Glycine conjugate detoxification profiling with sodium benzoate loading tests in a selected population of the North-West University

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ABSTRACT

The glycine conjugation system is an essential detoxification system needed to detoxify a variety of xenobiotics, including sodium benzoate, a very commonly used preservative. Recently more than 100 single nucleotide polymorphisms (SNP's) have been identified in the glycine N-acyltransferase enzyme system. These were identified during genetic research and none have been linked to a medical condition, although it may have a drastic effect on a person's detoxification and general health. No previous studies have been done to identify possible individuals with deficiencies in this system and possible complications due to such defects. The aim of this study was to determine glycine conjugation profiles of selected test groups after sodium benzoate loading tests, and to identify, according to our own classification, possible slow, medium and fast metabolizers. Hippuric acid, glycine, benzoylcarnitine and benzoic acid excretion in urine were monitored for 12 hours after the loading tests and an excretion profile for each test subject were obtained. A mean hippuric acid excretion curve was also obtained for every loading test and compared to that of the test persons. After the comparison possible slow, medium and fast metabolizers were identified which showed that there does indeed exists detectable variation in glycine conjugation efficiency. This opens a lot of possibilities for future research.

OPSOMMING

Die glisienkonjugerings-sisteem is 'n essensiële detoksifikasie-sisteem wat 'n verskeidenheid xenobiotikas detoksifiseer. Dit sluit natriumbensoaat, 'n algemene preserveermiddel, in. Huidig is daar al meer as 'n 100 enkel nukleotied polimorfismes (ENP's) in die glisien N-asieltransferase ensiemsisteem geïdentifiseer. Hierdie ENP's is tydens genetiese navorsing geïdentifiseer en is nie gekoppel aan enige siektetoestande nie. Dit kan egter 'n drastiese effek op 'n persoon se detoksifikasie en algemene gesondheid hê. Geen studies is al gedoen om individue met moontlike defekte in hierdie sisteem en die nagevolge daarvan te identifiseer nie. Die doel van die studie is om die glisien-konjugeringsprofiele van 'n geselekteerde toetsgroep na afloop van 'n natriumbensoaat-beladingstoets op te stel. Die profiele gaan dan gebruik word om moontlike stadige, medium en vinnige metaboliseerders, soos in die studie geklassifiseer, te identifiseer. Die uitskeiding van hippuursuur, glisien, bensoïelkarnitien en bensoësuur in uriene is vir 12 ure na die beladingstoets gemonitor. Hierna is 'n uitskeidingsprofiel vir elke proefpersoon opgestel. 'n Gemiddelde hippuursuur-uitskeidingskurwe is ook saamgestel vir elke beladingstoets en dit is vergelyk met die van die proefpersone. Na die vergelyking is moontlike stadige, medium en vinnige metaboliseerders geïdentifiseer. Dit wys waarneembare variasie is in die glisienkon-jugeringsweg se doeltreffendheid. Die observasie maak baie deure oop vir toekomstige studies.

LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
BA	Benzoic acid
CoA	Coenzyme A
cP450	Cytochrome P450
CPT	Carnitine palmitoyltransferase
Cr	Creatinine
ESI	Electrospray ionization
eV	Electron volt
g	Grams
Gly	Glycine
GLYAT	Glycine N-acyltransferase
HA	Hippuric acid
HCl	Hydrochloric acid
kg	Kilogram
l	Litre
mg	Milligram
min	Minutes
µl	Microlitre
ml	Millilitre
mM	Millimolar
MS	Mass spectrometry
nM	Nanomolar

NOAEL	No-observed-adverse-effect-level
Psi	Pounds per square inch
rpm	Revolutions per minute
sec	Second
SI	Stable isotope
SNP	Single nucleotide polymorphism
V	Volt

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CHAPTER 1:
BACKGROUND AND MOTIVATION

During the course of our life, we are all exposed to a great number of xenobiotics and toxins. Many of these substances are potentially damaging to the body. These substances include a variety of pharmaceuticals, pollutants and even substances present in food and drink (Liska, 1998). To metabolise and excrete these compounds, the body uses a variety of complex detoxification pathways. Detoxification increases the polarity of a compound to ease its excretion through urine. These enzyme systems generally function adequately to minimize the potential damage from xenobiotics, although many of them show little relationship to previously encountered metabolites (Murray, 2003). According to Liska (1998) these detoxification systems are highly complex, show a great amount of individual variability and are extremely responsive to the environment, lifestyle and genetic uniqueness of an individual.

One of these detoxification pathways conjugates the toxin with carnitine through a series of enzyme steps. Various defects associated with these conjugation enzymes can lead to a variety of metabolic diseases. An example of such a defect is the carnitine palmitoyltransferase 1 defect, or CPT1 defect, a well known metabolic disorder (John Hopkins University, 1995).

Another very important detoxification pathway involves conjugation with glycine (Meyer and Zanger, 1997). Glycine conjugation mainly takes place in the liver and the kidneys and is the main method of detoxification for a variety of substances. One of these substances is sodium benzoate, a preservative commonly found in our diets (JEFCA, 2005). During detoxification benzoate conjugates with glycine to form hippuric acid, which is freely excreted through the urine and possible negative side effects are thus limited. The enzyme used for this is glycine N-acyltransferase (Liska, 1998).

There is currently no metabolic disease described that involves a defect of the glycine conjugation pathway. Such defects are highly likely to exist because enzymes are involved and all enzymes are prone to mutations on DNA level. During routine metabolic investigations by the Laboratory for Inherited Metabolic Defects, School for Biochemistry, North-West University people with possible glycine N-acyltransferase

defects have been identified. Their urine contains very high levels of free benzoic acid, a metabolite that is usually present in very low concentrations in the urine of healthy individuals. Their urine also contains very low hippuric acid concentrations. Possible defects can however not be confirmed unless further research is done. This study attempts to address this issue.

CHAPTER 2:
LITERATURE OVERVIEW AND OBJECTIVES

2.1 METABOLISM

Metabolism describes the biochemical modification of all chemical compounds in living organisms. It can be defined as the total of all the enzyme-catalyzed reactions taking place within a cell. This includes the biosynthesis of complex organic molecules i.e. anabolism, as well as the breakdown of these molecules i.e. catabolism. Metabolism describes a sequence of enzymatic steps also known as metabolic pathways (Mathews *et al.*, 2000). Although there are relatively few metabolic pathways, they form a highly integrated network. That is, each individual metabolic pathway is linked through shared substrates to complex networks. Metabolic pathways can be broken down into individual, enzyme-specific, catalysed steps (Garrett and Grisham, 1999).

Mathews *et al.* (2000) state that metabolism has two main functions. First, it provides all the energy that is required to maintain the function and composition of the cell. Secondly, it provides the metabolites that are required to synthesize cell components and products.

Figure 2.1 is a schematic representation of a metabolic pathway. In figure 2.1 (i) A is the initial substrate, B and C are intermediate products and D is the final product. D is synthesized through a series of enzyme-catalyzed reactions. Product E can also be synthesized from substrate A by a minor route, but the amount of E produced is normally far less than D. However, if for example enzyme 3 is deficient, less C and D will accumulate, with a resultant increase in the levels of A, B and E, as shown in figure 2.1 (ii). The excess A, B and E can be toxic if alternative pathways are not available to counter their accumulation. If there is not a specific pathway to get rid of these molecules, the body will try to inactivate and excrete them (Mathews *et al.*, 2000; Teal and Saggers, 1997). Wilson (2002) describes this phenomenon as detoxification, i.e. the removal of toxins. A toxin is any substance that causes damage to the structure of cells or a disturbance in their function, leading to illness or death.

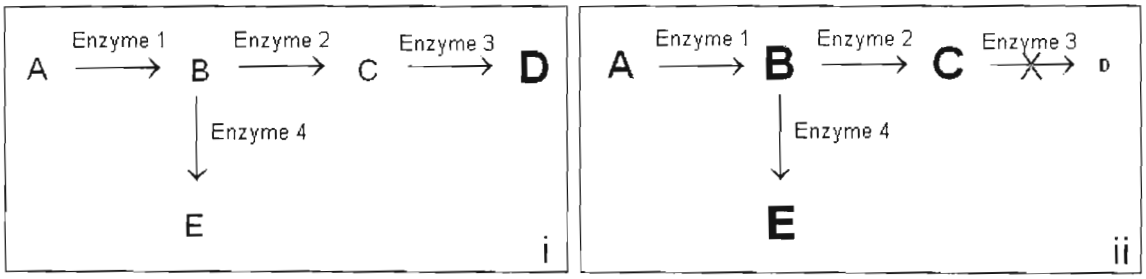

Figure 2.1. A schematic representation of a general metabolic pathway: normal metabolism (i) and impaired metabolism (ii) (adapted from Teal and Saggers, 1997)

Toxicity can be molecular or physiological and is present at every level of functionality and structure in the body. As a result, detoxification must also take place at appropriate levels in order to be efficient. Unfortunately multi-level detoxification is complicated because toxin elimination from the body has only a limited number of exit pathways i.e. the bowel, respiratory tract, skin and urinary tract. Water-soluble toxins are easily cleared by all four pathways. Oil soluble toxins, on the other hand, must enter the liver where they are degraded into water-soluble substances and eliminated through the urinary pathway. They can also remain fat soluble and are then carried in the bile through the intestinal tract and eliminated with ingested fibre (Wilson, 2002).

2.2 DETOXIFICATION

The human body is constantly exposed to a great number of toxins, known as xenobiotics. A xenobiotic is a chemical substance which is not natural to the organism. It includes naturally occurring compounds that are present in concentrations much higher than normal, drugs, environmental agents, carcinogens and insecticides (Duffus, 2005).

According to Liska (1998) most of these compounds, which the body is capable of detoxifying, have no relationship to previously encountered metabolites. In order to achieve this, the human body has detoxification systems that can adequately minimize the potential damage caused by xenobiotics. The detoxification system is extensive, highly complex and influenced by a myriad of regulatory mechanisms. There is large

individual variability in these pathways. They are also extremely responsive to the environment, lifestyle and genetic uniqueness of the individual.

Liska (1998) explains that it is likely that there is an association between impaired detoxification and disease. Thus, the ability of an individual to detoxify xenobiotics may play a role in the etiology of the exacerbation of a range of chronic conditions such as cancer, Parkinson's disease, chronic fatigue and immune dysfunction syndrome.

The primary line of defence of the body against metabolic toxicity is in the liver (Cabot, 2003). The liver has several roles: it filters the blood to remove large toxins, synthesizes and secretes bile full of cholesterol and other fat-soluble toxins and enzymatically neutralizes xenobiotics. These enzymatic processes usually occur in a two step process referred to as phase I and phase II detoxification (Broe and Broe, 2005). Phase I and II detoxification is schematically summarized in figure 2.2.

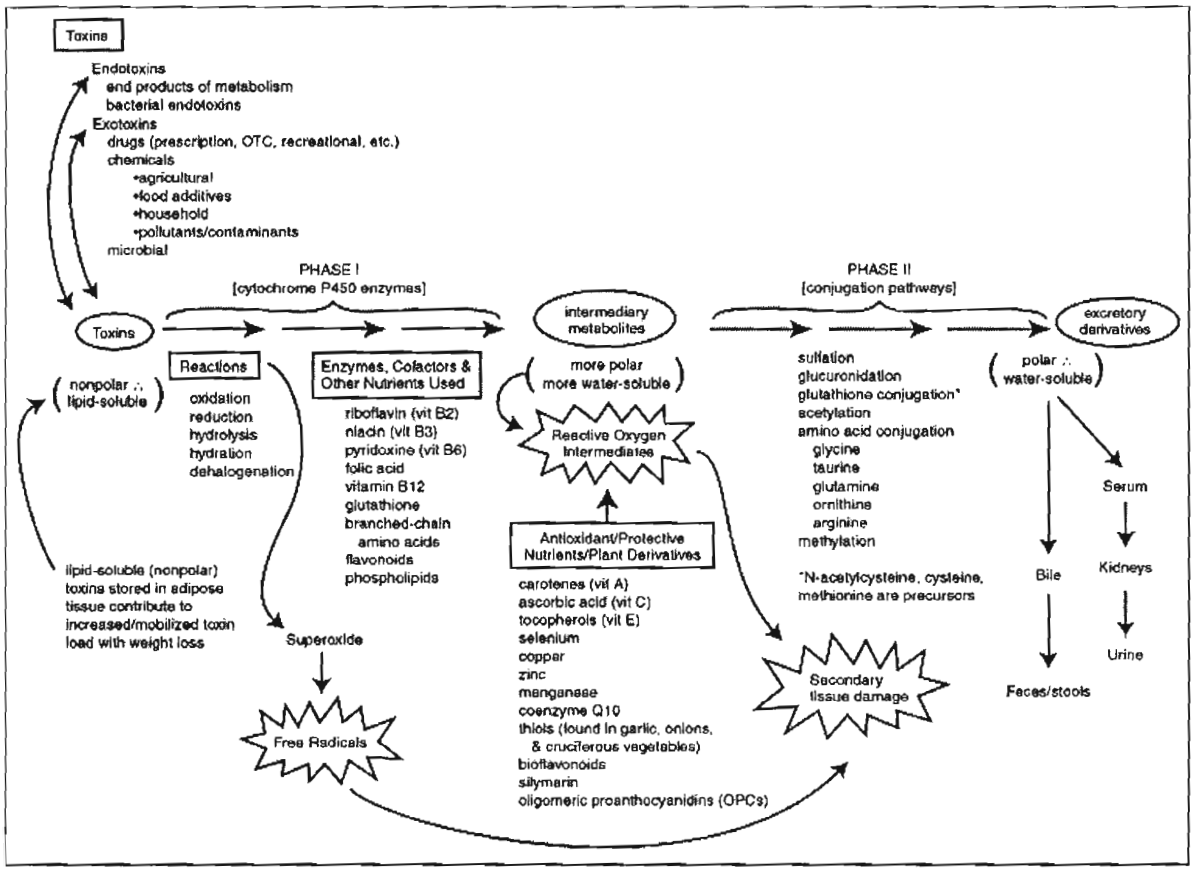

Figure 2.2. A summary of the two phases of detoxification (Liska, 1998)

During phase I detoxification, the xenobiotic compound is either directly neutralized or it is functionalized by oxidation, hydrolysis or reduction to form hydroxyl-, amino-, carboxyl- or thiol-containing molecules, i.e. the activated primary metabolites. In phase II, these primary metabolites undergo conjugation reactions with endogenous agents to form secondary metabolites. Phase II detoxification biotransformation does not only lead to an inactivation of the original agent and its primary metabolites, but also to increased hydrophilicity and thus enhanced excretion (Broe and Broe, 2005; Levsen *et al.*, 2004).

The primary metabolites are chemically much more active and therefore also potentially more toxic. If the phase II detoxification systems are not working properly, the accumulated intermediates can cause substantial damage and can be carcinogenic. An imbalance between phase I and phase II can occur if a person is exposed to large amounts of xenobiotics. Under these conditions, the critical nutrients needed for phase II detoxification are depleted, allowing the highly toxic activated intermediates to accumulate (Broe and Broe, 2005; Cabot, 2003).

2.2.1 Phase I detoxification

The phase I detoxification system composes mainly of the cytochrome P450 (cP450) super gene enzyme family. It is generally the first enzymatic defence against xenobiotics (Liska, 1998). These enzymes, situated in the mitochondrial membrane, mainly occur in the liver and to a lesser extend in the intestines and lungs (Alschuler, 2002). Almost 100 enzymes make up the cytochrome P450 system. The cP450 enzyme systems are quite diverse and a few examples of the enzymes involved in phase I detoxification are shown in table 2.1. Each enzyme preferably detoxifies a given xenobiotic. There is, however, overlapping activity (Broe and Broe, 2005).

Table 2.1. Examples of the Cytochrome P450 enzyme family (adapted from Liska, 1998)

P450 enzyme	% of total P450	Substrates
Cyp3A4,5	28.8±10.4	Cyclosporin Nifedipine Testosterone
Cyp2C8,9,18	18.2±6.7	R-mephenytoin Tolbytamide S-wafarin
Cyp1A2	12.7±6.2	Phenacetin Caffeine Aflatoxin B1
Cyp2E1	6.6±2.9	Ethanol Carbon tetrachloride Dimethylnitrosamine
Cyp2A6	4.0±3.2	Coumarin Dimethylnitrosamine
Cyp2D6	1.5±1.3	Debrisoquine Sparteine Bufuralol
Cyp2B6	0.2±0.3	Cyclophosfamide

Alschuler (2002) states that the main function of the cP450 system is to convert fat-soluble toxins into water-soluble, polarized compounds. These compounds can then be conjugated by phase II pathways and excreted in the bile or urine. In a typical phase I reaction, a cytochrome P450 enzyme uses oxygen and NADH to add a reactive group, such as a hydroxyl radical, to the xenobiotic. This produces reactive molecules which may be even more toxic than the parent molecule. If these reactive molecules are not further metabolized by phase II conjugation, they can cause widespread problems, especially stimulating carcinogenesis. In order to prevent this, the rate of production of activated intermediates in phase I must be balanced by the rate at which phase II finishes neutralizing them (Liska, 1998; Broe and Broe, 2005).

2.2.2 Phase II detoxification

According to Cabot (2003) phase II detoxification typically involves conjugation in which various enzymes in the liver attach an endogenous compound to the xenobiotic in order to render it less harmful. This either neutralizes the xenobiotic or makes it water-soluble to ease excretion through urine or bile. Phase II detoxification reactions may act directly on some xenobiotics, while others must first be activated by the phase I enzymes (Broe and Broe, 2005).

There are essentially six phase II detoxification pathways (table 2.2) which include glutathione and amino acid conjugation, methylation, sulfation, acetylation and glucuronidation (Broe and Broe, 2005). In contrast to exothermic phase I biotransformation, phase II conjugation reactions are endothermic processes. For phase II detoxification, either the conjugation agent or the xenobiotic compound must be activated (Levsen *et al.*, 2004). The xenobiotic is linked to the conjugation agent through a functional group that may be present on the original xenobiotic or which is the result of a phase I reaction. In many conjugation reactions, the proton present in a hydroxyl, amino or carboxyl group is replaced by the conjugation agent (Lohr *et al.*, 1998).

Table 2.2. Major phase II detoxification activities (adapted from Liska, 1998)

Reaction	Enzyme	Cellular localization	Substrate
Glutathione	Glutathione transferases	Microsomes	Electrophiles
Glucuronic acid (UDPGA)	Glucuronyl transferases	Microsomes	Phenols, thiols, amines, carboxylic acids
Sulfuric acid (PAPS)	Sulfotransferases	Cytosol	Phenols, thiols, amines
Methyl group (SAM)	N- and O- methyl transferases	Microsomes Cytosol	Phenols, amines
Acetic acid (Acetyl-CoA)	N-acetyl transferases	Cytosol	Amines
Amino acids (Acetyl-CoA, taurine, glycine)	Amino acid transferases	Microsomes	Carboxylic acids

(Abbreviations in brackets are the co-substrates: UDPGA = uridine-3′,5′-diphosphoglucuronic acid; PAPS = 3′-phosphoadenosine 5′-phosphosulfate; SAM = S-adenosylmethionine; CoA = coenzyme A)

Specific enzymes act on the conjugation molecules in order to catalyze conjugation. To function properly, these enzyme systems need nutrients for both their activation and to provide the small molecules they attach to the xenobiotic. In addition, the systems utilize metabolic energy to synthesize some of the small conjugating molecules. All these reactions require cofactors which must be replenished through dietary sources (Broe and Broe, 2005; Liska, 1998).

2.3 AMINO ACID CONJUGATION

Amino acid conjugation starts with coenzyme A (CoA). CoA is a large molecule derived from ATP, pantothenic acid and β-mercaptoethylamine (Mathews *et al.*, 2000). CoA activates the xenobiotic that it attaches to and provides a site where an amino acid can conjugate with the xenobiotic. Conjugation increases the hydrophilicity and molecular weight of a xenobiotic and makes it more amenable for excretion (Levsen *et al.*, 2004).

In humans glycine, taurine, glutamine, arginine and ornithine can be conjugated. According to Broe and Broe (2005) glycine is most commonly utilized during phase II amino acid detoxification. These conjugation reactions occur with substrates containing an alcohol or a carboxyl moiety, and especially those substrates with aromatic groups. The acid or alcohol combines with the amino acid to form an amide bond. The enzymes that facilitate these conjugation reactions are called transferases.

The general amino acid conjugation reaction is as follows (Lohr *et al.*, 1998)

$R(CO)OH + NH_2CH_2(CO)OH = R(CO)NHCH_2(CO)OH$

2.3.1 Glycine conjugation

Kasuya *et al.* (1996) state that many xenobiotic carboxylic acids undergo conjugation with glycine. This reaction forms an important route for detoxification of carboxylic acids such as aromatic, heteroaromatic, arylactetic and aryloxyacetic acids. Glycine conjugation detoxifies amongst others, sodium benzoate, a common preservative, and aspirin, a common medicine (Alschuler, 2002).

The pathway consists of two sequential reactions resulting in the joining of the carboxylic acid to the amino acid nitrogen (Van der Westhuizen *et al.*, 1999). During the first reaction the carboxylic acid is activated to an intermediate acyl-CoA in an ATP-dependent reaction catalyzed by acyl-CoA synthetase. The acyl group is then transferred to the amino group of glycine by glycine N-acyltransferases. It is not known whether the specificity of glycine conjugation is exerted at the activation step and/or at glycine transfer (Kasuya *et al.*, 1996).

There is wide variation in the activity of the glycine conjugation pathway. According to Broe and Broe (2005) this is due to genetic variation and availability of glycine in the liver. Glycine and the other amino acids that are used for conjugation, become deficient when a person is on a low-protein diet. This, together with chronic exposure to xenobiotics can cause depletion of the amino acids.

2.3.2 Glycine N-acyltransferase

An example of glycine conjugation is the formation of hippuric acid from benzoic acid and glycine (figure 2.3). Hippuric acid is more polar in solution, has different excretion rates than the original benzoic acid, and is readily excreted in the urine. The enzymes that catalyze the conjugation reactions are acyl-CoA synthetase [EC 6.2.1.3] and glycine N-acyltransferase (GLYAT) [E.C.2.3.1.13]. Both are present in liver and kidney mitochondria (Lohr *et al.*, 1998; Feoli-Fonseca, 1995).

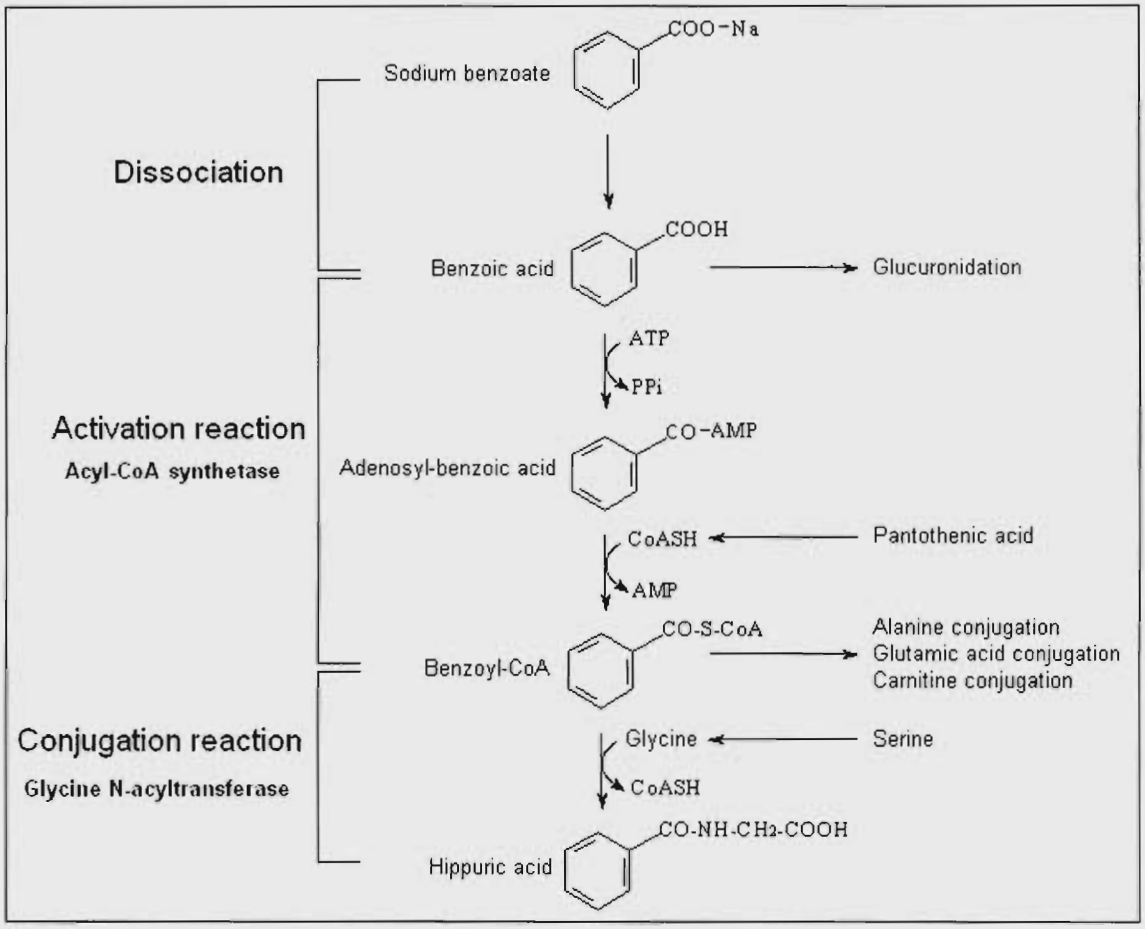

Figure 2.3. A summary of glycine conjugation of benzoate

Lohr *et al.* (1998) explain that two factors limit the rate of hippuric acid synthesis, depletion of coenzyme A and the availability of glycine. During conjugation glycine is consumed and Co A is regenerated each time a molecule of hippuric acid is synthesized. If glycine levels fall and limit the rate of this reaction, CoA is trapped as benzoyl CoA. These activated intermediates effectively denies CoA to other metabolic processes, including the production of more benzoyl CoA. In effect, this limits the supply of benzoyl CoA for conjugation, placing a limit on the reaction rate.

et al. 1999). Benzoylalanine is normally detected in urine of hyperammonemic patients

2.4 DIFFERENT COMPONENTS OF GLYCINE CONJUGATION OF BENZOIC ACID THAT ARE FACILITATED BY GLYCINE N-ACYLTRANSFERASE

The next section contains a brief discussion of some of the components involved in the detoxification of sodium benzoate.

2.4.1 Glucuronidation

It was formerly believed that at rates faster than the maximal rate of hippurate synthesis benzoic acid was shunted to the glucuronic acid conjugation pathway, as explained by Lohr *et al.* (1998). More accurate techniques for the measurement of excreted glucuronic acid conjugates showed that glucuronidation is found at very low levels of benzoic acid. Glucuronic acid conjugation with benzoic acid increases proportionally with benzoic acid concentration, showing no relationship with glycine availability or the maximal rate of hippuric acid formation. Glucuronidation of benzoic acid to form hippuric acid is not a reserve pathway for benzoic acid elimination but rather a second pathway operating completely independent of glycine conjugation.

2.4.2 Pantothenic acid and coenzyme A

Pantothenic acid (vitamin B_5), a component of coenzyme A, is essential in a variety of reactions. CoA was first named for its role in acetylation reactions. Most acetylated proteins are modified by the addition of an acetate group donated by CoA. Protein acetylation affects the 3-dimensional structure of proteins, potentially altering their function and activity (Garrett and Grisham, 1999).

2.4.3 Other amino acid substrates for Glycine N-acyltransferase

Glycine N-acyltransferase also facilitates the conjugation of alanine and glutamic acid. Conjugation of these amino acids with benzoyl-CoA is, however, extremely low relative to glycine conjugation. It is unlikely that alanine and glutamic acid will contribute significantly to detoxify benzoyl-CoA under normal circumstances (Van der Westhuizen *et al.*, 1999). Benzoylalanine is normally detected in urine of hyperammonemic patients treated with very large amounts of sodium benzoate (Shinka et al., 1985).

2.4.4 Carnitine

Carnitine conjugation is another competitive pathway for glycine conjugation through the CoA thioester. Benzoic acid is, however, a much better substrate for glycine conjugation. Carnitine transferase has more substrate specificity for the cyclic side chain carboxylic acids with fewer carbon atoms, while glycine N-acyl transferase has inverse specificity (Kanazu and Yamaguchi, 1997).

2.4.5 Glycine

The most important limiting factor in the biosynthesis of hippuric acid is the availability of glycine, which gets depleted easily with high intake of benzoate. (Pölönen *et al.*, 2000; Wibbertmann *et al.*, 2000). It gets depleted because there is no storage pool for glycine. In most mammals, glycine is constantly synthesised and catabolized via the glycine cleavage system (Rittenberg and Schoenheimer, 1938).

2.4.6 Serine

Mathews *et al.* (2000) state that serine is involved in the glycine cleavage system, the principal biosynthetic route of glycine. Serine and tetrahydrofolate is catalyzed by transhydroxymethylase to form glycine and 5,10-methylene-tetrahydrofolate respectively.

2.4.7 Sodium benzoate and benzoic acid

According to Reynolds (1993) benzoates possess antibacterial and antifungal properties and are commonly used preservatives in pharmaceuticals, cosmetics, food and drinks. The antimicrobial activity is due to the undissociated benzoic acid and is therefore pH-dependant i.e. it is relatively inactive above a pH of 5. Approximately 0,1% benzoic acid is usually sufficient to preserve a product that has been properly prepared and adjusted to a pH 4,5 or below. Benzoic acid is also naturally present in certain milk products, fruits, beans, cereals, soya flour and nuts (Wibbertmann *et al.*, 2000).

Wibbertmann *et al.* (2000) explain that under acidic conditions, like those in the stomach, sodium benzoate converts to undissociated benzoic acid. Sodium benzoate is about 200 times more soluble in water than benzoate and is therefore often used as an

alternative to benzoate. As a result, the metabolism and systemic effects of benzoic acid and sodium benzoate are viewed and evaluated together.

After oral uptake, benzoic acid are rapidly absorbed from the gastrointestinal tract, metabolized in the liver and excreted. Owing to rapid metabolism and excretion, an accumulation of benzoates or their metabolites are not expected (Kubota and Ishizaki, 1991; Reynolds, 1993). These substances are mainly metabolized by glycine conjugation and almost entirely excreted as hippuric acid (JEFCA, 2005).

When sodium benzoate is conjugated with glycine and excreted as hippuric acid it facilitates an alternative pathway of nitrogen excretion. It is therefore used in the treatment of hyperammonemia, particularly in infants with inborn errors of urea synthesis (Reynolds, 1993).

According to literature, like the summaries made by the World Health Organization, benzoate and its salts are only toxic at very high doses. The no observed adverse effect level (NOAEL) was calculated as 500mg/kg body weight (Wibbertmann *et al.*, 2000).

2.4.8 Hippuric acid

After glycination, benzoic acid converts to hippuric acid, and therefore hippuric acid can be used as a representative of glycine detoxification (Geng and Pang, 1998). Hippuric acid has the disadvantage that significant variation exists between individuals, depending on environmental factors and individual characteristics (Alvarez-Leite *et al.*, 1999). Because of this variation, a normal range of hippuric acid excretion cannot be determined.

2.4.9 The rate of glycine conjugation of benzoic acid

The rate of glycination of benzoic acid is high. In humans, the glycination of oral doses of between 40 and 160mg sodium benzoate/kg body weight is independent of the dose. This calculates to glycination of about 17-29 mg benzoate per kg body weight per hour. This corresponds with the NOAEL value of about 500mg/kg body weight per day (Kubota and Ishizaki, 1991; Wibbertmann *et al.*, 2000).

Benzoic acid is rapidly absorbed and rapidly excreted in the urine. 100% absorption can be assumed (JEFCA, 2005). In normal individuals, 50-85% of the given dose of

benzoate is excreted within two hours, 70-95% within four hours and the rest within two to three days (Van Sumere *et al.*, 1969; Wibbertmann *et al.*, 2000).

2.5 POSSIBLE DEFECTS IN THE GLYAT SYSTEM

According to Sheth and Brunton (2002) there exists large variability in drug metabolism. Its impact on inter-individual responsiveness to the same dose of a given drug has received considerable attention in past studies. Drug metabolism is affected by numerous factors of both environmental and genetic origin. A substantial portion of the population may have altered metabolism for certain substances due to genetic factors, such as mutations, that substantially affects their ability to metabolize specific drugs. These individuals may display diminished capacity for phase I and II detoxification and are normally referred to as slow metabolizers. Such individuals tend to accumulate substantially higher substance concentrations than normal metabolizers, which increases their risk for related adverse affects (Meyer, 2000; Sheth and Brunton, 2002).

It is important to recognize that each individual is genetically unique. As a result drug efficacy may vary up to 100 fold amongst individuals within a general population (West *et al.*, 1997). Inter-individual variability is also observed with regard to adverse effects following drug administration. Reductions in the rate of drug catabolism to inactive products may lead to an increased incidence of these undesirable effects. It has been shown that variability in responsiveness to the same dose of a given drug may result from mutations that alter the metabolism of drugs (Sheth and Brunton, 2002).

Genetic polymorphisms of drug metabolism are relatively common occurrences. Mutations in the genes of drug-metabolizing enzymes may result in enzyme variants with reduced or altered activity, or may even result in the absence of an enzyme (Meyer and Zanger, 1997). Based on the differences in drug metabolism, the general population may be subdivided into slow, normal and fast metabolizers (figure 2.4). Meyer (2000) explains that slow metabolizers are characterized by an increased metabolic ratio i.e. the ratio between parent substance concentration and an excreted metabolite concentration in the urine. Typically, the metabolic rate between parent drug concentration and metabolite concentration for drugs with genetic polymorphism exhibits a bimodal or trimodal frequency of distribution in the general population.

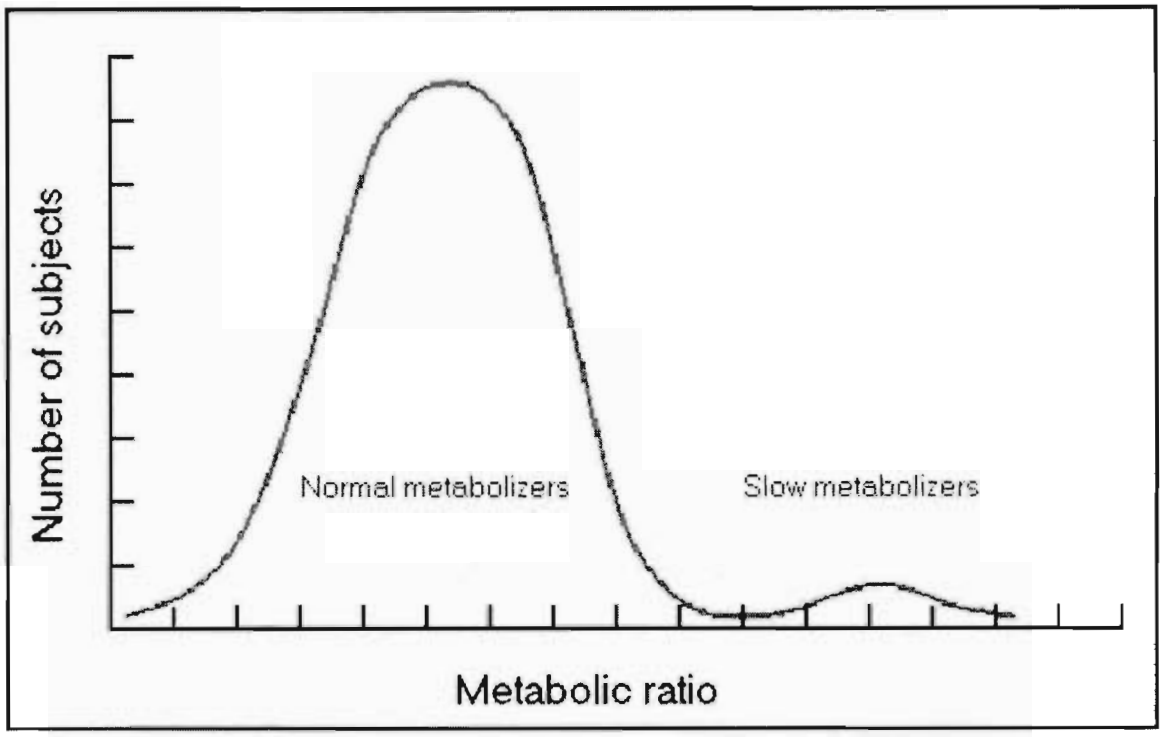

Figure 2.4. The occurrence of normal and slow metabolizers in medicine metabolism (Adapted from Sheth and Brunton, 2002)

The concept of slow metabolizers can be related to any metabolic pathway, including detoxification. Therefore, there will also be slow, normal and fast metabolizers for glycine conjugation. Slow metabolizers will show adverse effects if they are subjected to high concentration of sodium benzoate through diet. Although no polymorphisms relating to a glycine N-acyltransferase defect has been described, it is likely that mutations in this enzyme can cause severe defects. According to Genbank 118 SNP's have already been identified in the glycine N-acyltransferase gene, although none has been linked to a specific genetic condition (NCBI Entrez, 2007).

People with possible defects in the glycine conjugation pathway have also been identified through metabolic screening at the Laboratory for Inherited Metabolic Defects, School for Biochemistry, North-West University. Their organic acid urinary profiles show abnormally high concentrations of free benzoic acid and very low hippuric acid levels.

If the GLYAT enzyme system is put under pressure, it is possible that persons with decreased or differentiated enzyme activity can be identified. It was postulated that a

sodium benzoate loading test will put the glycine detoxification system under enough pressure so that individuals with abnormal glycine N-acyltransferase activity can be identified. These individuals will then be studied for possible defects in the glycine detoxification pathway.

2.6 AIM AND OBJECTIVES

Aim

To identify individuals with decreased or increased glycine N-acyltransferase activity to be used for further characterization of the possible errors in the GLYAT system.

Objectives

- To determine if a sodium benzoate loading test will put pressure on glycine N-acyltransferase system to differentiate between different test persons.
- To determine the excretion profile of hippuric acid, glycine, benzoylcarnitine and benzoic acid for test subjects after a sodium benzoate loading test.
- To get the mean excretion curve for test subjects that participated in the loading test.
- To identify possible slow, medium and fast metabolizers based on the mean excretion curve.

CHAPTER 3:
EXPERIMENTAL METHODS

3.1 STRATEGY

During an extensive literature search no studies describing individuals with possible deficiencies of the glycine detoxification system and complications related to a deficiency have been found. In this study glycine conjugation will be monitored as urinary excretion of hippuric acid, glycine, benzoylcarnitine and free benzoic acid following a sodium benzoate loading.

After the loading test changes in urinary hippuric acid, glycine, benzoylcarnitine and benzoate will be followed, with sampling every hour for the first 6 hours and then after 9 and 12 hours.

ESI/MS/MS will be used to measure hippuric acid and glycine, because no extraction is required. It is also a rapid and sensitive method to identify and quantify these metabolites in urine. Stable isotopes will be used for quantification. Benzoylcarnitine and benzoate will be quantified with methods standardized by the Laboratory for Inherited Metabolic Defects, School for Biochemistry, North-West University.

3.2 SUBJECTS

25 healthy volunteers participated in the study. They completed a questionnaire about their general health (Annexure A) and liver function tests (Serum gamma-GT; serum-ALT and serum-AST) were done by the laboratory of Drs. Du Buisson, Bruinette and Kramer.

The exclusion criteria were as follows:

(1) A history of liver disease,

(2) Insufficient liver function according to test results,

(3) The use of antibiotics or any dietary supplements within 4 weeks prior to the start of the study.

The study was only done after the procedures were fully explained to each subject and they gave written consent. The study protocol was approved by the Ethics Committee of the North-West University (project 06M03).

3.3 STUDY DESIGN

The loading tests were done under the supervision of Dr. GM Meyer (MP 0306401) in the Metabolic Unit of the North West University. Sodium benzoate was dissolved in 250ml water and administered orally. Initially a dose of 500mg sodium benzoate/kg body weight was given to 10 volunteers. Due to side effects this dose was discontinued. A dose of 250mg/kg was given to five volunteers. It was also discontinued due to side effects. Lastly a dose of 150mg/kg body weight was given to 10 test persons.

The volunteers had no breakfast on the day of the loading, and fasted for 6 hours after ingesting the sodium benzoate. They had water at lib.

Urine samples were collected at 0 (predose), 1, 2, 3, 4, 5, 6, 9 and 12 hours (postdose) and stored at -4°C till analysed.

3.4 HISTORIC BACKGROUND OF TANDEM MASS SPECTROMETRY

For the past few decades mass spectrometry and more recently tandem mass spectrometry has been used as a diagnostic tool to identify genetic diseases. Tandem mass spectrometry coupled with electrospray ionization has decreased turn around time dramatically. Amino aciduria, organic aciduria and β-oxidation defects are all examples of defects that can easily be diagnosed with tandem mass spectrometry (Hardy, 1999).

Mass spectrometry is an analytical method that identifies compounds on the bases of their mass and charge. Soft ionization techniques such as electrospray mass spectrometry result in ionization spectra with little or no fragmentation. Fragmentation must be induced by a special collision cell (Hardy, 1999).

Figure 3.1 is an example of fragments formed by ionization of hippuric acid. In this study the 105m/z ion was used as precursor ion for identification and quantification of hippuric acid.

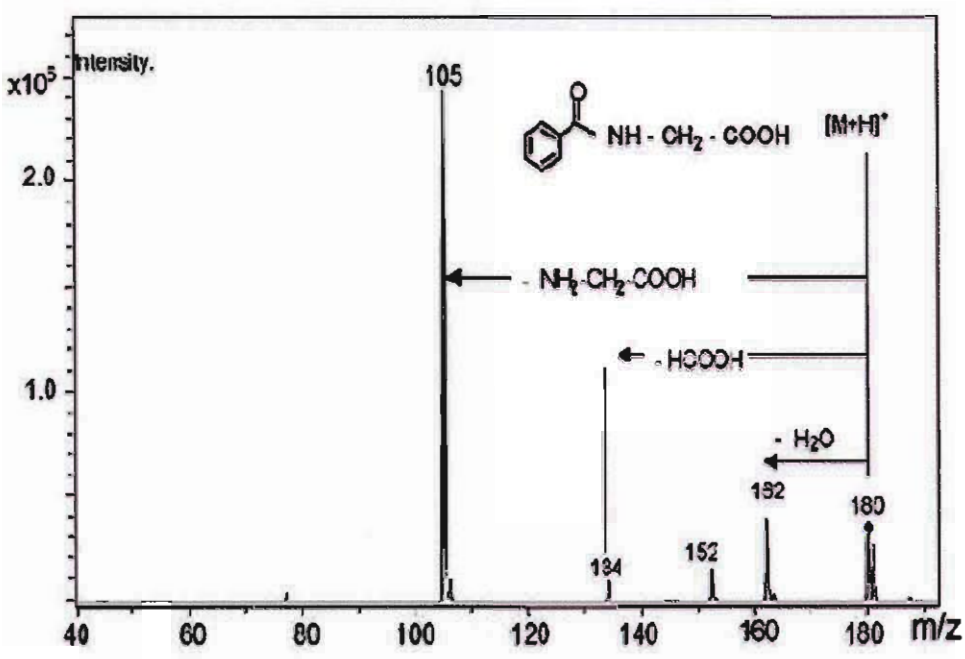

Figure 3.1. Electrospray ionization fragmentation spectrum of hippuric acid (Levsen, *et al.*, 2004)

3.4.1 Measuring hippuric acid with tandem mass spectrometry

For hippuric acid and its stable isotope the precursor ions of 105m/z and 110m/z were monitored in the range of 220 to 250m/z. By alternatively switching the scan function from parents of m/z 105 to parents of m/z 110, data from the stable isotope and the authentic hippuric acid were acquired simultaneously in the urine samples. The hippuric acid parent ion was 236m/z and the D5-stable isotope parent ion was 241m/z (figure 3.2).

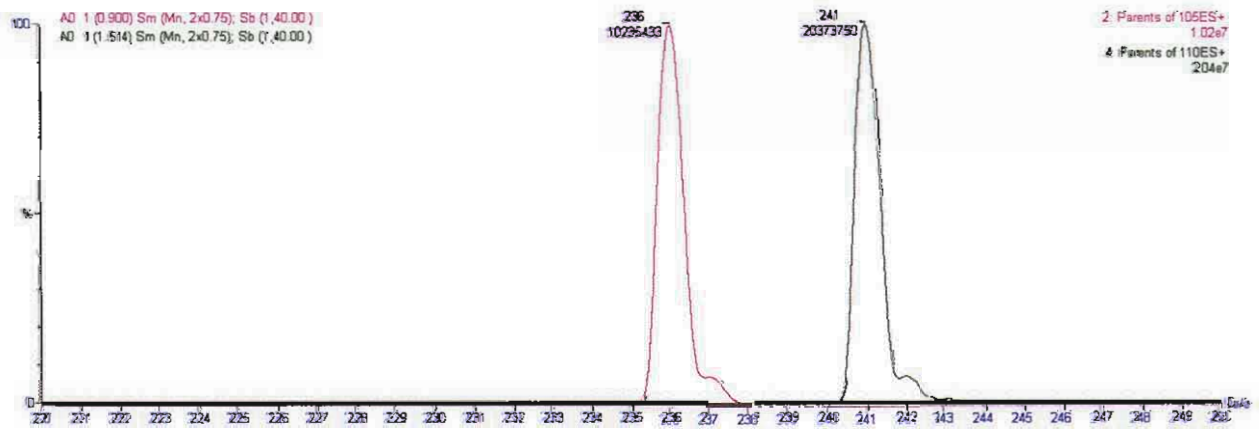

Figure 3.2. Example of hippuric acid (green) and hippuric acid stable isotope (purple) mass spectrum

3.4.2 Measuring glycine with tandem mass spectrometry

For glycine and its stable isotope a neutral loss of 56m/z was monitored in the range of 120 to 140m/z. The glycine parent ion was 131m/z and the D2-stable isotope parent ion was 133m/z (figure 3.3).

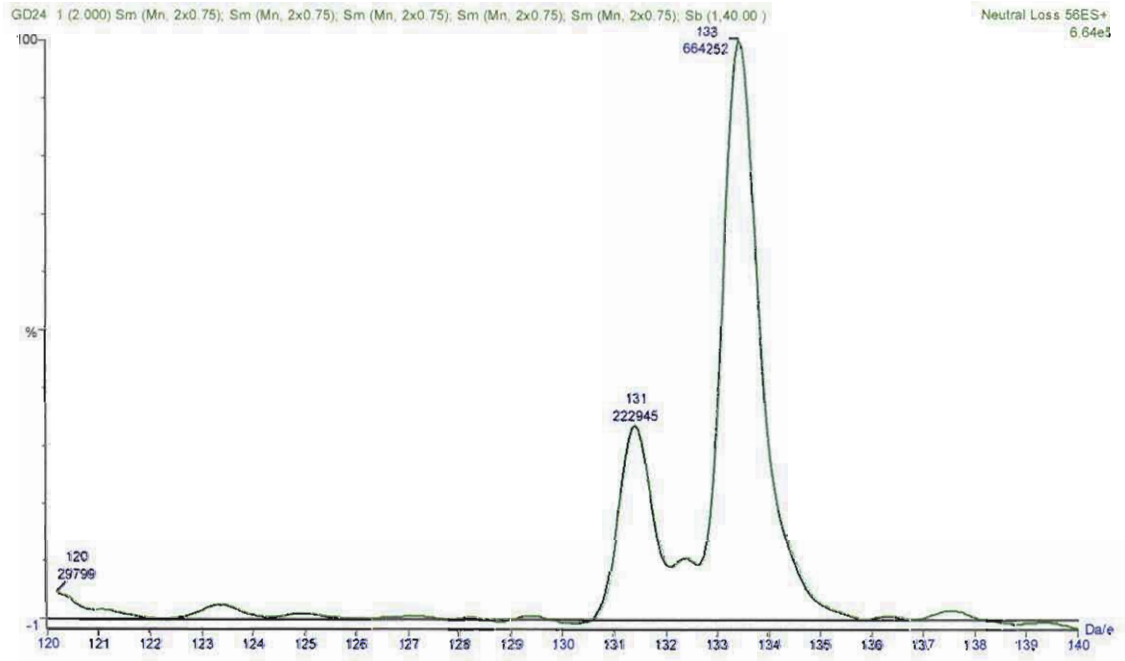

Figure 3.3. Example of glycine and glycine stable isotope ionization spectrum

3.5 HIPPURIC ACID AND GLYCINE ANALYSIS

3.5.1 Reagents, equipment and materials

Table 3.1. Reagents, equipment and material used for hippuric acid and glycine analysis

Reagent/Equipment/Material	Company
Eppendorf tubes	Merck BRAA780500
Hippurate-D5	Cambridge Isotope Laboratories, Inc
Glycine-D2	Euroisotop, Gif-sur-yvette
Acetonitrile	Merck BC15256Q
Butanol	Sigma 281549
Acetylchloride	Sigma 239577
Centrifuge	Optolabor BHG 1100
Evaporating adaptor with nitrogen	Afrox
Dri-Block	Techne DB-3
Electrospray tandem mass spectrometer	Waters Micromass Quatro micro API triple quadrupole mass spectrometer
High Pressure Liquid Chromatograph	Hewlett-Packard 1090
Software for data analysis	Masslynx^{TM} Micromass

3.5.2 Procedure

The urine samples were thawed at room temperature. 50µl urine and 50µl stable isotope solution was transferred into eppendorf tubes. This was then centrifuged at 13 000 x rpm for 20 minutes. 50µl of the supernatant was transferred to a new eppendorf tube, 50µl 80:20 acetonitrile:distilled water was added and it was again centrifuged at 13 000 x rpm for 20 minutes. The supernatant was carefully transferred to a new tube

to prevent disturbing the pellet. This final mixture was dried under nitrogen for 45 minutes at 65°C. When dried, 200µl butanolic HCl was added, and the samples were left to stand for 15 minutes at 65°C. After butylation the samples were dried for 1 hour. The remaining residue was dissolved in 100µl 80:20 acetonitrile:distilled water.

The stable isotope mixture for the hippuric acid analysis consisted of 1g/l hippurate-D5 (Cambridge Isotope Laboratories, Inc, Andover, MA, USA) dissolved in 80:20 acetonitrile:distilled water. The stable isotope solution for the glycine analysis was 0.5g/l glycine-D2 (Euroisotop, Gif-sur-yvette, France) dissolved in 80:20 acetonitrile:distilled water.

3.5.2.1 Preparation of butanolic HCl

A glass container was washed with butanol and then 50ml butanol was poured in the container. To prevent evaporation parafilm was used to cover the container. The butanol was left on ice for 5 minutes. 12,5ml acetylchloride was added drop-wise while continuously mixing. The butanolic HCl was again wrapped with parafilm and kept on ice for 20 minutes before use.

3.5.3 Electrospray tandem mass spectrometry specifications

Electrospray tandem mass spectrometry (Waters Micromass Quatro micro API triple quadrupole mass spectrometer) was used for analyses. Samples (30µl) were directly infused into the electrospray ion source via a Hewlett-Packard 1090 HPLC. Nitrogen gas was used as the nebuliser gas and argon as the collision gas at a pressure of 3 X 10^{-3} mbar.

Table 3.2. ESI/MS/MS specifications for hippuric acid analysis

FUNCTION 1:	
Parents of	105.0
Type	Parent Scan
Ion Mode	ES+
Start Mass	220.0

End Mass	250.0
Scan Time (sec)	0.5
InterScan Time (sec)	0.01
Start Time (min)	0.6
End Time (min)	0.9
Cone Voltage (V)	30.0
Repeats	5.0
Scans To Sum	1 000 000.0
Collision Energy (eV)	18.0

Table 3.3. ESI/MS/MS specifications for D5-hippuric acid analysis

FUNCTION 2:	
Parents of	110.0
Type	Parent Scan
Ion Mode	ES+
Start Mass	230.0
End Mass	250.0
Scan Time (sec)	1.0
InterScan Time (sec)	0.1
Start Time (min)	1.2
End Time (min)	1.5
Cone Voltage (V)	30.0
Repeats	5.0
Scans To Sum	1 000 000.0
Collision Energy (eV)	18.0

Table 3.4. ESI/MS/MS specifications for glycine and D2-glycine analysis

FUNCTION 1:	
Losses of	56.0
Type	Neutral Loss Scan
Ion Mode	ES+
Start Mass	120.0
End Mass	140.0
Scan Time (sec)	0.2
InterScan Time (sec)	0.01
Start Time (min)	0.5
End Time (min)	2.0
Cone Voltage (V)	20.0
Repeats	5.0
Scans To Sum	1 000 000.0
Collision Energy (eV)	14.0

3.5.4 Standardization and quantification of hippurate analysis

A standard curve was constructed to quantify hippuric acid in the urine. The standard curve (figure 3.4) was obtained using a standard series of hippuric acid dissolved in acetonitrile:distilled water. The series ranged from 100nM to 100mM hippuric acid. Each hippuric acid value was then expressed relative to the hippurate-D5 value. The lower detection limit was 1µmol/l.

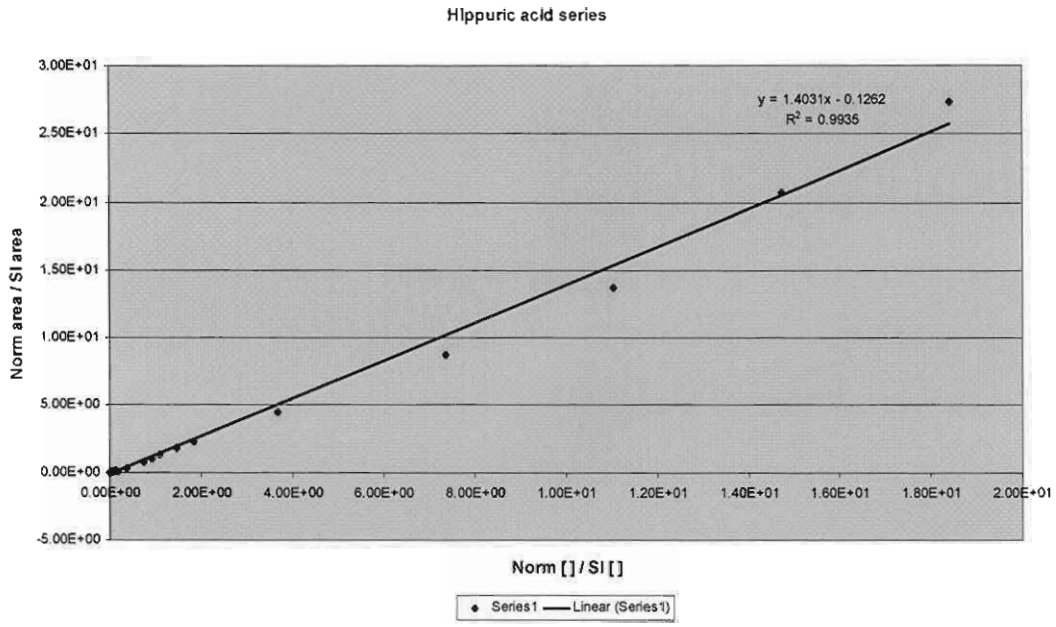

Figure 3.4. Standard curve to determine hippuric acid excretion

3.5.5 Standardization and quantification of glycine analysis

A standard curve was constructed to quantify glycine concentrations in urine. The standard curve (figure 3.5) was obtained using a standard series of glycine concentrations dissolved in acetonitrile:distilled water. The series ranged from 100µM to 2mM glycine. Each glycine value was expressed relative to the glycine-D2 value. The lower detection limit was 200µmol/l.

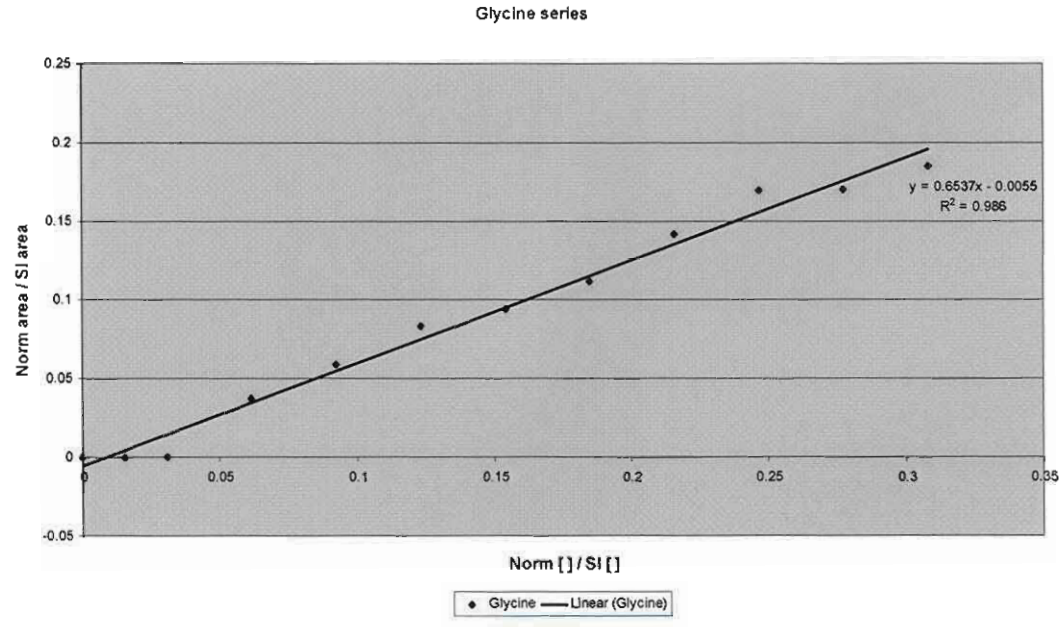

Figure 3.5. Standard curve to assess glycine excretion

3.6 BENZOYLCARNITINE QUANTIFICATION

3.6.1 Reagents, equipment and materials

Table 3.5. Reagents, equipment and material used for benzoylcarnitine analysis

Reagent/Equipment/Material	Company
Eppendorf tubes	Merck BRAA780500
Isovalerylcarnitine	Cambridge Isotope Laboratories, Inc
Methanol	Merck BC152506X
Acetonitrile	Merck BC15256Q
Butanol	Sigma 281549
Acetylchloride	Sigma 239577
Formic acid	Univar SAAR243800LC
Centrifuge	Optolabor BHG 1100
Evaporating adaptor with nitrogen	Afrox
Dri-Block	Techne DB-3
Mass spectrometer	VG quantro tandem MS (HRGC series)
High Pressure Liquid Chromatograph	Hewlett-Packard Series 2
Software for data analysis	Masslynx™ Micromass

3.6.2 Procedure

The urine samples were thawed at room temperature. 100µl urine was transferred into eppendorf tubes. This was then centrifuged at 13 000 x rpm for 30 minutes. 10µl of the supernatant was transferred to a new eppendorf tube and 410µl of the sable isotope mixture was added. This was again centrifuged at 13 000 x rpm for 20 minutes. The stable isotope mixture contained 0.0000347g/l isovalerylcarnitine D-9 (Cambridge Isotope Laboratories, Inc, Andover, MA, USA) dissolved in methanol. The supernatant

was carefully transferred to a new tube to prevent disturbing the pellet. The final mixture was dried under nitrogen for 45 minutes at 65°C. When dried, 200µl butanolic HCl was added, and the samples were left to stand for 15 minutes at 65°C. After butylation the samples were dried for 1 hour. The remaining residue were dissolved in 100µl 80:20 acetonitrile:distilled water and 1% formic acid.

3.6.3 Electrospray tandem mass spectrometry specifications

Electrospray tandem mass spectrometry (VG Quattro 2 4000 series tandem mass spectrometer) was used for analyses. Samples (25µl) were infused into the electrospray ion source via a Hewlett-Packard series 2 HPLC. Nitrogen gas was used as the nebuliser gas and argon as the collision gas at a pressure of 3×10^{-3} mbar.

Table 3.6. ESI/MS/MS specifications for benzoylcarnitine and D-9 isovalerylcarnitine analysis

FUNCTION 1:	
Parents of	85.20
Type	Parent Scan
Ion Mode	ES+
Start Mass	210.00
End Mass	580.00
Scan Time (sec)	2.0
InterScan Time (sec)	0.01
Start Time (min)	0.6
End Time (min)	1.5
Cone Voltage (V)	35.0
Repeats	5.0
Collision Energy (eV)	25.0

3.7 BENZOATE QUANTIFICATION

3.7.1 Reagents, equipment and materials

Table 3.7. Reagents, equipment and material used for benzoate analysis

Reagent/Equipment/Material	Company
Kimax tubes	Lasec 16125
5 M Hydrochloric acid	Rochelle chemicals
3-Phenyl butyric acid (mw 164.21)	Sigma T78243
Ethyl acetate HPLC grade/distilled	Merck BB101086J
Diethylether HPLC grade/distilled	Merck BB100946B
Na_2SO_4 anhydrous	Merck BB102644V
BSTFA	Sigma T1506
TMCS	Sigma T4252
Rotary mixer	Roto-torque 7637-10
Hotplate	Velp scientific
Evaporating adaptor with nitrogen	Afrox
Centrifuge	Optolabor BHG 1100
Hamilton syringes (10µl and 100µl)	Separations 80600
Gas chromatograph	Hewlett-Packard (6890)
Mass spectrometer	Hewlett-Packard (5973)
Software for data analysis	AMDIS Version2.1

3.7.2 Procedure

3.7.2.1 Organic acid extraction

The volume urine needed for analysis, depended on the creatinine content of urine used, was as follows:

- Creatinine > 100mg% use 0.5ml urine
- Creatinine < 100mg% use 1ml urine
- Creatinine < 5mg% use 2ml urine
- Creatinine < 2mg% use 3ml urine

The urine samples were thawed at room temperature. The required volume of urine was transferred into kimax tubes containing 250µl 5M HCl and 5x creatinine mg% (µl) internal standard (26.25mg 3-Phenyl butyric acid dissolved in a few drops NaOH and then added to 50ml distilled H_2O). 6ml ethyl acetate was added. The mixture was shaked for 30 minutes and then centrifuge for 3 minutes at 5 000 x rpm. The organic phase was aspirated into a clean kimax tube and 3ml diethylether added. The mixture was then again shaked for 10 minutes and centrifuge for 3 minutes at 5 000 x rpm. The organic phase was pooled with the ethyl acetate phase, to which two spatulas Na_2SO_4 was added. The mixture was centrifuged for 2 minutes. Finally the organic phase was poured into a small kimax tube and it was evaporated under Nitrogen at 40°C for 1 hour.

3.7.2.2 Derivatization

Two x creatinine mg% (µl) BSTFA and 0.4 x creatinine mg% (µl) TMCS was added to the dried sample and it was incubated for 1 hour at 60°C.

3.7.3 Gas chromatograph specifications

Samples were analysed with a Hewlett Packard 6890 Series Gas chromatograph and a Macherey-Nagel (MN 30962-52) column. 1µl air, 1µl external standard (C_{24} Tetracosanoic acid), 1µl air and 0.4µl sample were sequentially injected.

Table 3.8. GC specifications for benzoate analysis

FUNCTION 1:	
Inlet method	Splitless
Detector	Flame ionization detector
Carrier gas	Hydrogen (1ml/min, 3-4psi)
Make up gas	Nitrogen (30ml/min)
Oven temperature (°C)	70.0
Init temperature (°C)	70.0
Init time (min)	2.0
Rate (°C /min)	5.0
Final temperature (°C)	280.0
Final time (min)	3.0
Inj B temperature (°C)	280.0
Det A temperature (°C)	280.0
Oven max (°C)	280.0
Equib time (min)	1.0

CHAPTER 4:
RESULTS

4.1 INTRODUCTION

In this study the aim was to identify possible slow, medium and fast metabolizers of sodium benzoate and in the process possible defects of glycine conjugation. This will give a guideline for further studies on possible defects in the glycine N-acyltransferase enzyme system. To monitor the conjugation of benzoate and glycine tandem mass spectrometry was used. Tandem mass spectrometry was used because it is a very rapid and sensitive method for quantification of hippuric acid, glycine and benzoate excreted in the urine.

Three different loading tests 500, 250 and 150mg sodium benzoate/kg body weight were executed to test detoxification. The concentration of hippurate, glycine, benzoyl-carnitine and benzoate of every urine sample were determined to monitor detoxification of the test persons.

4.2 A COMPARISON OF THE DIFFERENT LOADING TESTS

The following figures compare the excreted concentrations of hippuric acid (blue), glycine (pink), benzoylcarnitine (yellow) and benzoic acid (turquoise) after the 500, 250 and 150mg sodium benzoate/kg body weight loading tests. In these figures all the excreted concentrations of the different substances for the specific loading test are included regardless of the specific time.

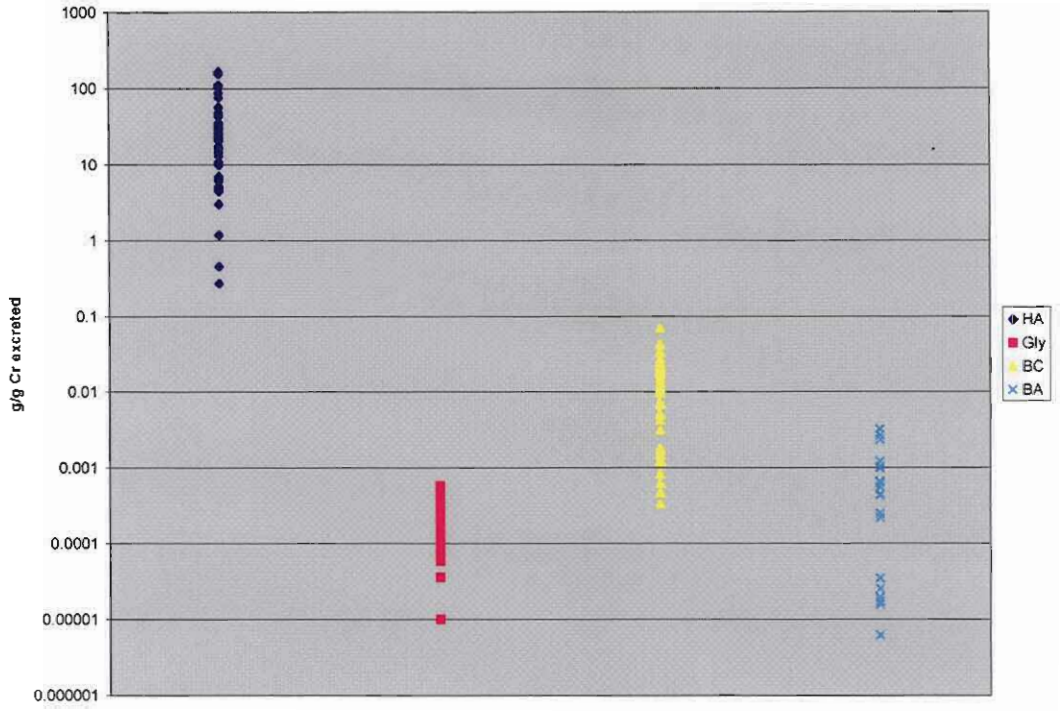

Figure 4.1. **Hippuric acid, glycine, benzoylcarnitine and benzoate excreted after a 500mg/kg body weight loading test**

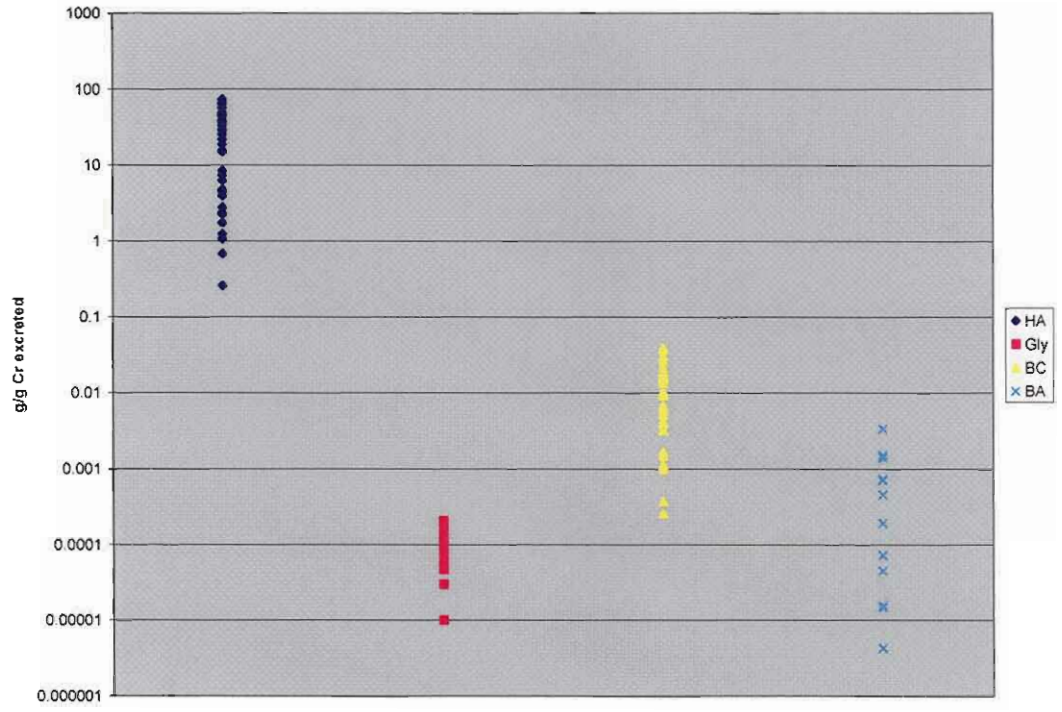

Figure 4.2. **Hippuric acid, glycine, benzoylcarnitine and benzoate excreted after a 250mg/kg body weight loading test**

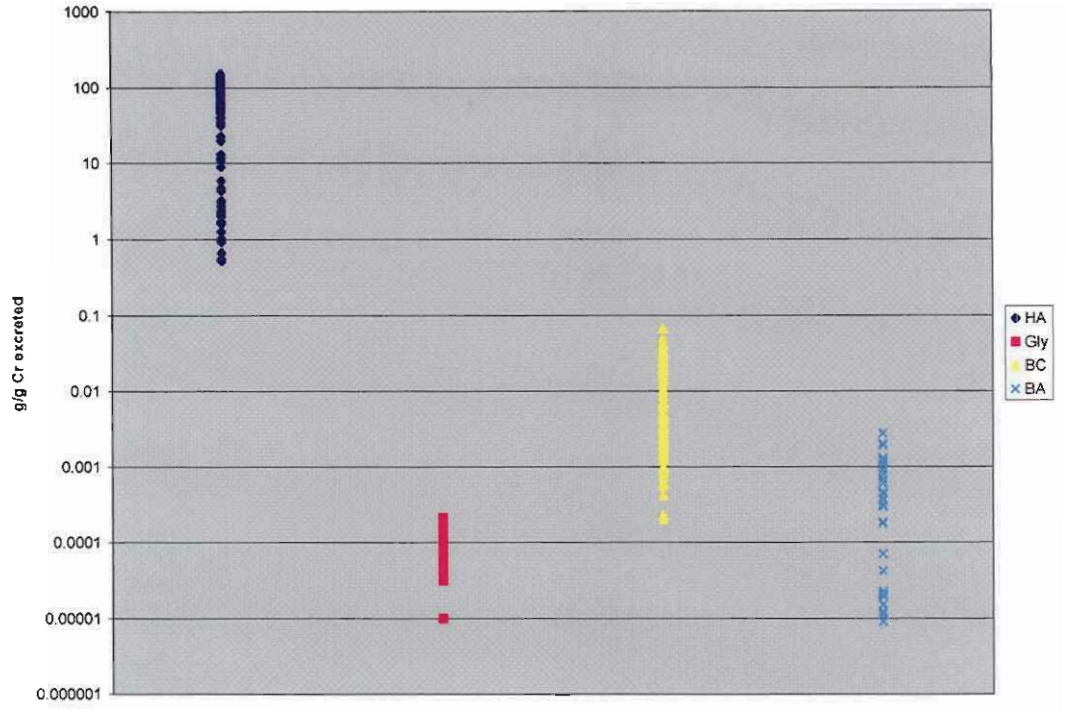

Figure 4.3. Hippuric acid, glycine, benzoylcarnitine and benzoate excreted after a 150mg/kg body weight loading test

4.3 500MG/KG BODY WEIGHT LOADING TEST

The first loading test was done on 10 test persons with a sodium benzoate concentration of 500mg/kg body weight. Urine was collected every hour for 6 hours after loading. Four test persons were eliminated because of vomiting. After this loading test it was decided that the test should be repeated with lower loading concentrations because of the unexpected side effects. These side effects included nausea, light sensitivity, dizziness and vertigo, none of which was mentioned in other studies with the same dosage.

4.3.1 Hippuric acid excretion after a 500mg/kg loading test

Hippuric acid excreted in the urine was identified with ESI/MS/MS and quantified with the use of a hippuric acid stable isotope. A standard curve for a series of hippuric acid concentrations was obtained, and it was used for quantification, as described in section 3.5.

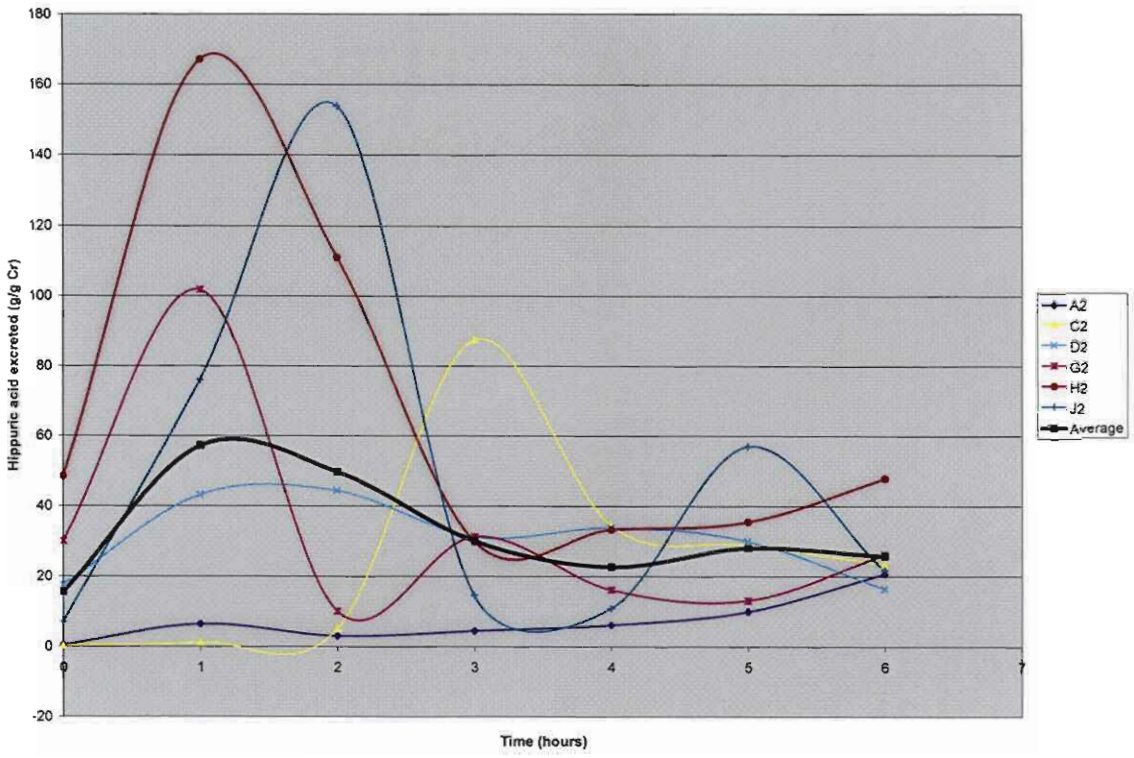

Figure 4.4. Hippuric acid excretion (g/g Cr) after the 500mg sodium benzoate loading test

Figure 4.4 shows the hippuric acid excretion curve (g/g Cr) over 6 hours for 6 test subjects after a 500mg sodium benzoate/kg body weight loading test. The black curve represents the average of all 6 test subjects.

Subject A2 is a possible slow metabolizer of sodium benzoate. Subjects C2 and G2 are examples of possible medium metabolizers, and subjects H2 and J2 of possible fast metabolizers during this loading test. Most of the test group reached peak concentrations 1-2 hours after loading.

4.3.2 Glycine excretion

Glycine excreted in the urine was identified with ESI/MS/MS and quantified with the use of a glycine stable isotope. As described in section 3.5, a standard curve for a series of glycine concentrations was obtained, and it was used for quantification.

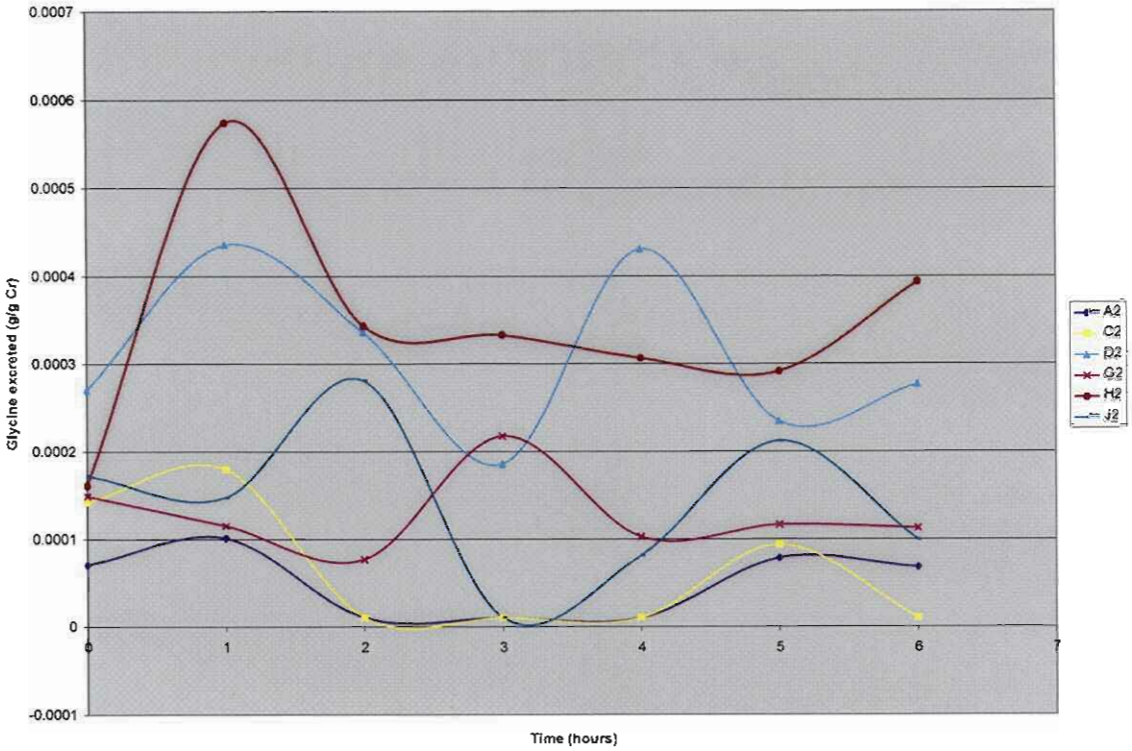

Figure 4.5. Glycine excretion (g/g Cr) after 500mg sodium benzoate loading test

Figure 4.5 shows the glycine excretion curve (g/g Cr) over 6 hours for 6 test subjects after a 500mg sodium benzoate/kg body weight loading test. The urinary glycine levels for all the test subjects remained constantly low during the duration of the test. Most of the subjects showed a decrease in excreted glycine levels after 2 hours.

4.3.3 Benzoylcarnitine excretion

Benzoylcarnitine excreted in the urine was identified with ESI/MS/MS and quantified with the use of an isovalerylcarnitine stable isotope, as described in section 3.6. The method used for quantification was already standardised.

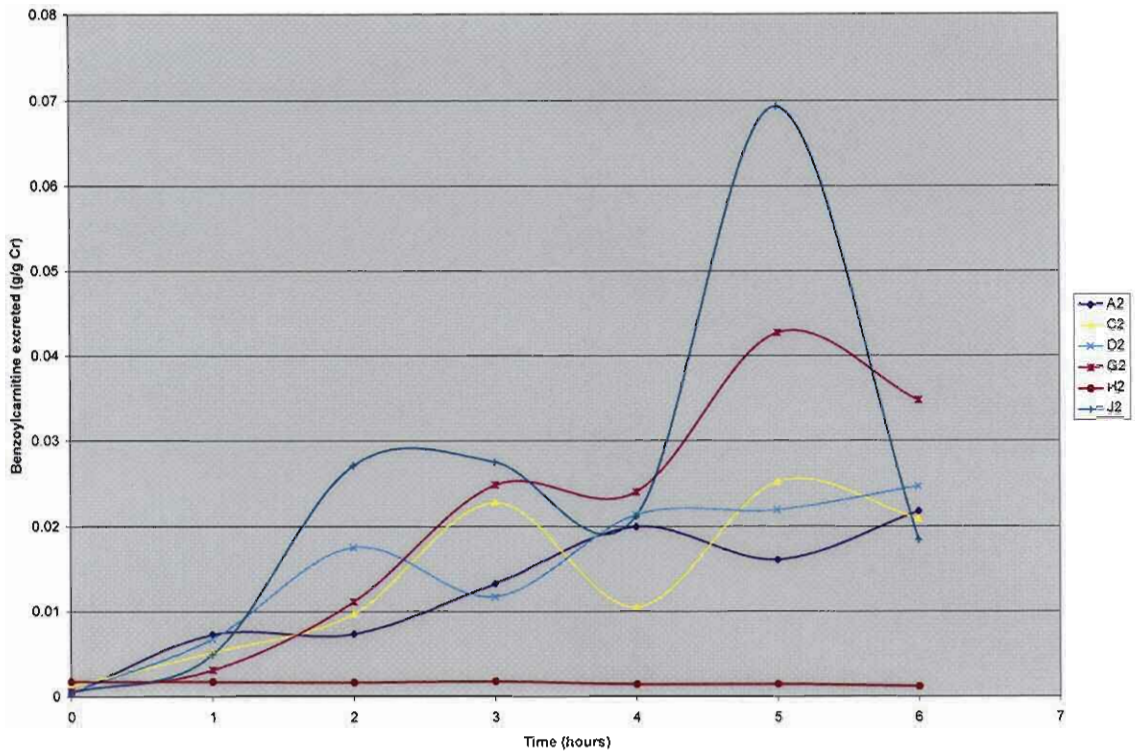

Figure 4.6. Benzoylcarnitine excretion (g/g Cr) after 500mg sodium benzoate loading test

Figure 4.6 shows the benzoylcarnitine excretion curve (g/g Cr) over 6 hours for the test group after a 500mg sodium benzoate/kg body weight loading test. H2, a possible fast metabolizer, has very low benzoylcarnitine levels, which does not increase over time. All the other subjects have increasing benzoylcarnitine excretion as time progress, although the levels remained very low in comparison to the excreted hippuric acid concentrations. It is possible that these values remain low because benzoate is not an ideal substrate for carnitine. Carnitine has a higher binding affinity for aliphatic molecules than aromatic molecules.

4.3.4 Benzoic acid excretion

Free benzoic acid excreted in the urine was measured by means of gas chromatography. With this method all urinary organic acids can be quantified. It was already standardised by the Laboratory for Inherited Metabolic Defects. See section 3.7.

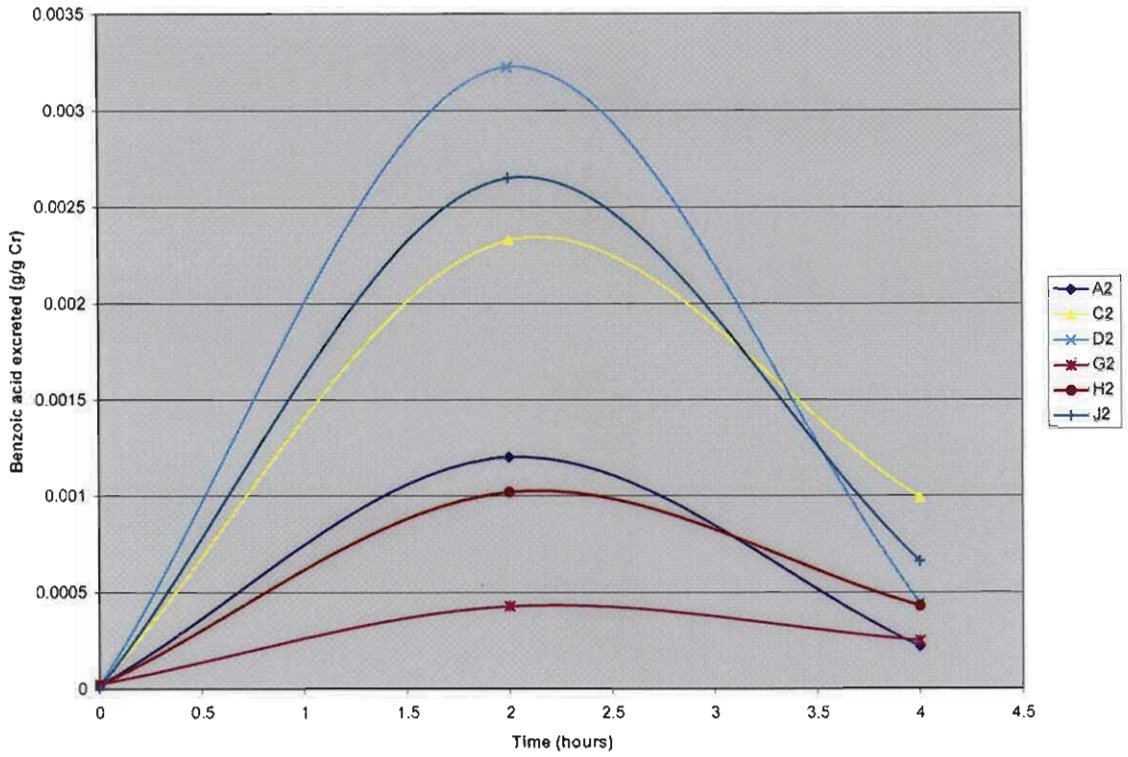

Figure 4.7. Benzoic acid excretion (g/g Cr) after 500mg sodium benzoate loading test

Figure 4.7 shows the benzoate excretion curve (g/g Cr) over 6 hours for 6 test subjects after a 500mg sodium benzoate/kg body weight loading test. All the samples showed a rise in free benzoic acid after the loading test. After 4 hours the excreted benzoate concentrations returned to normal. The levels of free benzoic acid in all the samples were very low in comparison to hippuric acid.

4.3.5 Different metabolizers after a 500mg sodium/kg body weight loading test

After determining the hippuric acid, glycine, benzoylcarnitine and benzoic acid concentrations excreted in the urine and comparing the results, possible slow, medium and fast metabolizers were identified based on their excretion profiles. The classification of slow, medium and fast metabolizers was assigned based on the excretion profiles of the test groups and must not be interpreted in context with the classifications used in the pharmaceutical disciplines. In the following figure the concentrations of hippuric acid excreted by the specifically identified individuals will be compared.

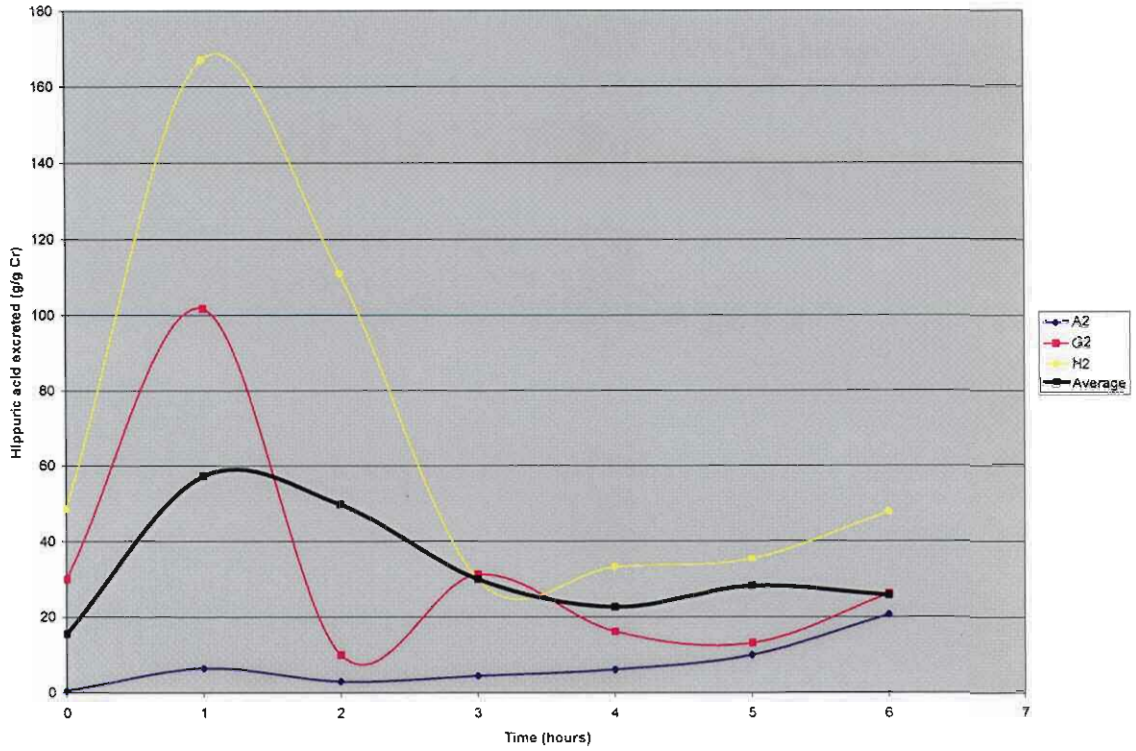

Figure 4.8. A possible slow (blue), medium (pink) and fast (yellow) metabolizer after a 500mg sodium benzoate loading test

Figure 4.8 shows the hippuric acid excretion (g/g Cr) curve for possible very slow (A2), medium (G2) and fast (H2) metabolizers after the 500mg/kg body weight loading test. Also included is the average hippuric acid excretion curve for all test subjects of the 500mg/kg loading test.

4.4 250MG/KG BODY WEIGHT LOADING TEST

Five test persons were used for the 250mg sodium benzoate/kg body weight loading test. One test person was eliminated due to vomiting. Urine was collected every hour for 6 hours after the loading test and then again after 9 and 12 hours.

The results of this loading test will not be discussed and can be seen in Annexure C. The results were discarded because this loading test was done to see if a 250mg/kg body weight loading still put the desired pressure on the detoxification system to identify different metabolizers, but with less negative side effects.

The results of the 250mg/kg loading test were comparable with the 500mg/kg loading test and a lot of the same side effects were seen. It was therefore decided to lower the loading concentration again.

4.5 150MG/KG BODY WEIGHT LOADING TEST

The 150mg sodium benzoate/kg body weight loading test was done on 10 test persons. With the 150mg/kg body weight the only negative side effect experienced by the test group was nausea. Urine samples were collected every hour for 6 hours, and 9 and 12 hours after the loading test.

4.5.1 Hippuric acid excretion

Again the hippuric acid excreted in the urine was identified with ESI/MS/MS and quantified with use of a hippuric acid stable isotope and a standard concentration curve, as described in section 3.5.

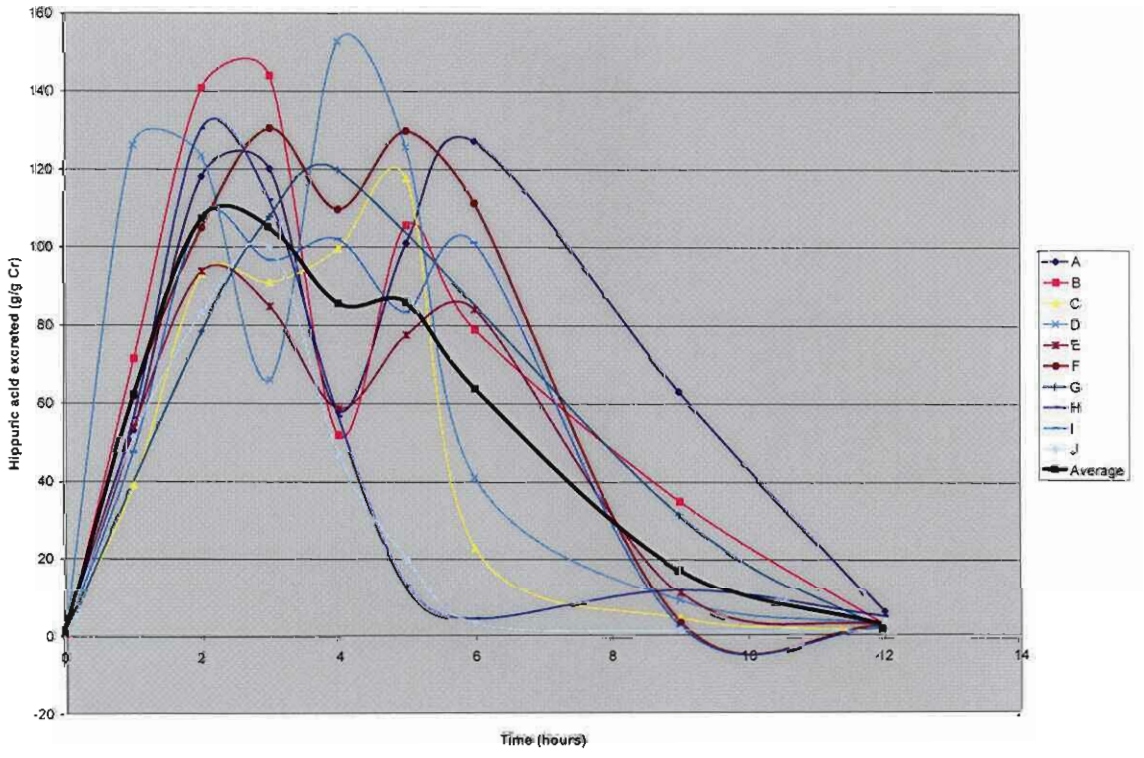

Figure 4.9. Hippuric acid excretion (g/g Cr) after 150mg sodium benzoate loading test

Figure 4.9 shows the hippuric acid excretion curve (g/g Cr) over 12 hours for 10 test subjects after a 150mg sodium benzoate/kg body weight loading test. The black curve represents the average of the whole test group.

Test subjects A, C and H are examples of possible slow, medium and fast metabolizers of sodium benzoate respectively based on this loading test. Most subjects' hippuric acid curve shows a first peak after 1 hour and a second peak after 3 hours. The two distinct concentration regions seen in most of the profiles is a very interesting observation, which merits further investigation.

4.5.2 Glycine excretion

Glycine excrete in the urine was identified with ESI/MS/MS and quantified with use of a glycine stable isotope and a standard concentration curve, as described in section 3.5.

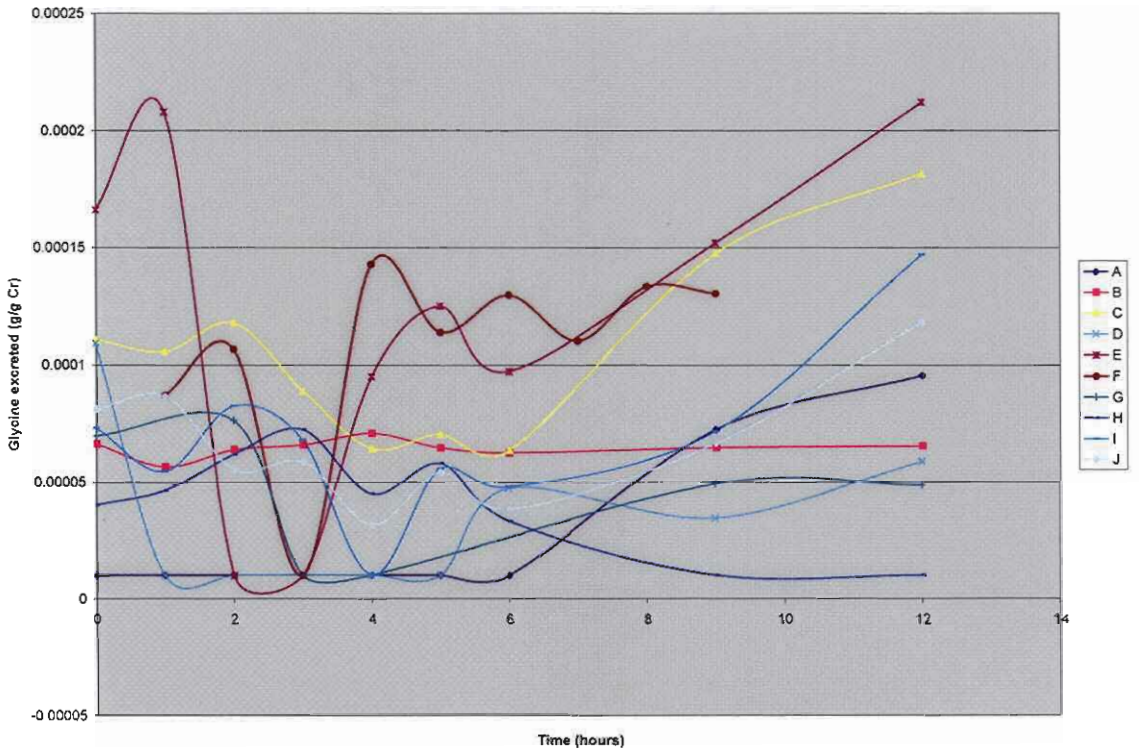

Figure 4.10. Glycine excretion (g/g Cr) after 150mg sodium benzoate loading test

Figure 4.10 shows the glycine excretion curve (g/g Cr) over 12 hours for 10 test subjects after a 150mg sodium benzoate/kg body weight loading test. The glycine levels for all the subjects showed a sharp decline after the loading test. Very low

glycine concentrations were detected for up to 6 hours after which the levels started to show a slight increase.

4.5.3 Benzoylcarnitine excretion

Benzoylcarnitine excreted in the urine was identified with a standardised ESI/MS/MS method and quantified with use of an isovalerylcarnitine stable isotope, see section 3.6.

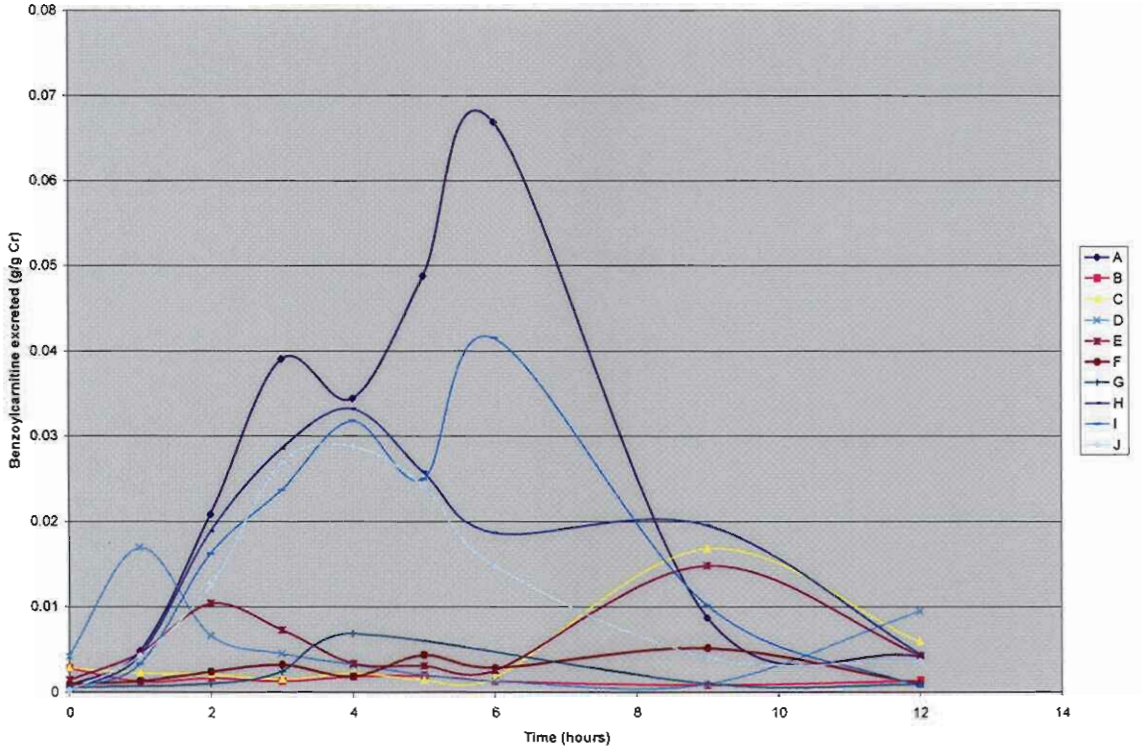

Figure 4.11. Benzoylcarnitine excretion (g/g Cr) after 150mg sodium benzoate loading test

Figure 4.11 shows the benzoylcarnitine excretion curve (g/g Cr) over 12 hours for 10 test subjects after a 150mg sodium benzoate/kg body weight loading test. Test subjects A, H, I and J, all possible medium or fast metabolizers, showed very high benzoylcarnitine levels, although the levels is low if compared to the excreted hippuric acid concentrations. The rest of the subjects have continuous low levels of benzoylcarnitine. The reason for the low levels of benzoylcarnitine can again be because of the higher affinity of the carnitine conjugation system to detoxify aliphatic molecules rather than aromatic molecules.

4.5.4 Benzoic acid excretion

Free benzoic acid excreted in the urine was measured and quantified with a standardised gas chromatograph method, as described in section 3.7.

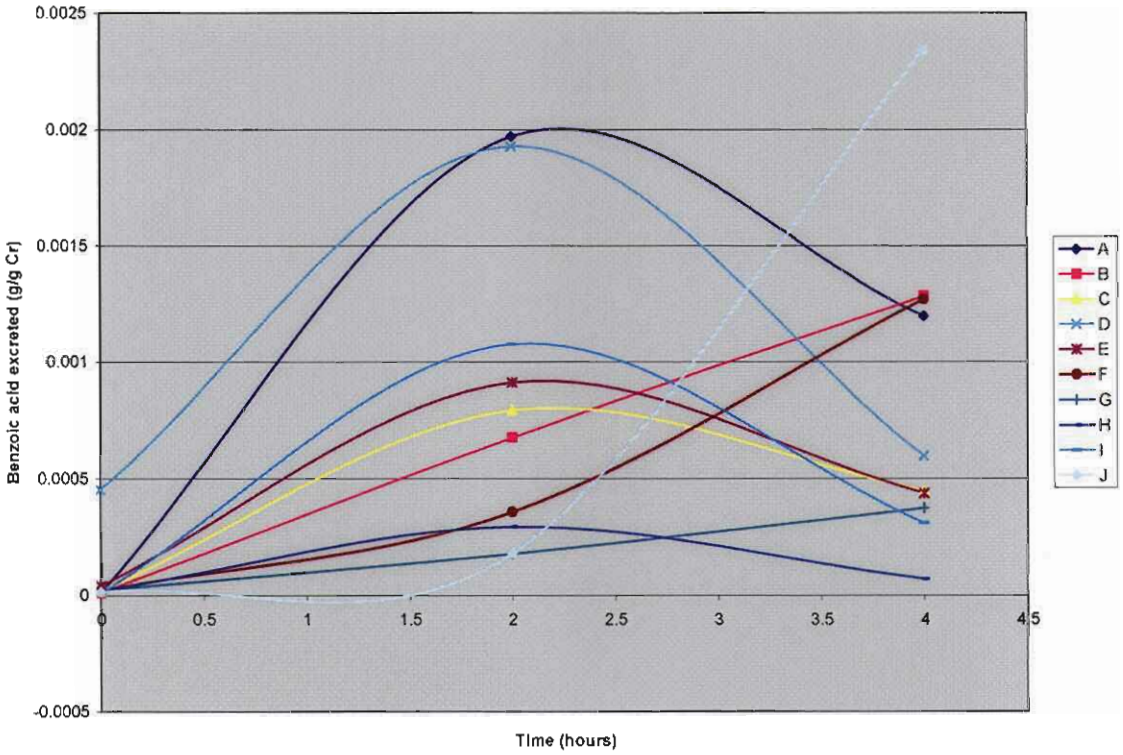

Figure 4.12. Benzoic acid excretion (g/g Cr) after 150mg sodium benzoate loading test

Figure 4.12 shows the benzoic acid excretion curve (g/g Cr) over 12 hours for 10 test subjects after a 150mg sodium benzoate/kg body weight loading test. Most of the test group showed an increase in free benzoic acid after the loading test but the values return to normal after 4 hours. The free benzoic acid values of test persons' B, F and J keeps showing an increase at 4 hours.

4.5.5 Different metabolizers after a 150mg sodium/kg body weight loading test

After determining the hippuric acid, glycine, benzoylcarnitine and benzoic acid concentrations excreted in the urine and comparing the results, possible slow, medium and fast metabolizers were identified based on their excretion curves. In the following figure these persons' excreted hippuric acid profiles will be compared.

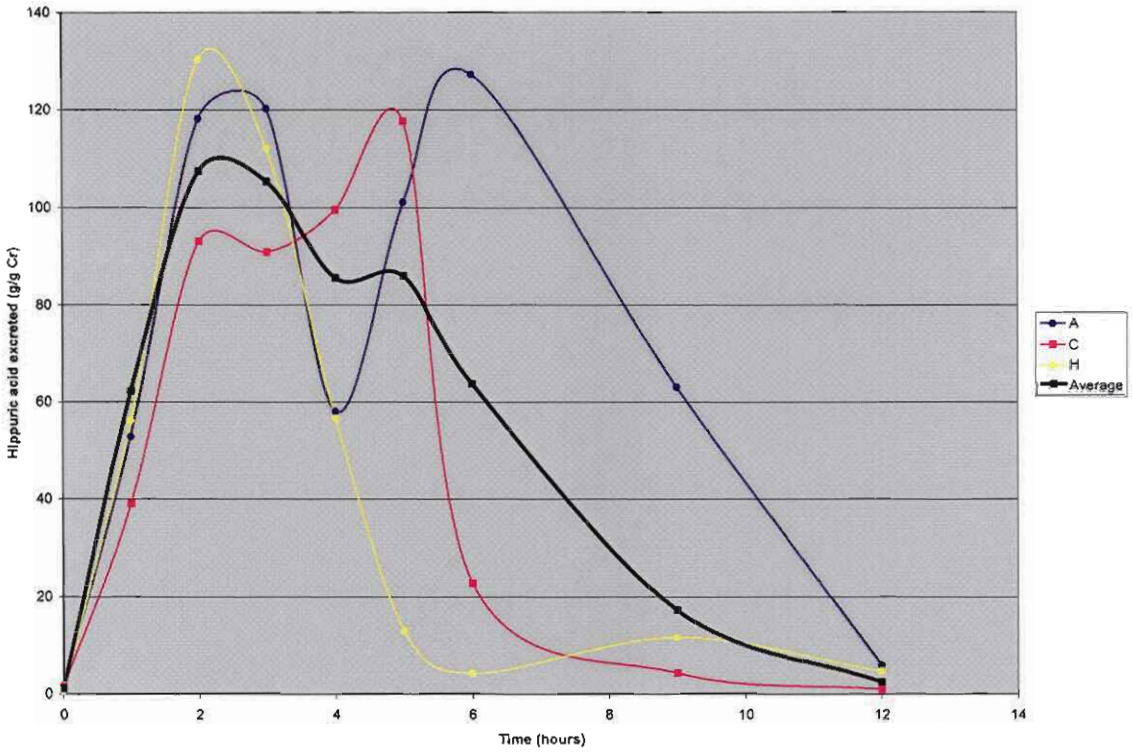

Figure 4.13. A possible slow (blue), medium (pink) and fast (yellow) metabolizer after a 150mg sodium benzoate loading test

Figure 4.13 shows the hippuric acid excretion (g/g Cr) curve for possible slow (A), medium (C) and fast (H) metabolizers after the 150mg/kg body weight loading test. Also included is the average hippuric acid excretion curve for the 150mg/kg loading test.

CHAPTER 5:
DISCUSSION

5.1 DEFINITIONS

In the following discussion the definitions of slow, medium and fast metabolizers are based on these particular loading tests only. Each of the different metabolizers discussed here was identified in comparison to the mean excretion curve for that specific loading test. To obtain precise definitions, as those used by Sheth and Brunton (2002), more test subjects and 24 hour urine samples are needed.

5.2 A COMPARISON OF THE DIFFERENT LOADING TESTS

Figures 4.1-3 clearly indicates that the range of excreted concentrations of hippurate, glycine, benzoylcarnitine and benzoic acid is comparable regardless of the concentration sodium benzoate used for loading. This indicates that the absorption and subsequent detoxification of sodium benzoate have a maximum rate regardless of the ingested dosage.

5.3 500MG/KG BODY WEIGHT LOADING TEST

The first sodium benzoate loading test (500mg/kg body weight) was done on ten voluntary test persons. This concentration was used because it was defined as the NOAEL by the World Health Organization (JEFCA, 2005). This dose of sodium benzoate caused various negative symptoms and four of the test persons were later eliminated due to vomiting. None of the negative effects was expected because it is not mentioned for this concentration in any of the studied literature. Quick (1931) did mention that toxic affects from benzoate can arise because of local irritation of the gastrointestinal mucosa (nausea and vomiting) and a effect on the central nervous system (headache, vertigo, dizziness and light sensitivity).

According to Wibbertmann *et al.* (2000), therapeutic doses of 250-500mg sodium benzoate/kg body weight per day given over several days, clinical signs of toxicity are rare and in most cases limited vomiting occurred.

Because of the negative side effects it was decided that the test will be repeated with a lower sodium benzoate concentration. The results of the six test persons that completed the test were however still acquired and compared.

5.3.1 A possible slow metabolizer

After comparing the hippuric acid excretion profiles of the six test persons it is obvious that test person A is a possible slow metabolizer. Test person A had the worst reaction after the loading and experienced the negative effects very severely. Test person A's hippuric acid excretion curve, figure 5.1 (blue), does not peak in the 6 hours of the study, and it reaches its highest concentration only after 6 hours. This is still not close to the highest level of the average hippuric acid excretion curve. It was decided that after the next loading test urine will be collected for longer to establish when a possible slow metabolizer will peak.

Test person A's glycine excretion profile, figure 5.1 (yellow) shows very low glycine levels. The predose level is lower than all other test persons. It falls to an undetectable low between hours 2 and 4, but starts to rise again after 4 hours. This can indicate possible glycine depletion shortly after loading and a later internal signal to increase glycine production.

Test person A's benzoylcarnitine levels, as shown in figure 5.1 (pink) are very close to the average for all the test persons. It is slowly rising over time, indicating that a small amount of benzoic acid has been detoxified through conjugation with carnitine.

Test person A's free benzoic acid (turquoise) increases after the loading, but after 4 hours the concentrations are returning to normal. The concentration of free benzoic acid stays a lot lower than the hippuric acid levels, which indicates that most of the benzoic acid is absorbed and detoxified sufficiently with glycine.

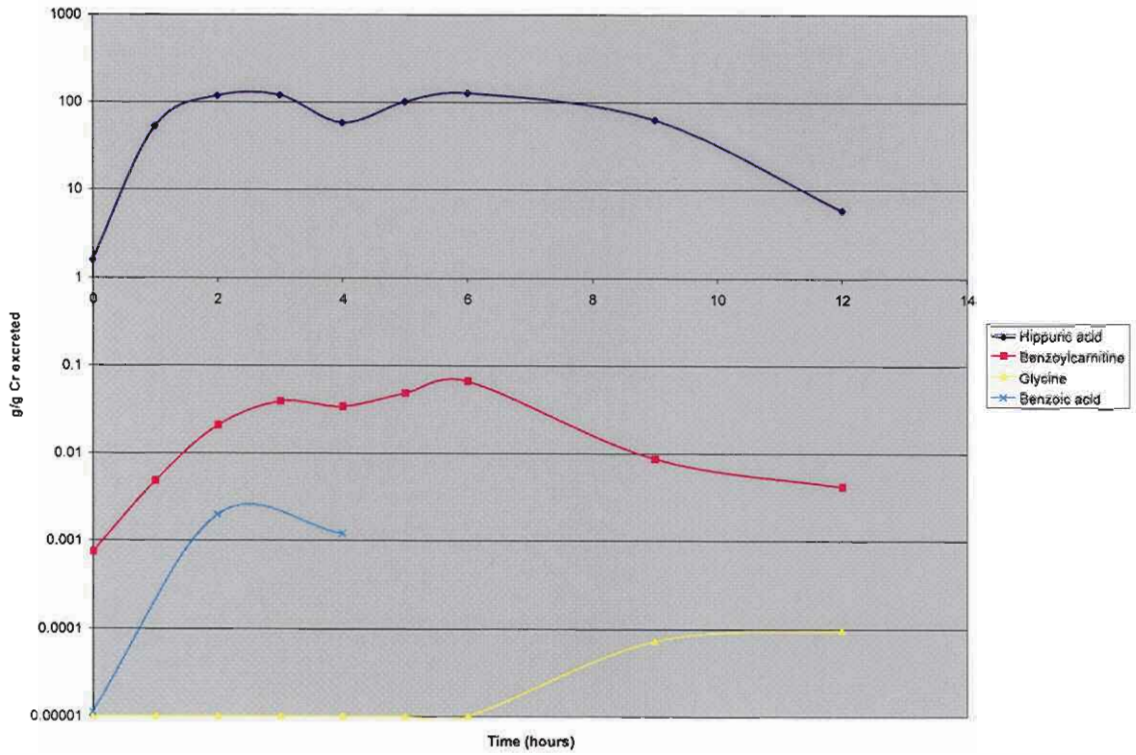

Figure 5.1. A possible slow metabolizer after a 500mg sodium benzoate loading test

5.3.2 A possible medium metabolizer

After comparing the hippuric acid excretion profiles of the six test persons, person G was identified as a possible medium metabolizer. The hippuric acid curve, as seen in figure 5.2 (blue), peaks after 1 hour and afterwards it falls and remains low. The 6 hour hippuric acid concentration is close to the predose value, and it can be concluded that all the sodium benzoate is detoxified and the concentration values returned to normal.

Test person G's glycine excretion profile, figure 5.2 (yellow) slowly falls for 2 hours after which it rises, which can again indicate glycine depletion and subsequent internal glycine production. After the spike at 3 hours, the glycine levels remain stable.

Test person G's benzoylcarnitine, figure 5.2 (pink), is comparable to all the benzoylcarnitine excretion profiles. It shows a slow and constant rise for the 6 hours the test took place. The levels do remain a lot lower than the hippuric acid excretion concentrations.

Test person G's free benzoic acid (turquoise) levels shows a slight rise after the loading test but the concentrations starts to decrease after 4 hours.

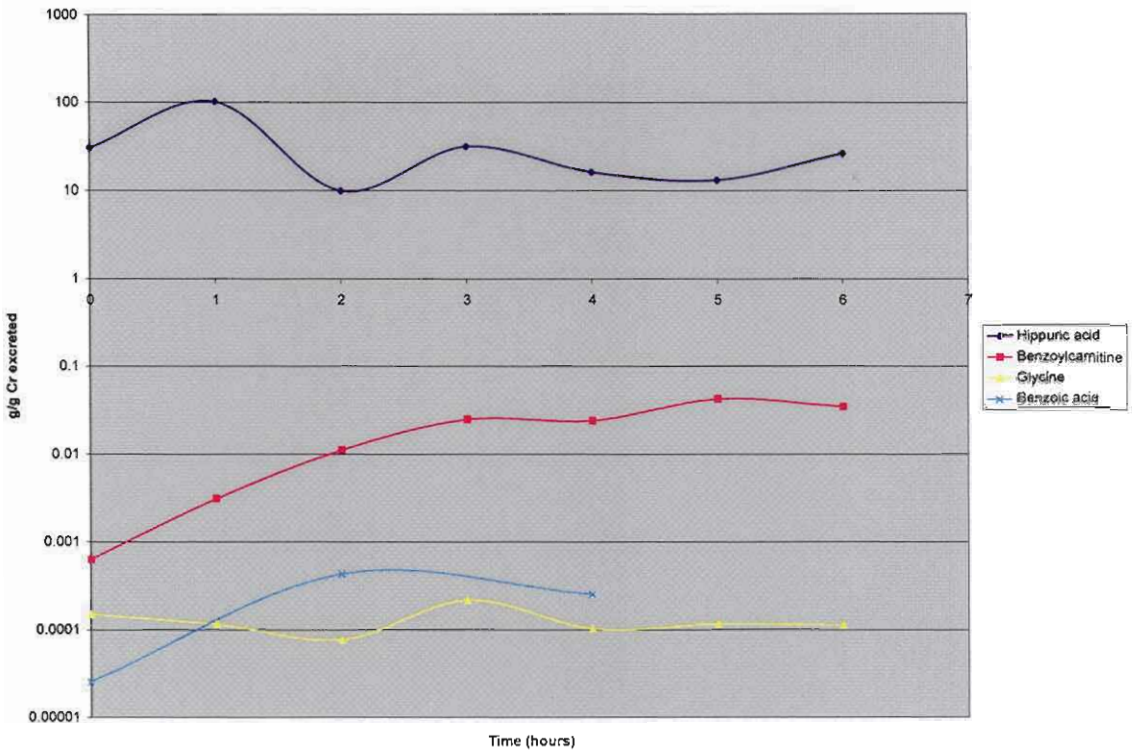

Figure 5.2. A possible medium metabolizer after a 500mg sodium benzoate loading test

5.3.3 A possible fast metabolizer

After comparing the hippuric acid excretion profiles of the six test persons, person H was identified as a possible fast metabolizer. The hippuric acid curve, figure 5.3 (blue), peaks after 1 hour, and it reaches the highest concentration of hippuric acid excreted by the test group. After an hour it slowly decreases until 3 hours, after which it remains low and stable, at a concentration comparable with the original predose concentration. It can be concluded that the sodium benzoate was completely detoxified after 3 hours.

Test person H's glycine excretion profile, figure 5.3 (yellow) also peaks after 1 hour, after which it decreases a bit and remains constant. Test person H's glycine concentration remains higher than the other test subjects. This might be an indication of an earlier and more effective trigger for glycine production, which might explain the faster hippuric acid formation. At 6 hours, the glycine levels start to rise which can indicate complete detoxification and that the excess glycine from internal production is excreted.

Test person H's benzoylcarnitine, figure 5.3 (pink), is very low in comparison to the other benzoylcarnitine excretion profiles. It remains very low for the duration of the test. This might be an indication that detoxification through hippuric acid is efficient enough and there is no need to activate this pathway because there is no excess benzoate to excrete.

Test person H's free benzoic acid (turquoise) is slightly increased after 2 hours but it decreases to the same level as the control values after 4 hours.

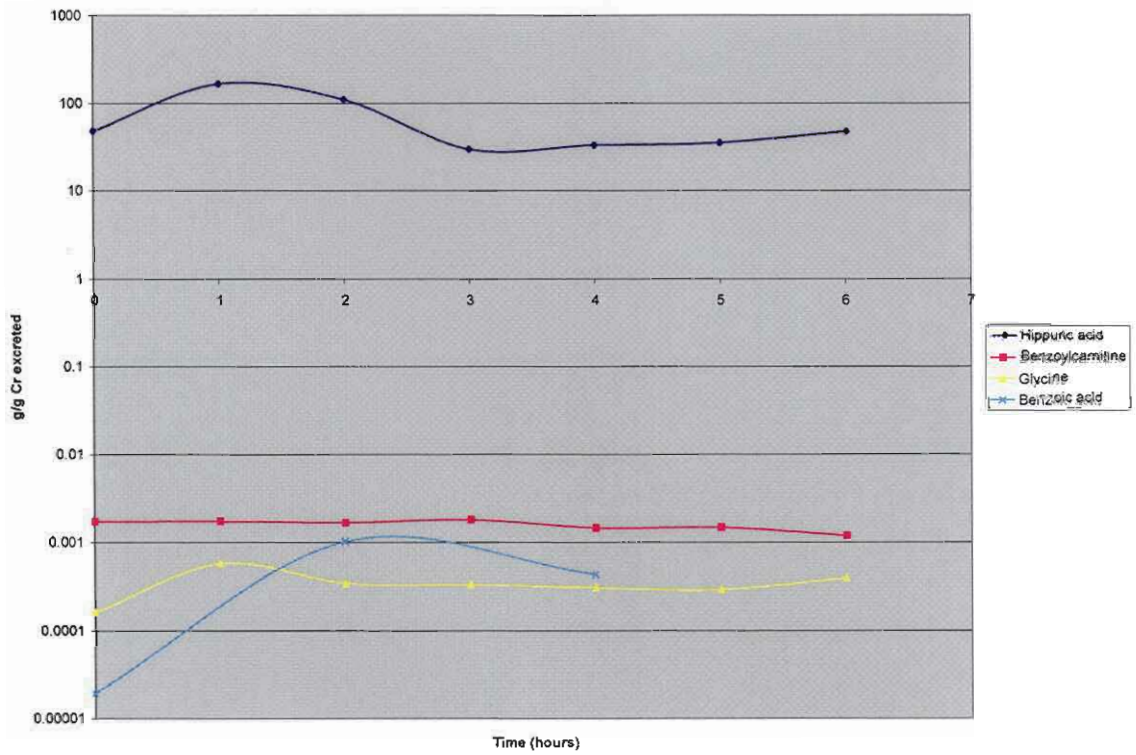

Figure 5.3. A possible fast metabolizer after a 500mg sodium benzoate loading test

5.4 250MG/KG BODY WEIGHT LOADING TEST

These results will not be discussed.

5.5 150MG/KG BODY WEIGHT LOADING TEST

The 150mg sodium benzoate/kg body weight loading test was done on ten voluntary test persons. This dose of sodium benzoate did not cause such drastic negative symptoms as the 500 and 250mg/kg body weight loading tests. The only side effect

experienced by the test group was nausea, possibly cause by local irritation of the gastrointestinal mucosa or the bad taste. All the volunteers completed this loading test.

To monitor detoxification, urine samples were collected every hour for 6 hours and again after 9 and 12 hours. The last two samples were collected to give a more complete picture of the detoxification.

5.5.1 A possible slow metabolizer

After comparing the hippuric acid excretion profiles of the ten test persons, subject A was identified as a possible slow metabolizer. Test person A's hippuric acid excretion curve, figure 5.4 (blue), shows 2 peaks, after 2 and 6 hours respectively. This might be an indication of internal up regulation of the enzyme system, or internal glycine production, but further investigation is needed to determine the specific underlying cause.

Test person A's glycine excretion profile, figure 5.4 (yellow) shows undetectable low concentrations for the first 6 hours. After 6 hours it starts to show a slight increase, which continues for the rest of the test. These low glycine levels might be why detoxification was delayed. The later rise of glycine may also be an indication of activation of internal glycine production.

Test person A's benzoylcarnitine levels, figure 5.4 (pink), remains very low throughout the study. It shows a slight increase and peaks after 6 hours after which it decreases again. The levels still remain a lot lower than the hippuric acid concentrations.

Test person A's free benzoic acid (turquoise) slightly increases after 2 hours. After 4 hours it is returning to the control T0 concentration. The concentration of free benzoic acid is a lot lower than the hippuric acid levels, which indicates that most benzoic acid is absorbed and detoxified sufficiently.

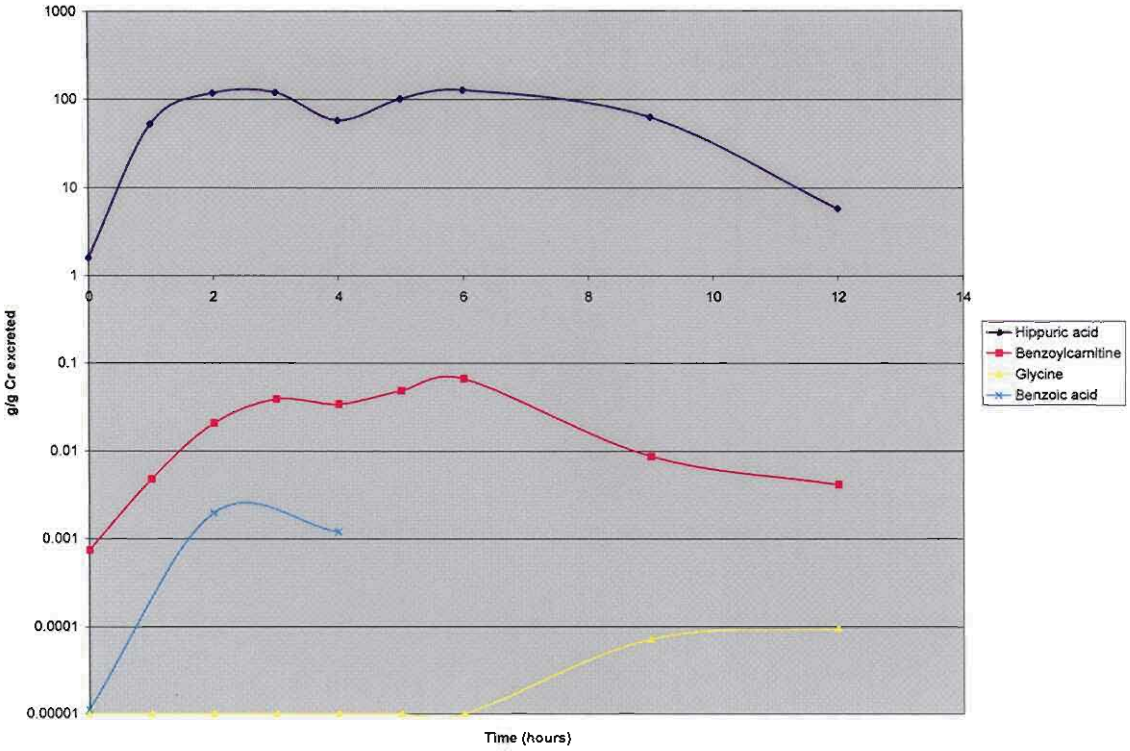

Figure 5.4. A possible slow metabolizer after a 150mg sodium benzoate loading test

5.5.2 A possible medium metabolizer

After comparing the hippuric acid excretion profiles of the ten test persons, person C was identified as a possible medium metabolizer. The hippuric acid curve, as seen in figure 5.5 (blue), only peaks after 5 hours and afterwards it falls and the concentrations return to the predose value. It can be concluded that all the sodium benzoate is detoxified because the concentration returned to normal.

Test person C's glycine excretion profile, figure 5.5 (yellow) slowly falls for 4 hours after which it rises slightly. This can indicate glycine depletion and the subsequent activation of internal glycine production. Glycine keeps rising and reaches relatively high levels after 12 hours.

Test person C's benzoylcarnitine, figure 5.5 (pink) is comparable to most of the benzoylcarnitine excretion profiles. It remains constant for 6 hours, after which it rises slightly. The levels still remain a lot lower than the hippuric acid excretion concentrations.

Test person C's free benzoic acid (turquoise) increases after the loading, but after 4 hours the concentrations return to normal. The benzoic acid concentrations remain very low if compared to the hippurate concentrations.

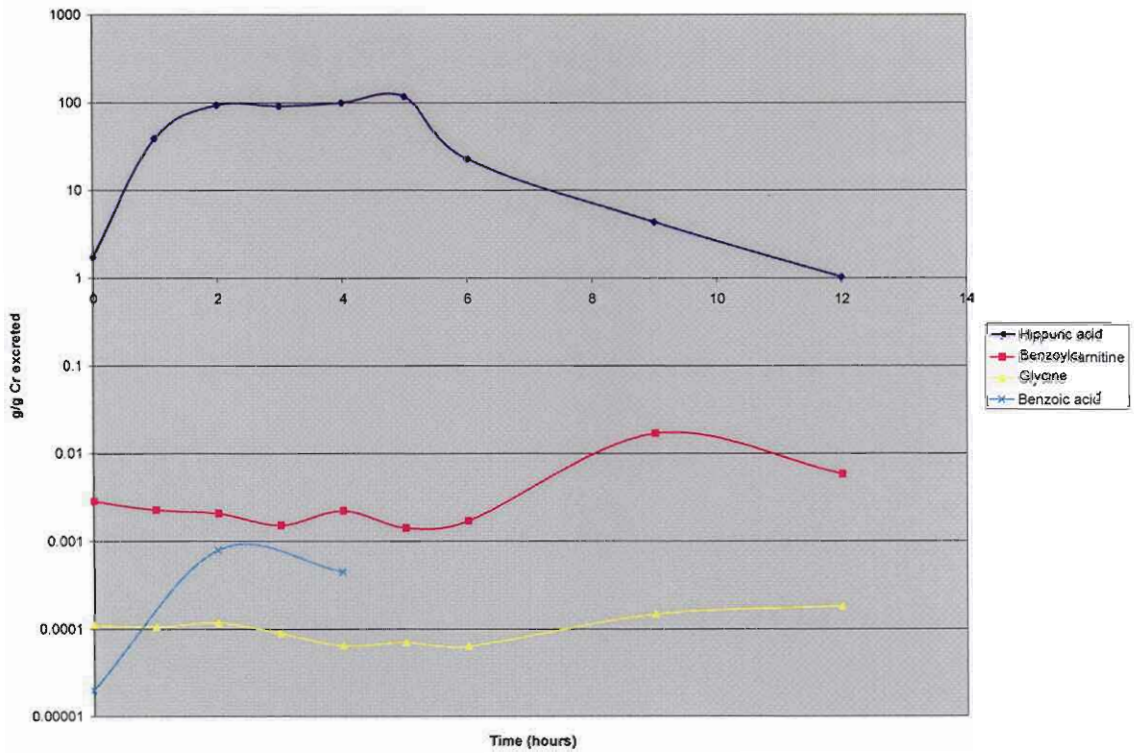

Figure 5.5. A possible medium metabolizer after a 150mg sodium benzoate loading test

5.5.3 A possible fast metabolizer

After comparing the hippuric acid excretion profiles of the ten test persons, person H was identified as a possible fast metabolizer. The hippuric acid curve, figure 5.6 (blue), peaks after 2 hours. After this it slowly decreases until 5 hours, after which it remains low and stable, and comparable with the original predose concentration. Again, it can be concluded that all the sodium benzoate was detoxified because the values returned to normal. Person H's excretion profile is also one of the few that that does not show 2 distinct peaks, it only peaks once and decreases completely to the predose values.

Test person H's glycine excretion profile, figure 5.6 (yellow) peaks after 3 hours, after which it slowly decreases and remains constant. The levels stay low until 12 hours. This might be an indication that there was no internal glycine production, and that glycine was depleted after detoxification.

Test person H's benzoylcarnitine, figure 5.6 (pink) is relatively high in comparison to the average benzoylcarnitine excretion profile. It peaks after 4 hours and then slowly decreases until it reached concentrations close to the T0 concentrations. The concentrations reached were a lot less that hippuric acid excretion values.

Test person H's free benzoic acid (turquoise) is slightly increased after 2 hours. After 4 hours it is returning to the control T0 concentration. The concentration of test person's H free benzoic acid remains lower than all the other test person's concentrations. This might be an indication of the complete detoxification of the ingested sodium benzoate.

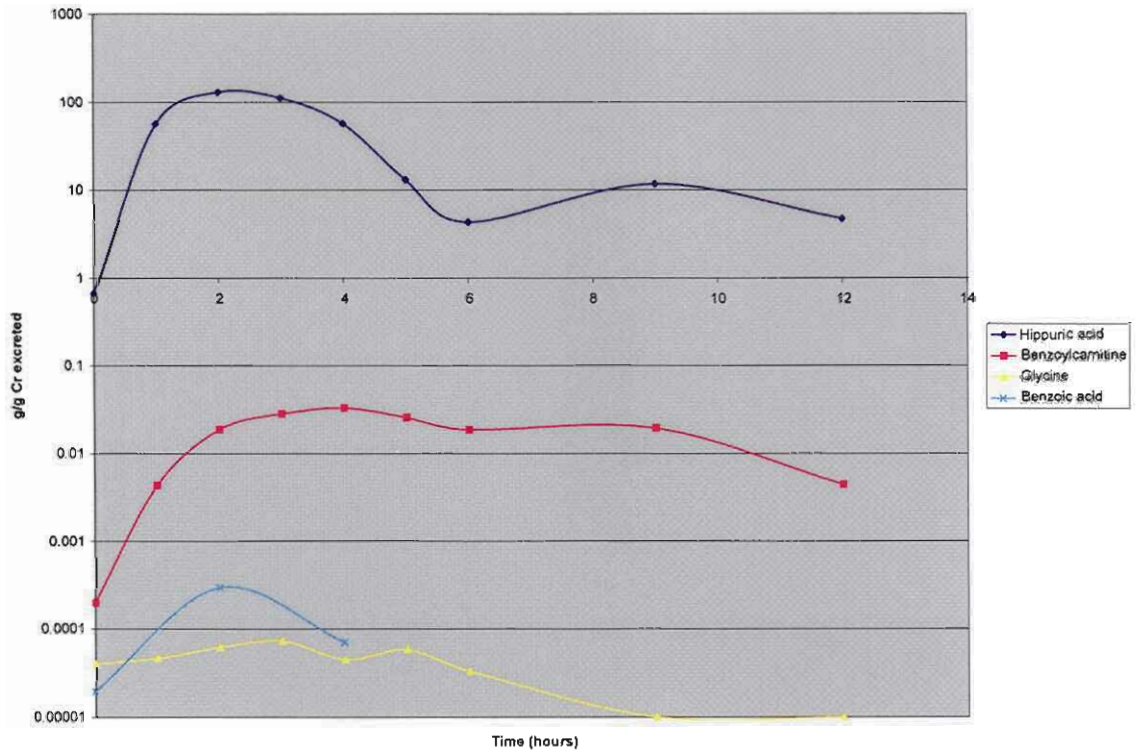

Figure 5.6. A possible fast metabolizer after a 150mg sodium benzoate loading test

CHAPTER 6:
CONCLUSION

The aim of this study was to identify individuals with decreased or increased glycine N-acyltransferase activity to be used for further characterization of the possible errors in the GLYAT system. Many SNP's has already been identified in this system but none has been linked to a specific genetic condition. Persons with altered glycine conjugation were identified by examining their excretion profiles of hippuric acid, glycine, benzoylcarnitine and benzoic acid after a sodium benzoate loading test. The use of loading tests to indirectly monitor the activity of the conjugation enzymes is a efficient way to test a person's detoxification capacity, because of the pressure it puts on the system. Three loading tests were done and after comparing the results it was clear that there is a maximum rate for hippuric acid formation, independent of the sodium benzoate dosage used. Therefore 150mg/kg body weight sodium benzoate is efficient to test glycine conjugation of benzoate and is preferable because of minimal side effects.

After compiling and comparing the test persons excretion profiles, it is clear that great inter individual variance exist in glycine conjugation - each test subject has a different capacity to detoxify sodium benzoate. Because only a few test subjects were used it is not possible to get a reference mean excretion curve for the population. Possible future studies can determine the range of variance and determine exact reference values. After comparing the individual excretion profiles, a mean excretion curve of hippuric acid was obtained for each loading test. This curve was then compared to each individual's excretion curve and possible slow, medium and fast metabolizers were identified. These definitions are not referring to these individuals as slow, medium and fast metabolizers in the general population; it is only based on this study.

One of the most significant findings of this study was the excretion profiles of hippuric acid which were characterized by two distinct concentration regions in most of the test subjects. This interesting observation merits further investigation regarding the underlying causes.

Blood samples can now be obtained from the identified test persons to do further genetic research. More research will also be done on some of the individuals identified by the Laboratory for Inherited Metabolic Defects.

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ANNEXURES

ANNEXURE A: QUESTIONNAIRE FOR TEST PERSONS

Natriumbensoaatbeladingstoets: **Nr.:**

Algemene inligting:

Naam en Van:	
Geboortedatum:	
Telefoonnommer:	

Uitsluitingsfaktore:

	Ja	**Nee**
Gebruik jy enige medikasie of aanvullings?		
Het jy enige geskiedenis van lewer probleme?		
Het jy enige geskiedenis van asma, allergiese rinitis of urtikaria?		

Belading:

Gewig:	kg
Beladingshoeveelheid:	mg natriumbensoaat

Toetsontwerp:

- **1 Week voor die beladingstoets:**

  Trek bloed by Drs. Du Buisson, Bruinette en Kramer Ingl, Kamer G01, Biochemie Gebou F3 vir 'n lewerfunksiebepaling.

- **Dag van die belading:**

  Die belading vind plaas by die Metaboliese Eenheid, Biologiese Blok

  1. Proefpersoon gee urienmonster vir 'n kontrole waarde (Houertjie 0)
  2. Proefpersoon word geweeg en hoeveelheid natriumbensoaat benodig vir belading word bepaal (150mg natriumbensoaat / kg liggaamsgewig)
  3. Natriumbensoaat word opgelos in water en gedrink
  4. Proefpersoon gee urienmonster elke uur vir die eerste 6 ure (Houertjie 1 – 6). Vul tyd op onderstaande tabel in.
  5. Proefpersoon gee twee verdere urienmonster, een na 9 en die ander na 12 uur (Houertjie 9 en 12) Vul tyd op onderstaande tabel in.

  Urienmonsters sal elke uur deur die navorser gekollekteer word en gevries word.

Natriumbensoaatbeladingstoets: **Nr.:**

Urienmonster	Tyd
0	
1	
2	
3	
4	
5	
6	
9	
12	

ANNEXURE B: THE 500MG/KG BODYWEIGHT SODIUM BENZOATE LOADING TEST

Test person	Date of birth	Nr.	Time	[Cr]mmol/L	Hippurate	Stable isotope	HA/SI	[HA]mmol/L	g HA/g Cr
Z Lindeque	01/14/1983	A2	0	10.476	9.63E+07	1.48E+08	6.51E-01	3.006510526	0.454667767
			1	2.933	1.81E+08	6.16E+07	2.94E+00	11.85966679	6.406006153
			2	2.611	1.28E+08	1.11E+08	1.15E+00	4.951099438	3.004150628
			3	4.712	2.00E+08	6.06E+07	3.30E+00	13.26067855	4.458485856
			4	5.211	3.02E+08	5.97E+07	5.06E+00	20.06528951	6.100301281
			5	8.012	6.71E+08	5.23E+07	1.28E+01	50.13985574	9.914460042
			6	7.862	7.99E+08	3.02E+07	2.65E+01	102.8768614	20.73058674
A Phoofolo	12/07/1983	C2	0	9.261	2.27E+08	8.07E+08	2.81E-01	1.576982232	0.269771361
			1	1.808	2.12E+08	9.49E+08	2.23E-01	1.352925833	1.185502545
			2	1.926	1.19E+08	8.02E+07	1.48E+00	6.230666769	5.125133345
			3	1.666	3.03E+08	1.28E+07	2.37E+01	92.09860113	87.58000469
			4	4.552	2.36E+08	9.29E+06	2.54E+01	98.8006122	34.38620988
			5	4.61	1.87E+08	8.62E+06	2.17E+01	84.44319703	29.01954971
			6	7.746	2.38E+08	8.07E+06	2.95E+01	114.6222863	23.44328685
C van Heerden	25/08/1983	D2	0	13.856	1.11E+09	2.81E+07	3.95E+01	153.3603649	17.53485527
			1	2.033	6.80E+08	4.79E+07	1.42E+01	55.42788183	43.19344375
			2	3.080	5.16E+08	2.33E+07	2.21E+01	86.19316265	44.33523627
			3	10.713	4.64E+08	8.53E+06	5.44E+01	211.0019924	31.20343306
			4	5.390	3.61E+08	1.22E+07	2.96E+01	115.002392	33.80220533
			5	9.117	6.24E+08	1.40E+07	4.46E+01	172.9799181	30.05873224
			6	8.315	1.99E+09	9.03E+07	2.20E+01	85.77415567	16.34259904
E van Dyk	05/11/1981	G2	0	10.039	5.49E+08	1.12E+07	4.90E+01	190.1876062	30.01364113
			1	5.167	7.40E+08	8.64E+06	8.56E+01	331.9469107	101.7787463
			2	6.948	4.55E+08	4.07E+07	1.12E+01	43.75254594	9.976321744
			3	6.471	2.98E+08	9.03E+06	3.30E+01	128.2027505	31.38722137
			4	8.650	3.22E+08	1.42E+07	2.27E+01	88.24478078	16.16217662
			5	5.532	1.78E+08	1.52E+07	1.17E+01	45.80815615	13.11860232
			6	9.229	3.58E+08	9.08E+06	3.94E+01	153.072179	26.27656224
J Pretorius	27/01/1982	H2	0	4.600	4.10E+08	1.13E+07	3.63E+01	140.9044011	48.52814089
			1	2.339	3.71E+08	5.83E+06	6.36E+01	246.7612576	167.1374148
			2	1.463	3.40E+08	1.29E+07	2.64E+01	102.4884506	110.9832731
			3	1.918	2.78E+08	3.01E+07	9.24E+00	36.23127116	29.92691126
			4	1.464	2.34E+08	2.99E+07	7.83E+00	30.77536752	33.30344026
			5	1.296	2.55E+08	3.46E+07	7.37E+00	29.01008633	35.4626305
			6	1.106	5.02E+08	5.90E+07	8.51E+00	33.41620907	47.86620317

L Zandberg	20/08/1982	J2	0	5.611	5.53E+08	8.91E+07	6.21E+00	24.50759946	6.919702648
			1	3.032	3.38E+08	9.01E+06	3.75E+01	145.6671648	76.11309776
			2	1.507	3.09E+08	8.21E+06	3.76E+01	146.1437732	153.6363535
			3	1.772	2.34E+08	5.74E+07	4.08E+00	16.26505804	14.54182209
			4	9.114	2.69E+08	1.69E+07	1.59E+01	62.08783659	10.79255897
			5	2.798	3.70E+08	1.43E+07	2.59E+01	100.6213167	56.97301638
			6	5.492	2.28E+08	1.18E+07	1.93E+01	75.26470677	21.7113972

Test person	Date of birth	Nr.	Time	Glycine	Stable isotope	Gly/SI	[Gly] mmol/L	g Gly/g Cr
Z Lindeque	01/14/1983	A2	0	2377728	22322176	0.106518648	0.00111179	7.04419E-05
			1	478232	12082176	0.039581612	0.000447437	0.000101257
			2	Undetectable	18402304	-	-	0.00001
			3	Undetectable	8835072	-	-	0.00001
			4	Undetectable	8537088	-	-	0.00001
			5	202889	2253236	0.090043386	0.000948272	7.8559E-05
			6	334928	4410880	0.07593224	0.000808218	6.82338E-05
A Phoofolo	12/07/1983	C2	0	1904768	9756672	0.195227225	0.001992226	0.000142786
			1	777856	17767424	0.043779897	0.000489105	0.000179559
			2	Undetectable	14283776	-	-	0.00001
			3	Undetectable	16612352	-	-	0.00001
			4	Undetectable	9125888	-	-	0.00001
			5	277360	4594688	0.060365361	0.000653716	9.41223E-05
			6	Undetectable	5460736	-	-	0.00001
C van Heerden	25/08/1983	D2	0	4333056	7712000	0.561858921	0.005631061	0.000269747
			1	1774272	13756416	0.12897778	0.001334698	0.000435762
			2	968064	6378752	0.151763856	0.00156085	0.000336368
			3	724416	2447104	0.296029919	0.002992697	0.00018542
			4	1053888	3035648	0.347170686	0.003500271	0.000431039
			5	557504	1747072	0.319107627	0.003221744	0.000234554
			6	596416	1735168	0.343722337	0.003466046	0.000276679
E van Dyk	05/11/1981	G2	0	1380032	6229248	0.221540706	0.002253389	0.000148987
			1	333008	3914752	0.085064903	0.00089886	0.000115467
			2	238720	3149568	0.075794522	0.000806852	7.70793E-05

			3	416336	1996608	0.208521653	0.002124174	0.000217883
			4	237056	1828800	0.129623797	0.001341109	0.000102909
			5	210192	2272000	0.092514085	0.000972794	0.000116719
			6	298480	1954880	0.152684564	0.001569988	0.000112913
J Pretorius	27/01/1982	H2	0	2585344	24131584	0.10713528	0.00111791	0.000161307
			1	4289024	21619712	0.198384881	0.002023566	0.000574237
			2	3098368	43790336	0.070754607	0.00075683	0.000343367
			3	2763776	30208000	0.091491525	0.000962645	0.000333136
			4	2579968	41177088	0.062655426	0.000676445	0.000306687
			5	2752000	53063680	0.051862215	0.000569322	0.00029158
			6	3205120	52887552	0.06060254	0.00065607	0.000393731
L Zandberg	20/08/1982	J2	0	2578176	18259968	0.141192799	0.001455932	0.000172228
			1	521456	8356096	0.062404262	0.000673953	0.000147538
			2	439696	7481856	0.058768306	0.000637866	0.000280944
			3	Undetectable	10018816	-	-	0.00001
			4	331680	3099136	0.107023377	0.001116799	8.13336E-05
			5	340800	4028928	0.084588258	0.00089413	0.000212108
			6	416064	5374208	0.077418663	0.000822971	9.94622E-05

Test person	Date of birth	Nr.	Time	Benzoylcarnitine	Stable isotope	BC/SI	[BC]mmol/L	g BC/g Cr
Z Lindeque	01/14/1983	A2	0	1407208	2691560	0.522822452	0.002390083	0.0003349
			1	7366304	2314984	3.182010761	0.014546562	0.0072794
			2	6816528	2382956	2.860534563	0.013076934	0.007351
			3	17463296	1876628	9.305678057	0.042540907	0.0132509
			4	25725376	1658384	15.5123156	0.070914551	0.0199738
			5	21757632	1133501	19.19507085	0.087750266	0.0160751
			6	39441472	1546788	25.49895138	0.116568456	0.0217617
A Phoofolo	12/07/1983	C2	0	1519168	867237	1.751733379	0.008008049	0.0012692
			1	3170876	2274716	1.393965664	0.006372514	0.0051732

			2	2958644	1055628	2.802733539	0.012812696	0.009764
			3	6749296	1190380	5.669866765	0.025919796	0.0228351
			4	8425296	1179064	7.14574951	0.032666794	0.0105329
			5	12962416	747489	17.34127994	0.079275661	0.0252397
			6	32688000	1355492	24.11522901	0.110242769	0.020889
C van Heerden	25/08/1983	D2	0	1314022	1362032	0.964751195	0.00441036	0.0004672
			1	3244320	1584648	2.047344268	0.009359434	0.0067571
			2	12865760	1599080	8.045726293	0.036781038	0.0175275
			3	18529888	989544	18.72568375	0.085604463	0.0117282
			4	13996624	816367	17.14501444	0.078378433	0.0213429
			5	28753280	965408	29.78355265	0.136155511	0.0219194
			6	26948224	879885	30.62698421	0.140011258	0.0247142
E van Dyk	05/11/1981	G2	0	1156960	1229992	0.940624004	0.004300063	0.0006287
			1	1678478	701090	2.394097762	0.010944618	0.0031089
			2	11535888	997734	11.56208769	0.052856084	0.0111656
			3	22927936	955499	23.99577184	0.109696671	0.024881
			4	22323520	719654	31.01979562	0.141806996	0.0240617
			5	35993088	1021628	35.23110956	0.161059017	0.0427315
			6	35557120	743004	47.8558931	0.218773215	0.0347924
J Pretorius	27/01/1982	H2	0	2172840	1843128	1.178887196	0.005389283	0.0017196
			1	1162903	1915328	0.607156059	0.002775614	0.0017417
			2	985308	2696596	0.36538955	0.001670378	0.0016758
			3	1179756	2273648	0.51888243	0.002372071	0.0018152
			4	800214	2524788	0.316943046	0.001448905	0.0014526
			5	921448	3235156	0.284823359	0.00130207	0.0014746
			6	748711	3809904	0.196517025	0.000898378	0.0011922
L Zandberg	20/08/1982	J2	0	780145	1964436	0.397134343	0.0018155	0.0004749
			1	3525824	1590128	2.21732087	0.010136482	0.0049069

			2	8528400	1400540	6.08936553	0.027837535	0.0271121
			3	9577968	1318565	7.263933139	0.03320707	0.027505
			4	28627392	995910	28.74495888	0.13140758	0.021162
			5	19793088	684740	28.90599059	0.132143736	0.0693177
			6	13774112	912644	15.09253553	0.068995526	0.0184389

Test person	Date of birth	Nr.	Time	Benzoic acid	mol/mol	g/g Cr
Z Lindeque	01/14/1983	A2	0	2.395	0.002395	1.96151E-05
			1			
			2	146.805	0.146805	0.001202334
			3			
			4	26.976	0.026976	0.000220934
			5			
			6			
A Phoofolo	12/07/1983	C2	0	2.095	0.002095	1.71581E-05
			1			
			2	284.745	0.284745	0.002332064
			3			
			4	121.033	0.121033	0.000991261
			5			
			6			
C van Heerden	25/08/1983	D2	0	1.919	0.001919	1.57166E-05
			1			
			2	393.732	0.393732	0.003224668
			3			
			4	54.646	0.054646	0.000447551
			5			
			6			
E van Dyk	05/11/1981	G2	0	3.087	0.003087	2.52826E-05
			1			
			2	52.539	0.052539	0.000430295

			3			
			4	30.58	0.03058	0.00025045
			5			
			6			
J Pretorius	27/01/1982	H2	0	2.346	0.002346	1.92138E-05
			1			
			2	124.533	0.124533	0.001019926
			3			
			4	52.763	0.052763	0.000432129
			5			
			6			
L Zandberg	20/08/1982	J2	0	0.754	0.000754	6.17527E-06
			1			
			2	323.633	0.323633	0.002650557
			3			
			4	80.572	0.080572	0.000659885
			5			
			6			

ANNEXURE C: THE 250MG/KG BODYWEIGHT SODIUM BENZOATE LOADING TEST

Test person	Date of birth	Nr.	Time	[Cr]mmol/L	Hippurate	Stable isotope	HA/SI	[HA]mmol/L	g HA/g Cr
T Venter	29/11/1982	B1	0	20.023	6.61E+07	9.14E+07	7.23E-01	3.285570486	2.60E-01
			1	11.353	8.45E+08	3.00E+07	2.81E+01	109.3093447	1.53E+01
			2	7.098	6.69E+08	1.74E+07	3.85E+01	149.6621399	3.34E+01
			3	6.194	5.50E+08	1.17E+07	4.69E+01	181.8778447	4.65E+01
			4	5.407	5.02E+08	7.78E+06	6.46E+01	250.4089318	7.34E+01
			5	8.617	4.91E+08	7.12E+06	6.89E+01	266.9888089	4.91E+01
			6	12.918	3.38E+08	8.57E+06	3.94E+01	152.898209	1.88E+01
			9	11.139	2.49E+08	2.21E+07	1.13E+01	44.18168203	6.28E+00
			12	4.907	9.46E+07	2.64E+07	3.59E+00	14.36529524	4.64E+00
J Pretorius	27/01/1982	D1	0	7.544	63084772	19079850	3.31E+00	13.28399814	2.79E+00
			1	8.275	225138000	5641444	3.99E+01	154.9319271	2.97E+01
			2	14.64	124163408	7091598	1.75E+01	68.24641643	7.39E+00
			3	12.342	130227312	7733590	1.68E+01	65.65605585	8.43E+00
			4	14.364	94262856	8568178	1.10E+01	43.06424345	4.75E+00
			5	12.025	59765648	6875591	8.69E+00	34.12814764	4.50E+00
			6	12.941	37217168	7688221	4.84E+00	19.22231461	2.35E+00
			9	17.4	23393508	6876046	3.40E+00	13.65481666	1.24E+00
			12	14.707	18540998	7576377	2.45E+00	9.959108558	1.07E+00
S Labuschagne	07/09/1982	E1	0	22.966	16669468	6852608	2.43E+00	9.902456303	6.83E-01
			1	8.546	45754596	1296932	3.53E+01	137.0185935	2.54E+01
			2	6.143	41739608	733568	5.69E+01	220.6893466	5.69E+01
			3	11.715	75617624	1817266	4.16E+01	161.5217084	2.18E+01
			4	13.791	57190172	645660	8.86E+01	343.2788535	3.94E+01
			5	11.363	49568424	609678	8.13E+01	315.1297408	4.39E+01
			6	7.805	36915884	1955705	1.89E+01	73.53854754	1.49E+01
			9	22.974	22266652	3488939	6.38E+00	25.18701779	1.74E+00
L Erasmus	16/08/1961	F1	0	9.437	10459022	3162965	3.31E+00	13.28538618	2.23E+00
			1	3.266	22410582	1155534	1.94E+01	75.54373687	3.66E+01
			2	5.291	23689216	610766	3.88E+01	150.5905882	4.51E+01
			3	4.975	18442468	342743	5.38E+01	208.727193	6.65E+01

			4	2.065	6848455	320468	2.14E+01	83.19099014	6.38E+01
			5	6.448	8296448	277214	2.99E+01	116.3096425	2.86E+01
			6	10.359	4760548	182782	2.60E+01	101.2824054	1.55E+01
			9	16.03	1961280	191097	1.03E+01	40.20726894	3.97E+00
			12	16.017	1315712	227188	5.79E+00	22.90070589	2.27E+00

Test person	Date of birth	Nr.	Time	Glycine	Stable isotope	Gly/SI	[Gly] mmol/L	g Gly/g Cr
T Venter	29/11/1982	B1	0	534144	6273280	0.085145889	9.00E-04	2.98233E-05
			1	215584	2285312	0.094334603	9.91E-04	5.79304E-05
			2	Undetectable	35889152	-	-	0.00001
			3	188176	2272256	0.082814612	8.77E-04	9.39285E-05
			4	270624	3061504	0.088395769	9.32E-04	0.0001144
			5	201168	1808576	0.111230051	1.16E-03	8.92407E-05
			6	275392	1565376	0.175927062	1.80E-03	9.25216E-05
			9	557184	5152256	0.108143695	1.13E-03	6.72102E-05
			12	842240	23191552	0.036316673	4.15E-04	5.61396E-05
J Pretorius	27/01/1982	D1	0	2805504	14458880	0.194033286	1.98E-03	0.000174241
			1	308192	2632960	0.117051531	1.22E-03	9.75634E-05
			2	Undetectable	96948224	-	-	0.00001
			3	900928	3428352	0.262787485	2.66E-03	0.000143203
			4	1587200	3675136	0.431875174	4.34E-03	0.000200593
			5	1392320	4550912	0.305943073	3.09E-03	0.00017062
			6	1117952	3027712	0.369239875	3.72E-03	0.000190765
			9	1871744	3798784	0.492721882	4.94E-03	0.00018863
			12	1960320	4331520	0.452570922	4.55E-03	0.000205185
S Labuschagne	07/09/1982	E1	0	1780288	4245760	0.419309617	4.22E-03	0.000121856
			1	402912	3414016	0.118017022	1.23E-03	9.52138E-05
			2	Undetectable	47312896	-	-	0.00001
			3	218304	2349568	0.092912399	9.77E-04	5.53406E-05
			4	217392	2075328	0.104750671	1.09E-03	5.26649E-05
			5	179712	1502016	0.119647194	1.24E-03	7.25545E-05
			6	440048	4279808	0.102819566	1.08E-03	9.14261E-05
			9	734528	3921152	0.187324541	1.91E-03	5.52919E-05

L Erasmus	16/08/1961	F1	0	694592	8373504	0.082951176	8.78E-04	6.17456E-05
			1	331600	7611648	0.04356481	4.87E-04	9.89669E-05
			2	Undetectable	33808384	-	-	0.00001
			3	Undetectable	5180672	-	-	0.00001
			4	Undetectable	22136832	-	-	0.00001
			5	216144	3053824	0.070778146	7.57E-04	7.79312E-05
			6	383776	3164672	0.121268808	1.26E-03	8.06178E-05
			9	626112	4817152	0.129975554	1.34E-03	5.56754E-05
			12	833344	7625216	0.10928792	1.14E-03	4.72119E-05

Test person	Date of birth	Nr.	Time	Benzoylcarnitine	Stable Isotope	BC/SI	[BC]mmol/L	g BC/g Cr
T Venter	29/11/1982	B1	0	1010229	903618	1.117982378	0.005110856	0.000374636
			1	6179680	713484	8.661273413	0.039595011	0.005118884
			2	11225648	627397	17.89241581	0.081795179	0.016913644
			3	19150272	784715	24.40411105	0.111563394	0.026436016
			4	24055680	980344	24.53799891	0.112175462	0.030449978
			5	37502208	784594	47.7982345	0.218509629	0.037218595
			6	61877440	825792	74.93102379	0.342547175	0.038919799
			9	69694400	1064279	65.48508427	0.299365063	0.039445766
			12	16108736	1170150	13.76638551	0.062933031	0.018823834
J Pretorius	27/01/1982	D1	0	1234325	960154	1.285548985	0.005876887	0.001143381
			1	7104288	865202	8.211132198	0.037537191	0.00665793
			2	18644192	818806	22.76997482	0.10409294	0.010435798
			3	17195456	990305	17.36379802	0.079378603	0.009439813
			4	12046512	990893	12.15722787	0.055576767	0.005678889
			5	8224752	901059	9.12787287	0.041728071	0.005093176
			6	7625584	1218594	6.25769042	0.028607032	0.00324452
			9	4139488	1076952	3.843707055	0.017571507	0.001482195
			12	3527564	964384	3.65784169	0.016721823	0.001668803
S Labuschagne	07/09/1982	E1	0	759048	860390	0.88221388	0.004033041	0.000257746
			1	4795920	895265	5.356983686	0.024489451	0.004205926
			2	8056544	915114	8.803869245	0.040246888	0.009616062
			3	19815040	645265	30.70837563	0.140383339	0.017588093
			4	23338048	662927	35.20455193	0.160937609	0.017128024

			5	21741056	792874	27.42056872	0.12535313	0.016191521
			6	33957184	1350328	25.14735975	0.114961155	0.021618407
			9	22608064	979935	23.07098328	0.105469	0.006738042
L Erasmus	16/08/1961	F1	0	1319708	951273	1.387307324	0.006342075	0.000986377
			1	2411456	1307348	1.844540245	0.008432316	0.00378945
			2	4871824	895201	5.442156566	0.024878819	0.006901404
			3	5298156	771424	6.868020699	0.031397157	0.009262809
			4	5306400	1268713	4.182506209	0.019120327	0.01359004
			5	14657248	997154	14.69908159	0.067196851	0.015295701
			6	25709312	1112539	23.10868383	0.105641348	0.014967925
			9	24322624	1039668	23.39460674	0.106948445	0.009792339
			12	12524624	1237904	10.11760524	0.046252632	0.004238388

Test person	Date of birth	Nr.	Time	Benzoic acid	mol/mol	g/g Cr
T Venter	29/11/1982	B1	0	1.893	0.001893	1.55037E-05
			1			
			2	89.159	0.089159	0.000730213
			3			
			4	85.542	0.085542	0.00070059
			5			
			6			
			9			
			12			
J Pretorius	27/01/1982	D1	0	1.79	0.00179	1.46601E-05
			1			
			2	23.447	0.023447	0.000192031
			3			
			4	8.849	0.008849	7.24734E-05
			5			
			6			
			9			
			12			
S Labuschagne	07/09/1982	E1	0	0.518	0.000518	4.24242E-06
			1			

			2	411.612	0.411612	0.003371106
			3			
			4	55.647	0.055647	0.000455749
			5			
			6			
			9			
L Erasmus	16/08/1961	F1	0	5.467	0.005467	4.47748E-05
			1			
			2	169.164	0.169164	0.001385455
			3			
			4	184.458	0.184458	0.001510713
			5			
			6			
			9			
			12			

ANNEXURE D: THE 150MG/KG BODYWEIGHT SODIUM BENZOATE LOADING TEST

Test person	Date of birth	Nr.	Time	[Cr]mmol/L	Hippurate	Stable Isotope	HA/SI	[HA]mmol/L	g HA/g Cr
N Homan	1980/08/31	A	0	4.306741	10243615	20378870	0.502658636	4.335191223	1.594727309
			1	1.213737	69015472	14683791	4.700112662	40.53623214	52.9109689
			2	0.46715	91399456	22629540	4.038944495	34.83397174	118.1336355
			3	0.787116	71932312	10389094	6.923829162	59.71472736	120.1903613
			4	1.949659	67789296	8181320	8.285862917	71.4616195	58.06856886
			5	1.180344	58283624	6675180	8.73139361	75.30410944	101.0734308
			6	2.079534	77352792	3991951	19.37718975	167.1190285	127.3171195
			9	6.043566	96088224	3443937	27.90069156	240.6301702	63.07884555
			12	13.57903	30670070	5269566	5.820226941	50.19668407	5.856433398
H Du Toit	1984/06/24	B	0	21.22	3814113	4428186	0.861326286	7.428528799	0.554605739
			1	10.71682	60707472	1083538	56.02708165	483.2068827	71.43221262
			2	6.993414	49875188	691858	72.08876388	621.7312387	140.8446574
			3	7.474671	51454936	653193	78.77447554	679.392316	143.9976737
			4	9.703647	60243968	1636483	36.81307291	317.4952127	51.83571808
			5	7.5	57778832	996191	57.99975306	500.2202337	105.6639687
			6	11.00811	84095304	1323543	63.53802181	547.9851627	78.86475642
			9	19.03749	102246480	2097075	48.75671113	420.5034013	34.99344923
			12	15.04813	11383235	4335944	2.625318731	22.64212311	2.383754579
F van der Spuy	1982/08/23	C	0	4.019	8007240	15752397	0.508318829	4.384007691	1.728145197
			1	4.1692	40651040	3411313	11.91653771	102.7744596	39.0534495
			2	6.158	42818600	1020242	41.96906224	361.9631639	93.12185496
			3	5.866261	64106988	1642404	39.03241103	336.6359464	90.91300948
			4	5.980243	68650104	1576100	43.55694689	375.6579124	99.5177702
			5	6.08156	68011128	1298880	52.36136364	451.5918115	117.6408056
			6	14.56687	53539800	2198963	24.34774937	209.9877368	22.83781843
			9	6.537487	16976118	8199865	2.070292377	17.85528527	4.326960018
			12	6.018237	4100403	9086863	0.451245166	3.891774539	1.024484314
D Conradie	1980/12/28	D	0	3.852	4305654	9254564	0.465246553	4.012529832	1.65028489
			1	0.81307	28021350	3729726	7.512978165	64.79585679	126.2543142
			2	1.073	22927770	2362028	9.706815499	83.71665845	123.6058685

			3	1.636272	33085726	4187454	7.90115569	68.14370298	65.97769795
			4	2.370821	53984624	2036580	26.50749001	228.6144707	152.767819
			5	5.283688	70202488	1443151	48.64528244	419.5423819	125.7956438
			6	11.16008	52040008	1553293	33.503021	288.9475922	41.01839844
			9	30.59568	58765476	2926927	20.07753388	173.1591629	8.966279959
			12	6.968085	18871296	13585162	1.389110855	11.98041922	2.72386452
M Cilliers	1981/05/16	E	0	19.068	16218112	5760697	2.815303773	24.28065357	2.017354199
			1	2.244174	37174888	4209644	8.830886412	76.16218745	53.76625775
			2	1.457	33572948	3360307	9.991035938	86.16792431	93.69426463
			3	1.193009	26159062	3534715	7.400614194	63.82677108	84.7590503
			4	3.81459	35175832	2145485	16.39528219	141.4014966	58.7263579
			5	3.700608	51400048	2447242	21.00325509	181.1430673	77.54888561
			6	5.663627	62037852	1779968	34.85335242	300.5935572	84.08372667
			9	16.21327	38699704	2986445	12.95845194	111.7604734	10.92055083
			12	12.41388	15049523	7020575	2.143631113	18.48779692	2.359416143
Zelda van Zweel	1983/07/21	F	0	19.772	3310561	4240620	0.780678533	6.732980358	0.53949034
			1	8.462513	52577240	1376458	38.1974895	329.4351461	61.67334569
			2	4.004559	50172888	1630840	30.7650585	265.3339702	104.9699732
			3	3.257345	34018876	1093071	31.12229306	268.4149481	130.5478929
			4	3.725937	46661632	1559017	29.9301624	258.1333892	109.7578531
			5	4.707912	53703640	1199701	44.76418708	386.0697837	129.9165273
			6	4.305428	83013960	2367212	35.06824061	302.4468655	111.2909077
			9	4.903404	10663403	9269565	1.150367142	9.921368461	3.205537259
			12	13.42456	14739948	6419886	2.295982826	19.80175785	2.336848795
Henru Mentz	1982/12/16	G	0	12.715	1570465	3237069	0.485150301	4.184190176	0.521341117
			2	7.295308	73584968	1760210	41.80465285	360.5452112	78.29662227
			3	3.661454	61470804	2128607	28.87841861	249.0626001	107.7660247
			4	1.361546	62626364	5244734	11.94080844	102.9837831	119.829418
			9	9.917203	131198816	5746780	22.82997017	196.8976144	31.4541663
			12	17,31141	9805824	8332606	1.176801591	10.14935299	0.928822746
Susan Lindeque	1983/07/21	H	0	39.24953	7081943	3714499	1.906567481	16.44323605	0.663712135
			1	15.54048	101439080	1587062	63.91626792	551.2473552	56.19646758
			2	9.618215	95267064	1037440	91.82898674	791.9812547	130.4509809

			3	8.617755	79469608	1124142	70.69356718	609.698332	112.0850761
			4	11.69963	68006704	1401877	48.51117751	418.3857908	56.6541498
			5	20.46228	46505820	2380344	19.53743661	168.5010813	13.04594659
			6	30.92402	27565096	2834811	9.723786171	83.86302241	4.296368187
			9	0.798068	16475505	24144650	0.682366694	5.885087593	11.68261222
			12	2.844986	5202412	5318523	0.978168563	8.436237765	4.697811698
Danie Visagie	1984/03/13	I	0	10.41	4586220	5916795	0.775118962	6.68503171	1.017371739
			1	9.112236	93112728	2941020	31.66001183	273.0525163	47.47316112
			2	5.777369	80622224	1770353	45.54019679	392.7624977	107.7027603
			3	6.283349	68252304	1536443	44.42228185	383.1210141	96.59879707
			4	5.535879	69928064	1691384	41.34369487	356.5696683	102.0433565
			5	10.0552	84926464	1385569	61.29356532	528.6277949	83.28872395
			6	10.17019	102391976	1360085	75.28351243	649.2844226	101.1423044
			9	16.01196	15691735	6029937	2.602304966	22.44364035	2.220627591
			12	10.48068	1401493	1088645	1.287373754	11.1029852	1.678329496
Riaan van Zyl	1984/02/07	J	0	17.134	3288996	4962263	0.662801629	5.71634823	0.528550931
			1	12.17111	84009288	1832760	45.83758266	395.3273092	51.45807744
			2	13.07958	76990216	965576	79.73501413	687.6765037	83.29468968
			3	8.560258	82993480	1325197	62.6272773	540.1304253	99.96286958
			4	14.32153	70697944	1442501	49.01067244	422.6936965	46.75879188
			5	16.55244	53948252	2237758	24.10817077	207.9214855	19.90051311
			6	29.86867	40104836	5942825	6.748446404	58.20213465	3.087094727
			9	38.17073	15693046	4460329	3.518360641	30.34418406	1.259423885
			12	18.82953	2864589	2142631	1.336949293	11.5305506	0.970146885

Test person	Date of birth	Nr.	Time	Glycine	Stable Isotope	Gly/SI	[Gly] mmol/L	g Gly/g Cr
N Homan	1980/08/31	A	0	Undetectable	102436864	-	-	0.00001
			1	Undetectable	175489024	-	-	0.00001
			2	Undetectable	276365312	-	-	0.00001
			3	Undetectable	183009280	-	-	0.00001
			4	Undetectable	100741120	-	-	0.00001
			5	Undetectable	171507712	-	-	0.00001

			6	Undetectable	92082176	-	-	0.00001
			9	2672640	43720704	0.061129848	0.000661304	7.26293E-05
			12	5801984	30308352	0.191431854	0.001954557	9.55396E-05
H Du Toit	1984/06/24	B	0	7746560	37191680	0.208287445	0.002121849	6.63702E-05
			1	2447360	28206080	0.086767108	0.000915755	5.67175E-05
			2	1576512	25222144	0.062505075	0.000674953	6.40602E-05
			3	1923264	27679744	0.069482724	0.000744207	6.60854E-05
			4	1900905	19198690	0.099012224	0.001037288	7.09526E-05
			5	2731008	39993344	0.068286563	0.000732335	6.48115E-05
			6	2708480	27267072	0.099331531	0.001040457	6.27358E-05
			9	3664384	20084736	0.182446212	0.001865374	6.50369E-05
			12	5181696	35889152	0.144380564	0.001487571	6.56144E-05
F van der Spuy	1982/08/23	C	0	6870016	110723072	0.062046833	0.000670405	0.000110719
			1	4025344	65466368	0.061487205	0.000664851	0.000105846
			2	3064320	29264896	0.104709752	0.001093836	0.000117901
			3	2752512	37298176	0.073797496	0.000787031	8.90501E-05
			4	362112	6825472	0.053053034	0.000581141	6.4501E-05
			5	2575872	43077632	0.059796044	0.000648066	7.07307E-05
			6	4054272	29922304	0.13549331	0.001399365	6.3763E-05
			9	7292160	51712000	0.141014851	0.001454166	0.000147641
			12	9161728	57049088	0.160593768	0.001648488	0.000181811
D Conradie	1980/12/28	D	0	5856256	100409344	0.058323815	0.000633454	0.000109152
			1	Undetectable	202407936	-	-	0.00001
			2	Undetectable	133951488	-	-	0.00001
			3	Undetectable	227262464	-	-	0.00001
			4	Undetectable	67776512	-	-	0.00001
			5	Undetectable	41656320	-	-	0.00001
			6	2570496	34484224	0.074541216	0.000794412	4.72479E-05
			9	4590848	29682688	0.15466416	0.001589636	3.44859E-05
			12	5513728	96948224	0.056872914	0.000619054	5.89683E-05
M Cilliers	1981/05/16	E	0	18456576	38793216	0.475768134	0.004776606	0.000166272
			1	8294144	126889984	0.065364844	0.000703337	0.000208023
			2	Undetectable	150355968	-	-	0.00001

			3	Undetectable	133488640	-	-	0.00001
			4	5117184	103223296	0.049573926	0.000546611	9.51118E-05
			5	4733440	73211904	0.064653967	0.000696281	0.000124886
			6	5091328	65130496	0.078171184	0.00083044	9.73234E-05
			9	14856192	40288256	0.368747458	0.003714422	0.000152063
			12	18661376	47312896	0.394424725	0.00396927	0.00021223
Zelda van Zweel	1983/07/21	F	0	7623680	29780992	0.255991473	0.002595314	8.71251E-05
			1	4216576	32061440	0.13151549	0.001359885	0.000106661
			2	Undetectable	56156160	-	-	0.00001
			3	2817280	43245568	0.065146098	0.000701165	0.000142876
			4	2855424	48472064	0.058908653	0.000639259	0.000113879
			5	2644224	30325760	0.08719399	0.000919992	0.000129706
			6	3252480	48910336	0.066498828	0.000714591	0.000110165
			9	5584640	59494400	0.09386833	0.000986235	0.000133502
			12	8794112	33808384	0.260116307	0.002636253	0.000130344
Henru Mentz	1982/12/16	G	0	6158080	47644672	0.129250129	0.001337401	6.9815E-05
			2	3132160	39481344	0.079332659	0.000841968	7.66047E-05
			3	Undetectable	57925632	-	-	0.00001
			4	Undetectable	130740224	-	-	0.00001
			9	3299072	47984640	0.068752668	0.000736961	4.93241E-05
			12	4792320	39186432	0.12229539	0.001268375	4.86316E-05
Susan Lindeque	1983/07/21	H	0	5340672	22835200	0.233878924	0.002375846	4.01779E-05
			1	2524672	24292352	0.103928677	0.001086084	4.63877E-05
			2	2443776	28737536	0.085037771	0.000898591	6.20114E-05
			3	2243328	25056256	0.089531652	0.000943193	7.26458E-05
			4	2076864	27949056	0.074308914	0.000792107	4.49382E-05
			5	3554816	20254720	0.175505561	0.001796488	5.82739E-05
			6	3971840	26374144	0.150595978	0.001549259	3.32531E-05
			9	Undetectable	200212480	-	-	0.00001
			12	Undetectable	150913024	-	-	0.00001
Danie Visagie	1984/03/13	I	0	3917056	35471360	0.110428695	0.001150597	7.33629E-05
			1	2130944	30374912	0.070154738	0.000750876	5.4695E-05
			2	1742400	26025984	0.066948477	0.000719054	8.26105E-05

			3	1632320	27437056	0.059493263	0.000645061	6.81418E-05
			4	Undetectable	43380736	-	-	0.00001
			5	1935040	24320000	0.079565789	0.000844282	5.57315E-05
			6	1924032	28139520	0.068374727	0.00073321	4.78523E-05
			9	4533760	26803200	0.169149952	0.001733408	7.18555E-05
			12	6677760	29239296	0.228383064	0.002321299	0.00014701
Riaan van Zyl	1984/02/07	J	0	4723200	22894592	0.206301995	0.002102144	8.14343E-05
			1	3132416	20217856	0.154933144	0.001592306	8.68361E-05
			2	2057664	19641344	0.104761874	0.001094354	5.55351E-05
			3	1843392	25950208	0.071035731	0.00075962	5.88998E-05
			4	1773632	28039168	0.063255515	0.000682401	3.16267E-05
			5	2797568	21294080	0.131377735	0.001358517	5.44762E-05
			6	3458560	20552704	0.168277614	0.00172475	3.83278E-05
			9	7522048	19698688	0.381855279	0.003844517	6.68521E-05
			12	6337792	19039232	0.332880654	0.003358442	0.000118387

Test person	Date of birth	Nr.	Time	Benzoylcarnitine	Stable isotope	BC/SI	[BC]mmol/L	g BC/g Cr
N Homan	1980/08/31	A	0	552259	1157415	0.477148646	0.002181285	0.000743377
			1	1638075	1878299	0.872105559	0.003986831	0.004821128
			2	2651148	1828095	1.450224414	0.006629701	0.020829702
			3	6582704	1435914	4.584330259	0.020957266	0.039078813
			4	13509952	1350676	10.00236326	0.045725804	0.034422957
			5	11389328	1327329	8.580636752	0.039226381	0.048777005
			6	21365856	1030412	20.73525541	0.09479122	0.066903305
			9	3468816	442418	7.840585148	0.035843235	0.008704812
			12	3198284	378609	8.447458988	0.038617559	0.004174088
H Du Toit	1984/06/24	B	0	5502144	620953	8.86080589	0.040507174	0.002801767
			1	786878	402727	1.953874461	0.008932137	0.001223305
			2	597300	357658	1.670031147	0.007634547	0.001602284
			3	514526	364550	1.411400357	0.006452217	0.001266958
			4	843662	320393	2.633209839	0.012037719	0.001820768
			5	807954	374469	2.15759916	0.009863465	0.001930251

			6	763085	358092	2.130974722	0.009741751	0.001298882
			9	904093	390034	2.317985099	0.010596669	0.000816968
			12	1260088	409585	3.076499384	0.014064217	0.001371761
F van der Spuy	1982/08/23	C	0	649082	379548	1.710144698	0.007817926	0.002855085
			1	503719	356602	1.412552369	0.006457483	0.002273296
			2	702161	368122	1.907413846	0.008719742	0.002078306
			3	489517	368117	1.329786454	0.006079119	0.001520984
			4	738691	372289	1.984187016	0.009070711	0.002226219
			5	492531	384464	1.281084835	0.005856479	0.001413407
			6	1516869	409007	3.708662688	0.016954151	0.001708265
			9	20525936	1252115	16.39301182	0.074940654	0.016824886
			12	2271316	430609	5.274659842	0.024113107	0.005880705
D Conradie	1980/12/28	D	0	996216	414716	2.402164373	0.010981494	0.004184279
			1	831878	405305	2.052474063	0.009382885	0.016937681
			2	459573	434144	1.058572732	0.004839265	0.0066195
			3	457470	418466	1.093207094	0.004997596	0.004482818
			4	424816	376147	1.129388244	0.005162998	0.003196309
			5	659285	418599	1.574979873	0.00720002	0.002000056
			6	861115	398911	2.158664464	0.009868335	0.001297843
			9	1477335	352238	4.194138622	0.019173505	0.000919786
			12	5737452	584162	9.821679603	0.044899808	0.009457504
M Cilliers	1981/05/16	E	0	1840718	448856	4.100909869	0.018747309	0.001443043
			1	669417	436436	1.533826265	0.007011887	0.004585892
			2	962325	427044	2.253456318	0.010301676	0.010377525
			3	555358	428744	1.295313754	0.005921527	0.007285099
			4	809345	422519	1.915523326	0.008756815	0.003369332
			5	869522	510185	1.704326862	0.00779133	0.003090182
			6	1273160	592086	2.150295734	0.009830077	0.002547462
			9	26216192	733514	35.74054756	0.163387913	0.014790907
			12	5463904	688645	7.93428254	0.036271573	0.004288489
Zelda van Zweel	1983/07/21	F	0	1334376	472376	2.824817518	0.012913653	0.000958615
			1	640003	389095	1.644850229	0.007519433	0.001304161
			2	749750	523513	1.432151637	0.006547081	0.002399598

			3	692604	443932	1.560157862	0.007132262	0.003213727
			4	513454	494489	1.038352724	0.004746829	0.001869879
			5	1770101	579688	3.05354087	0.013959262	0.004351907
			6	866178	472415	1.83351079	0.008381895	0.002857402
			9	1944440	516845	3.762133715	0.017198594	0.005148026
			12	864956	436981	1.979390408	0.009048783	0.000989317
Henru Mentz	1982/12/16	G	0	546238	513152	1.064476023	0.004866252	0.000561725
			2	541409	506725	1.068447383	0.004884407	0.000982684
			3	683013	518338	1.317698104	0.006023857	0.002414719
			4	624838	450736	1.386261581	0.006337295	0.006831516
			9	748088	515549	1.451051209	0.006633481	0.000981743
			12	1123375	485359	2.314523889	0.010580846	0.000897085
Susan Lindeque	1983/07/21	H	0	1860640	1610240	1.155504769	0.00528239	0.000197534
			1	11056688	1099836	10.05303336	0.045957442	0.004340471
			2	27704384	1025656	27.01138003	0.123482524	0.01884328
			3	37199104	1014472	36.66843836	0.167629766	0.028549761
			4	69241344	1195188	57.93343307	0.264842689	0.033224721
			5	93773568	1192648	78.62635748	0.359440393	0.025782103
			6	107382784	1247392	86.08583669	0.393541402	0.018678413
			9	7992640	3438164	2.324682592	0.010627286	0.019544653
			12	4686608	2489080	1.882867566	0.008607529	0.004440622
Danie Visagie	1984/03/13	I	0	1162769	1789800	0.649664208	0.00296994	0.000418738
			1	5775232	1287899	4.484227412	0.020499646	0.003301923
			2	15288032	1094723	13.96520581	0.063841938	0.016218902
			3	24239424	1093716	22.16244802	0.101315631	0.023666321
			4	28477952	1087304	26.19134299	0.119733724	0.031745004
			5	44668416	1193784	37.41750266	0.171054113	0.024968261
			6	53596352	851742	62.92557136	0.287664249	0.041514739
			9	31301696	1292310	24.22150722	0.11072862	0.010149879
			12	1565964	1620427	0.966389723	0.004417851	0.000618681
Riaan van Zyl	1984/02/07	J	0	687074	1169805	0.587340625	0.002685028	0.000230004
			1	8394544	1087372	7.720029576	0.035292115	0.004255915
			2	26003904	1058709	24.56189945	0.112284723	0.012600053

			3	36752960	1068372	34.40090156	0.157263721	0.026964178
			4	71127552	1157276	61.46118298	0.280969798	0.028794909
			5	89882368	1507926	59.6066173	0.272491651	0.02416221
			6	90860544	1387830	65.46950563	0.299293845	0.014707111
			9	27317824	1188629	22.98263293	0.105065106	0.004039927
			12	13252192	1310548	10.11194706	0.046226766	0.003603292

Test person	Date of birth	Nr.	Time	Benzoic acid	mol/mol	g/g Cr
N Homan	1980/08/31	A	0	1.361	0.001361	1.11466E-05
			1			
			2	240.577	0.240577	0.001970328
			3			
			4	146.219	0.146219	0.001197535
			5			
			6			
			9			
			12			
H Du Toit	1984/06/24	B	0	1.482	0.001482	1.21376E-05
			1			
			2	82.742	0.082742	0.000677658
			3			
			4	156.746	0.156746	0.001283751
			5			
			6			
			9			
			12			
F van der Spuy	1982/08/23	C	0	2.44	0.00244	1.99836E-05
			1			
			2	96.974	0.096974	0.000794218
			3			
			4	54.806	0.054806	0.000448862
			5			

			6			
			9			
			12			
D Conradie	1980/12/28	D	0	55.234	0.055234	0.000452367
			1			
			2	235.375	0.235375	0.001927723
			3			
			4	73.028	0.073028	0.0005981
			5			
			6			
			9			
			12			
M Cilliers	1981/05/16	E	0	5.126	0.005126	4.1982E-05
			1			
			2	111.382	0.111382	0.000912219
			3			
			4	53.439	0.053439	0.000437666
			5			
			6			
			9			
			12			
Zelda van Zweel	1983/07/21	F	0	2.74	0.00274	2.24406E-05
			1			
			2	43.802	0.043802	0.000358739
			3			
			4	155.095	0.155095	0.001270229
			5			
			6			
			9			
			12			
Henru Mentz	1982/12/16	G	0	2.556	0.002556	2.09337E-05
			2	21.742	0.021742	0.000178067
			3			

			4	45.593	0.045593	0.000373407
			9			
			12			
Susan Lindeque	1983/07/21	H	0	2.38	0.00238	1.94922E-05
			1			
			2	35.983	0.035983	0.000294701
			3			
			4	8.531	0.008531	6.9869E-05
			5			
			6			
			9			
			12			
Danie Visagie	1984/03/13	I	0	1.099	0.001099	9.00082E-06
			1			
			2	131.31	0.13131	0.00107543
			3			
			4	37.876	0.037876	0.000310205
			5			
			6			
			9			
			12			
Riaan van Zyl	1984/02/07	J	0	1.835	0.001835	1.50287E-05
			1			
			2	22.261	0.022261	0.000182318
			3			
			4	285.587	0.285587	0.00233896
			5			
			6			
			9			
			12			