MSc Chemistry
Analytical Sciences

Master Thesis

Ionization efficiency of LC-(ESI)MS/MS
&
LC-MS/MS determination of beta-oxidation acyl-CoA intermediates in cultured fibroblasts

by

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## Abbreviations

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<tbody>
<tr>
<td>Acyl-CoA</td>
<td>Acyl-coenzyme A</td>
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<tr>
<td>AF</td>
<td>Amniotic fluid</td>
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<tr>
<td>APCI</td>
<td>Atmospheric pressure chemical ionization</td>
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<tr>
<td>CACT</td>
<td>Carnitine/acylcarnitine translocase</td>
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<td>CAFSA</td>
<td>CSF free sialic acid</td>
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<tr>
<td>CE</td>
<td>Collision energy</td>
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<tr>
<td>CID</td>
<td>Collision induced dissociation</td>
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<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CPT1</td>
<td>Carnitine palmitoyltransferase 1</td>
</tr>
<tr>
<td>CPT2</td>
<td>Carnitine palmitoyl transferase 2</td>
</tr>
<tr>
<td>CXP</td>
<td>Collision cell exit potential</td>
</tr>
<tr>
<td>DATAN</td>
<td>Diacetyl-L-tartaric anhydride</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagles Medium</td>
</tr>
<tr>
<td>DP</td>
<td>Declustering potential</td>
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<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
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<tr>
<td>FA</td>
<td>Fatty acid</td>
</tr>
<tr>
<td>GABA</td>
<td>(γ-Aminobutyric acid)</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas chromatography combined with mass spectrometry</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks’ balanced salt solution</td>
</tr>
<tr>
<td>3HADs</td>
<td>3-hydroxyacyl-CoA dehydrogenases</td>
</tr>
<tr>
<td>D-2-HG</td>
<td>D-2-Hydroxyglutarate</td>
</tr>
<tr>
<td>D-2-HGA</td>
<td>D-2-hydroxyglutaric aciduria</td>
</tr>
<tr>
<td>D-2-HGDH</td>
<td>D-2-hydroxyglutarate dehydrogenase enzyme</td>
</tr>
<tr>
<td>D,L-2-HGA</td>
<td>Combined D,L-2-hydroxyglutaric aciduria</td>
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<td>L-2-HG</td>
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<tr>
<td>L-2-HGDH</td>
<td>L-2-hydroxyglutarate dehydrogenase enzyme</td>
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</table>
HClO₄  Perchloric acid
IDH2  Isocitrate dehydrogenase 2
IS  Internal standard
ISSD  Infantile free sialic acid storage disease
2-KG  2-Ketoglutarate
LC  Liquid chromatography
LCEH  Long-chain enoyl-CoA hydratases
LCHAD  Long-chain 3-hydroxyacyl-CoA dehydrogenase (C12-C16)
LCKAT  Long-chain 3-ketoacyl-CoA thiolase
LC-MS/MS  Liquid chromatography combined with tandem mass spectrometry
M  Molecule
M/z  Mass over charge ratio
MCA  Multi channel analysis
MCAD  Medium-chain acyl-CoA dehydrogenase
MCADD  Medium-chain acyl-CoA dehydrogenase deficient
MCKAT  Medium-chain 3-ketoacyl-CoA thiolase
MRM  Multiple reaction monitoring
MS/MS  Tandem mass spectrometry
MTHFR  Methylenetetrahydrofolate reductase
MTP  Mitochondrial trifunctional protein
NAD⁺  Nicotinamide adenine dinucleotide
NADH  Reduced nicotinamide adenine dinucleotide
NH₄AC  Ammonium acetate
(-)  Negative
OGS  Oligosaccharides
(+)  Positive
PPCA  Protective protein/cathepsin A
SA  Sialic acid
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>SAE</td>
<td>S-adenosylethionine</td>
</tr>
<tr>
<td>SAH</td>
<td>S-adenosylhomocysteine</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosylmethionine</td>
</tr>
<tr>
<td>SCAD</td>
<td>Short-chain acyl-CoA dehydrogenase</td>
</tr>
<tr>
<td>SCHAD</td>
<td>Short-chain 3-hydroxyacyl-CoA dehydrogenase</td>
</tr>
<tr>
<td>SCEH</td>
<td>Short-chain enoyl-CoA hydratases</td>
</tr>
<tr>
<td>S/N</td>
<td>Signal to noise</td>
</tr>
<tr>
<td>SPE</td>
<td>Solid-phase extraction</td>
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<tr>
<td>SRM</td>
<td>Selected reaction monitoring</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid</td>
</tr>
<tr>
<td>UHPLC</td>
<td>(Ultra)high-performance liquid chromatography</td>
</tr>
<tr>
<td>VUmc</td>
<td>VU University Medical Center</td>
</tr>
<tr>
<td>VLCAD</td>
<td>Very-long-chain acyl-CoA dehydrogenase</td>
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Abstract

In this research project two topics were studied, initiated with the effect of post-column mixed organic solvents on the ionization of several LC-(ESI)MS/MS methods. A well known effect in LC-MS/MS methods is ion suppression, which leads to a decreased sensitivity. This is for instance caused by co-eluting less volatile compounds with the analyte. However, post-column mixed organic solvents prior to the electrospray ionization- (ESI) interface, could possibly increase the ionization efficiency.

In order to examine effects for the positive and negative ionization mode, the VUmc metabolic laboratory LC-MS/MS methods for S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH), D- and L-2-hydroxyglutarate (D- and L-2-HG), sialic acid (SA) and homocysteine - methionine were selected for the post-column experiments. For every method a selection was made from the following organic solvents: 1- and 2-propanol, methanol, acetone, acetonitrile, tetrahydrofurane, ethanol and 2 mM acetyl acetone in 50% methanol. After post-column mixing at different flow rates of these solvents during measurements, peak areas were obtained, and for enhanced signals the signal to noise (S/N) ratios were calculated. Maximal enhancement of the signals was obtained during post-column mixing of 2-propanol for SAH. However, the S/N ratios did not show a similar elevation progress, indicating an increased noise level by post-column mixed 2-propanol. No general effects on ionization were observed for the examined LC-MS/MS methods during post-column mixing organic solvents. Furthermore, no clear differences were obtained between positive and negative ionization.

The second part of this research project focused on the LC-MS/MS measurement of mitochondrial beta-oxidation acyl-coenzyme A (acyl-CoA) intermediates, extracted from cultured fibroblasts. Fatty acids are stepwise degraded into acetyl-coA units by the mitochondrial beta-oxidation, in which process energy is obtained. The mitochondrial beta-oxidation pathway consists of various enzymatic conversions. An impaired enzyme of this pathway causes a beta-oxidation disorder, leading to accumulated intracellular
acyl-CoAs of a certain carbon chain length, and elevated extracellular acylcarnitine analogue levels. Former studies of beta-oxidation in cultured fibroblasts have often determined acylcarnitine levels in the culturing medium. By the measurement of intracellular acyl-CoAs and acylcarnitines in culturing medium of the same fibroblasts, differences could be shown between these methods.

During this project, fibroblasts were cultured in the presence of palmitate or L-palmitoylcarnitine. Furthermore, a LC-MS/MS method was developed which separates short (C2) from long chain (maximum C16) acyl-CoAs. Acyl-CoAs were measured in lysates from harvested cultured fibroblasts. Additionally, acylcarnitines were determined in culturing medium with the LC-MS/MS method of the VUmc metabolic laboratory. In experiments with L-palmitoylcarnitine clear signals originating from beta-oxidation acyl-CoA intermediates were measured. Additionally, a cultured medium-chain acyl-CoA dehydrogenase (MCAD) deficient fibroblast cell line was tested showing a difference in the pattern compared with control samples. In measurements of acyl-CoAs in cell lysates however, not all intermediates were observed and signals were often low. Obtained acyl-CoAs in the comparison of control- and MCAD deficient fibroblasts showed very low signals and no difference between the cell lines. This is possible caused by residual enzyme activity in obtained cell lysates. Therefore, the measurement of acylcarnitines in culturing medium, compared with acyl-CoAs in cultured fibroblast lysates, is at this moment a better method to determine beta-oxidation flux.

This is possible caused by degradation of acyl-CoAs through residual enzyme activity in obtained cell lysates. The measurement of acylcarnitines in culturing medium, compared to acyl-CoAs in cultured fibroblast lysates, is therefore currently a better method to determine beta-oxidation flux.
Introduction

1. Metabolic Laboratory

In the medical field, physicians can consult metabolic laboratories for patients with a clinical suspicion of an inborn-error of metabolism. Metabolic laboratories offer a broad range of different biochemical assays to determine the metabolic composition of body fluids, such as urine, blood, plasma, cerebrospinal fluid (CSF) and amniotic fluid (AF). If one or more metabolic markers show abnormal concentrations in the tested body fluids a metabolic disease might be present. Subsequently, metabolic laboratories can perform further analysis with enzymatic- and DNA tests to clarify the possible metabolic disorder. After the diagnosis of a metabolic disorder, it is sometimes possible to diminish the clinical features of the disorder e.g. by a therapy or diet. Additional periodical follow-ups are required to control the metabolic levels of the treated patients.

Research in metabolic laboratories mainly focuses on the knowledge about various metabolisms and the corresponding disorders.

At the metabolic laboratory of the VU University Medical Centre (VUmc) diagnostics are offered at metabolite, enzyme and DNA level. Furthermore, therapy control analyses are performed in samples of treated patients. Next to the diagnostics and therapy control analyses, translational research is performed on neurometabolic inborn errors and the metabolic causes of inborn or acquired heart and vascular diseases. The metabolic laboratory of the VUmc is specialised in the following metabolic sectors:

- GABA metabolism (γ-Aminobutyric acid)
- Pentose phosphate pathway
- Creatine metabolism and transport
- Homocysteine metabolism
- Pyridoxine-dependent epilepsy
Various analytical methods of a metabolic laboratory require highly purified biochemical agents, as for example (internal-)standards or substrates in enzyme reactions. Since many of these agents are not commercially available, one sub-division of the VUmc metabolic laboratory is an organic synthesis laboratory. This laboratory is specialized in the synthesis of several metabolic intermediates and their stable isotope labelled analogues. Next to the VUmc metabolic unit, also many other laboratories are provided with these unique compounds.

2. Analysis techniques to test metabolites
Metabolic investigations require analyses of a wide range of metabolites covering the possible disorders according to the patient’s clinical information. The methods of analysis vary from metabolite groups, such as the organic acids in urine, to single metabolite-methods as e.g. pipecolic acid in CSF. The technique of choice for a certain analytical method is based on the sample matrix, the chemical properties of the metabolite(-group) of interest and furthermore the required sensitivity and analysis time. Therefore, various analysis techniques such as colorimetry, electrophoresis, thin layer chromatography, high pressure liquid chromatography with photodiode array or fluorescence detection and gas chromatography with flame ionization detection are applied in metabolic laboratories. Another frequent applied technique is gas chromatography combined with mass spectrometry (GC-MS), to obtain sensitive and selective quantitative measurements. However, the GC-MS measurements cannot be performed directly in the body fluids as many metabolites are polar, non-volatile compounds and present in complex matrices. Therefore a time-consuming sample preparation is required prior to the GC-MS measurement. Another frequently applied technique in modern metabolic laboratories is liquid chromatography combined with tandem mass spectrometry (LC-MS/MS). This technique allows less sample pretreatment due to the polar environment of the mobile
phase in the liquid chromatography (LC) section. Another improvement is the ionization of analytes without the need of preliminary derivatisation. Since the introduction of LC-MS/MS on metabolic laboratories, many former GC-MS methods have been replaced by this technique. Nevertheless, not all metabolites show good analytical performances with LC-MS/MS, which proves that this technique is rather complementary than a substitute of GC-MS.

3. LC-MS/MS

The LC-MS/MS system is a technique which consists of a (ultra)high-performance liquid chromatography (UHPLC) system directly coupled to a tandem mass spectrometer, an example is shown in figure 1. Separation of the metabolites from the matrix occurs in the LC-section, afterwards the metabolites are ionized, selected on mass and detected in the tandem mass spectrometry (MS/MS) section.

Fig. 1: LC-MS/MS system at VUmc metabolic unit.
### 3.1 Liquid Chromatography

MS/MS is a separation and detection technique which can measure several analytes simultaneously without a preliminary separation of these analytes. Although the sensitivity of a MS/MS measurement can be considerably enhanced if the analyte is separated from its sample matrix. Sample matrices suppress the efficiency of ionization during MS/MS, leading to a reduced or even disappeared signal. The ionization suppression effect is further attended in the MS-interface section (paragraph 3.3). The UHPLC, the first part of a LC-MS/MS system, separates the metabolites of the injected sample by using an analytical column. Many separations are based on the difference in affinity of the sample analytes with the columns stationary phase in the presence of a certain mobile phase. For instance during reversed phase chromatography (figure 2), an apolar analyte has a high affinity with the apolar stationary phase and shows a high retention time during a reversed phase separation on a C\textsubscript{18}-packed column with a moderate polar mobile phase. If the mobile phase's polarity is decreased, the affinity of the analyte with the mobile phase increases, resulting in a lower retention time. Some analytes are easily separated from the sample matrix by the application of an isocratic mobile phase. Differently, separation in a single run of analyte mixtures with a large difference in a chemical property as e.g. polarity is possible by a gradient in time of different mobile phases.

The selection of a suitable analytical column for a certain separation method is often prescribed by the samples matrix and the chemical characteristics of an analyte. Therefore, other characteristics beside the polarity of an analyte, such as acidic or basic functional groups, chirality and the size of an analyte, can be explored to develop alternative separation strategies. Examples of other applied liquid chromatography mechanisms in combination with tandem mass spectrometry are normal phase-, hydrophilic-interaction-, paired-ion-, ion-exchange-, affinity-, chiral- and size exclusion chromatography. In this study, reversed phase chromatographic separations were applied in the examined LC-MS/MS methods.
Fig. 2: Schematic retention mechanism of apolar and polar analytes during reversed phase chromatography using a C\textsubscript{18} stationary phase and 5% methanol as mobile phase. Text in figure was modified [1].

3.2 Mass spectrometry - Interface

The tandem mass spectrometer, the second part of the LC-MS/MS, performs mass selective detection of molecules of interest after the LC-separation. The eluent flow can be directly coupled to the tandem mass spectrometer, with or without splitting the flow, which depends on the applied flow rate and ionization source type. The application of LC coupled to MS/MS requires an interface which transforms the dissolved sample molecules from the LC-section into ionized molecules in the gas phase. A frequently used ionization technique in LC-MS/MS is electrospray ionization (ESI, figure 3), with typical flow rates of 1 – 500 µL/min. In ESI experiments the mobile phase from the LC-section, containing the separated sample analytes, is sprayed at atmospheric pressure with a nano-spray needle. The involvement of an inert nebulizing gas stream (e.g. nitrogen), a positive (+) or negative (-) needle voltage and heating of the electrospray needle are important factors for the formation of dispersed positively or negatively charged droplets. An inert drying gas vaporizes the droplet solvent, causing a reduced droplet size and an increased droplet charge. If the charge becomes too high for the droplet size a coulombic explosion occurs which causes droplet fission. This process continues until the droplet solvent is
completely evaporated and free positively or negatively charged sample molecules remain. The composition of the LC sample solution can influence the adduct type formed with the analyte during the ionization. Frequently observed molecule (M) ion adducts during LC-MS/MS are for instance \([\text{M+H}]^+\), \([\text{M+Na}]^+\), \([\text{M+K}]^+\), \([\text{M+NH}_4]^+\) in ESI (+) and \([\text{M-H}]^-\) in ESI (-). ESI is known as a soft ionization technique since minor fragmentation is observed in the interface, making it suitable for the measurement of labile biomolecules. Another frequently applied ionization technique in LC-MS/MS is atmospheric pressure chemical ionization (APCI, figure 3), with typical flow rates from 50 to 2000 µL/min. During this technique the separated sample solution from the LC-section is nebulized in a heated tube, supported by a nitrogen gas flow, where the nano droplets can evaporate. The ionization is induced outside the heated tube by a corona discharge needle, in the orbit from the heated tube to the entrance of the MS (orifice). Although APCI is a soft ionization technique, more fragmentation in the interface is observed. A major part of MS/MS sensitivity depends on the efficiency of ionization, which is determined by the yield of loaded analytes after ionization. The ionization efficiency can be influenced by ion suppression, this effect is induced by several mechanisms. One of the suppression causes is, for instance, the co-elution of less volatile compounds [2]. These compounds negatively affect the formation of droplets and the evaporation, resulting in a reduced amount of loaded analytes. Beside this effect, competition to obtain charge between the molecule of interest and other co-eluting agents can lead to reduced ionization. In general the ionization of molecules with a lower mass is suppressed by larger molecules, and moderate to high polar analytes are more sensitive to ion suppression compared with apolar compounds. Furthermore, overloading of the LC-column could also result in a decreased MS/MS sensitivity [3].
The advantage of APCI is the possibility of application without splitting of the eluent flow at higher flow rates. Furthermore, this technique allows the use of apolar mobile phases since the ionization occurs in the gas phase. In general, APCI shows better ionization for relatively apolar molecules with a low to moderate mass, compared with ESI. ESI shows better ionization performances for polar to moderate apolar compounds at a wide molecular mass range, compared with APCI, covering the majority of metabolites. Therefore, ESI is the most applied ionization technique in the LC-MS/MS methods of the VUmc metabolic laboratory. In figure 4 the APCI and ESI suitability for ionization of molecules with different masses and polarities are shown.

Fig. 3: Schematic overview of ESI and APCI. Text in figure was modified [4] [5].

Fig. 4: Overview suitability of ESI and APCI for the ionization of analytes with various masses and polarities. Additionally, zones are shown for particle beam LC-MS and GC-MS, which techniques are not discussed in this report [6].
3.3 MS/MS

After ionization, the ions are forced towards the orifice by an electrical field, which allows the charged analyte to enter the high vacuum of the tandem mass spectrometer. Preparation of the ions for the first selection on quadrupole 1 occurs by several lenses, performing the transformation into a focused ion-beam. Subsequently, the analyte enters the first quadrupole which selects molecule ions based on their masses. A quadrupole consists of 4 parallel cylindrical rods, which create oscillating electrical fields. Charged molecules in high vacuum and an oscillating electrical field show an unique trajectory based on their mass over charge ratio (m/z). In a quadrupole the oscillating electrical field allows a selected m/z to pass the quadrupole and eliminates all other ratios, as shown in figure 5 (L). The second quadrupole, or collision cell, operates in the presence of a collision gas (e.g. nitrogen) at a lightly increased pressure. The analyte enters this quadrupole and collides with the collision gas molecules and breaks down into unique fragments. Selection of a second m/z, for instance of the analyte fragments after collision, is performed in quadrupole 3. A diagram of the quadrupoles and collision cell is illustrated in figure 5 (R). Detection of the charged analyte or fragments, and transformation into an electrical signal is achieved by an electron multiplier. This signal is send to the computer where obtained data can be analyzed.

Fig. 5: Schematic overview of molecule ion-selection (resonant ion) in a quadrupole by the mass based unique trajectory in an oscillating electrical field (L) [7]. Diagram of the quadrupoles and a collision cell (Q2) in a tandem mass spectrometer (R) [8].
3.4 Mass spectrometry – scanning modes

The tandem mass spectrometer can operate in several scanning modes (figure 6). During a product-ion scan the first quadrupole is selected on a m/z of the analyte of interest. After collision induced dissociation (CID) in the collision cell the fragments are scanned in the third quadrupole. Fragments obtained during a product-ion scan could be applied during measurements in the selected reaction monitoring (SRM) mode, where quadrupole 1 and 3 are selected on a m/z ratio. Although the initial signal of an analyte is reduced after CID, the application of SRM in an analytical method can yield a very high sensitivity. Due to the application of an unique m/z transition for a certain analyte, which leads to sensitive detection of nearly exclusively the selected m/z a low background signal can be obtained. Therefore, this mode is frequently applied for quantitative MS/MS methods. Beside single SRM m/z transitions it is also possible to measure multiple m/z transitions in a single run in the multiple reaction monitoring mode (MRM, not shown in figure). In the precursor-ion scanning mode the first quadrupole is in the scanning mode and the third quadrupole measures a selected m/z after the collision cell. This mode is applied to show the parent- or precursor-ion belonging to the selected fragment, for instance during acquiring a mass scan of acylcarnitines in a plasma sample. This group of metabolites all show a fragment m/z 85.1 after collision, the scan of the precursor-ions belonging to this fragment shows the different carbon-chain lengths acylcarnitines present in the sample. During neutral-loss scanning measurements the first and third quadrupole are scanning parallel with a certain m/z difference. This difference is a neutral group that leaves the analyte after CID in the collision cell. For instance in groups of metabolites such as different carbon-chain lengths acyl-coenzyme A (acyl-CoA), with a known specific leaving fragment-ion or neutral group, the precursor-ion or the neutral-loss respectively can be beneficial. Next to the different MS/MS scan modes, it is also possible to perform single-quadrupole scans without CID.
In MS/MS method development several parameters of the instrument can be optimized for the measurement of a specific analyte. For the source and interface the nebulizer and ion source gas, source temperature, ion spray voltage and the entrance potential can be tuned. Furthermore, inside the tandem mass spectrometer the declustering potential and curtain gas are adjustable parameters to decrease interferences of the analyte with sample matrix components. Important parameters for processes in the collision cell are the collision energy, the pressure of collision gas and the collision cell exit potential.

4. Research aims

During this research project, 2 separate topics were studied. The initial project focuses on LC-(ESI)MS/MS ionization efficiency, and in the second project a metabolic pathway (beta-oxidation) was studied in fibroblasts. Although these subjects are considerable different, a positive outcome of project 1 could be applied during project 2.
4.1 Research aim 1 – Ionization enhancement of LC-(ESI)MS/MS

The research aim of the first project is to study the ionization efficiency enhancement, in LC-(ESI)MS/MS measurements, by online post-column mixing of a wide range of organic solvents to the LC effluent, prior to the ESI-interface entrance. In order to study the effects for the positive and negative ionization mode, we tested LC-MS/MS methods for several metabolites. For our study we selected S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH), homocysteine and methionine, D- and L-2-hydroxyglutarate (D- and L-2-HG), and sialic acid (SA), which are regularly measured at the metabolic laboratory of the VUmc.

Further introduction for this initial project is described in the section of ionization enhancement of LC-(ESI)MS/MS, biochemical backgrounds of the selected metabolites are explained in appendices.

4.2 Research aim 2 – Beta-oxidation in fibroblasts

The aim of research project subject II is to develop a culturing method for fibroblasts, by obtaining energy via beta-oxidation and a method to analyze extracted acyl-CoA intermediates from these cells by LC-MS/MS. Additionally, the diagnostic application of this new method for diagnosis of a beta-oxidation disorder can be examined by culturing a fibroblast cell line with a known beta-oxidation disorder.

An additional introduction for subject II is given in the section of mitochondrial beta-oxidation.
Ionization enhancement of LC-(ESI)MS/MS

1. Sensitivity of mass spectrometry

As mentioned in chapter 1, one of the advantages of LC-MS/MS is the reduction of pretreatment steps of the sample, as it is possible to perform sensitive measurements in a vast amount of matrix components. However, these components can affect the measurement of the analytes of interest negatively due to ionization suppression. To investigate the ionization suppression of a LC-MS/MS method for a certain compound, the baseline has to be elevated by mixing a standard solution of this compound post column to the mobile phase prior to the ESI-interface. When ionization suppressing sample constituents elute from the LC-column under these conditions, a negative peak can be observed. Although ion suppression can negatively influence quantitative measurements, quantification of the obtained reduced signals can be corrected by using stable isotope labeled analogues as internal standards (IS). The application of these internal standards, which have nearly identical characteristics as the metabolite of interest, are therefore important to quantify the metabolite under ion suppression conditions.

MS/MS offers the possibility to acquire very selective and sensitive measurements, although this technique has minimal analyte concentration limits to obtain a signal.

We hypothesized that sensitivities of LC-MS/MS assays can be enhanced by improving the ionization efficiency. In a pilot experiment we have mixed organic solvents post-column to the LC effluent, prior to the ESI-interface entrance. During this experiment an enhancement of the overall MS/MS sensitivity of the metabolite SAH was observed. To our knowledge, the influence on ESI efficiency of mixing organic solvents post-column to the mobile phase has not been studied before.
2. Experimental approach

As described in the research aim, post-column experiments were performed on LC-MS/MS methods for SAM and SAH, homocysteine and methionine, D and L-2-HG and SA. The methods for sample preparation and LC-MS/MS measurement, which are regularly applied at the VUmc metabolic laboratory, are described in this paragraph. Furthermore, the post-column experiments and examined solvents are explained per method.

2.1 LC-MS/MS method for SAM and SAH in plasma and cerebrospinal fluid

The SAM and SAH (resp. S-adenosyl methionine and S-adenosyl homocysteine, figure 7) method was performed according to the SOP of the metabolic laboratory which is based on Struys et al. (2000) [10]. In order to prepare plasma and CSF for the LC-MS/MS measurement, samples were deproteinized by the addition of 312 µL 10% perchloric acid (HClO₄; v/v) to 500 µL sample. After mixing adequate, the mixture was centrifuged for 5 min. by 3345 g at 4°C. In a second test tube, 500 µL of the clear supernatant was mixed with 50 µL IS mixture containing 0.125 µM ¹³C₅-SAHI and 2.0 µM ²H₃-SAM. By the addition of 225 µL of 1 M phosphate buffer (pH 11.5) the acidic mixture was neutralized, subsequently 1 mL of water was added. The OASIS HLB solid-phase extraction (SPE) columns (60 mg, 3 mL, Waters) were conditioned by sequentially rinsing with 1 mL methanol, 750 µL 10 mM lauric acid in 0.1 M sodium hydroxide and 1 mL of water. The mixture was purified by application to the conditioned SPE column, followed by washing with 750 µL of water and elution with 800 µL of water-methanol (85:15, by volume), containing 0.1% formic acid (v/v).

Fig.7: Structural formulas of SAM (L) and SAH (R) [11] [12].
SAM and SAH were purchased by resp. Sigma-Aldrich and Abbott Laboratories. $^2$H$_3$-SAM was purchased by C/D/N Isotopes Inc. $^{13}$C$_5$-SAH is not commercially available and was therefore enzymatic synthesized. For both (internal) standards dilutions in milli-Q water were made from existing stock standards.

The analyses were performed on a 4000 Q trap tandem mass spectrometer (AB Sciex). Other instrumentation was an Acquity UHPLC system and sample manager (Waters). The separation of 10 µL sample was performed at room temperature on a SymmetryShield C$_{18}$ analytical column (2.1 x 100 mm; 3.5 µm bead size; Waters), with a mobile phase consisting of water-methanol (85:15, v/v) and 220 µL/L butyric acid, at a flow rate of 180 µL/min. After separation the flow was splitless connected to a turbo ion electrospray, operating in the positive mode. For optimal ionization conditions the ion spray voltage was set at 5000 V, the temperature of the turbo ion electrospray at 500°C and the ion source gas 1 and 2 were set at 40 and 20, respectively. The mass spectrometric analysis included fragmentation in a CID cell, using nitrogen at 10 as collision gas. Furthermore the following MS/MS settings were used: collision energy (CE), 26V (SAH) and 25V (SAM); declustering potential, 65V (SAH) and 45V (SAM); curtain gas flow, 15; entrance potential, 10V; collision cell exit potential, 5V.

The triple quadrupole tandem mass spectrometers were operated in the MRM mode. Protonated SAH ([M + H]$^+$), positively charged SAM ([M]$^+$) and the labeled analogues show signals in quadrupole 1. The signals of fragment 136.1 (protonated adenine) can be obtained after collision induced dissociation of charged SAH and SAM. This fragment is applied in quantitative measurements of SAH and SAM, table 1 shows the MRM transitions.
Table 1: Transition settings for the measurement of SAM and SAH.

<table>
<thead>
<tr>
<th>Compound</th>
<th>m/z quadrupole 1</th>
<th>m/z quadrupole 3</th>
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<tbody>
<tr>
<td>SAM</td>
<td>399.1</td>
<td>136.1</td>
</tr>
<tr>
<td>$^2$H$_3$-SAM</td>
<td>402.1</td>
<td>136.1</td>
</tr>
<tr>
<td>SAH</td>
<td>385.1</td>
<td>136.1</td>
</tr>
<tr>
<td>$^{13}$C$_5$-SAH</td>
<td>390.1</td>
<td>136.1</td>
</tr>
</tbody>
</table>

2.2 Post-column experiments for SAM and SAH

The ionization of SAM and SAH was studied by post-column mixing of several organic solvents, prior to the ESI-interface. The additional flow was generated by an external HPLC pump (Gynkotek) and connected with the LC-flow by a mixing tee, as showed in figure 8. During these experiments, measurements were performed on the same sample at different post-column mixing flow rates. Initially, a stable measurement was obtained without mixing organic solvent for each experiment. The additional flow rate-range and the different rates tested within this range were selected during the measurements, based on obtained effects. Effects on ionization of post-column mixing 2-propanol, methanol, acetone, acetonitrile, methanol (all obtained from VWR Chemicals) and tetrahydrofuran, 1-propanol, absolute ethanol (all acquired by Merck KGaA), were determined with the SAM and SAH LC-MS/MS method. Furthermore, post-column mixing of 2 mM acetyl acetone (Sigma-Aldrich) in 50% methanol was examined. The latter mixture was tested after a study using acetylacetone as modifier of the mobile phase of Siegel et al. (2013) [13]. During this study minimal increased signals were obtained by mixing acetylacetone with stocks of energy metabolites prior to directly infusion MS/MS measurements.

Peak areas and signal to noise (S/N) ratios were obtained with Analyst 1.4.2 software (AB Sciex). Prepared samples for SAM and SAH from the VUmc metabolic laboratory routine analysis were pooled and used as sample during the post-column experiments.
Fig. 8: Schematic overview of post-column mixing during LC-MS/MS. Original figure was modified [14].

2.3 Additional experiments SAM and SAH
Since effects obtained for SAM and SAH are different, some additional experiments were performed on the measurement of SAM.

2.3.1 Sample matrix
In order to show possible matrix effects of the samples for SAM with post-column mixing of 2-propanol, the experiments for the pooled samples were repeated with a standard solution in milli Q water of SAM and SAH.
Probable sample matrix effects were further investigated by injecting samples consisting of 15% methanol, 0.1 % formic acid and 15% methanol + 0.1 % formic acid, in order to reproduce the sample matrix after the SPE-eluting step.

2.3.2 SAM in LC-system
The background signal in the particular transitions for SAM might be caused by continuously eluting SAM from the LC-system and/or the analytical column. Tracing back a possible pollution of the LC-system was achieved by obtaining 149 MCA–scans (multi channel analysis) in 2.5 minutes in the production scan-mode for SAM (399.1), with a 2-propanol post column mixing flow of 70 µL/min. Measurements were performed with a 156 nM SAM standard in water, at the regular CE of 45V and a lower CE of 30V, in order to obtain the ratio of the production peak height at different CEs. Comparable
experiments were performed without injection to provide possible SAM production peaks from the mobile phase.

2.3.3 Transitions SAM
Proceeding on the observed MCA-scans with post-column mixing of 2-propanol, transition 399.1-102.0 was used to measure SAM. This production has a low signal in the mobile phase and might influence the S/N ratio of SAM positively. Measurements were performed at the following post-column mixing flows of 2-propanol: 0, 20, 40, 60, 80 µL/min.

2.4 S-adenosylethionine
Post-column experiments were also performed with S-adenosylethionine (SAE). The only difference of SAE with SAM is the side chain of the sulfur atom, which is an ethyl group instead of a methyl group, respectively. Therefore SAE could show similar ionization behavior compared with SAM during post column mixing of organic solvents. A standard solution of 50 nM SAE (Sigma) was prepared in milli Q water. Measurements of SAE were performed by using the SAM/SAH LC-MS/MS method, with transition settings of m/z 413.1 and m/z 136.1 for quadrupole 1 and 3, respectively. Effects on SAE ionization were examined by post-column mixing of 2-propanol, methanol, tetrahydrofurane, 1-propanol and ethanol. These solvents showed enhancement of SAM and SAH signals during post-column mixing. The mixing experiments were performed comparable as described for SAM and SAH.
2.5 LC-MS/MS method for D- and L-2-hydroxyglutarate in urine

The D/L-2-HG method was performed according to the SOP of the metabolic laboratory which is based on Struys et al. (2004) [15]. To prepare urine samples for the LC-MS measurement 20 µL urine was pipetted in a glass vial containing 250 µL of 0.004 mM $^2$H$_4$-D/L-2-HG in methanol as internal standard. The mixture was dried at 50°C in combination with a nitrogen stream. Preparation of di-acetyltartary derivatives was performed with the addition of 50 µL new-made 50 g/L diacetyl-L-tartaric anhydride (DATAN; Aldrich) in dichloromethane-acetic acid (4:1, v/v) to the residues. After the vial was capped and the content was mixed the mixtures were heated at 75°C for 30 minutes. This chiral derivatisation, shown in figure 9, was applied to allow the separation of D/L-2-HG and the internal standards by a nonchiral LC column. The vials were cooled to room temperature, and the content was dried at room temperature with a flow of nitrogen. The dried content was dissolved in 500 µL of milli-Q water. Portions of aqueous calibrators, containing 0, 50, 100, 200, 500, 1000 and 2000 pmol, were processed as described above. D- and L-2-HG were purchased by Sigma-Aldrich.

\[ \text{Fig. 9: Chemical reaction of } \text{D/L-2-HG with DATAN. The carbon atoms marked with an asterisk are chiral centers [15].} \]

The analyses were performed on the same LC-MS/MS system as described for SAM and SAH. For the separation 10 µL of the prepared sample or calibrator was injected on an XTerra RP C$_{18}$ analytical column (3.9 x 150 mm; 5 µm bead size; Waters) at room temperature. The mobile phase consisted of water-acetonitrile (93:7, v/v) and 125 mg/L ammonium formate, adjusted to pH 3.6 by the addition of formic acid, at a flow rate of 1 mL/min. After the column the flow was split at a ratio of 4:1, and this final 200 µL/min
flow was connected to a turbo ion electrospray, operating in the negative mode. For optimal ionization conditions the ion spray voltage was set at -4500V, the temperature of the turbo ion electrospray at 400°C, and the ion source gas 1 and 2 were set at 25. The mass spectrometric analysis included fragmentation in a collision induced dissociation cell, using nitrogen at 4 as collision gas. Furthermore the following MS/MS settings were used: CE, -7.5V; declustering potential, -10V; curtain gas flow, 10; entrance potential, -10V; collision cell exit potential, -15V. The tandem mass spectrometer was operated in the multi reaction monitoring mode. Deprotonated derivatives of D/L-2-HG and the labeled analogues show signals in quadrupole 1 at m/z -363.2 and m/z -367.2, respectively. After collision induced dissociation, signals of the hydroxyglutarate backbones of D/L-2-HG and labeled IS can be obtained, respectively, m/z -147.1 and m/z -151.1. Table 2 shows the applied MRM transitions for the quantitative measurement of D/L-2-HG.

Table 2: Transition settings for the measurement of D/L-2-HG.

<table>
<thead>
<tr>
<th>Compound</th>
<th>m/z quadrupole 1</th>
<th>m/z quadrupole 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>D/L-2-HG</td>
<td>-363.2</td>
<td>-147.1</td>
</tr>
<tr>
<td>²H₄-D/L-2-HG</td>
<td>-367.2</td>
<td>-151.1</td>
</tr>
</tbody>
</table>

2.6 Post-column experiments for D/L-2-HG

Organic solvents examined for effects on D- and L-2-HG ionization during post-column mixing were: 2-propanol, methanol, acetone, acetonitrile, tetrahydrofurane and ethanol. The post-column experiments were performed and analyzed as described for SAM and SAH (paragraph 2.2). During these experiments prepared D- and L-2-HG calibrators, obtained from the metabolic laboratory diagnostics, were used as sample.
2.7 LC-MS/MS method for sialic acid in cerebrospinal fluid, amniotic fluid and urine

The sialic acid (SA, figure 10) method was performed according to the SOP of the metabolic laboratory [16]. To prepare cerebrospinal fluid, amniotic fluid and urine for the LC-MS/MS measurement of free SA, 50 µL of the sample was pipetted in a Amicon Ultra centrifugal filter (10K, regenerated cellulose; Merck Millipore). This filter contained 50 µL 0.01 mM and 0.1 mM $^{13}$C$_3$-SA in milli-Q water, respectively for cerebrospinal or amniotic fluid and urine. The centrifugal filters were 15 minutes centrifuged at 20000 g. The supernatants were pipetted in GC vials with inserts and capped. For quantitative measurements, portions aqueous SA (Sigma-Aldrich) containing 0, 0.1, 0.2, 0.5, 1.0 and 2.0 nmol for cerebrospinal- or amniotic fluid and portions containing 0, 1.0, 2.0, 5.0, 10 and 20 nmol for urine were pipetted in vials with inserts. These calibrators were filled until 50 µL with milli-Q water, and the amounts of IS described above was added. For the LC-MS/MS measurement of total (free and bound) SA, 50 µL of the body fluid was pipetted in a glass vial, containing 20 µL 0.1 mM and 50 µL 0.1 mM $^{13}$C$_3$-SA in Milli-Q water, respectively for cerebrospinal- or amniotic fluid and urine. The hydrolysis of bounded SA was performed by the addition of 150 µL 63 mM sulfuric acid to the mixtures, after the vials were capped and mixed the mixtures were heated at 80°C for 60 minutes.

![Structural formula of SA](image)

Fig. 10: Structural formula of SA [17].

The LC-MS/MS system described by SAM and SAH was applied during measurements of SA. 2 µL of the prepared sample or calibrator was separated at room temperature by...
injection on an Xterra RP C₁₈ analytical column (3.9 x 150 mm; 5 µm bead size; Waters). The separation was performed by using a gradient of 2 mobile phases containing 10 mM ammonium formate in milli-Q water (A) and in 60% acetonitrile (B), the gradient is showed in table 3. After the column the flow of 0.6 mL/min was split at a ratio of 4:1, and this final 120 µL/min flow was connected to a turbo ion electrospray, operating in the positive ionization and MRM mode. For optimal ionization conditions the ion spray voltage was set at 4500V, the temperature of the turbo ion electrospray at 650°C, and the ion source gas 1 and 2 were set at 60 and 10, respectively. The mass spectrometric analysis included fragmentation in a CID cell, using nitrogen as collision gas tuned at 5.

Furthermore, the following MS/MS settings were used: CE, 25V; declustering potential, 55V; curtain gas flow, 10; entrance potential, 12V; collision cell exit potential, 10V. Protonated SA and the labeled analogue show signals in quadrupole 1 at m/z 310.2 and m/z 313.2, respectively. After collision induced dissociation, signals of fragments m/z 167.4 and m/z 170.4 can be obtained for non labeled SA and the labeled IS, respectively.

Table 3: Gradient in time of mobile phases A, milli-Q water, and B, 60% acetonitrile, both containing 10 mM ammonium formate, for the separation of SA.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>%A</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>2.5</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>5.1</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>
2.8 Post-column experiments for sialic acid

The effects of post-column mixed organic solvents on the ionization of SA and the $^{13}$C-labeled variant were examined with: 2-propanol, methanol, acetone, acetonitrile, tetrahydrofuran or 2 mM acetyl acetone in 50% methanol. These tests were performed and analyzed comparable with the post-column experiments explained for SAM and SAH (paragraph 2.2). Prepared samples, measured during post-column mixing, were obtained from the metabolic laboratory routine analysis.

2.9 LC-MS/MS method for homocysteine and methionine

Homocysteine and methionine (figure 11) are regularly measured for research purposes at the VUmc metabolic laboratory. The described method is based on Smith et al. (2013) [18]. This method was performed on a LC-MS/MS system consisting a Perkin-Elmer Series 200 HPLC and autosampler, connected with an API 3000 triple quadrupole tandem mass spectrometer (AB Sciex). Separation of 20 µL sample was performed by injection on a Symmetry C$_{18}$ analytical column (2.9 x 100 mm; 3.5 µm bead size; Waters) at room temperature. The mobile phase applied started with 2 minutes equilibration at 5% acetonitrile, followed by a linear gradient of 7 minutes to 50% acetonitrile. All mobile phases contained 5 mM nonapentanoic acid (ion pair reagent) and the flow rate was set at 0.3 mL/min. The LC effluent was splitless infused into the ESI-MS/MS, operating the MRM mode with positive ionization. Measured transitions were m/z 136.1 → 90.0 for homocysteine and m/z 150.1 → 104.0 for methionine. The ESI ion spray voltage was set at 5000V, and the electrospray was performed at 450°C. Nitrogen was used as turbo ion gas, at a rate of 8 L/min, and as nebulizer gas tuned at 8. The fragmentation was performed in a CID cell with nitrogen, set at 4, as collision gas. Further MS/MS settings were: CE, 19V; declustering potential, 21V; curtain gas flow, 8; entrance potential, 10V; collision cell exit potential, 14V.
2.10 Post-column experiments for homocysteine and methionine

The effect on homocysteine and methionine ionization of post-column mixing 2-propanol was examined to show whether similarities can be observed with the experiments for SAM and SAH. Post-column mixing was performed and analyzed as described for SAM and SAH (paragraph 2.2). During this experiment an aqueous 1 µM homocysteine and methionine standard, containing 6 µg/mL dithiothreitol to overcome disulfide bonds, was used as sample.

3. Results and Discussion

3.1 Post-column experiments for SAM and SAH

During post-column experiments for SAM and SAH, ionization enhancement of one or both analytes was obtained during post-column mixing of 2-propanol, methanol, tetrahydrofurane, 1-propanol and ethanol. However, measured effects were often minimal and not similar for both analytes. Furthermore enhancement of S/N ratios was low or not showed. By post-column mixing of 2-propanol, figure 12, maximal elevation of the SAH signal was obtained for the solvents examined. Also the S/N ratio of SAH was increased compared with the initial value. SAM was only slightly increased in the same experiment. Stable or decreased signals were obtained during experiments with post-
column mixing of acetone, 2 mM acetyl acetone (in 50% methanol) and acetonitrile, of which the latter is showed in figure 13.

Fig. 12: Area counts and S/N ratios for SAM and SAH peaks, obtained with and without post-column mixing of 2-propanol at different flow rates.

Fig. 13: Area counts for SAM and SAH peaks, obtained with and without post-column mixing of acetonitrile at different flow rates.

3.2.1 Additional experiments SAM and SAH - Sample matrix

Measurements of SAM and SAH in a standard mixture showed comparable effects during post-column mixing of 2-propanol, compared with obtained results with a sample. The
base signal was not altered after injections of diluted methanol, formic acid and the mixture.

3.2.2 Additional experiments SAM and SAH - SAM in LC-system

In MCA production scans of SAM, fragments were obtained at m/z 97, 102 and 136 after injection of a 156 nM standard. Altered CE tuning of 45V to 30V changed the peak heights to 35%, 147% and 78% of the initial value, for m/z 97, 102 and 136, respectively. The same fragments were observed during MCA production scans of SAM without injection of sample. Altered CEs changed peak heights of the same fragments to 56%, 133% and 50% of the initial values in this experiment, which could indicate SAM traces. The obtained signals were clearly visible after injection of a standard, however without injection signals were only observed at noise-level. A measurement without analytical column showed comparable signals without injection, indicating that possible pollution of the system is not derived from the column.

3.2.3 Additional experiments SAM and SAH - Transitions SAM

No major difference in effect of post-column mixed 2-propanol on the ionization efficiency was observed between SAM transitions m/z 399.1-102.0 and m/z 399.1-136.1. Although, signals obtained with the m/z 102.0 transition were less intense compared to a transition to m/z 136.1.

3.3 Post-column experiments for SAE

The post-column experiments performed during the measurement of SAE showed elevated peak areas with mixing 2-propanol, methanol, tetrahydrofuran, 1-propanol and ethanol. The obtained increased signals and S/N ratios were often comparable or lower than results for SAM. During mixing of 1-propanol the SAE signal was more increased compared to measurements of SAM, however, peak areas were only slightly higher
compared to post-column mixing of 2-propanol. In figure 14, area counts and the S/N ratio are represented, which were obtained during post-column mixing of 2-propanol.

Fig. 14: Area counts and S/N ratios for SAE peaks, obtained with and without post-column mixing of 2-propanol at different flow rates.

3.4 Post-column experiments for D/L-2-HG

The post-column experiments performed during measurements of D- and L-2-HG and the internal standard showed only slightly increased peak areas with the examined solvents. In figure 15, the obtained peak areas during post-column mixing of 2-propanol are shown for D- and L-2-HG and the internal standard. No S/N ratios were calculated for these experiments.

Fig. 15: Area counts for D- and L-2-HG peaks, obtained with and without post-column mixing of 2-propanol at different flow rates.
3.5 Post-column experiments for sialic acid

During mixing experiments with the SA LC-MS/MS method, maximal increased signals were determined with post-column mixing of 2-propanol, shown in figure 16. Further minor enhancements were found during post-column mixing of methanol and acetonitrile. Stable signals were detected during mixing of acetone and tetrahydrofuran, whereas during mixing of 2 mM acetylacetone in 50% methanol the signals were decreased at higher flow rates (40 and 80 µL/min). Since the minimal effects observed, no S/N ratios were calculated for these experiments.

![Fig. 16: Area counts for SA peaks, obtained with and without post-column mixing of 2-propanol at different flowrates.](image)

3.6 Post-column experiments for homocysteine and methionine

The effect of post-column mixing 2-propanol was examined twice, which results are shown in figure 17. In the first experiment a slightly elevated signal was observed for homocysteine and methionine. The second experiment showed decreasing signals for methionine, and for homocysteine after a minimal elevation. The measured S/N values showed an increasing curve only for methionine during the first test.
4. Conclusions

The performed experiments for research topic 1 showed no general effects on ionization during post-column mixing of 1- and 2-propanol, methanol, acetone, acetonitrile, tetrahydrofuran, ethanol and 2 mM acetyl acetone in 50% methanol for the LC-MS/MS methods examined. Maximal enhancement of MS/MS signals were obtained for SAH by post-column mixing of 2-propanol. However, the determined SAH S/N ratios for this experiment showed lower increases compared with the peak areas, indicating elevation of the noise level by post-column mixing 2-propanol. Experiments with other organic solvents, and experiments performed with the other LC-MS/MS methods showed less enhanced, stable or decreased signals. No specific differences in effects of post-column mixed organic solvents on ionization were observed between the LC-MS/MS methods operated in positive- (SAM and SAH, SA, homocysteine and methionine) and negative ionization mode (D/L-2-HG).
Mitochondrial beta-oxidation

1. Mitochondrial beta-oxidation of fatty acids and enzyme analysis

The human body produces energy from several sources, which provides the required energy under several circumstances, such as well-fed or fasting periods. The main sources of energy are sugars (carbohydrates), fats (fatty acids) and proteins, which are absorbed from food. Production of energy from these sources takes place at cellular level, via various metabolic pathways. Under well-fed conditions energy is mainly produced by the breakdown of carbohydrates via enzymatic conversions in the Krebs cycle. However, some organs, such as the heart, mainly obtain energy from fatty acids (FAs). In (bio)chemistry a FA consists of a carboxylic acid with an (un)saturated aliphatic carbon chain, which is mainly even numbered in our diet. The human body can retain FAs, stored in adipose tissue or at intracellular sites, which is required for energy production at low carbohydrate levels, for instance during fasting. Beside dietary uptake FAs can originate from these stored fats or from de novo synthesis.

The mitochondrial beta-oxidation is the main degradation route for energy production from FAs and consists of various enzymatic reactions. Degradation of FAs also occurs in the alpha- and omega-oxidation, however, the influence on energy production of these pathways is marginal. In human cells the beta-oxidation also occurs in the peroxisomes, in which a minor part of the FAs is degraded. However, since peroxisomal beta-oxidation is not completely similar with the mitochondrial, lacking completion of FA degradation, peroxisomes release chain-shortened FAs or carnitine esters. These peroxisomal products are further processed in the mitochondrial beta-oxidation. Another function of peroxisomal beta-oxidation compared with the mitochondrial is the ability to oxidate FA derivatives such as pristanic acid, the cholesterol derivatives di- and trihydroxycholestanolic acid and very long chain FAs (higher than C22).

During mitochondrial beta-oxidation, extracellular FAs are converted into intramitochondrial acyl-CoA analogues, followed by the enzymatically catalyzed
degradation into acetyl-CoA (C2) units. These acetyl-CoA units are further processed in the Krebs cycle, in figure 18 a schematic overview is shown of the pathway. The mitochondrial beta-oxidation route is initiated by the uptake of extracellular FAs, and the conversion into intracellular equivalent acyl-CoAs. At the outer membrane of the mitochondrion the carnitine palmitoyltransferase 1 (CPT1) enzyme converts acyl-CoAs into acylcarnitines. Subsequently, transport through the mitochondrial inner membrane and the conversion back into the acyl-CoA analogues is performed by carnitine/acylcarnitine translocase (CACT) and carnitine palmitoyl transferase 2 (CPT2), respectively. The degradation of acyl-CoA chains into acetyl-CoA units is catalyzed by 4 enzyme groups in the acyl-CoA oxidation system. Dehydrogenation of acyl-CoAs, yielding trans-2-enoyl-CoA, is catalyzed by chain length specific acyl-CoA dehydrogenases, namely short-chain (SCAD; C4), medium-chain (MCAD; C6-C12) and very-long-chain (VLCAD; C14-C16) acyl-CoA dehydrogenase. Although the name of the latter enzyme supposes the ability to catalyze reactions with very long chain acyl-CoAs (C22+), a low enzyme activity is observed with acyl-CoA C24 as substrate. Hydratation of trans-2-enoyl-CoAs, yielding L-3-hydroxyacyl-CoAs, is catalyzed by the second enzyme group, the short-chain (SCEH; C4-C8) and long-chain (LCEH; C10-C16) enoyl-CoA hydratases, of which the latter is a part of the mitochondrial trifunctional protein (MTP). The 3-hydroxyacyl-CoA dehydrogenases (3HADs) are involved in the oxidation of L-3-hydroxyacyl-CoAs with nicotinamide adenine dinucleotide [NAD⁺], which are converted into 3-ketoacyl-CoA and reduced NAD⁺ [NADH]. This enzyme group consists of a short-chain 3-hydroxyacyl-CoA dehydrogenase (SCHAD, C4-C10) and long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD, C12-C16), as another MTP component. Completion of the degradation process is implemented by 3-ketoacyl-CoA thiolase catalyzed thiolysis of 3-ketoacyl-CoAs, after which (n-2)acyl-CoA and acetyl-CoA is formed. The thiolytic cleavage of C4 – C8 and C10 – C16 3-ketoacyl-CoAs is catalyzed by medium-chain (MCKAT) and long-chain 3-ketoacyl-CoA thiolase (LCKAT), respectively, of which the LCKAT is the third element of MTP. After this last step of the mitochondrial beta-oxidation the shortened acyl-CoA
chains are reprocessed again, and the acetyl-CoA units are further degraded in the Krebs cycle.

Impairment of one of the beta-oxidation involved enzymes can lead to accumulation of acyl-CoAs with various carbon chain lengths, which depends on the specific enzyme affected. These accumulated acyl-CoAs can be reconverted into acylcarnitine analogues by CPT2 to allow removal of excesses from the mitochondrion via CACT. Subsequently, acylcarnitines can pass the mitochondrial outer membrane and the cell membrane to reach the plasma compartment. Consequently, deficiencies of a specific enzyme of the beta-oxidation pathway lead to elevated levels of acylcarnitines in plasma. Therefore, currently one of the basic laboratory tests during the diagnosis of patients with a clinical suspicion of a beta-oxidation disorder is the measurement of acylcarnitine levels in plasma.

After the measurement of elevated plasma acylcarnitine levels, indicating a certain beta-oxidation disorder, conformational enzyme analysis is required. Currently tests are available, which can selectively examine several supposed affected enzymes. In many of these tests incubations are performed on extracted mitochondria or cell homogenates, for instance prepared from fibroblasts, with an enzyme-selective substrate after which the formation of the enzyme-reaction products is measured. However these experiments indicate the (dys)function of a specific enzyme, the total beta-oxidation flux is not obtained as found in the plasma acylcarnitines. Especially if the specific enzyme tests did not show the defect, whole cell incubation studies can be informative. In the last decades various methods have been developed which focuses on the measurement of beta-oxidation end-products of cells incubated with labeled FAs. For instance the release of \(^{14}\text{C}\)-labeled CO\(_2\) or tritium after incubation was measured in order to obtain the total beta-oxidation flux. Furthermore, methods were developed measuring radioactively labeled acyl-CoAs and acylcarnitines of labeled FA incubated mitochondria or selectively permeabilized fibroblasts. Later on, methods were described measuring released stable isotope labeled acylcarnitines after incubating intact fibroblasts \([21]\). The application of
intact fibroblasts incubated with stable isotope labeled FAs offers a model which very closely approaches the cell processes of the human body. Acylcarnitines and acyl-CoAs are relatively easy measured by MS/MS, this technique is frequently applied for the analysis of these compounds during incubation studies with stable isotope labeled FAs. The examination of acylcarnitines released from incubated cells show often very distinctive profiles for beta-oxidation disorders. However, elevated acylcarnitine excretion instigated by a beta-oxidation disorder is a secondary effect of the initial accumulated intra mitochondrial acyl-CoAs. Since enzyme analysis in general prefers a minimal amount of conversions in between the initial substrate and the measured products, it would be interesting to show differences of acylcarnitine and acyl-CoA profiles of the same incubated cells.

Fig. 18: Schematic overview of beta-oxidation pathway, including the involved enzymes. See text for explanation [21].
2. Experimental approach

During this study, LC-MS/MS measurements of acyl-CoAs were performed in cultured fibroblast lysates. A LC-MS/MS method was developed, with the tandem mass spectrometer operating in the MRM mode. Beside this mode, also measurements have been performed in the neutral loss scan mode. Furthermore, precursor ion scans of released acylcarnitines in culturing medium of the fibroblasts were obtained with the LC-MS/MS method used at VUmc metabolic laboratory.

2.1 LC-MS/MS method for acyl-CoA measurements

In order to separate the acyl-CoAs from the sample matrix and short from long chain acyl-CoAs, a reversed phase chromatographic separation method was developed. The first measurements were performed with a C₈ analytical column and later experiments with a C₁₈ variant. After CID in the tandem mass spectrometer, all acyl-CoAs show a typical neutral loss of m/z 507 (phosphoadenosine diphosphate) and a second high abundant fragment at m/z 428. Positive ESI-MS/MS in the MRM mode measures all the transitions of the even chain length acyl-CoAs from beta-oxidation (C₂–C₁₆:0). Additionally, transitions were included of non beta-oxidation related acyl-CoA compounds, which were available as standard at the VUmc metabolic laboratory. The preparation of fibroblast lysates is described by the culturing tests.

The measurements were performed on a LC-MS/MS system consisting of an Acquity UHPLC system and sample manager (Waters), coupled to a 4000 Q trap tandem mass spectrometer (AB Sciex). A linear gradient of mobile phase A, consisting 2% acetonitrile, and mobile phase B, consisting 90% acetonitrile, both with added ammonium acetate, was mixed as shown in tables 4 and 5. During culturing experiment 1, a Zorbax SB C₈ (2.1 x 100 mm, 3.5 µm bead size, Agilent) analytical column was used, at a flow of 200 µL/min, coupled splitless to the tandem mass spectrometer. The measurements for the other experiments were performed with a XTerra RP C₁₈ (3.9 x 150 mm, 5 µm bead size, Agilent).
Waters) analytical column, at a flow of 800 µL/min. The latter analytical column was selected since the separation on the C₈ column was not stable during different experiments. After the C₁₈ column the flow was split at a ratio of 4:1, the yielded 160 µL/min was connected to the tandem mass spectrometer. MS/MS in the MRM mode was achieved with positive ESI at 525°C, with the ion spray voltage tuned at 5000V and the ion source gasses GS1 and GS2 at 45 and 20, respectively. CID was achieved by application of nitrogen as collision gas, tuned at 3. The transitions measured after the culturing experiments are shown in table 6, with corresponding settings for declustering potential (DP), collision energy (CE) and collision cell exit potential (CXP). Dwell times were set at 20 msec per transition in experiment 1-4 and at 10 msec in experiment 5-6. The dwell times for experiment 5-6 were altered in order to decrease elevated scan period times caused by the high number of transitions. Furthermore, the curtain gas flow was tuned at 10, entrance potential at 5 V and the autosampler temperature was set at 5°C. Data analysis was performed with Analyst 1.4.2 (AB Sciex).
Table 4: Linear gradient in time applied during measurements with Zorbax SB C<sub>8</sub> (2.1 x 100 mm, 3.5 µm bead size, Agilent) analytical column. Mobile phases A and B were consisting of 2% and 90% acetonitrile, respectively, both containing 5 mM ammonium acetate.

<table>
<thead>
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<th>%A</th>
<th>%B</th>
</tr>
</thead>
<tbody>
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<td>0</td>
</tr>
<tr>
<td>1</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
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</tr>
<tr>
<td>16</td>
<td>100</td>
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</tbody>
</table>

Table 5: Linear gradient in time applied during measurements with XTerra RP C<sub>18</sub> (3.9 x 150 mm, 5 µm bead size, Waters) analytical column. Mobile phases A and B were consisting of 2% and 90% acetonitrile, respectively, both containing 15 mM ammonium acetate.

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Table 6: MRM transitions with corresponding DP, CE and CXP, applied during measurements of obtained cell lysates after culturing experiments 1-6.

<table>
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<tr>
<th>Acyl-CoA Carbon chain</th>
<th>Q1 Mass (amu)</th>
<th>Q3 Mass (amu)</th>
<th>DP (V)</th>
<th>CE (V)</th>
<th>CXP (V)</th>
<th>Experiment</th>
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<td>Free CoA</td>
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<td>40</td>
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<td>40</td>
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</table>
2.2 LC-MS/MS method for acyl-CoA measurements - neutral loss scan mode

This method performs chromatographic separations only with the XTerra RP C<sub>18</sub> (3.9 x 150 mm, 5 µm bead size, Waters) analytical column as described for the MRM-method. MS/MS parameters for measurements in the neutral loss scanning mode were similar with the settings of the MRM-method, except of the DP, CE and the CXP. These parameters were tuned with start and stop values during a scan period, see table 7. In order to obtain neutral loss scans from acyl-CoAs, the neutral loss fragment was set on 507 amu. Scans were acquired in 0.5 s from 750 amu until 1050 amu with a step size of 0.1 amu.

Table 7: Start and stop values of DP, CE and CXP during a scan period in the neutral loss scanning mode.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>start – stop (V)</th>
</tr>
</thead>
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<td>DP</td>
<td>20 – 35</td>
</tr>
<tr>
<td>CE</td>
<td>40 – 55</td>
</tr>
<tr>
<td>CXP</td>
<td>35 - 40</td>
</tr>
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</table>

2.3 Comparison ammonium acetate concentrations in mobile phase

Added ammonium acetate (NH₄Ac) in mobile phase can increase the retention stability of acyl-CoAs during LC. However, high concentrations of NH₄Ac in the mobile phase could also suppress the ionization. The LC-behavior and ionization of acyl-CoAs was examined during application of mobile phases with different concentrations ammonium acetate. A standard mixture of 100 nM acetyl-CoA (C2-CoA), propionyl CoA (C3:0-CoA), 2-butenoyl CoA (C4:1-CoA), glutaryl CoA (C5-CoA), octanoyl-CoA (C8:0-CoA), palmitoyl-CoA (C16:0-CoA), all purchased from Sigma-Aldrich, and ²H₃-C2-CoA, ²H₃-C8:0-CoA and ²H₃-C16:0-CoA, all obtained from dr. H. J. ten Brink (organic synthesis laboratory VUmc), was prepared in milli-Q water and in the tested mobile phase A. Mobile phases A and B with concentrations of 5, 10, 15 and 20 mM NH₄Ac were prepared. After equilibration of
the LC-system, 10 µL of the standard was injected and measured in duplicate with the acyl-CoA MRM LC-MS/MS method, using the XTerra RP C_{18} (3.9 x 150 mm, 5 µm bead size, Waters) analytical column. The retention behavior was determined by visually evaluation of the peak shapes, effects on MS-ionization was obtained by measuring the peak areas.

2.4 Ion suppression
Eluting sample constituents can induce ion suppression during LC-MS/MS. Therefore, it is important to determine whether the ionization is suppressed during the measurement of a sample. The analytical method should be improved if ion suppression is present at the analyte retention time, for instance by the removal of suppressing agents during sample preparation or modifying the LC separation. The presence of ion suppression during measurements on samples was investigated by post-column mixing of an internal standard mixture. The elevated baselines of the internal standard transitions are strongly decreased if the ionization suppressing matrix elutes. An aqueous 100 nM internal standard mixture (\(^2\)H\(_3\)-C\(_2\)-CoA, \(^2\)H\(_3\)-C\(_8\):0-CoA and \(^2\)H\(_3\)-C\(_{16}\):0-CoA) was prepared and at a flow of 500 µL/min post column mixed with the eluted mobile phase. Of a residual fibroblast lysate volumes of 10 µL were injected and measured with MRM LC-MS/MS method, using the XTerra RP C\(_{18}\) analytical column.

2.5 LC-MS/MS method for acylcarnitines in culturing medium
During this study acylcarnitine scans were obtained from sampled culturing medium. Measurements were performed with the LC-MS/MS method of the VUmc metabolic laboratory for acylcarnitines in plasma, bloodspots and urine [22]. In this method, prepared butyl-ester analogues of the acylcarnitines are measured by direct infusion-MS/MS in the precursor ion- scan mode with positive ionization. This mode is applied since all butyl-ester derivatives of acylcarnitines show a similar fragment, m/z 85 [CH\(_2\)-CH=CH-COOH]\(^+\), after CID.
A 50 µL aliquot of culture medium was pipetted in an eppendorf tube containing 50 µL IS mixture of 20 µM \(^2\)H\(_3\)-carnitine, 1 µM \(^2\)H\(_5\)-propionylcarnitine, 0.25 µM \(^2\)H\(_3\)-octanoylcarnitine and 0.25 µM \(^2\)H\(_3\)-palmitoylcarnitine in acetonitrile. The stable isotope labeled carnitine and acylcarnitines were obtained from dr. H. J. ten Brink (organic synthesis laboratory VUmc). Deproteination of the mixtures was performed by mixing with 450 µL acetonitrile followed by 5 minutes centrifugation at 1640 g. The obtained supernatants were transferred into glass vials and dried by evaporation at 30°C with a nitrogen flow. Obtained residues were dissolved in 100 µL acetyl chloride-n-butanol (1:17, v/v), vials were capped and incubated for 15 minutes at 60°C, in order to convert the acylcarnitines into their butyl-ester derivatives. After reaction the mixtures were dried by evaporation with a flow of nitrogen at 30°C. The obtained residues were dissolved in 100 µL acetonitrile, transferred into glass vials with inserts and capped. 5 µL portions of the prepared samples were injected and directly infused in the tandem mass spectrometer by an eluent flow.

The measurements were performed on a LC-MS/MS system of a Perkin-Elmer Series 200 HPLC and autosampler, connected with an API 3000 triple quadrupole tandem mass spectrometer (AB Sciex). A constant flow of 200 µL/min 75% acetonitrile was splitless pumped into the mass spectrometer, operating in the precursor ion scanning mode with positive ionization. Electrospray was performed at 300°C and an ion spray voltage of 5500V, with nitrogen as turbo ion gas at a flow of 8L/min and the nebulizer gas set at 6. CID was achieved by a CE of 38V, with nitrogen as collision gas tuned at 2. Precursor ion scans were obtained from 215 amu to 515 amu of fragment 85.1 amu, with scan periods of 1 second and a stepsize of 0.1 amu. Further MS parameters were: curtain gas, 10; focusing potential, 340V; entrance potential, 10V; collision cell exit potential, 8V; declustering potential, 55V. Data analysis was performed with Analyst 1.4.2.
2.6 Acyl-CoAs in cultured fibroblasts

During culturing experiments, purchased control fibroblast cell lines GM00023 and GM08447 (respectively obtained from a 31 and 2 years old blank female, Coriell Institute) were used. Furthermore a known MCAD deficient (MCADD) fibroblast cell line was obtained from the VUmc metabolic laboratory sample storage.

2.6.1 Culturing test 1 and 2, determination of minimal required L-glutamine and D-glucose levels in culturing medium.

In order to start up the beta-oxidation during incubation tests with long chain fatty acid or acylcarnitines, culturing medium is required with relatively low glucose and glutamine concentrations in which the fibroblasts can survive for several days, as for instance described for glucose in Roe et al. (1999) [23]. Medium applied at our laboratory for fibroblast culturing usually contains 1.0 mM L-glutamine and 6.1 mM D-glucose. This experiment roughly determines the minimal concentrations of L-glutamine and D-glucose in medium, required for the survival of fibroblasts during 6 days of culturing without refreshment. Additionally, measurements were performed to determine acyl-CoAs in obtained cell lysates and acylcarnitines in culture medium samples.

Various concentrations of L-glutamine (Sigma) and D-glucose (Merck) were prepared in Dulbecco’s Modified Eagles Medium (DMEM) without L-glutamine, D-glucose, phenol red and sodium pyruvate (Gibco, Life technologies), see tables 8 and 9. Furthermore, all the medium conditions contained 10% fetal bovine serum (v/v; heat inactivated; Bodinco BV) and 1% penicillin/streptomycin mixture (v/v; Gibco, Life technologies). The survival tests were started in 75 cm² flasks with control fibroblasts at approximately 40% confluency, with 15 mL specific prepared medium added to a flask. The fibroblasts were cultured for 6 days in a humidified 5% CO₂ enriched air incubator at 37°C. During the first test one control fibroblast cell line (GM00023, passage 19) was cultured and the coverage was obtained after 3 and 6 days. The second test was performed with 2 control
fibroblast cell lines (GM00023, GM08447, resp. passage 22 and 11) and the coverage was obtained after day 1 and 6. After 6 days of culturing, medium samples were collected of all conditions and stored at -80°C upon analysis. Fibroblasts were harvested after rinsing with 10 mL Hanks’ balanced salt solution (HBSS; Gibco, life technologies) by using 2 mL 0.05% trypsin-EDTA (Gibco, life technologies) in HBSS at 37°C, subsequently 1 mL cold HBSS was added and the cell mixture was transferred into a clean tube and kept on ice. Cell pellets were prepared by centrifugation of the mixtures at 340 g, afterwards the HBSS-trypsin mixture was removed. The cells were transferred into eppendorf tubes on ice by suspension of the obtained pellets in 1 mL cold HBSS, and pellets formed by 3x pulse spinning. HBSS was removed and dry cell pellets were stored at -80°C upon analysis.

Preparation of cell lysates was performed by suspending the pellets of test 1 in 200 µL HBSS. Cell pellets of test 2 were suspended in 180 µL HBSS and 20 µL 1 µM mix of $^{2}$H$_3$-acetyl-CoA ($^{2}$H$_3$-C2-CoA), $^{2}$H$_3$-octanoyl-CoA ($^{2}$H$_3$-C8:0-CoA) and $^{2}$H$_3$-palmitoyl-CoA ($^{2}$H$_3$-C16:0-CoA) in milli Q water, since these internal standards were later available. Lysation of the cells was achieved by 3x 10 seconds ultrasonication of the mixtures using a Sonopuls ultrasonic homogenizer (Bandelin). Sample tubes were kept on ice during all these steps. For test 1 and control cell line GM00023 of test 2 the disrupted cell suspension was transferred into an Ultrafree centrifugal filter (0,22 µm pore size, Durapore-PVDF membrane, Merck Millipore), and centrifuged for 10 minutes in a cooled (4°C) ultra centrifuge at 20,000 g. The cell lysates of cell line GM08447 were first 5 minutes centrifuged in a cooled ultra centrifuge at 10,000 g, to remove a major part of solid cell material preventing for probable leaking of insoluble’s during filtration. Subsequently, the supernatant was transferred into the centrifugal filter, and centrifuged for 10 minutes in cooled ultra centrifuge at 4000 g. For both tests, obtained supernatants were transferred into glass vials with inserts, capped and stored in a cooled LC-autosampler upon analysis. 10 µL aliquots of lysates from culturing test 1 were injected on the Zorbax SB C8 analytical column for acyl-CoA measurements in the MRM mode.
Acyl-CoAs in lysates from test 2 were measured in this mode by injecting 30 µL volumes on an XTerra RP C_{18} analytical column. For test 2, neutral-loss scans were obtained by injections of 10 µL lysate. Furthermore, acylcarnitines were measured in culturing medium of test 1.

Table 8: Concentrations L-glutamine and D-glucose in corresponding medium conditions of test 1.

<table>
<thead>
<tr>
<th>Condition medium</th>
<th>L-glutamine (mM)</th>
<th>D-glucose (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>0.1</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 9: Concentrations L-glutamine and D-glucose in corresponding medium conditions of test 2 for control fibroblasts GM00023 and GM08447. For condition 6, different concentrations of L-glutamine were applied.

<table>
<thead>
<tr>
<th>Condition medium</th>
<th>L-glutamine (mM)</th>
<th>D-glucose (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>0.5</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>6 - GM00023</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>6 - GM08447</td>
<td>0.6</td>
<td>5</td>
</tr>
</tbody>
</table>
2.6.2 Culturing test 3, Palmitate and L-palmitoylcarnitine added to culturing medium

In order to enhance beta-oxidation, a culturing test was performed using medium with added palmitate, as for instance described in Roe et al. (1999) for the 16-2H₃-variant [23]. Furthermore, a comparable experiment was performed with, added L-palmitoylcarnitine to the culturing medium. The incubations were started with control fibroblasts (GM00023, GM08447, resp. passage 24 and 16) in 75 cm² flasks at approximately 40% confluency. Basis medium of DMEM (without L-glutamine, D-glucose, phenol red and sodium pyruvate), was supplemented with L-glutamine and D-glucose (end concentration 1 mM). Furthermore, basis medium contained 10% fetal bovine serum and 1% penicillin/streptomycin mixture (v/v). Two medium conditions were prepared per control fibroblast cell line in separated flasks. For palmitate 100 µL 15 mM (100 µM) in 70% ethanol and for L-palmitoylcarnitine 14 µL 429.1 mM (400 µM) in 70% ethanol was filled to 15 mL with basis medium. Additionally, one flask per condition was cultured with the same amount of basis medium and 70% ethanol as negative control. During this test the fibroblasts were 5 days cultured at 37°C in an incubator containing 5% CO₂ enriched humidified air. Sampling of medium and obtaining of cell pellets was performed as described by culturing test 1 and 2. The preparation of cell lysates, and the different analyses were performed as described for control cell line GM08447 of culturing test 2.

2.6.3 Culturing test 4, neutralized L-palmitoylcarnitine added to culturing medium

Prevention for cell death, possible induced by a pH change of the culturing medium, was tested by neutralizing the L-palmitoylcarnitine mixture in 70% ethanol to pH 6 with sodium hydroxide, prior to addition to the culture medium. Furthermore, the effect of neutralized L-palmitoylcarnitine on the fibroblasts beta-oxidation was determined at several concentrations in basis medium. Incubations of control fibroblasts (GM08447,
passage 16) in 75 cm² flasks initiated at approximately 40% confluency. Incubation conditions of several concentrations neutralized L-palmitoylcarnitine in basis medium were mixed (table 10) in separate flasks. The fibroblasts were cultured for 5 days, in the same incubator as the previous culturing tests. Medium samples and cell pellets were obtained similar with culturing test 1 and 2. Cell lysates were prepared as described for control cell line GM08447 in culturing test 2. The only difference for the analyses was a lysate volume of 30 µL injected for the neutral-loss scans.

Table 10: Concentrations L-palmitoylcarnitine in corresponding medium conditions of test 4.

<table>
<thead>
<tr>
<th>Condition medium</th>
<th>L-palmitoylcarnitine (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>400</td>
</tr>
<tr>
<td>2</td>
<td>299</td>
</tr>
<tr>
<td>3</td>
<td>201</td>
</tr>
<tr>
<td>4</td>
<td>103</td>
</tr>
</tbody>
</table>

2.6.4 Culturing test 5, $^2$H₃-palmitoylcarnitine and L-palmitoylcarnitine added to culturing medium

Exact tracing of beta-oxidation induced by adding long chain acylcarnitine to the culturing medium is possible by the application of a deuterium labeled analogue. To compare the effect between labeled and unlabeled acylcarnitine in culturing medium, dilutions of neutralized $^2$H₃-palmitoylcarnitine, obtained from dr. H. J. ten Brink (organic synthesis laboratory VUmc), and L-palmitoylcarnitine were prepared in basis medium (table 11). The culturing test started with 15 mL of a medium condition in a 75 cm² flask for 2 control fibroblast cell lines (GM00023, GM08447, resp. passage 21 and 18), at approximately 40% confluency. Additionally, one flask per cell line was cultured as negative control with normal basis medium. Culturing of the fibroblasts was performed for 4 days at 37°C in a similar incubator as for the previous tests. Sampling of medium and preparation of cell pellets was achieved by the method described for culturing test 1.
and 2. In order to prepare cell lysates, the negative controls and pellets obtained after incubation with unlabeled L-palmitoylcarnitine were suspended in 180 µL milli Q water + 20 µL 1 µM mix of $^{3}$H$_{3}$-C2-CoA, $^{3}$H$_{3}$-C8:0-CoA and $^{3}$H$_{3}$-C16:0-CoA in milli Q water. Pellets obtained after incubation with $^{3}$H$_{3}$-palmitoylcarnitine were suspended in 200 µL milli Q water, all tubes were kept on ice. The mixtures were transferred into glass vials and capped. Subsequently, the vials were heated for 2 minutes at 100°C to inactivate enzyme activity. After cooling the cell mixtures were transferred into clean eppendorf tubes on ice. Cell disruption, centrifugation and filtration of the suspensions were performed equally with control cell line GM00023 of culturing test 2. Aliquots of 20 µL lysate were injected on the column for MRM and neutral-loss LC-MS/MS, furthermore, acylcarnitines were measured in medium samples.

Table 11: Concentrations $^{3}$H$_{3}$-palmitoylcarnitine and L-palmitoylcarnitine in corresponding medium conditions of test 5 for control fibroblasts GM00023 and GM08447. For condition 2, different concentrations of $^{3}$H$_{3}$-palmitoylcarnitine were applied.

<table>
<thead>
<tr>
<th>Condition medium</th>
<th>$^{3}$H$_{3}$-palmitoylcarnitine (µM)</th>
<th>L-palmitoylcarnitine (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>75</td>
<td>0</td>
</tr>
<tr>
<td>2 – GM00023</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>2 – GM08447</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>75</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>103</td>
</tr>
</tbody>
</table>

2.6.5 Culturing test 6, culturing of MCAD deficient fibroblasts with added $^{3}$H$_{3}$-palmitoylcarnitine and L-palmitoylcarnitine

In order to examine possible differences of measured acyl-CoAs and acylcarnitines obtained of fibroblasts with a beta-oxidation disorder, a MCAD deficient cell line was cultured in the presence of deuterium labeled and unlabeled palmitoylcarnitine. Dilutions of neutralized $^{3}$H$_{3}$-palmitoylcarnitine and L-palmitoylcarnitine were prepared in basis
medium (table 12). Initially, 75 cm² culture flasks at 40% confluency of 2 controls and a MCAD deficient (MCADD) fibroblast cell line (GM00023, GM08447, resp. passage 24, 21, and +3 for MCADD), were filled with 15 mL of a medium condition. A negative control containing normal basis medium was cultured for each cell line. The fibroblasts were cultured for 5 days at 37°C in the incubator described previously. Sampling of medium, cell pellet and lysate preparation and the analyses were performed as described for culturing test 5.

Table 12: Concentrations \(^2\)H\(_3\)-palmitoylcarnitine and L-palmitoylcarnitine in corresponding medium conditions of test 6, for control fibroblasts GM00023, GM08447 and the MCADD fibroblast cell line.

<table>
<thead>
<tr>
<th>Condition medium</th>
<th>(^2)H(_3)-palmitoylcarnitine (µM)</th>
<th>L-palmitoylcarnitine (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>75</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>103</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

2.7 Milli Q water or HBSS used as solvent, and stability of acyl-CoAs at 37°C

During sample preparation of cultured fibroblasts, pellets were suspended in HBSS for experiments 1-4. In order to investigate the effect on acyl-CoA signal intensities of HBSS and milli Q water used as solvent, a test was performed with internal standard mixtures prepared in both solvents. Furthermore, the stability of \(^2\)H\(_3\)-C2-CoA, \(^2\)H\(_3\)-C8:0-CoA and \(^2\)H\(_3\)-C16:0-CoA at 37°C was obtained. A mixture of these deuterium labeled acyl-CoAs was prepared in milli Q water and in HBSS, at a concentration of 100 nM. Milli Q water and HBSS have typical pHs of 5 and 7, respectively. Possible effects of the pH in both solvents on obtained acyl-CoA intensities was determined by preparation of a mixture in milli Q water at pH 7 with sodium hydroxide and in HBSS at pH 5 with 10% acetic acid. In a period of 90 minutes, 10 µL portions of the internal standard solutions at 2 pHs were injected on the X Terra RP C\(_{18}\) analytical column with a 30 minutes interval. The acyl-CoA
MRM LC-MS/MS method was applied during this test, with the autosampler temperature set at 37°C.

2.8 Effect of pH and heat on acyl-CoA signal intensities

Residual enzyme activity in fibroblast lysates (paragraph 2.9) could induce the lack of many beta oxidation acyl-CoAs, and the low signals observed, during measurements of culturing experiments 1-4. Several methods can be applied to stop residual enzyme activity in cell lysates, for instance by acidifying or heating of the samples. However, utilizing these methods could cause break down of acyl-CoAs, or affect the measurements sensitivity. To investigate the effect of these methods on signals of acyl-CoAs, pH adjusted internal standard mixtures were measured during this experiment. Furthermore, the stability of acyl-CoAs exposed to a 2 minute heat shock was determined. 100 nM internal standard mixture of \( ^2\text{H}_3\)-C2-CoA, \( ^2\text{H}_3\)-C8:0-CoA and \( ^2\text{H}_3\)-C16:0-CoA in milli Q water was prepared at pH 2 and 4 (with formic acid), 5 (in milli Q water) and 7 (with sodium hydroxide). Additionally, a common enzyme deactivation method at our laboratory was applied, described in Wamelink et al. (2005) [24]. A 150 µL aliquot of a 1 µM internal standard mixture in milli Q water was mixed with 25 µL 5% HClO₄ (pH 1) and stored for 30 minutes at -20°C. Afterwards, the mixture was neutralized to pH 6 with 15 µL 1 M phosphate buffer (pH 11.5). Testing the effect of a high temperature on acyl-CoA stability was performed by heating a 150 µL portion of 1 µM internal standard mixture in milli Q water for 2 minutes at 100°C in a capped glass vial. Mixtures obtained from the enzyme deactivation method and the heat shock test were diluted until a concentration of 100 nM. In a separate experiment, 100 nM internal standard mixtures were prepared at pH 2 and 4 as described earlier and neutralized until pH 5 after 30 minutes with sodium hydroxide. Volumes of 10 µL of the prepared solutions were injected on the XTerra RP C₁₈ analytical column and measured in triplicate with the acyl-CoA MRM LC-MS/MS method.
2.9 Residual enzyme activity in cell lysates

Residual enzyme activity in fibroblast lysates was obtained by interval measurements of added acyl-CoA internal standard (IS) levels, during incubation at 37°C. In order to investigate deactivation of residual enzymes, measurements were performed at 37°C on initially and afterwards added IS mixtures to heat and HClO₄ exposed fibroblast lysates. For these experiments 2 pools were prepared of 2 x 6 control fibroblast cell pellets obtained from confluent 75 cm² flasks, cultured with HAM-F10 nutrient medium (Gibco, life technologies) containing 10% fetal bovine serum and 1% penicillin/streptomycin mixture (v/v). The pellets of pool 1, used for experiment 1 (IS mixture added at start of preparation), were suspended in a total volume of 870 µL milli Q water. The pellets of pool 2, used for experiment 2 (IS mixture added after preparation), were suspended in a total volume of 990 µL milli Q water. For experiment 1 140 µL portions of pool 1 were transferred into 2 eppendorf tubes and in a glass vial, all containing 20 µL 1 µM IS mixture of ²H₃-C2-CoA, ²H₃-C8:0-CoA and ²H₃-C16:0-CoA in milli Q water. A 40 µL volume of milli Q water was added to the cell mixture in one eppendorf tube (not deactivated) and to the glass vial, and mixed. For experiment 2 160 µL portions of pool 2 were transferred into 2 eppendorf tubes and in a glass vial, of which the vial and one eppendorf tube (not deactivated) contained 40 µL milli Q water. Furthermore a blank solution containing 180 µL milli Q water and 20 µL IS mixture was prepared in an eppendorf tube. The mixtures in eppendorf tubes of experiments 1 and 2 without added milli Q water were deactivated with 5% HClO₄ as described earlier. The glass vials were capped and for 2 minutes heat-deactivated at 100°C, after which the mixtures were cooled and transferred into clean eppendorf tubes. Lysation of the cell mixtures was performed by 3x 10 seconds ultrasonication with an ultrasonic homogenizer. All these steps were performed on ice. The disrupted cell suspensions were 5 minutes centrifuged in a cooled ultra centrifuge at 10,000g. Subsequently, the supernatant was transferred into a Ultrafree centrifugal filter (0,22 µm pore size, Durapore-PVDF membrane), and centrifuged for 10 minutes in cooled ultra centrifuge at 4000g. The obtained
supernatants of experiment 1 were transferred into glass vials with inserts, capped and stored at 4°C until the start of analysis. 150 µL supernatant aliquots obtained of experiment 2 were transferred into glass vials containing a suspension of 20 µL 1 µM IS mixture and 30 µL milli Q water, capped, mixed and stored at 4°C until the start of analysis. All samples of experiments 1 and 2 were prepared in duplicate. Portions of 10 µL prepared mixture were several times injected on the XTerra RP C18 analytical column. The acyl-CoA MRM LC-MS/MS method was applied during these experiments, with the autosampler temperature set at 37°C.

3. Results and Discussion

3.1 LC-MS/MS method acyl-CoAs in MRM mode: standard measurements

In order to show differences between the analytical columns, examples of chromatograms obtained after injection of 10 µL standard mixture with the MRM LC-MS/MS methods applied for culturing experiment 1 and 2 are shown in figure 19. The aqueous standard mixture contained 100 nM 2-butenoyl-CoA (C4:1-CoA), octanoyl-CoA (C8:0-CoA) and palmitoyl-CoA (C16:0-CoA). The acyl-CoAs show more retention by the first method, using a Zorbax SB C8 (2.1 x 100 mm, 3.5 µm bead size, Agilent) analytical column. The signals for second method are lower since the eluent flow is split before introduction to the tandem mass spectrometer. Separation of short and long chain acyl-CoAs is comparable for both columns.
3.2 Comparison ammonium acetate concentrations in mobile phase

No major differences could be obtained between the different concentrations NH₄Ac in the mobile phase. A higher concentration NH₄Ac in the mobile phase leads to more stable chromatographic behavior of analytes in the presence of sample matrix. Therefore, the mobile phase containing 15 mM NH₄Ac was used for measurements with the XTerra RP C₁₈ analytical column.

3.3 Ion suppression

An ion suppression effect is visible in the chromatogram, figure 20, obtained after injection of fibroblast lysate during post-column mixing of internal standard. Around 1.5
minutes after injection the signal is disappeared for every internal standard measured. The internal standard mixture was mixed to the eluent for approximately 4.5 minutes during this run, as can be noticed in the chromatograms. Acetyl-CoA, the first eluted acyl-CoA in our method, has a retention time of 2.1 minutes. This result proves that measurements of acyl-CoAs during this study are obtained outside the ion suppression region.

![Chromatograms showing ion suppression](image)

Fig. 20: Measurement of ion suppression during post-column mixing of \( ^2\text{H}_3\text{-C2-}, \ ^2\text{H}_3\text{-C8:0-}, \ ^2\text{H}_3\text{-C16:0} \) acyl-CoA (A/B/C, transitions m/z 813.3-306.1; 897.3-390.3; 1009.5-502.4, resp.). Suppressed ionization visible at 1.5 minutes, post-column mixing was performed until 4.6 minutes.

### 3.4 Culturing test 1 and 2, determination of minimal required L-glutamine and D-glucose levels in culturing medium.

After 3 days culturing (test 1) a coverage was obtained between 40% and 70%. Fibroblasts cultured with medium condition 1, 2 and 6 were deceased at the end of test 1. Culturing with medium condition 3, 4 and 5 resulted in 50-90% coverage, but showed poor cell conditions. Another test was required with higher levels of L-glutamine and D-glucose to increase the fibroblast survival after 6 days culturing. The only signal during measurements of acyl-CoAs was obtained for C2-CoA. Acylcarnitine scans in culturing medium showed signals for C2- and C4:0-carnitine, however, no difference between different conditions was found.
After 1 day culturing (test 2) a coverage was obtained between 50% and 80% for both fibroblast cell lines. For all conditions cell line GM00023 was covered between 80% and 100% at the last day. Fibroblasts of cell line GM08447 were death in condition 1 and 2 after 6 days, while the other conditions were 100% covered. Medium condition 4 (1 mM L-glutamine and D-glucose) was found as optimal for a 6 days culturing test with both cell lines, at minimal concentrations L-glutamine and D-glucose. Clear signals were visible for C2-, C14:0- and C16:0-CoA, as shown in figure 21 for medium condition 4 in fibroblast cell line GM08447. However, these signals were also obtained in a control fibroblast lysate cultured with normal DMEM containing 4 mM L-glutamine and 25 mM D-glucose. No additional signals were found in the neutral-loss scans.

![Chromatograms for C2-, C14:0 and C16:0 acyl-CoA in cell lysates of cultured control fibroblasts GM08447 at medium condition 4 during test 2. Peaks observed resp. at 2.14, 2.69 and 2.91 minutes (Transitions m/z 810.3-303.1; 978.4-471.4; 1006.5-499.4, resp.).](image)

3.5 Culturing test 3, Palmitate and L-palmitoylcarnitine added to culturing medium

After 5 days culturing, fibroblasts cultured with L-palmitoylcarnitine were deceased. The flasks with added palmitate were 100% covered. Flasks of the negative controls were 100% covered, however, cells of GM08447 were partially deceased. Acyl-CoA signals
were obtained for the C2, C14:0 and C16:0 chain length, however, no differences were determined between the incubated cells and the negative controls. No additional results for the acyl-CoAs were obtained by the neutral-loss scans of the cell lysates, compared with MRM results. Acylcarnitine scans showed only enhanced C12- and C14-carnitine signal in medium samples obtained after culturing with L-palmitoylcarnitine, shown in figure 22 for control fibroblasts GM00023.

![Fig. 22: Acylcarnitine scans from culturing test 3 of medium after culturing control fibroblasts GM00023 with L-Palmitoylcarnitine (L), and the corresponding negative control (R).](image)

### 3.6 Culturing test 4, neutralized L-palmitoylcarnitine added to culturing medium

After 3 days of culturing, cells of medium condition 1-3 were deceased. The flask of medium condition 4 was covered for 80% at day 5. In the lysate obtained from cells of this condition, acyl-CoAs of carbon chain C2, C4:0, C14:0 and C16:0 were measured. Additionally, acylcarnitine analogues of the beta-oxidation acyl-CoAs were measured in sampled medium of this condition, indicating conversion of L-palmitoylcarnitine. Both results are shown in figure 23. Measurements in the neutral-loss scan mode showed similar acyl-CoAs compared with the results of the MRM method.
Fig. 23: Chromatograms (L) for C2-, C4:0, C14:0 and C16:0 acyl-CoA in cell lysates of cultured control fibroblasts GM08447 at medium condition 4 during test 4. Peaks observed resp. at 2.13, 2.23, 2.69 and 2.90 minutes. (Transitions m/z 810.3-303.1; 838.3-331.1; 978.4-471.4; 1006.5-499.4, resp.) And the corresponding acylcarnitine scan of the culturing medium (R).

3.7 Culturing test 5, $^{2}$H$_{3}$-palmitoylcarnitine and L-palmitoylcarnitine added to culturing medium

Flasks of all medium conditions were 80-100% covered after 4 days culturing. Acyl-CoA analysis of lysates obtained from incubations with $^{2}$H$_{3}$-palmitoylcarnitine failed during preparation of the samples. Lysates obtained from fibroblasts cultured with unlabeled palmitoylcarnitine showed similar acyl-CoAs as measured in condition 4 of culturing test 4. In acylcarnitine scans of samples medium from condition 1 and 2 labeled acylcarnitine equivalents of beta oxidation acyl-CoAs were measured, showed in figure 24 for control GM00023.

Fig. 24: Acylcarnitine scan from culturing test 5 of medium after culturing control fibroblasts GM00023 with $^{2}$H$_{3}$-palmitoylcarnitine (medium condition 1).
3.8 Culturing test 6, culturing of MCAD deficient fibroblasts with added $^{2}$H$_3$-palmitoylcarnitine and L-palmitoylcarnitine

After 5 days of culturing, all flasks were covered for 80-100%. Acyl-CoA measurements of the fibroblast lysates showed minimal signals for the labeled and unlabeled C2, C4:0, C14:0 and C16:0 intermediates. Based on acyl-CoAs no difference could be obtained between the control and the MCAD deficient cell lines. Acylcarnitine analogues of the beta oxidation acyl-CoAs were determined in medium samples of condition 1 and 2 for the control and MCADD fibroblasts, showed in figure 25. In these scans a difference in patterns is shown, with elevated C8:0-carnitine and the absence of C2- and C4:0-carnitine in both medium conditions of the MCADD cell line.

![Fig. 25: Acylcarnitine scans from culturing test 6, of medium after culturing control GM00023 and MCADD fibroblasts with $^{2}$H$_3$-palmitoylcarnitine and L-palmitoylcarnitine (medium conditions 1 and 2, resp.).](image)

3.9 Milli Q water or HBSS used as solvent, and stability of acyl-CoAs at 37°C

Peak areas obtained of the measurements at 37°C are shown in figure 26 for the different labeled acyl-CoAs. Short and long chain acyl-CoAs solved in milli Q water and
HBSS are stable for at least 90 minutes at 37°C. Decreased signals are observed with $^2$H$_3$-C8:0-CoA solved in HBSS at pH 5 and $^2$H$_3$-C16:0-CoA, solved in HBSS at both pHs. Peak areas obtained for $^2$H$_3$-C2-CoA solved in both solvents show comparable counts at both pHs. In order to enhance signals of long chain acyl-CoAs, cell lysates were prepared in milli Q water (pH 5) during culturing experiments 5 and 6.

![Figure 26: Peak areas obtained during measurements at 37°C of $^2$H$_3$-C2-, $^2$H$_3$-C8:0-, $^2$H$_3$-C16:0 acyl-CoA dissolved in milli Q water and HBSS at different pHs.](image)

### 3.10 Effect of pH and heat on acyl-CoA signal intensities

At pH 2 and 4 lower signals are observed for all acyl-CoAs measured, see figure 27. In the second experiment, data not shown, the signal measured after neutralizing pH 2 remained decreased, although neutralizing of mixtures prepared at pH 4 showed signals comparable with mixtures at pH 5. The internal standard mixtures exposed to the heat shock and HClO$_4$ show comparable peak areas for $^2$H$_3$-C2-CoA and $^2$H$_3$-C8:0-CoA. However, $^2$H$_3$-C16:0-CoA signals were decreased after exposing to heat and HClO$_4$. The latter 2 methods were selected to examine deactivation of residual enzyme activity.

![Figure 27: Measured peak areas of a 100 nM mixture of $^2$H$_3$-C2-, $^2$H$_3$-C8:0-, $^2$H$_3$-C16:0 acyl-CoA, treated with different enzyme deactivation methods (pH, heat and HClO$_4$).](image)
3.11 Residual enzyme activity in cell lysates

In not deactivated fibroblast lysates a decreasing signal is visible for all tested acyl-CoAs, indicating residual enzyme activity, shown in figure 28A for experiment 2. $^2$H$_3$-C2-CoA measured in samples exposed to HClO$_4$ is more stable at 37°C in both experiments, shown in figure 28B for experiment 1. $^3$H$_3$-C8:0-CoA and $^3$H$_3$-C16:0-CoA exposed to HClO$_4$ show comparable or elevated peak areas at the initial measurement compared with not deactivated lysates of the same experiment. However, at 37°C the signals of these acyl-CoAs are also decreasing. The most stable signals were obtained in lysates exposed to heat, shown in figure 28C for experiment 1, indicating the best enzyme deactivation method for this study. This deactivation method was applied during sample preparation of culturing experiment 5 and 6.

Fig. 28: Measured peak areas in non treated (A), and HClO$_4$ and heat shock treated (A/B resp.) control fibroblast lysates for $^2$H$_3$-C2-, $^2$H$_3$-C8:0-, $^2$H$_3$-C16:0 acyl-CoA at 37°C.
4. Conclusions

A LC-MS/MS method is developed which separates low (C2) from high (maximal C16) carbon chain length acyl-CoAs in the LC-section. Subsequently, MS/MS was operated in the MRM and neutral loss scanning mode with positive ionization. In order to allow energy uptake via the beta-oxidation pathway, a fibroblast culturing method was setup using medium with low L-glutamine and D-glucose levels. Additional palmitate in the culturing medium showed no enhanced acylcarnitine analogues of beta-oxidation acyl-CoA intermediates. However, addition of L-palmitoylcarnitine and the $^3$H$_3$-variant resulted in clear signals of these analogues in culturing medium, indicating conversion by the beta-oxidation pathway. Nevertheless, not all acyl-CoAs, corresponding to the observed acylcarnitines, were determined during measurements of the cell lysates.

Residual enzyme activity in fibroblast lysates was shown by periodical measurements of samples with added $^3$H$_3$-C2-, $^3$H$_3$-C8:0- and $^3$H$_3$-C16:0 acyl-CoA at 37°C. A two minute heat shock of the samples was the best to method to deactivate the enzymes. However, during the last 2 culturing experiments, measurements of acyl-CoAs in cell lysates obtained after preparation including the heat shock procedure, showed no high acyl-CoA signals. Comparison of acyl-CoAs determined in cell lysates of control and MCAD deficient fibroblasts showed low signals and no difference between cell lines. In culturing medium samples of these cells, a clear difference was shown in acylcarnitine pattern. This indicates that at this moment, measurement of acylcarnitine levels in culturing medium of L-palmitoylcarnitine incubated fibroblasts is a better method for diagnosis of a beta-oxidation disorder. Additional investigation on preparation of cultured fibroblasts, in terms of direct enzyme deactivation during harvesting of cells, could possibly improve the method with measured acyl-CoAs. Furthermore, the developed incubation method using L-palmitoylcarnitine as substrate is not appropriate for detection of CPT1 deficiency.
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Appendix – Biochemical backgrounds

Appendix 1: One carbon metabolism

Methionine and homocysteine are important intermediates of the one carbon metabolism, which is subdivided into three processes: methylation, transsulphuration and remethylation. Methylation initiates with methionine, which is an essential α-amino acid characterized by a sulphur atom in its molecular backbone. Methionine can be converted into SAM by the enzyme methionine adenosyltransferase, in the presence of adenosine triphosphate. SAM functions as a methyl donor in many transmethylation reactions of compounds as for instance DNA, RNA, neurotransmitters and phospholipids. After donation of one methyl group SAH is yielded. SAH is hydrolyzed to homocysteine in a reversible reaction with SAH-hydrolase. Subsequently, homocysteine can either be remethylated to methionine or transsulphurated to cysteine. Remethylation of homocysteine mainly occurs via the enzyme methionine synthase, which is part of the folate metabolism [10]. One of the conversions in this pathway is the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which is catalyzed by methylenetetrahydrofolate reductase (MTHFR). In the homocysteine metabolism the enzyme MTHFR plays a key role during regulation. Another route for remethylation is via the betaine-homocysteine methyltransferase enzyme, which converts homocysteine in dimethylglycine and methionine.

The first transsulphuration step of homocysteine consists of the irreversible condensation with serine to cystathionine and is catalyzed by cystathionine β-synthase. Subsequently, cystathionine is cleaved into cysteine and α-ketoglutarate. Cysteine is further converted into glutathione, taurine and sulphate. In figure 29 the methionine-homocysteine pathway is shown [25].
1. SAM and SAH

SAM-dependent methyltransferases, which are involved in transmethylation reactions, can be inhibited by SAH. Under normal conditions this inhibition is negligible since the intracellular concentration of SAH is low. However, if homocysteine levels are increased reverse hydrolysis can lead to elevated intracellular SAH and decreased activity of SAM-dependent methyltransferases. Aberrant SAM and SAH concentrations are found in patients suffering from enzyme defects in the one carbon metabolism. Additionally, several studies have been performed which suggest the relation of altered SAM and SAH levels with other disorders such as HIV and neurological disease.

The diagnosis of the known SAM/SAH related diseases, and the research in other SAM/SAH associated diseases, requires a rapid and sensitive analytical method to measure SAM and SAH in body fluids. The applied analytical method at our laboratory is based on a sample preparation by solid-phase extraction (SPE) followed by a chromatographic separation coupled to MS/MS detection operating in the MRM mode with positive ionization [10].
2. Homocysteine and methionine

Methionine and homocysteine levels are measured on the LC-MS/MS during many studies involving the one carbon metabolism at our laboratory. Prior to the LC-MS/MS measurement the samples are cleaned by a cation exchange SPE. After a LC separation methionine and homocysteine are detected by MS/MS in the MRM mode with positive ionization [18].

Appendix 2: D- and L-2-hydroxyglutarate

D- and L-2-HG are enantiomeric compounds, and normal components of the urine. In the cell metabolism D- and L-2-HG are derivatives of mitochondrial 2-ketoglutarate (2-KG), an intermediate of the tricarboxylic acid (TCA) cycle. To date the role of both metabolites in the human metabolism is still unclear. The conversion of 2-KG into D-2-HG is catalyzed by the enzyme hydroxyacid-oxoacid transhydrogenase, whereas L-2-HG is the product of a non-specific reaction of L-malate dehydrogenase with 2-KG. D- and L-2-HG are both converted back into 2-KG via either D-2-hydroxyglutatarate dehydrogenase enzyme (D-2-HGDH) or L-2-hydroxyglutatarate dehydrogenase enzyme (L-2-HGDH), respectively, to overcome carbon loss in the TCA cycle and toxic effects of D- and L-2-HG. Several disorders are described for the pathways of D- and L-2-HG [26]. One of these diseases is D-2-hydroxyglutaric aciduria (D-2-HGA), which is subdivided in type I and II disorder. In the VUmc metabolic laboratory decreased activities have been shown for D-2-HGDH in lymphoblasts and fibroblasts of type I patients. Cells obtained from patients suffering from D-2-HGA type II have been demonstrated to have normal enzyme activities (Wickenhagen et al. (2009) [27]; Kranendijk et al. (2010) [28]). The accumulation of D-2-HG in D-2-HGA type II is the result of heterozygous mutations in isocitrate dehydrogenase 2 (IDH2). Wild type IDH2 converts isocitrate to 2-KG and vice versa, whereas the IDH2wt/R140Q-mutant catalyzes the pathway from 2-KG to D-2-HG. Both D-2-HGA subtypes demonstrate D-2-HG accumulation, biochemically a distinction can be observed by the levels of D-2-HG. The accumulation of D-2-HG in D-2-HGA type
II is more pronounced compared with type I, since an elevated reaction velocity of the IDH2\(^{WT/R140Q}\)-mutant raises above the reverse enzyme activity of D-2-HGDH.

Another disease is L-2-hydroxyglutaric aciduria (L-2-HGA), which is biochemically presented by impaired L-2-HGDH induced L-2-HG accumulation. In figure 30 an overview is shown of the described conversions of 2-KG into D- and L-2-HG and reverse. Additionally, cases of combined D,L-2-hydroxyglutaric aciduria (D,L-2-HGA) have been discovered. Furthermore, several other disorders have been published showing elevated D- and L-2-HG, such as: neoplastic disorders with IDH mutations, skeletal dysplasia, glutaric aciduria type II, succinic semialdehyde dehydrogenase deficiency and miscellaneous diseases related with 2-hydroxyglutarate [26].

To diagnose defects in the D- and L-HG metabolism and to investigate the relation of these metabolites in other diseases, a fast and sensitive LC-MS/MS method is applied. The method is based on a chiral derivatisation of D- and L-2-HG with diacetyl-L-tartaric anhydride (DATAN). The prepared samples are measured by LC-MS/MS, operating in the MRM mode with negative ionization [15].

![Fig. 30: Overview of different enzymatic conversions from 2-KG to D- and L-2-HG and reverse, see text for explanation [26].](image)

**Appendix 3: Sialic acid**

Sialic acid (SA), or N-acetylneuramic acid, is synthesized from glucose by several enzymatic steps in the cytosol. SA is converted in cytidine monophosphate-SA, which is part of silylation reactions of oligosaccharides (OGS) in the golgi apparatus. Subsequently, SA is released after breakdown of these sialyl-OGS in the lysosome.
Several lysosomal enzyme defects cause bound or free SA accumulation, which is released into the body fluids. Elevated levels of SA in the body fluid are markers to screen for certain lysosomal disorders. A defect of the lysosomal enzymes β-galactosidase and α-neuraminidase causes galactosialidosis, a disorder with increased bound SA levels. These two enzymes form a complex with protective protein/cathepsin A (PPCA), and are involved in the hydrolysis of sialyl-OGS. The impairment of these enzymes is a secondary effect of affected PPCA, and causes the progressive storage of sialyl-OGS in the lysosome. Another deficiency showing these elevated bound SA levels is sialidosis, which is the result of defect α-neuraminidase only.

The transport of SA through the lysosomal membrane is performed by the protein sialin. If this protein is impaired, it shows infantile onset in the disorder called infantile free sialic acid storage disease (ISSD), while a later onset is observed for Salla disease. Since the accumulation of free SA is observed in these diseases, they can be distinguished with the (galacto)sialidoses, in which bound SA is progressively stored. However, if elevated free SA in CSF is observed this can be an indication for the disease cerebellar ataxia with elevated CSF free sialic acid (CAFSA) [25] [29].

For the diagnosis of SA related disorders, a LC-MS/MS method is applied to measure SA in urine, CSF and amniotic fluid. In order to measure total SA (free and bound), a hydrolysis is performed with a sulfuric acid solution. The prepared samples are measured by LC-MS/MS, operating in the MRM mode with positive ionization [16].
References


