# You Are What You Eat

**Mass spectrometry in paediatric kinetic studies using stable isotopes**

# Je bent wat je eet

**Massa spectrometrie in pediatrische kinetiek studies met behulp van stabiele isotopen**

**You are what you eat - Mass spectrometry in paediatric kinetic studies using stable isotopes**

**Thesis, Erasmus University, Rotterdam, The Netherlands**

The work presented in this thesis was performed at the Division of Neonatology, Department of Paediatrics, Erasmus MC, University Medical Center, Rotterdam, The Netherlands.

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Mass spectrometry in paediatric kinetic studies
using stable isotopes

## Je bent wat je eet

Massa spectrometrie in pediatrische kinetiek studies
met behulp van stabiele isotopen

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ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
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CHAPTER

# 1

## General introduction, outline, and aims of the thesis

Partly based on:

H.Schierbeek, CHP van den Akker, LB Fay and JB van Goudoever
Mass Spec Rev
Submitted 2009

## Introduction

Historically, attempts at unravelling metabolic pathways in health and disease made use of the study of metabolites such as amino acids, proteins, carbohydrates and fats. Occasionally the nitrogen or energy balance was studied as well. The emphasis though was largely on concentration measurements, which may not necessarily provide useful information when kinetic data are absent. A more dynamic insight is obtained from the quantification of utilization and synthesis rates. This is most reliably done with the use of isotopic tracers. In paediatric research, however, we must restrict ourselves to non-invasive techniques – so radioactive tracers are not allowed. We may nevertheless use stable isotopes almost without restrictions as they are harmless and already present in the human body in small amounts. Furthermore, high precision mass spectrometry will quantify very low amounts of stable isotopes, so we need to administer only small doses of tracer. Precise and accurate measurements can be performed in small volumes, which is of great benefit in paediatric studies.

In paediatric research, stable isotopes are mainly applied in metabolic in vivo kinetic studies. Here, mass spectrometry has proven to be an essential tool in a wide range of research disciplines related to intestinal diseases, obesity, severe cerebral palsy, oxidative stress and foetal metabolism[1-11]. Most of these studies dealt with the interaction between the exogenous influx of nutrients via the diet and the endogenous metabolism of the same or related metabolites. Endogenous metabolism is regulated and affected by an exogenous flux into the pool. Stable isotopes in combination with mass spectrometry can be used to study the level of regulation and affection. Several metabolic interactions occur in metabolic compounds such as carbohydrates, peptides, fats, proteins and amino acids. These interactions include synthesis, oxidation, excretion or conversion into metabolic products. Due to the diversity and complexity of the different metabolites involved in these studies, there is a great need for sophisticated mass spectrometric instruments. The different types of these instruments will be discussed here as well as their application in paediatric research.

## Stable Isotopes

The term "isotope" is derived from the Greek words "isos", which means equal, and "topos", which means place. Stable isotopes are elements sharing the same place in the periodic table. They have the same chemistry, i.e. the same number of protons, but differ in their atomic mass due to different number of neutrons (Table 1). Different stable isotopes can be isotopomers (having the same number of each isotopic atom but are only differing in their positions) or isotopologues (differ in their isotopic composition) according to the "International Union of Pure and Applied Chemistry" (1994).

The major benefit of stable isotopes lies in the fact that they are non-radioactive and therefore present no risk when used in human in vivo studies. Common organic elements such as H, C, N, S and O each have a stable isotope counterpart such as $^{2}H$, $^{13}C$, $^{15}N$, $^{32}S$ and $^{18}O$.

***Table 1*** *List of common organic elements such as H, C, N, S and O with their isotope counterpart such as $^{2}H$, $^{13}C$, $^{15}N$, $^{32}S$ and $^{18}O$.*

| Element | *m/z* | %Natural Abundance |
|---|---|---|
| $^{1}H$ | 1.008 | 99.985 |
| $^{2}\mathbf{H}$ | 2.014 | 0.015 |
| $^{12}C$ | 12.000 | 98.89 |
| $^{13}\mathbf{C}$ | 13.003 | 1.11 |
| $^{14}N$ | 14.003 | 99.63 |
| $^{15}\mathbf{N}$ | 15.000 | 0.37 |
| $^{16}O$ | 15.995 | 99.759 |
| $^{17}O$ | 16.999 | 0.037 |
| $^{18}\mathbf{O}$ | 17.999 | 0.204 |
| $^{28}Si$ | 27.977 | 92.21 |
| $^{29}Si$ | 28.976 | 4.70 |
| $^{30}Si$ | 29.974 | 3.09 |
| $^{32}S$ | 31.972 | 95.05 |
| $^{33}S$ | 32.968 | 0.76 |
| $^{34}S$ | 33.967 | 4.22 |

**Isotopic enrichment**

Mass spectrometry analyses yield the ratio of the labelled compound fragment versus the unlabeled compound fragment: the tracer / tracee ratio (TTR), also called the relative enrichment. The absolute enrichment is calculated from the TTR values of the naturally enriched sample (TTR0) and tracer enriched sample (TTRs) expressed as the molar percent excess (MPE).

To this end, TTR is first corrected for the natural abundance of the tracer:

TTRs -TTR0 = TTRe

MPE = TTRe/(1- TTRe) X 100

In isotope ratio mass spectrometry (IRMS), however, the isotopic enrichment is obtained by determining the ratio of the heavy atom to the light atom isotopes in the sample. The isotopic abundance of a sample is always calculated relatively to a reference. The variation in isotopic ratio at natural abundance is so small that it often amounts to more than the third or fourth significant digit after the decimal point. Therefore, variation of isotopic ratio relative to the $\delta^{13}C$ value is expressed in per mil (‰). The delta ‰ notation is defined as $\delta\ ^{13}C_{\ sample} = [(R_s / R_{st}) - 1] \times 1000$, where $R_s$ is the ratio of $^{13}C$ in the sample and $R_{st}$ is the ratio of the international standard used.

The result of this calculation is a relative δ calibrated against the international standard. The reference standard material used to be PDB, the acronym for Pee Dee Belemnite (, a carbonate from a rock in South Carolina, U.S.A.. This has long been exhausted, however, and was replaced by another carbonate with the same isotopic ratios for $^{13}C$ and $^{18}O$ isotopes, i.e. VPDB (i.e. Vienna PDB)[12-13]. The practical advantage of using the $\delta^{13}C$ (‰) notation instead of the $^{13}C/^{12}C$ ratio (TTR) notation is that small variations after the fifth digit after the decimal point are easier to handle. As an example, a sample with $\delta^{13}C$= -1‰ corresponds to a TTR of 0.0112260 ($R_{vpdb}$ = 0.0112372) and a sample with $\delta^{13}C$= -3‰ corresponds to a TTR of 0.0112036. A negative $\delta^{13}C$ value indicates that the molecule is depleted in the heavy isotope relative to the standard. On the other hand, a positive $\delta^{13}C$ value indicates that the molecule is enriched in the heavy isotope relative to the standard.

From the $\delta^{13}C$ value the Atom Percent (AP) can be calculated:

$$AP = \left[ \frac{100 \times R \times \left( \left( \delta 13C / 1000 \right) + 1 \right)}{1 + R \times \left( \left( \delta 13\mathrm{C} / 1000 \right) + 1 \right)} \right]$$

where R is the ratio of ($^{13}C/^{12}C$) of International Standard of Pee Dee Belemnite, R=0.0112372.

To calculate the Atom Percent Excess, which is an absolute measurement of the isotopic enrichment, AP (background) is subtracted from AP (sample). APE can subsequently be transformed in MPE (Molar Percent Excess) using the following formula: MPE = APE x ($C_{Total}/C_{labelled}$), where $C_{Total}$ is the total number of carbon atoms in the molecule and $C_{labelled}$ is the number of labelled carbon atoms within the molecule. It is usually the MPE that is used in calculations for kinetic studies.

**High precision compound-specific GC/LC/(IR)MS analyses**

Isotope ratio mass spectrometry (IRMS) and organic mass spectrometry (MS) are the two most mature techniques for isotopic analysis of compounds. The first approach to GC online combustion MS (GC/C/IRMS) was reported by Sano et al as early as 1976[14]. $CO_2$ was still measured, however, with a conventional magnet instrument with multiple ion detection. Nevertheless Sano's group was able to study the metabolic pathway of administered $^{13}C$ labelled aspirin in humans. In 1978 Matthews and Hayes published the first paper on measurements with a computer-controlled beam switching IRMS. They managed to measure carbon and nitrogen ratios at natural levels. Isotopic enrichments in complex mixtures could also be determined with Gas Chromatography Mass Spectrometry (GCMS) and selected ion monitoring (SIM).This technique, applied for more than twenty years in many metabolic tracer studies, is suitable for quantification as well[15].

Regarding sample introduction, gas chromatography (GC) coupled to either IRMS or MS is state-of-the-art technique for compound-specific isotopic analysis of volatile compounds. However, liquid chromatography (LC) can be considered as a tool for the sample introduction into IRMS or MS for$^{13}C$ isotopic analyses of non-volatile compounds at natural abundance as well as for$^{13}C$-labelled compounds.

**LC/MSMS**

Although GCMS is much more used for measurement of isotopic enrichment, LC coupled to MS (or: LC/MS) – and especially LC/MSMS with electro spray injection (ESI)(Figure 1) – has proven to be suitable for numerous applications and has advanced rapidly since its development in the 1970s. Its sensitivity of detection can reach very low levels. In environmental studies, the sensitivity level is in the nanomole (nM) range and in proteomics analyses a few femtomoles of peptide can be routinely detected.

Because the LC/MSMS system measures the characteristic ions of each component separately, there is no need for chromatographic baseline resolution. The run time is much shorter therefore. The system uses a shorter analytical column with a narrower bore and a reduced mobile-phase flow, thereby also reducing solvent and reagent usage. It also allows the measurement of characteristic daughter ions generated from the primary ion for each compound, which enormously increases the signal noise ratio and the specificity.

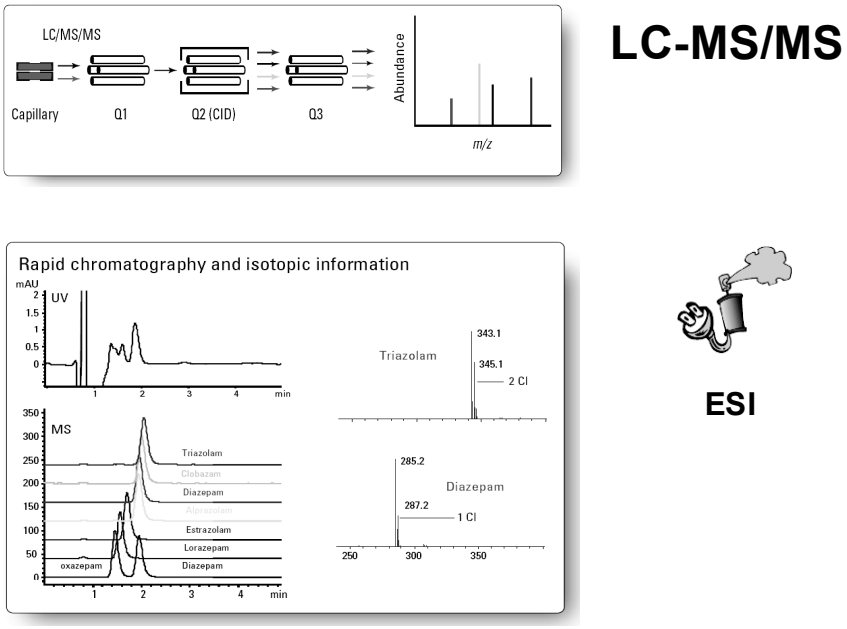


***Figure 1*** *Schematic overview of LC/MSMS device using electro spray injection.*

**GC/MS**

For decades, GC/MS has been the state of art to determine enrichments of stable isotopes at a relatively high enrichment level. The biggest limitation is the relatively high low-level limit of quantification (LOQ) necessary for precise measurement. Typically the enrichment level should exceed 0.5% APE. However, multiple labelled substrates could be used. In these applications the natural isotopic abundances are very low and enrichment levels down to 0.1% APE could be measured accurately. The metabolites are separated by gas chromatography, ionized in the ion source and fragmented to charged fragments with different masses. The produced fragments are focused and separated by a magnetic field according to their mass charge ratio (*m/z)*. Fragmentation may result in the loss of one or more labelled atoms. Selected ion monitoring (SIM) (Figure 2) requires the choice of a suitable charged fragment containing the labelled tracer so that the isotopic distribution of the fragment can be measured. This isotopic distribution is dependent on the elemental composition of the fragment and consists of the intensity of mass M+0, M+1, M+2, et cetera. M+0 represents the probability that all elements are present as the most abundant lightest species ($^{12}C$, $^{1}H$, $^{16}O$, $^{14}N$). M+1 is one mass unit higher; representing the situation that one higher isotope is present. The abundance of M+1 is mainly determined by the contribution of $^{13}C$, being 1.1% per C-atom. A molecule containing 10 C-atoms will show a

M+1 contribution of about 11 (10x 1.1%)%. M+2 represents the probability that 2 $^{13}$C-atoms are present and its contribution (1%) is much lower than that of M+1. Then, M+3, M+4 and so on follow in decreasing abundances based on the probability that 3, 4 and so on $^{13}$C atoms are present.

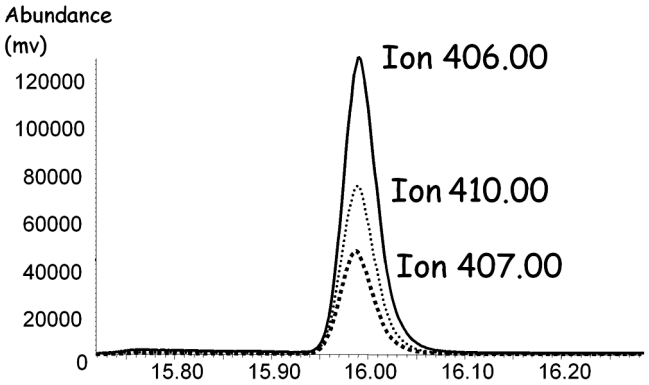


***Figure 2*** *Example of a SIM analysis. Three different ion signals were selected for determination of the enrichment and concentration of cysteine.*

Using the GC/MS technique in SIM has many advantages. The enrichment measurements are independent on the type of isotope. Derivatization does not reduce the level of enrichment although it may affect the baseline isotopic abundance as is the case in silicon-containing derivatives. Silicon has high abundant higher mass isotopes.

The two major advantages of GC/MS are its very sensitivity in terms of required amount of metabolite necessary for accurate measurement and the possibility of simultaneous measurement of multiple tracers. Pico gram quantities are often sufficient, so the technique can be applied at low metabolite concentration levels n small samples. The sensitivity can even be manipulated through type of derivatization and ionization technique. Multiple isotope labelling of the substrate does not increase the enrichment of the metabolite, but moves the measurement to a higher isotopologue with a lower natural background. This improves the signal to background ratio in enriched samples, which enables a more accurate measurement. The relatively high low-limit enrichment needed for precise and accurate measurement (>0.5MPE) is the major drawback of GC/MS.

**GC/C/IRMS**

In general, compound specific isotope analysis in biological matrices at low enrichment level will make use of GC interfaced to isotope ratio mass spectrometry through a reactor interface. In case of $^{13}$C abundance measurements, organic compounds are combusted on-line to $CO_2$ and water with a capillary combustion furnace (GC/C/IRMS) (Figure 3). The addition of

a reduction furnace provides for $^{15}N/^{14}N$ isotope ratio measurements of nitrogen-containing compounds such as amino acids.

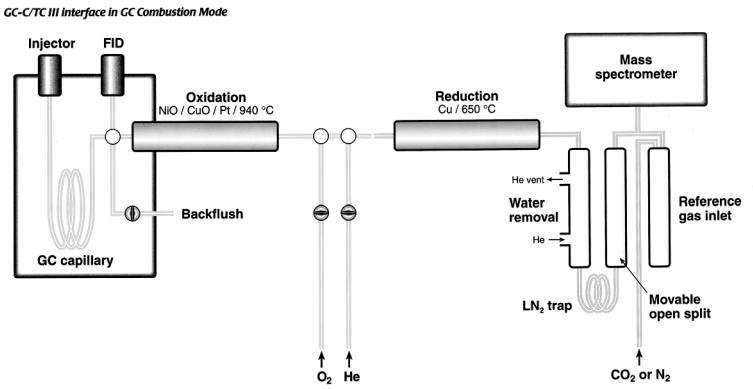


***Figure 3*** *Schematic overview of GC/C/IRMS device. After separation by GC the components are thermally oxidized. After separation of water vapour the components are introduced into the source of the IRMS by means of an open split device.(Thermo fisher, Bremen, Germany)*

Deuterium and $^{18}O$ enrichment in metabolites can be determined using a high temperature pyrolyis interface (GC/HT/IRMS) converting the metabolite to carbon monoxide and hydrogen. On-line transfer of produced hydrogen and $^{18}O$ into the IRMS allows measurement of $^{2}H/^{1}H$ isotope ratios and $^{18}O/^{16}O$. The on-line technique coupled to IRMS permits measurement of low enrichment levels, though relatively much metabolite is needed to assure accuracy. The required amount of metabolite is determined by the number of isotope atoms in the molecule and the natural isotope abundance of the heavy isotope. Carbon is prominently present in organic molecules and the $^{13}C$ natural abundance is near 1.1 at%. This supports a sensitive method in terms of the required amount of material. Roughly 5 ng carbon is the lower limit for accurate $^{13}C$ determination. Hydrogen is also prominently present but the natural abundance of 0.015 at% is very low. The minimally required amount of organic metabolite is around 100 ng. The natural abundance of $^{15}N$ is intermediate (0.34 at%) but organic molecules like amino acids normally contain no more than one or two nitrogen atoms. Therefore at least 200 ng organic substance is needed for accurate $^{15}N$ measurements. GC/C/IRMS and GC/HT/IRMS normally require derivatization, which might lead to isotope fractionation and dilution of the enrichment.

**LC/IRMS**

A new development is the introduction of commercial instrumentation for LC/IRMS. This opened the way to $^{13}C$ isotope abundance measurements of metabolites after separation on high pressure liquid chromatography (HPLC) or direct injection (Fig 4). The LC/IRMS device became commercially available in 2004[16] after several other experiments over the last decade to link (or hyphenate) LC to IRMS[17-21].

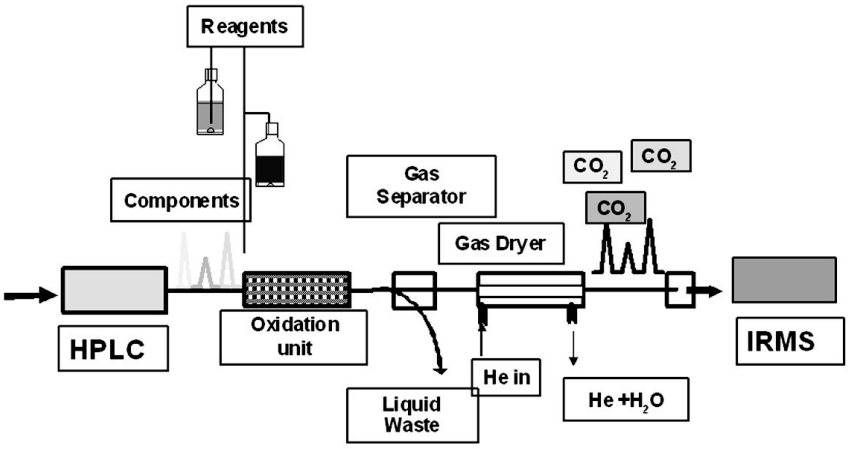


***Figure 4*** *Schematic overview of LC/IRMS device. Samples separated by LC are transferred to $CO_2$ by means of chemical oxidation. $CO_2$ is transported over a membrane using a He flow and introduced into the IRMS system.*

This new device is based on a wet chemical oxidation process and presents few analytical constraints interfering with LC method development. Typically the LC flow rate must be lower than 600 µL/min, organic buffers are prohibited and the presence of salt and low pH facilitates $CO_2$ extraction. Various LC separations were developed for targeted compounds such as underivatized amino acids[22-23] and underivatized carbohydrates[24].

The past three years have seen applications in various research areas including paediatrics[25-33]. The publications show the power and robustness of LC/IRMS in analyzing amino acids, peptides, carbohydrates, fatty acids and volatile fatty acids in biological matrices. So far the technique provides for combustion and $^{13}C$-analysis and only 5 ng of carbon is needed to measure enrichments accurately

**FIA/IRMS**

The above mentioned LC/IRMS system can also be used without column separation. Samples are then directly injected into the flow path of the system to determine the bulk $^{13}C$ abundance. Accurate measurement requires 5 to 10ng of carbon.

**TC/EA/IRMS**

In the classical methods, water is isolated from biological fluids by cryo-distillation and converted into $HD/H_2$ and $C^{18}O_2/C^{16}O_2$ gases[34]. Hydrogen is produced through reduction of the collected water using for example zinc, platinum or manganese[35-41]. The $C^{18}O_2/C^{16}O_2$ gases are produced by water equilibration overnight with un-enriched $CO_2$ gas to achieve $^{18}O$ enrichment in $CO_2$ gas similar to that present in the water samples. These doubly labelled water ($D_2{}^{18}O$) techniques are laborious, time-consuming and require relatively voluminous samples (a few hundred microliters or even a few millilitres). Meanwhile, new methods have been developed for measuring deuterium and $^{18}O$ enrichments[42-43].

The most recent system for the analysis of $^2H/^1H$ enrichments is the thermal conversion elemental analyzer (TC/EA), which converts micromoles of water sample into $H_2$ by reaction in a glassy carbon tube reactor at 1420ºC[42-46].This system was used to measure $^2H/^1H$ and $^{18}O/^{16}O$ in plasma samples of rats[42]. The procedure is largely automated, so that samples can be analyzed routinely. The major disadvantages of TC/EA are its high purchase price and costs of consumables, as well as the need to repack the ceramic tube with glassy carbon chips after every 300–400 samples. On the other hand, it is well suited for simultaneous determination of the enrichment in deuterium and $^{18}O$ of urine and saliva samples in paediatric studies – i.e. in terms of non-invasive sample collection, smaller sample size, and minimal sample preparation.

**Metabolic kinetic studies**

In life sciences the commercial availability of labelled precursors has increased the number of stable isotope applications in various disciplines such as medicine, nutrition, and metabolic studies. Stable isotopes are preferred to radioisotopes as they are safer, do not emit radiation, and are stable. This is particularly relevant for paediatric, in vivo, metabolic studies. On the other hand, the use of stable isotopes requires expensive mass spectrometers and trained staff. This may explain why stable isotopes have not yet replaced radioisotopes in all applications.

Being the most precise, sensitive, and accurate for analysis of most elements in the periodic table, mass spectrometry (MS) is today considered the best technique to measure isotopic ratios and enrichments in paediatric kinetic studies. However, for light elements (e.g., C, H, N, and O) the most suitable device for the isotopic ratio acquisition is the isotope ratio mass spectrometer (IRMS) (Table 2). Typically, the low limit analytical range for isotopic precision (measured as APE) of IRMS is in the order of $10^{-4}$ % enrichment. For MS it is in the order of $10^{-2}$ % enrichment. This means that

the absolute $^{13}C/^{12}C$ ratio determination in the MS in the single ion-monitoring mode (SIM) must be higher than 0.05%. Using the same scale, the IRMS isotopic ratio can be precisely measured as 0.0002%. This illustrates on the one hand the ''high-precision'' domain covered by the IRMS, and on the other hand the ''low precision'' domain covered by the MS. Still we must consider the limits of quantification (LOQ) of both systems. The LOQ for IRMS is in the low nmol range whereas for MS it is in the high fmol range. Furthermore, the method of measurement differs between the two techniques. MS measures isotopic ratios of ionized molecules, whereas IRMS measures the $^{13}C/^{12}C$ isotopic ratio after conversion of organic molecules into ionized $CO_2$. This implies that in IRMS the selectivity is only established by the separation method. In MS the detector is also selective for the compound to be measured. Baseline resolution of components is mandatory with IRMS and co elution of compounds is a minor issue in MS.

***Table 2*** *Comparison of the MS / IRMS techniques*

| MS | IRMS |
|---|---|
| **Large mass range: 1 - +2000 m/z.**<br><br>**Structural information of each compound.**<br><br>**Compound dependent detection.**<br><br>**Measurement of fragment masses possible.**<br><br>**Measurable Atom% range: 0.1% - 100%**<br><br>**Minimum detectable sample amount for good precision: Picogram range**<br><br>**Precision: +/- 0.2%** | **Limited mass range: 1-150 m/z.**<br><br>**No structural information possible.**<br><br>**Compound independent detection.**<br><br>**Measurement of the sumof one element.**<br><br>**Measurable Atom% range: 0% - 20%.**<br><br>**Minimum detectable sample amount for good precision: Nanogram range.**<br><br>**Precision:+/- 0.0002%** |

Commonly most of the IRMS applications are related to determinations of natural abundance variations and metabolic studies using stable isotope-enriched tracers. For tracer studies, the ranges of precision of both molecular MS and IRMS techniques are complementary because they measure isotopic enrichment in various physiological pools presenting different isotopic dilutions and different ranges of isotopic values. For ''high precision'' $^{13}C$

isotopic ratio measurements, GC coupled to IRMS (GC/C/IRMS) is still the state of the art technique. Two issues have long limited the association of LC with magnetic sector instruments. These are (a) lack of an efficient step for solvent removal as organic solvents will result in very high baseline background signal; and (b) lack of an efficient step for analytic transformation into $CO_2$ and transportation to the MS source gas without any isotopic effect. Due to these limitations, the interface enabling LC coupled with IRMS was only commercialized in 2004.

The chemical diversity and concentration in physiological fluids of the targeted molecules (from macro molecules to small molecules, from hydrophobic to hydrophilic, and from acidic to basic molecules) in complex biological samples faces the analytical chemist with the question when to use which MS technique (Table 3).

**Study designs**

The principles of metabolic kinetic studies using stable isotopes have been described extensively by Wolfe[47], including the different manners of tracer administration like single bolus and primed continuous infusion techniques. Bolus administration is recommended in a single pool under ideal conditions. The TTR will first increase and then gradually decrease over time. The most popular tracer administration technique is primed continuous infusion technique (Figure 5). The continuous tracer infusion is preceded by administration of a small bolus. Under these conditions a steady state (Figure 6)can be quickly reached and kinetic parameters can be determined with only limited sampling, which is of benefit in paediatric studies.

An additional technique for tracer administration is the so-called staggered infusion protocol. Pertaining to all living species, and notably to premature infants, the number of samples must be as low as possible and is limited to only one sample e.g. at surgery or when scarifying an animal. One strategy to achieve this is the use of different tracers with similar metabolic characteristics in an overlapping – or staggered – infusion protocol. Dudley et al., for example, aimed to measure the FSR of lactase phlorizin hydrolase in porcine gut mucosa[48]. At six consecutive time points they started a primed continuous infusion of different stable isotopomers (2 of leucine and 4 of phenylalanine). In this concept, a single biopsy is taken after the enriched precursor pool of the last infusion is assumed to have reached steady state. Precursor steady state can be confirmed as the isotopomers within each amino acid should be equal. In combination with the enrichments of all infused isotopomers in the product, which theoretically should increase with the duration of infusion, the FSR can thus be calculated. Apart from documenting feasibility, these researchers showed that the multiple tracer, single sample approach gave the same results as the conventional single tracer, multiple consecutive samples method.

***Table 3*** *Comparison of the different MS techniques used for applications in pediatric research.*

| | GC/MS | LC/MSMS | G/C/IRMS | LC/IRMS | FIA/IRMS | TCEA/IRMS | IRMS |
|---|---|---|---|---|---|---|---|
| **LOQ** | 100 pg | 1 pg | 5 ηg | 5 ηg | 5 ηg | 5 ηg | 1 μg |
| **Range % APE** | 0.1-100% | 0.1-100% | 0.0005-20% | 0.0005-20% | 0.0005-20% | 0.0005-20% | 0.0001-20% |
| **Precision** | 0.2% | 0.2% | 0.0002% | 0.0002% | 0.0002% | 0.0002% | 0.00002% |
| **Application** | Volatile components | Volatile and non volatile components | Volatile components | Volatile and non volatile components | Volatile and non volatile components | Water | CNOS Gasses |
| **Robustness** | +++++ | ++++ | ++++ | +++ | ++++ | ++++ | +++++ |
| **Sample introduction** | Liquid | Liquid | Liquid | Liquid | Liquid | Liquid | Gas |
| **Costs instrument** | 50-100 K€ | 200-250 K€ | 180-220 K€ | 200 K€ | 200-250 K€ | 200-250 K€ | 50-100 K€ |
| **Costs analysis** | 8€ | 15€ | 12€ | 12€ | 10€ | 12€ | 5€ |

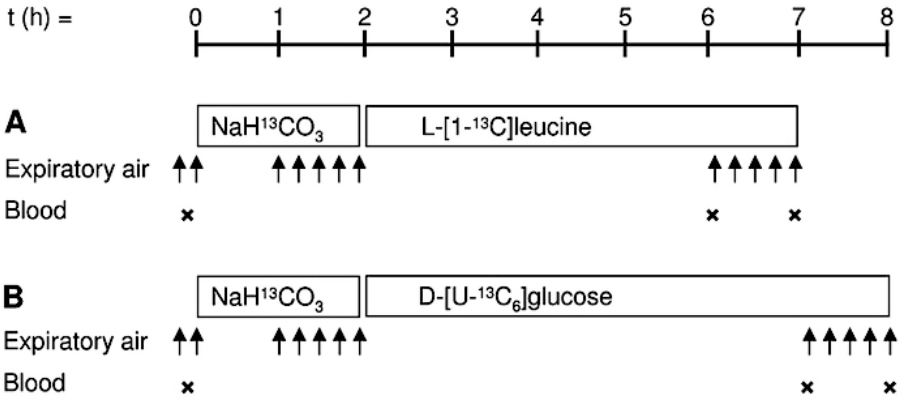


***Figure 5*** *Example of a study design. Infants in both the control and intervention group were subjected to either the labelled leucine (A) or the labelled glucose (B) protocol on postnatal day 2. The bicarbonate pool was also enriched with a primed (15 µmol/kg) continuous $NaH^{13}CO_3$ infusion (15 µmol/(kg·h)). After 2 h, the infusion was replaced by a primed (10 µmol/kg) continuous D-[U-$^{13}C_6$]glucose infusion (5 µmol/(kg·h)) lasting for 6 h (Figure 1B).*

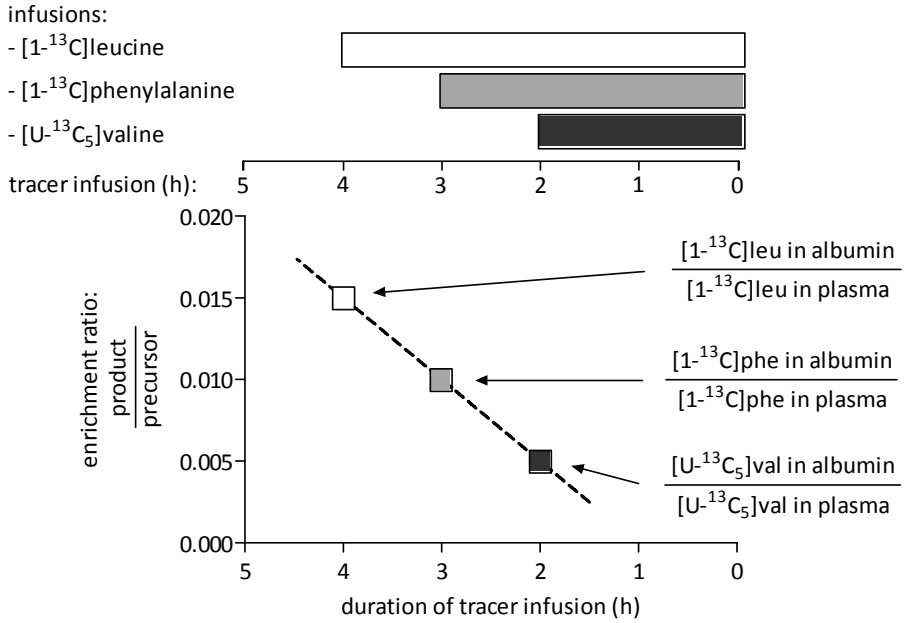


***Figure 6*** *Study design. Pregnant women received 3 different stable isotope-labeled amino acid infusions starting at different times before caesarean delivery. In maternal and umbilical cord blood, sampled at t=0, we measured the product/precursor enrichment ratio of each of the 3 infused amino acids. These ratios were plotted in a graph against the moment the corresponding isotope infusion was started. Because labelled leucine had the longest infusion time, its incorporation into albumin will be highest. The albumin fractional synthesis rate (%/d) was calculated as: slope of the trend line × -1 × 24h ×100%.*

As fewer samples are needed, this method reduces not only the burden to the research subject, but also the analytical workload and the risk of measurement artefacts. Disadvantages include the higher costs of multiple tracers. Potential volume infusion restrictions in premature infants can be overcome by dissolving isotopes in dextrose or other compounds of parenteral nutrition instead of saline, so that tracer volume can be subtracted from the parenteral nutrition infusion volume. Besides, choice for and limitations of the mass-spectrometry machinery must be considered closely if measuring the FSR of specific proteins is aimed for. For enrichments in the product-protein are usually low, the more so when proteins have a longer turnover time. So we must consider that a GC/MS, while being able to measure multiple isotopomers of different masses simultaneously, has limited precision for measuring very low enrichments. Indeed, a GC/C/IRMS is well suited for this purpose. However, it cannot simultaneously measure two isotopomers with carbon-labelled atoms in different positions as the combustion product (carbon dioxide) will be indistinguishable from the original isotopomer. Theoretically, a GC/C/IRMS provides for measuring isotopomer enrichments of different labelled elements, e.g. [1-$^{13}$C]leucine, [$D_7$]leucine, and [$^{18}$O]leucine, but measuring the latter two elements in this example presents great technical challenges. Nitrogen-labelled branched chain amino acids (such as leucine) are not suited because of rapid label loss due to transamination. For that matter, in the above-mentioned study Dudley et al. still managed to measure product enrichment by means of GCMS. This was possible on account of the very high lactase phlorizin hydrolase FSR (on average 128 %/d) that resulted in sufficiently high product enrichment.

Paolini et al. applied a multiple infusion start time protocol in pregnant women to confirm foetal leucine steady state as only one foetal (umbilical cord) blood sample could be taken during cordocentesis[49]. The protocol prescribed continuous infusion of primed [1-$^{13}$C]leucine at thirty-minutes intervals, at each time point followed by [5,5,5-$D_3$]leucine. Infusion at the same rate should result in equal plasma enrichments in the foetus after equilibrium has been reached. From one sample, the enrichments of both leucine isotopomers, measured with GC/MS, are representative of two time points.

Recently, we applied a multiple infusion protocol to measure FSRs of albumin in the foetus[50]. As again only one blood sample could be obtained from the umbilical cord, we sequentially started [1-$^{13}$C]leucine, [1-$^{13}$C]phenylalanine, and [U-$^{13}C_5$]valine prior to caesarean section. We then measured in umbilical cord blood sampled immediately after birth, both the tracer plasma enrichments (precursor) and the tracer enrichments in hydrolyzed albumin (product). From the three product/precursor enrichment ratios, a graph was plotted of which the slope represented the FSR (Figure 7). Because albumin was not expected to have an FSR as high as that of lactase phlorizin hydrolase, we needed to use GC/C/IRMS in view of the low

product enrichments. As explained above, a single amino acid strategy with labels from different elements could not be used, so we resorted to different carbon labelled amino acids. Dudley et al. showed that leucine and phenylalanine can be used interchangeably for a staggered FSR measurement. As valine is also a branched chain amino acid, it was assumed that the three amino acids were equally handled as albumin precursors. Very high linear regression coefficients ($r^2 \sim 0.99$) of the trend line through the enrichment ratios proved the assumption to be valid. This staggered infusion method is thus of benefit in all situations where multiple sampling is impossible or inconvenient. In organ protein metabolism studies (for example liver, bowel, or muscle protein synthesis) the number of tissue biopsies can be reduced to one – from the two or three required in many currently used models[51-52]

A recent study by Brown et al. provides another example of a staggered infusion protocol applied to decrease sample burden in neonates[53]. Two infusates, each containing either an m+1 or m+6 tracer of mannose and inositol, were started 15 minutes apart. Two blood samples taken at the end of the infusion were thus representative of four time points so that steady state could be confirmed precisely. Plasma enrichments were sufficiently high to measure enrichments using GC/MS.

The examples presented here clearly show that staggered infusion protocols are more convenient to the research subject, which is very important of course in the neonatal and paediatric populations. Regrettably they still come with technical limitations and these have probably limited their application in biomedical research so far.

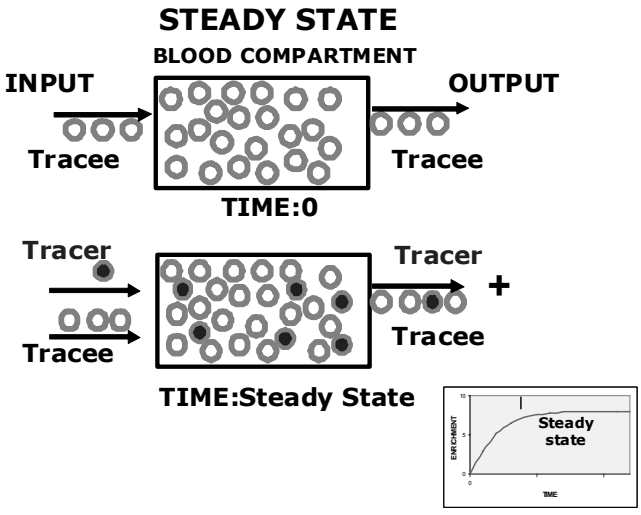


***Figure 7*** *Example of a steady state. A plateau was reached after three hours. In this time period three sample were taken for determination of the kinetic parameters.*

**Precision**

The isotopic precision is determined by multiple measurements of one sample. We may distinguish two types of precision. One is intra-assay precision, which expresses the precision under the same operating conditions over a short interval of time, for example measurements in one single day. The other is inter-assay precision, which expresses variation: for example measurements performed on different days. These precisions are generally assessed by calculating the standard deviation (SD).

**Accuracy**

The accuracy determines the resemblance of the experimental value to the reference value. This measure is a reflection of the systematic error. In tracer measurement, the isotopic accuracy originating from the analytical instrument as well as the sample preparation is assessed by plotting a curve (theoretical excess of isotopic enrichment versus the measured one). Curves are based on samples corresponding to a known amount of unlabeled material mixed with a known amount of labelled material with a known isotopic enrichment. Thus, the measured isotopic data are corrected to obtain the accurate isotopic values. (Figure 8)

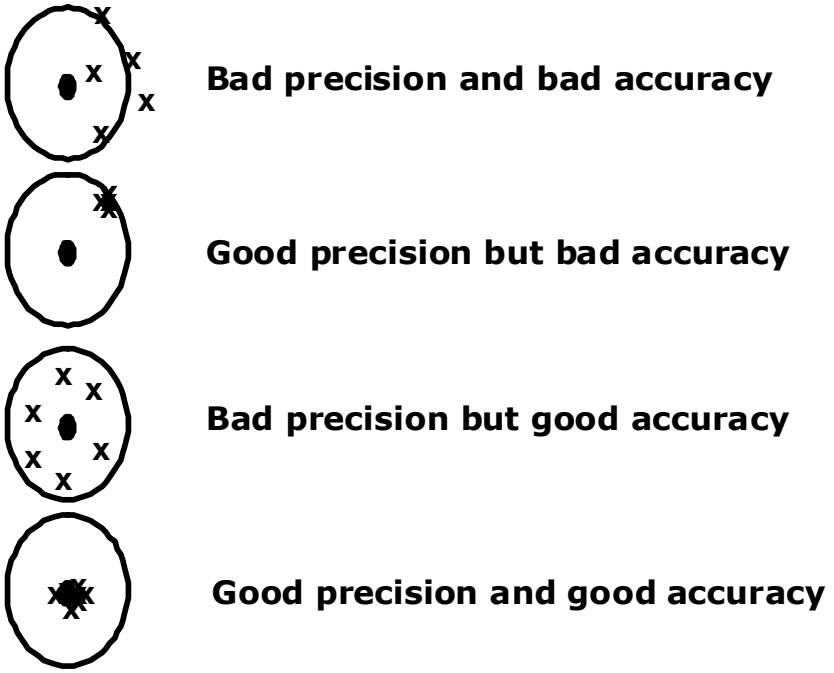


***Figure 8*** *In this figure the difference is pointed out between accuracy and precision.*

## Applications in paedriatic research

### Carbohydrate metabolism

Carbohydrates are important in human metabolism. Changes in plasma glucose concentrations are the result of several simultaneously occurring processes[54-55]. Blood glucose concentration is stable in the fasting condition. Stability is maintained through balancing glucose production in the liver with its subsequent release into the systemic circulation and its removal from the blood by insulin-independent body tissues, e.g. muscle, brain, kidney, gut and erythrocytes. Glucose can be stored as glycogen. Glucose can be converted to fat as well. Both glycogen and fat can be reconverted again into glucose if blood levels decrease during fasting or physical hardship. Long periods of fasting or physical exercise require additional production of glucose. Metabolites like pyruvate, lactate, glycerol and amino acids can be converted to glucose (gluconeogenesis). The liver is the regulating organ for the glucose pathways. The glycolysis, gluconeogenesis and fatty acid synthesis from glucose must be up or down regulated depending on physical exercise and nutritional intake. Insulin plays an important role in this process.

The endogenous production of glucose is generally measured through continuous infusion of deuterated glucose (6,6-$^{2}H_2$-glucose). Deuterium enrichment in the fasting state is determined by the rate of isotope infusion and the rate of production. In the fed situation it is determined by the total production rate, consisting of the rate of infusion and the sum of endogenous production and the influx from the intestine. Stable isotopic labelled glucose is used to gain insight into glucose kinetics.

Many clinical and metabolic studies have used [1-$^{13}$C]glucose[56-57], [U-$^{13}C_6$]glucose[58] or [6,6-$^{2}H_2$]glucose[2, 59-61] to measure glucose turnover. The various methods to determine plasma glucose enrichments involve different cleanup and derivatization techniques, such as trimethyl silyl[59-60, 62-64], pentaacetate[65-66], butyl boronic acid[67-69] and aldonitrile pentaacetate[70-71] derivatization in combination with GC-MS measurement in electron impact (EI)[57] or chemical ionisation (CI) mode[72-73].

Plasma glucose concentration and enrichment are usually analyzed by different methods, using separate aliquots of the same sample[74]. LC/IRMS offers the possibility of simultaneous measurement of plasma concentration and $^{13}$C glucose enrichment using only low sample volumes and administration of very low levels of $^{13}$C glucose enriched tracer[75]

### Energy metabolism and body composition

Energy utilization is measured using the doubly labelled water ($^{2}H_2$ $^{18}$O) technique. This technique was first suggested by Lifson et al. and applied by Schoeller in the early 1980s. It is based on the knowledge that after administration $^{18}$O is lost in body water and $CO_2$, whereas $^{2}$H is lost only in

water. The energy expenditure therefore can be calculated from the decays of $^{2}H$ and $^{18}O$ enrichment in body water. The same technique serves to calculate total body water composition from either $^{2}H$ or $^{18}O$ enrichment. Total body water volume is calculated as the distribution volume and lean body mass by the known relationship between body water mass and lean body mass. Fat mass is determined by subtraction of body water mass plus lean body mass from the total body mass. These method is frequently used in clinical nutrition to assess total body water composition (TBW)[37, 39, 76-78] and total energy expenditure (TEE)[36, 79-82].

Recently we have validated a method for simultaneous measurement of deuterium and $^{18}O$ enrichment in urine and saliva samples, enabling to determine children's total body water composition and energy expenditure[83]. Sample preparation is much simpler than with the classical methods. The analysis process is fully automated, with very small samples (0.1 µL) directly injected into a TC/EA/IRMS system equipped with a liquid auto sampler. Samples are converted into hydrogen and carbon monoxide gases that are transferred on-line using helium gas into the directly coupled isotope ratio mass spectrometer. The TC/EA/IRMS system provides for accurate and simultaneous measurement of $^{2}H$ and $^{18}O$ enrichment of saliva and urine samples. Although the results did not differ between the two sample types (Figure 9), sampling of saliva is preferred because its production time can be determined almost exactly. In addition, the sampling of saliva is less invasive than blood, which is an important issue in paediatric studies.

This methodology is a good alternative to the laborious off-line IRMS measurements. By saving on labour and analysis time it also lowers costs of analysis. The accuracy, simplicity and robustness of the TC/EA-IRMS using the doubly labelled water dilution technique in saliva samples can be a great support to assess body composition and energy expenditure in all subjects in which blood collection is less desirable.

**Specific protein metabolism**

**Albumin synthesis**

Weight gain in children requires a positive protein balance; in other words: the synthesis rate must be higher than the breakdown rate. Catabolic disease states due to inflammation or other illness may increase protein breakdown and thus lead to a negative protein balance. In adults the protein balance must be neutral; in other words, equal synthesis and breakdown rates. Synthesis and breakdown are measured using labelled amino acid tracers in a continuous infusion mode. Whole body protein metabolism is measured solely by isotope dilution in the amino acid pool. Dilution of label occurs by input of

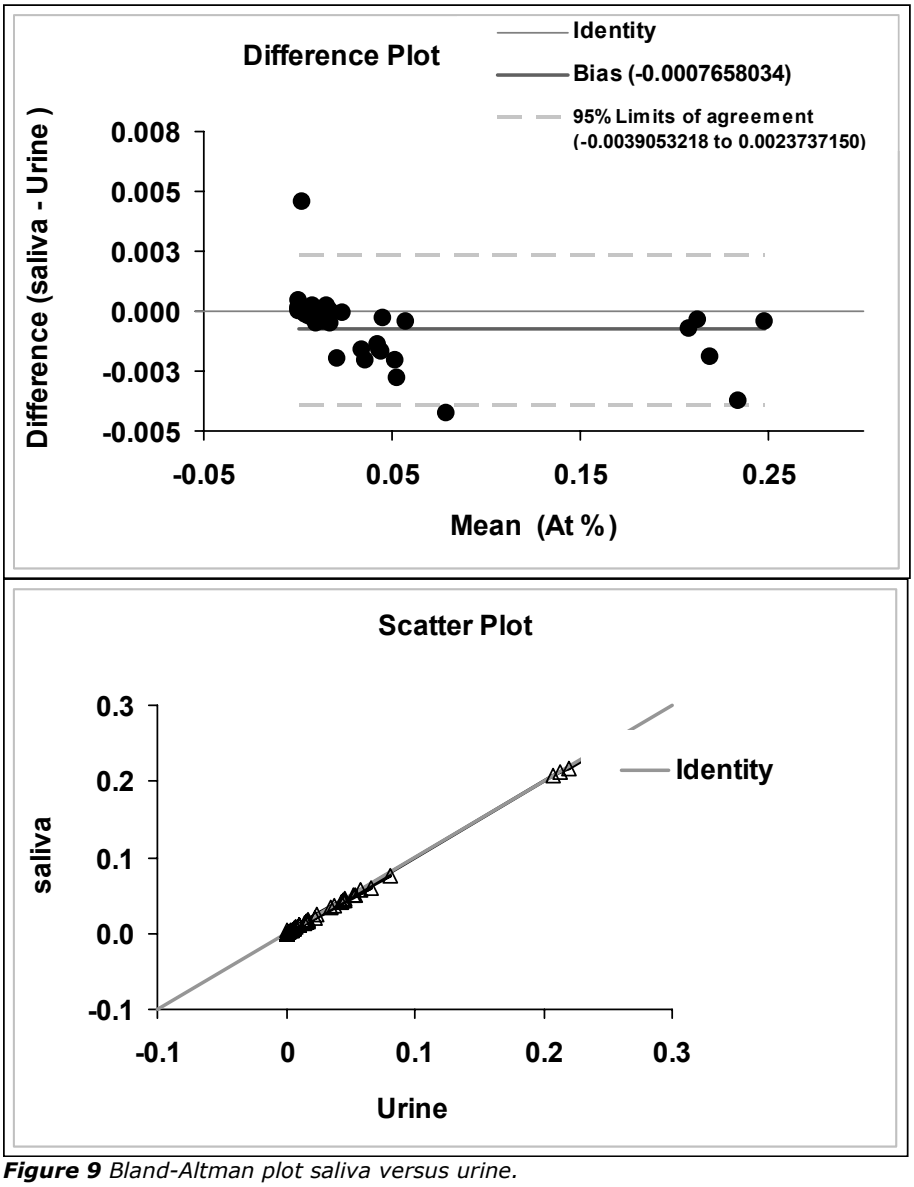


***Figure 9*** *Bland-Altman plot saliva versus urine.*

unlabeled amino acid by protein breakdown. Protein synthesis is mostly studied with the use of $^{13}C$ labelled leucine. Measurement of synthesis and breakdown of specific proteins requires collection of a protein sample from blood sample or specific tissue such as muscle. Specific amino acid pathways are also subject of stable isotope related research.

Whole body amino acid kinetics are often quantified by means of primed continuous stable isotope infusions followed by plasma sampling during steady state. Outcome variables in these experiments include

proteolysis, protein synthesis, or amino acid catabolic (oxidation) rates. These processes are the averages of protein metabolism in the whole body. Counteracting changes in for example muscle versus liver metabolism, however, will not be noted. Specific knowledge on organ kinetics thus adds much to a deeper understanding of metabolism. Knowledge can be obtained either by performing arteriovenous balancing techniques across a single organ[84-87] or by directly biopsying muscle, liver, or bowel tissue samples, for example[86, 88]. From an ethical point of view, however, it is hardly ever possible in the paediatric setting to insert the required deep catheters or to obtain organ biopsies.

Proteins excreted from organs, such as albumin from the liver, could provide organ-specific kinetic data. Moreover, they are easily sampled from an accessible compartment, i.e. the circulation. The fractional synthesis rate of a protein can then be calculated from the increase of tracer enrichment over time in multiple plasma samples. In paediatric studies, however, the concentration of a protein of interest may not be high enough to isolate enough material. In adults larger blood volumes can more easily be drawn to measure synthesis rates of a wide variety of plasma proteins or e.g. complete leukocytes[89-90].

Experiments quantifying in vivo albumin synthesis rates started as early as the 1960s[91]. The focus on albumin is probably due to its important function in medicine[92], its high concentration providing enough material for mass-spectrometry analyses, and a relatively easy purification method. Albumin was first isolated from plasma using absolute ethanol based on the principle of different solubility of albumin and other proteins in plasma[93-94]. Soon it was recognized that some other proteins were soluble in alcohol as well[95-96]. Some groups added saturated ammonia sulfate to precipitate immunoglobulins[97-98], although especially contaminating apolipoprotein A-1 could affect results[99]. Isolation procedures using specific human serum albumin antigens are thus preferable[50].

Albumin is one of the few plasma proteins whose concentration is sufficiently high to permit analysis of small blood volumes (<100 µL), a mandatory criterion in neonates. Measuring the albumin synthesis rate gives a good indication of general liver activity and is more responsible to nutrition than simple concentrations are. Although albumin forms over half of the total plasma protein content, albumin plasma concentration is an insensitive marker of nutritional status. Only 40% of the total albumin mass resides intravascular. However, when albumin synthesis is reduced, as reflected in lower plasma albumin concentration, albumin from the interstitium increases its lymphatic return into the intravascular compartment, so that a measurable drop in albumin concentration will not be observed initially[87, 92].

On the other hand, inflammatory events might increase the transcapillary albumin escape rate so that albumin concentrations will drop despite the liver's higher albumin production rate[100]. Correlations between

albumin concentrations and mortality[101] or necrotizing enterocolitis[102] in premature neonates thus do not necessarily result from lower liver activity. The albumin synthesis rate provides more detailed information on the specific organ effects of supplemented nutrition. The albumin FSRs in healthy adults are approximately 6-8 %/d[103-106]. Studies in premature neonates have found FSRs ranging from 12 to 23 %/d depending on nutritional state[98, 107-108], consistent with the general finding that younger individuals have higher metabolic rates than adults. Synthesizing albumin at very high rates during early life could, however, also be a response to physiological changes. Compared to intrauterine life, infants are exposed to a higher lipid intake through breast milk, a surge in bilirubin disposal, and an environment with a higher oxygen tension. The physiological functions of albumin include transporting of fatty acids and bilirubin, and acting as an antioxidant[92], leading to an increased albumin demand. The G/C/IRMS is the method of choice for measuring low levels of enrichment in the newly produced albumin.

**Glutathione synthesis**

Reactive oxygen species (ROS) are normally present in the human body at low concentrations and play a role in regulating gene expression. In foetal development they help eliminate pathogens during inflammation. In high concentrations, however, ROS can cause serious damage to cells. This damage can be prevented by antioxidants. One important oxidant operating intracellularly is glutathione (GSH). It can be synthesized within the cell, but is mainly produced in the liver.

Glutathione metabolism has been studied in a variety of species and experimental settings[109-113]. The emphasis, though, was on concentration measurements, with a minor role for measuring kinetics using stable isotope tracers. GSH has a rapid turnover, as reflected by its high FSR (Figure 10). In healthy adult volunteers, FSR is around 65-83 %/d[113-114], which implies that all GSH is completely renewed in 36 hours. Determining utilization and synthesis rates of GSH provides a dynamic insight into its metabolism under pathological conditions such as oxidative stress or in response to interventions. GC/MS techniques are used to study FSR with stable isotope tracers ($^{13}C$ or $^{2}H$ incorporated into glycine or cysteine respectively) and a primed continuous infusion for 6 to 8 hours[115]. FSR determination of GSH in erythrocytes requires suitable methods to measure both low level of isotopic enrichment in GSH and in its precursor. Moreover, determination in neonates is complicated because only small amounts of blood can be sampled. Studies in neonates would benefit form a method that can deal with small samples.

GC/MS and stable isotope dilutions techniques have been frequently used in metabolic kinetic studies[5, 8, 115-118] or concentration measurements[15, 119] (gold standard method).

Several papers have been published over the past four years[22-25, 28-29, 31-33], showing the power and robustness of LC/IRMS analyzing amino acids, carbohydrates, fatty acids and volatile fatty acids.

Recently we have developed a method for measurement of $^{13}C$-glutathione as its dimeric form (GSSG) (Figure 11) using LC/IRMS[26]. Still, this method needed to be complemented with glycine measurement on GC/MS or GC/C/IRMS. Although this method was successfully applied in a kinetic studies in preterm infants[120] it was further modified into a single analysis for both GSSG and glycine[27]. This simultaneously measures GSSG) and its precursor [1-$^{13}C$] glycine in erythrocytes, and thus reduces sample preparation time and sample volume.

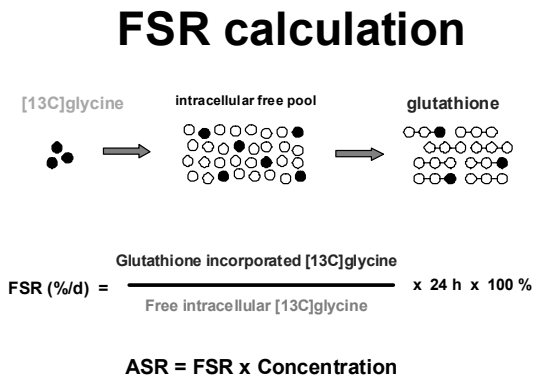


***Figure 10*** *Example of a FSR calculation for Glutathione.*

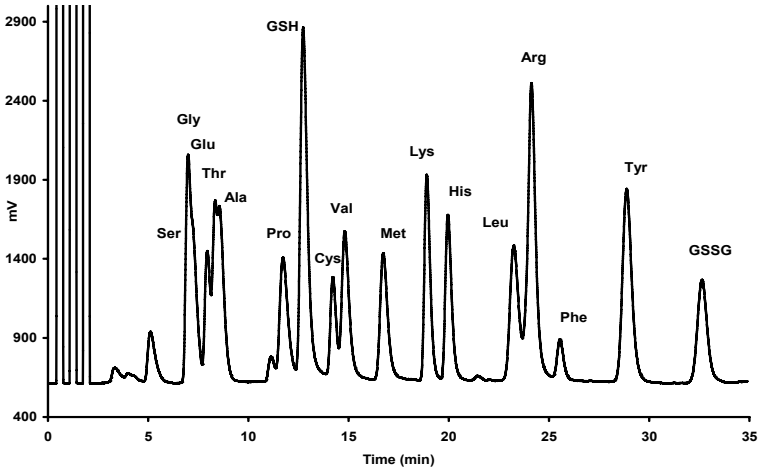


***Figure 11*** *LC-IRMS chromatogram of a 0.25 mmol hydrolysate amino acid standard mixed with 0.5 mmol glutathione standard solution at low pH.*

**Aims and outline of the thesis**

The studies described in this thesis primarily aimed at the developing and fine tuning of mass spectrometric techniques for the quantification of stable isotopes in various disciplines of metabolic research in paediatrics. These techniques can be helpful in addressing clinical hypotheses.

The main hypotheses that will be tested are:

- Glutathione is the major intracellular anti oxidant. Preterm infants suffer from increased oxidative stress, with subsequently increased rates of morbidity and mortality. Can we increase GSH by modulating the diet of preterm infants?

- Nutrition for the preterm neonate is based upon the composition of human milk and the factorial approach. We have hardly any insight in foetal metabolism. Foetal metabolism may provide novel insights in the way we can provide preterm infants with substrates. Furthermore, foetal protein synthesis rates can be taken as a gold standard as we consider the intra-uterine environment as optimal. These rates can than be thrived for in preterm infants.

- Energy intake and expenditure are two sides of the balance when considering body composition. Especially in handicapped people, energy expenditure is difficult to determine reliably, although many disables children are either overweight or underweight. The double labelled water technique offers the possibility to measure energy expenditure in these special children over a prolonged time frame enabling the physician to customize nutritional intake for each child.

**Chapter 1** gives an overview of the subject of this thesis and describes current knowledge and research questions.

**Chapter 2** describes a novel LC/IRMS method for studying glucose metabolism compared with other existing methods at very low enrichments.

**Chapter 3** reports the validation of a new technique for measurement of $^{2}H$ and $^{18}O$ in several human body fluids.

**Chapter 4** applies the technique described in chapter 3 to determine energy expenditure and total body water composition in children with severe cerebral palsy. Suitability of saliva and urine was assessed.

**Chapter 5** describes a novel method using stable isotope techniques for studying glutathione metabolism in extremely small sample volumes, such as is required for measurements in preterm infants.

**Chapter 6** introduces a modification of the method described in chapter 5. The precursor and the product can now be analyzed in one single method, saving costs and labour time

**Chapter 7** describes a randomized clinical trial determining stimulatory effects of early amino acid administration on glutathione synthesis rates and its potential to lower oxidative stress in preterm infants.

**Chapter 8** describes a randomized clinical trial which addresses the hypothesis that a high dose of cysteine stimulates glutathione synthesis as compared to a lower dose in preterm infants – based on the premise that cysteine is an essential amino acid in very preterm infants.

The next part of this thesis describes several studies in which pregnant women received multiple stable isotope infusions in the hours prior to caesarean section. After birth, umbilical cord blood was sampled and analyzed for the amino acid concentrations and enrichments.

In **chapter 9** the foetal albumin synthesis rate is quantified using a relatively novel stable isotope model.

In **chapter 10** the foetal metabolic pathways of phenylalanine and tyrosine are quantified.

**Chapter 11** is the general discussion and includes future perspectives

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CHAPTER

# 2

# Analysis of [U-$^{13}C_6$]glucose in human plasma using Liquid Chromatography Isotope Ratio Mass Spectrometry (LC/IRMS) compared with two other mass spectrometry techniques.

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**Abstract**

The use of stable isotopic labelled glucose enables insight into glucose metabolism. The $^{13}C$ isotopic enrichment of glucose is usually analysed by gas chromatography mass spectrometry (GC/MS) or gas chromatography combustion isotope ratio mass spectrometry (GC/IRMS). However, in both techniques the samples must be derivatized prior to analysis, which makes sample preparation more labour intensive and increases the uncertainty on the measured isotopic composition. A novel method for the determination of isotopic enrichment of glucose in human plasma is using liquid chromatography isotope ratio mass spectrometry (LC/IRMS) has been developed. Using this technique, for which hardly any sample preparation is needed, we showed that both the enrichment and concentration could be measured with very high precision using only 20 µL of plasma.

In addition, a comparison with GC/MS and GC/IRMS showed best performances were achieved with the LC/IRMS method making it the method of choice to measure $^{13}C$ isotopic enrichment in plasma samples.

## Introduction

Changes in plasma glucose concentrations are the result of several simultaneously occurring processes[1, 2]. Blood glucose concentration is stable in the fasting condition. Stability is maintained through balancing glucose production in the liver with its subsequent release into the systemic circulation and its removal from the blood by insulin independent tissues of the body, e.g. muscle, brain, kidney, gut and erythrocytes.

Stable isotopic labelled glucose is used to gain insight into glucose kinetics. Many clinical and metabolic studies have used [1-$^{13}$C]glucose[3, 4], [U-$^{13}C_6$]glucose[5] or [6,6-$^{2}H_2$]glucose[6-9] to measure glucose turnover. The various methods to determine plasma glucose enrichments involve different cleanup and derivatization techniques, such as trimethyl silyl[6, 7, 10-12], penta acetate[13, 14], butyl boronic acid[15-17] and aldonitrile penta acetate[18, 19] derivatization in combination with GC/MS measurement in electron impact (EI)[4] or chemical ionisation (CI) mode[20, 21]. Plasma glucose concentration and isotopic enrichment are usually analysed by different methods, using separate aliquots of the same sample[22]. A novel method, liquid chromatography isotope ratio mass spectrometry (LC/IRMS), offers the possibility of simultaneous measurement using only low tracer infusion rates, which makes it cost-friendly. In addition, as derivatization is not needed, sample preparation is easier. Since the introduction of LC/IRMS by Krummen et al. in 2004[23], several studies have documented its power and robustness in the analysis of amino acids, small peptides, carbohydrates, and volatile fatty acids[24-31].

Our aim was to develop an accurate, simple and rapid method for the simultaneous measurement of $^{13}$C glucose enrichment and concentration in human plasma. We assumed that a LC/IRMS technique could meet these demands with administration of low amounts of label.

## Experimental Section

### Chemicals and reagents

Glucose and phosphoric acid (85 % v/v) were purchased from Sigma (St Louis, USA). Sodium peroxodisulfate (p.A.) and sodium hydroxide solution (50%) were purchased from Fluka (Buchs, Switzerland). Perchloric acid (70% v/v), potassium hydroxide, $Na_2HPO_4$ and $H_3PO_4$ were purchased from Merck (Darmstadt, Germany). [U-$^{13}$C] glucose was purchased from Cambridge Isotope Laboratories (Buchem, Apeldoorn, the Netherlands). Hydroxylamine hydrochloride and acetic anhydride were purchased from Pierce Chemical Company (Rockford, IL, USA). Freshly prepared Milli-Q water (18.2 Mohm, DOC free; Millipore, Bedford, USA) was used in all experiments.

The $^{13}$C-enriched glucose reference standards IAEA-309A (certified at $d^{13}C$ = 93.9 ± 1.0‰) and IAEA-309B ($d^{13}C$ = 535 ± 5‰) were purchased from the International Atomic Energy Agency (IAEA, Vienna, Austria)

### Analytical methods

#### LC/IRMS.

High performance anion-exchange chromatography was carried out on a Thermo Surveyor system consisting of a HPLC pump (MS Pump Plus) and an autoinjector (Autosampler Plus; Thermo Fisher, Bremen, Germany), fitted with a CarboPac PA20 guard and narrow-bore analytical column (3 x 150mm; Dionex Benelux, Amsterdam, the Netherlands) and eluted at 300 µL min$^{-1}$ isocratically with 1mM NaOH. Injection volume was 20 µL.

The HPLC system was coupled to the IRMS by a LC Isolink interface (Thermo Fisher, Bremen, Germany). The technique of the Isolink interface is based on wet oxidation of organic components with peroxodisulfate under acidic conditions. The $CO_2$ produced is subsequently separated from the mobile phase in a capillary gas exchanger flushed with helium gas, dried and led into the ion source of the mass spectrometer in a helium stream via the open split interface. The temperature of the oxidation reactor was set at 99.9 $^{o}$ C. The flow rates of the acid (1.5 M $H_3PO_4$) and oxidant reagents (0.7 M $Na_2S_2O_8$) were 50 µL min$^{-1}$ each.

Isotopic ratio measurements were carried out on a Delta V Advantage IRMS (Thermo Fisher, Bremen, Germany). The LC/IRMS system and data collection were controlled using Isodat 2.5 SP 1.13 software. Baseline corrections were made with the basic algorithm provided by the Isodat software and manually optimized when necessary. Peak identification was based on retention times.. Concentration measurements was based on the peak areas of the m/z 44, 45, 46 signals of the separated compound, using external standards for calibration. Samples were analyzed in duplicate.

**GC/MS**

The mass spectrometric analyses were performed on an Agilent 5975 C mass spectrometer coupled with an Agilent 7890 A gas chromatograph (Agilent, Amstelveen, the Netherlands). A chemically bonded DB 5ms (J&W Scientific Folsom, CA USA) capillary column with a length of 30 m, an internal diameter of 0.25 mm and a film thickness of 0.40 μm was used for the chromatographic separation. Aldonitrile penta acetate derivatives were used for the analysis of the $^{13}C$ enrichment of glucose in human plasma[18, 32, 33]. The mass spectrometer interface was set at 280° C; the ion source and analyser temperature were set at 200° C.

The injector temperature was set at 280° C. The selected column temperature program for aldonitrile penta acetate was 180° C for 1 min. then raised to 280° C at 15° C/min and held at 280° C for 4 min. The carrier gas was helium at 85 mbar. A split injection method with a split ratio of 1:20 was used for sample introduction. The intensities of the fragments 314.2 and 319.2 were selected for the aldonitrile penta acetate derivative. Fragment 314.2 is representing the non enriched glucose and fragment 319.2 is representing [U-$^{13}C_6$]glucose. All measurements were carried out in selective ion monitoring (SIM) mode using electron ionization set at 70 eV, with an emission current of 0.200 mA. Samples were analyzed in duplicate.

**GC/C/IRMS**

The $^{13}C/^{12}C$ ratio measurement of glucose was performed on a Delta-XP isotope ratio mass spectrometer coupled online with a Trace gas chromatograph and a combustion interface type 3 (Thermo Fisher, Bremen, Germany). Aliquots of 0.5 μL of the chloroform solution containing the glucose derivative were introduced to the GC system by a CTC PAL autosampler (CTC, Zwingen, Switzerland). The flow was set at a constant rate of 1mL/min and samples were introduced in splitless mode.

A Sil-24ms (Varian, Middelburg, the Netherlands) capillary column length 30m and an internal diameter of 0.25 mm was used for the chromatographic separation. The injector temperature was set at 250° C and the oven temperature programme was 160° C for 1 min to 230° C at 5° C/min and held at 230° C for 5 min.

After being separated by means of capillary gas chromatography, glucose aldonitrile penta acetate was online combusted at 940° C and introduced as $CO_2$ into the isotope ratio mass spectrometer, where the $C^{13}/C^{12}$ ratio was measured. $NO_x$ formed by incomplete oxidation was reduced to $N_2$ and also $O_2$ bleed from the oxidation oven was removed by the reduction reactor operating at 650° C. The produced water was removed by an online nafion capillary. Each sample was analyzed two times, along with an external $CO_2$ reference gas for calibration. The $\delta$ values were calculated using the Isodat 2.0 (Thermo Fisher) data software.

**Sample preparation for LC/IRMS**

An aliquot of 20 uL plasma was diluted 10 times with Milli-Q. The diluted samples were filtered with an ultrafiltration membrane with a nominal molecular weight limit (NMWL) of 5.000 Da on an ultrafree-MC centrifugal filter (Millipore Corporation, Bedford, USA). centrifuged at 5.000 g for 60 minutes at a temperature of 4° C.

**Sample preparation for GC/MS and GC/C/IRMS:**

2 mL cold (4° C) methanol was added to an aliquot of 100 µL plasma. After having been shaken vigorously for 30 seconds the mixture was kept at 4° C for 30 min. Next the samples were centrifuged and the supernatant was collected and dried under a gentle stream of nitrogen at 50° C. 1 mL dichloromethane was added and evaporated to remove the last traces of water.

Standard curves were prepared by mixing aqueous solutions of natural and labelled glucose for both enrichment and concentration determination.

**Derivatization:**

Preparation of aldonitrile pentaacetate derivatives:

An aliquot of 100 µL of a 2% solution (w/v) of hydroxylamine-HCl in pyridine was added to the dried samples and heated at 90° C for 30 min. After cooling, 50 µL of acetic anhydride was added and heated at 90° C for another 30 min. Next it was dried under a gentle stream of nitrogen and resolved in 100 µL of chloroform. Aliquots of 0.5 µL were injected.

**Calibration and isotopic rearrangements**

For calibration, two reference $CO_2$ gas pulses with an interval of 20 s were introduced at the beginning of each run, the $CO_2$ flow was set in order to obtain a signal of 3.0 ± 0.2 V on cup one (resistor 300 MΩ).

The $^{13}C/^{12}C$ abundance ratio was expressed as $\delta\ ^{13}C$ values calibrated against the international standard of Vienna Pee Dee Belemnite (VPDB). The delta notation is defined as $\delta\ ^{13}C_{\ sample} = [(R_s / R_{st}) - 1] \times 1000$, where $R_s$ is the $^{13}C/^{12}C$ ratio of the sample and $R_{st}$ is the $^{13}C/^{12}C$ ratio of a reference standard.

Atom % was calculated as:

$$\text{Atom \%} = \left[ \frac{100 \times Rst \times \left( \left( \delta 13C / 1000 \right) + 1 \right)}{1 + Rst \times \left( \left( \delta 13\text{C} / 1000 \right) + 1 \right)} \right]$$

where $R_{st}$ is the $^{13}C/^{12}C$ ratio of the reference standard to which the value is relative to, in this study it is the VPDB, $R_{st}$=0.0112372.

Atom % Excess (APE) is defined as: Atom % (sample) minus Atom % (natural abundance).

## Clinical study design



### Study

The included infants were a subset of those included earlier in a study determining safety and efficacy of high-dose early amino acid administration[34]. The study was designed as a randomized open trial and was performed in the neonatal intensive care unit of the Erasmus MC–Sophia Children's Hospital, Rotterdam, the Netherlands. The study was investigator initiated with no funding from industry. The protocol was approved by the Erasmus MC Medical Ethical Review Board and parental consent was obtained before the study.

### Subjects

Subjects in the earlier study were 32 prematurely born infants with a birth weight <1500 g who were born in the Erasmus MC–Sophia Children's Hospital, were mechanically ventilated, had an arterial catheter, and were expected to be completely dependent on parenteral nutrition for the first 2 days of life. Exclusion criteria were known congenital abnormalities, chromosome defects, and metabolic, endocrine, renal, or hepatic disorders. Directly after birth they were randomly assigned to receive either glucose only during the first 2 days (control group, n = 16) or glucose supplemented with 2.4 g of protein/kg/d as amino acids (Primene 10%, Baxter, Clintec Benelux N.V., Brussels, Belgium) within 2 h postnatally (intervention group, n = 16). The first eight subjects of both groups comprised the subset for the present study. Amino acid and/or glucose solutions were constantly infused without interruptions during the study. Lipids and/or (minimal) enteral feedings were not administered until after the study period. For all infants, we recorded birth weight, gestational age, SD scores for weight[35], antenatal corticosteroid usage, and severity of illness at entry of the study by means of Apgar and CRIB scores[36]. We also assessed blood gases and nitrogen balances as described previously[34].

Infants in both the control and intervention group were subjected to the labelled glucose protocol on postnatal day 2. In this study, the bicarbonate pool was also enriched with a primed (15 μmol/kg) continuous $NaH^{13}CO_3$ infusion (15 μmol/kg/h). After 2 h, the infusion was replaced by a primed (10 μmol/kg) continuous D-[U-$^{13}C_6$]glucose infusion (5 μmol/kg/h) lasting for 6 h (Fig. 1). Tracers were infused with a Perfusor fm infusion pump (Braun Medical B.V., Oss, the Netherlands) along the same infusion route as the parenterally administered nutrients.

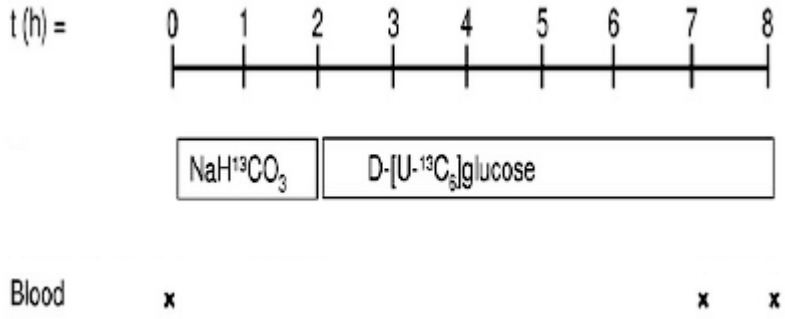


***Figure 1*** *Study design. Infants in both groups were subjected to the labelled glucose protocol on postnatal day 2.*

**Measurement of isotopic enrichments in plasma.**
Arterial blood samples were drawn once before the isotope infusions (baseline) and twice during the last hour of glucose tracer infusion. After collection, the samples were immediately put on melting ice and centrifuged, after which the plasma was collected and stored at –80°C until analysis.

## Results and discussion

### LC/IRMS measurement of glucose $^{13}$C-enrichment and concentration in plasma.

The enrichment of $^{13}$C glucose was determined by comparing the $^{12}$C/$^{13}$C ratios using standard curves between 0% and 0.5% APE from known fractions of [U-$^{13}C_6$]glucose. Linear relationships were obtained for glucose with a regression coefficient ($R^2$) of 0.9995 (Fig 2c) with the LC/IRMS. The linear relationships of the enrichment curves of $^{13}$C glucose obtainend with the GC/MS (Fig 2a) and the GC/C/IRMS (Fig 2b) techniques showed a regression coefficient of 0.9985 and 0.9994 respectively. The concentration of the analyte is an important parameter in every metabolic study. Four glucose standards were measured between 1 and 7.5 nmol. A linear relationship was obtained ($y = 6.781x - 0.0625$). The regression coefficient ($R^2$) was calculated at 0.9992 (Fig 3). The concentration of glucose was measured with a good reproducibility (coefficient of variation (CV) of 1.66%, when measured as estimates of the duplicates). The mean glucose concentration (7.15 ± 0.24 µmol/mL) was consistent with values reported in literature[25,37]. These findings show that LC/IRMS can analyze concentration of metabolites in blood with good precision (Sd = 0.12 µmol/mL) and a limit of quantification (LOQ) of 0.2 nmol absolute. A typical chromatogram of a LC/IRMS analysis of glucose in plasma is shown in Fig. 4.

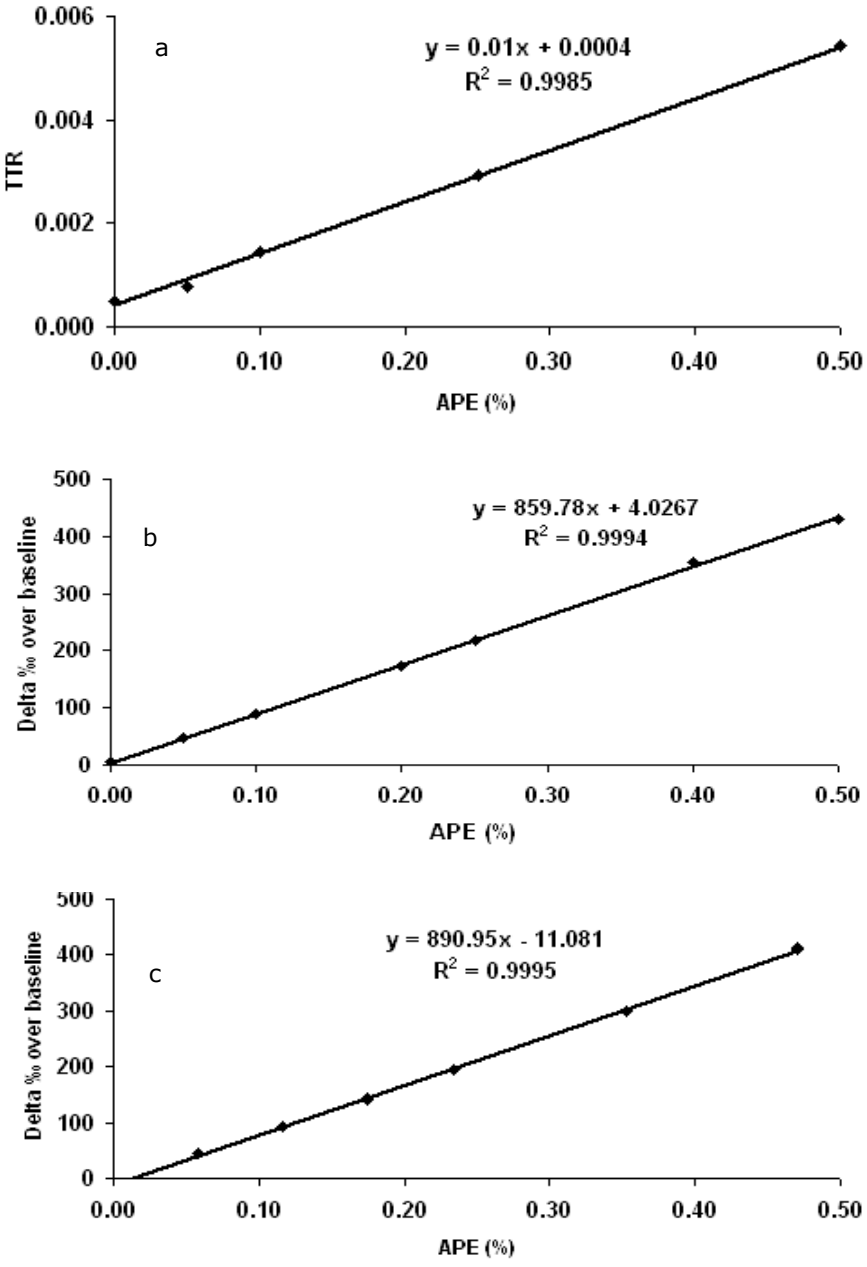


***Figure 2*** *Calibration curves for measurement of [U-$^{13}C_6$]glucose enrichment in human plasma analysed with GC/MS (a) expressed as tracer/tracee ratio's or as δ over base line , GC/C/IRMS (b) and LC/IRMS (c).*

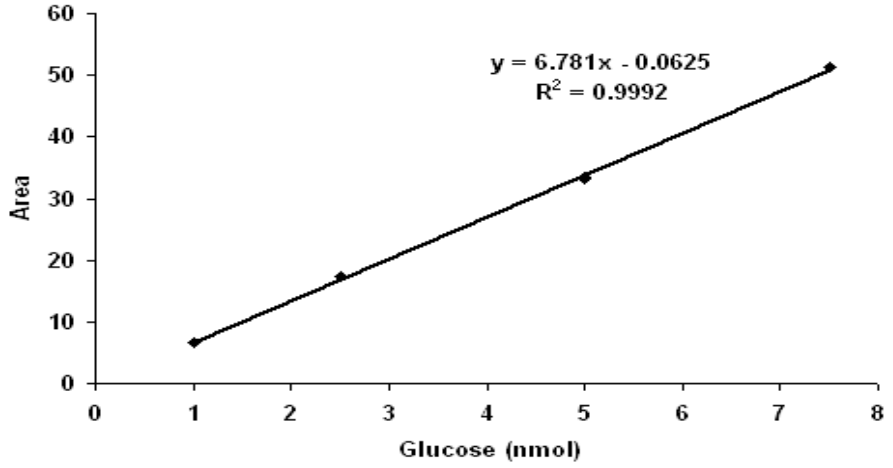


***Figure 3*** *Calibration curve of the absolute glucose concentration in the range 1-7.5 nmol.*

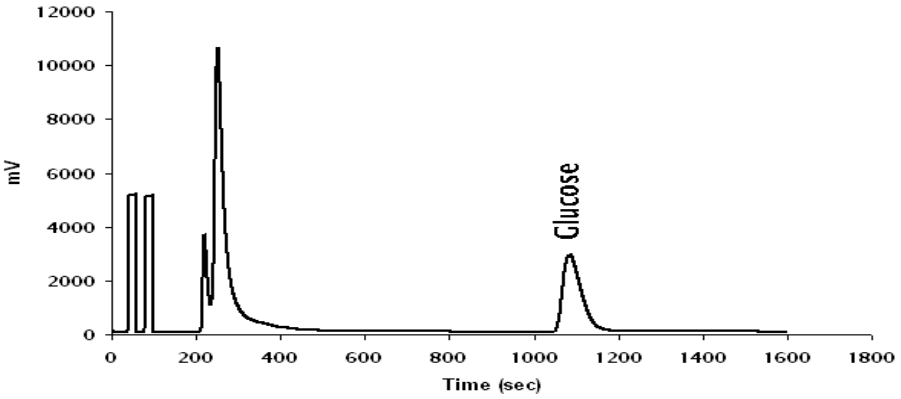


***Figure 4*** *LC/IRMS chromatogram of glucose in plasma. The signal of the y axis is expressed as milliVolt.*

**Accuracy and precision of isotopic measurement**

As illustrated in Table 1, the intra-assay repeatability was excellent for the certified IAEA standards 309a (93.74 ± 0.37 ‰, CV = 0.39% (n = 10)) and 309b (533.03 ± 0.53 ‰, CV = 0.10% (n = 10)). The inter-assay repeatability values (Table 2) measured at five different days in a two-week period, were also excellent. For standard 309a the Sd was 0.37 with a variation (CV) of 0.39% (n = 5); for standard 309b the Sd was 0.71 with a

variation (CV) of 0.13% (n = 5). Accuracy of isotopic measurement was assessed during the intra-assay repeatability as well as the inter-assay repeatability analysis (Table 1 and 2). Both show accurate values for the measurement of the two standards. For each standard, the δ $^{13}$C glucose values were close to the certified value, i.e. -0.42‰ (CV = 0.45%) for standard 309a and -3.36‰ (CV = 0.63%) for standard 309b. Table 3 shows the results of the analysis of sixteen subjects at three different time points. Time 0 = just before administration of the tracer and after 5 and 6 hours are at steady state. The values of the measurement of physiological samples show also nice correlations (mean Sd = 0.36‰ with a CV of 0.61%). These values show excellent isotopic precision as well as accuracy of isotopic measurement at both enriched abundance and natural abundance. Table 3 also shows that there is only little difference between each set of values at time points 5 and 6, which means that for all subjects a plateau was obtained.

***Table 1*** *Intra-assay precision assessed by replicate analysis of IAEA standards 309a and 309b on a single day.*

| Standard | δ ‰ Value IAEA | δ ‰ Measured | Accuracy | Standard | δ ‰ Value IAEA | δ ‰ Measured | Accuracy |
|---|---|---|---|---|---|---|---|
| 309a | 93.90 | 92.92 | -1.09 | 309b | 535.30 | 533.41 | -1.89 |
| | | 93.68 | -0.22 | | | 532.91 | -2.39 |
| | | 94.03 | 0.13 | | | 533.07 | -2.23 |
| | | 93.70 | -0.20 | | | 533.00 | -2.30 |
| | | 93.56 | -0.35 | | | 532.78 | -2.52 |
| | | 93.76 | -0.14 | | | 532.55 | -2.75 |
| | | 93.89 | -0.02 | | | 532.32 | -2.98 |
| | | 93.97 | 0.06 | | | 532.12 | -3.18 |
| | | 93.97 | 0.07 | | | 532.38 | -2.92 |
| | | 94.04 | 0.14 | | | 531.59 | -3.71 |
| Mean | | 93.74 | -0.16 | | | 533.03 | -2.69 |
| Sd | | 0.365 | | | | 0.532 | |
| Precision (CV) | | 0.39% | | | | 0.10% | |
| Accuracy (CV) | | | 0.17% | | | | 0.50% |

***Table 2*** *Inter-assay precision assessed by replicate analysis of IAEA standards 309a and 309b on four different days.*

| Standard | δ ‰ Value IAEA | Day | δ ‰ Measured | Accuracy | Standard | δ ‰ Value IAEA | δ ‰ Measured | Accuracy |
|---|---|---|---|---|---|---|---|---|
| 309a | 93.90 | 1 | 93.74 | -0.16 | 309b | 535.30 | 533.03 | -2.27 |
| | | 3 | 93.53 | -.037 | | | 531.23 | -4.07 |
| | | 7 | 93.38 | -0.52 | | | 531.43 | -3.87 |
| | | 10 | 93.83 | -0.07 | | | 531.81 | -3.49 |
| | | 14 | 92.91 | -0.99 | | | 532.19 | -3.11 |
| Mean | | | 93.48 | -0.42 | | | 531.94 | -3.36 |
| Sd | | | 0.37 | | | | 0.71 | |
| Precision (CV) | | | 0.39% | | | | 0.13% | |
| Accuracy (CV) | | | | 0.45% | | | | 0.63% |

***Table 3*** *Glucose $^{13}C$ isotopic enrichment measured with LC/IRMS of sixteen different subjects showing the standard deviation (Sd) and the coefficient of variation CV (%). Samples taken at 0, 5 and 6 hours were measured in duplicate.*

| Subject | T=0 δ‰ | Sd (n=2) | T=5 δ‰ | Sd (n=2) | T=6 δ‰ | Sd (n=2) |
|---|---|---|---|---|---|---|
| 1 | -12.5 | 0.01 | | | 189.8 | 0.19 |
| | -12.5 | | | | 190.1 | |
| 2 | -12.2 | 0.03 | 140.1 | 0.14 | 142.4 | 0.21 |
| | -12.3 | | 139.9 | | 142.1 | |
| 3 | -10.6 | 0.05 | 230.9 | 0.61 | 228.3 | 0.22 |
| | -10.5 | | 230.0 | | 228.0 | |
| 4 | -11.0 | 0.23 | 275.3 | 0.17 | 279.8 | 0.72 |
| | -11.3 | | 275.6 | | 280.8 | |
| 5 | -12.1 | 0.07 | 242.0 | 1.63 | 251.2 | 0.61 |
| | -12.2 | | 244.4 | | 250.3 | |
| 6 | -12.9 | 0.18 | 262.3 | 0.33 | 253.4 | 0.31 |
| | -13.2 | | 262.7 | | 253.8 | |
| 7 | -10.8 | 0.15 | 179.3 | 1.99 | 181.5 | 0.02 |
| | -10.6 | | 182.1 | | 181.5 | |
| 8 | -10.1 | 0.13 | 246.4 | 0.68 | 250.0 | 0.44 |
| | -10.0 | | 245.4 | | 250.6 | |
| 9 | -11.5 | 0.18 | 154.5 | 1.42 | 172.0 | 0.03 |
| | -11.3 | | 156.5 | | 172.0 | |
| 10 | -11.0 | 0.01 | 178.7 | 0.34 | 163.3 | 0.14 |
| | -11.0 | | 179.1 | | 163.5 | |
| 11 | -13.0 | 0.25 | 240.5 | 0.14 | 249.5 | 0.85 |
| | -13.4 | | 240.3 | | 248.3 | |
| 12 | -13.7 | 0.42 | 211.7 | 0.42 | 219.7 | 0.26 |
| | -13.1 | | 212.3 | | 219.4 | |
| 13 | -11.4 | 0.17 | 131.5 | 0.50 | 131.7 | 0.71 |
| | -11.2 | | 130.8 | | 132.7 | |
| 14 | -10.9 | 0.23 | 111.4 | 0.15 | 117.4 | 0.80 |
| | -10.6 | | 111.2 | | 118.5 | |
| 15 | -11.7 | 0.15 | 172.6 | 0.17 | 165.7 | 0.40 |
| | -11.5 | | 172.4 | | 166.3 | |
| 16 | -11.2 | 0.28 | 214.2 | 1.56 | 208.6 | 0.39 |
| | -10.8 | | 216.4 | | 208.0 | |
| Mean | -11.63 | 0.16 | 199.69 | 0.64 | 200.32 | 0.27 |
| CV% | | 1.37 | | 0.32 | | 0.13 |
| Mean Sd (δ‰) | 0.36 | | | | | |
| | 0.61 | | | | | |

**Comparison of LC/IRMS with GC/MS and GC/C/IRMS**

All samples were analyzed as duplicates using three different types of mass spectrometric techniques. We compared the results obtained with the novel LC/IRMS method with those of a GC/MS method and those of a GC/C/IRMS method – visualized in two Bland-Altman plots (Fig. 5a and 5b).

The plots clearly show that the agreement between LC/IRMS and GC/IRMS (Limits of agreement -0.0125 – 0.0175) is better than that between LC/IRMS and GC/MS (Limits of agreement -0.0485 – 0.0288). Table 4 gives the mean precision of each technique for human plasma measurements. The values for the standard deviation and the variation are 0.0114%APE and a CV of 4.75% for GC/MS, 0.0016% APE and a CV of 0.69% for GC/C/IRMS, and 0.0004 APE and a CV of 0.19% for LC/IRMS.

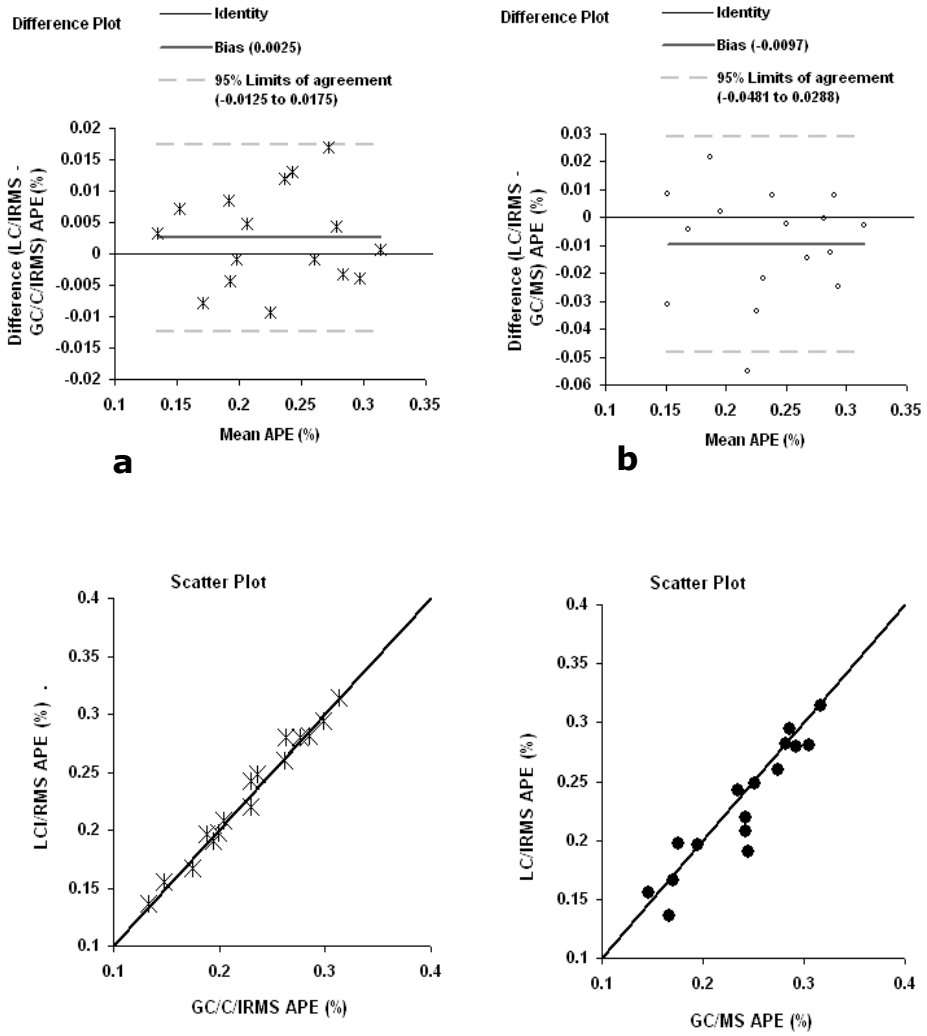


***Figure 5*** *Bland Altman plots comparing the LC/IRMS technique with (a) GC/C/IRMS and (b)GC/MS . The unit of the x and y axes are expressed as APE.*

***Table 4*** *Comparison of the precision of the three investigated MS techniques.*

| Technique | Mean APE (%) | Precision (n=96) Sd APE (%) | CV |
|---|---|---|---|
| GCMS | 0.23912 | 0.01137 | 4.75% |
| GCIRMS | 0.22695 | 0.00156 | 0.69% |
| LCIRMS | 0.22977 | 0.00044 | 0.19% |



**Conclusion**

This new LC/IRMS method for measuring kinetics of glucose shows to be a powerful tool in metabolic studies in neonates. Only little pre-purification is necessary and the analyses reported here were fully automated. The measurements of both glucose concentrations and $^{13}C$ isotopic enrichments gave excellent results. Even more since only 20 µL of plasma was needed, which is of extremely high relevance for studies in neonates or in small animals. Glucose concentrations corresponded to values measured by other techniques in our lab and to those reported in literature. The precision and accuracy of the measurement of the isotopic composition at natural abundance and at higher enrichment do not show any notable isotopic fractionation during sample preparation and analysis.

In this experiment the precision of the LC/IRMS technique proved to be superior to the GC/MS and GC/C/IRMS techniques at enriched as well as at natural levels. The better precision for the LC/IRMS technique is mainly due to the fact that it does not require correction for derivatization. Compared to the GC/MS technique, the LC/IRMS technique requires lower amounts of label to obtain accurate data, which reduces the costs of the experiment. This novel LC/IRMS method could therefore become the technique of choice when measuring $^{13}C$ glucose isotopic enrichment in blood plasma.

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CHAPTER

# 3

# Validation of deuterium and oxygen[18] in urine and saliva samples from children using online continuous flow isotope ratio mass spectrometry

H [illegible]arbeek [1]
R [illegible]en[2,3]
K [illegible]orst[1]
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**Abstract**

The doubly labelled water method is valuable for measuring energy expenditure in humans. It usually involves blood or urine sampling, which might be difficult in neonates and children with cerebral palsy or other disabilities. We therefore aimed to validate a method making use of saliva samples analyzed by automated thermal conversion elemental analyzer in combination with isotope ratio mass spectrometry (TC-EA/IRMS). The subjects received labelled water orally and urine and saliva samples were collected and analyzed. Deuterium as well as oxygen$^{18}$ was measured in one single run using a peak jump method. Excellent linearity was found for measurement of enrichments of deuterium ($R^2$=0.9999) and oxygen$^{18}$ ($R^2$=0.9999). Both the intra-assay precision and the inter-assay precision of the measurement of two standards were good for both deuterium and oxygen$^{18}$.

The variation between urine and saliva samples was small (4.83% for deuterium and 2.33% for oxygen$^{18}$ n=40). Saliva sampling is to be preferred, therefore, as it can be easily collected and is non-invasive. Moreover, its time of production is almost exactly known. The TC-EA/IRMS method is a good alternative to the more laborious off-line IRMS measurements.

## Introduction

The past decade has seen the development of new interfaces for measuring deuterium and $^{18}O$ enrichments[1, 2]. In the classical methods, water is isolated from biological fluids (plasma and urine) by cryo-distillation and converted into $HD/H_2$ and $C^{18}O_2/C^{16}O_2$ gases[3]. The former are produced through reduction of the collected water using zinc, platinum or manganese for example[4-10]. The latter are produced by water equilibration overnight with un-enriched $CO_2$ gas to achieve $^{18}O$ enrichment in $CO_2$ gas similar to that present in the water samples. $HD/H_2$ and $C^{18}O_2/C^{16}O_2$ are then transferred off-line into the isotope ratio mass spectrometer for measuring their deuterium and $^{18}O$ enrichments. These doubly labelled water ($D_2{}^{18}O$) techniques are laborious, time-consuming and require relatively voluminous samples (a few hundred microliters or even a few millilitres). Yet they are frequently used in clinical nutrition to assess patients' total body water composition (TBW)[6, 8, 11-13] and total energy expenditure (TEE)[5, 14-17], following the administration of a single dose of $D_2{}^{18}O$ or $D_2O$. Quantities of fat-free mass and fat-mass can be derived from TBW estimates by assuming that all water is contained in the lean body mass and that the composition of the lean body mass is constant. TEE determination is accomplished by the following principle. Deuterium leaves the organism only as water, whereas $^{18}O$ leaves the organism as water and carbon dioxide. The difference in the elimination rates of deuterium and $^{18}O$ is therefore a measure of the body's $CO_2$ production rate, which in its turn can be converted into energy expenditure.

The usual sample medium is plasma, because full equilibration of the tracer in plasma occurs very quickly. As plasma sampling may raise ethical and practical concerns, urine and saliva have been validated as alternative media. Urine, however, has the disadvantage that it takes longer to reach an isotopic equilibrium. Furthermore, it may be harder to collect in specific populations such as children suffering from severe generalized cerebral palsy . This condition is characterized by a moderate to severe intellectual disability in combination with a severe motor handicap, and most of the patients are incontinent. Consequently the exact time of urine production is often difficult to determine, which has an impact on the precision of the results. By contrast, saliva is collected almost immediately after it is produced. Isotopic equilibration in saliva is also faster than in urine[12]. Generally, data on the feasibility of sample collection in children are lacking.

For paediatric studies there is a need to simplify sample preparation with a minimum of sample volume (a few microlitres). This allows a high-throughput (10 measurements / hour) Continuous-flow isotope ratio mass spectrometry (CF-IRMS)[18-21] systems are designed for high productivity and for streamlining the lengthy procedures associated with off-line sample preparation and dual-inlet mass spectrometry measurements. To this aim a

helium carrier transports the gas to be measured from the preparation device to the directly coupled isotope ratio mass spectrometer. The most recent system for the analysis of $^{2}H/^{1}H$ enrichments is the thermal conversion elemental analyzer (TC/EA), which converts micromoles of water sample into $H_2$ by reaction in a glassy carbon tube reactor at 1420º C[1, 2, 22-24]. This system was used to measure $^{2}H/^{1}H$ and $^{18}O/^{16}O$ in plasma samples of rats[2]. The procedure is largely automated, so that samples can be analysed on a routine basis. The major disadvantages of TC/EA are its high purchase price and costs of consumables, as well as the need to repack the ceramic tube with glassy carbon chips after every 300–400 samples.



We report a study aimed at developing and validating a robust and quick procedure using the TC/EA-IRMS online technique for accurate simultaneous measurement of deuterium and $^{18}O$ in body fluids of children obtained in a non-invasive way. The simultaneous determination of the enrichment in deuterium and $^{18}O$ of urine and saliva samples offers great advantages in terms of non-invasive sample collection, smaller sample size, minimal sample preparation and high throughput which will be a major advantage for total body water and energy expenditure studies in neonates and children with cerebral palsy or other disabilities.

## Experimental

### Chemicals and materials

$D_2{}^{18}O$ was purchased from Cambridge Isotope Laboratories (Buchem, Apeldoorn, Netherlands). Mini centrifuge filters 0.22 µm were bought from Millipore BV (Bedford, MA, USA). The $H_2$ and CO working reference gases (Linde, quality 6.0 and 4.7 respectively) were calibrated with known reference waters, i.e. Standard Light Antarctic Precipitation (SLAP) and Greenland Ice Sheet Precipitation (GISP), purchased from the International Atomic Energy Agency (IAEA, Vienna, Austria). Working standards GS 47, GS 49 and HDW1 were used for calculation of each batch of analyses.

### Clinical study design

Ten children with severe cerebral palsy who participated in a larger nutritional study were selected to validate the method. They met the following inclusion criteria: age between 2 and 19 years; IQ<55; and a motor impairment, defined as hypertonic or hypotonic generalized cerebral palsy or a motor developmental delay to such an extent that the subject could at best crawl. Exclusion criteria were: active infection or an altered water balance (oedema or dehydration as confirmed by a physician).

The study design was approved by the Erasmus MC Medical Central Committee on Research Involving Human Subjects and informed parental consent was obtained prior to the study.

**Doubly labelled water administration/ urine and saliva sampling**

One dose (3 g/kg) of doubly labelled water ($^2H_2O$: 10%, $H_2{}^{18}O$: 5%) was administered orally, or via gastrostomy. Saliva and urine samples were collected just before administration and after an equilibration period of 4 h, in which children remained fasted. Over the following two weeks another five urine and saliva samples (days 1, 5, 8, 11 and 15) were collected and stored until analysis. Patients were not allowed to drink for 30 min prior to saliva sampling. Urine was extracted from diapers with cotton batting pads and stored in 30 mL glass urine bottles. Saliva was sampled by swabbing a dry cotton rod in the child's mouth for 2-5 minutes and then putting the cotton rod in a plastic container (Salivette, Sarstedt, Etten-Leur, The Netherlands). The container was then centrifuged (4000 g) and a clear, fluid sample (0.25 – 1.5 mL) was transferred into a glass vial of 2 mL. Both urine and saliva sample bottles were flushed with nitrogen to reduce isotope exchange in the sample container. Also for this reason we stored the samples in glass rather than plastic containers, as the latter are semi-permeable. All sample containers were stored frozen at -20º C prior to analysis.

**TC-EA/IRMS**

Experiments were carried out on a high-temperature thermal conversion elemental analyzer (TC-EA) (Thermo Fisher, Bremen, Germany) coupled with a Delta XP isotope ratio mass spectrometer (Thermo Fisher, Bremen, Germany) via a Conflo-III Interface (Thermo Fisher, Bremen, Germany). The IRMS instrument was operated at an accelerating voltage of 5 kV. The ion source was held at a pressure of $3.0 \times 10^{-6}$ Torr, and ions generated by electron impact at 70 eV. Subsequently, two sets of faraday cup detectors monitored signals for the ions at m/z 2 ($^1H/^1H$) and m/z 3 ($^2H/^1H$) ion beams of $H_2$ gas, as well as the *m/z* 28 ($C^{16}O$) and *m/z* 30 ($C^{18}O$) ion beams of CO. The $^2H/^1H$ ratios were corrected for the H3+ effect. The dynamic range of the instrument is between 0.2 and 50 V. The reactor consists of a glassy carbon tube filled with carbon chips (IVA, Meerbusch, Germany). The following conditions were used: reactor temperature 1420º C, GC column temperature 90º C, helium flow 110 mL/min, and two reference gases, hydrogen 6.0 and carbon monoxide 4.7.

**Analytical conditions**

Samples were thawed and aliquots of 50 µL were transferred to a mini centrifuge filter tube (0.22 µm) and centrifuged at 4000 g for 5 min. The filtrated fluid was collected and transferred to a sample vial with a 100 µL insert. Aliquots of 0.1 µL were injected by an auto sampler into the TC-

EA/IRMS system. Samples were analyzed in the dual measurement mode. Each analytical cycle consists of three pulses of the hydrogen reference gas introduced by the Con Flow III unit followed by measurement of the eluting hydrogen peak. After a quick swap to a different cup setting, the eluting carbon monoxide was measured, followed by three pulses of CO reference gas. Each sample was measured five times and calculated against the reference gases injected in the same run. The deuterium and oxygen isotope abundances of the water samples are expressed in delta per mil (δ pm). The $^{2}H/^{1}H$ ratios were corrected for the $H3^{+}$ effect, which was determined before each sequence.



**Calculations**

TEE can be calculated by using the equation by De Weir[25, 26] :

$$\text{TEE (kcal/day)} = \frac{3.9rCO_2(\text{liter/day})}{RQ} + 1.11rCO_2$$

where $rCO_2$ is expressed in L/day and can be converted from mol/day by multiplying by 22.4. RQ is oxygen consumption/$rCO_2$.

RQ can be measured by performing indirect calorimetry for at least 20 minutes. $rCO_2$ can be calculated using the following equation:

$$rCO_2 = 0.4554N(1.01K_o - 1.04K_h)$$

where $K_o$ and $K_h$ the rate constants at which $^{2}H_2O$ and $^{18}O$ are lost from the pool.

The dilution space for deuterium or $^{18}O$ or TBW can be calculated using the following equation:

$$N\ (\text{mol}) = \left[\frac{WA}{(18.02)a}\right] X \left[\frac{(da\text{-}dt}{(ds\text{-}dp)}\right]$$

where N is the pool space, *W* is the amount of water used to dilute the labelled water, *A* is the weight of labelled water administered, *a* is the diluted dose for analysis, and d is the enrichment of dose (*a*), dilution water (*t*), post dose sample (*s*) and pre dose baseline (*p*) samples[11, 25]. Total body water can be determined using either deuterium or $^{18}O$. To correct for isotopic fractionation the deuterium dilution space must be divided by 1.04 and the $^{18}O$ diluting space by 1.01.[6]

**Statistics**

Calculations were made using Microsoft Office - Excel software (version 2003; Microsoft Corp, Redmond, WA, USA). Statistical analysis was performed using GraphPad Prism software (version 4.0; San Diego, CA, USA). The intra-assay precision was determined by a multiple measurement (n=10) of standards on a given day. Samples of these standards were also injected together with each series of analyses, and the enrichments determined in this way provided the inter-assay precision. The Bland-Altman method[27] was used for the comparison of the two different methods.



## Results and discussion

### Calibration

Three well defined standard solutions of water with known deuterium and $^{18}O$ enrichments (GS 47, GS 49 and HDW1) were used to establish the calibration curve for correction of the values. They were in a range of -50.6 δ ‰ up to 136.7 δ ‰ for $^{18}O$ and of -400.2 δ ‰ up to 918.5 δ ‰ for HD respectively. These set of standards were analyzed at the beginning and end of each sequence of 28 samples. An excellent correlation was obtained for both $^{18}O$ and HD (Fig 1a and 1b).

### Sample measurement

Memory effects are known to occur when measuring samples using this technique[1, 22, 24, 28, 29]. When injecting biological samples containing varying enrichments of D and $^{18}O$ into the IRMS, trace amounts of the previous sample will be carried over, resulting in a memory effect

To minimize this effect, each sample is injected five times. The first two measured enrichments are excluded from the final analysis. After each injection the full volume of the syringe is flushed five times with air in order to avoid cross contamination of the samples. Finally, the syringe is flushed with one syringe volume of sample prior to injection. Samples were analyzed in dual measurement mode with a jump calibration between H and CO measurements. After each 150 injections the septum of the injector was replaced and after each 300 injections, the glassy carbon reactor was exchanged with a new one.

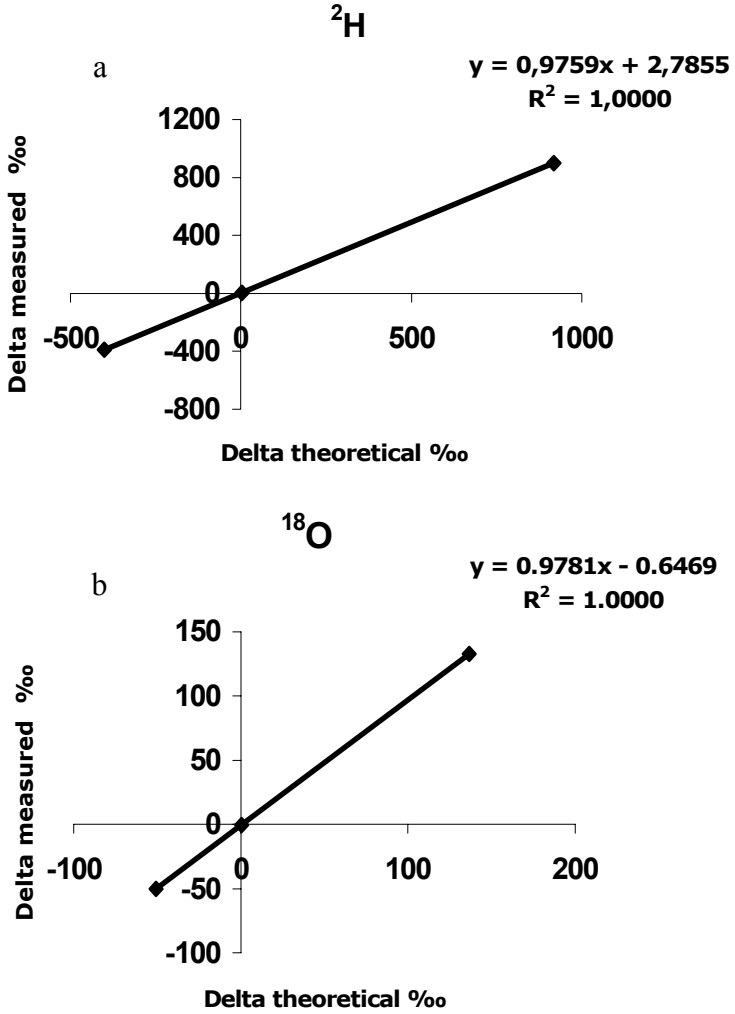


***Figure 1*** *Calibration curve for measurement of $^{2}H_2$ enrichment (a) and $^{18}O$ enrichment (b).*

**Accuracy and precision**

The intra-assay precisions of the $^{18}O/^{16}O$ and $^{2}H/^{1}H$ analyses were determined using three well defined doubly labelled water standards. Standards were determined ten times on a given day, and these results provided the intra-assay precision (Table 1). Samples of this water were also injected together with each series of analyses, and the enrichments determined in this way provided the inter-assay precision (Table 2). The variability of the D enrichment was higher than that of the $^{18}O$ enrichment, in line with findings from the literature[2]. The accuracy of the TC-EA/IRMS instrument was determined by injecting

the two certified reference waters, i.e. SLAP and GISP several times (Table 3). The measured D and $^{18}O$ enrichments for these two water standards are in close agreement with the values determined by the IAEA against the international reference, V-SMOW. These data demonstrate the reliable performance of the system at the level of accuracy reported in the literature.

***Table 1*** *Intra-assay precision assessed by replicate analysis of specimen aliquots on a single day.*

**Intra assay precision (n=9)**

| | **δ Deuterium ‰** | **δ Deuterium ‰** | **δ Oxygen-18 ‰** | **δ Oxygen-18 ‰** |
|---|---|---|---|---|
| | **Gs 49** | **HDW-1** | **Gs 49** | **HDW-1** |
| | 6.985 | 873.745 | 2.341 | 132.226 |
| | 6.077 | 873.538 | 2.409 | 132.768 |
| | 6.09 | 874.994 | 2.331 | 133.271 |
| | 7.567 | 880.998 | 2.469 | 133.522 |
| | 7.238 | 881.97 | 2.489 | 132.879 |
| | 7.458 | 883.443 | 2.49 | 133.514 |
| | 5.841 | 878.854 | 2.648 | 133.799 |
| | 6.285 | 880.165 | 2.879 | 133.9 |
| | 5.899 | 880.871 | 2.712 | 133.74 |
| **Mean** | **6.60** | **878.73** | **2.53** | **133.29** |
| **Sd** | **0.701** | **3.713** | **0.182** | **0.560** |

***Table 2*** *Inter-assay precision assessed by replicate analysis of specimen aliquots on several days.*

**Inter assay precision (n=9 days mean)**

| | **δ Deuterium ‰** | **δ Deuterium ‰** | **δ Oxygen-18 ‰** | **δ Oxygen-18 ‰** |
|---|---|---|---|---|
| | **Gs 49** | **HDW-1** | **Gs 49** | **HDW-1** |
| | 6.38 | 874.09 | 2.16 | 132.76 |
| | 7.42 | 882.14 | 2.15 | 133.31 |
| | 6.01 | 879.96 | 2.75 | 133.81 |
| | 5.80 | 860.44 | 2.75 | 132.90 |
| | 5.23 | 860.64 | 2.62 | 131.54 |
| | 6.57 | 881.28 | 2.54 | 131.10 |
| | 5.12 | 870.75 | 2.38 | 131.75 |
| | 6.06 | 865.55 | 2.44 | 133.67 |
| | 6.45 | 878.91 | 2.59 | 133.71 |
| **Mean** | **6.12** | **872.64** | **2.49** | **132.73** |
| **Sd** | **0.705** | **8.707** | **0.225** | **1.025** |

***Table 3*** *Two international standards, GISP and SLAP, were assessed by replicate analysis to determine the accuracy.*

| Standard | δ Deuterium Value IAEA | δ Deuterium Measured | Accuracy | δ Oxygen18 Value IAEA | δ Oxygen18 Measured | Accuracy |
|---|---|---|---|---|---|---|
| | ‰ | ‰ | ‰ | ‰ | ‰ | ‰ |
| SLAP | -428.00 | -425.12 | 2.88 | -55.50 | -54.83 | 0.67 |
| | | -425.46 | 2.54 | | -54.99 | 0.51 |
| | | -426.44 | 1.56 | | -54.91 | 0.59 |
| | | -426.32 | 1.68 | | -55.15 | 0.35 |
| | | -426.68 | 1.32 | | -55.21 | 0.29 |
| GISP | -189.30 | -188.47 | 0.83 | -24.77 | -24.27 | 0.50 |
| | | -188.09 | 1.21 | | -24.33 | 0.44 |
| | | -187.82 | 1.48 | | -24.38 | 0.39 |
| | | -187.37 | 1.93 | | -24.24 | 0.53 |
| | | -187-91 | 1.39 | | -24.46 | 0.31 |
| Mean SLAP | | -426.00 | 2.00 | | -55.02 | 0.48 |
| Sd | | 0.675 | | | 0.160 | |
| CV % | | -0.16 | | | -0.29 | |
| Mean GISP | | -187.93 | 1.37 | | -24.34 | 0.43 |
| Sd | | 0.401 | | | 0.088 | |
| CV % | | -0.21 | | | -0.36 | |

**Analysis of human saliva and urine samples**

Deuterium and $^{18}O$ enrichments were measured in urine and saliva from 10 subjects during a period of two weeks. Values measured in saliva and urine both showed a linear decline in enrichment as shown in the logarithmic presentation (Fig 2). The enrichments of D and $^{18}O$ of the 20 saliva and 20 urine samples showed a good correlation with only a small variation of 5.9% (sd= 0.026) for deuterium and 0.95% for $^{18}O$ (sd= 0.002). The average enrichments for deuterium (4.83% n=40) and for $^{18}O$ (2.33% n=40) were consistently lower for saliva (Table 4, Fig 3). While saliva production and collection occur roughly at the same time, the time between urine production and collection is often unknown in incontinent subjects. However, it is safe to assume that the latter time is longer, resulting in a higher value for the enrichments of deuterium and $^{18}O$ in urine.

Experiments for measurement of deuterium and $^{18}O$ have also been assessed with other techniques, i.e. using a laser and IRMS[30, 31] and high temperature conversion IRMS[32, 33], but these techniques show to have less precision and accuracy compared to the technique using TC-EA/IRMS.

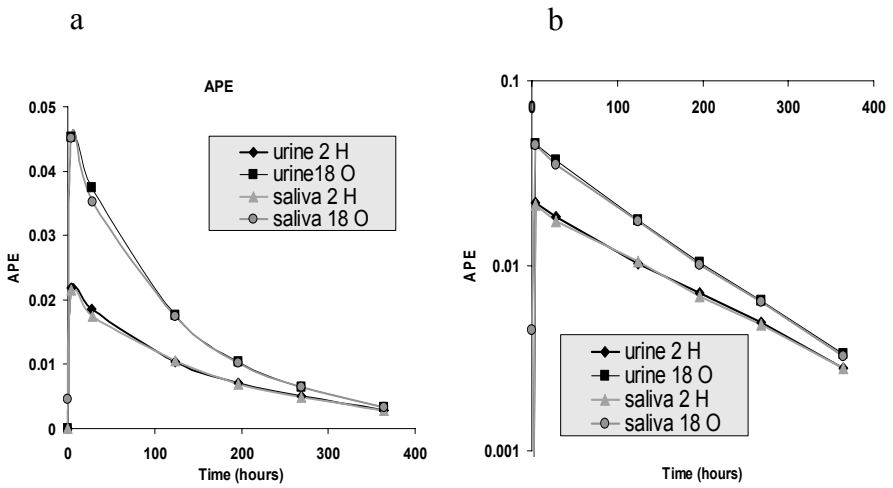


***Figure 2*** *The decline of $^{2}H_2$ enrichment and $^{18}O$ enrichment after administration of an oral dose $D_2{}^{18}O$ (a) and the same values expressed logarithmic (b).*

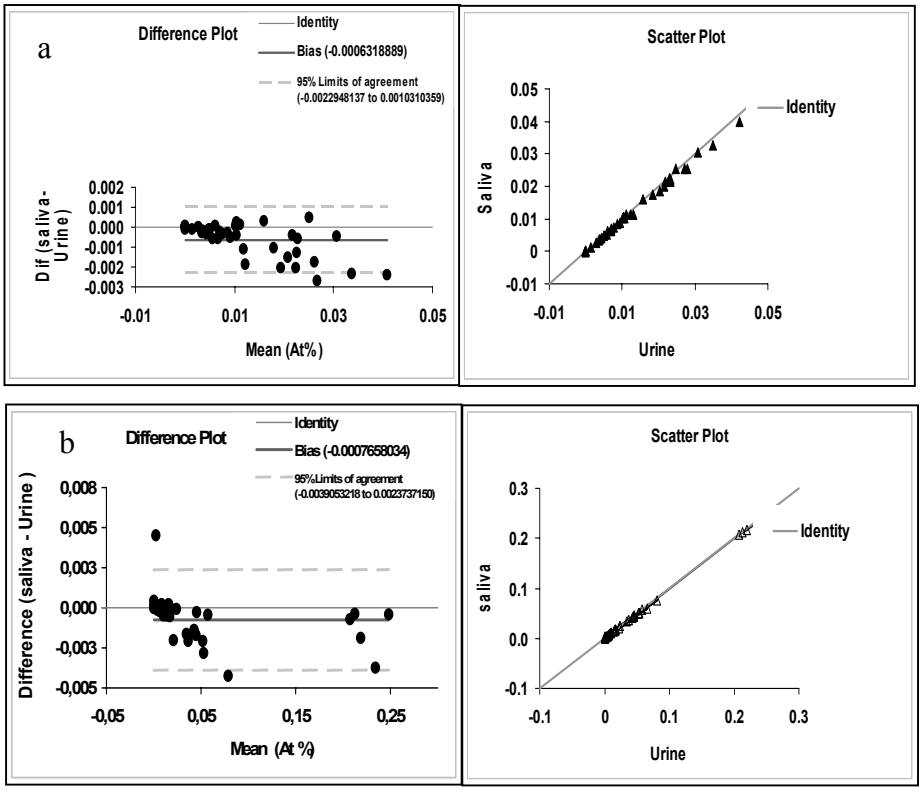


***Figure 3*** *Comparison a) deuterium measurement and b) oxygen$^{18}$ of both urine and saliva (Bland Altman plot)*

***Table 4*** *Comparison of urine and saliva analysis of ten subjects after administration of an oral dose $D_2{}^{18}O$.*

| Subject | Time (hours) | Type | At% D | Sd (n=2) | At% 18O | Sd (n=2) |
|---|---|---|---|---|---|---|
| 506 | 0.0 | Urine | 0.0150237 | 0.0000113 | 0.1993150 | 0.0031997 |
| | 0.0 | Saliva | 0.0150397 | | 0.2038400 | |
| | 4.2 | Urine | 0.0369177 | 0.0002854 | 0.2446730 | 0.0002006 |
| | 4.1 | Saliva | 0.0365140 | | 0.2443893 | |
| | 196.2 | Urine | 0.0221397 | 0.0001706 | 0.2097630 | 0.0002001 |
| | 196.3 | Saliva | 0.0218983 | | 0.2094800 | |
| | 364.2 | Urine | 0.0178130 | 0.0000067 | 0.2026390 | 0.0000622 |
| | 364.3 | Saliva | 0.0178225 | | 0.2025510 | |
| 505 | 0.0 | Urine | 0.0153223 | 0.0000005 | 0.1995747 | 0.0001223 |
| | 0.0 | Saliva | 0.0153230 | | 0.1997477 | |
| | 3.5 | Urine | 0.0407120 | 0.0012398 | 0.2502970 | 0.0014632 |
| | 3.5 | Saliva | 0.0424653 | | 0.2523663 | |
| | 196.0 | Urine | 0.0247163 | 0.0003578 | 0.2133520 | 0.0003352 |
| | 195.0 | Saliva | 0.0242103 | | 0.2128780 | |
| | 363.5 | Urine | 0.0190680 | 0.0001214 | 0.2038390 | 0.0001042 |
| | 363.5 | Saliva | 0.0188963 | | 0.2036917 | |
| 516 | 0.0 | Urine | 0.0154283 | 0.0000181 | 0.1996250 | 0.0000207 |
| | 0.0 | Saliva | 0.0154540 | | 0.1995957 | |
| | 3.8 | Urine | 0.0503500 | 0.0045839 | 0.2651190 | 0.0044354 |
| | 3.8 | Saliva | 0.0438673 | | 0.2588463 | |
| | 196.5 | Urine | 0.0312137 | 0.0002041 | 0.2235687 | 0.0000431 |
| | 196.7 | Saliva | 0.0315023 | | 0.2235077 | |
| | 365.8 | Urine | 0.0156610 | 0.0049578 | 0.2001403 | 0.0065219 |
| | 365.7 | Saliva | 0.0226723 | | 0.2093637 | |
| 507 | 0.0 | Urine | 0.0155183 | 0.0000693 | 0.2002643 | 0.0000354 |
| | 0.0 | Saliva | 0.0154203 | | 0.2003143 | |
| | 3.2 | Urine | 0.0468937 | 0.0030479 | 0.2606437 | 0.0057624 |
| | 4.0 | Saliva | 0.0425833 | | 0.2524943 | |
| | 192.8 | Urine | 0.0257623 | 0.0000212 | 0.2158477 | 0.0001558 |
| | 192.9 | Saliva | 0.0257923 | | 0.2160680 | |
| | 361.6 | Urine | 0.0205453 | 0.0000627 | 0.2064480 | 0.0000097 |
| | 360.8 | Saliva | 0.0204567 | | 0.2064617 | |
| 515 | 0.0 | Urine | 0.0153497 | 0.0000057 | 0.1991937 | 0.0000354 |
| | 0.0 | Saliva | 0.0153417 | | 0.1991437 | |
| | 3.8 | Urine | 0.0574973 | 0.0017147 | 0.2798440 | 0.0029974 |
| | 3.9 | Saliva | 0.0550723 | | 0.2756050 | |
| | 196.3 | Urine | 0.0222773 | 0.0004106 | 0.2095947 | 0.0003460 |
| | 196.3 | Saliva | 0.0216967 | | 0.2091053 | |
| | 362.8 | Urine | 0.0168043 | 0.0000790 | 0.2009243 | 0.0000387 |
| | 362.7 | Saliva | 0.0166927 | | 0.2009790 | |
| Mean | | | 0.0260934 | 0.0015348 | 0.2191274 | 0.0020916 |
| CV% | | | 5.88 | | 0.95 | |

## Conclusions

We have validated a method for simultaneous measurement of deuterium and $^{18}O$ enrichment in urine and saliva samples, enabling to determine children's total body water composition and energy expenditure. Sample preparation is much simpler than with the classical methods. The analysis process is fully automated, with very small samples (0.1 µL) directly injected into a TC-EA/IRMS system equipped with a liquid auto sampler. Samples are converted into hydrogen and carbon monoxide gases that are transferred on-line using helium gas into the directly coupled isotope ratio mass spectrometer. The TC-EA/IRMS system provides for accurate and simultaneous measurement of D and $^{18}O$ enrichment of saliva and urine samples. Although the results did not differ between the two sample types, sampling of saliva is preferred because its production time of saliva can be determined almost exactly. In addition, the sampling of saliva is less invasive than blood, which is an important issue in paediatric studies. This methodology is a good alternative to the laborious off-line IRMS measurements. This method is saving labour and analysis time and therefore also lowering the analysis costs. The accuracy, simplicity and robustness of the TC/EA-IRMS using the doubly labelled water dilution technique in saliva samples can be a great support to assess body composition and energy expenditure in all subjects in which blood collection is less desirable.

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CHAPTER

# 4

# Measuring energy expenditure and total body water in children with severe cerebral palsy and intellectual disability using the doubly labeled water method: comparability of urine and saliva sampling

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**Abstract**

Background: Energy expenditure and body composition are accurately measured by the doubly labeled water (DLW) method. However, information on the comparability of its outcomes based on either urine or saliva samples in children with severe cerebral palsy (CP) and intellectual disability (ID), is lacking.
Objective: Our aim is to evaluate agreement of the outcomes of the DLW method based on urine and saliva sampling and its feasibility in children with severe CP and ID.
Design: Thirteen children (aged 3-15 y) received a single dose of DLW. Total energy expenditure (TEE) and total body water (TBW) were calculated based on urine and saliva samples. TBW was calculated using multi-point (TBW-MP) and two-point analysis (TWB-2P). To correct for age and weight, TEE was expressed as a percentage of recommended daily allowance (%TEE of RDA) and TBW as a percentage of weight (%TBW). Agreement between the outcomes of urine and saliva sampling was evaluated using Bland and Altman analyses.
Results: 90.1% of urine and 86.8% of saliva samples were successfully analyzed. Limits of agreement (mean ± 2SD) between urine and saliva samples were favorable for the outcomes %TEE of RDA (mean difference –0.1% ± 9.7%) and %TBW-MP (mean difference –1.8% ± 2.7%), but less favorable for %TBW-2P (mean difference –2.1% ± 8.4%).
Conclusions: Both urine and saliva are acceptable sample media in the DLW method in children with severe CP and ID. While saliva sampling deserves preference from a theoretical standpoint, one should be aware of hyposalivation in some children with severe CP.

## Introduction

Malnutrition is a major health problem in children with severe cerebral palsy (CP)[1] and has led to a considerable body of research into the variations in energy expenditure and body composition in this group of children. The technique that is considered to be a method of reference in this particular area of research is the doubly labeled water (DLW) method.[2-4] This method requires administering stable isotopes (tracers) of deuterium ($^2H_2O$) and labeled oxygen ($H_2O^{18}$) and provides accurate and reliable measurements of total energy expenditure (TEE) and total body water (TBW).[5, 6]

TEE and TBW can be calculated most accurately by measuring isotope enrichment in serum, because full equilibration of the tracer in serum transpires very quickly.[7] However, because of its invasiveness and because of practical concerns, urine and saliva sampling are more patient friendly for research purposes and were found to be valid alternatives compared with serum in the past.[7, 8] Urine sampling is most commonly used in the DLW method in children with CP,[2, 3, 9] but has a number of disadvantages. First of all, it takes longer for the tracers to equilibrate in the bladder contents.[6] Secondly, because almost all children with severe generalized CP are incontinent, urine has to be collected from a urine collection bag or cotton batting pads in the diaper. The time from the initial production at the renal level and the actual collection of urine is therefore not known. Both problems add imprecision to the measurement.

Saliva sampling, however, is not affected by these issues since saliva can be collected almost immediately after it is produced. Isotopic equilibration in saliva is also faster than in urine.[7] However, some children with severe CP produce large amounts of saliva that are not swallowed. Because of these excessive amounts of saliva, children may be treated with botulinum injections or surgery, resulting in a restricted salivary production. In addition, the saliva may potentially be diluted by the large amounts of bronchial slime that these children can cough up or by esophageal refluxate that reaches the oral cavity. In addition to these problems, some children have oral hypersensitivity to such an extent that they might not even allow access to the mouth to collect saliva.

In the past, urine has been sampled most often in the DLW method in children with CP[2-4, 9], but, more recently, Sullivan et al.[10] used both urine and saliva samples in their study evaluating the effect of gastrostomy tube feeding on weight gain and overfeeding in 40 children with spastic quadriplegic CP. However, they did not report whether the clinical outcomes of the DLW method were comparable between urine and saliva samples and how they dealt with differences of these outcomes, if present. The question is whether in this group of complex children, in whom sampling urine or saliva is not always easy, it would be warranted to assume comparability of outcomes using urine and saliva samples.

The aim of this study is therefore to establish, when applying the DLW method in children with severe CP and intellectual disability (ID) in a field setting, (1) whether saliva and urine sampling result in the same clinical outcomes and (2) report on the feasibility of both urine and saliva sampling in this specific group of children.

**Subjects & Methods.**

**Subjects**

Thirteen children with severe CP and ID (seven males and six females) were recruited from a large children's care center in Rotterdam. Inclusion criteria were: age between 2 and 19 years; a moderate to severe ID with an estimated IQ below 55; and a motor impairment, defined as hypertonic or hypotonic generalized CP, or a motor developmental delay to such an extent that a child could at best crawl. The motor impairment had to be the equivalent of Gross Motor Functioning Classification System level four or five. (11) Children that had contracted an active infection or had an altered water balance (edema or dehydration) at the time of the measurements were excluded. The parents or legal guardians of the children provided written informed consent and the Dutch Central Committee on Research Involving Human Subjects approved the study protocol.

**Doubly labeled water method**

Subjects were studied in the daycare center at least 3 h after their regular morning feeding. Body weight (BW) was measured with an electronic wheelchair scale (Universal PM 7050, Lopital, Oisterwijk, Netherlands) to the nearest 0.1 kg. They then received a single dose of DLW ($^2H_2O$: 10%, $H_2O^{18}$: 5%, Cambridge Isotope Laboratories, distributed by Buchem BV, Apeldoorn, The Netherlands) of 3 g/kg BW orally or via gastrostomy. The dose container was washed out with 50 mL of plain tap water and administered to the child. Special care was taken to avoid spillage in children receiving the DLW orally. Saliva and urine samples were taken just before administration of the DLW and after an equilibration period of four hours, in which children remained fasting. In the subsequent two weeks, preferably in the morning, five additional urine and saliva samples (day 1, 5, 8, 11 and 15) were taken in order to calculate TEE. The researcher (RR) collected most samples, but the caregivers were also responsible for collection in some instances.

**Urine and saliva sampling**

Diapers with cotton batting pads were used for collection of urine samples. Approximately 5 mL of urine was retained at each time point and stored in 30 mL glass urine bottles. Saliva was sampled by swabbing a cotton rod in the mouth of the child for 2-5 minutes and then putting it in a plastic container (Salivette, Sarstedt, Nümbrecht, Germany). Children were not allowed to drink any liquids 30 minutes prior to the saliva sampling to avoid dilution of the sample. The saliva container was then centrifuged (3000 rpm) and a clear, fluid sample (0,25 – 1,5 mL) was pipetted into a glass vial of 2 mL. The vial, containing saliva, and the urine bottle were flushed with nitrogen in order to reduce isotope exchange inside the sample containers. Glass, instead of plastic, vials were used to store saliva samples, because of the semi permeability of plastic containers. All sample containers were stored frozen at -20° C prior to analysis.

To assess feasibility of both urine and saliva sampling in these children, the occurrence and reasons for failed sampling or failed analysis were recorded.

**Analysis and calculations**

Enrichments of the purchased DLW and the collected urine and saliva samples were measured in a high-temperature conversion elemental analyzer (TC-EA) coupled with a Delta XP isotope ratio mass spectrometer via a Conflo-III Interface (Thermo Fisher, Bremen, Germany).

Only children, in whom a minimum of four out of a possible six successfully analyzed samples of urine as well as saliva could be obtained, were entered in the final analysis. Excluded samples contained either insufficient material or caused a notable spike in the residuals of the elimination curves of the isotopes.

TBW was calculated by estimating isotope dilution spaces (IDS) by two different techniques. In the multi-point procedure, distribution space is calculated by determining the isotope dilution at time zero by back extrapolation using the same data as that used to measure the slopes of the elimination of both isotopes (TBW-MP). In the two-point methodology volume is calculated from determining isotopic dilution at a plateau four hours after administering the DLW (TBW-2P). In either situation, the isotope dilution spaces of $^{2}H$ and $^{18}O$ were calculated by using the following formula:

$$IDS(kg) = \frac{d}{MW} x \frac{APE}{100} x 18.02$$

Where d is the dose of the isotope in grams, MW is the molecular weight of the tracer, and APE is the atom percent excess.

Using the IDS of both $^{2}H$ and $^{18}O$, TBW is calculated using the formula:

$$TBW(kg) = \frac{\frac{IDS_{2H}}{1.041} + \frac{IDS_{18O}}{1.007}}{2}$$

The constants 1.007 and 1.041 were included to adjust for the differences between the isotope dilution spaces and TBW due to isotope exchange.[12] The observed $IDS_{2H}$ and $IDS_{18O}$ values were normalized by a fixed factor of 1.034.[13]

TEE can be calculated by using the following equation, adapted from DeWeir[14]:

$$TEE(kcal/day) = \frac{3.9 rCO_2 (liter/day)}{RQ} + 1.11 rCO_2$$

where $rCO_2$ is expressed in liter/day and Respiratory Quotient (RQ) is oxygen consumption/$rCO_2$.

RQ was measured for each child by performing indirect calorimetry (Deltatrac II MBM-200, Datex Division Instrumentarium, Helsinki, Finland) for at least 20 minutes. If an individual RQ was not available, the individual Food Quotient (FQ) was calculated using three-day food questionnaires. FQ most closely approximates RQ.[15]

$rCO_2$ was calculated using the following equation, which is an adapted version by Racette[12] of the original formula by Schoeller[7]:

$$r_{CO_2} = (N/2.078)(1.007K_O - 1.041K_D) - 0.0246R_{Gf}$$

where N is total body water (TBW); $K_O$ and $K_D$ are the $^{18}O$ and $^{2}H$ isotope disappearance rates, respectively; and rGf is the rate of water loss through gaseous routes subject to isotope fractionation. The latter is estimated as 1.05 N (1.007 KO – 1.041 KD).

To account for the broad diversity in age of the children studied, TEE was also expressed as a percentage of Recommended Daily Allowance (%TEE of RDA) based on national recommendations of daily energy intake published by the Health Council of the Netherlands.[16] To account for body size, TBW was expressed as a percentage compared to total body weight of the child calculated by the multi-point procedure (%TBW-MP) and the two point method (%TBW-2P).



**Statistical analysis**

The absolute differences of TEE, TBW-MP and TBW-2P between urine and saliva samples were expressed as percentages of the outcome based on the urine samples. Correlations of these absolute outcomes and those corrected for age and body weight (TEE of RDA, %TBW-MP and %TBW-2P) of the two sampling methods were calculated using intraclass correlation coefficients (ICCs). To study the agreement of these outcomes between urine and saliva, Bland and Altman limits of agreement analyses were performed.[17] With this method, a pair-wise comparison is used to show the mean difference and the limits of agreement (mean difference ± 2 SD of the difference) between the outcomes based on urine and saliva samples by plotting their mean difference against the mean of the two sampling methods. All analyses were done using SPSS 15.0 software (SPSS Inc, SPSS for Windows, Chicago, Illinois, United States).

## Results

**Characteristics of subjects**

General characteristics of the seven male and six female subjects are summarized in Table 1. Ages ranged from 3 to 15 years. Eleven children were non-ambulatory (GMFCS 5), while two could walk with a walking aid for a short distance or crawl (GMFCS 4). Twelve children received food through a gastrostomy and had a severe intellectual disability (ID). One child received food orally and had a moderate ID.

**Feasibility**

In total, urine was collected and analyzed successfully in 90,1% of cases and saliva in 86,8%. Reasons for failed urine and saliva collection or analysis are summarized in Table 2. The condition that four or more samples had to be analyzed successfully was not met in one child (subject 10). In subject 10 the number of adequate saliva samples was insufficient because of

hyposalivation. A TBW-2P calculation was not possible in subject 13, because a second urine sample could not be obtained in this child.

***Table 1*** *General characteristics*

| | |
|---|---|
| Total number (n) | 13 |
| Mean (± Sd) age (years) | 7.1 ± 4.0 |
| Gender | 7 m, 6 f |
| Mean (± Sd) weight (kg) | 21.8 ± 9.6 |
| Etiology (n) | |
| Congenital | 3 |
| Perinatal | 6 |
| Acquired | 2 |
| Combination | 2 |

*Sd= standard deviation*

***Table 2*** *Reasons for missing samples*

| Urine | | Saliva | |
|---|---|---|---|
| Failed sampling | 5 / 91 (5.5%) | Failed sampling | 7 / 91 (7.7%) |
| Child had not urinated | 4 | Not enough saliva in cotton rod | 3 |
| Sampling forgotten by caregiver | 1 | Sampling forgotten by caregiver | 1 |
| | | Drank before sampling | 1 |
| | | Unknown reason | 2 |
| Samples excluded * | 4 / 86 (4.6%) | Samples excluded * | 5 / 84 (6.0%) |
| Total | 9 / 91 (9.9%) | Total | 12 / 91 (13.2%) |

* Samples were excluded if they caused a spike in the residuals of the elimination curves of the isotopes

**Comparison of clinical outcomes**

The absolute scores of TEE, TBW-MP and TBW-2P for all 13 children are listed in Table 3. Table 4 shows the group means of the absolute outcomes and those corrected for age and weight for urine and saliva sampling. Subject 10 was excluded from the agreement analyses for the outcomes TEE and TBW-MP, because she only produced three successfully analyzed saliva samples. For the TBW-2P analysis, three children were excluded; one child (subject 13), because no second sample of urine could be collected on the first day and another two children (subjects 1 and 9) because the TBW calculated from the urine samples was 5.7 (52%) and 20.3 kg (156%) higher than that determined from saliva samples. This could either indicate that in these children the bladder contents had not reached isotopic equilibrium or that the urine sample had been contaminated with ambient water, as described earlier by Schoeller et al.[6]

***Table 3*** *Raw data of clinical outcomes*

| patient | Age (y) | TEE (kcal) urine | TEE (kcal) saliva | TBW - MP (kg) urine | TBW - MP (kg) saliva | TBW - 2P (kg) urine | TBW - 2P (kg) saliva |
|---|---|---|---|---|---|---|---|
| 1 | 15 | 870 | 849 | 11.8 | 12.5 | 33.3 | 13.0 |
| 2 | 7 | 664 | 502 | 9.0 | 9.0 | 9.3 | 11.0 |
| 3 | 5 | 745 | 725 | 7.1 | 7.3 | 7.5 | 7.2 |
| 4 | 3 | 894 | 928 | 6.7 | 6.8 | 6.4 | 6.5 |
| 5 | 4 | 424 | 449 | 5.3 | 5.4 | 4.3 | 5.0 |
| 6 | 11 | 1143 | 1026 | 17.6 | 18.4 | 18.0 | 19.0 |
| 7 | 5 | 672 | 621 | 8.8 | 8.7 | 7.8 | 8.6 |
| 8 | 13 | 950 | 1110 | 15.7 | 17.2 | 16.7 | 16.3 |
| 9 * | 5 | 1438 | 1567 | 10.2 | 10.9 | 16.7 | 11.0 |
| 10 ** | 4 | 851 | --- | 7.3 | --- | 8.6 | 7.7 |
| 11 | 6 | 585 | 531 | 8.8 | 9.4 | 9.3 | 10.3 |
| 12 | 11 | 785 | 819 | 11.2 | 11.9 | 11.5 | 12.2 |
| 13 * | 3 | 962 | 1026 | 7.6 | 7.8 | --- | 8.0 |

TEE= Total energy expenditure, TBW – MP = Total body water, calvulated by multi-point analysis, TBW – 2P = Total body water, calculated by two-point analysis
* excluded from two-point analysis
** excluded from multi-point analysis, because of insufficient number of saliva samples

***Table 4*** *Clinical outcomes*

| | Mean ± Sd urine | Mean ± Sd Saliva | Mean Difference ± 2 Sd | ICC |
|---|---|---|---|---|
| *Absolute* | | | | |
| TEE (kcal) (n=12) | 844 ± 268 | 846 ± 317 | -1.8 ± 186.4 | 0.949 |
| TBW-MP (kg) n=12) | 9.9 ± 3.6 | 10.4 ± 4.0 | -0.5 ± 0.9 | 0.993 |
| TBW-2P (kg) (n=10) | 9.9 ± 4.4 | 10.4 ± 4.4 | -0.4 ± 1.6 | 0.984 |
| *Relative to age and weight* | | | | |
| % TEE of RDA (n=12) | 48.0 ± 17.6 | 48.1 ± 20.5 | -0.1 ± 9.7 | 0.968 |
| % TBW – MP (n=12) | 46.8 ± 6.5 | 48.5 ± 6.4 | -1.8 ± 2.7 | 0.977 |
| % TBW – 2P (n=10) | 45.5 ± 5.0 | 47.7 ± 5.1 | -2.1 ± 8.4 | 0.651 |

Mean difference = urine minus saliva, TBW – MP= total body water, calculated by multi-point analysis, TBW – 2P = Total body water, calculated by two-point analysis, Sd = standard deviation, ICC = intraclass correlation coefficient, %TEE of RDA = percentage of expected TEE based on Dutch guidelines, %TBW = percentage total body water of body weight

On average, the outcome TEE is 0.8% (SD11.0%) higher when saliva samples are used and the outcome TBW-MP and TBW-2P 4.0% (SD3.2%) and 5.2% (SD9.1%) respectively. ICCs for the clinical outcomes with TEE, TBW-MP and TBW-2P were all above 0.9 as were the corrected outcomes %TEE for RDA and %TBW-MP. In contrast, the ICC for %TBW-2P was below 0.7. The limit of agreement of the mean differences between measurements of TBW-2P and %TBW-2P were higher than those for TBW-MP and %TBW-MP (1.6 and 8.4 versus 0.9 and 2.7). Figure 1 depicts Bland and Altman plots of the comparison between outcomes of urine and saliva for %TEE for RDA (1a), %TBW-MP (1b) and %TBW-2P (1c) respectively.

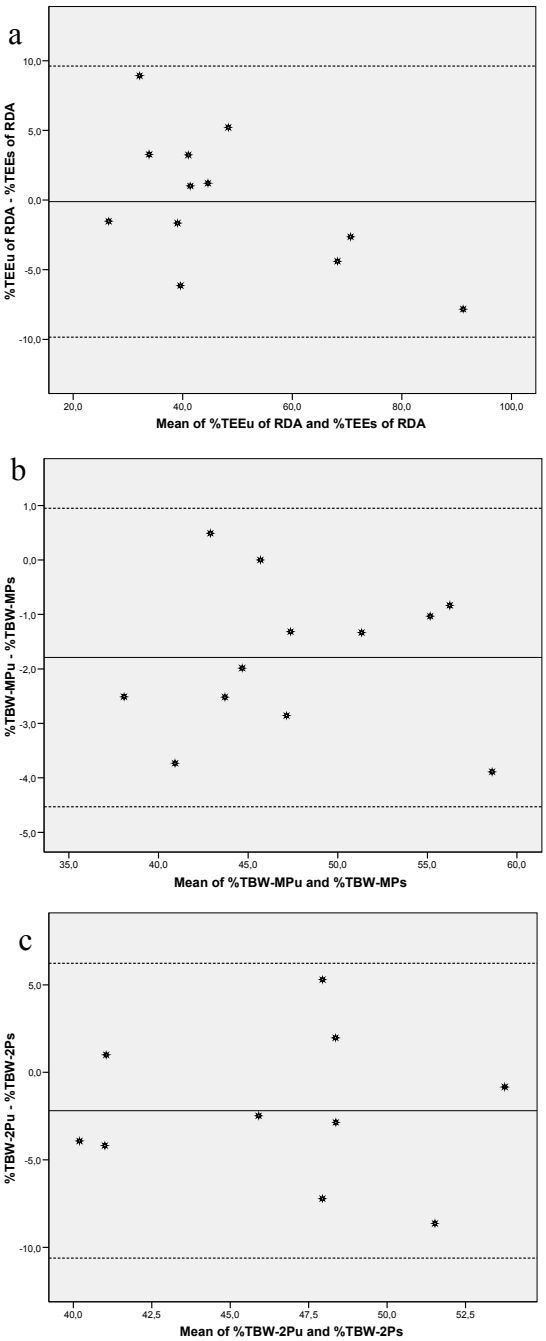


***Figure 1*** *Bland and Altman plot of a) %TEE of recommended daily allowance (RDA) b) percentage total body water of body weight using multi-point analysis c) percentage total body water of body weight using the two-point analysis*

## Discussion

This study shows that urine and saliva samples for the DLW method can be reliably obtained in most children with severe CP and ID and successfully analyzed in the laboratory. However, because of hyposalivation, one child that had produced insufficient saliva samples was excluded. Fortunately, energy expenditure and total body water outcomes between urine and saliva samples agreed favorably, which would seem to allow calculation of clinical outcomes based on either sampling medium if the other fails.

TBW seems to be structurally higher using saliva samples than using urine samples. An explanation for this observation may be that the time between the production and collection of the urine sample is unknown, while saliva is immediately collected after its production. It can therefore be assumed that even when urine and saliva are collected at the same time, enrichments for urine are higher since they might have been produced some time before the collection time noted. Consequently, the quantity of tracer eliminated will appear to be lower and as a result the clinical outcomes will be lower relative to the outcomes for saliva. However, TEE is not affected by this phenomenon as this outcome is based upon the difference between elimination rates of both tracers, which is independent of the precise enrichments at the different sampling times.

The way the urine and saliva samples are collected has most probably not added to the variability. Cotton balls have been found to be suitable for urine collection in children for the doubly labeled water technique, if the volume of urine that is expressed from the cotton ball is more than 5 mL.[18] Schoeller et al[7] used cotton rolls to collect saliva samples in order to validate the enrichment of deuterium and labeled oxygen of the sample expressed from these rolls to those of serum samples. Although they found that enrichments were slightly higher in saliva compared with serum, the authors did not attribute this to the collection method. It was probably the result of evaporation of water in the mouth that preferentially removes the lighter isotopes of hydrogen and oxygen resulting in a relatively more enriched saliva sample. The only difference between their experiments and ours is that we could not dry our cotton sampling material in an oven and store them in a dessicator before sampling, because our sampling was done in the daycare center. Failure to dry the cotton rolls could have possibly resulted in a 1% to 2% relative error in total body water determination, according to Schoeller's experiments.[7] However, since we used cotton sampling material in both urine and saliva, it probably did not add to the variability between these two media. It may, however, have produced a small error compared to more controlled hospital settings.

In order to measure TBW, the use of the multi-point procedure leads to less variability than the 2-point method. In our study, this is demonstrated by the larger limits of agreement and lower ICC between urine and saliva using the two-point method compared with the multi-point procedure. In their experiments comparing enrichments from urine and saliva samples with serum samples in five healthy women, Jankowski et al[8] found that DLW takes longer to equilibrate in urine (2.5-5 h) than in saliva (2.0-4.0 h), which has also been described by Schoeller et al.[6] This creates the possibility that the tracers have not fully equilibrated in urine after four hours, while they have in saliva. A possible solution to this problem is to sample urine or saliva several

times around the four hour mark in half or full hour intervals, as suggested by Jankowski et al.[8] However, this doesn't circumvent the problem that urine sampling is based on spontaneous production and might therefore not be available at the desired time intervals. It also adds further complexity and burden, especially in children with CP.

**Conclusion**

Both urine and saliva sampling are acceptable choices for measuring the clinical outcomes TEE and TBW using the DLW technique in children with severe CP and ID. However, saliva sampling in our view deserves preference, primarily because the time between production and collection is minimal and because saliva agreed more favorably with serum in an earlier study by Jankowski.[8] However, while in most children with CP saliva is collected as easily and non-invasively as in urine, in some children issues such as hyposalivation can impair proper collection and analysis.

If time or money are constrained, we recommend standard saliva sampling in children with severe CP and ID and reserving urine sampling for children that are known to hyposalivate or have severe oral hypersensitivity.



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RR, CP, HvG, HME and DT conceived the study and were responsible for the study design. RR was responsible for the data collection, the statistical analysis and the manuscript preparation. HS performed the mass spectrometry analyses. HS, HvG, DT, HME, and CP reviewed and commented on drafts of the paper. None of the authors has a personal or financial conflict of interest to declare.

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CHAPTER

# 5

# A novel method for measurement of glutathione kinetics in neonates using liquid chromatography coupled to isotope ratio mass spectrometry

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**Abstract**

A novel analytical method using liquid chromatography coupled to isotope ratio mass spectrometry (LC-IRMS) was developed for measuring glutathione (GSH) fractional synthesis rate (FSR) in neonates after infusion of [1-$^{13}$C]glycine as a tracer. After transformation of GSH into GSSG, its dimeric form, the intra erythrocytic concentration and $^{13}$C- isotopic enrichment of GSH were determined using 200 µL of blood. The results showed that using LC-IRMS, the concentration (range of µmol/mL) was reliably measured using norvaline as internal standard with precision better than 0.1 µmol/mL. In addition, the $^{13}$C-isotopic enrichment measured in the same run gave reliable values with excellent precision (with sd lower than 0.3 ‰) and accuracy (measured between 0 and 2 APE). The inter assay repeatability of $\delta^{13}$C of norvaline used as internal standard with *in vivo* samples was assessed at -26.07 ± 0.28 ‰ with CV at 1.1%. The FSR calculated either with GSH or GSSG showed similar results with slightly higher values for GSSG (41.6 ± 4.7 and 46.5 ± 4.4 respectively). Successfully used in a clinical study, this rapid and reliable method opens up a variety of kinetic studies with relatively low administration of tracer infusates, reducing the total cost of the study design. Besides, the small volume of blood needed enables studies even in extremely small subjects, such as premature infants as reported in this study.

## Introduction

Glutathione (GSH), or L-γ-glutamyl-L-cysteinyl-glycine, is a tripeptide consisting of glutamate, cysteine and glycine. It is synthesised *de novo* in nearly all mammalian cells. Glutathione is present in its monomeric, reduced form (GSH), and its dimeric, oxidized form (GSSG). GSH can be oxidized non-enzymatically *in vivo* to GSSG by electrophilic substances, such as reactive oxygen species. GSH is regenerated from GSSG by the enzyme glutathione reductase. Glutathione is synthesized de novo in two steps: first, catalyzed by glutamylcysteine synthetase, a glutamate is covalently bound to a cysteine residue – the rate-limiting step – and second, catalyzed by glutathione synthetase, glycine is added [1, 2]. Nearly all mammalian cells produce glutathione in large amounts, reaching intracellular concentrations within the millimolar range [3]. In plasma, it is mainly present in its oxidized form such as GSSG or protein GSH disulfite, at levels in the micromolar range (2-20 µmol/L). The monomeric form, GSH, is actively involved in many cellular functions, both as a cofactor for several enzymes and independently [4]; it acts as antioxidant by scavenging radicals or xenobiotics (such as acetaminophen), plays a role in the synthesis of leukotrienes and prostaglandins, in cell proliferations, and is involved in many diseases (such as cancer).

It is quite common to measure GSSG concentration in addition to GSH in biological samples, as the GSH/GSSG ratio is considered to be inversely related to oxidative stress [5]. To this aim, high performance liquid chromatography methods using electrochemical [6] or fluorometric detection[7] or nuclear magnetic resonance have been reported[8]. However, concentration of GSSG in erythrocytes is very low and possible artefacts in sample handling might produce misleading results[9].

Glutathione metabolism has been studied in a variety of species and experimental settings [10-14]. The emphasis, though, was largely on concentration measurements, with a minor role for measuring kinetics using stable isotope tracers. GSH has a rapid turnover, as reflected by its high fractional synthesis rate (FSR). In healthy adult volunteers, FSR is around 65-83 %/d[14, 15], which implies that all GSH is completely renewed in 36 hours. Therefore, static parameters such as concentration may not necessarily provide useful information if kinetic data are absent. Quantifying utilization and synthesis rates of GSH provides a dynamic insight into its metabolism under pathological conditions such as oxidative stress or in response to interventions. Nowadays, gas chromatography-mass spectrometry (GC-MS) techniques are used to study FSR with stable isotope tracers ($^{13}C$ or $^{2}H$ incorporated into glycine or cysteine respectively) and a primed continuous infusion for 6 to 8 hours[16]. In addition, FSR determination in erythrocytes requires suitable methods to measure both low level of isotopic enrichment in GSH and in its precursor. Typically, this information is

obtained with the incorporation rate of tracer into GSH, using erythrocyte-free glycine or cysteine as the precursor pool from which erythrocytes synthesise GSH[11]. Studies in neonates are limited by the small amounts of blood that can be sampled. Then, only small amounts of stable isotope tracer can be used, lest metabolism is disturbed.

Although gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) is now the state of the art to measure low isotopic enrichment in tracer studies, the targeted compound needs to be volatile after derivatisation[17]. Thus, for isotopic analyses of a tripeptide such as glutathione, the GC introduction might be quite laborious. Then, liquid chromatography coupled to isotope ratio mass spectrometry (LC-IRMS) needs to be envisaged as well. The LC-IRMS device became commercially available in 2004 [18] after several other experiments to link (or hyphenate) LC to IRMS over the last decade[19-23]. This coupling is based on a wet chemical oxidation process and presents few analytical constraints interfering with LC method development. Typically the LC flow rate must be lower than 600 µL/min, organic buffers are prohibited and the presence of salt and low pH facilitates $CO_2$ extraction. Various LC separations were developed using LC-IRMS for targeted compounds such as underivatised amino acids[18, 24, 25] and underivatised carbohydrates[26] but none specifically for routine isotopic analysis and concentration measurement in the frame of metabolic studies. Our aim here was to develop a novel and robust method to determine low GSH $^{13}$C-isotopic enrichments requiring a low volume of blood, for use in e.g. premature infants. Therefore, a new method was designed based on stable isotope technique using liquid chromatography coupled to isotope ratio mass spectrometry (LC-IRMS). To this purpose, we assessed GSH kinetics through the measurement of the $^{13}$C-isotopic enrichment and the concentration of GSSG entity chemically produced by oxidation of GSH.



## Experimental Section

### Chemicals and reagents

Glutathione, glycine, norvaline, norleucine and phosphoric acid (85 % v/v) were purchased from Sigma (St Louis, USA). Sodium peroxodisulfate (p.A.) was purchased from Fluka (Buchs, Switzerland). Perchloric acid (70% v/v), potassium hydroxide and sodium hydroxide were purchased from Merck (Darmstadt, Germany). The [1-$^{13}$C]-labelled glycine was purchased from Cambridge Isotope Laboratories (Buchem, Netherlands). [1,2-$^{13}$C, $^{15}$N] glutathione was a gift from Cambridge Isotope Laboratory, CIL (Andover, MA, USA). Sterile water was purchased from Baxter BV (Utrecht, Netherlands) dithiothreitol (DTT) and N-ethylmaleimide (NEM) were purchased from Sigma (St Louis, USA).

**Instruments**

Experiments were carried out on a Delta XP (Thermo Electron, Bremen, Germany). The IRMS was operated at an accelerating voltage of 5 kV. The ion source was held at a pressure of 3.0 x $10^{-6}$ Torr, and ions generated by electron impact at 70 eV. Three faraday cup detectors monitored simultaneously and continuously the $CO_2^{+\cdot}$ signals for the three major ions at *m/z* 44 ($^{12}CO_2$), *m/z* 45 ($^{13}CO_2$ and $^{12}C^{17}O^{16}O$) and *m/z* 46 ($^{12}C^{18}O^{16}O$). The dynamic range of the instrument is between 0.2 and 50 V. The $CO_2$ working reference gas (Linde, quality 5.3) was calibrated with known reference gasses (Messer Griesheim, Krefeld, Germany) against δ $^{13}C_{VPDB}$ (Vienna Pee Dee Belemnite, VPDB). In order to increase filament lifetime, the oxygen signal (*m/z* 32) produced by oxidant reagent should not exceed 20 V measured on the first faraday cup (resistor 300 MΩ).

An LC-Isolink® interface (Thermo Electron, Bremen, Germany) was coupled to the Delta XP with slight modifications. The mixing "Tee" was replaced by a "Tee" with a smaller dead volume (Bester BV, Amstelveen, Netherlands) and the stainless steel connections for the reagents and acid were replaced by "No-Ox" material (1/8'' x 1.5 mm). A degasser (Alltech, Breda, Netherlands) was placed between the solvent bottles and the pumps to remove traces of air in the eluents. "No-Ox" material was used to prevent re-gassing of solvents. The temperature of the interface reactor was set at 99.9 °C. PEEK® tubing was used for connections between the auto sampler the LC column and the interface. In addition, the reagent bottles were degassed with helium during analysis. To avoid crystallisation of reagents the pump heads of the oxidant and acid pumps were rinsed with water several times a day. A filter of 0.2 µm (Vici, Bester BV, Netherlands) was placed before the mixing chamber of the interface to avoid any blockage of particles in the system.

The LC-IRMS interface was coupled to an LC system consisting of two Knauer pumps (Berlin, Germany) and a Midas auto sampler (Spark, Emmen, Netherlands) and controlled by Sparklink software (version 3.10, service pack 2, Spark). Figure 1 shows a schematic view of the system used for the analysis of glutathione.

The isotopic enrichment of $^{13}$C-glycine in human erythrocytes was measured on the same Thermo Finnigan Delta-XP isotope ratio MS coupled online with a trace gas chromatograph (Thermo Electron, Germany) and a combustion interface type III (Thermo Finnigan, Germany) equipped with a CTC PAL auto sampler (CTC Switzerland). After separation using a capillary GC column VF1701, 30 m x 0,25 mm ID and 0,5µm film thickness (Varian B.V. Middelburg, Netherlands), derivatised amino acids were combusted online at 940°C and introduced as $CO_2$ into IRMS ion source, where the $C^{13}/C^{12}$ ratio was measured.

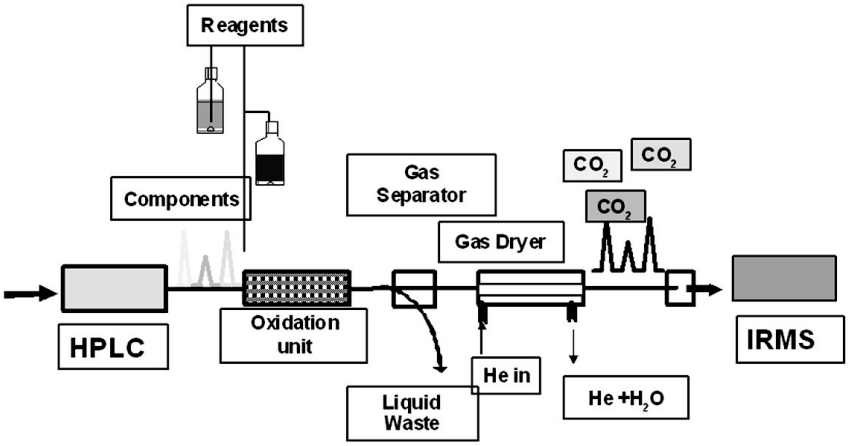


***Figure 1*** *Schematic overview of LC-IRMS device.*



**Isotopic calibration and isotopic measurement**

$CO_2$ reference gas was introduced at regular intervals at the beginning of each run for 20 s at a level of 3.0 ± 0.2 V on cup one (resistor 300 MΩ) and used for calibration.

The $^{13}C/^{12}C$ abundance ratio was expressed as δ $^{13}C$ values calibrated against the international standard. The delta notation is defined as δ $^{13}C_{sample}$ = $[(R_s / R_{st}) - 1] \times 1000$, where $R_s$ is the ratio of $^{13}C$ in the sample and $R_{st}$ is the ratio of the international standard used. The result of this calculation is a relative δ calibrated against the international standard.

Atom % was calculated as:

$$\text{Atom \%} = \left[\frac{100 \times R \times \left(\left(\delta 13C / 1000\right)+1\right)}{1 + R \times \left(\left(\delta 13\text{C} / 1000\right)+1\right)}\right]$$

where R is the ratio of ($^{13}C/^{12}C$) of International Standard of Pee Dee Belemnite, R=0.0112372.

Atom % Excess (APE) is defined as:

Atom % (sample) minus Atom % (background).

APE can be transferred to Mol % Excess (MPE) using the next formula:

$$\text{MPE} = \left[\frac{APE}{100 + APE} x100\right]$$

**Clinical study design**

The study design was approved by the Erasmus MC Medical Ethical Review Board Committee and informed parental consent was obtained prior to the

study. The study population consisted of very low birth weight infants (birth weight <1500 g) admitted to the neonatal intensive care unit. A primed (40 µmol/kg) continuous infusion of 1-$^{13}$C labelled glycine (20 µmol/kg/h) was administered intravenously for 6 hours. Blood samples were taken after respectively 4, 5 and 6 hours (steady state). Portions of 400 µl freshly drawn EDTA blood were centrifuged at 900 x G for 10 min at 4 °C. The upper layer was discarded and the lower layer containing primarily erythrocytes and other cells was reconstituted to the original volume with distilled water and stored at -80 °C until further analysis**.**

**Glutathione determination**

Before analysis erythrocytes were disrupted by freezing and thawing [27]. Then 40 µl of a mixture of 1,525 µmol/mL norleucine and 8.54 µmol/mL norvaline was added as internal standard and the samples were deproteinized by adding 200 µl of 6 % g/v perchloric acid, left for 10 min on ice and finally centrifuged at 10'000 G for 20 min. The supernatant was transferred in a new tube and the pH was adjusted to 8 - 9 with approximately 20 µl of 4 M KOH. Excess of perchloric acid was precipitated and removed by centrifugation at 10'000 G for 10 min. 100 µl of 0.5M $NaHPO_4$ was added to maintain pH 9. The supernatant was filtered through 0.2 µm Nylon membrane filters (Nylon, Alltech, Breda, Netherlands). Then, 150 µl was transferred in a sample vial and 20 µl was injected for analysis of both glutathione concentration and $^{13}$C-isotopic enrichment by LC-IRMS. The remaining supernatant was used for analysis of both concentration and $^{13}$C- isotopic enrichment of glycine by GC-C-IRMS.

**Glycine determination**

Aliquots of 200 µl of the remaining supernant were poured on a 1 ml AG50 W-X8, $H^+$ cat ion exchange column (Biorad, Richmond, Virginia, USA). Columns were washed thoroughly with 5 ml water to remove non amino acid contaminants. Amino acids were eluted with 3 ml 6 M $NH_4OH$ and dried under nitrogen at 50°C. Then, the dried residue containing glycine among other amino acids was converted to its *N*-ethoxycarbonylethyl ester derivative [28, 29]. Finally aliquots of 1 µL of the chloroform suspension containing amino acid derivatives were introduced into the GC-C-IRMS system described previously.

**Analytical LC-IRMS conditions**

Samples were introduced using a Midas auto sampler (Spark) and analysed with a linear high-pressure gradient (as reported in Table 1). Glutathione analyses (concentration and isotopic enrichment) were performed on a Sielc primesep 100 mixed mode column (250 × 3.2 mm, 5 µm), (Aurora Borealis, The Netherlands) at room temperature, (24 ± 2) °C. The LC flow rate was 500 µLmin$^{-1}$. The LC gradient was linearly increased from 4% to 40 % 1 molar $H_3PO_4$ (pH 2.2) in 15 min, followed by a linear increase to 80 % 1

molar $H_3PO_4$ (pH 2.2) in 5 min and held for 8 min. Sodium peroxodisulfate 0.84 mol/L in sterile water was used as oxidation solution. Acid reagent was prepared as 1.5 M phosphoric acid solution in sterile water. The flow rate of the acid and oxidant reagents in the LC interface was 30 µL/min each.

**Calculations and data evaluation**

The incorporation of labelled glycine in glutathione was determined by measurement of the $^{13}C$ enrichment of glutathione in erythrocytes. The rate of incorporation of tracer is a reflection of the fractional synthesis rate (FSR) of glutathione and was calculated according to the equation described below (equation 1):

$$FSR = \frac{\text{slope}\, E_{[1-^{13}C]GSSG_{t4,5,6}}}{E_{[1-^{13}C]\text{glycine}}} \times 24h \times 100\%$$

$^{13}C$ isotopic enrichment of GSSG was measured by LC-IRMS. Slope $E_{[1\text{-}13C]GSSGt4,5,6}$ represents the increase / hour in isotopic enrichment of GSSG between 4 and 6 hours of infusion, expressed in Molar Percent Excess (MPE). $E_{[1\text{-}13C]glycine}$ represents the isotopic enrichment of intra-erythrocitic glycine, the precursor, in MPE at steady state.

**Results and discussion**

In this study, a novel LC-IRMS method was developed to separate underivatised amino acids, underivatised GSH and GSSG with a phosphoric acid gradient condition in 2000 sec (33 min) as illustrated in Figure 2.
In order to obtain better chromatographic resolution between GSH and amino acids eluted near GSH peak (Pro and Cys), helium flow in the separation unit was increased (from 1 to 2 mL/min) to reduce peak width (from 85 sec to 60 sec). This modification affected the overall sensitivity of the analyses without compromising GSH isotopic and concentration analyses in blood erythrocytes.

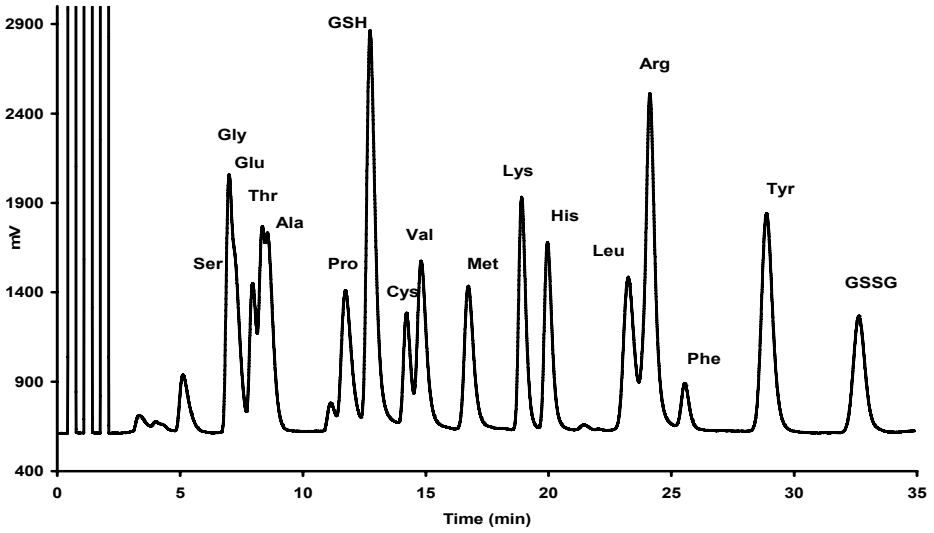


***Figure 2*** *LC-IRMS chromatogram of a 0.25 mmol hydrolysate amino acid standard mixed with 0.5 mmol glutathione standard solution at low pH.*



**Transformation of GSH into GSSG and its separation in LC-IRMS**

Reducing agents such as dithiothreitol (DTT) or N-ethylmaleimide (NEM) are normally used to reduce GSSG entity present in low amounts in blood or to prevent oxidation immediately after sampling, respectively. However, these reagents evoked disturbing peaks when analysing them with LC-IRMS (very abundant peaks at the beginning of chromatogram causing elevated baseline background). Moreover, GSH is very susceptible to oxidation, which necessitates strict sample collection and storage protocols. A recent paper indeed reported the artifactual oxidation of GSH after sample treatment with different deproteinizing agents and subsequent alkalinisation [30]. We found that GSH is completely oxidized as early as after 4 hours at room temperature at pH 8-9. In addition, the area where GSH elutes in the chromatogram was not totally free of other co-eluting compounds (as illustrated in Figure 2), which increases the risk of incorrect measurements (either concentration or isotopic enrichment). Therefore, to avoid the problematic baseline separation of GSH, all GSH was completely oxidized and measured as GSSG. The complete transformation of GSH into GSSG was performed in 4 hours and monitored with the disappearance of GSH peak by LC-IRMS (as illustrated in Figures 3a and 3b). GSSG was eluting in a part of the chromatogram free of other co-eluting compounds as shown in Figure 3b. $^{13}$C-isotopic enrichment as well as concentration of the total glutathione pool was then reliably measured in this condition.

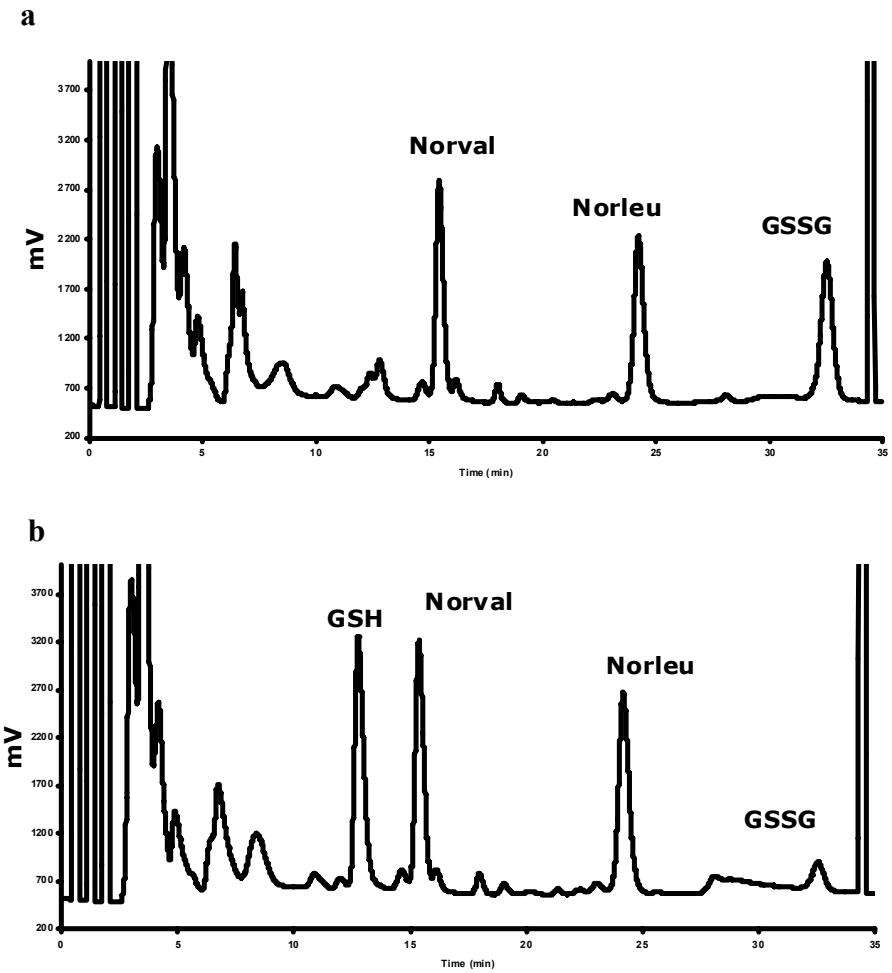


***Figure 3*** *LC-IRMS chromatograms of (a) glutathione analysis in erythrocytes of human blood at pH 4-5 and (b) of glutathione analysis in erythrocytes of human blood as its oxidized form GSSG (pH 8-9).*

**Measurement of concentration of GSSG**

The concentration of the tracee is an important parameter in any metabolic study. Therefore, two internal standards (I.S.) (norvaline and norleucine) were added and eluted closely to the GSH and GSSG peaks, respectively. This spiking of I.S. allowed to obtain the GSH and GSSG ratios versus I.S. and thus to assess GSH and GSSG concentrations in blood. Five standard solutions of GSSG (using GSSG over I.S. area plotted against the µmol/mL of GSSG injected) were measured between 0.2 to 2 mg/mL. A linear relationship was obtained and gave y = 1.4506 x + 0.008. The regression coefficient ($r^2$) was calculated at 0.998 (Figure 4). The area of GSSG measured was between 17.7 ± 0.25 V.s to 191 ± 1.6 V.s. Table 1 shows

GSSG concentrations in blood measured with only 200 µL of samples for four premature neonates. The concentration of GSSG measured through different days and showed good reproducibility (CV of 12.3 %, when measured as duplicates). The mean value (0.54 ± 0.07 µmol/mL) of GSSG equivalent to the concentration of GSH was consistent with values reported in the literature[31]. These findings show that LC-IRMS can analyze concentration of metabolites in blood with good precision (better than 0.1µmol/mL) and a limit of detection of 5µmol/mL.

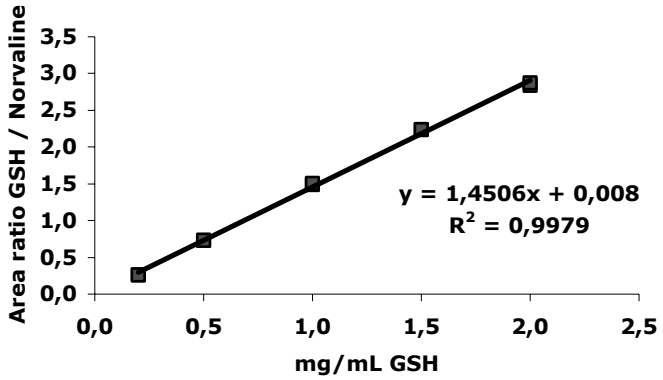


***Figure 4*** *Calibration curve for measurement of glutathione concentration in human blood erythrocytes.*

***Table 1*** *Concentration and $^{13}C$- isotopic enrichments of GSSG in 4 different neonates (sd: standard deviation, CV (%) = sd /mean x100)*

| | Time (h) | Level (µmol/mL) of GSSG | Sd (n=2) | $\delta^{13}C$ (‰) of GSSG | Sd (n=2) |
|---|---|---|---|---|---|
| Neonate A | 4 | 0.55 | 0.06 | 5.48 | 0.68 |
| | 5 | 0.58 | 0.001 | 8.54 | 0.55 |
| | 6 | 0.61 | 0.004 | 13.51 | 0.64 |
| Neonate B | 4 | 0.61 | 0.02 | -0.70 | 0.03 |
| | 5 | 0.45 | 0.001 | 4.23 | 0.30 |
| | 6 | 0.52 | 0.003 | 7.86 | 0.18 |
| Neonate C | 4 | 0.49 | 0.02 | 10.86 | 0.03 |
| | 5 | 0.45 | 0.0003 | 15.61 | 0.44 |
| | 6 | 0.44 | 0.003 | 22.81 | 0.04 |
| Neonate D | 4 | 0.53 | 0.02 | -2.22 | 0.75 |
| | 5 | 0.60 | 0.02 | 3.67 | 0.15 |
| | 6 | 0.59 | 0.02 | 8.51 | 0.33 |
| Mean | | 0.54 | | | 0.07 |
| | | | | CV (%) | 12.3 |

**Repeatability and accuracy of isotopic measurement**

As illustrated in Figure 5, the intra-assay repeatability assessed with norvaline measured with three different blood samples from the same neonate showed a sd measured at -25.95 ± 0.20 ‰ and a CV calculated at 0.8 % (n=6). The inter-assay repeatability measured over different days was measured at -26.07 ± 0.35 ‰ (n = 24). The reproducibility (CV) was calculated at 1.3 %. For each neonate, the δ $^{13}$C of norvaline are close. These values were in the same range as obtained with standard injection of I.S showing excellent isotopic precision as well as reproducibility of isotopic measurement at natural abundance.

Accuracy of isotopic measurement was assessed using standard calibration curve performed with various amounts of $^{13}$C labelled GSH added to a fixed amount of natural GSH. By plotting measured APE versus theoretical APE between 0 and 2.5 APE (or δ $^{13}$C values between -8.7 to 170.5 ‰), the curve was linear and a slope of 1.0124 was found (Fig. 6). This shows that no isotopic fractionation occurred in the sample preparation and the analysis. The regression coefficient ($r^2$) was calculated at 1.000, showing excellent linearity.



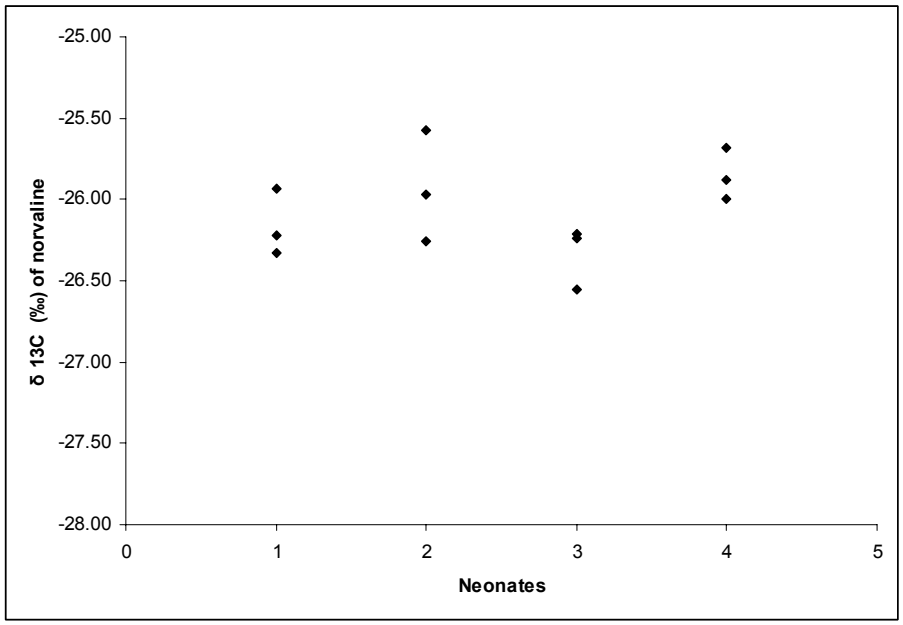


***Figure 5*** *Variation of δ $^{13}$C (‰) of norvaline added as internal standard and spiked in blood erythrocyte for 4 different neonates. Blood was collected at three time points and corresponding values are shown. Each point corresponds to duplicate injections.*

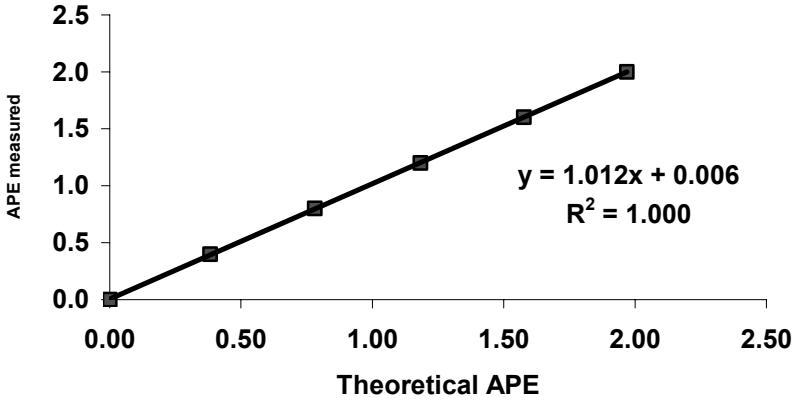


***Figure 6*** *Calibration curve for measurement of [7-$^{13}$C]-glutathione enrichment in human blood erythrocytes using [1,2-$^{13}$C- $^{15}$N] glutathione.*



**Fractional synthesis rate (FSR) of glutathione**

Blood samples where taken at regular intervals (4h, 5h and 6h) when erythrocyte [1-$^{13}$C ] glycine levels were in steady state (according to results obtained by GC-C-IRMS and not shown). Figures 7a and 7b show a linear rise in time in both GSH APE and GSSG APE. FSR of GSH and GSSG were calculated from these measurements using equation 1 reported before. Values for four patients are reported in Table 2.

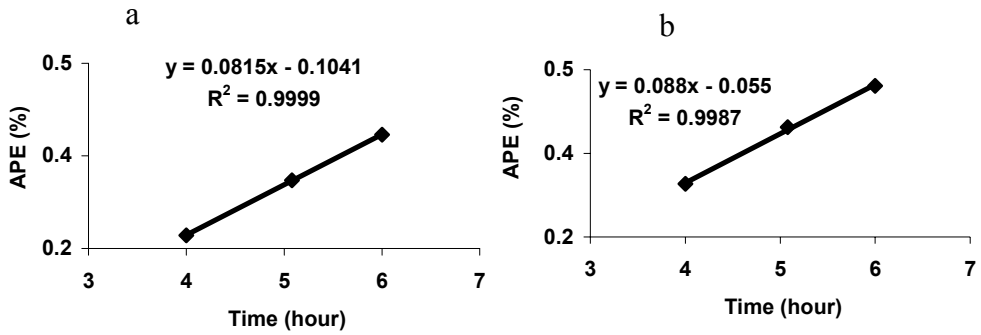


***Figure 7*** *Graphs of the increase of (a) enriched GSH of a subject expressed in APE / hour and (b) enriched GSSG of the same subject as shown in (a) expressed in APE / hour.*

It follows that the glutathione pool of these patients was renewed approximately every two days. The calculated FSR slightly, but acceptably, differs for either of the forms of glutathione. In all cases the FSR for GSH is

slightly lower than for GSSH. FSR of GSH was 41.6 ± 4.7 with a reproducibility (CV) was calculated at 11.2 % and FSR of GSSG was 46.5 ± 4.4 with a reproducibility (CV) was calculated at 9.6 % (n = 8). A possible explanation for the lower FSR for GSH is dilution of another co eluting (non amino acid) compound. For this reason, and also because the concentration is more precisely determined when measured as a single GSSG peak, we decided to measure GSSG, the oxidized form of glutathione.

***Table 2*** *Comparison of the fractional synthetic rates (FSR) of GSH and GSSG in four different subjects; GSH, GSSG and glycine analyses were carried out in duplicate.*

| Sample | Compound | Precursor MPE in steady state | Slope APE %/h | Slope MPE %/h | FSR %/day |
|---|---|---|---|---|---|
| Neonate 1 | GSH | 3.90 | 0.0815 | 0.0754 | 46.36 |
| | GSSG | 3.90 | 0.0880 | 0.0809 | 49.77 |
| Neonate 2 | GSH | 2.93 | 0.0578 | 0.0546 | 44.76 |
| | GSSG | 2.93 | 0.0656 | 0.0616 | 50.43 |
| Neonate 3 | GSH | 3.66 | 0.0627 | 0.0590 | 38.70 |
| | GSSG | 3.66 | 0.0736 | 0.0686 | 44.97 |
| Neonate 4 | GSH | 3.53 | 0.0570 | 0.0539 | 36.66 |
| | GSSG | 3.53 | 0.0641 | 0.0602 | 40.95 |
| GSH | Mean | 41.6 | Sd | 4.7 | |
| GSSG | Mean | 46.5 | Sd | 4.4 | |



## Conclusion

This novel LC-IRMS method for measuring kinetics of glutathione using its oxidized form (GSSG) showed to be a powerful tool in metabolic studies in neonates. Only little pre-purification was necessary and the analyses reported here were fully automated. The measurements of both concentration and $^{13}C$ isotopic enrichment gave excellent results with only 200 µL of blood required, which is extremely important factor in neonatal studies. GSSG concentrations were found to be in agreement with results reported in literature. The precision and accuracy of isotopic enrichment at natural abundance and at higher isotopic enrichment gave excellent results showing no isotopic fractionation and an isotopic precision for GSSG in blood assessed at 0.3 ‰.

## Acknowledgments

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CHAPTER

# 6

# Simultaneous analysis of $^{13}C$-glutathione as its dimeric form GSSG and its precursor [1- $^{13}C$] glycine using Liquid Chromatography Isotope Ratio Mass Spectrometry (LC-IRMS)

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**Abstract**

Determination of glutathione kinetics using stable isotopes requires accurate measurement of the tracers and tracees. Previously, the precursor and synthesized product were measured with two separate techniques, liquid chromatography isotope ratio mass spectrometry (LC-IRMS) and gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS). In order to reduce sample volume and minimize analytical effort we developed a method to simultaneously determine $^{13}$C-glutathione as its dimeric form (GSSG) and its precursor [1-$^{13}$C] glycine in a small volume of erythrocytes in one single analysis.

After having transformed $^{13}$C-glutathione into its dimeric form GSSG, we determined both the intra-erythrocytic concentrations and the $^{13}$C-isotopic enrichment of GSSG and glycine in 150 µL of whole blood using liquid chromatography coupled to LC-IRMS. The results show that the concentration (range of µmol/mL) was reliably measured using cycloleucine as internal standard, i.e. with a precision better than 0.1 µmol/mL. The $^{13}$C-isotopic enrichment of GSSG and Glycine measured in the same run gave reliable values with excellent precision (SD < 0.3 ‰) and accuracy (measured between 0 and 5 APE).

This novel method opens up a variety of kinetic studies with relatively low dose administration of tracers, reducing the total cost of the study design. In addition, only minimal sample volume is required, enabling studies even in very small subjects, such as preterm infants.

## Introduction

Glutathione metabolism has been studied in several different experimental settings and species[1-5]. Glutathione is primarily an intracellular antioxidant, with extremely high cellular concentration (low millimolar range) in mammalian tissue. Quantification of the utilization and synthesis rates provides a dynamic insight into its metabolism under pathological conditions, such as oxidative stress, or in response to interventions. Glutathione is present in two forms: the monomeric, reduced or active form (GSH), and the dimeric, oxidized form (GSSG). GSH is synthesized in a series of two reactions. In the first, rate-limiting step, a glutamate is covalently bound to a cysteine residue. During the second reaction, glycine is added to complete the synthesis [2, 6, 7]. Glutathione typically has a rapid turnover. The fractional synthesis rate (FSR) reported in healthy adult volunteers varied from 63 to 83%/day in the various studies[5, 8, 9]. In neonates it was found to be much lower, from 35 to 55%/day[8, 10]. Nevertheless, in all cases the total pool is renewed within three days.

FSR determination of GSH in erythrocytes requires suitable methods to measure both low level of isotopic enrichment in GSH and in its precursor. Moreover, determination in neonates is complicated because only small amounts of blood can be sampled. Studies in neonates would benefit form a method that can deal with small samples. Gas chromatography-mass spectrometry (GC-MS) and stable isotope dilutions techniques have been frequently used in metabolic kinetic studies[11-16] or concentration measurements[17, 18] (gold standard method). Several papers have been published over the past four years [19-27], showing the power and robustness of LC-IRMS analyzing amino acids, carbohydrates, fatty acids and volatile fatty acids.

Recently we have developed a method for measurement of GSSG using liquid chromatography coupled to isotope ratio mass spectrometry (LC-IRMS) [28]. Still, this method needed to be complemented with glycine measurement on GCMS or GC/C/IRMS. This new method was successfully applied in a kinetic study in preterm infants[10]. In the present study we aimed to develop a new method for simultaneous measurement of $^{13}$C-glutathione as its dimeric form (GSSG) and its precursor [1-$^{13}$C] glycine in erythrocytes, in order to reduce sample preparation and sample volume.

## Experimental Section

### Chemicals and reagents

Glutathione, glycine, cycloleucine and phosphoric acid (85% v/v) were purchased from Sigma (St Louis, USA). Sodium peroxodisulfate (p.a.) was purchased from Fluka (Buchs, Switzerland). Perchloric acid (70% v/v), potassium hydroxide, $Na_2HPO_4$, $H_3PO_4$ and sodium hydroxide were purchased from Merck (Darmstadt, Germany). [1-$^{13}$C] glycine was purchased from Cambridge Isotope Laboratories (Buchem, Apeldoorn, Netherlands). [1,2-$^{13}$C, $^{15}$N] glutathione was a gift from Cambridge Isotope Laboratory, CIL (Andover, MA, USA). Milli-Q (18.2 MΩ) water was produced with a milli-Q system Millipore BV (Bedford, MA, USA).

### Analytical methods

Experiments were carried out on a Delta XP (Thermo Fisher, Bremen, Germany). The IRMS was operated at an accelerating voltage of 5 kV. The ion source was held at a pressure of 3.0 x $10^{-6}$ Torr, and ions generated by electron impact at 70 eV. Three faraday cup detectors monitored simultaneously and continuously the $CO_2^{+\cdot}$ signals for the three major ions at *m/z* 44 ($^{12}CO_2$), *m/z* 45 ($^{13}CO_2$ and $^{12}C^{17}O^{16}O$) and *m/z* 46 ($^{12}C^{18}O^{16}O$). The dynamic range of the instrument is between 0.2 and 50 V. The $CO_2$ working reference gas quality 5.3 (Linde, Schiedam, Netherlands) was calibrated with known reference gases (Messer Griesheim, Krefeld, Germany) against $\delta^{13}C_{VPDB}$ (Vienna Pee Dee Belemnite, VPDB). In order to increase filament lifetime, the oxygen signal (*m/z* 32) produced by oxidant reagent should not exceed 15 V measured on the first faraday cup (resistor 300 MΩ). A LC-Isolink® interface (Thermo Fisher, Bremen, Germany) was coupled to the Delta XP with slight modifications. The apparatus conditions were the same as described previously except for some minor modifications[28]. The temperature of the interface reactor was set at 99.9 °C. PEEK® tubing was used for connections between the autosampler, the LC column and the interface. In addition, the reagent bottles were degassed with helium during analysis. To avoid crystallisation of reagents the pump heads of the oxidant and acid pumps were rinsed with water several times a day. A filter of 0.2 µm (Vici, Bester BV, Netherlands) was placed between the analytical column and the mixing chamber of the interface to avoid any blockage of particles in the system. Also, a 0.2 µm filter was placed after the reagent pumps to prevent blocking of the oxidation oven by impurities or crystallization deriving from the reagents.

The LC-IRMS interface was coupled to an LC system consisting of two Knauer pumps (Berlin, Germany) and a Midas auto sampler (Spark, Emmen, Netherlands) and controlled by Sparklink software (version 3.10, service pack 2, Spark). The isodat software was used to control the IRMS system.

**Calibration and isotopic rearrangements**

$CO_2$ reference gas was introduced at regular 20s intervals at the beginning of each run at a level of 3.0 ± 0.2 V on cup one (resistor 300 MΩ) to calibrate peaks eluting during the run.

The $^{13}C/^{12}C$ abundance ratio was expressed as δ $^{13}C$ values calibrated against the international standard. The delta notation is defined as δ $^{13}C_{sample}$ = $[(R_s / R_{st}) - 1] \times 1000$, where $R_s$ is the ratio of $^{13}C$ in the sample and $R_{st}$ is the ratio of the international standard used. The result of this calculation is a relative δ calibrated against the international standard.

Atom % was calculated as:

$$\text{Atom \%} = \left[ \frac{100 \times R \times \left( \left( \delta 13C / 1000 \right) + 1 \right)}{1 + R \times \left( \left( \delta 13\text{C} / 1000 \right) + 1 \right)} \right]$$

where R is the ratio of ($^{13}C/^{12}C$) of International Standard of Pee Dee Belemnite, R=0.0112372.

Atom % Excess (APE) is defined as:

Atom % (sample) minus Atom % (background)

APE can be transferred to Mol % Excess (MPE) using the next formula:

$$\text{MPE} = \left[ \frac{APE}{100 + APE} x100 \right]$$

**Glutathione and Glycine measurement**

Before analysis erythrocytes were disrupted by freezing and thawing and subsequently sonicated for 5 min in an ultra sonification bath. Then 20 µL of 8 µmol/mL cycloleucine was added as internal standard to an aliquot of 200 µL erythrocyte solution and the samples were deproteinated by adding 100 µL of 2 M perchloric acid, incubated for 10 min on ice and finally centrifuged at 10000 x g for 20 min. The supernatant was transferred in a new tube and the pH was adjusted to 8 - 9 with approximately 20 µL of 4 M KOH. Excess perchloric acid was precipitated and removed by centrifugation at 10000 x g for 10 min. 50 µL of 1 M $Na_2HPO_4$ was added to maintain pH 9. The supernatant was filtered through 0.2 µm Nylon membrane filters (Grace Alltech, Breda, Netherlands). Then, 200 µL was transferred in a sample vial and 50 µL was injected for analysis of both glutathione concentration and $^{13}C$- isotopic enrichment by LC-IRMS.

**LC-IRMS conditions**

Samples were introduced using a Midas autosampler (Spark) and analysed with a linear high-pressure gradient. Glycine and glutathione analyses (concentration and isotopic enrichment) were performed on a Sielc primesep A mixed mode column (250 × 3.2 mm, 5 µm), (Aurora Borealis,

Schoonebeek,The Netherlands) at room temperature, (20 ± 2) °C. The LC flow rate was 500 µL-min$^{-1}$. The chromatographic separation is based on both ion exchange and hydrophobic interactions. After flushing the column with solvent A (Table 1) for 2.1 min, the LC gradient was increased in multiple steps from 0% to 80% using solvent B (1 M $H_3PO_4$, pH 2.2) (Table 1). Sodium peroxodisulfate 0.84 mol/L in sterile water served as oxidation solution. Acid reagent was prepared as 1.5 M phosphoric acid solution in sterile water. The flow rate of the acid and oxidant reagents in the LC interface was 25 µL/min each.

***Table 1*** *LC-IRMS conditions for analysis of glycine and glutathione*

| | | | | | | |
|---|---|---|---|---|---|---|
| Analytical conditions: | | | | | | |
| Column: | Sielc primesep A, 250x3.2mm, 5µm | | | | | |
| Solvent A: Milli-Q 18.2MΩ Water | Solvent B: 1M $H_3PO_4$ | | | | | |
| Column temp.: 20°C | Column flow rate: 0.5mL/min | | | | | |
| | | | | | | |
| LC-IRMS parameters | | | | | | |
| Reactor temperature | 99.9 °C | | | | | |
| Acid reagent | $H_3PO_4$, 1.5M, 25µl | | | | | |
| Oxidant Reagent | $Na_2S_2O_8$, 0.8M, 25µl | | | | | |
| Injection volume | | | | | | |
| | | | | | | |
| Gradient profile used for GSSG analysis | | | | | | |
| Time (min) | 0 | 32 | 32.1 | 42 | 57 | 57.1 |
| %B | 0 | 0 | 10 | 80 | 80 | 0 |

**Kinetic measurements**

The fractional synthesis rate (FSR) is the percentage of the total renewal of a product per day. The incorporation of labelled glycine in glutathione was determined by measurement of the $^{13}C$ enrichment of glutathione in erythrocytes. The rate of incorporation of tracer is a reflection of the FSR of glutathione and was calculated according to the equation described below:

$$FSR = \frac{\text{slope}\, E_{[1-^{13}C]GSSG_{t4,5,6}}}{E_{[1-^{13}C]glycine}} \times 24h \times 100\%$$

The $^{13}C$-isotopic enrichment of GSSG was measured by LC-IRMS. Slope $E_{[1\text{-}13C]GSSGt4,5,6}$ represents the increase / hour in isotopic enrichment of GSSG between 4 and 6 hours of infusion, expressed in Molar Percent Excess (MPE). $E_{[1\text{-}13C]glycine}$ represents the isotopic enrichment of intra-erythrocyte glycine, the precursor, in MPE at steady state.

Subsequently, the intravascular absolute synthesis rate of GSH ($ASR_{GSH}$) can be calculated using the following equation:

$$ASR_{GSH}(mg/kg.d)) = FSR_{GSH}/100 \text{ X conc X } 307 \text{ X ht X } 0.075$$

where conc is the GSH concentration in mmol/L of the erythrocyte fraction, 307 the molecular weight of GSH, ht the hematocrit content in L/L and 0.075 the estimated circulating blood volume in a neonate, expressed as L/kg.

**Clinical study design**

The study design was approved by the Erasmus MC Medical Ethical Review Board. The study population consisted of very low birth weight infants (birth weight <1500 g) admitted to the neonatal intensive care unit. Informed parental consent was obtained prior to the study. A primed (40 µmol/kg) continuous infusion of [1-$^{13}$C] glycine (20 µmol/kg/h) was administered intravenously for 6 hours. Blood samples were taken after 4, 5 and 6 hours (steady state). Portions of 400 µL freshly drawn EDTA blood were centrifuged at 900 x g for 10 min at 4 °C. The upper layer was discarded and the lower layer – containing primarily erythrocytes and other cells – was reconstituted to the original volume with distilled water and stored at -80 °C until further analysis.



**Results and Discussion**

**Chromatographic separation**

Using this new LC-IRMS method, glycine and GSSG clearly stand out from the other eluting compounds, as shown in Figures 1 and 2. Retention times were stable with just a little variation in drift during the serial measurements: 29.6 +/- 0.5 min for glycine and 58.1 +/- 0.8 min for GSSG, respectively, evidence of robust chromatographic conditions. Cycloleucine was chosen as internal standard because its chromatographic properties under the conditions used are better than those of e.g. norvaline and norleucine.

In our previous study we encountered the problem of glycine co-eluting with threonine and glutamate[28]. The use of a slightly different column, a Sielc primesep A instead of a primesep 100, and a modified gradient has solved this problem.

Additionally, in the method described here several other amino acids, like threonine and aspartate, were nicely separated. This finding can be relevant to other neonatal studies dealing with stable isotopes.

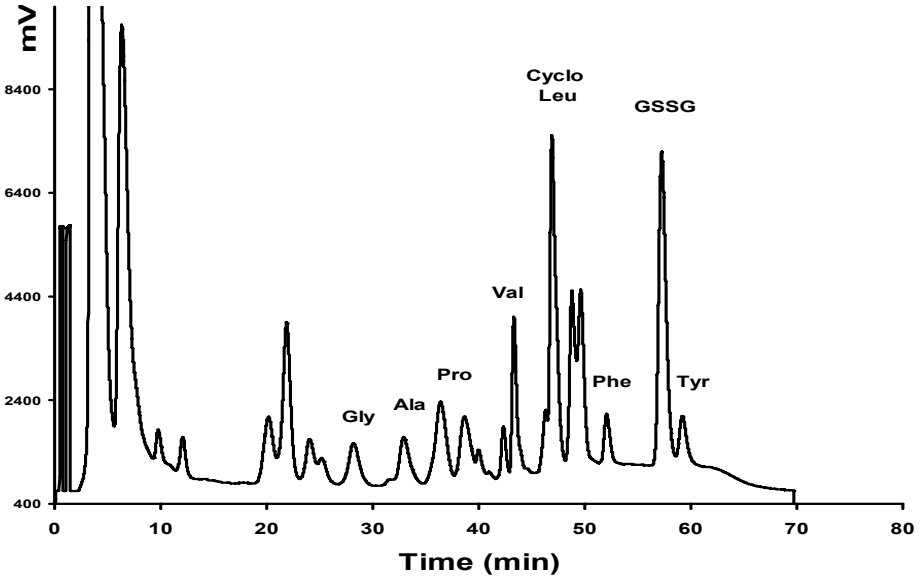


***Figure 1*** *LC-IRMS chromatogram of 0.25 mmol hydrolysate amino acid standard mixed with 0.5 mmol glutathione standard solution.*



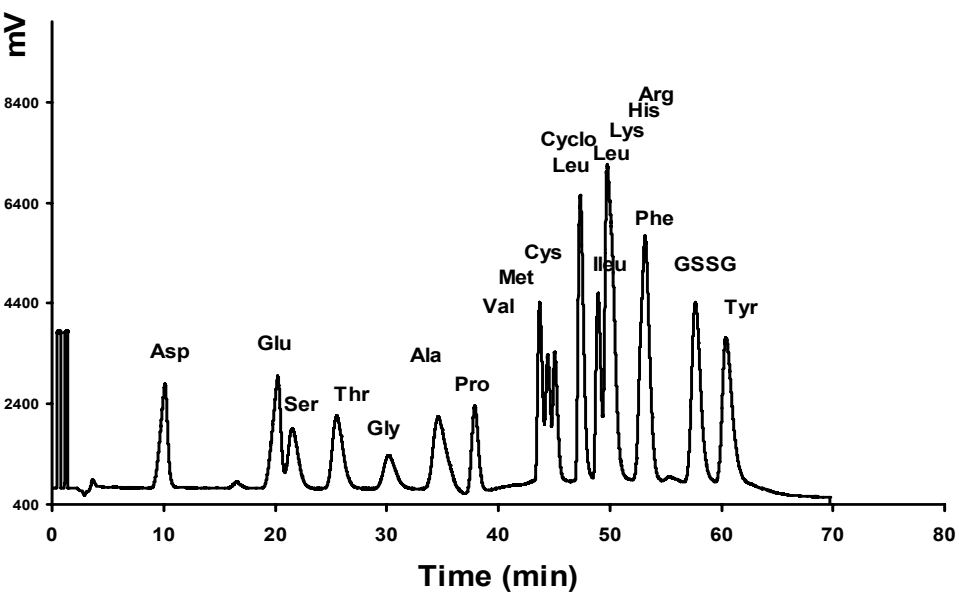


***Figure 2*** *LC-IRMS chromatogram of glycine and glutathione in erythrocytes of human blood as its oxidized form GSSG.*

**Measurement of concentration of GSSG**

The concentration of the tracee is an important parameter in every metabolic study. Therefore, a known amount of cycloleucine was added to each sample to be analyzed. This spiking of an internal standard (I.S.) made it possible to obtain the GSSG ratio versus I.S. and thus to assess GSSG concentrations in erythrocytes.

Five standard solutions of GSSG (using GSSG over I.S. area plotted against the mmol/L of GSSG injected) were measured between 0.1 to 2 mmol/L. A linear relationship was obtained (y = 1.8081 + 0.004). The regression coefficient ($r^2$) was calculated at 0.999 (Fig 3). The area of GSSG measured was between 15.7 ± 0.21 V.s to 189 ± 1.5 V.s. Table 2 shows GSSG concentrations in erythrocytes per mL blood measured with only 150 µL of sample at three different time points in four subjects. The concentration of GSSG was measured with a good reproducibility (CV of 14.3 %, when measured as duplicates). The mean value (0.54 ± 0.08 µmol/mL) of GSSG equivalent to the concentration of GSH was consistent with values reported in our previous paper[28] and in other literature[29]. These findings show that LC-IRMS can analyze the concentration of metabolites in blood with good precision (better than 0.1 µmol/mL) and a limit of detection (LOD) of 5 µmol/mL.

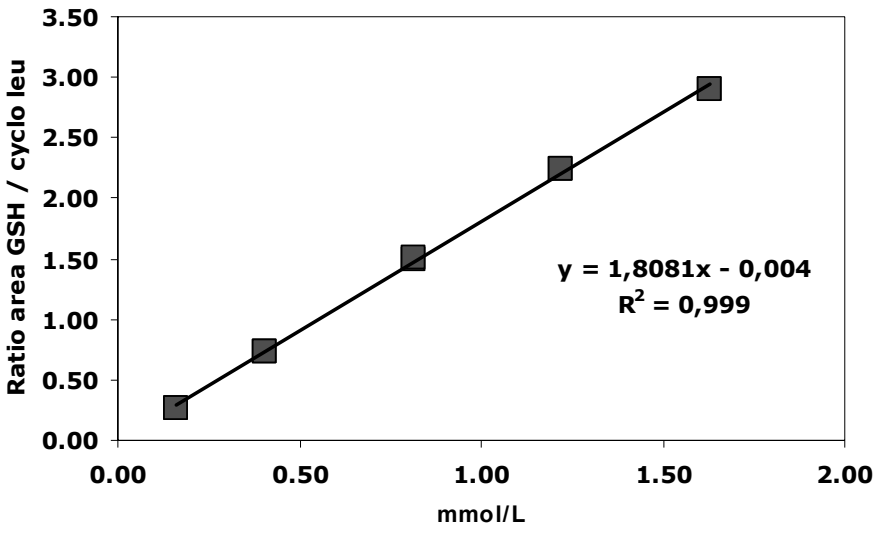


***Figure 3*** *Calibration curve of glutathione concentration in human blood erythrocytes.*

***Table 2*** *Concentration and $^{13}C$- isotopic enrichments of glutathione in four different subjects showing the standard deviation (sd) and the correlation variation CV (%). Samples taken at 4, 5 and 6 hours were measured in duplicate.*

| Subject | Time (hour) | GSH μmol/mL | Sd (n=2) | APE GSH | Sd (n=2) |
|---|---|---|---|---|---|
| 1 | 4a | 0.49 | 0.007 | 0.214 | 0.000 |
| | 4b | 0.48 | | 0.215 | |
| | 5a | 0.44 | 0.000 | 0.263 | 0.005 |
| | 5b | 0.45 | | 0.270 | |
| | 6a | 0.44 | 0.003 | 0.346 | 0.000 |
| | 6b | 0.44 | | 0.345 | |
| 2 | 4a | 0.54 | 0.007 | 0.065 | 0.008 |
| | 4b | 0.53 | | 0.076 | |
| | 5a | 0.60 | 0.007 | 0.134 | 0.002 |
| | 5b | 0.61 | | 0.137 | |
| | 6a | 0.58 | 0.002 | 0.191 | 0.004 |
| | 6b | 0.58 | | 0.186 | |
| 3 | 4a | 0.45 | 0.018 | 0.166 | 0.005 |
| | 4b | 0.47 | | 0.160 | |
| | 5a | 0.54 | 0.002 | 0.209 | 0.001 |
| | 5b | 0.55 | | 0.208 | |
| | 6a | 0.48 | 0.007 | 0.270 | 0.001 |
| | 6b | 0.49 | | 0.271 | |
| 4 | 4a | 0.65 | 0.022 | 0.155 | 0.002 |
| | 4b | 0.62 | | 0.152 | |
| | 5a | 0.62 | 0.001 | 0.212 | 0.005 |
| | 5b | 0.62 | | 0.219 | |
| | 6a | 0.61 | 0.004 | 0.260 | 0.003 |
| | 6b | 0.61 | | 0.255 | |
| Mean | | 0.54 | 0.007 | 0.208 | 0.003 |
| Sd | | 0.077 | | | |
| CV % | | 14.31 | | | |



**LC-IRMS measurement of glycine and GSSG $^{13}C$ enrichment in erythrocytes.**

After being oxidized, using the method described in the experimental section, a amino acid hydrolysate standard with a known added amount of GSH (Fig 1) as well as the erythrocyte samples (Fig 2) were analysed using the LC-IRMS system. Because there is a little chromatographic shift in eluting time, when measuring $^{12}C$ /$^{13}C$, it is necessary to have a good separation of the compound of interest and other eluting compounds for a reliable determination of the ratio. As shown in Figure 1 and 2, it can be seen that glycine and GSSG were eluting in a part of the chromatogram free of other eluting compounds. The enrichment of $^{13}C$ glycine and $^{13}C$ GSSG was determined by comparing the $^{12}C$ /$^{13}C$ ratio's using standard curves between 0% and 3% APE from known fractions of [1-$^{13}C$] glycine and [1,2-$^{13}C$- $^{15}N$]

glutathione. Linear relationships were obtained for glycine with a regression coefficient ($R^2$) calculated at 0.9999 (Fig 4) as well as for GSH with a regression coefficient ($R^2$) calculated at 0.9998 (Fig 5).

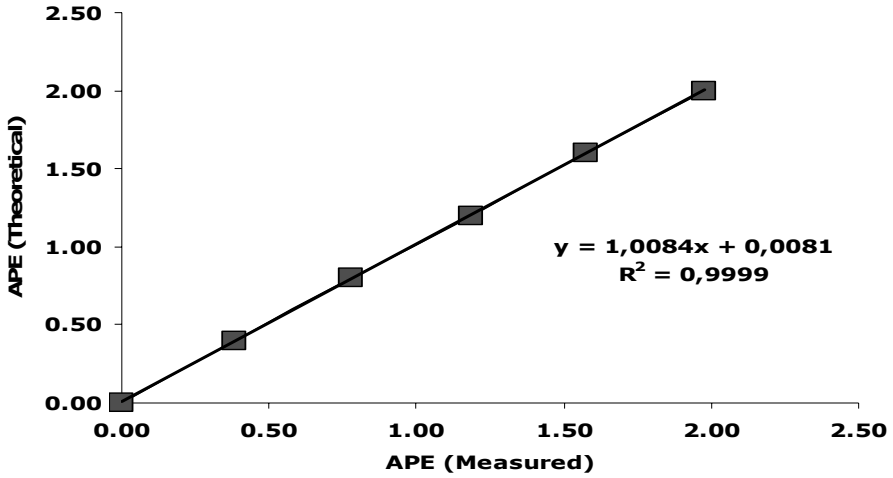


***Figure 4*** *Calibration curve for measurement of [7-$^{13}$C]-glutathione enrichment in human blood erythrocytes using [1,2-$^{13}$C-$^{15}$N] glutathione.*



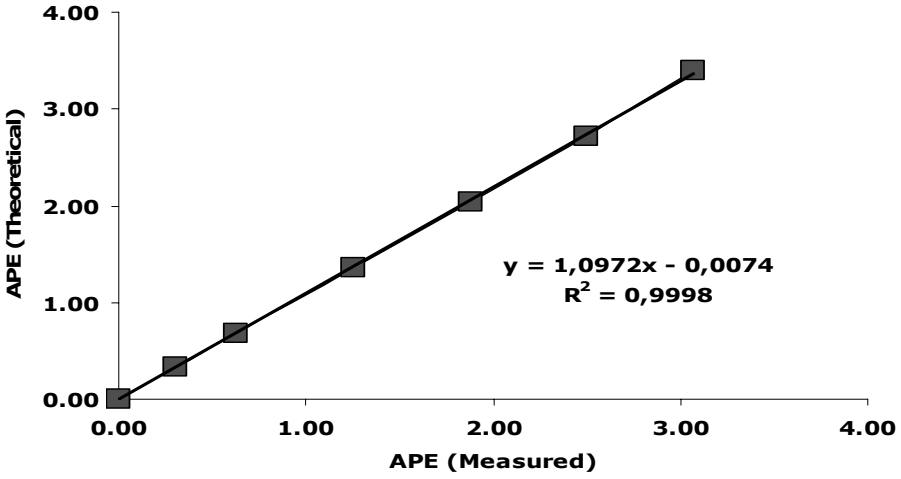


***Figure 5*** *Calibration curve of $^{13}$C-glycine enrichment in human blood erythrocytes using [1-$^{13}$C] glycine.*

**Accuracy and precision of isotopic measurement**

As illustrated in Figure 6, the intra-assay repeatability assessed with cycloleucine measured with different blood samples from the same subject showed a sd of 0.09 measured at -27.03± 0.08 ‰ and a reproducibility (CV)

of 0.4 % (n=6). The inter-assay repeatability measured over different days was -27.01 ± 0.12 ‰ (n = 21). The CV was 0.8 %. For each subject, the δ $^{13}$C values of cycloleucine were close to the mean value. These values were in the same range as obtained with standard injection of I.S., evidence of excellent isotopic precision as well as accuracy of isotopic measurement at natural abundance.

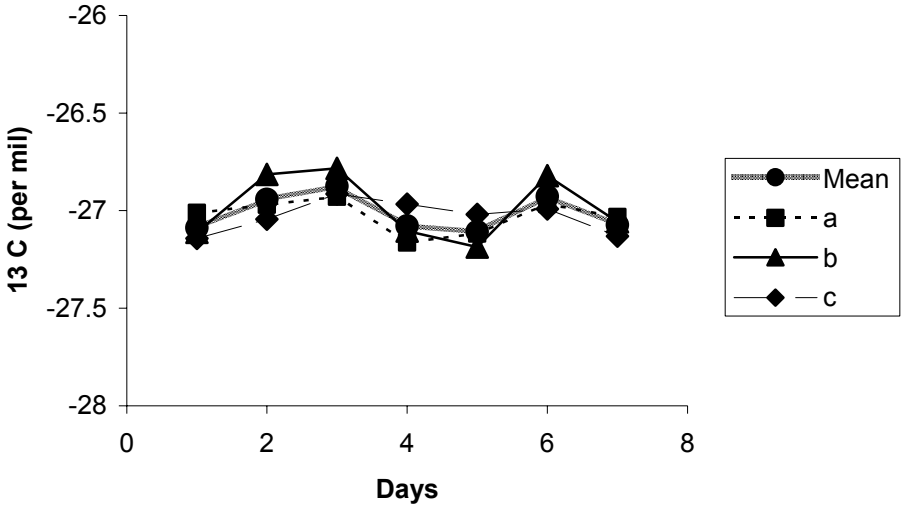


***Figure 6*** *Variation of δ $^{13}$C (‰) of cycloleucine added as internal standard and spiked in blood erythrocyte for 4 different subjects. Blood was collected at three time points (a, b, c) and corresponding values are shown. Each point corresponds to duplicate injections.*

The accuracy of isotopic measurement was assessed using standard calibration curve performed with various amounts of $^{13}$C labelled GSH added to a fixed amount of natural GSH. By plotting measured APE versus theoretical APE between 0 and 2.5 APE, the curve was linear at a slope of 1.0084 (Fig. 4). This shows that no isotopic fractionation occurred in the sample preparation and the analysis. The regression coefficient ($R^2$) was 0.9999, showing excellent linearity.

**Calculation of the FSR of glutathione**

During the steady state period of erythrocyte 1-$^{13}$C glycine levels, blood samples where taken at regular intervals (4h, 5h and 6h). The enrichments of glycine and GSH were determined in these erythrocyte samples. Figure 7 shows a linear rise in time in GSSG APE. From these measurements FSR GSSG was calculated using the above equation 1. Values for four patients are reported in Table 3.

It would seem that the glutathione pool of these patients was renewed approximately every two days. FSR of glutathione was 44.5 ± 2.4%/day with

a reproducibility (CV) of 5.4 % (n = 4). These values were in the same range as reported previously[28].

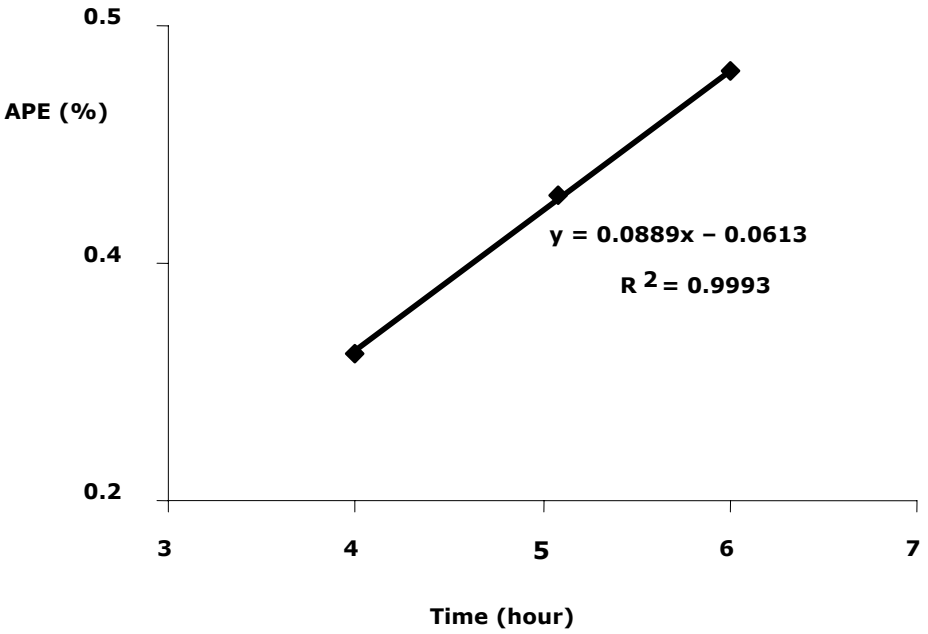


***Figure 7*** *Graph of the increase of enriched glutathione of a subject expressed in APE / hour.*

***Table 3*** *The fractional synthetic rate (FSR %/d) of glutathione in four different subjects; GSH and glycine analyses were carried out in duplicate.*

| Subject | Glycine MPE | GSH APE %/Hour | GSH MPE %/Hour | GSH FSR %/day |
|---|---|---|---|---|
| **1** | 3.80 | 0.0791 | 0.0733 | 46.27 |
| **2** | 3.73 | 0.0732 | 0.0682 | 43.86 |
| **3** | 4.07 | 0.0756 | 0.0703 | 41.43 |
| **4** | 3.59 | 0.0748 | 0.0696 | 46.53 |
| **Mean** | 3.80 | 0.0757 | 0.0703 | 44.53 |
| **Sd** | 0.202 | 0.002 | 0.002 | 2.386 |
| **CV%** | 5.31 | 3.28 | 3.05 | 5.36 |

## Conclusion

This new LC-IRMS method for measuring kinetics of glutathione in its oxidized form (GSSG) shows to be a powerful tool in metabolic studies in neonates. Only little pre-purification is necessary and the analyses reported here were fully automated. The simultaneously measurement of glycine and GSH, for both concentration and $^{13}C$ isotopic enrichment, gave excellent results. The more so as only 150 µL of blood was needed, which is of extremely high relevance for neonatal studies or studies in small animals. GSH concentrations corresponded to those reported in the literature. The precision and accuracy of isotopic enrichment at natural abundance and at higher isotopic enrichment gave excellent results without isotopic fractionation.

**Acknowledgments**
We would like to thank Ko Hagoort for his editorial critique

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CHAPTER

# 7

# Glutathione synthesis rates after amino acid administration directly after birth in preterm infants

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**Abstract**

Availability of glutathione, the main intracellular antioxidant, is compromised in preterm neonates. A possible explanation is low substrate availability for synthesis, as many neonatologists for fear of intolerance are reluctant to administer amino acids in the direct postnatal period.

The objective of the study was to determine effects of amino acid administration directly following birth on glutathione synthesis rates and markers of oxidative stress.

Premature infants (<1500 g) received from birth onwards either dextrose (control group, n=10) or dextrose and amino acids (2.4 g/(kg·d)) (intervention group, n=10). On postnatal day 2, [1-$^{13}$C]glycine was administered to determine glutathione fractional synthesis rates ($FSR_{GSH}$) and absolute synthesis rates ($ASR_{GSH}$) in erythrocytes. In plasma, advanced oxidized protein products (AOPP) and dityrosine, both markers of oxidative stress, were determined. Results are expressed as mean ± SD.

The $FSR_{GSH}$ was not different between groups (44 ± 6 and 48 ± 9 %/d in the control and intervention group respectively (p=0.28)). The concentration of erythrocyte glutathione in the intervention group (2.28 ± 0.35 mmol/L) was higher than in the control group (1.73 ± 0.37) (p<0.001). The $ASR_{GSH}$ was 6.5 ± 1.5 and 11.3 ± 1.9 mg/(kg·d) in the control and intervention group, respectively (p<0.001).

AOPP and dityrosine concentrations were not different between groups. In conclusion, amino acid administration directly following birth increases $ASR_{GSH}$ in preterm infants. Our data are consistent, however, with higher glutathione concentration rather than a higher $FSR_{GSH}$. Greater availability of glutathione, nevertheless, did not bring down markers of oxidative stress.

## Introduction

Birth coincides with a sharp increase in oxygen exposure. The formation of reactive species, such as superoxide, hydrogen peroxide and hydroxyl radicals evokes upregulation of antioxidant defense systems in full term infants[1] or rabbits[2]. This, however, does not seem to occur in preterm infants, as reflected by poor antioxidant availability and presence of protein and lipid (per)oxidation products[3, 4]. Yet, upon their unanticipated transition to the extrauterine world, preterm neonates frequently receive, though not necessarily require, ventilation with high concentrations of oxygen. This may result in oxidative stress, which is strongly associated with neonatal diseases such as bronchopulmonary dysplasia, retinopathy of prematurity, and periventricular leukomalacia[5-8].

With concentrations in the millimolar range, glutathione (GSH) is the most important intracellular antioxidant. Most cells are equipped with the enzymatic machinery to synthesize this tripeptide of glutamate, cysteine and glycine. Moreover, the enzymatic apparatus is present and active already in the second trimester of pregnancy and thus not a limiting factor for GSH synthesis in preterm infants[9, 10]. GSH concentrations in erythrocytes and plasma of preterm infants are high immediately after birth, but then drop to significantly lower levels than found in term neonates in the neonatal period [3, 11]. This shortage may be due to the fact that preterm infants do not tolerate significant amounts of enteral nutrition in the first days of life, and are, therefore, given parenteral nutrition, typically starting off with dextrose only. Meanwhile, however, the safety of early amino acid (AA) administration in preterm infants has been well established, as relevant studies found no abnormal blood gas values or abnormal plasma AA profiles[12, 13]. In addition, infants' catabolic state when receiving dextrose only was found to convert into an anabolic state representing true growth upon AA administration[14]. Despite these findings, AA administration directly after birth is still not uniformly standard of care.

We hypothesized that GSH production in preterm infants is compromised by shortage of substrates, and that AA administration will stimulate GSH synthesis rates. To test this hypothesis, we conducted a stable isotope study designed to determine synthesis rates of GSH in infants receiving either dextrose only or dextrose and AAs. Degree of oxidative stress was established by measuring concentrations of advanced oxidized protein products (AOPP)[15] and dityrosine [16, 17], both markers of oxidative stress. We hypothesized that preterm infants receiving dextrose only would show the highest concentrations of AOPP and dityrosine.

## Methods

### Design

The study was designed as a randomized clinical trial performed in the neonatal intensive care unit of the Erasmus MC – Sophia Children's Hospital, Rotterdam, the Netherlands. The study was investigator initiated with no funding from industry. The protocol was approved by the Erasmus MC Medical Ethical Committee and informed written parental consent was obtained prior to the study.

### Subjects

Subjects were premature infants with a birth weight <1500 g, born in the Erasmus MC–Sophia Children's Hospital, had an indwelling arterial catheter for clinical purposes, and were expected to be completely dependent on parenteral nutrition for the first 2 days of life. Directly after birth they were randomly assigned to receive either only dextrose during the first 2 days (control group), or dextrose and AAs (2.4 g/(kg·d)) (Primene 10%, Baxter, Clintec Benelux N.V., Brussels, Belgium) within 2 hours postnatally (intervention group). The composition of the AA solution can be found in Table 1. AA and dextrose solutions were infused constantly without interruptions during the study. Lipids were not administered until after the study period. Exclusion criteria included erythrocyte transfusions within 12 hours prior to the study or during the study, known congenital abnormalities, chromosome defects, and metabolic, endocrine, renal, or hepatic disorders. For all infants, we recorded birth weight, gestational age, birth weight Z-scores, severity of illness at entry of the study by means of Apgar and CRIB scores[18]. We recorded plasma AA concentrations, and caloric intake and AA intake. In addition, we recorded fractions of inspired oxygen, blood glucose levels, and incidence of sepsis as evidenced by bacteremia. According to our policy at the NICU, all infants being ventilated receive prophylactic antibiotics. Therefore, all infants included received antibiotics. Administration of these antibiotics are stopped after 48-72 hours whenever C-reactive protein concentrations are low and blood cultures are negative.

***Table 1*** *Composition of the amino acid solution Primene 10% (Baxter, Clintec Benelux N.V., Brussels, Belgium)*

| Amino acid | Value g/L | Amino acid | Value g/L |
|---|---|---|---|
| L-Isoleucine | 6.70 | L-Alanine | 8.00 |
| L-Leucine | 10.00 | L-Aspartate | 6.00 |
| L-Valine | 7.60 | L-Cysteine | 1.89 |
| L-Lysine | 11.00 | L-Glutamate | 10.00 |
| L-Methionine | 2.40 | Glycine | 4.00 |
| L-Phenylalanine | 4.20 | L-Proline | 3.00 |
| L-Threonine | 3.70 | L-Serine | 4.00 |
| L-Tryptophane | 2.00 | L-Tyrosine | 0.45 |
| L-Arginine | 8.40 | L-Ornithine-HCl | 3.18 |
| L-Histidine | 3.80 | Taurine | 0.60 |

**Tracer infusion protocol and sample collection**

[1-$^{13}$C]Glycine (99% enriched, sterility and pyrogenicity tested) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA) and was dissolved in a 0.9% saline solution, filtered (0.2 µm) and sterilized by the hospital's pharmacy. The final product was tested for identity, content, sterility and pyrogenicity.

On postnatal day 2 neonates received a primed (40 µmol/kg) continuous (20 µmol/(kg·h)) infusion of [1-$^{13}$C]glycine for 6 hours. Blood samples (400 µL each) were drawn from an indwelling arterial catheter after 4, 5, and 6 hours and collected in EDTA containing microtainers to quantify erythrocyte free glycine enrichment, GSH-bound glycine enrichment, GSH concentration, plasma oxidative stress markers, and plasma GSH precursor AA concentrations.

Samples were immediately put on melting ice after centrifugation at 3500 x G for 10 min at 4°C. After the plasma fraction was removed, the lower layer containing primarily erythrocytes was reconstituted to its original volume with ice-cold distilled water to disrupt cell membranes. The plasma and cell fractions were subsequently stored at -80°C until further analysis.

**Glutathione enrichments and concentration**

Enrichments of GSH and its precursor glycine as well as GSH concentrations were determined to measure fractional and absolute synthesis rates. For this purpose, we used a new technique described previously, using an LC-Isolink® interface (Thermo Electron, Bremen, Germany) coupled to a Delta XP isotope ratio mass spectrometer (Thermo Electron, Bremen, Germany) (LC-IRMS)[19]. This highly sensitive method requires only a very small sample volume, whereas no derivatization is required. Briefly, erythrocytes were disrupted by freezing and thawing. Next, 40 µL of a mixture of 0.2 mg/mL

norleucine and 1.0 mg/mL norvaline was added as internal standards and the samples were deproteinized by adding 200 µL of 6 % g/v perchloric acid, left on ice for 10 minutes and finally centrifuged at 10.000 x G for 20 minutes. The supernatant was transferred to a clean tube and the pH was adjusted to 8 - 9 with a KOH (4 mol/L) solution. Excess perchloric acid was precipitated and removed by centrifugation at 10.000 x G for 10 minutes. 100 µL of $NaHPO_4$ (0.5 mol/L) was added to maintain pH 9. The supernatant was filtered through a 0.2 µm Nylon membrane filter (Nylon, Alltech, Breda, The Netherlands). An aliquot of 150 µL was transferred to a sample vial and 20 µL was injected for each analysis of both GSH concentration and $^{13}C$-isotopic enrichment by LC- IRMS. The remaining supernatant was used for analysis of $^{13}C$- isotopic enrichment of glycine by gas chromatography – combustion – isotope ratio mass spectrometry, similar to an earlier developed method for measurement of the isotopic enrichment of threonine[20]. Plasma concentrations of direct GSH precursors glutamate, glycine, and cysteine (measured as cystine), and indirect precursors glutamine, methionine and serine, were determined with a Biochrom 30 amino acid analyzer, using ninhydrin detection (Biochrom Ltd, Cambridge, England).

**Calculations**

The $FSR_{GSH}$ represents the fraction of the total intraerythrocytic GSH pool that is renewed per unit of time, and is expressed as %/d. It was measured according to the product/precursor- equation:

$$FSR_{GSH}\ (\%/d) = \frac{\text{slope}\, E_{[1\text{–}^{13}C]\text{glutathione}_{t4,5,6}}}{E_{\text{intraerythrocytic}[1\text{-}^{13}C]\text{glycine}}} \times 24h \times 100\%$$

where E stands for enrichment expressed as mole percent excess (MPE). The nominator (product) of this equation represents the hourly increase of incorporated [1-$^{13}$C]glycine into GSH as calculated from the increase in enrichment between 4 and 6 hours of infusion. The denominator (precursor) represents the intraerythrocytic free [1-$^{13}$C]glycine enrichment at isotopic steady state. A steady state plateau was defined as an insignificant change with time in intraerythrocytic enrichment. Subsequently, the intravascular absolute synthesis rate ($ASR_{GSH}$) was calculated by the following equation:

$$ASR_{GSH}\ (mg/(kg{\cdot}d)) = FSR_{GSH}/100 \times conc \times 307 \times ht \times 0.075$$

where conc is concentration in mmol/L of packed erythrocytes, 307 is the molecular weight of GSH, ht is hematocrit, and 0.075 is the estimated circulating blood volume in a preterm neonate, expressed as L/kg.

**Oxidative stress markers**
We measured AOPP in plasma by the spectrophotometric assay described by Witko-Sarsat et al[15]. Dityrosine concentrations were measured by the method described by Abdelrahim et al., based on liquid-liquid extraction, reversed-phase chromatography and fluorescence detection[16].

**Glutathione kinetics in healthy adults**
Since kinetic measurements require venous access and an indwelling arterial catheter, healthy term neonates cannot serve as controls. GSH kinetics so far have only been studied in older infants and adults, as relatively high blood volumes are needed for precise determination of enrichment and concentration. In two studies on GSH kinetics in healthy adults, mean $FSR_{GSH}$ was found to be 65%/d and 83%/d, respectively[21, 22]. GSH concentrations, however, were found to vary substantially between studies[23]. Even minimal manipulation of samples can result in loss of GSH, thereby creating false assumptions with respect to the in vivo situation. To correct for different methods yielding different results, we determined GSH kinetics in healthy adults by exactly the same method we used for preterm infants. Studies were conducted after an overnight fast, and subjects remained in the fasting state throughout the study.

**Statistics**
Statistical analyses were performed using SPSS version 15.0 (SPSS Inc, Chicago, IL, USA) and GraphPad Prism version 4 (GraphPad Software, San Diego, CA, USA). Data are expressed as means ± SD or as medians (min – max). Primary outcome of the study was the glutathione fractional synthesis rate. Based on an abstract by Shew et al.[24], we calculated that with an α of 0.05, a power of 0.80 and a difference in $ASR_{GSH}$ of 0.22 mmol/(L·d) with an SD of 0.06, group size needed to be at least 3 to detect a difference. We included 10 infants in each group in order to increase power.

Differences between groups were determined using independent t-tests or Mann-Whitney tests in case of normal or skewed distribution of the study groups, respectively. A P value of $<0.05$ was considered as statistically significant.

**Results**

Clinical characteristics are listed in Table 2.
All infants received additional oxygen as part of their treatment. We found no correlation between fractions of inspired oxygen and GSH concentration or $FSR_{GSH}$. Nutritional intakes before and during the study are shown in Table 3.
Blood glucose levels were not different between groups and none of the infants received insulin during or prior to the study (data not shown). Also,

there were no differences in incidence of hyperglycemia. In one blood culture micrococcus species was isolated, which was considered contamination. None of the other infants had either a rise in C-reactive protein concentrations or positive blood cultures before or during the study.

***Table 2*** *Clinical characteristics*[1]

| | control | intervention |
|---|---|---|
| N (M:F) | 10 (7:3) | 10 (4:6) |
| birth weight (g) | 990 ± 205 | 916 ± 150 |
| gestational age (wks) | 27 $^{1}/_{7}$ ± 2 | $27^{5}/_{7}$ ± $2^{1}/_{7}$ |
| birth weight Z-score (SD) | -0.4 ± 1.3 | -1.4 ± 0.9 |
| mode of delivery vaginal:cesarean section | 5:5 | 5:5 |
| Apgar score (5 min) | 8 (6 – 10) | 7 (4 – 9) |
| CRIB score[2] | 5 (1 – 7) | 4 (1 – 8) |
| $FiO_2$ minimum | 21 (21 – 29) | 21 (21 – 24) |
| maximum | 54 (24 – 100) | 45 (28 – 72) |

[1] Values are expressed as either mean ± SD or median (min-max).
There were no statistical differences between groups (Student's T-test or Mann-Whitney, depending on distribution).
[2]The CRIB score (Clinical Risk Index for Babies) indicates the degree of illness. The score is positively correlated with the severity of illness.

***Table 3*** *Nutritional intakes before and during the study*[1]

| | control (n=10) | intervention (n=10) | *P* value |
|---|---|---|---|
| nonprotein energy intake (kcal/(kg·d)) | 38 ± 4 | 42 ± 8 | 0.22 |
| amino acid intake (g/(kg·d)) [2] | 0 ± 0 | 2.5 ± 0.1 | <0.001 |

[1] Values are expressed as mean ± SD (Student's T-test).
[2] As anticipated by study design, the control group did not receive amino acids during or prior to study.

**Concentrations of plasma precursor AAs**

Table 4 shows plasma concentrations of all AAs involved in GSH synthesis with reference values obtained from healthy term breast-fed infants[25]; AA concentrations were significantly higher in the intervention group. Concentrations of glutamate, cystine, and methionine in the control group were below reference ranges. Although the AA solution administered contained cysteine (1.89 g/L), cystine concentrations still were low in the intervention group as well.

***Table 4*** *Plasma concentrations of amino acids*[1,2]

| | control (n=10) | intervention (n=10) | *P* value | term infants |
|---|---|---|---|---|
| glutamate | 19 ± 5 | 72 ± 47 | 0.017 | 76 – 551 |
| cystine | 14 ± 9 | 27 ± 10 | 0.011 | 33 – 55 |
| glycine | 206 ± 71 | 341 ± 154 | 0.047 | 66 – 432 |
| glutamine | 280 ± 167 | 566 ± 243 | 0.012 | 147 – 623 |
| serine | 94 ± 31 | 189 ± 91 | 0.022 | 79 – 227 |
| methionine | 12 ± 5 | 36 ± 24 | 0.026 | 21 – 55 |

[1] Amino acids that are directly or indirectly involved in glutathione synthesis with a reference range of term breast-fed infants[25].
[2] Values are expressed as mean ± SD (Student's T-test).

**GSH concentrations and synthesis rates**

In Figure 1 precursor and product enrichments are plotted against duration of tracer infusion. Free intraerythrocytic [1-$^{13}$C]glycine, considered as the precursor for GSH synthesis, reached a plateau after 4 hours of infusion defined as no significant increase in enrichment. Precursor enrichments did not differ between the groups (3.6 ± 0.7 MPE in each). Levels of [1-$^{13}$C]glycine bound to GSH linearly increased between 4 and 6 hours of infusion, with a mean $R^2$ of 0.996 and 0.994 between the values measured for the control and intervention group, respectively. The LC-IRMS method used in this study quantified [1-$^{13}$C] enrichment of the total GSH pool, since IRMS is known to combust all carbon elements of GSH into $CO_2$. We, therefore, were not able to discriminate between [1-$^{13}$C] enrichment of GSH originating from glycine or cysteine. We studied, however, the cysteine peak in our chromatogram, and we did not find any enrichment, thereby excluding possible overestimation of the FSR.

GSH kinetic data are shown in Figure 2. $FSR_{GSH}$ did not differ between groups. The concentration of erythrocyte GSH in the intervention group was higher, however, than that in the control group. As a result, also $ASR_{GSH}$ was higher in the intervention group.

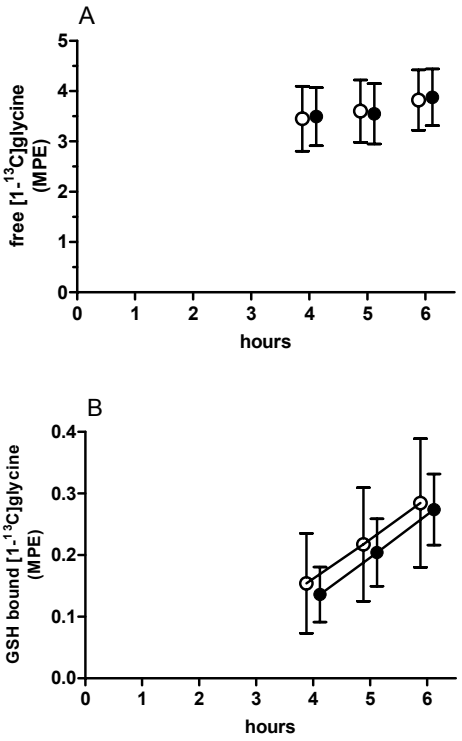


***Figure 1*** *Means and SD's of isotopic steady state of [1-$^{13}$C]glycine (precursor) enrichments (A) and [1-$^{13}$C]glutathione (product) enrichments (B) in the control (o, n=10) and intervention (•, n=10) groups. There were no significant differences in either isotopic steady state of [1-$^{13}$C]glycine enrichment or increase in [1-$^{13}$C]glutathione enrichment (Student's T-test).*



**Plasma concentrations of oxidative stress markers**

Plasma concentrations of AOPP are shown in Figure 3. We found no differences between groups for both markers. These results are in agreement with earlier studies on AOPP levels in preterm infants and indicate the presence of oxidative stress[4, 26]. Increased GSH availability as a result of AA administration did not result in lower AOPP levels on postnatal day 2.

**GSH kinetics in healthy adults**

We included 5 healthy non smoking adults. They had an age of 34 ± 8 years and a Body Mass Index of 22.5 ± 1.2 kg/m$^2$ (mean ± SD). We found a mean concentration of 1.43 ± 0.13 mmol/L which is comparable to the concentrations reported by Darmaun and colleagues[22]. The mean $FSR_{GSH}$ in erythrocytes was 62 ± 2 %/d resulting in a mean $ASR_{GSH}$ of 7.7 ± 1.1 mg/(kg·d). Thus, the $ASR_{GSH}$ of fasted adults is lower than that of fed preterm infants.

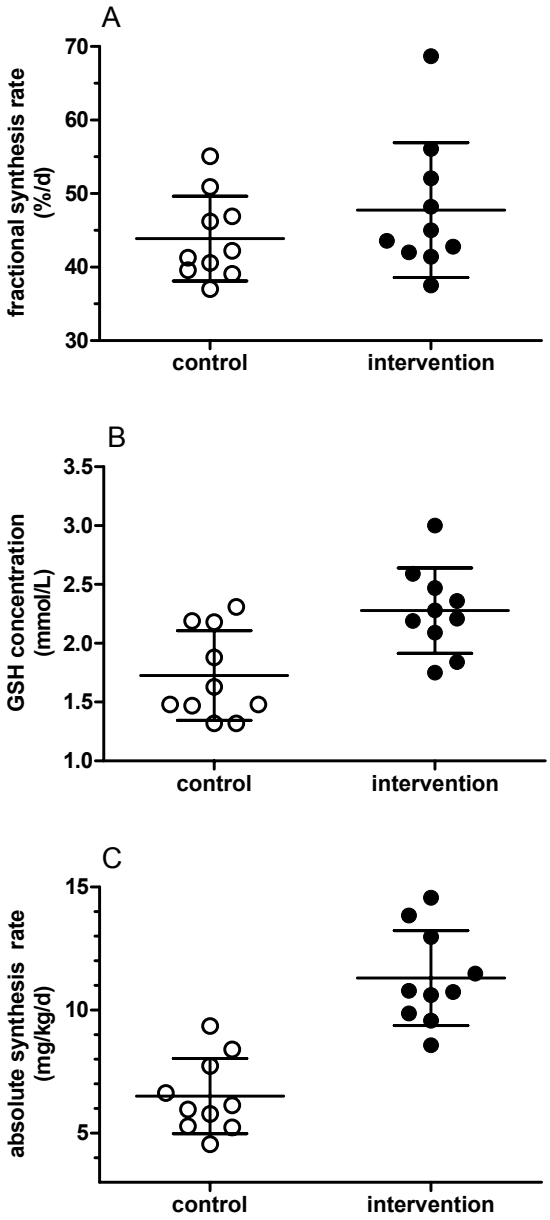


***Figure 2*** *Glutathione fractional synthesis rates (A), concentrations (B) and absolute synthesis rates (C) in erythrocytes in the control (○, n=10) and intervention (●, n=10) groups expressed as individual cases with horizontal lines representing means and SD's. There were no significant differences in fractional synthesis rates between groups (Student's T-test). Glutathione concentrations and absolute synthesis rates were higher in the dextrose + AA group (P<0.001 for both concentration and absolute synthesis rate, Student's T-test).*

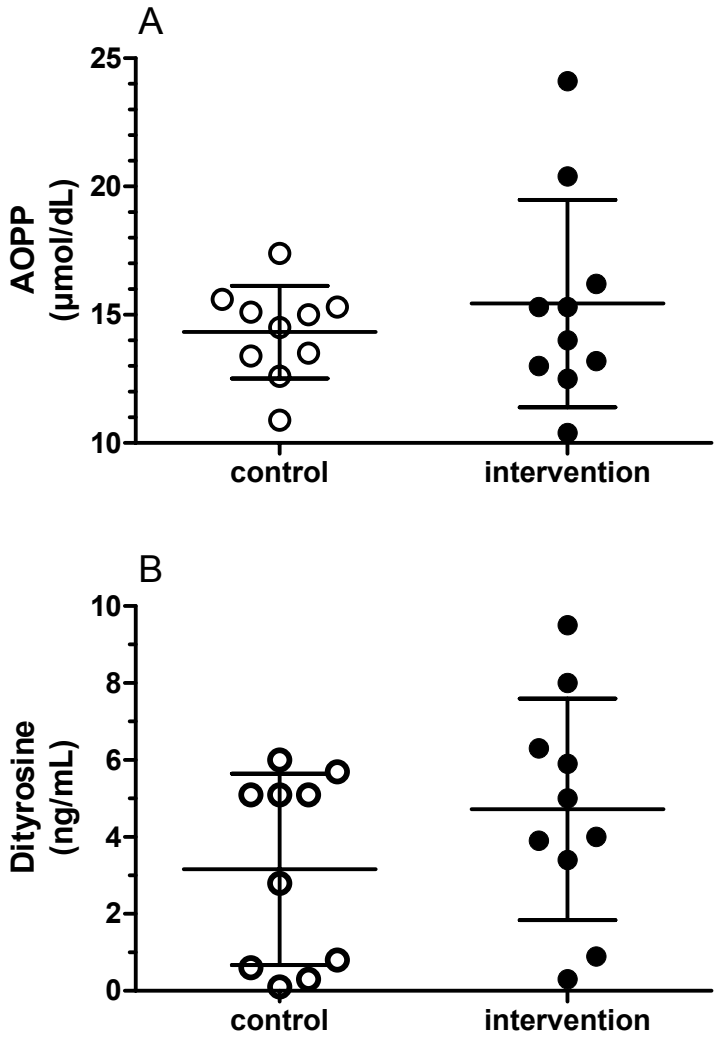


***Figure 3*** *Plasma concentrations of advanced oxidized protein products (AOPP) (A) and dityrosine (B) in the control (○, n=10) and intervention (●, n=10) groups expressed as individual cases with horizontal lines representing means and SD's. There were no statistically significant differences for either AOPP or dityrosine concentrations (Student's T-test).*



**Discussion**

We showed AA administration to be a safe, simple and efficacious means of increasing GSH synthesis rates. We demonstrated a 32% increase in erythrocyte GSH concentration and a 74% increase in the $ASR_{GSH}$ following

administration of 2.4 g AAs/(kg·d) as compared to dextrose administration solely. These results clearly bring out the discrepancy between, on the one hand, demands of the body and, on the other hand, the scarce availability of substrates if only dextrose is administered. Antioxidant defense build-up was not associated, however, with lower concentrations of oxidative stress markers.

The observed increase of the $ASR_{GSH}$ can almost exclusively be attributed to the increase in concentration. This is fascinating, since in adults and children alike increases in $ASR_{GSH}$ seem to arise primarily from increases in $FSR_{GSH}$ rather than from elevated concentrations[21, 27, 28]. We have two explanations. For one, the $FSR_{GSH}$ might have been increased already on the first day of life, and then have dropped as a result of negative feedback. A study performed on the first day of life under the same conditions could provide more insight. Second, the lack of increase in $FSR_{GSH}$ might be related to the function of GSH as an AA reservoir. Plasma glycine concentration rose 65% after AA administration (Table 3). It follows that plasma glycine enrichment must have been lower in the intervention group, since infusion rates of [1-$^{13}$C]glycine were identical for both groups. Despite theoretically lower plasma glycine enrichment in the intervention group as compared to the control group, intraerythrocytic enrichment did not differ between groups, as shown in Figure 2. Seeing that GSH itself is the major intraerythrocytic pool of glycine, the most plausible explanation is decreased breakdown of intracellular GSH in the intervention group compared to the control group, as proposed earlier[29]. Note that GSH is an important AA reservoir, its intracellular concentration being in the millimolar range whereas the free fractions of its constituent AAs, especially cysteine, are in the micromolar range. Altogether, these data strongly suggest decreased consumption of GSH in the intervention group, possibly resulting – as we showed recently – from increased synthesis of other antioxidants such as albumin[30].

An explanation for the overall low $FSR_{GSH}$ in preterm neonates as compared to adults is increased recycling of GSSG to GSH in neonates. Normally, GSSG concentrations are kept very low to protect the cell from a shift in redox equilibrium. This is achieved by either reducing GSSG to GSH or exporting it to the extracellular space, dependent on the availability of the enzyme glutathione reductase and NADPH[31]. Indeed, increased recycling of GSSG into GSH in erythrocytes of preterm neonates as opposed to adults was observed earlier[32, 33]. More efficient recycling could very well decrease the need for de novo synthesis and perfectly fits our data, i.e. a relatively low $FSR_{GSH}$ in preterm infants as compared to adults.

Blood glucose concentrations and incidence of sepsis were not different between groups. This is relevant, seeing that both hyperglycemia and sepsis are known to produce oxidative stress[34, 35].

The importance of GSH in maintaining health and preventing oxidative stress has been widely studied. Chessex et al. studied the individual effects of

hyperoxia and nutrient restriction on liver and lung GSH availability in preterm guinea pigs[36]. They concluded that total parenteral nutrition not only increased both liver and lung GSH concentrations but also protected against hyperoxic lung injury and associated mortality. In agreement, Welty et al. and Yeung et al. recommended administration of antioxidants or their precursors as soon as possible following birth in order to prevent oxidative stress related diseases, such as bronchopulmonary dysplasia[37, 38].

GSH kinetics were measured using a new technique which transforms all GSH to its dimeric form (GSSG). We did not, therefore, discriminate between GSH and GSSG. However, since the fractional synthesis rate is a relative measure, it will not be influenced by a difference in redox state. We measured GSH kinetics in erythrocytes, which are readily accessible as opposed to other tissues. Moreover, erythrocytes can protect other tissues, such as the lung, by providing intracellular antioxidants[39] or by directly taking up ROS[40]. In a very recent study, Giustarini et al. provided strong evidence for a role for erythrocytes as GSH donor for other tissues[41]. They demonstrated active GSH export towards the plasma, indicating that, besides the liver, erythrocytes might significantly contribute to the extracellular GSH pool. These studies, however, fail to elucidate the mechanism by which the GSH is exported, since GSH is assumed unable to cross the cellular membrane intact.

We measured AOPP and dityrosine as markers of oxidative stress. Tyrosine is oxidized to dityrosine in response to oxidative stress and can be considered a good endogenous marker. Experiments that expose protein to oxygen-free radicals have demonstrated the formation of dityrosine. Dityrosine has been recognized as an oxidative stress product of pathological response to disease or other environmental stress[17, 42].
The AOPP concentrations were higher than those found in older, more stable preterm infants as well as healthy adults[4, 15].

Cysteine is generally assumed to be the rate limiting substrate for GSH synthesis. It is also considered to be an essential AA in the preterm neonate on the grounds of high cystathionine concentrations[43] and low cystathionase activity impeding the conversion of methionine into cysteine[44]. Although evidence is mounting that preterm infants are in fact able to synthesize cysteine[45, 46], demands may still exceed capacity to synthesize. Indeed, earlier we showed that plasma cyst(e)ine concentrations were below reference values, both in group not receiving any AAs and in a group receiving AAs including cysteine[14]. The AA solution we used provides methionine and cysteine, delivered as cysteine-HCl. Premixed AA solutions can only contain modest amounts of cysteine-HCl due to instability of cysteine at higher pH.

We demonstrated that AA administration to preterm infants directly postnatally is a safe and efficient way to increase GSH synthesis rates. Our data suggest that this does not increase GSH consumption, as intracellular

GSH breakdown seems to decline. Levels of oxidative stress markers nevertheless remained high. Plasma cystine concentrations rose upon AA administration, but still remained low. Worthy of further research is the question whether higher doses of AAs, or additional cysteine in particular, would further increase GSH synthesis rates.

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CHAPTER

# 8

# High dose cysteine does not increase the synthesis of the antioxidant glutathione in parenterally fed preterm infants

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**Abstract**

Cysteine is thought to be the rate limiting substrate for glutathione synthesis, the major intracellular antioxidant. Our aim was to evaluate whether administration of additional cysteine is safe and stimulates glutathione synthesis in preterm infants' early life.

We conducted a prospective, randomized clinical trial in infants with a birth weight <1500 gram (n=20). They were randomly assigned to receive either a standard dose (45 mg/(kg·d)) or a high dose of cysteine (81 mg/(kg·d)). Intakes of other amino acids were similar, providing a total protein intake of 2.4 g/(kg·d) in both groups. We recorded base requirements in the first 6 days of life. On postnatal day 2, we conducted a stable isotope study to determine glutathione concentrations and synthesis rates in erythrocytes.

Base requirements were higher in the high dose cysteine group on days 3, 4 and 5. Despite an 80% increase in cysteine intake, plasma cystine concentration did not increase. Glutathione concentrations and glutathione synthesis rates did not increase upon additional cysteine administration.

Administration of a high dose of cysteine (81 mg/(kg·d) to preterm infants appears clinically safe but does not stimulate glutathione synthesis as compared to a lower dose (45 mg/(kg·d)). Further research is required to prove whether or not there is significant benefit associated with cysteine supplementation.

## Introduction

Cysteine, a nonessential amino acid (AA) synthesized de novo from methionine and serine, has been considered essential in preterm infants for the last decades. This was primarily based on experiments in human fetal tissues demonstrating lack of cystathionase activity, the enzyme catalyzing the final step in the cysteine synthesis pathway (1-3). Although it has recently been demonstrated that capacity of cysteine synthesis is present in both enterally (4, 5) and parenterally (6) fed preterm infants, this does not necessarily imply that exogenous cysteine administration is not clinically relevant. The cysteine synthesizing pathway might not be fully active (7), and demands may very well exceed synthesis capacity directly after birth. Indeed, apart from being a substrate for protein synthesis, cysteine is considered to be the rate limiting substrate for glutathione (GSH) synthesis. GSH, a tripeptide synthesized from glutamate, cysteine and glycine, is required to prevent oxidative stress. The latter is strongly associated with a number of serious diseases observed in the neonatal period, such as bronchopulmonary dysplasia and periventricular leukomalacia (8). Due to concerns of metabolic intolerance and instability in parenteral solutions, most neonatal AA solutions contain only little amounts of cysteine or none at all. On the other hand, providing cysteine might decrease the need for methionine, which is associated with hepatotoxicity (9). In addition, it increases calcium and phosphorus solubility by lowering the pH of the AA solution (10). Hypothesizing that cysteine results in increased GSH synthesis rates, we conducted a randomized clinical trial in very low birth weight (VLBW) infants comparing effects of a standard versus a high dose of parenterally administered cysteine.

## Patients and Methods

### Design

The study was designed as a randomized clinical trial performed in the neonatal intensive care unit of the Erasmus MC – Sophia Children's Hospital, Rotterdam, the Netherlands between March 2007 and September 2007. The study was investigator initiated with no funding from industry. The protocol was approved by the Erasmus MC Medical Ethical Review Board and informed written parental consent was obtained prior to the study.

### Patients

Subjects were inborn premature infants with a birth weight <1500 g who had an indwelling arterial catheter for clinical purposes, and were expected to be exclusively dependent on parenteral nutrition for the first 2 days of life. Besides glucose, all neonates received AAs directly following birth (2.4 g/(kg·d)) (Primene 10%, Baxter, Clintec Benelux N.V., Brussels, Belgium). Standard cysteine concentration of Primene is 1.89 g/L, thus providing a daily dose of 45 mg/kg (370 µmol/kg) at an AA intake of 2.4 g/(kg·d). Directly after birth, infants were randomly assigned using a random-number table to receive either AAs including this standard amount of cysteine (CYS) or AAs including additional cysteine ($CYS^+$) (Hospira, Inc. Lake Forest, IL, USA, and SICOR Pharmaceuticals, Inc., Irvine, CA, USA) providing a total daily cysteine intake of 81 mg/kg (670 µmol/kg). The additional cysteine was infused directly into the amino acid solution by the attending nurse within an hour prior to administration. This was performed in a non-blinded fashion, since the study was not placebo-controlled. Intake of all other AAs did not differ between groups. Glucose and AAs were administered in separate solutions. They were administered either through the umbilical venous catheter or peripherally inserted central catheter. Lipids and vitamins were not administered until after the stable isotope study on postnatal day 2 had been concluded. Exclusion criteria included erythrocyte transfusions within 12 hours prior to the study or during the study, known congenital abnormalities, chromosome defects, and metabolic, endocrine, renal, or hepatic disorders.

### Study endpoints

Primary endpoint with respect to efficacy of cysteine administration was GSH synthesis rate in erythrocytes on postnatal day 2. Secondary endpoint was safety of cysteine administration, as reflected by base requirements in the first six days of life to prevent metabolic acidosis. Our unit has a guideline that a metabolic acidosis should be corrected when pH is <7.25 with $pCO_2$ values within a normal range.

For all infants, we recorded birth weight, gestational age, birth weight Z-scores, and severity of illness at entry of the study by means of Apgar and CRIB scores (11). Furthermore, we monitored use of supplemental oxygen

and also documented antibiotics administration and incidence of sepsis, as sepsis is known to contribute to oxidative stress (12). We daily recorded actual nutritional intakes. We recorded relevant plasma AA concentrations on the second day of life both as a safety and an efficacy parameter.

**Tracer infusion protocol and sample collection**

[1-$^{13}$C]Glycine (99% enriched, sterility and pyrogenicity tested) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA) and was dissolved in a 0.9% saline solution, filtered (0.2 µm) and sterilized by the hospital's pharmacy. The final product was tested for identity, content, sterility and pyrogenicity.

On postnatal day 2 neonates received a primed (40 µmol/kg) continuous (20 µmol/(kg·h)) infusion of [1-$^{13}$C]glycine for 6 hours. Blood samples (400 µL each) were drawn from an indwelling arterial catheter after 4, 5, and 6 hours of stable isotope infusion and collected in EDTA containing microtainers. Samples were immediately put on melting ice after centrifugation at 3500 x G for 10 min at 4°C. The plasma fraction was removed and stored separately for measurement of individual AA concentrations. The lower layer containing primarily erythrocytes was reconstituted to its original volume with ice-cold distilled water to disrupt cell membranes. The plasma and cell fractions were subsequently stored at -80°C until further analysis.

**Glutathione and amino acid measurements**

We measured GSH as total GSH, i.e. GSH + oxidized glutathione. Enrichments of GSH-bound glycine and GSH concentrations were determined according to a recently developed technique, using an LC-Isolink interface (Thermo Electron, Bremen, Germany) coupled to a Delta XP isotope ratio mass spectrometer (Thermo Electron, Bremen, Germany) (LC-IRMS) (13). Gas chromatography-combustion-isotope ratio mass spectrometry was used for analysis of $^{13}$C- isotopic enrichment of intraerythrocytic glycine, similar to an earlier developed method for measurement of the isotopic enrichment of threonine (14). Plasma concentrations of the direct GSH precursors glutamate, glycine, and cysteine (in vitro oxidized and measured as cystine), and indirect precursors glutamine, methionine and serine, as well as taurine, the product of cysteine catabolism, were determined with a Biochrom 30 amino acid analyzer using ninhydrin detection (Biochrom Ltd, Cambridge, England).

**Calculations**

The GSH fractional synthesis rate ($FSR_{GSH}$) was measured according to the product/precursor equation. As opposed to concentration, which is a static parameter, the FSR is a dynamic parameter providing information on metabolic changes over time. Accordingly, the $FSR_{GSH}$ represents the fraction

of the total intraerythrocytic GSH pool that is renewed per unit of time, and is expressed as %/d.

$$FSR_{GSH}\ (\%/d) = \frac{\text{slope}\, E_{[1\text{-}^{13}C]GSH_{t4,5,6}}}{E_{\text{intraerythrocytic}[1\text{-}^{13}C]\text{glycine}}} \times 24h \times 100\%$$

where E stands for enrichment expressed as mole percent excess (MPE). The nominator (product) of this equation represents the hourly increase of incorporated [1-$^{13}$C]glycine into GSH as calculated from the increase in enrichment between 4 and 6 hours of infusion. The denominator (precursor) represents the intraerythrocytic free [1-$^{13}$C]glycine enrichment at isotopic steady state. The endogenous background of $^{13}$C was assumed equivalent for all infants. The accuracy of the isotopic enrichments at isotopic plateau was tested by calculating the coefficient of variation for the three blood samples. A coefficient of variation <10% was considered as a valid plateau. Subsequently, the intravascular absolute synthesis rate ($ASR_{GSH}$) was calculated by the following equation:

$$ASR_{GSH}\ (mg/(kg{\cdot}d)) = FSR_{GSH}/100 \times conc \times 307 \times ht \times 0.075$$

where conc is concentration in mmol/L of packed erythrocytes, 307 is the molecular weight of GSH, ht is hematocrit, and 0.075 is the estimated circulating blood volume in a preterm neonate, expressed as L/kg.

**Statistics**

Statistical analyses were performed using SPSS version 15.0 (SPSS Inc, Chicago, IL, USA) and GraphPad Prism version 4 (GraphPad Software, San Diego, CA, USA). Data are expressed as mean ± SE or SEM or as median (range). Primary outcome of the study was the glutathione synthesis rate. Based on an abstract by Shew et al. (15), which describes a similar study, we calculated that with an α of 0.05, a power of 0.80 and a difference in $FSR_{GSH}$ of 30%/d with a SE of 10%, group size needed to be at least three to detect a difference. We included 10 infants in each group in order to increase power. Differences between groups were determined using independent t-tests or Mann-Whitney tests. A P value of <0.05 was considered as statistically significant.

**Results**

We included twenty VLBW infants, equally distributed between the two groups. Twenty-seven infants were initially assessed for eligibility, of whom seven infants were excluded due to not meeting inclusion criteria (n=2) or parental refusal (n=5). Group characteristics are shown in Table 1. Stable isotope infusion was initiated at 30 ± 7 hours and 30 ± 6 hours postnatally in the CYS and CYS$^+$ group, respectively. In none of the infants stable isotope

infusion had to be delayed because of requirements for erythrocyte transfusion. As anticipated by study design, daily cysteine intakes were significantly higher in the $CYS^+$ group during the entire study period. Actual daily AA and non protein calorie intakes were not different between groups during the whole study period. On the day of stable isotope infusion, infants in the CYS group received 2.5 ± 0.1 g AA/(kg·d) and a total non protein calorie of 39 ± 10 kcal/(kg·d); infants in the $CYS^+$ group received 2.5 ± 0.2 g AA/(kg·d) and 41 ± 5.0 kcal/(kg·d).

***Table 1*** *Clinical characteristics of the included infants.*
*Values are expressed as means ± SE or medians (range) when appropriate.*
*There are no differences between groups.*

| | CYS | $CYS^+$ |
|---|---|---|
| N (M:F) | 10 (6:4) | 10 (8:2) |
| birth weight (g) | 978 ± 274 | 1006 ± 120 |
| gestational age (wks) | 28 ± 1 $^5/_7$ | 27 $^3/_7$ ± 1 $^2/_7$ |
| birth weight Z-score (SD) | -1.1 ± 1.7 | -0.3 ± 1.5 |
| mode of delivery vaginal:cesarean section | 5:5 | 3:7 |
| Apgar score (5 min) | 9 (5) | 8 (4) |
| CRIB score | 3 (9) | 2 (4) |

None of the infants developed sepsis during the study. All infants were ventilated and therefore received prophylactic antibiotics according to our NICU's policy. Administration of these antibiotics was stopped in all infants after 48-72 hours since C-reactive protein concentrations were low and blood cultures were negative.

All infants received additional oxygen during or prior to the stable isotope infusion period. No differences between groups were observed (data not shown).

Figure 1 shows base requirements during the first 6 days of life. Infants in the $CYS^+$ group required more base administration on days 3 ($p=0.048$), 4 ($p=0.002$), and 5 ($p=0.002$).

Relevant plasma AA concentrations are shown in Table 2; they did not differ between groups. In particular concentrations of cysteine, measured as its dimer cystine, were not higher in the $CYS^+$ group, despite an almost doubled intake. Also concentration of taurine, a product of cysteine catabolism, was not significantly increased in the $CYS^+$ group.

***Table 2*** *Plasma amino acid concentrations.*
*Values are expressed as means ± SE. There are no significant differences between groups.*

| | CYS | $CYS^+$ |
|---|---|---|
| glutamate | 57 ± 28 | 61 ± 27 |
| cystine | 32 ± 10 | 33 ± 14 |
| glycine | 325 ± 120 | 281 ± 106 |
| glutamine | 570 ± 190 | 516 ± 176 |
| serine | 179 ± 54 | 166 ± 37 |
| methionine | 45 ± 24 | 33 ± 11 |
| taurine | 73 ± 55 | 85 ± 54 |

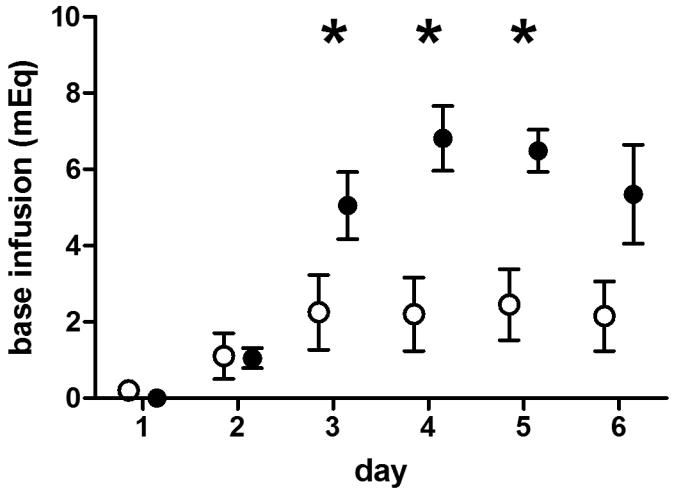


***Figure 1*** *Base infusion in preterm infants receiving a standard (o) or a high parenteral cysteine intake (•). Values are expressed as means ± SEM. * Statistically significant difference.*

**Glutathione concentration and synthesis rates**

All infants in both groups reached isotopic steady state after 4 hours of infusion (Figure 2). Mean precursor enrichments were 3.6 ± 0.6 in the CYS and 3.3 ± 0.6 in the $CYS^+$ group, with a coefficient of variation of 5.1 ± 2.0 % and 6.5 ± 2.5 %, respectively. $FSR_{GSH}$ was identical: 48 ± 11 %/d and 48 ± 8 %/d in the CYS and $CYS^+$ group, respectively (Figure 3a). GSH concentration was 1.83 ± 0.28 mmol/L in the CYS group and 2.02 ± 0.18 mmol/L in the $CYS^+$ group (p=0.10) (Figure 3b). Consequently, $ASR_{GSH}$ was 8.9 ± 2.2 mg/(kg·d) and 9.6 ± 2.1 mg/(kg·d) (p=0.49) (Figure 3c). We did not detect a significant gender specific difference in either plasma cystine concentrations or GSH kinetics.

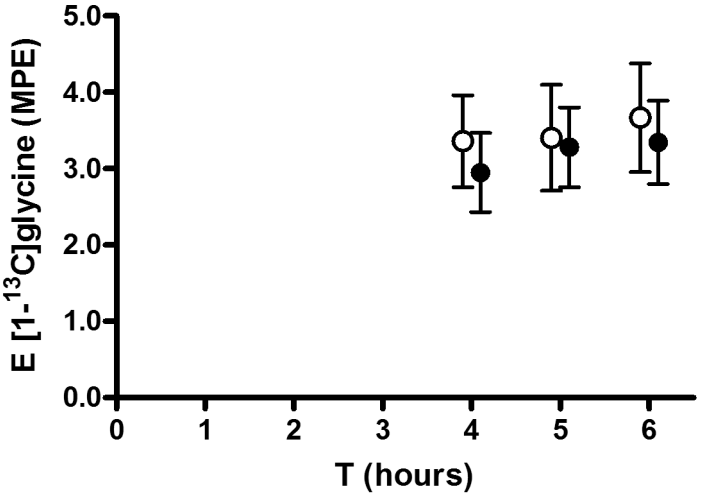


***Figure 2*** *Isotopic steady state was obtained in the standard dose (o) and the high dose group (•) after 4 hours of infusion, as represented by a non significant rise in intraerythrocytic [1-$^{13}$C]glycine enrichment. Values are expressed as means ± SE.*

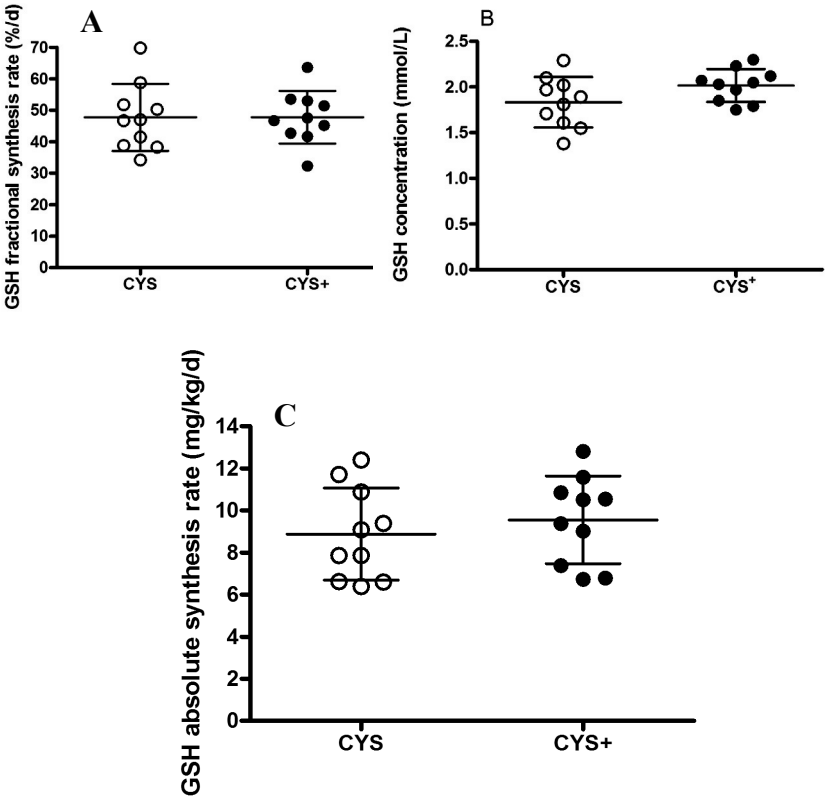


***Figure 3*** *GSH fractional synthesis rate (**A**), GSH concentration (**B**), and GSH absolute synthesis rate (**C**) in the standard dose (CYS) and high dose (CYS$^+$) group. Individual values are shown. The horizontal lines represent means ± SE. No differences were observed between the groups.*



## Discussion

Parenteral AA solutions contain only low amounts of cysteine or none at all. Few products offer separate cysteine solutions to be added to the AA mixture just prior to administration. In this randomized clinical trial, we demonstrate that it appears safe to administer cysteine at a dose of 81 mg/(kg·d), although infants in the high dose group did require more base administration on days 3, 4, and 5. Furthermore, there was no apparent clinical benefit. In a previous trial we showed that GSH synthesis was stimulated upon administration of 2.4 g/(kg·d) of protein including cysteine at a dose of 45 mg/(kg·d) (16). The higher dose of cysteine in the present trial nevertheless does not result in a higher GSH synthesis rate as compared to the lower dose, nor does it increase plasma cystine concentrations. There might be an

effect on GSH concentration (p=0.10), although our study was not powered to detect a difference in GSH concentration.

There are a number of explanations to explain the lack of effect of a high dose cysteine. First, the additional cysteine might be oxidized. However, plasma cysteine concentrations are in both groups still at the lower end of the reference range, so oxidation does not really seem an obvious metabolic pathway for the additional cysteine. Second, a higher cysteine dose might very well preserve GSH through inhibition of its breakdown, as one of the main functions of GSH is being a cysteine reservoir. Since GSH synthesis is feedback inhibited, GSH concentrations might be already adequate in the CYS group. In agreement, Cho et al. observed that feeding cystine above the supposed requirements did not increase GSH of any tissue in growing rats (17).

A third explanation for the lack of increase in GSH synthesis rate might be that cysteine is used for other purposes. In the present study, the additional cysteine does not seem to be broken down to form taurine, as taurine concentrations hardly rose upon a higher cysteine dose. Besides for GSH synthesis, cysteine is likely to be rate limiting for a number of proteins as well and, consequently, a higher cysteine availability might be preferentially incorporated into proteins such as albumin. Albumin has major antioxidant capacities apart from other functions and is rapidly upregulated upon AA administration in preterm infants directly following birth (18). In addition, cysteine is abundantly present in acute phase proteins (19). Using cysteine for other purposes than GSH would also explain why plasma cystine concentrations failed to rise in the $CYS^+$ group.

Compromised bioavailability and stability of cysteine in preterm infants might be a fourth explanation why GSH synthesis did not increase. For example, when simultaneously delivered with glucose in a TPN bag, cysteine is known to form adducts which have diminished bioavailability (20). Although glucose and AAs are infused in separate bags at our NICU, they are infused at the same intravenous site, which might affect bioavailability. To overcome stability issues, stable cysteine analogues such as N-acetylcysteine (NAC) have been administered to improve GSH availability or clinical outcome in preterm infants. Although NAC has been shown to be very effective as GSH enhancing substrate in rodents and humans (21-23), preterm infants have a relative inability to deacetylate NAC (24). So far there are no data on long term safety aspects of these acetylated products.

A limiting factor for GSH synthesis, other than cysteine, might be another explanation for the present findings. Since the enzymes necessary for GSH synthesis are present already during midgestation and are readily upregulated in case of increased GSH requirements, these are unlikely to be limiting GSH synthesis (25, 26). With respect to availability of other substrates: glycine has been found to be essential in preterm infants in a number of studies. Van Lingen et al., using [$^{15}$N]glycine, found that hardly

any added tracer was detectable in urinary urea, particularly in small for gestational age infants (27). Indeed, glycine might be limiting under particular circumstances. Still, plasma concentrations of both glycine and glutamate, which are used as cysteine in equimolar amounts to synthesize GSH, are much higher than cystine concentrations in the present as well as other studies (22, 28).

Our high dose group ($CYS^+$) received cysteine at a dose of 81 mg/(kg·d). Although this may be considered 'high', a few other studies have used higher amounts up to 92 mg/(kg·d) (6). Indeed there is a lack of data, making it hard to draw any firm conclusions on the upper limit of cysteine intake in preterm infants. However, we explicitly chose not to seek for the upper limits of cysteine intake.

We performed our measurements in erythrocytes. Erythrocyte transfusions are commonly performed in premature neonates beyond the first few days of postnatal life. Although this will likely affect cysteine availability or GSH synthesis, there are little data available in the literature. In blood samples obtained from preterm infants within three hours before and after erythrocyte transfusion it was found that it did not significantly affect oxidative stress as well as protein sulphydryl groups and total antioxidant capacity (29).

A limitation of this study is that we did not include a group of infants receiving no cysteine at all. Based on the ESPEN/ESPGHAN guidelines on pediatric parenteral nutrition (30), we chose to use an AA solution at our NICU which includes cysteine several years ago. At the onset of the study we therefore decided not to incorporate a study group receiving no cysteine at all.

Another limitation of this study is that all infants were in a relatively healthy state during the study period. None of the infants suffered from sepsis. However, from both animal and human data it is known that sepsis augments oxidative stress which increases GSH demands (31, 32). Further research should be directed towards defining whether exogenous cysteine administration is clinically relevant in the parenterally fed preterm neonate in the direct postnatal phase, particularly in disease states such as sepsis. Ideally, this would include a control group not receiving cysteine at all.



**Conclusions**

Cysteine administered at a dose of 81 mg/(kg·d) appears to be safe, but does not increase GSH synthesis rates nor concentrations as compared to a dose of 45 mg/(kg·d). It has yet to be proven that there is a significant clinical benefit associated with cysteine supplementation.

**Acknowledgements**

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CHAPTER

# 9

# Human fetal albumin synthesis rates during different periods of gestation

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**Abstract**

Background: Despite nutritional intervention, albumin concentrations are often low in critically ill premature neonates.
Objective: Our aim was to quantify albumin synthesis rates during early life under physiologic circumstances. Human fetuses thereby reflect the developmentally related optimal condition.
Design: Pregnant women undergoing elective cesarean section received three different labeled amino acid infusions starting at different times prior to surgery. Using mass spectrometry techniques, this novel model enabled us to quantify fetal albumin synthesis from a single blood sample taken from the umbilical cord after cesarean delivery. The fractional synthesis rate reflects the fraction of the albumin pool that is daily renewed. The absolute synthesis rate is the absolute amount of albumin that is daily synthesized. Results are expressed as median ($25^{th}$-$75^{th}$ percentile).
Results: We studied 8 fetuses at 29.9 (28.4-35.4) weeks gestation and 8 fetuses around term. Fractional synthesis rates in premature fetuses (17.5 (12.1-24.4) %/d) were higher (p=0.02) than in mature fetuses (10.4 (9.1-13.7) %/d). Absolute synthesis rates were also higher (p=0.02) in premature than in mature fetuses: 280 (227-365) versus 205 (184-238) mg/(kg·d).
Conclusions: On a weight basis, albumin synthesis rates in premature fetuses were higher than in fetuses at term and higher the rates previously found in neonates after preterm birth. Considering that the premature fetal liver has the capacity to synthesize albumin at a high rate, the observed hypoalbuminemia in premature infants therefore would seem to suggest that current (nutritional) therapies fail to meet requirements necessary to sustain an optimum in albumin synthesis rates.

## Introduction

Albumin concentrations are considered a marker of nutritional status; albumin synthesis rates a measure of liver activity. Albumin is the major export protein produced by the liver and forms more than half of the total plasma protein mass. Albumin has been described as "the body's tramp steamer, shuttling cargo of various kinds between ports of call"[1]. Its load includes bilirubin, cysteine, free fatty acids, calcium, and drugs. Besides, albumin preserves the colloid osmotic pressure and is an important antioxidant.

Recently, we determined albumin synthesis rates in premature infants immediately after birth and who received only glucose[2]. These rates almost doubled in response to additional intravenous amino acid administration[3]. Despite this increase, plasma albumin concentrations were still very low. However, having knowledge of albumin synthesis rates during early life under physiologic circumstances, i.e. pregnancy, would enable us to relate the intrauterine with the extrauterine synthesis rates. To this aim, we employed a stable isotope model allowing measurements on the human fetal albumin synthesis rates.

It has been long known that animal[4,5] and human[6] fetuses are capable of endogenous albumin synthesis from early pregnancy on. Besides, all albumin in the fetus is from fetal origin since albumin does not cross the hemochorial placenta as demonstrated in the rat[7], guinea pig[8], and the in vitro dually perfused human placenta[9]. But also after intravenous injection of radioiodinated albumin to pregnant women, only trace amounts were found in umbilical cord blood[10,11]. Furthermore, fetal plasma albumin concentrations at term are often higher than in maternal plasma[12,13], which suggests no passive materno-fetal transport. In addition, normal concentrations of fetal plasma albumin were found during mild or severe maternal hypoalbuminemia[13,14].

The only available kinetic information on albumin synthesis, however, is in the ovine fetus, where the albumin fractional synthesis rates (FSRs) were determined[15,16]. The albumin FSR reflects the fraction of the intravascular albumin pool that is renewed per unit of time. The FSR is usually calculated by infusing one stably labeled amino acid and obtaining multiple blood samples at consecutive time points. From the increase of tracer incorporation in albumin over time, one can calculate its synthesis rate. In humans, however, the insertion of catheters in the fetus or umbilical cord for repetitive blood sampling is impossible on ethical grounds. Obtaining blood from both the umbilical vein and artery is only possible at birth. We therefore modified the staggered infusion protocol proposed by Dudley and colleagues[17] into a simplified model enabling us to measure the synthesis rate of albumin from a single blood sample taken at birth.

**Subjects and Methods**:

**Setting and subjects**

The study was performed at the Mother and Child Center of the Erasmus MC – Sophia Children's Hospital after approval by the Dutch (CCMO, The Hague) and the institutional medical ethical review board. Pregnant women scheduled to undergo elective cesarean section (repeat, breech, or multiple pregnancy) were eligible. We aimed to include fetuses who were close to term as well as fetuses who were still premature. Exclusion criteria were obesity (preconceptional body mass index > 30 kg/$m^2$), diabetes, or known fetal anomalies. Participants gave written consent after having been fully informed about the study.

**Experimental design**

L-[1-$^{13}$C,$^{15}$N]leucine, L-[1-$^{13}$C]phenylalanine, and L-[U-$^{13}C_5$]valine were bought from Buchem BV, Apeldoorn, The Netherlands (local distributor of Cambridge Isotope Laboratories, Andover, MA, USA) (all 99% enriched and tested for sterility and pyrogenicity). Our hospital pharmacy dissolved the isotopes in 0.9% saline after which the solution was filtered (0.2 μm) and sterilized. Tests were performed to reassure the correct identity, concentration, and a sterile and pyrogen free product.

Pregnant women received primed continuous stable isotope infusions of L-[1-$^{13}$C,$^{15}$N]leucine (8 μmol/(kg·h)), L-[1-$^{13}$C]phenylalanine (5 μmol/(kg·h)), and L-[U-$^{13}C_5$]valine (5 μmol/(kg·h)), starting at least 4, 3, and 2 hours prior to planned surgery, respectively. The priming doses were half of the hourly doses. Tracers were given in a forearm vein with three separate Perfusor® fm infusion pumps (B|Braun Medical B.V., Oss, the Netherlands) until surgery was completed.

Maternal blood was sampled before the tracer infusions had begun (baseline) and from a contralateral maternal forearm vein immediately before anesthesia started. Fetal blood was sampled from both the vein and arteries of a doubly clamped segment of the umbilical cord immediately after delivery. After collection, blood samples were centrifuged (2000×g) in heparin tubes and plasma was frozen at -80°C until analysis.

**Blood sample analyses**

To isolate albumin from plasma, we used anti-human serum albumin affinity resin kits (Vivascience – Sartorius Group, Hannover, Germany). Fetal and adult albumin are indistinguishable[18]. Enclosed spin columns were filled with 400 μL affinity resin and 25 μL of thawed plasma. According to the included protocol, the column was washed three times with a tris-buffer and albumin was thereafter eluted from the affinity resin with 0.1 mol glycine/L (acidified to pH 2.5 with HCl). Eluted albumin was precipitated with 750 μL of 2 mol $HClO_4$/L. A washing step was performed with 0.2 mol $HClO_4$/L by

resuspending and precipitating the pellet again. The protein pellet was then hydrolyzed in 140 µL of 6 mol HCl/L for 22 hours at 110°C. Following hydrolyzation, the acid was evaporated using a speedvac, after which the dried amino acids were dissolved in $H_2O$. Samples were derivatized using propylchloroformate (commercial kits: Phenomenex for hydrolysates, EZ:Faast, Bester BV, Amstelveen, The Netherlands) and measured in triplicate on a gas chromatograph – combustion – isotope ratio mass spectrometer (Delta XP, Thermo Electron, Bremen, Germany)[2].

The enrichments of the true albumin precursors (intrahepatic amino-acyl tRNA) can obviously not be measured in the human fetus or mother. Because keto acids are intracellularly derived metabolites of amino acids, their enrichment has been advocated as a surrogate precursor[19,20]. However, keto acids are also transported transplacentally and it is thus not possible to discriminate whether the keto acids have undergone intracellular metabolism in the maternal, placental, or fetal compartment. Therefore, we chose to use plasma amino acid enrichments as the albumin precursors. As keto acid enrichment can only be lower than amino acid enrichment, the use of the latter results in a slight underestimation of synthesis rates.

Amino acids were extracted from plasma and derivatized using the same Phenomenex kits which were also used for product (albumin) sample preparation. Enrichments of plasma leucine, phenylalanine, and valine were measured in triplicate on a gas chromatograph – combustion – isotope ratio mass spectrometer as well.

Plasma albumin concentrations in maternal and umbilical plasma were measured on a Roche Hitachi 917 (Roche Diagnostics, Basel, Switzerland). Hematocrit was measured on an Advia 120 (Bayer Diagnostics, Leverkussen, Germany).

**Calculations**

Baseline enrichment in the fetus could not be measured but was considered to be identical to that in the pregnant woman since the fetus consists of what the mother eats.

The fetal liver is perfused with blood directly from the umbilical vein (70%) and with blood which first passes the ductus venosus and then reenters the liver through the portal vein (20%) and hepatic arteries (10%)[21,22]. Blood from the portal vein and hepatic arteries has theoretically the same composition as in the umbilical arteries. Thus, the fetal liver is perfused with blood from both umbilical cord vessels. However, plasma amino acid enrichment in the umbilical arteries is slightly lower than that in the umbilical vein due to isotopic dilution by unlabeled amino acids released from fetal protein breakdown. Therefore, we calculated the precursor enrichment as the mean of umbilical venous and arterial plasma enrichment.

The enrichment of amino acids incorporated in fetal albumin was very similar in blood from the umbilical vein and arteries, which indicates no

materno-fetal albumin transport. Nevertheless, we averaged the values. In each subject, the separate leucine, phenylalanine, and valine product/precursor enrichment ratios were plotted in a graph against the moment the corresponding infusion was started (Figure 1). Using computer software, the slope and the correlation coefficient of the linear trend line were calculated. The FSR was then derived using the following equation:

$$\text{FSR (\%/d)} = \text{slope of trend line} \times -1 \times 24\text{h} \times 100\%$$

The absolute synthesis rate (ASR) represents the absolute amount of albumin that is produced per unit of time and can be calculated with the following equation:

$$\text{ASR (mg/(kg·d))} = \text{FSR} \times C_{alb} \times vol_{pl} \times \text{weight}^{-1}$$

where $C_{alb}$ is the plasma albumin concentration in g/L, $vol_{pl}$ is the plasma volume in mL, and weight is the maternal actual weight or infant's birth weight in kg. Maternal plasma volume was estimated from data by Whittaker et al.[23] according to the following equation:

$$\text{plasma volume (mL)} = 36.1 \times \text{height (cm)} + 11.0 \times \text{weight (kg)} - 3029$$

Fetal plasma volume (including placental and umbilical blood) was calculated by multiplying (1-hematocrit) with an estimated 105 mL blood/kg fetal body weight[24].

In our model, the use of one single amino acid with three different isotopomers (e.g. [1-$^{13}$C]leucine, [$D_7$]leucine, and [$^{18}$O]leucine) could theoretically be preferred over infusing three different labeled amino acids as in our study. However, since the enrichment of incorporated amino acids in albumin is very low (ranging from 0.01 mole percent enrichment (MPE) for valine to 0.17 MPE for leucine in our study), enrichments can only be analyzed accurately by using GC-C-IRMS. Measuring hydrogen and oxygen on a GC-C-IRMS is technically very challenging. Besides, leucine with an oxygen label was at time of the study prohibitively expensive. Unfortunately, [$^{15}$N]leucine could not be used because of label loss due to transamination. Owing to these technical difficulties and financial constraints, the single amino acid strategy could not be utilized. We thus have chosen to use carbon labels only, which necessitates using different amino acids when measuring with a GC-C-IRMS. In addition, because of anticipated low enrichment in the last infused amino acid, valine was uniformly labeled to increase measurement accuracy.

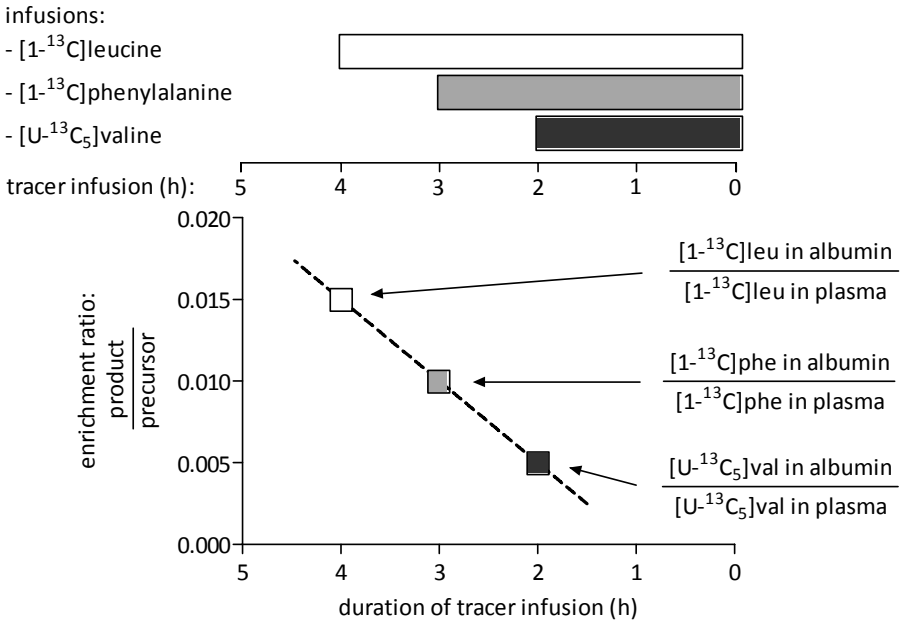


***Figure 1*** *Study design. Pregnant women received three different stable isotopically labeled amino acid infusions starting at different times prior to cesarean section. In maternal and umbilical cord blood, sampled at t=0, we measured the product/precursor enrichment ratio of each of the three infused amino acids. These ratios were plotted in a graph against the moment the corresponding isotope infusion was started. As labeled leucine had the longest infusion time, its incorporation into albumin will be highest. The slope of the trend line determines the albumin FSR.*

**Statistics**

Calculations were made with Microsoft Office - Excel software (version 2007; Microsoft Corp, Redmond, WA, USA) and statistical tests were done in GraphPad Prism software (version 4.0; San Diego, CA, USA). Because of our small groups, normality distribution of our data could not be determined or assumed. Therefore non-parametric data analysis was performed. Consequently, values are expressed as median ($25^{th}$ – $75^{th}$ percentile) and Mann-Whitney tests were used to detect statistical differences. Significance level was set at $p<0.05$.

**Results**

We included eleven pregnant women, of whom eight delivered at term, one at 31 weeks gestation, one delivered a triplet at 35 weeks (two identical, one non-identical), and one delivered a quadruplet at 28 weeks (all non-identical). We thus studied 16 fetuses, classified into two groups: premature

(<37 weeks gestation) and mature. Maternal age, preconceptional and current body mass index, and parity are shown in Table 1. Descriptive characteristics of fetuses/neonates, which include birth weight, gestational age, birth weight Z-score[25], sex, umbilical pulsatility index, and Apgar score are shown in Table 2.

***Table 1*** *Maternal characteristics (n=11).*

| Characteristic | Value |
|---|---|
| Age (y) | 35.0 (27.5 – 36.5) [1] |
| Preconceptional BMI (kg/m$^2$) | 22.8 (20.3 – 24.6) |
| Actual BMI (kg/m$^2$) | 29.4 (25.2 – 31.6) |
| Parity (0:1:2:3) (n) | (6:1:2:2) |

[1] Median (25$^{th}$ – 75$^{th}$ percentile) (all such values).

***Table 2*** *Characteristics of the premature (<37 weeks gestation) and mature group of fetuses.*

| | Premature (n=8) | Mature (n=8) |
|---|---|---|
| Gestational age (wks) | 29.9 (28.4 – 35.4) [1] | 38.5 (37.6 – 38.9) |
| Birth weight (kg) | 1.3 (1.2 – 1.9) | 3.3 (2.7 – 3.4) |
| Birth weight Z-score (SD) [2] | -0.19 (-0.70 – 0.22) | -0.11 (-0.86 – 0.52) |
| Sex (M:F) | 3:5 | 4:4 |
| P.I. [3] | 1.28 (1.18 – 1.36) | 0.89 (0.78 – 0.96) |
| Apgar score at 5 min [4] | 9 (9 – 10) | 10 (10 – 10) |

[1] Median (25$^{th}$ – 75$^{th}$ percentile) (all such values).

[2] Birth weight corrected for gestational age (reference 28).

[3] The umbilical pulsatility index (P.I.) is a Doppler ultrasound derived index on the blood stream velocity profile through the umbilical arteries and is a marker of fetal well-being. A normal P.I. decreases slightly over gestation.

[4] The Apgar score is a postnatal scoring scale ranging from 0-10.



Table 3 and Table 4 show the enrichments of the three infused labeled amino acids both incorporated in albumin and free in plasma, respectively. Figure 2 displays the trend lines through the leucine, phenylalanine, and valine product/precursor enrichment ratios in each studied subject. The median linear regression coefficients ($r^2$) of these trend lines were 0.995 (0.985 – 0.999) in pregnant women, 0.988 (0.981 – 0.993) in premature fetuses, and 0.996 (0.985 – 0.998) in mature fetuses. In Figure 3, the maternal, premature fetal, and mature fetal albumin FSRs are outlined. They were all significantly different from each other; pregnant women had the lowest FSRs, premature fetuses the highest.

Maternal albumin concentrations were 32.0 (29.5 – 34.5) g/L. Concentrations in premature fetuses were 28.8 (27.3 – 30.8) g/L and in mature fetuses 33.5 (32.6 – 34.6) g/L, which is significantly different (p=0.003). Hematocrit in umbilical cord blood (mean of venous and arterial blood) was 0.43 (0.40 – 0.50) in the premature fetuses and 0.46 (0.46 – 0.48) in the mature group. The albumin ASRs are shown in Figure 4. Similar to the fractional values, premature fetuses had the highest ASRs, followed by the mature fetuses and the pregnant women.

***Table 3*** *Enrichments of the infused amino acids incorporated into albumin (product enrichments) in the maternal and fetal (mean of arterial and venous umbilical cord plasma) compartment.* [1]

| | **Pregnant women (n=11)** | **Premature fetuses (n=8)** | **Mature fetuses (n=8)** |
|---|---|---|---|
| [1-$^{13}$C]leucine | 0.075 (0.061 – 0.083) | 0.105 (0.097 – 0.119) | 0.096 (0.089 – 0.112) |
| [1-$^{13}$C] phenylalanine | 0.063 (0.054 – 0.078) | 0.103 (0.089 – 0.116) | 0.095 (0.087 – 0.108) |
| [U-$^{13}C_5$]valine | 0.015 (0.013 – 0.022) | 0.019 (0.017 – 0.024) | 0.025 (0.023 – 0.030) |

[1] Enrichment is expressed in mole percent excess (MPE). All values are median (25$^{th}$ – 75$^{th}$ percentile).

***Table 4*** *Enrichments of the infused amino acids in plasma (precursor enrichments) in the maternal and fetal (mean of arterial and venous umbilical cord plasma) compartment.*[1]

| | **Pregnant women (n=11)** | **Premature fetuses (n=8)** | **Mature fetuses (n=8)** |
|---|---|---|---|
| [1-$^{13}$C]leucine | 8.89 (8.27 – 9.27) | 5.40 (4.84 – 6.12) | 6.55 (5.98 – 7.24) |
| [1-$^{13}$C] phenylalanine | 12.7 (11.1 – 13.0) | 8.55 (8.27 – 8.96) | 10.1 (9.19 – 11.4) |
| [U-$^{13}C_5$]valine | 6.73 (6.02 – 7.06) | 3.75 (3.24 – 4.34) | 4.97 (4.56 – 5.20) |

[1] Enrichment is expressed in mole percent excess (MPE). All values are median (25$^{th}$ – 75$^{th}$ percentile).

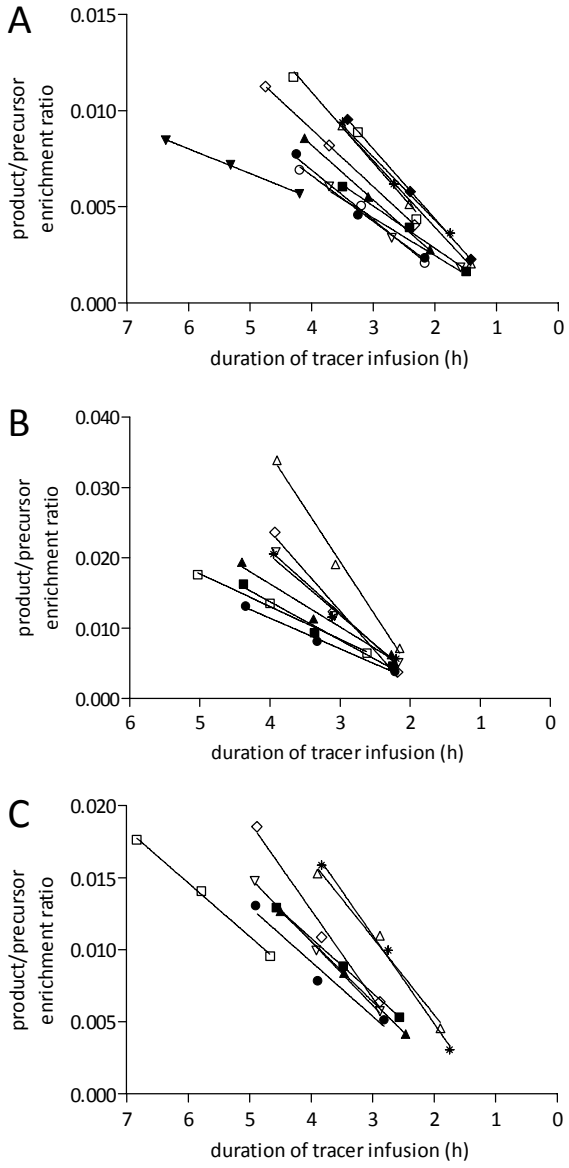


***Figure 2*** *Individual trend lines through the three product/precursor trend lines in (A) pregnant women (n=11), (B) premature fetuses (n=8), and (C) mature fetuses (n=8). In each case, leucine had the longest infusion time, followed by phenylalanine and valine, respectively.*

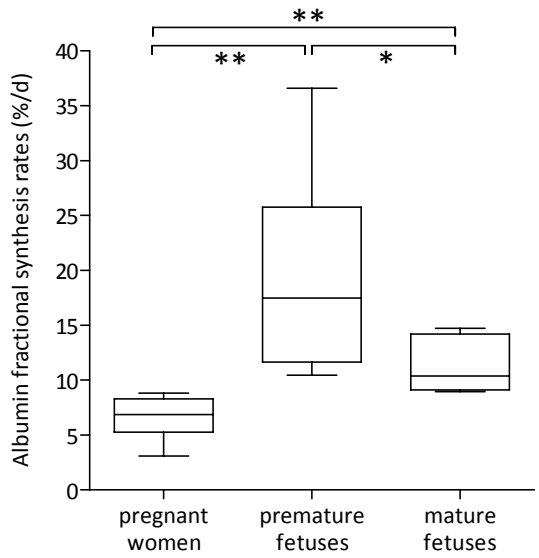


***Figure 3*** *Albumin fractional synthesis rates in pregnant women (n=11), premature fetuses (<37 weeks gestation, n=8), and mature fetuses (n=8). Boxes and whiskers indicate the medians, and interquartile and outer ranges. * Significantly different (Mann-Whitney), p<0.05; ** Significantly different (Mann-Whitney), p<0.001.*

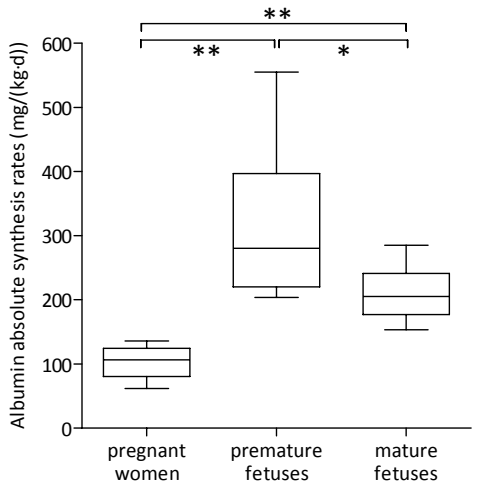


***Figure 4*** *Albumin absolute synthesis rates in pregnant women (n=11), premature fetuses (<37 weeks gestation, n=8), and mature fetuses (n=8). Boxes and whiskers indicate the medians, and interquartile and outer ranges. * Significantly different (Mann-Whitney), p<0.05; ** Significantly different (Mann-Whitney), p<0.001.*

## Discussion

This is the first study addressing albumin synthesis rates in human fetuses. These values are of great importance since they give guidance as what to strive for in aiming optimal nutrition for premature infants. The fetal measurements were possible due to a relatively novel multiple stable isotope infusion method. This enabled us to measure a protein's synthesis rate from a single blood sample. Given the high correlation coefficients, our method proves to be valid.

In this study, we compared albumin synthesis between pregnant women, fetuses at term, and fetuses that were still premature. In mothers of the latter group, however, elective cesarean sections are rarely performed as these are usually in the acute setting because of sudden (worsening of) fetal or maternal distress. Thus, there is usually no time for obtaining informed consent followed by a four-hours-lasting infusion protocol for research purposes. Still, we were able to include three women who underwent a planned cesarean section before term, and whose infants were assumed to be in relatively good condition. One woman had to give early birth because of maternal cervical carcinoma, the two other women because of anticipated complications due to triplet and quadruplet pregnancy. Whether the results in the premature group of fetuses were influenced the effects of multiple pregnancy itself or by genetic relationships remain unknown. However, a common genetic background does not imply having equal fetal metabolic nutrient availability. In normal pregnancy, the latter depends more on placental activity in each individual than on maternal nutrient availability. Thus, amongst multiplets, it is likely that the intrauterine metabolic environments are different, which was also reflected by different synthesis rates between siblings.

As the maternal blood sample used for calculation of the albumin FSR was taken before spinal anesthesia was initiated and surgery had started, the latter two procedures could not have influenced our results. It is unknown, however, to what extent maternal surgery influences fetal metabolism. Yet, surgery until the infant was born only lasted some ten minutes, which is only a short period relative to the total infusion time. Thus, potential effects of maternal surgery would only minimally influence fetal synthesis rates.

The maternal plasma albumin concentrations in this study are low as compared with those in non-pregnant individuals, but a 10 g/L drop in concentration starting early in pregnancy is common[23]. However, rather than simple dilution because of a pregnancy-associated plasma volume expansion, actual alterations in albumin metabolism during pregnancy have been observed. During late gestation, albumin FSRs and ASRs as well as the total intravascular albumin pool were found to be higher than those in non-pregnant women[23,26]. Our measured maternal synthesis rates were very similar to the rates in those studies. Increased synthesis could be necessary

to compensate for the albumin loss caused by placental uptake and subsequent degradation, thereby releasing free amino acids available for transport to the fetus[27,28].

Two of the mature fetuses had birth weights that were only on the 5$^{th}$ percentile. These two small for gestational age infants had the lowest two albumin FSRs and ASRs. When nutrient availability is compromised, ultimately leading to reduced growth, oxygen and nutrient rich blood entering the fetus through the umbilical vein is shunted away from the liver through the ductus venosus towards the upper body half[29]. Bypassing the fetal liver ensures a more or less constant supply of essential substrates to the myocardium and brain. Underperfusion of the fetal liver, however, results in diminished liver growth. Small for gestational age infants are known to have smaller liver volumes, also when corrected for total body weight[30,31]. Interestingly, these two fetuses did not upregulate albumin synthesis rates so as to compensate their supposedly smaller liver size. In fact, the opposite was true as the albumin synthesis rates were the lowest. This could have important implications as impaired liver functioning might have lifelong effects on metabolism. Summarized as the 'fetal origins of adult disease' or 'Barker hypothesis', compromised growth during early life of organs such as the liver, pancreas, spleen, kidneys and adrenal glands, predisposes an individual to cardiovascular disease, stroke, and type two diabetes[32,33].

Considering the functions of albumin, which include acting as an antioxidant and transporting bilirubin and free fatty acids, one may wonder why normally grown fetuses, especially earlier in gestation, have such high synthesis rates. During intrauterine life, oxygen tension in blood is low, thereby generating only low amounts of radicals, which could damage albumin. The low oxygen tension is compensated for by the increased oxygen affinity of fetal hemoglobin. After birth, fetal hemoglobin is rapidly broken down, thereby releasing large amounts of bilirubin that should be transported off by albumin. Also, during the beginning of the third trimester, fatty acid concentrations are low and will be of no burden to albumin. The surge in albumin synthesis would therefore be expected just prior to term birth, as a preparation against an elevated radical exposure and for a higher transport load consisting of hemoglobin breakdown products and fatty acids, the latter found in high amounts in postnatal nutrition (breast milk). In addition, all mothers of the prematurely born infants had received corticosteroids in the two days prior to their planned cesarean section. Antenatally given steroids accelerate fetal lung maturation in preparation for postnatal life. These stress hormones, however, can also elicit a catabolic response in the fetus. Albumin synthesis might therefore even have been downregulated in the premature group at time of the measurements.

The reason for a decreasing albumin synthesis rate during gestation could either be functional or depend on the general metabolic rate. During ovine pregnancy, fetal whole body protein synthesis rates decrease

significantly throughout gestation[34]. Oxygen consumption by the ovine fetal liver has also been shown to decrease[35]. In human preterm infants, whole body protein metabolic rates are also higher when compared to infants born at term[36]. However, human fetal liver volume as percent of body weight does not decrease as much throughout gestation as it does in fetal sheep[37].

The albumin ASR in intravenously fed premature babies (27 wks gestation) was 228 (187 – 289) mg/(kg·d)[3]. The ASRs of premature fetuses measured in the current study are higher than the postnatal values from premature infants. Having low albumin concentrations and ASRs after birth is an unfortunate situation considering that sick premature infants experience more oxidative stress after high oxygen pressure ventilation and have to deal with increased bilirubin and drug transport. In our previous study, we showed that albumin synthesis in premature neonates is responsive to parenteral nutrition[3]. Yet, the current recommended nutrient intakes for premature infants still not appear to be sufficient to increase the albumin synthesis rates to levels observed in fetuses. This can be speculated since premature infants should however, theoretically, be able to synthesize albumin in larger quantities as they also did while still in utero. Although the traditional method of measuring an FSR used in premature infants is different from our employed infusion model, the two should theoretically give comparable results.

In conclusion, we showed that mature fetuses produce twice as much albumin as their mothers per kg bodyweight and premature fetuses three times as much. Premature fetuses have higher albumin synthesis rates than parenterally fed premature neonates indicating that postnatal synthesis capacity is reduced or that recommended nutrient intake is not sufficient. Our employed method is not only applicable in fetal research, but could be of benefit in all situations where multiple sampling is impossible or inconvenient to a subject. In organ protein metabolism studies (for example liver, bowel, or muscle protein synthesis) the required number of tissue biopsies can be reduced to one, instead of two or three with many currently used models[38,39]. In addition, our single sample method shortens sample preparation and analysis time and reduces risk on measurement artifacts.



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The authors' responsibilities were as follows: CvdA, JD, ES, and JvG participated in the design and implementation of the study, including recruitment of patients; AV prepared and tested all intravenous stable

isotope solutions; CvdA collected and prepared blood samples for analysis; HS and TR provided technical supervision of blood sample preparation; HS performed mass spectrometry analyses; CvdA, HS, and JvG analyzed the data; CvdA wrote the manuscript draft; and all authors reviewed the manuscript and approved the final version. None of the authors had a personal or financial conflict of interest.

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CHAPTER

# 10

# Human fetal amino acid metabolism at term gestation: phenylalanine and tyrosine kinetics

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**Abstract**

Background: Knowledge on human fetal amino acid (AA) metabolism, largely lacking so far, is pivotal in improving nutritional strategies for prematurely born infants. Phenylalanine kinetics are of special interest since there is debate as to whether neonates will adequately hydroxylate phenylalanine to the semi-essential AA tyrosine.
Objective: Our aim was to quantify human fetal phenylalanine and tyrosine metabolism.
Design: Eight fasted, healthy pregnant women undergoing elective cesarean section at term received primed continuous stable isotope infusions of [1-$^{13}$C]phenylalanine and [*ring*-$D_4$]tyrosine starting prior to surgery. Umbilical blood flow was measured by ultrasound. Maternal and umbilical cord blood was collected and analyzed by gas chromatography mass spectrometry for phenylalanine and tyrosine enrichments and concentrations. Data are expressed as median ($25^{th}$ – $75^{th}$ percentile).
Results: Women were in catabolic state for which net fetal AA uptake was responsible for at least one quarter. Maternal and fetal hydroxylation rates were 2.6 (2.2 – 2.9) and 7.5 (6.2 – 15.5) μmol phenylalanine/(kg·h), respectively. Fetal protein synthesis rates were higher than breakdown rates: 92 (84 – 116) vs. 73 (68 – 87) μmol phenylalanine/(kg·h), respectively, indicating an anabolic state. The median metabolized fraction of available phenylalanine and tyrosine in the fetus was less than 20% for both AA.
Conclusions: Around term gestation, fetuses still show considerable net AA uptake and AA accretion (converted to tissue ~12 g/(kg·d)). The low metabolic uptake (AA usage) implies a very large nutritional reserve capacity of nutrients delivered through the umbilical cord. Fetuses at term are quite capable of hydroxylating phenylalanine to tyrosine.

## Introduction

These days, many premature infants survive – yet sometimes at the cost of impaired outcome[1,2]. Inappropriate nutrition is at least partially responsible for suboptimal outcome, as it negatively affects neonatal growth and brain development[3,4]. Since several decades, the international pediatric nutritional goals are to feed premature infants so that they grow at the same rate they would have had while in utero and thereby mimicking the fetal tissue composition or quality. Many infants do not reach these targets, however, as growth lags behind. Moreover, the body composition of preterm-born infants is often more adipose at term corrected age[5]. Seeing that hardly anything is known about human fetal metabolism itself, it is not surprising that fetal accretion rates are often not met. It would seem, therefore, that better mimicking of fetal growth could be achieved by putting more effort in unraveling human fetal metabolism. The obtained knowledge could then lead to improved nutritional strategies.

Current knowledge on fetal metabolism is mostly derived from animal data. Technical difficulties and ethical issues are of course causal to the lack of knowledge on human fetal metabolism. However, the use of stable isotopes to study protein metabolism during human pregnancy provides a safe research tool.

The quantification of fetal phenylalanine and tyrosine kinetics is of particular importance. It does not only give information on fetal protein breakdown and synthesis rates in general, but also quantifies the metabolic conversion (hydroxylation) rate of the essential amino acid phenylalanine to tyrosine. Hydroxylation occurs in the liver and kidneys[6]. It is important for two reasons: it disposes of excess phenylalanine, and provides an alternative source of tyrosine if tyrosine is absent in the diet, for example due to poor tyrosine solubility in parenteral nutrition. Parenterally fed neonates thus depend on hydroxylation for their tyrosine requirements necessary for net protein accretion. Yet, the enzymatic activity of phenylalanine hydroxylase might be suboptimal in neonates and even older infants, making tyrosine a conditionally essential amino acid[7]. Tyrosine that is not incorporated into proteins can be degraded and oxidized through the formation of fumarate and acetoacetate. The amount of tyrosine used as a precursor of the catecholamines dopamine, norepinephrine, and epinephrine, is quantitatively negligible.

In this study, our aim was to investigate several aspects of fetal phenylalanine and tyrosine kinetics by analyzing umbilical cord blood after having infused pregnant women with stable isotopically labeled amino acids prior to elective cesarean section at term.

## Subjects and Methods

### Setting and subjects

The study was performed at the Mother and Child Center of the Erasmus MC – Sophia Children's Hospital after approval by both the institutional medical ethical review board and the Dutch central committee on research involving human subjects (CCMO, The Hague). Pregnant women undergoing elective cesarean section (repeat or breech pregnancy) under spinal anesthesia at term were eligible. Exclusion criteria were maternal obesity (preconceptional BMI >30), preeclampsia, diabetes, severe fetal growth restriction (> -2 SD), or known fetal anomalies. Participating women gave written consent after having been fully informed about all study details.

### Experimental design

To determine the blood flow necessary for our calculations (see below), blood flow velocity and vessel diameters were measured in the umbilical vein with an ultrasound machine (iU22, Philips Medical Systems, Eindhoven, the Netherlands) as previously described[8]. Ultrasound measurements were made in the late afternoon on the day preceding the cesarean section; sections were all performed at approximately 8.00 a.m. after an overnight fast.

At least 3 hours to planned surgery, the women received a priming dose of L-[1-$^{13}$C]tyrosine (0.5 μmol/kg) directly followed by a primed continuous infusion of L-[1-$^{13}$C]phenylalanine (2.5 μmol/kg; 5 μmol/(kg·h)) through a forearm vein. One hour later a primed continuous infusion of L-[*ring-2,3,5,6*-$D_4$]tyrosine (1.5 μmol/kg; 3 μmol/(kg·h)) was started along. Isotopes (all >99% enriched and tested for sterility and pyrogenicity) were bought from Buchem BV, Apeldoorn, the Netherlands (local distributor of Cambridge Isotope Laboratories, Andover, MA, USA). Our hospital pharmacy dissolved the isotopes in 0.9% saline and the solutions were filtered (0.2 μm) and sterilized. Tests were performed to ensure the correct identity, concentration, and a sterile and pyrogen free product. Tracers were given using Perfusor® fm (B|Braun Medical B.V., Oss, the Netherlands) and Graseby 3000 (Graseby Medical Ltd, Watford, UK) infusion pumps for the phenylalanine and tyrosine tracers, respectively. Maternal blood was sampled before the tracer infusions started (baseline), then immediately before anesthesia and, if possible (n=4), also about 20 minutes later just before surgery started. Fetal blood was sampled after birth from both the vein and arteries of a doubly clamped segment of the umbilical cord. The fact that there are two arteries in the umbilical cord does not affect our results as the concentrations of the amino acids and their enrichments in the blood of both arteries should be equal. After collection in heparin tubes, blood was centrifuged and plasma was frozen at -80°C until analysis.

**Blood sample analysis**

As calculations in a veno-arterial balance model (as on the umbilical cord in the fetus, see below) largely depend on the small differences in concentration and enrichment between the vein and arteries, rather than on the absolute values, measurements must be extremely precise. To minimize the effects of potential analytical measurement errors, samples were prepared for analysis twice using two different derivatization methods (PCF and MTBSTFA, see below). Each derivatized sample was analyzed in triplicate on two different gas chromatography mass spectrometers (GCMS) (see below). Enrichments were calculated from the mean of all twelve analyses; concentrations could be calculated from the mean of the six analyses using the MTBSTFA derivative only.

PCF (propylchloroformate) derivatization on samples was performed using commercial kits (EZ:Faast for hydrolysates, Phenomenex, Bester BV, Amstelveen, the Netherlands) according to the enclosed protocol. As internal standards for concentration determinations, [$D_8$]phenylalanine and [U-$^{13}C_9$,$^{15}N$]tyrosine were added to the samples to be derivatized with MTBSTFA. Concentration calibration curves were prepared using MTBSTFA as well. Two different enrichment calibration curves were made with either PCF or MTBSTFA derivates. Samples and calibration curves were analyzed with a MSD 5975C Agilent GCMS (Agilent Technologies, Amstelveen, the Netherlands) on a VF-17ms, 30m x 0.25mm ID capillary column (Varian Inc., Middelburg, the Netherlands) and a Thermo DSQ GCMS (Thermo Fisher, Breda, the Netherlands) on a VF-1701ms, 30m x 0.25mm ID capillary column (Varian Inc., Middelburg, the Netherlands).

**Calculations**

For the calculation of maternal whole body phenylalanine and tyrosine kinetics, including hydroxylation rates, we used the Clarke and Bier model[9], in combination with the adjustments proposed by Thompson et al.[10]. To control for pregnancy, we added an extra parameter to the rate of disappearance. In our model, amino acids disappear not only through hydroxylation (or oxidation) or incorporation into protein synthesis, but also through net transport to the fetus. The latter is calculated as the umbilical veno-arterial concentration difference multiplied with the umbilical blood flow per kg maternal weight. If maternal blood was sampled twice prior to surgery, enrichments were averaged.

We quantified fetal whole body kinetics by using the concept of an umbilical veno-arterial balance model. To do so, we rewrote the leucine arteriovenous balance model by Tessari et al.[11] and the phenylalanine hydroxylation equation proposed by Nair et al.[12] into a phenylalanine and tyrosine model suitable for fetal studies.

The model is outlined in Figure 1 and its determinants are calculated using the following equations, where kg in all units denotes fetal weight (=birth weight):

Rate of phenylalanine delivery from umbilical vein to the fetus in μmol/(kg·h):

$$\text{delivery} = [\text{phe}]_{\text{vein}} \times \text{BF} \quad (1.1)$$

where [phe] is the total (labeled + unlabeled) phenylalanine concentration (μmol/L) and BF the umbilical blood flow (L/(kg·h)). Subscripts indicate whether blood was sampled from the umbilical vein or arteries (as below).

Rate of phenylalanine release from fetus to umbilical artery in μmol/(kg·h):

$$\text{release} = [\text{phe}]_{\text{art}} \times \text{BF} \quad (1.2)$$

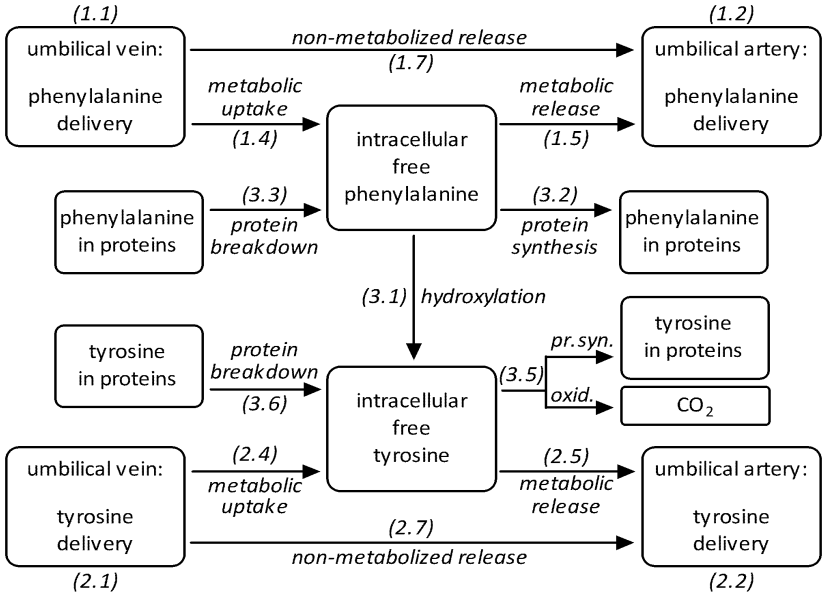


***Figure 1*** *Schematic model of fetal phenylalanine and tyrosine metabolism. Phenylalanine and tyrosine are delivered to the fetus through the umbilical vein (1.1) and (2.1). Part of these amino acids are taken up from the fetal intravascular system into the fetal cells (1.4) and (2.4), whereas the remainder of the intravascular amino acids are transported back to the placenta through the umbilical arteries (1.7) and (2.7). Amino acids are constantly released from proteins due to proteolysis (3.3) and (3.6). Part of the available phenylalanine is hydroxylated to tyrosine (3.1), incorporated in proteins (3.2), or released into the vascular system (1.5). Tyrosine is either used for protein synthesis or oxidation (3.5), or also released into the vascular system (2.5). Finally phenylalanine and tyrosine are transported back to the placenta through the umbilical arteries (1.2) and (2.2).*

*Numbers in brackets also correspond to the equations in the methods section and the fluxes outlined in table 5.*

Fraction of phenylalanine in the umbilical vein that is metabolized intracellularly in %:

$$\text{metabolized fraction} = \left(1 - \frac{[^{13}\text{C}\bullet\text{phe}]_{\text{art}}}{[^{13}\text{C}\bullet\text{phe}]_{\text{vein}}}\right) \times 100\% \quad (1.3)$$

where [$^{13}$C·phe] is the labeled phenylalanine concentration (μmol/L).

Rate of phenylalanine inflow from umbilical vein into intracellular compartment in μmol/(kg·h):

$$\text{metabolic inflow} = \text{Eq}(1.1) \times \text{Eq}(1.3) \quad (1.4)$$

Rate of phenylalanine outflow from intracellular compartment into umbilical artery in μmol/(kg·h):

$$\text{metabolic outflow} = \text{Eq}(1.2) + \text{Eq}(1.4) - \text{Eq}(1.1) \quad (1.5)$$

Net fetal phenylalanine uptake in μmol/(kg·h):

$$\text{net uptake} = \text{Eq}(1.1) - \text{Eq}(1.2) = \text{Eq}(1.4) - \text{Eq}(1.5) \quad (1.6)$$

Rate of phenylalanine directly released from umbilical vein to artery without being metabolized in μmol/(kg·h):

$$\text{non-metabolized release} = \text{Eq}(1.1) - \text{Eq}(1.4) \quad (1.7)$$

Equations (1.1) through (1.7) can also be used for calculations of tyrosine kinetics (using tyrosine concentrations and the [$D_4$]tyrosine enrichments), yielding equations (2.1) through (2.7).

Rate of phenylalanine hydroxylation to tyrosine in μmol/(kg·h):

$$H_{PT} = \text{Eq}(2.1) \times \left(\left(\frac{D_4\bullet\text{tyr}\bullet E_{\text{vein}}}{D_4\bullet\text{tyr}\bullet E_{\text{art}}} \times \frac{^{13}\text{C}\bullet\text{tyr}\bullet E_{\text{art}}}{^{13}\text{C}\bullet\text{phe}\bullet E_{\text{art}}}\right) - \frac{^{13}\text{C}\bullet\text{tyr}\bullet E_{\text{vein}}}{^{13}\text{C}\bullet\text{phe}\bullet E_{\text{art}}}\right) \quad (3.1)$$

where $D_4$·tyr·E is the [$D_4$]tyrosine enrichment (in MPE). Other enrichments are abbreviated accordingly.

Rate of intracellular phenylalanine incorporation into protein (synthesis) in μmol/(kg·h):

$$S_{\text{phe}} = \text{Eq}(1.4) \times \frac{^{13}\text{C}\bullet\text{phe}\bullet E_{\text{vein}}}{^{13}\text{C}\bullet\text{phe}\bullet E_{\text{art}}} - \text{Eq}(3.1) \quad (3.2)$$

Rate of phenylalanine release from proteolysis (breakdown) into the intracellular space in μmol/(kg·h):

$$B_{\text{phe}} = \text{Eq}(3.1) + \text{Eq}(3.2) - \text{Eq}(1.6) \quad (3.3)$$

Rate of net phenylalanine accretion in μmol/(kg·h):

$$\text{accretion}_{\text{phe}} = \text{Eq}(3.2) - \text{Eq}(3.3) \quad (3.4)$$

In our model, we could not discriminate between the two major intracellular pathways of tyrosine metabolism, i.e. incorporation into protein and oxidation. This is why we used the sum of the latter two rates in μmol/(kg·h):

$$S_{tyr} + O_{tyr} = Eq(2.4) \times \frac{D_4 \bullet tyr \bullet E_{vein}}{D_4 \bullet tyr \bullet E_{art}} \quad (3.5)$$

Rate of tyrosine release from proteolysis into the intracellular space in μmol/(kg·h):

$$B_{tyr} = Eq(3.5) - Eq(3.1) - Eq(2.6) \quad (3.6)$$

Phenylalanine and tyrosine protein synthesis and breakdown rates can be converted from molar rates into grams of protein and grams of tissue under the assumption that one gram protein contains 246 μmol phenylalanine and 158 μmol tyrosine[13] and new tissue contains 14% protein[14].

Hydroxylation rates in several previously performed whole body experiments have also been calculated without tyrosine tracer infusion in order to measure tyrosine kinetics or proteolysis rates[10,15]. The latter are then estimated by multiplying the actual phenylalanine proteolysis rate with an average tyrosine/phenylalanine breakdown ratio ($^{Btyr}/_{Bphe}$) measured in similar studies or with the theoretical tyrosine/phenylalanine molar content ratio of total body protein. No equations were as yet available for an arteriovenous balance model, and these were therefore developed ourselves, using analogous derivations to the whole-body model by Thompson et al.[10]. These equations are outlined beneath and enable to compare our hydroxylation rates with those of Chien et al.[16] in spite of the fact that this group did not infuse labeled tyrosine to their subjects.

The rate of phenylalanine released from proteolysis (equation 3.3) can also be calculated as follows (mathematically the same):

$$B_{phe} = Eq(1.1) \times \left( \frac{^{13}C \bullet phe \bullet E_{vein}}{^{13}C \bullet phe \bullet E_{art}} - 1 \right) \quad (4.1)$$

The total rate of tyrosine appearance ($Ra_{tyr}$), defined as Eq(2.1)+Eq(3.1)+Eq(3.5), can normally be calculated after labeled tyrosine infusion according to the following equation:

$$Ra_{tyr} = Eq(2.1) \times \frac{D_4 \bullet tyr \bullet E_{vein}}{D_4 \bullet tyr \bullet E_{art}} \quad (4.2)$$

Equation (3.1) can then be rewritten into:

$$H_{PT} = Ra_{tyr} \times \frac{^{13}C \bullet tyr \bullet E_{art}}{^{13}C \bullet phe \bullet E_{art}} - Eq(2.1) \times \frac{^{13}C \bullet tyr \bullet E_{vein}}{^{13}C \bullet phe \bullet E_{art}} \quad (4.3)$$

However, if no labeled tyrosine is infused, Eq(4.2) cannot be used so that $Ra_{tyr}$ has to be calculated alternatively using a known ratio $^{Btyr}/_{Bphe}$ (in our case the mean of the other seven fetuses):

$$Ra_{tyr} = H_{PT} + Eq(2.1) + Eq(4.1) \times {}^{B_{tyr}}\!\!\diagup\!\!_{B_{phe}} \quad (4.4)$$

Equations (4.3) and (4.4) can then be combined and rewritten into:

$$H_{PT} = \frac{Eq(4.1) \times {}^{B_{tyr}}\!\!\diagup\!\!_{B_{phe}} \times {}^{13}C\bullet tyr\bullet E_{art} + Eq(2.1) \times \left( {}^{13}C\bullet tyr\bullet E_{art} - {}^{13}C\bullet tyr\bullet E_{vein} \right)}{{}^{13}C\bullet phe\bullet E_{art} - {}^{13}C\bullet tyr\bullet E_{art}} \quad (4.5)$$

Thus, using the latter equation it is possible to calculate hydroxylation rates in a balance model if no labeled tyrosine has been infused.

In our model, we make the following assumptions: 1, a labeled molecule will not be discriminated from an unlabeled molecule; 2, the labeled molecule will trace the movement of the unlabeled molecules; 3. the administration of the labeled molecules will not affect the kinetics of the unlabeled molecules.

**Statistics**

Calculations were made using Microsoft Office - Excel software (version 2007; Microsoft Corp, Redmond, WA, USA). Statistical analysis was performed using GraphPad Prism software (version 4.0; San Diego, CA, USA). Because the number of included subjects was relatively small (n=8), normality distribution of our data could not be determined or assumed. All results are therefore expressed as median ($25^{th}$ – $75^{th}$ percentile). Consequently, however, by presenting our data as medians, all rates as outlined in table 5 do not add up correctly as outlined in our model (figure 1). The fluxes of each individual subject still do, nonetheless.

**Results**

We included eight feto-maternal dyads. Maternal age, preconceptional and actual body mass index (BMI), and parity are shown in table 1. Five of the cesarean sections were performed because of breech presentation; three because of a cesarean section in the patient's medical history. There were no complications during any of the cesarean sections. Visual inspection of the placentas and umbilical cords showed no abnormalities. Fetal characteristics in terms of gestational age, birth weight, birth weight *z*-score[17], sex, umbilical blood flow, pulsatility index (P.I.), umbilical arterial pH and base excess, and Apgar score are shown in table 2. None of the infants had congenital anomalies and all were discharged from the hospital in good health together with the mother at the fifth day of life.

***Table 1*** *Maternal characteristics (n=8).*

| Characteristic | Value |
|---|---|
| Age (y) | 33.0 (28.8 – 38.0) [1] |
| Preconceptional BMI (kg/m$^2$) | 21.9 (20.3 – 24.5) |
| Actual BMI (kg/m$^2$) | 30.5 (23.3 – 31.6) |
| Parity (0:1:2:3) (n) | (4:1:2:1) |

[1] Median (25$^{th}$ – 75$^{th}$ percentile) (all such values).

***Table 2*** *Fetal characteristics (n=8).*

| Characteristic | Value |
|---|---|
| Gestational age (wks) | 38.5 (37.6 – 38.9) [1] |
| Birth weight (kg) | 3.3 (2.7 – 3.4) |
| Birth weight *z*-score (SD) [2] | -0.11 (-0.86 – 0.52) |
| Sex (m:f) (n) | 4:4 |
| Umbilical blood flow (mL/(kg·min)) | 101 (90 – 110) |
| P.I. [3] | 0.89 (0.78 – 0.96) |
| Umbilical arterial pH | 7.30 (7.28 – 7.32) |
| Umbilical arterial Base Excess (mmol/L) | -1.5 (-2.0 – -1.0) |
| Placental weight (kg) | 0.590 (0.558 – 0.649) |
| Apgar score at 5 min [4] | 10 (10 – 10) |

[1] Median (25$^{th}$ – 75$^{th}$ percentile) (all such values).

[2] Birth weight corrected for gestational age (reference 17).

[3] The umbilical pulsatility index (P.I.) is a Doppler ultrasound derived index on the blood stream velocity profile through the umbilical arteries and is a marker of fetal well-being.

[4] The Apgar score is a postnatal scoring scale ranging from 0-10.

In four women, we obtained two blood samples before surgery had started at an approximately 20-minutes interval. Phenylalanine and tyrosine enrichments had not changed over this time interval, even though spinal anesthesia had been started meanwhile. Therefore, steady state was assumed. Only one blood sample could be withdrawn from the other four women, which was before anesthetics were initiated. Concentrations and enrichments of phenylalanine and tyrosine in maternal and fetal plasma are shown in table 3. The median coefficients of variance for all measurements of the phenylalanine and tyrosine concentrations were 0.007 (0.002 – 0.011) and 0.012 (0.007 – 0.019), respectively. The coefficients of variance for the enrichments of [1-$^{13}$C]phenylalanine, [1-$^{13}$C]tyrosine, and [*ring*-$D_4$]tyrosine amounted 0.022 (0.011 – 0.040), 0.091 (0.060 – 0.123), and 0.026 (0.011 – 0.058), respectively. The feto-maternal enrichment ratios across the maternal and umbilical veins were 0.90 (0.80 – 0.92) for phenylalanine and 0.72 (0.67 – 0.75) for tyrosine.

***Table 3*** *Phenylalanine (phe) and tyrosine (tyr) concentrations (μmol/L) and enrichments (MPE) measured in the maternal vein, umbilical vein, and umbilical artery (n=8 for each).*[1]

| | **Maternal vein** | **Umbilical vein** | **Umbilical artery** |
|---|---|---|---|
| phe concentration | 64.7 (58.5 – 67.2) | 87.0 (83.4 – 93.6) | 84.1 (78.6 – 88.4) |
| tyr concentration | 48.0 (42.7 – 49.4) | 65.8 (61.5 – 76.2) | 66.9 (65.2 – 71.6) |
| [1-$^{13}$C]phe enrichment | 11.5 (10.9 – 11.9) | 9.8 (9.0 – 10.2) | 8.4 (7.6 – 8.7) |
| [1-$^{13}$C]tyr enrichment | 0.94 (0.89 – 1.08) | 1.04 (0.97 – 1.12) | 1.02 (0.97 – 1.13) |
| [$D_4$]tyr enrichment | 8.3 (7.7 – 8.6) | 5.8 (5.3 – 6.1) | 4.9 (4.7 – 5.4) |

[1] All values are median ($25^{th}$ – $75^{th}$ percentile).

Table 4 shows maternal phenylalanine and tyrosine kinetics. Since women were in fasting state, phenylalanine released from protein breakdown equaled the total flux. The tyrosine/phenylalanine breakdown ratio ($^{Btyr}/_{Bphe}$) was 0.75 (0.74 – 0.79). The fraction of the maternal net catabolic state that could be explained by net fetal uptake was 26 (23 – 40)%.

***Table 4*** *Maternal phenylalanine and tyrosine kinetics (n=8).* [1]

| **Flux** | **Value** |
|---|---|
| Phenylalanine released from proteolysis | 38.2 (36.6 – 40.5) |
| Phenylalanine used for protein synthesis | 34.2 (33.4 – 37.8) |
| Net phenylalanine balance | -3.8 (-4.7 – 2.8) |
| Phenylalanine hydroxylation | 2.6 (2.2 – 2.9) |
| Tyrosine released from proteolysis | 29.5 (28.5 – 31.2) |

[1] All values are expressed in μmol/(kg·h) as median ($25^{th}$ – $75^{th}$ percentile).

Figure 1 and table 5 display fetal phenylalanine and tyrosine kinetics. Although gravidae were catabolic, their infants had a positive phenylalanine accretion balance. The fetal $^{Btyr}/_{Bphe}$ was 0.75 (0.70 – 0.81), slightly higher than the theoretical ratio of 0.64 (=158/246) calculated from the molar expressed amino acid content of protein in deceased fetal bodies[13]. Conversion to protein turnover rates from phenylalanine kinetics reveals a protein synthesis rate of 9.0 (8.2 – 11.3) g/(kg·d) and a proteolysis rate of 7.1 (6.7 – 8.5) g/(kg·d). Accretion rates are 1.7 (0.8 – 3.0) g protein/(kg·d) or 12.2 (5.4 – 21.3) g tissue/(kg·d). Conversion from tyrosine kinetics yield a proteolysis rate of 7.9 (7.2 – 11.4) g/(kg·d).

***Table 5*** *Fetal phenylalanine and tyrosine kinetics (n=8).*

| Flux | Phenylalanine | Tyrosine |
|---|---|---|
| Umbilical vein delivery (1.1 & 2.1) [1] | 559 (456 – 603) [2] | 454 (325 – 492) |
| Umbilical artery output (1.2 & 2.2) | 509 (443 – 567) | 449 (310 – 472) |
| Metabolized fraction (%) (1.3 & 2.3) | 18 (17 – 20) | 16 (12 – 17) |
| Metabolic uptake from umb. vein (1.4 & 2.4) | 91 (80 – 111) | 69 (55 – 78) |
| Metabolic output into umb. artery (1.5 & 2.5) | 59 (57 – 66) | 55 (49 – 67) |
| Net uptake (1.6 & 2.6) | 23 (16 – 47) | 2.4 (-3.9 – 9.2) |
| Non-metabolized release (1.7 & 2.7) | 449 (371 – 499) | 374 (253 – 422) |
| Hydroxylation (3.1) | 7.5 (6.2 – 15.5) | |
| Protein synthesis (3.2 & 3.5) | 92 (84 – 116) | 77 (63 – 93) [3] |
| Release from protein breakdown (3.3 & 3.6) | 73 (68 – 87) | 52 (47 – 75) |
| Net accretion (3.4) | 17.5 (7.8 – 30.6) | |

[1] Numbers between brackets indicate the used equation and flux for phenylalanine and tyrosine kinetics, respectively, as they are also outlined in figure 1.
[2] All values are expressed in μmol/(kg·h) as median ($25^{th}$ – $75^{th}$ percentile) unless indicated otherwise.
[3] Includes tyrosine oxidation.

**Discussion**

In this study, we described several aspects of human maternal and fetal phenylalanine and tyrosine metabolism. We aimed to add to the minute knowledge on fetal amino acid metabolism, so as to stimulate and aid the development of better nutrition for premature infants.

Whether the fetus is a significant drain on maternal substrate availability during fasting is a semantic issue. Umbilical phenylalanine uptake per kilogram maternal weight was 1.0 (0.6 – 1.9) μmol/kg/h. This amount can easily be realized through a small increase in the maternal proteolysis rate or a reduction in protein synthesis as these rates are approximately 30 times higher than net umbilical uptake. On the other hand, when considering the effects on the gravida's net catabolic state, one-fourth is attributable to

net umbilical uptake. This fetal attribution to maternal catabolism is even underestimated since it does not include the extra amino acid consumption of other conceptus tissues such as the placenta.

How the metabolic load of the total conceptus is handled by the gravida is not exactly known. In rats, maternal protein stores initially increase during early pregnancy, and are later catabolized to sustain fetal demands necessary for rapid growth[18,19]. In humans, there is no such evidence. However, since protein intake does not seem to be increased substantially in pregnant women, other mechanisms probably also account for the total accumulation of 925 gram protein in various tissues and fetus during pregnancy[20]. Some studies showed unchanged oxidation[21-23], but others showed reduced amino acid oxidation and urea synthesis rates[24,25], or reduced nitrogen excretion rates[22,24,26], probably all to spare nitrogen necessary for fetal growth[26]. Maternal phenylalanine hydroxylation rates in our subjects were lower than those found in non-pregnant individuals, but comparable to those in other pregnant women[15,21,27]. Relating the other kinetic rates measured in this study to non-pregnant women is difficult. For one thing, the differences are probably more subtle. Moreover, changes in body weight and composition during pregnancy, and the contribution of the feto-placental compartment to maternal metabolism, distort comparisons to non-pregnant women.

Whereas net umbilical uptake of all essential amino acids was considerable in the hereafter cited studies, tyrosine uptake in the term human fetus has been shown to be small or even slightly negative[16,28-30]. During the second trimester of gestation, fetal tyrosine uptake was also found to be absent[31], or small at most[29]. Although placental amino acid transporters are capable of transporting tyrosine to the fetus, the in vitro measured tyrosine influx is strongly inhibited by the presence of several other amino acids, even at physiological concentrations[32,33]. The $K_i$ value (giving half-maximal inhibition) of tyrosine transport across the maternal facing trophoblastic membrane was found to be 68±4.0 μM with phenylalanine[32]. This value does not deviate much from the observed maternal phenylalanine concentrations (table 3). In this light, it is interesting that mothers of growth-restricted fetuses have elevated plasma amino acid concentrations, including phenylalanine[34]. Whether this could result in further inhibition of materno-fetal tyrosine transport remains speculative.

Because net fetal tyrosine uptake is probably low, it thus seems that the fetus is largely dependent on endogenous tyrosine synthesis from phenylalanine. Some early in vitro studies reported substantial enzymatic activity in liver extracts from first or second trimester aborted human fetuses[35-37]. Tyrosine formation in premature neonates immediately after death has also been described[38]. One other study describes disappearing phenylalanine hydroxylase capacity during the second half of pregnancy[39] and the absence was confirmed in a deceased premature infant[40]. Next to

these in vitro studies, of which none measured renal hydroxylating capacity, only Chien and colleagues attempted to quantify in vivo phenylalanine hydroxylation rates in fetuses at term[16]. They did not simultaneously infuse labeled tyrosine, however, and Nair et al.[12] had not published their balance model by then, resulting in a highly simplified model to determine hydroxylation rates. But when using their enrichment results in combination with our equation (4.5) (online section), hydroxylation rates are very high, i.e. 42.6 μmol/(kg·h) using our median $^{Btyr}/_{Bphe}$ of 0.75, or 40.8 μmol/(kg·h) using the theoretical breakdown ratio of 0.64 from deceased fetuses[13]. As these rates are much higher than the net umbilical phenylalanine uptake, they seem improbable. Hydroxylation rates in premature and term infants have been measured in several studies during the last 15 years. Mean rates in fasting premature infants receiving only glucose range from 6 to 17 μmol/(kg·h)[41-45]. If amino acids are also supplemented, some studies report no change[41], other show a small increase with means ranging from 11 to 22 μmol/(kg·h)[42-44]. Shortland et al. even measured hydroxylation rates of 48 μmol/(kg·h) after amino acid supplementation[45], but these rates appear to be overestimated[46]. Rates in healthy term infants do not seem to be different from rates in preterm infants and range from 8 to 13 μmol/(kg·h), irrespective of nutrient administration[42,47]. Our observed hydroxylation rates of 7.5 (6.2 – 15.5) μmol/(kg·h) are in concordance with the postnatal values.

Fetal growth velocities around 38 weeks gestation are around 8 to 10 g/(kg·d). Our observed growth rates (12.2 (5.4 – 21.3) g tissue/(kg·d)), calculated from the accretion rate of one amino acid, are not much different. Potential errors in the conversions to proteins and body weight and measurement errors are probably responsible for the small difference.

Much to our surprise, the metabolized fraction of available fetal phenylalanine and tyrosine was only approximately 20% for both amino acids. Calculations on data from Chien et al. in term human fetuses as well[16], reveal a metabolic uptake of 26% for phenylalanine and 36% for leucine. In ovine fetuses, we calculated from available data[48] a fraction of approximately 25% for leucine, which does not seem to differ between normally grown and growth-restricted fetal animals. This implies that most amino acids entering the fetus through the umbilical cord remain intravascular before returning to the placenta through the umbilical arteries. It thus seems that the placenta provides the fetus with an enormous reserve capacity of these amino acids. Intrauterine growth restriction would, therefore, not seem to be primarily caused by a lack of amino acids. The cause of fetal growth restriction could then lie in a reduced potential or necessity to internalize amino acids from the fetal tissue arterioles into the tissue or organ, because of a lack of secondary metabolites necessary for cellular inward transport or growth (e.g. ATP, oxygen, or sodium), or through hormonal influences. Insufficient oxygen supply to the ovine fetus, for example, decreases protein synthesis more than breakdown, so that net protein accretion becomes compromised[49]. Fetal

amino acid concentrations increased during this four-hours lasting experiment, whereas placental leucine transport decreased. Whether the latter is primarily the result of hypoxemia too or a compensatory mechanism in order to avoid hyperaminoacidemia is speculative.

On the other hand, growth-restricted fetuses show reduced umbilical plasma concentrations[34]. Besides, placentas of fetuses with intrauterine growth restriction (IUGR) have reduced transporter activity[50,51] which has also been described in human in vivo research[52]. Although there seems to be a large overcapacity of phenylalanine and tyrosine available for fetal growth, protein accretion is determined by the first limiting amino acid. It might well be that for one of the other essential amino acids, the surplus is less abundant. Reduced placental functioning might then induce a lower availability of this first limiting amino acid and consequently slower growth. Further studies on other amino acids and studies in growth-restricted fetuses are necessary to support these hypotheses.

All our concentration and enrichment measurements were done in the plasma compartment, rather than in whole blood, due to analytical advantages. Many studies, however, reported rapid equilibrium between erythrocyte and plasma concentrations of various amino acids, including phenylalanine and tyrosine[53-55]. The role of erythrocytes in organ amino acid delivery is thus as important as the role of the plasma compartment. Compared to normal organ balance studies, the circulation time of blood in fetal balance studies is relatively long since blood from the umbilical vein flows through the whole fetus before returning to the umbilical arteries. By then complete mixing can be expected. Even in single organ balance studies, many groups chose to use plasma sampling in combination with whole blood flows rather than plasma flows[56-59]. The latter would reduce all kinetic rates by approximately 40% (~ hematocrit) and yield improbably low kinetic rates.

Whether maternal anesthesia and surgery would have any consequences for fetal metabolism remains speculative. Spinal anesthesia might result in maternal hypotension and blood flow redistribution, but these effects can be prevented by using a lateral wedge. Furthermore, blood pressure monitoring allows for prompt correction if necessary. Besides, the pulsatility index of the umbilical artery does not seem to be influenced by spinal anesthesia[60]. Konje et al. measured flow using a transonic time flowmetry technique on a exteriorized loop of the umbilical cord during cesarean surgery[61]. Their flow values halfway during surgery correspond well to our flow measurements. Besides, umbilical blood flow after vaginal delivery has been reported to be stable for the first 100 postnatal seconds[62]. We thus assume that umbilical blood flow is fairly constant during surgery. The fetal metabolic response to maternal surgery remains speculative. A maternal noradrenalin surge after an invasive procedure did not seem to reach the human fetus[63]. In mice, however, noradrenalin was suggested to have transferred the placenta[64].

To conclude, we showed that the fetus at term receives considerable amounts of phenylalanine from the placenta. Nevertheless, the fetus actively uses only a relatively small part; two-thirds of which are used for net protein synthesis and one-third for hydroxylation to the semi-essential amino acid tyrosine. Whether these findings would also hold true for the growth restricted fetus or a fetus earlier in gestation remains to be elucidated.

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CHAPTER

# 11

# General Discussion

Partly based on:

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Mass Spec Rev
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## General discussion

Applications of stable isotopes in paediatric research cover carbohydrate and amino acid metabolism as well as body composition, energy expenditure, and the synthesis of specific proteins such as glutathione and albumin. The main focus of all studies is on the interactions between exogenous influx of nutrients and endogenous metabolism in the body pool and how these affect a growing infant's health. Research in this area is very important, considering that the composition of tissue gain has huge impact on metabolism during later life. Rapid advances have been made meanwhile. The major issues on metabolic level are synthesis and catabolism rates, which are easily obtained with stable isotope labelled tracers.

Organic mass spectrometry (MS) and isotope ratio mass spectrometry (IRMS) are the two most mature techniques for the isotopic analysis of compounds. Direct physical coupling of gas chromatography (GC) to either IRMS or MS is the most robust technique for specific isotopic analysis of volatile compounds. In addition, liquid chromatography (LC) is now gaining a reputation as a tool for sample introduction of both volatile and non-volatile compounds into IRMS or MS. Its applications include $^{13}C$ isotopic analyses at natural abundance and analysis of $^{13}C$-labelled enriched compounds.

Small sample numbers and volumes are a must in paediatric research. So sample size restriction is an important issue as well as the availability of stable isotope-labelled substrates for measurement of kinetics and concentrations in metabolic studies. During the last decade more of these substrates have become available. As also the accuracy precision and sensitivity of existing techniques such as GC/IRMS have improved and new techniques like LC/IRMS were introduced, we saw new possibilities to tackle these limitations.

## Major findings

Changes in plasma glucose concentrations are the result of several simultaneously occurring processes. that disturb stability in the fasting condition[1-2]. Stability is maintained through balancing glucose production in the liver with its subsequent release into the systemic circulation and its removal from the blood by insulin-independent tissues of the body, e.g. muscle, brain, kidney, gut and erythrocytes.

The glucose metabolism might be unravelled with the use of stable isotopic labelled glucose . The $^{13}C$ isotopic enrichment of glucose is usually analysed by GC/MS or GC/IRMS. However, in both techniques the samples must be derivatized prior to analysis, which makes sample preparation more labour intensive and casts a doubt on the correctness of the measured isotopic composition. The novel method for the determination of isotopic

enrichment of glucose in human plasma using LC/IRMS as described in **chapter 2** is a new step forward in measurement of very low levels of enrichment in *in vivo* studies. In comparison with GC/MS and GC/IRMS it has better precision and requires less preparation effort. The precision and accuracy of the measurement of the isotopic composition at natural abundance and at higher enrichment do not show any notable isotopic fractionation during sample preparation and analysis.

In **chapter 3** we have validated a method for simultaneous measurement of deuterium and $^{18}O$ enrichment in urine and saliva samples. With this doubly labelled water method we can determine not only children's total body water composition but also energy expenditure. It usually involves blood or urine sampling, which might be difficult in neonates and children with cerebral palsy or other disabilities. We therefore aimed to validate a method making use of saliva samples analyzed by automated thermal conversion elemental analyzer in combination with IRMS (TC-EA/IRMS). Sample preparation is much simpler than with the classical methods. The analysis process is fully automated, with very small samples (0.1 µL) directly injected into a TC-EA/IRMS system equipped with a liquid auto sampler. Deuterium and $^{18}O$ enrichment of saliva and urine samples can therefore be measured accurately and simultaneously. We did not find significant differences between the two sample types, but would prefer saliva because its production time can be determined almost exactly. In addition, the sampling of saliva is less invasive than blood, which is an important issue in paediatric studies. Saliva values are also more in agreement with the serum values than urine values found in an earlier study by Jankowski[3]. The 'saliva" method has been successfully applied to group of children suffering from severe cerebral palsy (CP) as described in **chapter 4**. In a field setting, we investigated whether the DLW method applied on saliva and urine in children with severe CP and intellectual disability would result in the same clinical outcomes. We reported on the feasibility of both urine and saliva sampling in this specific group of children. Both urine and saliva sampling were acceptable choices for measuring the clinical outcomes TEE and TBW using the DLW technique in children with severe CP and ID. Because of the more exact timing, saliva sampling is preferential in children with severe CP and ID.One should reserve urine sampling for children that are known to hyposalivate or have severe oral hypersensitivity.

Glutathione, one of the major anti-oxidants in the human body, has been studied in a large variety of species and study designs[4-9]. The emphasis, though, was largely on concentration measurements, and rather not on kinetics. However, full insight into glutathione metabolism requires information on the kinetics as well. We therefore designed a new method (**chapter 5**) based on stable isotope technique using LC-IRMS. To this purpose, we measured the $^{13}C$-isotopic enrichment and the concentration of glutathione in its oxidized form (GSSG). This new method proved to be a

powerful tool in metabolic studies in neonates (**chapter 7 and 8**). Only little pre-purification was necessary and the analyses reported here were fully automated. Nevertheless, even though this innovation was a big step forward in unraveling glutathione, it needed to be complemented with glycine measurement on GCMS or GC/C/IRMS. In **chapter 6** a new method is presented for simultaneous measurement of GSSG and its precursor [1-$^{13}$C] glycine in erythrocytes. The simultaneously measurement of glycine and GSH, for both concentration and $^{13}$C isotopic enrichment, gave excellent results. The more so as only 150 µL of blood was needed, which is of very high relevance for neonatal studies or studies in small animals. GSH concentrations corresponded to those reported in the literature.

**Chapter 7** Although glutathione is the main intracellular antioxidant, it is important to realize that the total antioxidant defence is the result of the interplay between other intracellular and extracellular antioxidants, such as vitamin C and vitamin E. Glutathione synthesis is primarily dependent on the presence of its synthesizing enzymes, activity of AA transport mechanisms across cellular membranes, and availability of substrates. Our data agree with results from earlier studies demonstrating that these enzymes were present in leukocytes obtained from tracheal aspirates and cord blood as well as in autopsy samples of foetal liver, lung, and kidney[4, 10]. In addition, even during starvation, glutathione synthetic enzymes appear to be maintained for readiness of glutathione synthesis upon substrate availability[11].

Uptake of glutathione substrates is another requirement for glutathione synthesis. Lavoie et al. determined L-[$^{35}$S]cysteine uptake capacity in leukocytes of cord blood and tracheal aspirates of infants of varying gestational ages[12]. As the maturity of female but not male newborns was positively correlated with cysteine uptake, the authors concluded that cysteine uptake might be compromised in preterm infants. However, we did find active glutathione synthesis in erythrocytes of preterm infants, which increased dramatically upon amino acid (AA) administration. This can only be achieved by uptake of substrates, including cysteine. The final requirement for glutathione synthesis is availability of substrate. Collectively, the findings from our studies suggest substrate availability to be the most important determinant in glutathione availability in preterm infants in the immediate postnatal phase.

From our studies in preterm human infants we can conclude that the compromised glutathione availability in erythrocytes is primarily due to nutritional deprivation. It would seem, however, that more aggressive nutritional support easily results in adequate concentrations upon. Similarly, Chessex et al. fed neonatal guinea pigs either intravenous glucose or glucose and AAs. When challenged with oxygen therapy, only those in the group who had received supplemental AA show increased liver glutathione concentration, i.e. twofold[22].

We demonstrated that $FSR_{glutathione}$ on the second day of life was not higher in the group receiving AA from birth onwards as compared to infants receiving glucose only, suggesting feedback inhibition of glutathione synthesis following decreased glutathione consumption. This suggestion is supported by the fact that in glutathione synthesis did not further increase upon administration of a higher dose of cysteine, generally considered as the rate limiting substrate for glutathione synthesis[24-25] (**chapter 8**). Indeed, cysteine concentrations in several tissues are significantly lower than glutamate and glycine concentrations that are used in equimolar amounts in the GSH synthesis process. Although glycine demands are probably increased in preterm infants, being involved in the synthesis of many other AAs, utilization of glycine for erythrocyte GSH synthesis represented less than 2% of the plasma glycine flux[7].

In **chapter 8** we studied the effects of a high cysteine dose on erythrocyte glutathione kinetics as compared to a standard dose. We did not find any difference in either glutathione concentration or synthesis rates. Surprisingly, also plasma cystine concentrations did not rise. Because cysteine is unstable in solutions, it bioavailability has been questioned. Moreover, cysteine in plasma is thought to be auto-oxidized to cystine, thus releasing reactive species[39]. If this were to be true, we might expect increased levels of certain protein oxidation markers such as advanced oxidized protein products. However, we found no difference in concentrations of these products between infants receiving either the high or the standard cysteine dose. Still, for the very reason of its instability in solutions ???cysteine must be added to an AA solution just prior to administration. The finding that plasma cystine concentrations were not increased might reflect its use for other purposes such as protein synthesis, or its catabolism to sulfate. High dose cysteine (81 mg/(kg·d)) appears safe but does not increase glutathione concentration or synthesis rate in erythrocytes of preterm infants as compared to a lower dose (45 mg/(kg·d)).

Using a relatively new multiple tracer infusion protocol (modified from Dudley et al.[64]), we were able to measure albumin synthesis rates in foetuses of different gestational ages in a single sample taken from the umbilical cord immediately after birth (**chapter 9**)[65]. Figure 2 depicts these synthesis rates. They ranged widely and were surprisingly high in premature foetuses. From a physiological developmental point of view we cannot figure out why this should be so. For many of the functions of albumin, such as fatty acid and bilirubin transport or anti-oxidant defence, pertain more to the postnatal than the prenatal phase. Nevertheless, the most important finding is that the liver of a premature foetus is capable of synthesizing albumin at very high rates. In theory this capability should still be present after birth. It seems, however, that the postnatal values are lower than the prenatal ones, and that it would be possible to raise the synthesis rates by more nutrition.

We were able to measure the protein synthesis rate in the feto-maternal dyads at term using a phenylalanine isotope. From this we could calculate the percentage of protein synthesis spent on albumin. Departing from the knowledge that albumin contains 6.87% phenylalanine on a weight basis and combining the from **chapters 9 and 10** we arrived at the conclusion that 3.4 (3.1 – 4.4) and 5.2 (4.4 – 5.9) % of total protein synthesis is albumin synthesis in term foetuses and their mothers, respectively. These values are close to the values we found in premature infants (~4%). These figures are not only interesting from a metabolic point of view, but also give credence to our methods used in the foetal study as they are entirely different from those used in neonates, yet give similar values.

**Foetal amino acid metabolism**

Quantitative data on human in vivo foetal amino acid metabolism are very scarce[66]. By interpreting enrichment ratios, researchers formed ideas on intrauterine growth restriction and placental transport characteristics[67-70]. Most of their study designs did not require steady state assumptions and blood was often sampled in a relatively unstressed situation, i.e. by cordocentesis from the umbilical vein. Quantitative balance studies such as those performed by Chien et al.[66] and our group in **chapter 10**, however, require sampling from both the umbilical vein and arteries. Therefore, the type of research we performed is limited to the metabolism just prior to birth. In this context, caesarean sections are probably less stressful to the infant, but most importantly, if elective, they are of course scheduled. On the other hand, the elective nature largely limits this type of research to term infants. Most research in the foetal phase so far has therefore been in animals – apart from rodents predominantly in sheep. Contrary to pigs, which are mostly used in neonatal research, ovine pregnancy rears mostly singletons and allows foetal surgical manipulation without instigating litter death. Animal models on the whole provide for an (almost) completely unstressed and physiologic situation in which multiple catheters can be inserted into various foetal and maternal blood vessels. Then, after a few days recovery, blood sampling and flow measurements is possible from all sites simultaneously. Even more so, this can be done during different periods of gestation and of course during numerous experimental settings, e.g. fasting, hormonal infusion, and induced foetal growth restriction.

Nevertheless, despite all advantages of animal studies, we expressly wished to explore human foetal metabolism. Animal research is not always ideal due to the many interspecies and human research provides for direct comparisons to postnatal research results, just like we did with the albumin synthesis rates. For example, as mentioned above, we determined the foetal

whole body protein synthesis rate on the basis of phenylalanine kinetics in **chapter 9**. In foetuses around term it amounted to 9.0 g/(kg·d). Assuming a mean 120 g protein per kg body weight, we calculated a fractional synthesis rate of foetal proteins at term of 7.4 %/d. This fractional protein turnover rate seems to be slightly lower than that of the premature infant on the second day of life (8.9%/d) as reported above.

**Conclusions**

High precision isotopic ratio analyses are gaining a foothold in ever more scientific disciplines, including metabolic kinetic studies in paediatrics. Innovations in mass spectrometry techniques and tracer administration techniques have made mass spectrometry the instrument of choice for the analysis of isotopic labelled compounds. Techniques for high precision measurements of deuterium and oxygen but also for 13C isotopic analysis have progressed, too, as outlined in this thesis. Especially the coupling of LC with IRMS has created new possibilities for paediatric research, particularly when low sample volume is a must, such as in newborn babies. An increasing number of papers using on LC-IRMS using stable isotopes in metabolic research have demonstrated the robustness of this technique. Still, it has the disadvantage of being suitable for $^{13}C$ isotopic measurement only. A major future challenge therefore is development of a technique that enables measurement of all common stable isotopes.

**Future perspectives**

Although new techniques have been developed and existing techniques have been improved there are still experimental disciplines uncovered. The coupling of LC to IRMS was a major step towards unravelling metabolic kinetics – made feasible by direct measurement of isotopes in a wide range of low molecular weight compounds as well as macromolecules ranging from natural abundance till highly enriched samples. Its strength lies in straightforward analysis of underivatized components. The main drawbacks of this method are the relatively low sensitivity (nanogram range) and its restriction to measure $^{13}C$ isotopic measurement only. The low sensitivity can be a problem when measuring components in low concentrations, such as vitamins and hormones, or when samples are small like in preterm infants or small rodents.

A welcome improvement would be a new technique of measuring $^{15}N$ isotopes with LC/IRMS, which would open up new possibilities for studying metabolic pathways including those of arginine and ornithine. Even with the advances made so far, there are still many topics in metabolic kinetic studies

to be elucidated. As a reassuring thought, however, the growing availability and the decreasing costs of stable isotopes will make it possible to broadly explore human metabolic kinetics worldwide.

The improvements of the sensitivity and robustness of LC/MSMS systems have opened up new possibilities for studying macromolecules like peptides, hormones, vitamins and small proteins. Still a width range of applications have to be developed in several disciplines using this technique.

Also new developed techniques like infra red techniques for measurement of isotopically labelled compounds are gaining access in many biomedical applications were stable isotopes are involved. The most important advantages compared to isotope ratio mass spectrometry are its low costs and simplicity. The latest developed instruments show similar precision as IRMS. The recent development are the instruments based on wavelength scanned cavity ring down spectroscopy (WS-CRDS analyzer). These instruments show similar precision as show for IRMS but need less sample for example when measuring a3C values in CO2. These instruments require little or no sample preparation, short analysis time (few minutes) and a minimum of skills is needed to operate these machines. However, these instruments still need to be thoroughly tested in a biomedical environment.

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CHAPTER

# 12

## Summary & Samenvatting

## Summary

### Chapter 1

In the introductory chapter of this dissertation, an overview will be presented about applications of stable isotopes in paediatric research. Mass spectrometry has proven to be an essential tool for unraveling kinetic studies in a large range of different research disciplines related to intestinal diseases, severe cerebral palsy, oxidative stress and fetal metabolism. Due to the diversity and complexity of the different metabolites involved in these studies, there is a high demand on sophisticated mass spectrometric instruments. The different types of mass spectrometric instruments will be discussed here as well as several applications in pediatric research utilizing these techniques. The applications cover amino acid metabolism as well as body composition, energy expenditure and the synthesis of specific proteins such as glutathione and albumin. Finally, the aims and outline of this dissertation are covered.

### Chapter 2

In this chapter the development is described of a novel method for $^{13}C$ isotopic enrichment of glucose in human plasma, using liquid chromatography isotope ratio mass spectrometry (LC/IRMS). The $^{13}C$ isotopic enrichment of glucose is usually analysed by gas chromatography mass spectrometry (GC/MS) or gas chromatography combustion isotope ratio mass spectrometry (GC/IRMS). However, in both techniques the samples must be derivatized prior to analysis, which makes sample preparation more labour intensive and increases the uncertainty on the measured isotopic composition. Using this new LC/IRMS technique, for which hardly any sample preparation is needed, we showed that both the enrichment and concentration could be measured with very high precision using only 20 µL of plasma.

In addition, a comparison with GC/MS and GC/IRMS showed best performances were achieved with the LC/IRMS method making it the method of choice to measure $^{13}C$ isotopic enrichment in plasma samples.

### Chapter 3

For measuring energy expenditure and total body water composition in humans the doubly labelled water method is a valuable technique. It usually involves blood or urine sampling, which might be difficult in neonates and

children with cerebral palsy or other disabilities. We therefore aimed to validate a method making use of saliva samples analyzed by automated thermal conversion elemental analyzer in combination with isotope ratio mass spectrometry (TC/EA/IRMS). The subjects received labelled water orally and urine and saliva samples were collected and analyzed. Deuterium as well as oxygen$^{18}$ was measured in one single run using a peak jump method. Saliva sampling is to be preferred, therefore, as it can be easily collected and is non-invasive. Moreover, its time of production is almost exactly known. The TC-EA/IRMS method is a good alternative to the more laborious off-line IRMS measurements.

**Chapter 4**

In this chapter an application is described using the validated doubly water technique as described in chapter 3. Malnutrition is a major health problem in children with severe cerebral palsy (CP) and has led to a considerable body of research into the variations in energy expenditure and body composition in this group of children. The technique that is considered to be a method of reference in this particular area of research is the doubly labeled water (DLW) method. This method requires administering stable isotopes (tracers) of deuterium ($^2H_2O$) and labeled oxygen ($H_2O^{18}$) and provides accurate and reliable measurements of total energy expenditure (TEE) and total body water (TBW). Saliva can be collected almost immediately after it is produced. Isotopic equilibration in saliva is also faster than in urine.

The question is whether in this specific group of children, in whom sampling urine or saliva is not always easy, it would be warranted to assume comparability of outcomes using urine and saliva samples.

The aim of this study is therefore to establish, when applying the DLW method in children with severe CP and intellectual disability (ID) in a field setting, whether saliva and urine sampling result in the same clinical outcomes and report on the feasibility of both urine and saliva sampling in this specific group of children. The result of the study shows that both urine and saliva can be used in this field setting, but saliva is preferred above because the determination of the production time of saliva is more exact than that of urine.

**Chapter 5**

Quantifying utilization and synthesis rates of GSH provides a dynamic insight into its metabolism under pathological conditions such as oxidative stress or in response to interventions. A novel analytical method using liquid chromatography coupled to isotope ratio mass spectrometry (LC/IRMS) was

developed for measuring glutathione (GSH) fractional synthesis rate (FSR) in neonates after infusion of [1-$^{13}$C]glycine as a tracer was presented in this chapter. After transformation of GSH into GSSG, its dimeric form, the intra erythrocytic concentration and $^{13}$C- isotopic enrichment of GSH were determined using 200 µL of whole blood. The results showed that using LC/IRMS, the concentration (range of µmol/mL) was reliably measured using norvaline as internal standard. However, gas chromatography-mass spectrometry (GC/MS) techniques was still needed to measure intracellular$^{13}$C glycine enrichment in erythrocytes in order to determine FSR. This rapid and reliable method opens up a variety of kinetic studies with relatively low administration of tracer infusates, reducing the total cost of the study design. Besides, the small volume of blood needed enables studies even in extremely small subjects, such as premature infants.

**Chapter 6**

Determination of glutathione kinetics using stable isotopes requires accurate measurement of the tracers and tracees. Previously, the precursor and synthesized product were measured with two separate techniques, liquid chromatography isotope ratio mass spectrometry (LC/IRMS) and gas chromatography combustion isotope ratio mass spectrometry (GC/C/IRMS). In order to reduce sample volume and minimize analytical effort we developed a method to simultaneously determine $^{13}$C-glutathione as its dimeric form (GSSG) and its precursor [1-$^{13}$C] glycine in a small volume of erythrocytes in one single analysis. After having transformed $^{13}$C-glutathione into its dimeric form GSSG, we determined both the intra-erythrocytic concentrations and the $^{13}$C-isotopic enrichment of GSSG and glycine in 150 µL of whole blood using LC/IRMS.

This novel method opens up a variety of kinetic studies with relatively low dose administration of tracers, reducing the total cost of the study design.

**Chapter 7**

Early AA administration resulted in increased production of albumin. To investigate whether early AA administration stimulates GSH synthesis, we performed a clinical trial, described in Chapter 7, in which we determined GSH concentrations and synthesis rates in infants either receiving glucose only during the first two postnatal days, or infants receiving glucose and AAs directly following birth. In addition, we quantified levels of AOPP and dityrosine. The results showed that AA administration directly following birth increased GSH absolute synthesis rate by more than 70%. Our data are

consistent, however, with higher GSH concentration rather than a higher fractional synthesis rate. Greater availability of GSH, nevertheless, did not bring down markers of oxidative stress.

**Chapter 8**

In chapter 8 we studied the effects of doubling the amount of cysteine administered parenterally on GSH concentration and synthesis rate. Although it appeared metabolically safe, a high dose cysteine did not increase plasma cysteine concentrations. GSH concentration and synthesis rate were not altered upon the additional cysteine administration.

**Chapter 9**

In chapter 9 and chapter 10 two studies are described where amino acid and protein metabolism is measured in feto-maternal dyads just prior to birth. These exploratory studies give insight in the physiological metabolic capabilities of an individual during early life.

In this chapter the fetal albumin synthesis rate was measured in fetuses at term and preterm gestation (n=8 in each group). To do so, a relatively new model was applied whereby multiple tracers were administered to pregnant women starting at different times in the hours prior to cesarean section. From a single blood sample, taken at birth from the umbilical cord, the fetal albumin synthesis rates could be quantified. Whereas the functions of albumin during intrauterine life are not as clear as during postnatal life, fetuses synthesized very large amounts of albumin, especially earlier in gestation. The fact that a fetus of approximately 30 weeks gestation synthesizes under physiological circumstances large amounts of albumin but does not seem to continue this rate after birth (chapter 4), could indicate that postnatal nutritional strategies for premature infants do not provide enough substrates necessary for the high albumin synthesis rate. Another explanation can be that the other circumstances such as illness, specific medication use reduces albumin synthesis rates

**Chapter 10**

In the study described in this chapter, whole-body protein metabolism was measured in 8 fetuses that were at term gestation. By measuring the umbilical blood flow using ultrasound and infusing appropriate tracers of phenylalanine and tyrosine prior to cesarean section, several metabolic rates could be quantified amongst which the protein synthesis and breakdown

rates. In addition, we could measure the conversion (hydroxylation) of phenylalanine into tyrosine, a process which is said to be hampered in young and critically ill individuals.

Where fetuses showed considerable net uptake of phenylalanine from the placenta, tyrosine uptake was negligible. Fetal phenylalanine uptake was even responsible for one-fourth of the net catabolic state the mother was at while fasting prior to cesarean section. High protein synthesis rates do explain at least partly the high amounts of amino acids utilized. Converted to tissue, fetuses with a gestational age of 38 weeks had a net accretion rate of 12 g/(kg·d).Furthermore, the fetuses showed considerable tyrosine production, indicating that phenylalanine hydroxylation occurs unrestricted.

**Chapter 11**

This chapter provides a general discussion in which all results of this dissertation are again critically analyzed against the current literature. Furthermore, some considerations for future research are presented.

**Samenvatting**

**Hoofdstuk 1**

In het inleidende hoofdstuk van dit proefschrift, krijgt men een overzicht van de toepassingen van stabiele isotopen onderzoek in de kindergeneeskunde. Massaspectrometrie is een essentieel instrument voor een groot aantal verschillende onderzoeksdisciplines die betrekking hebben op darm ziekten, ernstige mentaal gehandicapte kinderen, oxidatieve stress en foetaal metabolisme voor het oplossen van metabole vraagstukken. Vanwege de diversiteit en complexiteit van de verschillende metabolieten betrokken bij deze studies, is er een grote vraag naar geavanceerde massaspectrometrische instrumenten. De verschillende soorten massaspectrometrische instrumenten zullen hier worden behandeld alsmede verschillende toepassingen in pediatrisch onderzoek met behulp van deze technieken. De toepassingen van deze technieken op aminozuur stofwisseling, energie huishouding en de synthese van specifieke eiwitten zoals glutathione en albumine zullen worden besproken. Ten slotte zullen de doelstellingen en de opbouw van dit proefschrift worden uitgelicht.

**Hoofdstuk 2**

In dit hoofdstuk wordt de ontwikkeling van een nieuwe methode voor 13 C isotopen verrijking van glucose in menselijk plasma, met behulp van vloeistof chromatografie isotoop verhouding massaspectrometrie (LC/IRMS) beschreven. 13 C isotopen verrijking van glucose wordt meestal geanalyseerd door gaschromatografie massaspectrometrie (GC/MS) of door gaschromatografie verbrandings isotoop ratio massaspectrometrie (GC/C/IRMS). In beide technieken moeten de monsters worden gederivatiseerd voorafgaand aan de analyse, wat de voorbereiding meer arbeids intensief maakt en daarnaast verhoogt het de onnauwkeurigheid van de gemeten isotopen samenstelling. Met deze nieuwe LC/IRMS techniek, voor welke nauwelijks monster voorbereiding nodig is, hebben we laten zien dat de verrijking en de concentratie met zeer hoge precisie in slechts twintig µL plasma kunnen worden gemeten.

Daarnaast werd deze nieuwe techniek vergeleken met twee andere massa spectrometrie technieken n.l. GC/MS en GC/IRMS. De beste resultaten werden verkregen met de LC/IRMS methode, zodat dit nu de methode van voorkeur is voor het meten van de isotopen 13 C verrijking van glucose in plasma monsters.

**Hoofdstuk 3**

De dubbel gelabeld water methode is voor de meting van de energie huishouding en de samenstelling van totale lichaams water bij de mens een waardevolle techniek. Het gaat meestal om bloed of urine monster afname, welke moeilijk uit te voeren zijn bij zuigelingen en kinderen met een handicap. Wij hebben ons daarom gericht om een metode te valideren voor het analyseren van speeksel monsters, met behulp van een geautomatiseerd hoge temperatuur conversie verbrandings unit in combinatie met de isotoop ratio massaspectrometer (TC-EA/IRMS). De onderzoeksgroep kreeg dubbel gelabeld water oraal toegediend waarna vervolgens urine en speeksel monsters werden verzameld en geanalyseerd. Deuterium alsmede zuurstof 18 werden gemeten in één enkel uitgevoerde analyse met behulp van een piek sprong methode. Analyse van speeksel heeft de voorkeur boven plasma en urine, omdat deze eenvoudig verzameld kan worden en niet belastend is. Bovendien is de tijd van productie bijna precies bekend. De methode van TC-EA/IRMS is een goed alternatief voor de meer bewerkelijke off line IRMS metingen.

**Hoofdstuk 4**

De dubbel gelabeld water methode zoals in het voorgaande hoofdstuk is beschreven is toegepast bij kinderen met geestelijke beperkingen. Ondervoeding is een groot gezondheidsprobleem in kinderen met ernstige hersenbeschadiging (CP) en heeft geleid tot een breed onderzoek naar variaties in de samenstelling van energie huishouding en de lichaamswater samenstelling bij kinderen. De techniek die wordt beschouwd als de "gouden standaard methode" op dit gebied van onderzoek is de dubbel gelabeld water methode (DLW). Bij deze methode is toediening vereist van stabiele isotopen (tracers) deuterium ($^2H_2O$) en gelabeld zuurstof ($H_2O^{18}$) voor correcte en betrouwbare metingen om de totale energie huishouding(TEE) en het totaal lichaams water (TBW) te kunnen bepalen. Speeksel kan bijna onmiddellijk nadat deze is geproduceerd worden verzameld. Het isotopisch evenwicht wordt in speeksel sneller bereikt dan in urine.

De vraag is of het gerond is om bij deze specifieke groep kinderen, waarbij het afnemen van urine of speeksel niet altijd gemakkelijk is, aan te nemen dat de resultaten voor speeksel en urine vergelijkbaar zijn.

Het doel van deze studie is daarom, vaststellen of, wanneer de DLW methode in de praktijk wordt toegepast bij kinderen met ernstige CP en een intellectuele handicap (ID), speeksel en urine afname dezelfde klinische resultaten geven en het bepalen van de uitvoerbaarheid van zowel urine als speeksel afname in deze specifieke groep. De resultaten laten zien dat bij bovengenoemde toepassingen speeksel de voorkeur heeft boven urine omdat het moment van productie bij speeksel beter vast te stellen is.

**Hoofdstuk 5**

Het in kaart brengen van glutathion productie geeft een dynamische inzicht in het metabolisme onder pathologische omstandigheden zoals oxidatieve stress of als reactie op de interventies. Een nieuwe analysemethode met behulp van de vloeistof chromatografie gekoppeld aan de isotoop ratio massaspectrometer (LC/IRMS) is ontwikkeld voor het meten van glutathion (GSH) productie snelheden na infusie met gelabeld [1-$^{13}$C]glycine. Na de omzetting van GSH tot GSSG, zijn de intracellulaire concentratie en verrijking gemeten in 200 µL bloed. Uit de resultaten is gebleken dat met behulp van LC/IRMS, de concentratie (bereik µmol/ml) op betrouwbare wijze gemeten kon worden. Gaschromatografie-massa-spectrometrie (GC/MS) was echter nog steeds nodig om de verrijking van $^{13}$C glycine in erytrocyten te kunnen meten en zodoende de FSR te kunnen bepalen. Deze snelle en betrouwbare methode biedt perpectief voor een groot aantal kinetisch onderzoeken met relatief lage toediening van label en daardoor vermindering van de totale kosten van het onderzoek. Bovendien maken de kleine hoeveelheid bloed die

nodig is voor de analyse studies mogelijk in neonaten en kleine onderzoeksdieren.

**Hoofdstuk 6**

De bepaling van de glutathion kinetiek met stabiele isotopen vereist een nauwkeurige meting van tracer en tracee. Voorheen werden de precursor en het door synthese verkregen product gemeten met twee afzonderlijke technieken, n.l. vloeistof chromatografie isotoop ratio massaspectrometrie (LC/IRMS) en gaschromatografie verbrandings isotoop ratio massaspectrometrie (GC/C/IRMS). Om het monstervolume en de analytische inspanning te beperken, hebben we een methode ontwikkeld om gelijktijdig $^{13}$C glutathion, als ook het dimeer GSSG en zijn precursor 1-$^{13}$C glycine in een klein volume erythrocyten en in één analytische run te bepalen. Zowel de intra-erythrocyt concentratie als de $^{13}$C isotopische verrijking van GSSG en glycine kunnen worden bepaald in 150μL bloed met behulp van LC/IRMS.
Deze nieuwe methode biedt perspectief voor verschillende kinetische studies met relatief lage toediening van tracers, waardoor de totale onderzoekskosten lager worden.

**Hoofdstuk 7**

Hier wordt een observationele studie beschreven in premature pasgeborenen met een geboortegewicht kleiner dan 1000 gram waarin glutathion concentraties en aanmaaksnelheid worden bepaald op levensdag twee (wanneer kinderen slechts glucose kregen) en op dag zes (wanneer kinderen zowel parenterale voeding als voeding via de natuurlijk weg werd toegediend). In het bloedplasma werd de mate van oxidatieve stress bepaald. Op zowel dag twee als dag zes waren de oxidatieve stress markers verhoogd. Dit werd echter niet gecompenseerd door een toegenomen glutathion productie. Mogelijk wordt deze discrepantie veroorzaakt door het feit dat glutathion bepaald werd in rode bloedcellen, terwijl oxidatieve stress bepaald werd in bloedplasma door gebrek aan voldoende rode bloedcellen.

**Hoofdstuk 8**

Van cysteïne, één van de drie aminozuren waaruit glutathion is opgebouwd, wordt algemeen aangenomen dat het de limiterende bouwsteen is voor de glutathion aanmaak. Het heeft van deze drie aminozuren de laagste bloed concentratie. De veiligheid van het toedienen van het losse aminozuur cysteïne heeft volgens sommigen mogelijk bijwerkingen tot gevolg. Daarnaast is het onzeker of het lichaam het in losse vorm kan verwerken. In hoofdstuk 8 wordt een studie beschreven waarin we een groep premature

pasgeborenen de standaard hoeveelheid cysteïne aanwezig in de aminozuuroplossing toedienden en vergelijken dit met een groep die de dubbele hoeveelheid ontving. Hoewel er geen aanwijzingen werden gevonden voor intolerantie van de hogere concentratie cysteïne, was de concentratie van cysteïne in bloedplasma niet hoger in de groep die meer cysteïne ontving. Ook waren glutathion concentratie en aanmaak snelheid niet verhoogd.

**Hoofdstuk 9**

In hoofdstuk 9 en hoofdstuk 10 worden twee studies beschreven waar het aminozuur en eiwit metabolisme wordt gemeten in de foeto-maternale twee-eenheid vlak voor geboorte. Deze exploratieve studies geven inzicht in de fysiologische metabole processen van een individu gedurende het vroege leven.

In dit hoofdstuk wordt de albumine synthese snelheid gemeten in foetussen na ongeveer driekwart en aan het einde van de normale zwangerschapsduur (n=8 in iedere groep). Dit was mogelijk met behulp van een relatief nieuw onderzoeksmodel waarbij verscheidene tracers werden geïnfundeerd aan zwangere vrouwen en welke startten op verschillende tijdstippen in de uren voor een keizersnede. Door bloed af te nemen na geboorte vanuit de navelstreng, kon de foetale albumine synthese snelheid berekend worden. Terwijl de functies van albumine gedurende het foetale leven niet zo duidelijk omschreven zijn als gedurende het leven na geboorte, werden zeer grote hoeveelheden albumine geproduceerd door de foetus, vooral in de maanden voor de uitgerekende datum. Het feit dat de foetus zoveel albumine synthetiseert bij ongeveer 30 weken zwangerschapsduur, maar deze snelheid niet lijkt te continueren na premature geboorte, kan een aanwijzing zijn dat de voedingsstrategieën voor prematuren niet genoeg substraten leveren voor een snelle albumine synthese. Een andere verklaring kan zijn dat onder omstandigheden als ziekte of gebruik van specifieke medicatie de albumine synthese verlaagd is.

**Hoofdstuk 10**

In de studie beschreven in dit hoofdstuk werd het eiwitmetabolisme op geheel lichaamsniveau gemeten in 8 foetussen dicht bij de uitgerekende datum. Dit was mogelijk door zowel de bloedstroomsnelheid in de navelstreng te meten als de geschikte tracers van phenylalanine en tyrosine aan zwangere vrouwen te geven in de uren voor een keizersnede. Uit het navelstrengbloed konden we de eiwitsynthese en –afbraak snelheden berekenen. Tevens was het mogelijk de omzetting (hydroxylatie) van

phenylalanine in tyrosine te meten, een proces dat mogelijk minder verloopt in jonge en zeer zieke individuen.

Terwijl de foetus een behoorlijke opname vanuit de placenta liet zien, was de opname van tyrosine verwaarloosbaar klein. De foetale phenylalanine opname was zelfs verantwoordelijk voor één vierde van de netto katabole toestand van de moeder terwijl zij aan het vasten was als voorbereiding op de keizersnede. De foetus gebruikte de aminozuren voor een hoge eiwitsynthesesnelheid. Omgerekend naar weefsel, hadden foetussen een netto groeisnelheid van 12 gram/dag per kilo lichaamsgewicht bij een zwangerschapsduur van 38 weken. Voorts lieten de foetussen een aanzienlijke tyrosine productie zien, welke indicatief was dat de phenylalanine hydroxylatie onproblematisch verliep.

**Hoofdstuk 11**

In dit hoofdstuk wordt een algemene discussie gegeven waarin alle resultaten uit deze dissertatie nogmaals kritisch bekeken worden in het licht van de huidige literatuur. Verder worden er enkele aanbevelingen gedaan voor toekomstig onderzoek.

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Hopelijk kan ik nog veel heugelijke momenten met jullie allen delen.

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**Curriculum Vitae**

Henk Schierbeek was born on July 28th, 1956 in Coevorden, the Netherlands. After completing grammar school (lyceum "de baander" in Emmen), he finished his study in clinical chemistry at the Hanze Hogeschool in Groningen in 1979. After his graduation and his service in the army he started his research career at the department of Pediatrics in Groningen in 1980. After 11 year he left the university and accepted a position as specialist for mass spectrometry at the department of nutrition of the Nestlé research centre in Lausanne, Switzerland. In 1995 he returned to the Netherlands and started two enterprises in the restaurant business. After a year of traveling around the world he returned to mass spectrometry research in 2003 having a position at the joined mass spectrometry laboratory of neonatology and internal medicine, participating in several research projects headed by Prof. Dr. J.B. van Goudoever at the division of neonatology of the department of pediatrics in Erasmus MC - Sophia Children's Hospital in Rotterdam.

**Portfolio**

**Courses:**

- 1980: Biochemistry, High School for Technical Education, Groningen, The Netherlands.

- 1981: Gas Chromatography,Hewlett Packard, Amstelveen, The Netherlands.

- 1983: Mass Spectrometry, Hewlett Packard, Amstelveen, The Netherlands.

- 1985: Programme in basic, University of Groningen, Department of Mathematics, Groningen, The Netherlands.

- 1989: Writing of Scientific text, University of Groningen, Dep. of specialised training, Groningen, The Netherlands.

- 1990: Isotope Ratio Mass Spectrometry, Finnigan training group Finnigan MAT, Bremen, Germany.

- 2004: GC-EA/IRMS, Thermo Electron, Bremen, Germany.

- 2006: LC/IRMS, Thermo Fisher, Bremen, Germany.

- 2006: Intensive Course in Tracer Methodology in Metabolism, Stockholm, Sweden.



**Memberships:**

- 1983: NVMS (Nederlands Vereniging Voor Massaspectrometrie).

- 1990: GASIR (German Association for Stable Isotope Research).

- 2003: BASIS (Benelux Association for Stable Isotope Scientists).

- 2008: BMMS Britisch Mass Spectrometry Society.

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- 1992: **Henk Schierbeek**: Authenticity in food flavours. 2nd Seminar on modern methods of analysis. Lausanne (Switzeland) (Oral Presentation)

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- 2003: 16th International Mass spectrometry conference, Edinburgh (United Kingdom).

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- 2004 **H. Schierbeek**, J.W.O. van den Berg , H.L.Tjiong , T. Rietveld , G.R. Swart and M.W.J.A. Fieren. Determination of the albumin fractional sythesis during CCPD by GC-Combustion-IRMS using 1-13C Leucine as a tracer. Benelux Association of stable isotopes, Utrecht (The Netherlands) (Oral presentation)

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ASMS, Indianapolis (USA). (Poster presentation)

Société de Isotope Stable, Annuel meeting Lyon (France)

Benelux Association of stable isotopes, Leuven, (Belgium)

GASIR Meeting (German Association for Stable Isotope Research) Bayreuth, (Germany)

- 2008 **Schierbeek H**, Rook D, te Braake FW, Dorst KY, Voortman G, Godin JP, Fay LB, van Goudoever JB: Simultaneous analysis of (13)C-glutathione as its dimeric form GSSG and its precursor [1-(13)C]glycine using liquid chromatography/isotope ratio mass spectrometry. Jesium meeting, Presque ile de Giens, France (Poster presentation)

  Benelux Association of stable isotopes, Arnhem, (The Netherlands)

- 2009: **Henk Schierbeek**, Denise Rook, Frans W.J. Te Braake, Kristien Y. Dorst, Gardi Voortman, Johannes B. Van Goudoever. Simultaneous measurement of both concentration and 13C enrichment of glutathione and glycine in one single run using Liquid Chromatography coupled to Isotope Ratio Mass Spectrometry (LC-IRMS) Britisch Mass Spectrometry Society, BMMS meeting Glasgow, (Great Brittain) (Poster presentation)

  45th Anniversary meeting of the Dutch society for mass spectrometry, Kerkrade (The Netherlands)

  **Henk Schierbeek**. On-line mass spectrometry techniques for compound specific analysis in metabolic studies using stable isotopes. (An overview). Benelux Association of stable isotopes, Bruges, (Belgium) (Invited keynote lecture)

  GASIR Meeting (German Association for Stable Isotope Research) Potsdam, (Germany)

**List of abbreviations**

**$AgNO_3$:** Silver nitrate

**APE:** Atom Percent Excess

**APCI:** Atmospheric Pressure Chemical Ionisation

**APPI:** Atmospheric Pressure Photoionsation

**ASR:** Absolute Sythesis Rate

**BSIA:** Bulk Specific Isotope Analysis

**CO2:** Carbon dioxide

**CP:** Cerebral Palsy

**CRI:** Chemical Reaction Interface

**CuO:** Cupric oxide

**CSIA:** Compound Specific Isotope Analysis

**CV:** Coefficient of variation

**EA:** Elemental Analyzer

**EA/IRMS:** Elemental Analyzer-Isotope Ratio Mass Spectrometry

**ECF:** Ethyl choloroformate reagent

**EI:** Electron impact

**ESI:** Electrospray Ionization

**eV:** electron Volt

**FIA:** Flow Injection Analysis

**FIA/IRMS:** Flow Injection Analysis-Isotope Ratio Mass Spectrometry

**FSR:** Fractional Synthesis Rate

**GC:** Gas Chromatography

**GC/C/IRMS:** Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry

**GC/MS:** Gas Chromatography-Mass Spectrometry

**Glc:** Glucose

**Gly:** Glycine

**GMP:** Good Manufacturing Pratice

**GSH:** Glutathione

**GSSG:** Glutathione disulfide

**$H_2$:** Hydrogen

**IAEA:** International Agency Energy Atomic

**IRMS:** Isotope Ratio Mass Spectrometry

**KIE:** Kinetic Isotope Effect

**kV:** kilo Volt

**LC:** Liquid Chromatography

**LC-IRMS:** Liquid Chromatography-Isotope Ratio Mass Spectrometry

**LC/C/IRMS:** Liquid Chromatography-Combustion-Isotope Ratio Mass Spectrometry

**LOD:** Limit of Detection

**LOQ:** limit of quantitation

**Min:** Minute

**MPE:** Molar Percent Excess

**MS:** Mass Spectrometry

**mV:** milli Volt

**MW:** Molecular Weight

***m/z*:** mass over charge

**n:** Number of replicates

**Ni:** Nickel

**$O_2$:** Oxygen gas

**P:** Phosphorous

**PDB:** Pee De Belenmite

**Phe:** Phenyl alanine

**P&T:** Purge and Trap

**rms:** root mean square

**Rs:** Chromatographic resolution

**SD:** Standard Deviation

**sec:** Second

**SPME:** Solid Phase Micro Extraction

**TBW:** Total body water

**TEE:** Total energy expenditure

**Thr:** Threonine

**TOF:** Time-of-Flight

**Tyr:** Tyrosine

**V:** Volt

**Val:** Valine

**VPDB:** Vienna Pee De Belemnite

$\delta^{13}$**C, ‰:** Delta per mil

**$13_C$:** Carbon 13

**$18_O$:** Oxygen 18