13.60	13.16	13.60	5.9		
	Batch 2	21-1		11.28				
		21-2		11.91				
		22-1		11.89				
		22-2		12.36				
		23-1		11.56				
		23-2	13.60	11.57	11.76	3.2		
	Batch 3	31-1		11.76				
		31-2		11.86				
		32-1		12.00				
		32-2		11.91				
		33-1		11.95				
		33-2	13.60	12.16	11.94	1.1	7.9	-8.6
QC-M	Batch 1	11-1		85.22				
		11-2		85.56				
		12-1		86.16				
		12-2		84.03				
		13-1		82.89				
		13-2	84.13	80.90	84.13	2.3		
	Batch 2	21-1		74.07				
		21-2		74.29				
		22-1		74.14				
		22-2		75.00				
		23-1		73.10				
		23-2	84.13	73.10	73.95	1.0		
	Batch 3	31-1		74.85				
		31-2		76.90				
		32-1		76.55				
		32-2		77.28				
		33-1		72.39				
		33-2	84.13	75.05	75.50	2.4	6.2	-7.5
QC-H	Batch 1	11-1		501.36				
		11-2		495.44				
		12-1	500.82	513.06	500.82	1.5	5.2	-6.1

		12-2		504.19				
		13-1		492.06				
		13-2		498.82				
	Batch 2	21-1		442.66				
		21-2		449.04				
		22-1		449.89				
		22-2		449.39				
		23-1		436.36				
		23-2	500.82	447.39	445.79	1.2		
	Batch 3	31-1		458.98				
		31-2		460.75				
		32-1		458.65				
		32-2		457.47				
		33-1		472.91				
		33-2	500.82	479.92	464.78	2.0		

Table 18: Results of the validation of short term stability. The three QC levels were measured after 0, 25 and 69 hours of storage in the autosampler at 5°C. Each QC level had three aliquots per batch and each aliquot was measured two times.

Sample ID			Storage time	Nominal concentration	Measured concentration	Average measured concentration	Intra-batch precision	Stability
			[h]	[µM]	[µM]	[µM]	[%]	[%Diff]
QC-L	Batch 1	11-1			14.68			
		11-2			14.57			
		12-1			13.11			
		12-2			12.97			
		13-1			13.12			
		13-2	0	13.60	13.16	13.60	5.9	0.0
	Batch 2	11-3			14.02			
		11-4			13.23			
		12-3			11.87			
		12-4			11.83			
		13-3			11.38			
		13-4	25	13.60	11.78	12.35	8.4	-9.2
	Batch 4	11-5			13.56			
		11-6			13.41			
		12-5			11.92			
		12-6			12.00			
		13-5			11.77			
		13-6	69	13.60	11.57	12.37	7.1	-9.0
QC-M	Batch 1	11-1			85.22			
		11-2			85.56			
		12-1			86.16			
		12-2			84.03			
		13-1			82.88			
		13-2	0	84.13	80.90	84.13	2.3	0.0
	Batch 2	11-3			75.67			
		11-4			76.04			
		12-3			76.11			
		12-4			75.43			
		13-3			73.65			
		13-4	25	84.13	73.37	75.05	1.6	-10.8
	Batch 4	11-5			76.60			
		11-6			76.62			
		12-5			76.48			
		12-6			76.31			
		13-5			73.54			
		13-6	69	84.13	73.08	75.44	2.2	-10.3
QC-H	Batch 1	11-1			501.36			
		11-2			495.44			
		12-1			513.06			
		12-2	0	500.82	504.19	500.82	1.5	0.0

		13-1			492.06			
		13-2			498.82			
	Batch 2	11-3			460.39			
		11-4			454.99			
		12-3			458.33			
		12-4			463.64			
		13-3			443.55			
		13-4	25	500.82	439.58	453.41	2.1	-9.5
	Batch 4	11-5			458.68			
		11-6			452.31			
		12-5			463.86			
		12-6			456.73			
		13-5			445.41			
		13-6	69	500.82	446.24	453.87	1.6	-9.4

Table 19: Results of the validation of longterm stability of unprocessed samples. Each QC level had three aliquots per batch and each aliquot was measured two times. The samples were stored unprocessed at -80°C for 4 and 7 weeks.

Sample ID			Storage time	Nominal concentration	Measured concentration	Average measured concentration	Intra-batch precision	Stability
			[h]	[ng/ml]	[ng/ml]	[ng/ml]	[%]	[%Diff]
QC-L	Batch 1	11-1			14.68			
		11-2			14.57			
		12-1			13.11			
		12-2			12.97			
		13-1			13.12			
		13-2	0	13.60	13.16	13.60	5.9	0.0
	Batch 5	51-1			12.88			
		51-2			12.6			
		52-1			12.97			
		52-2			12.73			
		53-1			13.4			
		53-2	646.45	13.60	12.72	12.88	2.2	-5.3
	Batch 6	61-1			13.48			
		61-2			13.52			
		62-1			13.9			
		62-2			14.06			
		63-1			13.05			
		63-2	1178.87	13.60	13.75	13.63	2.6	0.2
QC-M	Batch 1	11-1			85.22			
		11-2			85.56			
		12-1			86.16			
		12-2			84.03			
		13-1			82.88			
		13-2	0	84.13	80.90	84.13	2.3	0.0
	Batch 5	51-1			83.8			
		51-2			85.00			
		52-1			83.87			
		52-2			81.43			
		53-1			84.97			
		53-2	646.45	84.13	81.59	83.44	1.9	-0.8
	Batch 6	61-1			85.82			
		61-2			87.04			
		62-1			83.95			
		62-2			84.71			
		63-1			84.67			
		63-2	1178.87	84.13	84.13	85.05	1.4	1.1
QC-H	Batch 1	11-1			501.36			
		11-2			495.44			
		12-1	0	500.82	513.06	500.82	1.5	0.0

		12-2			504.19			
		13-1			492.06			
		13-2			498.82			
	Batch 5	51-1			509.67			
		51-2			512.31			
		52-1			497.82			
		52-2			505.40			
		53-1			497.97			
		53-2	646.45	500.82	508.4	505.26	1.2	0.9
	Batch 6	61-1			537.07			
		61-2			535.04			
		62-1			548.90			
		62-2			551.63			
		63-1			525.63			
		63-2	1178.87	500.82	533.78	538.68	1.8	7.6

Table 20: Results of the validation of longterm stability of processed samples. Each QC level had three aliquots per batch and each aliquot was measured two times. The samples were processed and stored at -80°C for 4 and 7 weeks.

Sample ID			Storage time	Nominal concentration	Measured concentration	Average measured concentration	Intra-batch precision	Stability
			[h]	[ng/ml]	[ng/ml]	[ng/ml]	[%]	[%Diff]
QC-L	Batch 1	11-1			14.68			
		11-2			14.57			
		12-1			13.11			
		12-2			12.97			
		13-1			13.12			
		13-2	0	13.60	13.16	13.60	5.9	0.0
	Batch 5	151-1			11.91			
		151-2			11.65			
		152-1			12.29			
		152-2			12.29			
		153-1			12.41			
		153-2	646.45	13.60	12.51	12.18	2.7	-10.5
	Batch 6	161-1			13.47			
		161-2			13.42			
		162-1			13.65			
		162-2			13.43			
		163-1			12.93			
		163-2	1178.87	13.60	13.04	13.32	2.1	-2.0
QC-M	Batch 1	11-1			85.22			
		11-2			85.56			
		12-1			86.16			
		12-2			84.03			
		13-1			82.88			
		13-2	0	84.13	80.90	84.13	2.3	0.0
	Batch 5	151-1			77.01			
		151-2			76.06			
		152-1			79.36			
		152-2			80.63			
		153-1			74.75			
		153-2	646.45	84.13	78.81	77.77	2.8	-7.6
	Batch 6	161-1			83.96			
		161-2			84.09			
		162-1			83.63			
		162-2			84.66			
		163-1			83.40			
		163-2	1178.87	84.13	82.97	83.79	0.7	-0.4
QC-H	Batch 1	11-1			501.36			
		11-2			495.44			
		12-1			513.06			
		12-2	0	500.82	504.19	500.82	1.5	0.0

		13-1			492.06			
		13-2			498.82			
	Batch 5	151-1			476.21			
		151-2			466.29			
		152-1			478.00			
		152-2			469.58			
		153-1			467.21			
		153-2	646.45	500.82	478.47	472.63	1.2	-5.6
	Batch 6	161-1			522.58			
		161-2			519.98			
		162-1			512.93			
		162-2			516.72			
		163-1			511.7			
		163-2	1178.87	500.82	506.74	515.11	1.1	2.9

Table 21: Results of the validation of freeze-thaw stability of unprocessed samples. The QC samples passed up to 3 freeze-thaw cycles and were compared to directly measured samples. Each QC level had three aliquots per batch and each aliquot was measured two times. The samples were stored at -80°C.

Sample ID			Freeze-Thaw cycles	Nominal concentration	Measured concentration	Average measured concentration	Intra-batch precision	Stability
				[ng/ml]	[ng/ml]	[ng/ml]	[%]	[%Diff]
QC-L	Batch 1	11-1			14.68			
		11-2			14.57			
		12-1			13.11			
		12-2			12.97			
		13-1			13.12			
		13-2	0	13.60	13.16	13.60	5.9	0.0
	Batch 2	21-1			11.58			
		21-2			11.92			
		22-1			11.89			
		22-2			12.36			
		23-1			11.56			
		23-2	1	13.60	11.57	11.81	2.7	-13.1
	Batch 3	34-1			12.01			
		34-2			11.81			
		35-1			11.88			
		35-2			11.93			
		36-1			11.81			
		36-2	2	13.60	11.86	11.88	0.6	-12.6
	Batch 4	41-1			12.30			
		41-2			12.17			
		42-1			11.76			
		42-2			12.08			
		43-1			12.08			
		43-2	3	13.60	12.30	12.12	1.7	-10.9
QC-M	Batch 1	11-1			85.22			
		11-2			85.56			
		12-1			86.16			
		12-2			84.03			
		13-1			82.88			
		13-2	0	84.13	80.90	84.13	2.3	0.0
	Batch 2	21-1			74.07			
		21-2			74.29			
		22-1			74.14			
		22-2			74.99			
		23-1			73.10			
		23-2	1	84.13	73.10	73.95	1.0	-12.1
	Batch 3	34-1			74.14			
		34-2			74.65			
		35-1	2	84.13	76.21	75.32	1.3	-10.5

		35-2			76.78			
		36-1			74.93			
		36-2			75.18			
	Batch 4	41-1			77.29			
		41-2			77.32			
		42-1			78.03			
		42-2			79.07			
		43-1			78.52			
		43-2	3	84.13	79.62	78.31	1.2	-6.9
QC-H	Batch 1	11-1			501.36			
		11-2			495.44			
		12-1			513.06			
		12-2			504.19			
		13-1			492.06			
		13-2	0	500.82	498.82	500.82	1.5	0.0
	Batch 2	21-1			442.66			
		21-2			449.04			
		22-1			449.89			
		22-2			449.39			
		23-1			436.36			
		23-2	1	500.82	447.39	445.79	1.2	-11.0
	Batch 3	34-1			452.83			
		34-2			472.44			
		35-1			453.31			
		35-2			460.58			
		36-1			466.04			
		36-2	2	500.82	465.72	461.82	1.7	-7.8
	Batch 4	41-1			486.04			
		41-2			483.33			
		42-1			458.61			
		42-2			456.59			
		43-1			472.23			
		43-2	3	500.82	468.69	470.92	2.6	-6.0

Table 22: Results of the validation of freeze-thaw stability of processed samples. The QC samples passed one freeze-thaw cycle and were compared to directly measured samples. Each QC level had three aliquots per batch and each aliquot was measured two times. The samples were processed and stored at -80°C.

Sample ID			Freeze-thaw cycles	Nominal concentration	Measured concentration	Average measured concentration	Intra-batch precision	Stability
				[ng/ml]	[ng/ml]	[ng/ml]	[%]	[%Diff]
QC-L	Batch 1	11-1			14.68			
		11-2			14.57			
		12-1			13.11			
		12-2			12.97			
		13-1			13.12			
		13-2	0	13.60	13.16	13.60	5.9	0.0
	Batch 5	151-1			11.91			
		151-2			11.65			
		152-1			12.29			
		152-2			12.29			
		153-1			12.41			
		153-2	1	13.60	12.51	12.18	2.7	-10.5
	Batch 6	151-3			12.20			
		151-4			12.02			
		152-3			12.98			
		152-4			13.36			
		153-3			13.32			
		153-4	2	13.60	13.41	12.88	4.8	-5.3
QC-M	Batch 1	11-1			85.22			
		11-2			85.56			
		12-1			86.16			
		12-2			84.03			
		13-1			82.88			
		13-2	0	84.13	80.90	84.13	2.3	0.0
	Batch 5	151-1			77.01			
		151-2			76.06			
		152-1			79.36			
		152-2			80.63			
		153-1			74.75			
		153-2	1	84.13	78.81	77.77	2.8	-7.6
	Batch 6	151-3			82.35			
		151-4			83.84			
		152-3			81.32			
		152-4			84.25			
		153-3			82.12			
		153-4	2	84.13	82.98	82.81	1.3	-1.6

QC-H	Batch 1	11-1			501.36			
		11-2			495.44			
		12-1			513.06			
		12-2			504.19			
		13-1			492.06			
		13-2	0	500.82	498.82	500.82	1.5	0.0
	Batch 5	151-1			476.21			
		151-2			466.29			
		152-1			478.00			
		152-2			469.58			
		153-1			467.21			
		153-2	1	500.82	478.47	472.63	1.2	-5.6
	Batch 6	151-3			505.50			
		151-4			513.62			
		152-3			511.65			
		152-4			526.64			
		153-3			507.05			
		153-4	2	500.82	507.33	511.97	1.5	2.2

Table 23: Flowchart of the quantification of BHB

Needed:

- 3% BSA in Elomel
- Standard Solutions (1mM)
  - Calibration Standards (Table 16)
- Sodium fluoride/potassium oxalate plasma

Sample preperation

- 100 µl plasma
  + 25 µl $^{13}C_2$-BHB (200 µM)
  + 400 µl methanol (-20°C)
- Mixing
- Incubation (4°C, 10 minutes)
- Centrifugation (3000g, 4°C, 10 minutes)
- 200 µl supernatant is evaporated with nitrogen at ambient temperature
- Redissolve in 100 µl methanol/MilliQ (20/80, v/v)

LC/MS method

Chromatography:

- UHPLC
- Acquity UPLC BEH C_{18} column (1.7 µm, 150 mm x 2.1 mm)
- Gradient flow of 0.2 ml/min at 35°C (timetable see table 1)
  - Solvent A: MilliQ/FA (100/0.02, v/v)
  - Solvent B: absolute methanol

Mass spectrometry:

- Parameters:

Delta EMV (-):	300
Polarity:	negative
Source	
Gas Temperature:	290°C
Gas Flow:	11 l/min
Nebulizer:	20 psi
Sheath Gas Temp:	250 °C
Sheath Gas Flow:	11 l/min
Negative Capillary:	3000 V
Nozzle Voltage:	1500 V
iFunnel Parameters	
High Pressure RF:	90 V
Low Pressure RF:	60 V

- Transitions:

Compound	Precursor Ion > Product Ion	Retention Time [min]
BHB	103 > 59.1	3.3
$^{13}C_2$-BHB	105 > 60.2	3.1
ACAC	101 > 57.3	2.9 and 3.2

Table 24: BHB concentrations of clinical study for diabetic drugs

		Visit 2	Visit 4	Visit 6	Visit 8
Subject	**Plasma glucose [mmol/l]**	**Calc. Conc. [µmol/l]**	**Calc. Conc. [µmol/l]**	**Calc. Conc. [µmol/l]**	**Calc. Conc. [µmol/l]**
R101	5.5 (low insulin)	15.20	23.18	21.46	16.52
	11.1	145.98	84.53	22.28	263.82
	5.5 (high insulin)	7.31	7.24	7.30	12.49
	3.5	5.27	5.78	5.40	8.98
	2.5	7.04	11.72	6.70	19.96
R102	5.5 (low insulin)	21.00	26.74	9.57	35.27
	11.1	292.36	195.54	69.32	182.11
	5.5 (high insulin)	9.98	8.12	3.88	7.33
	3.5	6.87	7.55	3.35	5.39
	2.5	8.33	9.04	3.97	5.92
R103	5.5 (low insulin)	20.08	22.40	884.06	66.20
	11.1	22.57	30.47	430.75	56.80
	5.5 (high insulin)	5.30	6.89	14.50	11.38
	3.5	4.99	6.39	9.88	10.34
	2.5	5.60	8.54	15.90	10.87
R104	5.5 (low insulin)	41.87	82.70	103.83	82.59
	11.1	210.79	469.24	242.57	67.96
	5.5 (high insulin)	19.03	38.52	28.52	22.17
	3.5	17.73	26.57	25.30	17.12
	2.5	17.53	25.92	23.89	17.19
R105	5.5 (low insulin)	61.06	45.24	32.90	33.75
	11.1	38.50	29.05	29.62	31.10
	5.5 (high insulin)	16.83	13.74	11.48	10.22
	3.5	12.85	10.82	10.84	10.29
	2.5	31.38	27.92	15.49	23.46
R107	5.5 (low insulin)	30.31	26.73	25.40	235.22
	11.1	12.78	13.97	17.20	56.70
	5.5 (high insulin)	5.65	6.63	7.76	11.01
	3.5	4.50	6.01	6.72	8.57
	2.5	4.87	7.96	10.37	11.70
R108	5.5 (low insulin)	39.25	29.15	24.20	42.68

	11.1	10.93	13.82	14.87	25.19
	5.5 (high insulin)	4.99	5.70	5.80	8.59
	3.5	4.94	6.41	6.46	7.28
	2.5	3.42	5.92	5.69	6.51
R109	5.5 (low insulin)	153.05	55.08	38.85	38.64
	11.1	31.11	27.79	22.46	22.35
	5.5 (high insulin)	5.85	8.57	4.61	8.14
	3.5	6.22	8.13	5.60	8.89
	2.5	6.03	8.50	5.49	8.85
R110	5.5 (low insulin)	18.73	13.47	23.55	12.98
	11.1	10.64	10.70	18.46	7.54
	5.5 (high insulin)	5.78	5.00	6.01	4.13
	3.5	5.52	4.69	6.13	4.03
	2.5	5.06	5.02	6.16	4.47
R111	5.5 (low insulin)	26.92	71.11	17.70	32.80
	11.1	fehlt	112.12	11.21	44.80
	5.5 (high insulin)	7.51	11.07	7.12	10.14
	3.5	8.92	10.87	7.75	11.48
	2.5	8.11	8.10	8.98	10.44
R113	5.5 (low insulin)	131.61	816.45	81.81	38.31
	11.1	449.86	520.55	520.62	73.03
	5.5 (high insulin)	17.92	23.48	20.94	11.11
	3.5	11.45	13.79	12.72	9.26
	2.5	24.58	54.05	19.45	12.57
R114	5.5 (low insulin)	39.75	12.12	16.01	44.06
	11.1	32.48	11.14	8.49	28.23
	5.5 (high insulin)	6.83	3.07	3.54	8.25
	3.5	7.96	2.63	2.88	7.46
	2.5	63.31	6.55	3.22	33.29
R115	5.5 (low insulin)	113.28	104.87	39.01	169.35
	11.1	384.25	155.81	86.63	58.16
	5.5 (high insulin)	14.65	9.43	9.40	8.72
	3.5	10.13	8.74	9.43	8.16
	2.5	8.09	8.87	9.69	7.63
R116	5.5 (low insulin)	256.88	108.90	86.89	61.09
	11.1	108.07	82.23	54.70	33.66

	5.5 (high insulin)	20.81	17.17	14.26	18.38
	3.5	15.62	16.85	12.83	18.53
	2.5	15.38	15.20	11.63	14.14
R117	5.5 (low insulin)	46.63	105.76	38.19	33.14
	11.1	82.42	50.35	97.10	28.62
	5.5 (high insulin)	4.98	5.12	7.87	7.55
	3.5	3.81	5.22	7.40	6.45
	2.5	5.12	5.99	8.62	7.26
R118	5.5 (low insulin)	43.93	51.54	45.90	21.07
	11.1	54.91	52.62	41.29	21.68
	5.5 (high insulin)	5.55	13.15	13.34	9.06
	3.5	5.14	12.12	12.36	9.49
	2.5	7.99	14.07	13.71	10.48
R119	5.5 (low insulin)	24.40	72.61	29.21	42.54
	11.1	13.01	22.87	11.15	15.20
	5.5 (high insulin)	3.91	6.25	4.67	5.72
	3.5	4.09	6.14	4.76	5.58
	2.5	3.58	5.61	4.51	6.08

JOANNEUM RESEARCH HEALTH

Development of an Enzyme Activity Assay for β-Hydroxybutyrate Dehydrogenase using LC/MS

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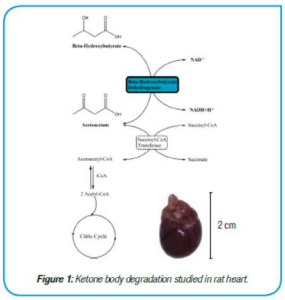

Figure 1: Ketone body degradation studied in rat heart.

Introduction

The ketone body β-hydroxybutyrate (BHB) is reversibly oxidized to acetoacetate (ACAC) by the enzyme *β-hydroxybutyrate dehydrogenase* (BDH) which is a key enzyme in the ketone body pathway. Ketone bodies serve as an energy source for the organism by converting ACAC to acetyl-CoA which then enters the citric cycle.

The aim of this experiment was to implement an enzyme activity assay based on liquid chromatography and mass spectrometry (LC/MS) to directly measure the product of BDH. This approach was used to determine the turnover of BHB to ACAC in rat tissue.

Methods

Assay conditions
were adapted from Lehninger et al.[1]:
- pH 8.2; 25 °C
- Substrate: BHB with a concentration of 20 mM; added after 15 minutes

Liquid chromatography
- Sample preparation by precipitation
- Acquity UPLC BEH C18 column (2.1 x 150 mm, 1.7 µm) (Waters)
- Mobile phase (A) containing 0.2% formic acid and (B) methanol, gradient elution

Massspectrometric detection
- Agilent Triple Quad (6495)
- Negative ionization mode
- Internal standard (ISTD): [1,3-^{13}C2]-BHB
- Multiple reaction monitoring
  - BHB: m/z 103 → m/z 59.1
  - ACAC: m/z 101 → m/z 57.3
  - ISTD: m/z 105 → m/z 60.2

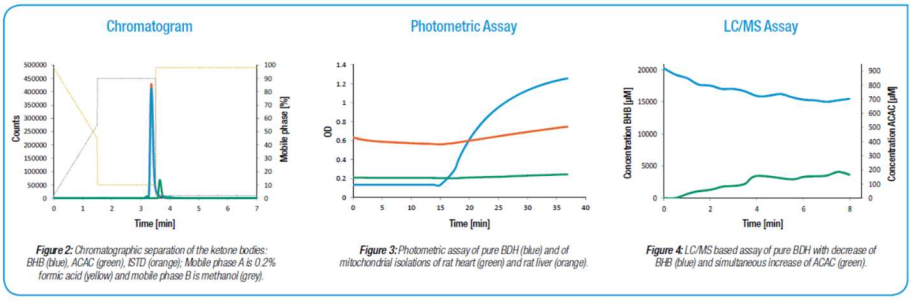

Figure 2: Chromatographic separation of the ketone bodies: BHB (blue), ACAC (green), ISTD (orange); Mobile phase A is 0.2% formic acid (yellow) and mobile phase B is methanol (grey).

Figure 3: Photometric assay of pure BDH (blue) and of mitochondrial isolations of rat heart (green) and rat liver (orange).

Figure 4: LC/MS based assay of pure BDH with decrease of BHB (blue) and simultaneous increase of ACAC (green).

Results

The photometric assay shows an increase of NADH whereas the LC/MS based results indicate the specific substrate and product of BDH. A baseline for the photometric assay is needed to reduce the unspecific rise of NADH. Additionally, activity of BDH is traceable in mitochondrial isolations of rat heart and rat liver.

Conclusion

- **In contrast to the conventional method, our novel approach offers a direct and quantitative measurement of BHB and ACAC and a better selectivity and accuracy of the enzyme kinetic of BDH.**
- **The enzyme assay can also be used to study the effect of drugs such as SGLT2 inhibitors on BDH.**
- **The newly implemented LC/MS method can additionally be used to assess the ketone body metabolism in living animal models and human studies when combined with stable isotope labeling.**

References

[1]
Lehninger, A. L.; et al (1960):
D-β-Hydroxybutyric Dehydrogenase of Mitochondria. The Journal of Biological Chemistry. 235-8: 2450-2455

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Figure 16: Poster for the HPLC 2017