Methods for the measurement of urinary biomarkers of oxidative stress- application to type 1 diabetes mellitus

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Abstract

The principal aim of the study was to develop methods for the measurement of potential urinary biomarkers of oxidative stress using liquid chromatography/tandem mass spectrometry with minimum sample preparation to avoid artefact formation. Initially the development of an assay to measure the urinary concentrations of isoprostanes (8-isoPGF2α) was attempted but this did not prove to be sufficiently sensitive and gave non-reproducible results. An assay to measure the intact sulphate and glucuronide conjugates of urinary metabolites of vitamin E [α-tocopheronolactone (α-TLHQ) and α-carboxy-ethyl-hydroxychroman (α-CEHC)] was then developed, as it has been suggested that α-TLHQ with an oxidised chroman ring might be an indicator of oxidative stress. A novel method was also developed to quantitate urinary amino acids associated with $NO^{\bullet}$ metabolism (L-arginine - precursor, L-citrulline - product, L-ADMA –inhibitor of nitric oxide synthase and L-homocysteine – reduces bioavailability of nitric oxide). This method was extended to quantitate seven additional amino acids. The latter two methods were applied to 32 children with type 1 diabetes and compared with age and sex matched controls. The mean concentrations of all the α-THLQ conjugates were highly significantly increased in the diabetic subjects ($p<0.002$). The concentrations of the α-CEHC conjugates were also increased but not to the same degree of significance ($p<0.05$). When the diabetic children were divided into those who were poorly (n=24) and adequately (n=8) controlled, the α-THLQ conjugates remained highly significantly increased ($p<0.002$) in the poorly controlled group compared to controls. However, the concentrations of the α-CEHC conjugates were not significantly different. The diabetic subjects had a highly significantly increased concentration ($p<0.0001$) of all the urinary amino acids studied compared to controls. These results suggest that the measurement of urinary α-TLHQ conjugates may provide a useful biomarker of oxidative stress. The clinical relevance of the increased concentrations of urinary amino acids in children with type 1 diabetes requires further investigation.

I, Gayatri Sharma, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis

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Abbreviations

8-OHdG	8-hydroxydeoxyguanosine
ACN	Acetonitrile
ADMA	Asymmetric dimethyl arginine
AGE	Advanced glycation end products
AP	Abasic site
ATP	Adenosine triphosphate
CEHC	Carboxy-ethyl-hydroxychroman
CMBHC	Carboxy-methyl-butyl-hydrochroman
C.V.	Coefficient of variation
CVD	Cardiovascular disease
DDAH	Dimethylarginine dimethylaminohydrolase
DHA	Dehydroascorbate
ECD	Electrochemical detection
EIA	Enzyme immuno assay
ELISA	Enzyme-linked immunosorbent assay
EDRF	Endothelium-derived relaxing factor
EPR	Electron paramagnetic resonance
ER	Endoplasmic reticulum
ESI	Electrospray ionisation
ESI-MS/MS	Electrospray triple quadrupole tandem mass spectrometry
ESR	Electron spin resonance
ETC	Electron transport chain
FADH	Flavin adenine dinucleotide-reduced
FMOC	9-fluorenylmethylchloroformate
GC-MS	Gas chromatography-mass spectrometry
GC-NICI-MS	Gas chromatography- negative ion chemical ionization–mass spectrometry
GSH	Reduced glutathione
GSSG	Oxidised glutathione
H_2O_2	Hydrogen peroxide
HbA1c%	Glycosylated/Glycated haemoglobin
HDL	High density lipoprotein
HPLC	High performance liquid chromatography
HPLC-MS/MS	Liquid chromatography tandem mass spectrometry
IsoPs	Isoprostanes
IS	Internal standard
$L^{\bullet}$	Initial radical
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LC-ESI-MS	Liquid chromatography tandem mass spectrometry
LDL	Low density lipoprotein
LH	Polyunsaturated fatty acyl chain
$LOO^{\bullet}$	Peroxyl radical

LOOH	Lipid hydroperoxide
MDA	Malondialdehyde
MeOH	Methanol
MRM	Multiple reaction monitoring mode
MS/MS	Triple quadrupole tandem mass spectrometry
NHS	National health scheme
NICE	National institute for health and clinical excellence
$NO^{\bullet}$	Nitric oxide
NOS	Nitric oxide synthase
NRP	Non radical addunct
O-O	Singlet oxygen
$^{\bullet}$O-O	Superoxide radical
$OH^{\bullet}$	Hydroxyl radical
OPA	Ortho-phthalaldehyde
$ONOO^{-}$	Peroxynitrite
PG	Prostaglandin
PKC	Protein kinase C
PUFA	Polyunsaturated fatty acids
Q1	Mass analyser
q2	Collision cell
Q3	Second mass analyser
QqQ	Triple quadrupole system
r^2	Coefficient of determination
RAGE	Receptor for advanced glycation end products
RCS	Reactive chlorine species
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RP	Reverse phase
SDA	Semidehydroascorbate
SDMA	Symmetric dimethylarginine
SO_3^{-}	Sulphite ion
SOD	Superoxide dismutase
SPE	Solid phase extraction
TA	Tocopheronic acid
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
THQ/TQ	Tocopherylquinone
TL	Tocopheronolactone
TLC	Thin layer chromatography
TLHQ	Tocopheronolactone
TMP	Tocopherol mediated peroxidation
$TO^{\bullet}$	α-Tocopherol radical
TOH	α-Tocopherol
TTP	Tocopherol transfer protein
VCAM-1	Vascular cell adhesion molecule type 1
VLDL	Very low density lipoprotein

Chapter 1

Introduction

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1 Introduction

The overall aim of this study was to develop methodologies to quantitate possible urinary biomarkers of oxidative stress that could be utilised in human diseases. The methods developed were aimed to be specific and rapid using reverse phase high pressure liquid chromatography together with tandem mass spectrometry as the principal methodology used in this current study. This section of the thesis will introduce the concept of oxidative stress, discuss the current biomarkers of oxidative stress and conclude with the principles of the methodology.

1. 1 Background information

1.1. 1 Oxidative stress

Oxidative stress refers to the situation where there is an imbalance between the production of reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) and antioxidant defences (Halliwell and Gutteridge 1999). Sies (1986), first introduced the term in the 1985 book entitled, "Oxidative Stress". He re-defined it in 1991 in the introduction to the second edition as "a disturbance in the prooxidant-antioxidant balance in favour of the former, leading to potential damage" (Sies 1991).

In principle, oxidative stress can result from:

1. Increased production of ROS/ RNS (Halliwell & Gutteridge 1999)

Or

2. Diminished concentrations or activities of antioxidants (Halliwell & Gutteridge 1999)

Therefore, in order to understand oxidative stress it is necessary to consider the chemistry of oxygen and reactive oxygen and nitrogen species, and antioxidant defence mechanisms.

1.1. 2 Oxygen

Oxygen (O_2) exists in the air as a diatomic molecule, which strictly should be called dioxygen. Over 99% of the O_2 in the atmosphere is the isotope oxygen-16 but there are traces of oxygen-17 (approximately 0.04%) and oxygen-18 (approximately 0.2%). O_2 appeared in significant amounts in the earth's atmosphere over 2.5 x 10^9 years ago, and geological evidence suggests that this was due to the evolution of photosynthesis by blue green algae (cyanobacteria). Except for certain anaerobic unicellular organisms, all animals, plants and bacteria require O_2 for efficient production of energy by the use of O_2

dependent electron transport chains, such as those in the mitochondria of eukaryotic cells (Gilbert 1981).

1.1. 3 Activation of oxygen

One of the paradoxes of life on this planet is that the molecule that sustains aerobic life, oxygen, is not only essential for energy metabolism and respiration, but has also been implicated in many diseases and degenerative conditions (Marx 1985). A common factor in

these conditions is the suggestion that partially reduced forms of oxygen and chemical species derived from oxygen acting as free radicals may be implicated (Gutteridge 1996). A free radical is any chemical species capable of independent existence that contains one or more impaired electrons. The presence of one or more unpaired electrons results in free radicals being highly reactive chemical species (Gutteridge 1996). The following section describes the current understanding of the general principles of activated oxygen.

Atmospheric oxygen in its ground-state is distinctive among the gaseous elements because it is a biradical, or in other words it has two unpaired electrons. This feature makes oxygen paramagnetic; it also makes oxygen extremely unlikely to participate in reactions with organic molecules unless it is "activated". The requirement for activation occurs, because the unpaired electrons in oxygen have parallel spins. According to Pauli's exclusion principle, this precludes reactions with a divalent reductant, unless this reductant also has two unpaired electrons with parallel spins opposite to that of oxygen, which is a very rare occurrence. This spin restriction means that the most common mechanisms of oxygen reduction in biochemical reactions are those involving transfer of only a single electron (monovalent reduction) (Afanas'ev 1985).

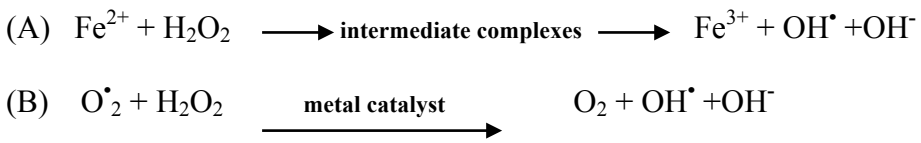
Activation of oxygen may occur by two different mechanisms: either by absorption of sufficient energy to reverse the spin of one of the unpaired electrons or by univalent reduction. The biradical form of oxygen is in a triplet ground state because the electrons have parallel spins. If triplet oxygen absorbs sufficient energy to reverse the spin of one of its unpaired electrons, it will form the singlet state (Kuehl 1994), in which the two electrons have opposite spins (Fig.1.1.1). This activation overcomes the spin restriction and singlet oxygen can consequently participate in reactions involving the simultaneous transfer of two

electrons (divalent reduction). Since paired electrons are common in organic molecules, singlet oxygen is much more reactive towards organic molecules than its triplet counterpart (Afanas'ev 1985). If a single electron is added to the ground state O_2 molecule, the product is the superoxide radical ($^{\bullet}$O-O) (Fig.1.1.1). With only one unpaired electron, superoxide is less of a radical than oxygen. Addition of another electron gives rise to the peroxide ion, which is not a radical. Further reductions give rise to the hydroxyl radical and then water.

Triplet oxygen (ground state)	**$^{\bullet}$O-O$^{\bullet}$**
Singlet oxygen	**O-O:**
Superoxide	**$^{\bullet}$O-O:**
Peroxide ion	**:O-O:**
Perhydroxyl radical	**$^{\bullet}$O-O : H**
Hydrogen peroxide	**H : O-O : H**
Hydroxyl radical	**H : O$^{\bullet}$**
Hydroxyl ion	**H : O :**
Hypochlorous acid	**HOCl**
Ozone	**O_3**

Fig.1.1. 1 Various forms of reactive oxygen

Hydrogen peroxide is noteworthy because it readily permeates membranes and it is therefore not compartmentalised in the cell. Numerous enzymes (peroxidases) use hydrogen peroxide as a substrate in oxidation reactions. The well-known reactivity of hydrogen peroxide is not due to its reactivity per se but requires the presence of a metal reductant (Fenton 1899) which following a series of reactions (Fenton and Haber Weiss reactions) which results in the production of the highly reactive hydroxyl radical ($OH^{\bullet}$) (Balentine 1982). The net Haber-Weiss reaction is shown below in scheme 1.1.1



Scheme 1.1. 1 (A) Fenton and (B) Haber-Weiss (1935) reactions

Hydroxyl radicals are responsible for a large part of the damage done to the cellular DNA, proteins and lipids by ionising radiation (Von Sontang 1987).

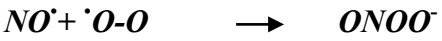
The oxidation of organic substances by $OH^{\bullet}$ may proceed by two possible reactions: either by an addition of OH to the organic molecule, or the abstraction of a hydrogen atom from an organic substrate. In the addition reaction, the hydroxyl radical forms a stable, oxidised product. In the abstraction reaction, an organic radical and water is formed. The organic radical has a single unpaired electron and thus can react with oxygen leading to the formation of a peroxyl radical. This in turn can readily abstract hydrogen from other organic molecules leading to the formation of a second carbon radical. This, therefore

results in a chain reaction which is why oxygen free radicals cause damage far in excess of their initial concentration (Haber and Wiess 1934).

A point to be noted is that not all products of activated oxygen can be referred to as free radicals, as some do not have unpaired electrons. Reactive oxygen species (ROS) is a collective term often used to include not only the oxygen derived free radicals but also other non-radical derivatives of oxygen. Other examples of ROS are shown in Fig 1.1.1 and include the perhydroxyl radical ($^{\bullet}$O-O : H), the hydroxyl radical (H : O$^{\bullet}$), hypochlorous acid (HOCl), an oxidizing and chlorinating agent produced by activated phagocytes and ozone (Boger et al. 1996); (Halliwell & Gutteridge 1999). Other reactive species such as the reactive nitrogen species are described below.

1.1. 4 Reactive nitrogen species

Some oxides of nitrogen such as nitric oxide ($NO^{\bullet}$) and nitrogen dioxide ($NO_2^{\bullet}$) are also free radicals and just as the term ROS has been introduced in the earlier section, there also exists a field of biology where products (radicals) of nitrogen react with reactive oxygen species resulting in a whole range of products which have been termed reactive nitrogen species (RNS). Some other examples of RNS are the non-radicals nitrous acid (HNO_2), dinitrogen trioxide (N_2O_3), dinitrogen tetroxide (N_2O_4), the nitronium (nitryl) ion (NO_2^+) and peroxinitrite (Sharpe and Cooper 1998). An important and biologically relevant reaction involving RNS is the fast reaction of the nitric oxide radical ($NO^{\bullet}$) and superoxide ($^{\bullet}$O-O) to form peroxynitrite (scheme.1.1.2) (Beckman and Koppenol 1996).



Scheme 1.1. 2 Reaction of nitric oxide and activated oxygen

Peroxynitrite itself is also a strong oxidant and can react directly with electron-rich groups, such as sulfhydryls in general (Radi 1991) and the active sulfhydryl site in tyrosine phosphatases (Takakura et al. 1999), iron-sulfur centers (Castro 1994) and zinc-thiolates (Crow 1995). Although peroxynitrite is a strong oxidant, the anion also reacts directly with nucleophiles, molecules with a partial positive charge (Sharpe & Cooper 1998). One example of major importance is carbon dioxide-forming carbonate. Carbonate radical is more selective than hydroxyl radical but will initiate many of the damaging reactions commonly attributed to hydroxyl radical in the biological literature and is perhaps more significant as a biological oxidant (Michelson and Maral 1983). Peroxynitrite can also produce novel products such as nitrotyrosine, nitrotryptophan, and nitrated lipids that serve as important biological markers *in vivo* (Radi 2001).

Nitric oxide and its biological importance will be discussed in more detail in chapter 4.

1.1. 5 Defence mechanisms

In order to prevent damage caused by oxygen *in vivo*, the body has an array of antioxidant compounds and enzymes, which in healthy subjects are able to scavenge or prevent production of these highly reactive oxygen species. These antioxidants function in a variety of different ways and are localised within specific areas of the cell. They include enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase (Burton and Ingold 1984), as well as small molecules such as water-soluble vitamin C (ascorbate), glutathione and lipid-soluble vitamin E (Packer 1979). These antioxidants will be briefly considered below:

1.1.5. 1 Superoxide dismutase (SOD)

Superoxide dismutase (SOD) was first isolated by Mann and Kleilin (Mann and Kleilin 1938) and thought to be a copper storage protein. SOD is now known to catalyse the dismutation of superoxide to hydrogen peroxide and oxygen by the following reaction (Fridovich 1995):

$$^{\bullet}O_2^- + {}^{\bullet}O_2^- + 2H^+ \xrightarrow[\text{Superoxide Dismutase}]{} H_2O_2 + O_2$$

Scheme 1.1. 3 Reaction catalysed by superoixde

The activity of this enzyme therefore determines the relative proportions of two constituents ($^{\bullet}O_2^-$ and H_2O_2) of the Haber-Weiss reaction that generates hydroxyl radicals.

SOD curtails the damaging reactions of superoxide, thus protecting the cell from superoxide toxicity. The reaction of superoxide with non-radicals is limited in biological systems, which means its main reactions are with itself (dismutation) or with another biological radical such as nitric oxide or a metal. SOD is important because superoxide reacts with sensitive and critical cellular targets. For example, as discussed above it can react with $NO^{\bullet}$ to produce peroxynitrite, which is a powerful oxidising agent (Murray-Rust 2001). Since SOD is present in all aerobic organisms and most subcellular compartments that generate activated oxygen, it has been assumed that SOD has a central role in the defence against oxidative stress. In humans (as in all other mammals and most chordates), three forms of superoxide dismutase are present: SOD1-3. The three distinct types of SOD are classified on the basis of the metal cofactor: SOD1 is a dimer containing copper and zinc and is located in the cytoplasm. SOD2 is a tetramer containing manganese in the centre (its active site) with copper and zinc- present in the mitochondria. SOD3 is also a tetramer containing copper and zinc and is found in the extracellular (Beyer 1991;Bowler and Inze 1992;Scandalias 1993).

1.1.5. 2 Catalase

H_2O_2 is removed by aerobes by two types of enzymes- catalases and peroxidases. Catalase is a heme-containing enzyme that catalyses the conversion of hydrogen peroxide into water and oxygen (scheme 1.1.4)

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

Scheme 1.1. 4 Catalase reaction mechanism

The enzyme is found in all aerobic eukaryotes and is important for the removal of hydrogen peroxide generated in peroxisomes by oxidases such as that involved in ß-oxidation of fatty acids, and purine catabolism. Catalase was one of the first enzymes to be isolated in a highly purified state (Hugo 1984). Examination of the structure of beef liver catalase has shown four NADPH binding sites per catalase tetramer (Fita and Rossmann 1985).

1.1.5. 3 Glutathione peroxidase

Selenium containing glutathione peroxidase (GPX) is the general name of an enzyme family with peroxidase activity whose main biological role is to protect the organism from oxidative damage (Richard and Raymond 1976). Eight different isoforms of glutathione peroxidase (GPX1-8) have been identified in humans of which glutathione peroxidase 1 (GPX1) the most abundant peroxidase *in vivo* (Prohaska and Ganther 1977). It is found in the cytoplasm of nearly all mammalian tissues, and its preferred substrate is hydrogen peroxide. Glutathione peroxidase 4 (GPX4) has a preference for lipid hydroperoxides and is expressed in nearly every mammalian cell. Glutathione peroxidase 2 is an intestinal and extracellular enzyme, while glutathione peroxidase 3 is predominantly an extracellular enzyme, especially abundant in plasma (Michiels 1994).

Glutathione peroxidase removes H_2O_2 by coupling its reduction to H_2O with oxidation of reduced glutathione to its oxidised form (GSSG) (Mills 1957).

H_2O_2 + 2GSH ⟶ GSSG + $2H_2O$

Scheme 1.1. 5 Reaction of glutathione peroxidase

1.1.5. 4 Ascorbate

L-ascorbic acid (vitamin C) is an important vitamin in the human diet. The most striking chemical property of ascorbate is its ability to act as a reducing agent. The physiological and biochemical actions of vitamin C are due to its action as an electron donor. It is noteworthy that when vitamin C donates electrons, they are lost sequentially (see scheme 1.1.6). The species formed after the loss of one electron is a free radical, semidehydroascorbic acid or ascorbyl radical. The ascorbyl radical is relatively stable and is fairly unreactive. This property explains why ascorbate may be a preferred antioxidant. Upon the loss of a second electron, dehydroascorbate (DHA) is formed. Dehydroascorbate is unstable and decomposes to L-threonic and oxalic acid (Halliwell & Gutteridge 1999b)

Ascorbate —(radical attack)→ semidehydroascorbate (Dinsdale 2008) radical

2SDA —(disproportionation)→ ascorbate + dehydroascorbate (DHA)

DHA —(rapid non-enzymatic breakdown)→ L-threonic and oxalic acid

Scheme 1.1. 6 Oxidation and degradation of ascorbate (Halliwell & Gutteridge 1999)

In vitro, ascorbate has been shown to have multiplicity of antioxidant properties, protecting various biomolecules against damage by ROS and RNS (Halliwell & Gutteridge 1999b). The levels of ascorbate found *in vivo* (30-100 μM in human plasma) are sufficient to exert such antioxidant effects, though direct evidence of ascorbic acid as an antioxidant *in vivo* is not supported consistently by currently available clinical research. Vitamin C may have favourable effects on vascular dilatation, possibly through its antioxidant effects on $NO^{\bullet}$ (Gokce 1999;Khassaf 2003) but these findings were not consistent (Duffy 1999). It has also been postulated to improve impaired endothelial vasodilation restoring nitric oxide activity. This phenomenon was first documented by Taddei *et al.,* (1998) where 14 hypertensive subjects were shown to have improved vasodilation after infusion of intrabrachial vitamin C (2.4 mg/100 mL forearm tissue per minute). This was reversed by the nitric oxide synthase inhibitor N^{G}-monomethyl-L-arginine. These findings supported the hypothesis that nitric oxide inactivation by oxygen free radicals contributes to endothelial dysfunction in essential hypertension. Moreover, in most studies, the vitamin C-induced effects on vasodilatation occurred when vitamin C was administered intra-arterially and whether vasodilatation occurs at physiologically relevant concentrations of vitamin C is uncertain (Choi 2010).

Several lines of evidence suggest that vitamin C is a powerful antioxidant in biological systems *in vitro*. However, its antioxidant role in humans has not been supported by currently available clinical studies (Ye and Song 2008). Zheng *et al.,* (2008) in a cohort study of vitamin C supplementation (30 mg/day for a period of 15 years) did not find any significant reductions in the CHD risks. Additionally, a randomised, double-blinded, placebo-controlled trial in more than 14,000 older men found that vitamin C supplementation (500 mg/day) for an average of eight years had no significant effect on major cardiovascular events, total myocardial infarction or cardiovascular mortality (Sesso 2008).

1.1.5. 5 Glutathione

Glutathione is a tripeptide composed of cysteine, glutamic acid and glycine. Its active group is the thiol (-SH) of cysteine. Oxidised glutathione (GSSG) consists of two GSH molecules joined by disulfide bridge (Meister 1989;Meister 1994). GSH functions as an antioxidant primarily as a component of the enzyme system containing GSH oxidase and reductase. GSH is oxidised to GSSG by selenium containing glutathione peroxidase (section 1.1.5.3 and scheme 1.1.5) and removing hydrogenperoxide. GSH reductase, which contain flavin adenine dinucleotide, a derivative of riboflavin, reduces GSSG:

$$GSSG + NADPH + H^{+} \xrightarrow{\text{reductase}} NADP^{+} + 2GSH$$

Scheme 1.1. 7 Reaction of glutathione reductase

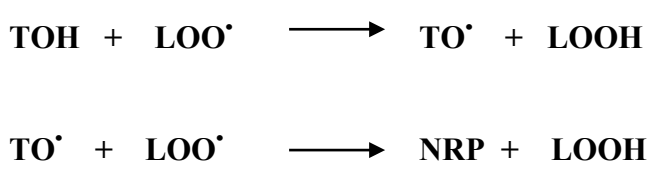
GSH also functions as an antioxidant independent of enzymes. For example it donates hydrogen to repair damaged DNA. For this reason, GSH and other thiol compounds might be important for protecting against damage from free radicals (Tanaka 2002).

1.1.5. 6 Vitamin E (α-tocopherol)

Vitamin E is a generic term for the tocopherols and tocotrienols, which have saturated and unsaturated side chains, respectively. Each group has α, β, γ and δ forms that differ according to the position and the number of the methyl groups on the hydroxychroman ring

with α-tocopherol being the most abundant form of vitamin E *in vivo* (Burton 1983). The structure and nomenclature of tocotrienols and tocopherols will be discussed in more detailed in chapter 3. An important biological activity of α-tocopherol is its ability to act as an antioxidant. α-tocopherol is considered to be the principal lipid soluble chain breaking antioxidant *in vivo* (Burton 1983).

Peroxyl radicals are probably the principal oxidants scavenged by α-tocopherol in biological systems. Phenols, such as α-tocopherol, typically trap lipid peroxyl radicals, for example during lipid peroxidation by a two-step mechanism as indicated in general terms in Scheme 1.1.8. First a peroxyl radical abstracts a hydrogen atom from tocopherol to produce a hydroperoxide and a tocopheroxyl radical. The tocopheroxyl radical is then able to scavenge another lipid peroxyl radical to form a non-radical adduct. The tocopheroxyl radical is unusually stable, owing to resonance stabilisation of the chroman ring, and therefore less likely to propagate the radical chain. Overall, each α-tocopherol molecule in capable of scavenging two peroxyl radicals (Burton 1983).



Scheme 1.1. 8 Reaction of α-tocopherol (TOH) with lipid peroxyl radicals (LOO•)

(**TO•-** tocopheroxyl radical, **NRP**- non- radical adduct and LOOH- lipid hydroperoxide)

In the event of the failure of the defence mechanisms to scavenge the free radicals, oxidative stress may result. The consequences of oxidative stress are discussed in the section below.

Fig.1.1. 2 Lipid peroxidation (Sevanian & Hochstein 1985)

1.1. 6 Oxidative damage

1.1.6. 1 Oxidative damage to proteins

Oxidative attack on proteins can result in a number of consequences such as site-specific amino acid modifications, fragmentation of the peptide chain, aggregation of cross-linked reaction products, altered electrical charge and increased susceptibility to proteolysis. The amino acids in a protein differ in their susceptibility to attack, and the various forms of activated oxygen differ in their potential reactivity. In spite of this complexity, some generalisations can be made. Sulphur containing amino acids, including those with thiol groups, are highly susceptible sites. Activated oxygen can abstract an H atom from cysteine residues to form a thiyl radical that will cross-link to a second thiyl radical to form disulphide bridges (Markesbery and Lovell 2007).

Many amino acids undergo specific irreversible modifications when a protein is oxidised. For example, tryptophane is readily cross-linked to form bityrosine products (Davies 1987a;Davies 1987b). Histidine, lysine, proline, arginine and serine form carbonyl groups on oxidation (Stadtman 1986;Stadtman 1993). The oxidative degradation of protein is enhanced in the presence of metal cofactors that are capable of redox cycling, such as iron. In these cases, the metal binds to a divalent cation-binding site on the protein. The metal can then react with hydrogen peroxide to form a hydroxyl radical that can rapidly oxidise an amino acid residue at or near the cation-binding site of the protein (Radak 2011).

Oxidative modification of specific amino acids is one mechanism of marking a protein for proteolysis (Stadtman 1986). In *E. coli* there are specific proteases that degrade oxidised proteins (Farr and Kogoma 1991a;Farr and Kogoma 1991b) and similar specificity is hypothesised to occur in humans, but no direct evidence has been put forward to date

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1.1.6. 2 Oxidative damage to DNA and carbohydrates

Oxidative damage to DNA is a result of interaction of DNA with reactive oxygen species (ROS), in particular the hydroxyl radical. Superoxide and hydrogen peroxide are normally not reactive towards DNA. However, in the presence of ferrous or cuprous ions (the Haber-Weiss reaction), both superoxide and hydrogen peroxide are converted to the highly reactive hydroxyl radical. The hydroxyl radical can produce a multiplicity of modifications in DNA. For example, oxidative attack by $OH^{\bullet}$ radical on the deoxyribose moiety will lead to the release of free bases from DNA, generating strand breaks with various sugar modifications and simple abasic (AP) sites, where a DNA base is lost (Sies H 1991b;Sies 1986). The C4-C5 double bond of pyrimidines in DNA are particularly sensitive to attack by the $OH^{\bullet}$ radical, generating a spectrum of oxidative pyrimidine products including thymine glycol, uracil glycol, urea residue, 5-OHdU (5-hydroxyuridine), 5-OHdC (5-hydroxycytosine), hydantoin and others (Kasai et al. 1984). Similarly, the interaction of $OH^{\bullet}$ radical with purines will generate 8-hydroxydeoxyguanosine (8-OHdG), 8-hydroxydeoxyadenine (8-OHdA), formamidopyrimidines and other less characterized purine oxidative products (Loft 2008). The biological consequences of many of the oxidative products are known. For example, unrepaired thymine glycol is a block to DNA replication and is thus potentially lethal to cells (Dizdaroglu 1985). 8-OHdG causes abundant oxidative damage to guanine, is readily by-passed by the DNA polymerase and is

highly mutagenic resulting in base mispairings (European standards committee on Oxidative DNA damage (ESCODD) 2003).

In the case of oxidative damage to carbohydrates, it has been observed that polysaccharides such as hyaluronic acid can be degraded by oxidative attack and superoxide dismutase was found to be capable of protecting hyaluronic acid against depolymerisation in synovial fluid (Duan and Kasper 2011). Proteoglycans have also been shown by (Rees et al. 2008) to be susceptible to oxidative breakdown in a similar manner to carbohydrate damage. Another key carbohydrate associated with oxidative stress is the aldehyde malonaldehyde which can be used as an index of lipid peroxidation, as discussed in section 1.1.8.

1.1. 7 Significance of oxidative stress in disease

As stated earlier, disease-associated oxidative stress could result from either (Suarna et al. 1995) diminished antioxidants (Golden 1987) or is an increased production of ROS/RNS (Halliwell & Gutteridge 1999). The latter mechanism is usually thought to be more relevant to disease and is frequently the target of attempted therapeutic interventions (Halliwell & Gutteridge 1999). However, in most human diseases, oxidative stress is likely to be a consequence and not the primary cause of the disease process. Oxidative stress can result in either adaptation, where the target completely, partially or overtly protects itself against damage, or tissue damage where molecular targets like DNA and protein are targeted or cell death where necrosis and/or apoptosis is observed. A point to be noted is that these three events follow each other chronologically, whereby the first line of action against

oxidative stress is defence, when this fails tissue damage can occur which is then followed by either necrosis and/or apoptosis of the cell (Halliwell & Gutteridge 1999b).

A large increase in the production of oxidants, leading to oxidative stress has been hypothesised to be implicated in the aetiology of number of diseases such as atherosclerosis, diabetes and rheumatoid arthritis (Burton 1983). Some human conditions may result directly from oxidative stress. For example, ionising radiation generates $OH^{\bullet}$, by splitting water molecules and many of the biological consequences of exposure to excess radiation are probably due to oxidative damage to proteins, DNA and lipids. Tissue damage by for example infection, trauma, toxins and abnormally high or low temperatures, usually leads to the formation of increased amount of putative 'injury mediators', such as prostaglandins, leukotrienes, interleukins and other cytokines such as tumour necrosis factor (TNFs). The presence of free radicals in these situations would exaggerate or aid in furthering tissue damage (Halliwell and Gutteridge 1990).

Oxidative stress has been implicated in the aetiology of numerous diseases and for this thesis purpose I have selected the more prominent diseases that have been implicated with oxidative stress. The section below details the role of oxidative stress in the aetiology of vascular disease, neurodegenerative diseases and diabetes.

In the case of vascular diseases, it is thought that oxidative stress contributes markedly to endothelial dysfunction. For example the, bioavailability of NO (nitric oxide) is reduced as a result of its reaction with superoxide (O_2^-) to peroxynitrite (Halliwell 2000). In addition, there is evidence that persisting oxidative stress will render endothelial nitric oxide synthase (eNOS) dysfunctional such that it no longer produces NO but superoxide and thus continues the cycle of destruction (Forstermann 2010). Evidence of this has been obtained

in rat aorta (Laursen 2001), endothelial cells treated with low-density lipoprotein (Pritchard 1995) and stroke prone spontaneously hypertensive rats (Kerr 1999). eNOS dysfunction has also been observed in patients with endothelial dysfunction due to hypercholesterolemia (Stuehr 2001), diabetes mellitus (Heitzer 2000) and in chronic smokers(Heitzer 2001a).

The neurodegenerative diseases - Alzheimer's disease (AD) and Parkinson's disease (PD), are age-related disorders characterized by the deposition of abnormal forms of specific proteins in the brain. AD is characterized by the presence of extracellular amyloid plaques and intra-neuronal neurofibrillary tangles in the brain (Jomovo 2010). Biochemical analysis of amyloid plaques revealed that the main constituent is fibrillar aggregates of a 39–42 residue peptide referred to as the amyloid-b protein (Ab) (Selkoe 2001). PD is associated with the degeneration of dopaminergic neurons in the substantia nigra pars compacta. One of the pathological hallmarks of PD is the presence of intracellular inclusions called Lewy bodies that consist of aggregates of the presynaptic soluble protein called α-synuclein (Latha and Hindupur 2010).

There are various factors influencing the pathological depositions, and in general, the cause of neuronal death in neurological disorders appears to be multifactorial. However, it appears, that the underlying factor in the neurological disorders is increased oxidative stress substantiated by the findings that the protein side-chains are modified either directly by reactive oxygen species (ROS) or reactive nitrogen species (RNS), or indirectly, by the products of lipid peroxidation (Jomova 2010). The increased level of oxidative stress in AD brain is reflected by the increased brain content of iron (Fe) and copper (Cu) both capable of stimulating free radical formation (e.g. hydroxyl radicals via the Fenton reaction), increased protein and DNA oxidation in the AD brain, enhanced lipid peroxidation, and decreased levels of cytochrome c oxidase and advanced glycation end products (AGEs), carbonyls, malondialdehyde (MDA), peroxynitrite, and heme oxygenase-1 (HO-1). AGEs,

mainly through their interaction with receptors for advanced glycation end products (RAGEs), further activate signaling pathways, inducing formation of proinflammatory cytokines such as interleukin-6 (IL-6) (Bush 2003). The conjugated aromatic ring of tyrosine residues is a target for free-radical attack, and accumulation of dityrosine and 3-nitrotyrosine has also been reported in AD brain (Valko 2005).

The oxidative stress linked with PD is supported by both postmortem studies and by studies showing an increased level of oxidative stress in the substantia nigra pars compacta, demonstrating thus the capacity of oxidative stress to induce nigral cell degeneration (Jomova 2010). Increased concentration (statistically significant) of markers of lipid peroxidation was observed, which included 4-hydroxytrans- 2-nonenal (HNE), 4-oxo-trans-2-nonenal (4-ONE), acrolein, and 4-oxo-trans-2-hexenal, all of which are well recognized neurotoxic agents (Kraystberg 2006). In addition, other important factors, involving inflammation, the toxic action of nitric oxide (NO), defects in protein clearance and mitochondrial dysfunction appears to contribute to the aetiology of PD (Jomova 2010).

Section 5.1 will focus on the aetiology of oxidative stress in diabetes, the condition which has been investigated in this study.

1.1. 8 Biomarkers of oxidative stress

Oxidative stress is propagated by free radicals (Section 1.1.6). Free radicals are extremely reactive and short-lived and it is difficult to measure these species, and therefore, oxidative stress directly. Electron spin resonance (ESR) can be used to detect free radicals (Gilbert 1981c). This method is however often too insensitive to detect directly such radicals such

as $O_2^{\bullet-}$ and $OH^{\bullet}$ in living systems. Direct ESR of biological material can only detect less-reactive radicals such as the ascorbyl radical. In practice it has been found to be necessary to use spin trapping methods to detect radical adducts but this method is not feasible for application to sample batches in clinical trials. Most methodologies therefore, measure products of oxidative stress. There are numerous potential products of oxidative stress, because as discussed previously free radicals attack all cell components resulting in peroxidation of lipids, oxidation of protein and carbohydrates and oxidative damage to DNA. Guanine is the DNA base most prone to oxidative damage resulting in the formation of 8-hydroxydeoxy guanosine (8-OHdG). 8-OHdG has been studied widely in both cellular DNA analyses and in non-invasive urinary analysis (Helbock 1998). The most commonly used analytical procedures are high performance liquid chromatography with electrochemical detection (HPLC-ECD) (Von Sontang 1987a;Von Sontang 1987b), gas chromatography-mass spectrometry (GC-MS) (Jenner 1998) and enzyme-linked immunosorbent assay (ELISA) (Tsuboi 1998). The major disadvantage of these assays is that it is labour intensive requiring multiple steps which include enzymatic digestion, an elaborate extraction and separation steps for 8-OHdG isolation. The HPLC procedure has its advantages over the GC-MS method as it determines the free 8-OHdG in plasma and urine without enzymatic digestion (Lengger 2000). In the case of the ELISA assay, (Shimoi 2002) found in their study that ELISA estimates were about twofold higher than that of the HPLC on original urine. For reasons not known, 10% of the urine samples showed more than a fourfold increase in value produced by ELISA.

Other frequently used markers of oxidative stress are products of lipid peroxidation. A direct approach would involve the measurement of primary products of peroxidation, such as the hydroperoxides. However, hydroperoxides are unstable, so indirect measures are

frequently used which employ determination of secondary or end products derived from further oxidation of the hydroperoxides. The most frequently quoted index of lipid peroxidation is the aldehyde, malondialdehyde (MDA). MDA is a three carbon, low-molecular weight aldehyde that can be produced from free radical attack on polyunsaturated fatty acids. Documented methods to measure MDA either involve the measurement of free MDA or an MDA derivative. The most common and simple method employs measurement of an MDA derivative, where MDA reacts with thiobarbituric acid (TBA) at low pH and elevated temperature to produce fluorescent and pigmented adducts, referred to as thiobarbituric acid reactive substances (TBARS). This method is however non-specific, as other low molecular weight aldehydes are also able to react with TBA Asakawa and Matsushita, 1980 (Asakawa Matsushita 1980a). HPLC methods have also been developed for the specific and direct quantitation of plasma and urinary MDA but these methods were found to require long elution times of almost 1 hr 90 min and were also observed to elute non specific MDA-related compounds which results in the over estimation of the metabolite (Janero 1990). To compensate the problem of overestimation, Khoschsorur (Khoschsorur 2000) et al., (2000) used an HPLC method with spectrofuorimetric detection to quantitae urinary MDA. The major advantage of the method was its high selectivity, accuracy and reproducibility compared to the existing methods but the sample preparation protocols still required a labourious derivatisation step, which though yet to be proved could potentially lead to overestimation (Janero 1990).

A hydrocarbon breath test is a method to measure exhalation of a group of volatile hydrocarbons, the alkanes, formed by in vivo lipid peroxidation of polyunsaturated fatty acids (PUFAs). The alkanes, most frequently measured, include ethane and pentane. In Kivits et al., 1981 (Kivits 1981)demonstrated that an increased recovery of exhaled ethane

resulted from an increased oxidation of ω-3-PUFA, and oxidation of ω-6-PUFAs was responsible for increased pentane recovery. Practical limitations of this non-invasive approach include the lack of standardised methods of collecting, processing and analysing expired air. In addition, ambient concentrations of ethane and pentane in the atmosphere are greater than in expired air and, can therefore easily contaminate the breath samples.

Oxidative products of proteins are measured by protein carbonyl (CO) groups. Carbonyl (CO) groups (aldehydes and ketones) are produced on protein side chains (especially of Proline, Arginine, Lysine and Threonine) when they are oxidised. These moieties are chemically stable, which is useful for both their detection and storage (le-Donne 2003). The proteins (plasma) are then quantated by spectrophotometry (Levine 1990), spectrophotometry coupled with HPLC (Gladstone 1994) or ELISA (Buss et al. 1997). The spectrophotometry assay was observed by (Fagan 1999) to be unreliable for quantitating carbonyl content in protein extracts that contain high amounts of chromophore that absorbs at 370 nm (e.g., haemoglobin, myoglobin, retinoids). Also the assay does not provide any information on the extent of oxidative damage to a particular protein in a complex mixture like plasma, tissue homogenates, or cellular extracts; it requires more protein than may be available from clinical samples; it is labor intensive; and washing steps can give rise to variability (Lyras 1996;Reznick and Packer 1994). In the case of spectrophotometry coupled with HPLC assay one major disadvantage observed was that protein mixture fractionation by HPLC cannot completely separate proteins of close molecular weights. On the other hand, it is a highly sensitive technique for the quantitation of protein oxidative damage, especially for investigating purified proteins, but is less useful in protein mixtures due to problems with resolution (Agarwal and Sohal 1995). The drawback of the ELISA test is that it requires somewhat expensive and specialized

equipment and, like the spectrophotometric assay, does not provide any information on the extent of oxidative damage to a particular protein in a complex mixture (Le- Donne 2003).

The oxidative products of carbohydrates especially deoxyribose has been exploited for the development of an assay of the formation of free radicals. In the tiobarbiturate assay, deoxyribose yields a degrading product that is almost identical to the product obtained with malondialdehyde (MDA) (Halliwell 1992). The use of MDA, its advantages and disadvantages have been covered earlier in this section.

Many studies have measured a group of compounds called the F_2-isoprostanes, a family of eicosanoid like structures, as a sensitive marker of *in vivo* oxidative stress. The F_2-isoprostanes are lipids which are non-enzymatically derived isomers of the prostaglandins. They are formed *in vivo* by free radical mediated oxidation of arachidonic acid. Increase in the F_2-isoprostanes have been well documented in a number of disease conditions (section 2.1.2). F_2-isoprostanes will be discussed in more detail in chapter 2 where I aimed to establish a novel method for the measurement of isoprostanes in urine. In summary the established method of quantitating urinary and plasma isoprostanes is a GC-MS method which requires an extraction protocol followed by a derivatisation step and a chromatographic separation prior to analysis using gas chromatography. The process is laborious and could generate artefacts. The ELISA and HPLC methods developed have been indicated to be inaccurate and are discussed in detail in section 3.1.3.

In conclusion, even though oxidative stress has been implicated in either the aetiology and/or a feature of various acute and chronic disease conditions, definitive evidence for this association is lacking. This may be due to the short comings with the biomarkers and/or

methods available to assess oxidative stress status in humans. The existing methodologies discussed above either lack validity or are too cumbersome to be used as a routine clinical procedure. Even though isoprostanes are currently considered to be the "gold standard" for quantitating oxidative stress, the established methodology is labour intensive and could cause artefact formation. Shorter HPLC and ELISA methods are inaccurate and thus there is a critical need to develop new methodologies to accurately quantitate biomarkers of oxidative stress.

In this study I attempted to develop a new and faster method of accurately measuring isoprostanes using HPLC MS/MS. In addition I also developed methods to measure other two other possible markers of oxidative stress namely conjugated metabolites of vitamin E and amino acids associated with NO metabolism. The reasoning behind selecting these markers will be explained in the following sections of the thesis.

Chapter 2

Materials and Methods

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2 Materials and Methods

2. 1 Principles of methodology

In this study the development of methods using liquid chromatography/ tandem mass spectrometry and the principles of this methodology will now be described.

2.1. 1 High performance liquid chromatography (HPLC)

High performance liquid chromatography (HPLC) is a physical method of separation in which the components to be separated are distributed between two phases, one is solid and stationary while the other is a mobile liquid. In practice the solid stationary phase is packed into a HPLC column. The liquid mobile phase is pumped at a high pressure (500-2000 psi) through the column, where individual components of the sample separate from each other based on their individual affinities for the solid and mobile phase, with each emerging from the column at separate points in time. This separation in time may be manipulated by changing the physico-chemical properties of the mobile phase (Sandie Lindsay 1992).

In the current study, reverse phase chromatography was applied to separate the metabolites. Reverse phase means that the polarity of the stationary phase is less than that of the mobile phase and as a result solutes are eluted in order of polarity with the most polar eluting first. The retention times of the solute can be controlled by for example, changing the polarity of the mobile phase. The advantages of using reverse phase chromatography are that it has a very broad scope that allows components with wide ranges of polarity to be separated, it uses relatively inexpensive solvents, and equilibration of the mobile phase with the column is rapid (Sandie Lindsay 1992).

In order to analyse and quantitate the metabolites of interest (isoprostanes, vitamin E and amino acids) in urine, HPLC was coupled to an electrospray triple quadrupole tandem mass spectrometer (ESI-MS/MS). The HPLC separates the metabolites of interest from the urine and then introduces them singularly to the ESI-MS/MS. This was a particularly important step in the case of the vitamin E metabolites, as the mass spectrometer cannot differentiate between the two important metabolites α-TLHQ (Pope et al. 2000) and α-CEHC (carboxy-ethyl-hydrochroman) because they have the same molecular weight. (the alternative method would include deconjugation but method has been implicated with artifact formation-which has been detailed in the discussion)

2.1. 2 Electrospray triple quadrupole tandem mass spectrometry (Fig.2.1.1)

Tandem mass spectrometry (MS/MS) is a rapid and sensitive method that is used routinely to permit the identification of metabolites in biological samples with minimal sample preparation (Griffiths et al. 2001a;Griffiths et al. 2001b). The basic principles of mass spectrometry and the usefulness of tandem mass spectrometry in identifying and characterising metabolites are discussed below.

2.1.2. 1 Basic principles of mass spectrometry

Mass spectrometers are essentially composed of three components (see Fig.2.2.1):

i ion source

ii mass analyser

iii detector

Fig.2.1. 1 Diagrammatic representation of HPLC-MS/MS

In order to obtain a mass spectrum, ions must be produced in the gas phase. These ions are then accelerated to a specific velocity using electric fields and projected into a mass analyser that separates the ions according to their mass. Finally, each charged particle of a particular mass is detected sequentially in time.

The appearance of the mass spectrum obtained is dependent largely on the ionisation method used. So-called soft or low energy ionisation methods such as electrospray or matrix assisted laser desorption, produce simple spectra which contain peaks corresponding mainly to the masses of molecular ions. In contrast, high energy ionisation techniques, such as electron impact, produce complicated spectra due to the large number of fragment ions produced. Electrospray ionisation was used in this study which is discussed below.

2.1.2. 2 Electrospray ionisation (ESI)

High-energy ionisation techniques, such as electron impact (EI), cause decomposition of thermally labile biomolecules, making such techniques unsuitable for the analysis of underivatised biological samples. In contrast, electrospray ionisation (ESI) is able to ionise intact non-volatile, thermally labile biomolecules and transfer them into the gaseous phase where they can be subjected to mass analysis.

ESI was originally described over 40 years ago (Dole et al. 1968) but the first description of the technique coupled to MS was not published until almost two decades later

(WhitehouseC.M. et al. 1985). In conventional ESI, the sample is dissolved in a solution and then sprayed through a thin capillary needle, which is maintained at a high voltage (2-5 kV) (Fig.2.1.2). At the end of the needle the solution disperses into a mist of highly charged droplets containing the analyte molecules of interest. As the charged droplets travel down a pressure and potential gradient towards an orifice in the high vacuum system of the mass spectrometer, they desolvate and reduce in size aided by the application of dry gas and/or heat. The surface area of the droplet decreases until either the solvent is completely removed (Dole, Mack, Hines, Mobely, Ferguson, & Alice 1968) or the charge density on the surface of the droplet reaches the Raleigh limit and the ion is ejected (desorbed) (Iribarne J.V. and Thomson 1976;Thomson and Iribarne J.V. 1979). The ion can then be transported to the mass analyser.

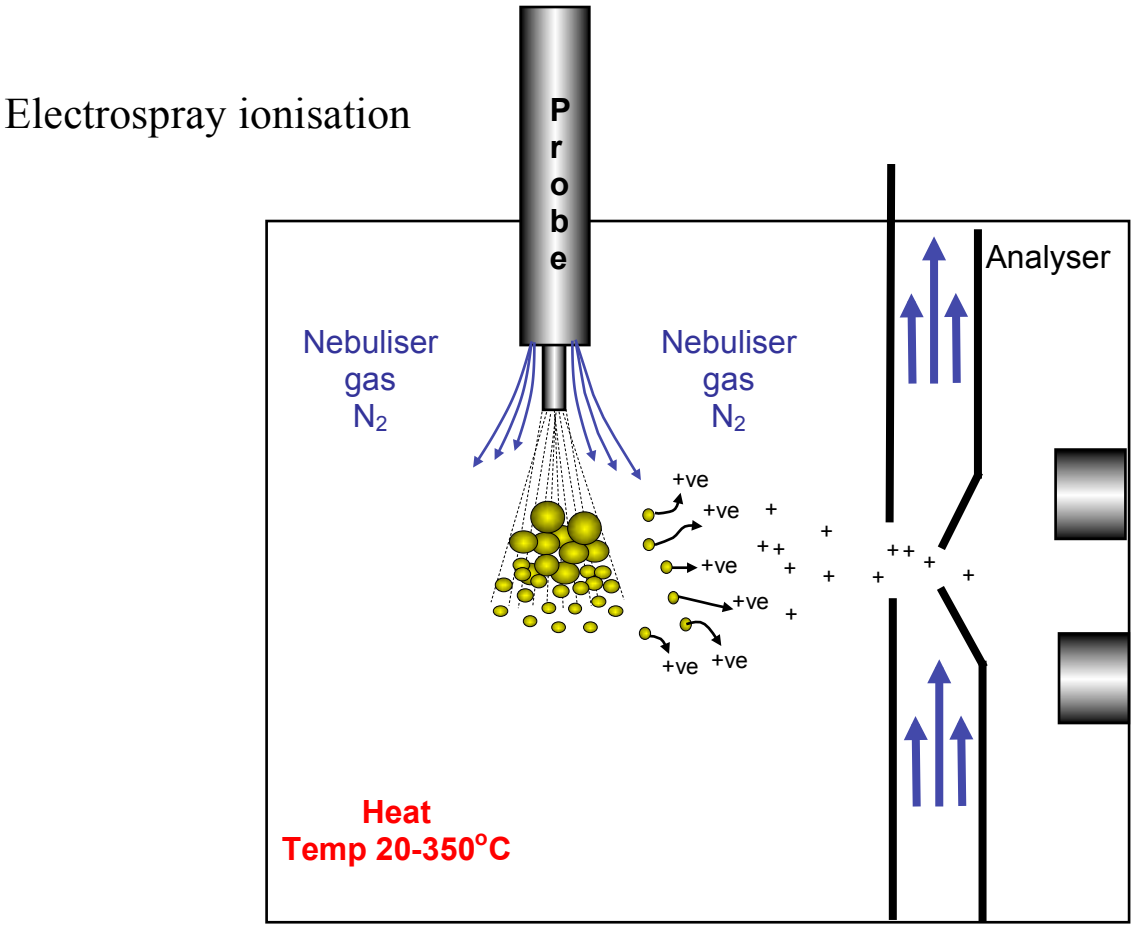

Fig.2.1. 2 Features and schematic of ioinisation in an ESI source (Dr. Kevin Mills)

An important feature of ESI, as with other soft ionisation methods, is the fact that following ionisation, it produces minimal fragmentation of the molecule of interest allowing its molecular mass to be determined. In certain situations e.g. quantitative mass spectrometry, this may be advantageous but it also has implications for the identification and structural characterisation of molecules within a complex biological sample owing to the isobaric (equal mass) nature of many biological molecules. It is, therefore, often necessary to separate the different species present in a biological sample either prior to (e.g. using liquid chromatography-mass spectrometry (LC-MS)) or after ionisation (e.g. tandem mass spectrometry (MS/MS). Although, MS/MS does not physically 'separate' isobaric components it may allow them to be detected individually and unambiguously due to specific fragmentations. In this study liquid chromatography and tandem mass spectrometry

was used to characterise the individual vitamin E metabolites and amino acids detected in urine.

2.1.2. 3 Basic principles of tandem mass spectrometry

The most commonly used type of tandem mass spectrometer consists of two quadrupole mass analysers coupled together in series. In a typical tandem mass spectrometric analysis (MS/MS), ions are selected by the first mass analyser (Q1) and focused into a collision cell (q2) preceding a second mass analyser (Q3) (Fig. 2.1.3). The second or middle quadrupole (q2) performs no mass analysis and serves as a high pressure gas collision cell, which promotes fragmentation of the ions selected by Q1. The second mass analyser (Q3) detects the fragment ions produced in the collision cell. Therefore, a tandem mass spectrometer not only gives information about the molecular mass of individual components but also allows structural information to be obtained by studying characteristic fragment ions.

2.1.2. 4 Possible configurations of a tandem mass spectrometer (Fig.2.1.3)

The basis of MS/MS is a process known as collision-induced dissociation (CID) (Chowdhury et al. 1990). In this process as mentioned above, ions of a selected mass (precursor or parent ions) are transmitted by the first mass analyser (Q1) into the collision cell, q2, where they collide with the neutral atoms of an inert gas usually argon, helium or nitrogen. As a result of these collisions, the internal energy of the parent ion is increased causing the molecule to fragment. The subsequent fragment (product or daughter ions) are then analysed by the second mass analyser, Q3.

The first and second mass analysers (Q1 and Q3) can be operated in either fixed mass mode, whereby only ions of a certain mass are measured, or in scan mode, where ions of a range of masses are measured sequentially. By operating Q1 and Q3 in various combinations of fixed mass and scan mode, a number of different types of tandem mass spectrometric analyses are possible.

Straight scan mode (Fig.2.1.3.A)

In order to produce a simple spectrum of all the species in a sample, the tandem mass spectrometer can be operated as a simple, single quadrupole mass spectrometer by utilising only the first mass analyser in scan mode. This analysis gives an overview of all the chemical species in a sample and allows fine tuning of the mass spectrometer for further analysis using the collision cell (q2).

Product ion scan (Fig.2.1.3.B)

The simplest reaction in MS/MS is the dissociation of a selected precursor ion into product ions. This reaction can be monitored by selecting one particular ion out of the ions generated in the ion source Q1, transferring that ion to the collision cell and then analysing the product ions with Q3. This enables a characteristic fragment ion spectrum to be produced for each compound of interest e.g. product ions from 357 in the case of the vitamin E metabolites (α-TLHQ and α-CEHC).

Precursor ion scan (Fig.2.1.3.C)

In a precursor ion scan, Q3 is fixed on one particular fragment ion produced by dissociation of precursor ions in the collision cell (q2). Scanning of Q1, while Q3 is fixed, allows all precursor ions which fragment to give a specific product ion to be analysed.

Fig.2.1. 3 Examples of possible analysis using a triple quadrupole mass spectrometer. CID=Collision induced dissociated

A precursor ion scan can be used to detect groups of compounds that produce a common product ion. An example is the analysis of sulphated metabolites of vitamin E by the detection of the characteristic product ion, m/z 80. In this way, if Q3 is set to m/z 80 and Q1 is scanned, the resulting spectrum will display all sulphated metabolites in that sample.

Neutral loss scan (Fig.2.1.3.D)

Many fragmentations in the collision cell produce neutral products that cannot be directly analysed by mass spectrometry owing to their lack of charge. However a neutral loss scan, where the two mass analysers, Q1 and Q3, scan simultaneously with a fixed mass difference between them allows neutral losses to be analysed.

An example of the use of a neutral loss scan is in the analysis of glucuronides metabolites of vitamin E, which typically fragment with a neutral loss of 176. If Q3 is set to scan 176 mass units below Q1 e.g. if Q1 scans from 300 to 600 and Q3 scans simultaneously between 124 and 424, the resulting spectrum will display all glucuronidated metabolites in the sample.

By combining the information obtained from these different types of tandem mass spectrometric analyses it is possible to identify and characterise even minor metabolites in urine.

2.1. 3 Internal standards

In order to quantitate compounds of interest using HPLC-MS/MS it is necessary to add known amounts of internal standards as early as possible in the analysis. Internal standards are compounds that are chemically and/ or structurally similar to the molecules intended for quantitation and are added in a known amount at the start of the extraction. They compensate for sample handling variations, mainly due to losses during preparation or variation of injection volumes. The types of internal standards are as follows:

Type 1: These are usually deuterated or ^{13}C labeled compounds and are chemically identical to the molecule intended for analysis. They behave almost identically to the compound it is intended to quantitate and can only be distinguished from this molecule by use of a mass spectrometer. They are the ideal internal standards.

Type 2: These are compounds structurally similar to the class of molecule to be quantitated.

Type 3: These compounds are not structurally or chemically similar to the compounds to be quantitated and are the least favourable choice of internal standards.

The specific internal standards used in this study will be discussed in more detail in the relevant sections.

2.1. 4 Urinary creatinine expression

In this study, urinary concentrations of the compounds of interest were corrected for dilution differences by measuring the urinary creatinine concentration as creatinine is filtered but not re-absorbed by the kidneys. Creatinine is a breakdown product of creatine phosphate found in muscle a spontaneously formed cyclic derivative of creatine. If the filtering of the kidney is deficient, creatinine blood levels rise. Therefore, creatinine levels in blood and urine may be used to calculate the creatinine clearance (CrCl), which reflects the glomerular filtration rate (GFR). The GFR is clinically important because it is a measurement of renal function. However, in cases of severe renal dysfunction, the creatinine clearance rate will be "overestimated" because the active secretion of creatinine will account for a larger fraction of the total creatinine (Gross et al. 2005). The urinary concentration of creatinine in this study of children and young people with type 1 diabetes mellitus were normal limits.

Creatinine was measured by mass spectrometry using deuterated creatinine as the internal standard in the urine samples. This method was developed in-house by the biological mass spectrometry unit.

Spot urines were used in this study and the concentrations of the compounds of interest were expressed per mmol of creatinine.

2. 2 Isoprostanes

2.2. 1 Chemical reagents

8-iso-$PGF_{2\alpha}$ and the internal standard d_4-8-iso-$PGF_{2\alpha}$ were purchased from Cayman Chemical Company (Ann Arbor, Michigan, USA). All the other reagents were of LC-MS grade or equivalent and obtained from the Sigma-Aldrich Chemical Company (Poole, Dorset, UK). The working solution of d_4-8-iso-$PGF_{2\alpha}$ and 8-iso-$PGF_{2\alpha}$ were made up to a concentration of 100 µmol/l in ethanol.

2.2. 2 Urinary samples

Method development was performed on a urinary pool obtained from 12 laboratory volunteers from the UCL Institute of Child Health (6 males and 6 females). The samples were stored in -20^{o}C.

2.2. 3 LC-MS/MS analysis

2.2.3. 1 Sample preparation prior to analysis

Neat urine (150 µl) was spun for 5 min at 1500 rpm/ 214 RCF (relative centrifugal force) and spiked with 10 µl of (100 µmol/l) of the internal standard d_4-8-iso-$PGF_{2\alpha}$. Samples were vortexed and 15 µl was injected onto the LC-MS for analysis. The LC-MS/MS specifications are detailed below (section 2.2.3.2 and 3):

2.2.3. 2 Liquid chromatography (LC)

8-iso-$PGF_{2\alpha}$ was desalted and/ or separated prior to mass spectrometry using a Waters 2795XE high performance liquid chromatography unit with 100 mm X 2.1 mm (5 μ) HyPURITY C8 column fitted with a guard column containing the same stationary phase (Phenomenex UK). The mobile phase consisted of water and acetonitrile: methanol (1:2) with a flow rate of 0.25 ml/min.

The LC gradient is shown in Table 2.4.1.The mobile phase consisted of a step change gradient where the mobile phase consisted of 80% HPLC grade water and 20% ACN:MeOH (1: 2, v/v) which was gradually changed to 100% ACN:MeOH (1: 2, v/v) over a period of 7.5 min. The mobile phase was maintained at 100% ACN:MeOH for 2.5 min. The column was then reconditioned and re-equilibrated before the next injection with 80% water and 20% ACN:MeOH (1: 2, v/v) for 4 min. The total analysis time between each injection was 14 min. The injection volume was 40 μl.

Time	Water %	Acetonitrile:Methanol (1:2) %	Flow ml/min
0.0	80	20	0.25
2.00	55	45	0.25
7.49	20	80	0.25
7.50	0	100	0.25
10.00	0	100	0.25
10.01	80	20	0.25
14.00	80	20	0.25

Table 2.2. 1 Chromatographic conditions

2.2.3. 3 Electrospray ionisation-tandem mass spectrometry (ESI-MS/MS)

Mass spectrometry was carried out using a triple quadrupole Quattro Micro instrument (MicroMass, Waters, U.K.) fitted with an electrospray ionisation source in negative ion mode. The source and desolvation gas temperature were held constant at 150 and 350 ^{0}C respectively, with flow rates of 900 and 25 litres of nitrogen per hour, respectively. The optimal collision energy was found to be 36 eV with an optimal gas cell pressure of 7.66 X 10^{-3} mbar. The MS/MS parameters remained constant throughout the method development in this chapter. Table 2.4.2 below shows the mass spectrometry parameters.

	Transition	Dwell time (Sec)	Cone Voltage	Collision Voltage
8-iso-$PGF_{2\alpha}$	353>193	0.50	42	26
d_4-8-iso-$PGF_{2\alpha}$	357>197	0.50	25	36

Table 2.2. 2 Mass spectral specifications

2.3. 1 Chemical reagents

The internal standards used in this study to quantitate the vitamin E metabolites were lithocholic acid sulphate and androsterone glucuronide which were purchased from Sigma-Aldrich Chemical Company (Poole, Dorset, UK). The working solutions of the internal standards were made up to a concentration of 100 μmol/l in methanol. Vitamin E metabolites were kindly synthesised and supplied by Simon Pope and were made up to a concentration 100 μmol/l in methanol. All the other reagents were of LC-MS grade or equivalent and obtained from the Sigma-Aldrich Chemical Company (Poole, Dorset, UK).

2.3. 2 Sample preparation prior to analysis

Neat urine (150 μl) was spun for 5 min at 1500 rpm/ 214 RCF (relative centrifugal force) and spiked with 10 μl of 100 μM of the internal standards, lithocholic acid sulphate and androsterone glucuronide. Samples were vortexed and 15 μl was injected into the LC-MS for analysis. The LC-MS/MS specifications are detailed below:

2.3. 3 Liquid chromatography (LC)

Vitamin E metabolites were desalted and/ or separated prior to mass spectrometry using a Waters 2795XE high performance liquid chromatography unit fitted with a 100 mm X 2.1 mm (5 μ) HyPURITY C8 column plus a guard column containing the same stationary phase (Phenomenex UK). The mobile phase consisted of methanol, 4 mM ammonium acetate (containing 0.01 % formic acid) and methanol: acetonitrile (2:1), using flow rates of 0.25-0.55 ml/min.

The chromatographic conditions used are tabulated in Table 2.3.1. The mobile phase consisted of a step change gradient where the LC gradient started with 5% 2:1 mixture of methanol: acetonitrile (MeOH: ACN) and 95% 4mM ammonium acetate (containing 0.01% formic acid) for 4 min. Water then replaced ammonium acetate in the gradient and MeOH: ACN was increased to 20% with 80% water for 1 minute and further increased to 45% where it was held until 10.49 minutes. It was at this gradient that all the vitamin E metabolites eluted between 7-10 minutes. The gradient was then increased and held at 100 % until 14 minutes. The column was reconditioned before the next injection for 4 min by re-equilibration with 5 % MeOH: ACN and 95% 4mM ammonium acetate (0.01% formic acid). The total analysis time between each injection was 20 minutes with a flow rate of 0.25-0.55 ml/min.

Time	Water %	4 mM Ammonium acetate (0.01 Formic acid)	Acetonitrile: Methanol (1: 2, v/v) %	Flow ml/min
0.0	0	95	5	0.50
1.00	0	95	5	0.50
1.01	0	95	5	0.25
4.00	80	0	20	0.25
5.00	55	0	45	0.25
10.49	20	0	80	0.25
10.50	0	0	100	0.50
14.00	0	0	100	0.50
16.01	0	95	5	0.55
20.00	0	95	5	0.55

Table 2.3. 1 Chromatographic conditions

2.3. 4 Electrospray ionisation-tandem mass spectrometry (ESI-MS/MS)

Mass spectrometry of the vitamin E metabolites was carried out using a triple quadrupole Micro Quattro instrument (MicroMass, Waters, U.K.) fitted with an electrospray ionisation source. The source and desolvation gas temperatures were held constant at 150^{o}C and 350^{o}C respectively, with flow rates of 950 and 60 liters of nitrogen per hour. The optimum gas cell pressure was set at 4 X 10^{-3} mbar. The vitamin E metabolites were detected using multiple reaction monitoring mode (MRM) of the transitions at 356.97>80.37 and

453.02>112.8 m/z for the CEHC/TLHQ sulphate and CEHC/TLHQ glucuronide, respectively. Table 3.3.2 below shows the mass spectrometry parameters. Data was acquired over a period of 2-20 min, in multiple channel acquisition mode and with a dwell time for each ion species of 50 ms. Using the divert valve, the HPLC mobile phase containing salts and other contaminants were diverted away from the mass spectrometer during the first 3 minutes. In this way at least 300 analyses could be performed before the ion source required routine maintenance.

The method developed is an in-house method and no changes were made.

	Transition	**Dwell (Sec)**	**Cone Voltage**	**Collision Voltage**
α-CEHC/TLHQ sulphate	356.9>79.7	0.50	42	26
α-CEHC/TLHQ glucuronide	453>112.8	0.50	25	36
Lithocholic acid sulphate	455.3>97	0.50	49	31
Androsterone glucuronide	465.3>113.2	0.50	156	46

Table 2.3. 2 Mass spectral specifications

2. 3 Amino acids associated with nitric oxide ($NO^{\cdot}$)

2.4. 1 Chemical reagents

All reagents were LC-MS grade or equivalent and obtained from the Sigma-Aldrich Chemical Company (Poole, Dorset, UK). The internal standard used in this study to quantitate the amino acids was $^{13}C_6$ arginine which was purchased from CDN isotopes (Thaxted, Essex, UK).

2.4. 2 Preparations of standards and internal standard for MS/MS

The working solution of each of the amino acids and internal standard was made up to a concentration of 100 μmol/l in methanol and was derivatised as follows: 15 μl of the 100 μmol/l working solution was added to 100 μl water. 250 μl 0.1 M borate buffer (pH 10.4) and 250 μl FMOC (5.8 mmol/l in acetone) were then added to the mixture, vortexed and left at room temperature for 15 min. The derivatised compounds were then desalted on a C18 Sep-Pak column (LiChrolut RP-18-500 mg) as follows:

1. Prime column with 3 ml 50 % acetonitrile- discard
2. Prime column with 3 ml water- discard
3. Apply sample to column and collect eluant
4. Reapply eluant and collect eluant
5. Wash with : 3 ml water- collect eluant

   3 ml 20 % acetonitrile – collect eluant

   3 ml 40 % acetonitrile – collect eluant

   3 ml 60 % acetonitrile – collect eluant

   3 ml 100 % acetonitrile – collect eluant

The total volume (15 ml) of the collected eluant was frozen (-20^{o}C) and used as standards in the validation procedures.

2.4. 3 Urinary samples

Method development was performed on a urinary pool obtained from laboratory volunteers from the UCL Institute of Child Health. Neat urine (150 µl) was spun for 5 min at 1500 rpm/ 214 RCF (relative centrifugal force) and spiked with 10 µl of (100 µmol/l) of the internal standard. The protocol for the urinary derivatisation of the urinary amino acids was as follows.

1. 10 µl of 100 µmol/l internal standard ($^{13}C_6$ arginine) was added to
2. 50 µl of urine and vortexed
3. 150 µl of 0.1 mol/l borate buffer, pH 10.4, was added and vortexed
4. 150 µl FMOC (5.8 mmol/l in acetone) was added and vortexed
5. The final reaction mixture was left for 15 minutes at room temperature prior to analysis by mass spectrometry

The derivatisation protocol was found to be >95% complete and any losses would be corrected for by the addition of the internal standard. The samples and metabolites were also found to be stable when experiments were conducted on spiked urine (data not shown).

2.4. 4 LC-MS/MS data analysis

15 µl of the sample prepared as described in section 2.4.3 was injected onto the LC-MS for analysis. The LC-MS/MS specifications are detailed below.

2.4.4. 1 Liquid chromatography

The amino acids were desalted and/ or separated prior to mass spectrometry using a Waters 2795XE high performance liquid chromatography unit. Rapid analyses were achieved using a 100 mm X 2.1 mm with a 5 μ particle size HyPURITY C8 column and a guard column containing the same stationary phase (Phenomenex UK) and using flow rates of 0.25 ml/min. The LC gradient used was procedure 2 as described previously (Table 4.2.4) which consisted of ammonium acetate containing 0.01 % formic acid and acetonitrile. The masses of all the amino acids were observed between 5-11 min. The total analysis time between each injection was 20 min.

2.4.4. 2 Electrospray ionisation-tandem mass spectrometry (ESI-MS/MS)

Mass spectrometry of the amino acids was carried out using a triple quadrupole Micro Quattro instrument (MicroMass, Waters, U.K.) fitted with an electrospray ionisation source in negative mode. The temperatures of the source and desolvation gas were held constant at 150 ^{o}C and 350 ^{o}C respectively, with flow rates of 950 and 60 liters of nitrogen per hour. The optimum gas cell pressure was set at 4 X 10^{-3} mbar. The amino acids were detected using multiple reaction monitoring mode (MRM) of the appropriate transitions. Table 4.3.1 shows the MRMs and mass spectrometry parameters for the amino acids analysed. Data was acquired over a period of 2-20 min, in multiple channel acquisition mode with a dwell time for each ion species of 50 ms. Using the divert valve, the HPLC mobile phase containing salts and other contaminants were diverted away from the mass spectrometer during the first 3 min. In this way at least 300 analyses could be performed before the ion source needed routine maintenance.

	Transition	Dwell (Sec)	Cone Voltage	Collision Voltage
FMOC L-arginine	394.7>172.9	0.50	18	8
FMOC L-citrulline	396.1>173.9	0.50	18	8
FMOC L-ADMA	422.8>201.0	0.50	16	13
FMOC L-homocysteine	356.1>159.8	0.50	9	9
FMOC L-taurine	346.0>123.7	0.50	16	21
FMOC L-serine	326.1>129.7	0.50	11	11
2(FMOC) L-cystine	683.5>151.8	0.50	12	13
FMOC L-cysteine	342.0>145.7	0.50	15	7
FMOC L-phenylalanine	386.6>190.1	0.50	14	13
FMOC L-glutamic	368.4>172	0.50	24	10
FMOC L-glycine	296.5>73.8	0.50	17	5
FMOC L-$^{13}C_6$arginine	400.6>178.9	0.50	18	8

Table 2.4. 1 Mass spectral specifications

Chapter 3

Isoprostanes

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3 Isoprostanes

The aim was to establish and validate a rapid non-invasive method for the measurement of 8-isoPGF$_{2\alpha}$ isoprostane in urine. The chapter describes the development of an assay and its potential use as a measure of oxidative stress.

3. 1 Isoprostanes as markers of oxidative stress

One of the greatest problems in the field of free radical research has been the absence of a reliable non-invasive method to assess oxidative stress in humans (Milne 2007). It has also been recognised that the methods developed previously for this purpose tended to lack specificity, sensitivity, or were too invasive for human investigation (Milne 2005). In(Morrow et al. 1990), Morrow *et al.,* reported the formation of isoprostanes (IsoPs), which are prostaglandin F_2 like compounds that are produced in vivo in man by the non-enzymatic free radical- induced peroxidation of arachidonic acid. The notion that prostaglandin (PG) - like compounds could be generated *in vitro* non-enzymatically as products of autoxidation of fatty acids was first demonstrated over 30 years ago (Nugteren 1976) and today isoprostanes are considered to be the "gold standard" for the assessment of oxidative stress *in vivo* (Milne and Morrow 2006).

3.1. 1 Chemistry and metabolism of the isoprostanes (IsoPs)

The F_2-isoprostanes consist of four regioisomers (classes III, IV, V, VI) (Fig.3.1.1), which have been observed to exist in eight racemic diastereomeric forms (Fig.3.1.2). This allows for the possibility of 64 different isoprostane isomers to be generated. The most studied of these are the isoprostanes isomeric to $PGF_{2\alpha}$, especially the class IV F_2-isoprostanes ($iPF_{2\alpha}$-III) species, 8-iso $PGF_{2\alpha}$ (Fig.3.1.2). The metabolic fate of 8-iso$PGF_{2\alpha}$ in humans has been explored using radiolabelled 8-iso$PGF_{2\alpha}$ (Robert 1997). Interestingly, approximately 43% of the excreted radioactivity was unextractable into ethyl acetate, suggesting the presence of very polar material, perhaps polar conjugates (Morrow 1994). The major urinary metabolite of 8-iso$PGF_{2\alpha}$ was identified as 2,3-dinor-5,6-dihydro-8-iso$PGF_{2\alpha}$ (Robert 1997).

Fig.3.1. 1 Formation of the four regioisomeric classes of F2-isoprostane

Fig.3.1. 2 Structure of prostaglandin PGF2α and the eight racemic diastereomeric forms of class III F2-isoprostanes

3.1. 2 Value of measuring isoprostanes to assess oxidative stress in vivo

Most methods available to assess oxidative stress, which are adequate for *in vitro* purposes such as malondialdehyde (MDA), thiobarbituric reactive substances (Seghrouchni et al. 2002) and 8-oxo-2' deoxyguanosine, have suffered from a lack of sensitivity and/or specificity or are unreliable when applied to *ex-vivo* complex biological fluids and tissues (Milne 2005). However, a considerable body of evidence has been obtained that suggests that the measurement of IsoPs represents an important advance in our ability to assess the status of oxidative stress *in vivo* (Robert 1997).

Firstly, it is important to point out that IsoPs are almost entirely products of lipid peroxidation. However, it is known that minute quantities of the F_2-IsoP, 8-isoPGF$_{2\alpha}$ can be produced as a minor by-product of cyclooxygenase activity (Robert 1997). More recently, it was demonstrated that small quantities of 8-isoPGF$_{2\alpha}$ are formed by prostaglandin H synthase-1 (PGH synthase-1) during aggregation of human platelets *in vitro* and by PGH synthase-2 in human monocytes (Pratico 1998). Importantly, it was demonstrated that the administration of high doses of cyclooxygenase inhibitors to normal humans does not significantly reduce 8-isoPGF$_{2\alpha}$ concentrations (Morrow 1990). This indicates that the relative contribution of the enzymatic generation of 8-isoPGF$_{2\alpha}$ *in vivo* is negligible compared to the amounts formed non-enzymatically (Robert 1997). Further evidence that 8-isoPGF$_{2\alpha}$ is predominantly formed non- enzymatically comes from pathologic/disease situations (e.g. cardiovascular disease, diabetes) associated with increased cyclooxygenase activity, where enzymatic generation of 8-isoPGF$_{2\alpha}$ remains insignificant in relation to the amounts formed non-enzymatically *in vivo* (Robert 1997).

Initial suggestions that the measurement of IsoPs may provide a valuable approach to assess the status of oxidative stress *in vivo* emerged from some of the early studies carried out by (Morrow 1990). Importantly, measurable levels of IsoPs can be detected in virtually every animal and human biological fluid and tissues that have been analysed. This allows the definition of a normal range such that even small increases in the formation of IsoPs can be detected. Normal ranges of F_2-IsoPs have been defined by (Milne 2007) as follows: plasma - 35 ± 6 pg/ml, urine - 1.6 ± 0.6 ng/mg creatinine and cerebrospinal fluid - 23 ± 1 pg/ml.

An increased production of IsoPs has been documented to occur in settings where oxidative stress has been implicated. Thus many studies have shown that the F_2-isoprostanes are reliable indicators of lipid peroxidation (Pratico 2000). Elevated levels have been detected in patients with cardiovascular disease (Reilly 1996), where lipid peroxidation is proposed to be involved in atherogenesis. Enhanced ROS production from a dysfunctional mitochondrial respiratory chain has been implicated in atherosclerosis and other vascular diseases (Madamanchi 2005). Human investigations also support the oxidative stress hypothesis of atherogenesis. Lipid peroxidation has also been suggested to play a role in the pathogenesis or consequence of neurodegeneration in many neurological disorders, and elevated concentrations of F_2-isoprostane have been reported in Alzheimer's disease (Montine 1999;Pratico 1998) and elevated levels of 8,12-iso-iP$F_{2\alpha}$-IV have been found in the urine of patients with Down's syndrome (Pratico 1998). Asthma is a chronic inflammatory disease of the airways, and elevated levels of the isoprostane, 8-isoP$GF_{2\alpha}$ have been found in urine and plasma following allergen challenge, which suggests that oxidant injury of the lungs may occur (Dworski 2001). Elevated concentrations of urinary F_2-isoprostanes have been reported in both type-1 and type-2 diabetes patients (Davi

1999a;Devaraj 2001). It was found that the elevated levels of F_2-isoprostanes seen in type-2 diabetic patients could be significantly reduced by supplementation with α-tocopherol (Devaraj 2001). Oxidative stress plays a secondary role in diabetes and has been dealt with in section 6.1.4. Increased concentrations of IsoPs in human body fluids and tissues have been found in a diverse array of human disorders indicating a possible increase in oxidative stress as shown in Table 3.1.1

Table 3.1. 1 Disorders in which increased concentration of F2-IsoPs have been reported

3.1. 3 Methods for the measurement of isoprostanes

Over recent years, several methods have been developed to quantify isoprostanes. The two most commonly used methods for measuring urinary F2-isoprostanes are by GC-MS and enzyme immuno-assay (EIA).

The most widely used method uses gas chromatography–negative ion chemical ionization–mass spectrometry (GC-NICI-MS) with a stable isotope of d_4-8-iso-$PGF_{2\alpha}$ as an internal standard (Morrow 1994). For *quantitation* purposes, $F_{2\alpha}$IsoP and other F_2-IsoPs that co-elute with 8-iso-$PGF_{2\alpha}$ were measured together. The main advantage of this technique over other approaches is its high sensitivity, which yields quantitative results in the low picogram range. The method can also detect the metabolite in low quantities in urine (Obrosova 2002) and plasma (MacRury 1993). The major drawback of this method is that it is labour intensive as it requires an extraction, separation and derivatisation procedure taking approximately 6-8 hours to analyse 12-15 samples.

Several alternative GC/MS assays have been developed by different investigators including (Pratico 1997) and Lawson *et al.,* (1998). Like the GC-NICI-MS assay developed by Morrow and co-workers (1994), all these methods require solid phase extraction (SPE), thin layer chromatographic (TLC) purification and/or chemical derivatisation. These methods measure other isomers of F_2-IsoPs in addition to 8-iso-$PGF_{2\alpha}$. These methods appear to be comparable to that of Morrow and co-workers (1994) in terms of sensitivity and specificity.

Enzyme immunoassay (EIA) is a biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample. A typical method involves

binding the antigen within the sample to a surface, and then a specific antibody is applied over the surface so that it can bind to the antigen. This antibody is linked to an enzyme, and in the final step another molecule or compound to the enzyme to create a detectable signal, most commonly a colour change in a chemical substrate. Antibodies have been generated against 8-iso-$PGF_{2\alpha}$ and currently at least three commercial immunoassay kits are available. A potential drawback of these methods is there is some debate regarding the accuracy of these techniques due to over-estimation of the metabolite (Milne 2005a).

The GC-MS and EIA methods have been compared by two independent research groups. (Proudfoot et al. 1999) found that GC-MS gave greater 8-iso$PGF_{2\alpha}$ concentration than EIA, which they explained by GC-MS failing to discriminate between a number of different F_2-isoprostane isomers, whereas EIA was specific for a particular isomer. In contrast, (Bessard et al. 2001) reported lower concentrations of 8-iso$PGF_{2\alpha}$ using GC-MS compared to EIA, and consequently concluded that EIA was less specific.

The fundamental difference between the GC-MS and EIA techniques is that EIA measures F2-isoprostanes that bind to an antibody raised against 8-iso$PGF_{2\alpha}$, whereas GC-MS measures only the isomers with common retention times to the internal standard d_4-8-iso$PGF_{2\alpha}$. Thus in theory, the 8-iso$PGF_{2\alpha}$ isomers that may not be present in the GC-MS peak could have cross-reactivity with the antibody, whereas the isomers that co-elute may not react with the antibody of the EIA.

In addition to these GC-MS and EIA assays, methods using liquid chromatography tandem mass spectrometry (LC-MS/MS) for the quantitation of F_2-IsoPs have also been developed. The most detailed of these methods has been reported by (Liang 2000) using SPE without a

derivatising procedure and (Bohnstedt et al. 2003) using a liquid/liquid extraction procedure. (Sircar and Subbaiah 2007)) overcame the labour intensive SPE or liquid/liquid extraction procedure by using an immuno affinity column to isolate the metabolite prior to the LC-MS/MS analysis. (Haschke 2007) developed an LC-MS/MS assay for 8-iso-$PGF_{2\alpha}$ with an automated online extraction procedure. (Teerlink 2007) prior to LC-MS/MS analysis. All these methods require less sample preparation time as compared to the established GC-MS method by Morrow *et al.,* (1994) but still involve a clean-up, separation/concentration procedure and required at least 3-5 ml of urine. In all the methods mentioned above, the sensitivity of the analysis of complex biological fluids was 4-5 fold lower than the established GC-MS method. It should also be noted that the antibody and the internal standard are supplied by a single commercial supplier- Caymen Chemicals U.S.A.

The methods described above involve multiple steps, including extensive sample preparation, derivatisation, and clean-up, that are not only labour intensive but could potentially lead to contamination, artefact generation and poor recoveries. The aim of the current study was to develop a method for the analysis of urinary isoprostanes with minimal sample preparation to reduce artefact formation and a short run time to increase the sample throughput.

3. 2 Method development for isoprostanes

3.2. 1 Principles of methodology

3.2.1. 1 Solid phase extraction

Solid phase extraction (SPE) is the separation or removal of an analyte or analytes from a mixture of compounds by selective partitioning of the compounds between a solid phase (sorbent) and a liquid phase (Hearn and Grego 1984; Jones Chomatography 1998).

Most solid phase extraction cartridges are based on silica chemistry. Cross-bonded silanols form the bases of the solid phase with changes in the reactive (R) group affecting specificity (Fig.3.2.1.A). The most commonly used SPE cartridges are probably C18 SPEs. These consist of the silanol backbone with octadecyl side-chains allowing hydrophobic interactions to occur with the analytes of interest. Sorbents containing other functional groups, which form polar or ionic interactions, are sometimes used to allow greater selectivity. Priming the cartridges with an organic solvent such as methanol stops the aggregation of the hydrophobic side chains and thereby increases the surface area for the hydrophobic interactions to occur (Fig.3.2.1.B).

The six steps involved in a typical solid phase extraction are as follows:

1. Sample pre-treatment. This may for example involve removal of solid material from the sample, ionisation/ deionisation of the sample to increase/ decrease interactions with the solid phase or derivatisation.
2. The solvation or priming (conditioning) of the sorbent bed of the SPE cartridge. This involves adding an organic solvent, such as methanol, to the cartridge so that

interactions between the branches of the solid phase are minimised allowing greater interaction with analytes. As mentioned above the hydrophobicity of solid phase such as C18 tends to make the chains aggregate together if they are not first primed.

3. The cartridge is washed with the solvent that will be used to apply the sample.
4. The sample is then loaded onto the column.
5. Interfering compounds are washed off, usually using aqueous solution.
6. The analytes are eluted using a suitable solvent, which is often the initial priming solvent. Variations can include sequential elution using greater amounts of organic modifier in order to achieve a better separation.

Fig.3.2. 1 (A) The structure of silanol based SPE cartridges. (B) Priming of the cartridges to increase retention capacity

3. 3 Method development

Initial analysis of isoprostane metabolites was conducted using standard solutions (100µmol/l) of d_4-8-iso-$PGF_{2\alpha}$ (internal standard) and 8-iso-$PGF_{2\alpha}$. The compounds were commercially available standards which aided the standardisation procedures for the assay. Straight scans were conducted to check the purity of the standards and check their amenability to electrospray ionisation MS/MS. After checking the suitability of isoprostanes for analysis by ESI-MS/MS, a proposed scheme for developing the assay was implemented (Fig 3.3.1), which is typical for the method development of metabolites using LC-MS/MS. The first steps involved mass spectral analysis which included determining the molecular weight and the integrity of the metabolite of interest, followed by fragmentation studies which give distinct progeny/daughter ions unique to each metabolite (but not their isomers). The last step in the method development involved separating the metabolite of interest not only from the biological fluid (Tsikas 2003b) but also from its isomers by using reverse phase high performance liquid chromatography. Following the method development the assay was tested and validated on laboratory control samples.

Fig.3.3. 1 Plan of preliminary studies used in method development

3.3. 1 Preliminary studies

100 µmol/l of Isoprostanes (d_4-8-iso-$PGF_{2\alpha}$ and 8-iso-$PGF_{2\alpha}$) were analysed by electrospray triple quadrupole mass spectrometry operated in negative ion mode. No signal was detected using positive ion mode. Straight scans were conducted to check purity, to see if the standards had degraded during transportation and storage, and to see if the masses of the standards corresponded to their theoretical molecular weights.

The tandem mass spectral analysis of the metabolite 8-iso-$PGF_{2\alpha}$ and internal standard d_4-8-iso-$PGF_{2\alpha}$ were obtained in scan mode over the mass range of 50 to 600 m/z. The major peaks were observed at 353.6 m/z and 357.3 m/z which corresponded to the molecular weight [M-H^-] of the metabolite (8-iso-$PGF_{2\alpha}$)and internal standard (d_4-8-iso-$PGF_{2\alpha}$) (Fig. 3.3.2 A and B).

Fig.3.3. 2 Parent ion scans of (A) 8-iso-PGF2α m/z 353.6 and m/z357.3 (B) d4-8-iso-PGF2α

Each metabolite was further characterised by fragmentation studies using the mass spectrometer operated in the product ion scan mode. The fragmentation analysis for both the 8-iso-$PGF_{2\alpha}$ and internal standard d_4-8-iso-$PGF_{2\alpha}$ were observed over the mass range 2-360 m/z. The fragmentation studies of 8-iso-$PGF_{2\alpha}$ led to the identification of a major peak at the intensity of 193 m/z with minor peaks at 247 m/z and 309 m/z (Fig.3.3.3 A). Fragmentation analysis of the internal standard (d_4-8-iso-$PGF_{2\alpha}$) revealed the presence of a major product ion at 197 m/z with minor peaks at 251 m/z and 313 m/z (Fig. 3.3.3 B).

Product ions at 193 m/z (Fig.3.3.3.A) and 197 m/z (Fig.3.3.3.B) were observed to be the most abundant product ions and were specific for 8-iso-$PGF_{2\alpha}$ and d_4-8-iso-$PGF_{2\alpha}$ respectively. Therefore, precursor/product ion pairs (transitions) for the detection of 8-iso-$PGF_{2\alpha}$ and d_4-8-iso-$PGF_{2\alpha}$ using MRM mode were determined to be m/z 353>193 and m/z 357>197 respectively.

Fig.3.3. 3 Product ion scan mass range 2-360 m/z of 8-iso-PGF2α (A) -major peak at the intensity of 193 m/z with minor peaks at 247 m/z and 309 m/z and d4-8-iso-PGF2α (B) major product ion at 197 m/z with minor peaks at 251 m/z and 313 m/z

3.3. 2 Development of the HPLC methodology: procedure-1

In the case of 8-iso-$PGF_{2\alpha}$, there are four regioisomers that cannot be distinguished from each other by using the MRM mode as all the isomers show the same fragmentation patterns. They must therefore be separated chromatographically prior to MS/MS analysis to allow for specific *quantitation*. As discussed in section 3.1.3, previous methods have relied on gas chromatography for this separation. However, these assays require extensive sample preparation, including solid phase extraction (SPE), thin layer chromatography (TLC), and a derivatising step to protect the polar group. Thus in the current methodology a high performance liquid chromatographic step was investigated, as it required little or no sample preparation without a derivatising step.

Initially, a 5cm X 2.1mm Discovery HSF5 (5µm) column with a guard column containing the same stationary phase (Supleco USA) was used with a flow rate of 0.20 ml/min. The initial HPLC gradient procedure, termed HPLC procedure-1 is shown in Table 2.3.1. The mobile phase consisted of a step change gradient where the LC gradient initially consisted of 5% ammonium acetate containing 0.01% formic acid and 95% methanol for the first 4 min, which was then changed to 30% ammonium acetate containing 0.01% formic acid and 70% methanol for another 6 min. The column was then reconditioned before the next injection by re-equilibration with 5% ammonium acetate (containing 0.01 % formic acid) and 95% methanol for 2 min. The total analysis time between each injection was 12 min. Each injection consisted of 40 µl of sample from a vial of 150 µl of neat urine plus 10 µl of 100µmol/L d_4-8-iso-$PGF_{2\alpha}$.

Time	Ammonium acetate (containing 0.01 % Formic Acid) %	Methanol %	Flow rate ml/min
0.00	5	95	0.20
4.00	5	95	0.20
4.01	30	70	0.20
10.00	30	70	0.20
10.01	5	95	0.20
12.00	5	95	0.20

Table 3.3. 1 HPLC procedure-1

The ESI-MS/MS parameters remained constant for the entire method development as described in section 3.4.3.3.

100 µmol/L of internal standard and the metabolite (in ethanol) were detected in the chromatogram Fig. 3.3.4. Though, using this procedure, neither 8-iso-$PGF_{2\alpha}$ nor d_4-8-iso-$PGF_{2\alpha}$ could be detected in the chromatogram (Fig. 3.3.5). The method was found to have a problem with sensitivity as the signal to noise ratio was very high. It was therefore proposed that either concentration and/or purification of the sample might enable the quantitation of the isoprostanes. It was thought therefore that the method should include either a metabolite extraction procedure or a different HPLC assay which may increase the sensitivity of the method. This is discussed in the next section.

Fig.3.3. 4 Elution of d4-8-iso-PGF2α (A) at 7.29 min and 8-iso-PGF2α (B) at 7.27 in ethanol using HPLC procedure 1

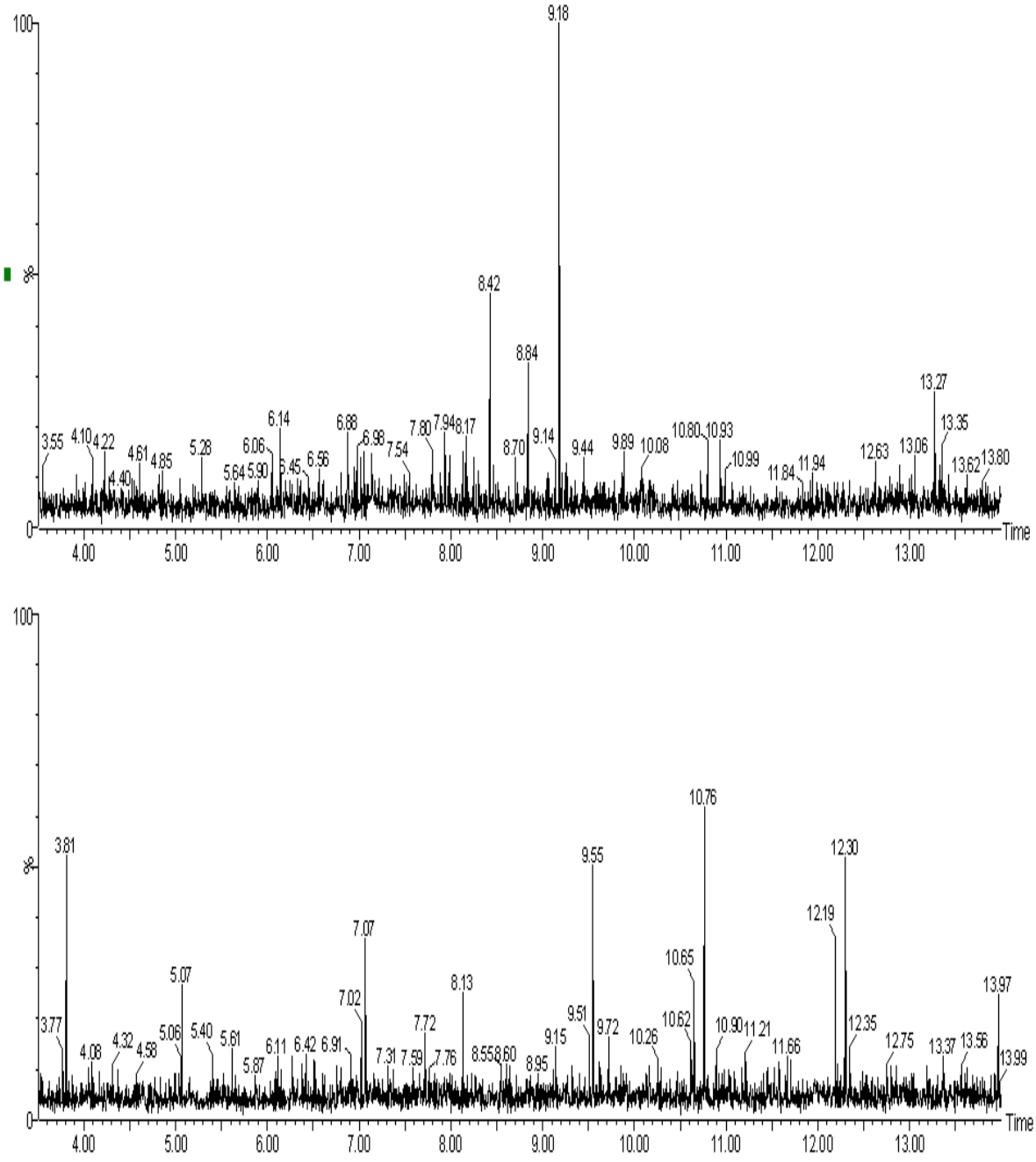

Fig.3.3. 5 Absence of peaks in the elution of d4-8-iso-PGF2α and 8-iso-PGF2α (B) in neat urine using HPLC procedure-1

3.3. 3 Development of the HPLC methodology: procedure-2

A possible reason for the lack of sensitivity of HPLC procedure-1 was a high matrix suppression effect caused by other metabolites eluting at the same time as the isoprostanes. The instability of the standards both in isolation and in urine as a reason for the lack of sensitivity was ruled out as firstly, the metabolite and internal standard were observed in ethanol (Fig.3.3.4) and secondly when a larger concentration (500µmol/l) was added to urine, elution of the metabolite was observed (data not shown). To eliminate possible matrix suppression effects and to concentrate the metabolite of interest an extraction procedure using SPE was evaluated.

To purify and concentrate the isoprostanes, solid phase extraction (SPE) was carried out using C-18 cartridges with 1 ml of urine based on the method of (Zhang and Saku 2007) as illustrated in Fig 3.3.6. For the HPLC step a- HyPurity C8 column was used instead of the HSF5 column, but all the other parameters of HPLC procedure-1 were retained for this experiment. This method was termed HPLC procedure-2

On analysis, only the mass for the internal standard (7.60 min) with a signal to noise ratio of 3:1 was detected, but no 8-iso-$PGF_{2\alpha}$ could be detected in the urine (Fig. 3.3.7). The signal intensity for the internal standard was very low indicating poor sensitivity and thus further steps were required to optimise the method. It was postulated that changes to the mobile phase of the HPLC solvents might lead to an increase in the sensitivity of the method, which is also discussed in the following section.

Fig.3.3. 6 Procedure for solid phase extraction (SPE) based on Zhang et al., 2007

Fig.3.3. 7 Elution of d4-8-iso-PGF2α at 7.60 min (A) and 8-iso-PGF2α (B) in neat urine using HPLC procedure 2

3.3. 4 HPLC procedure-3

It was postulated that changes to the HPLC parameters based on the method of Zhang *et al.,* 2007 might increase the sensitivity of the method. Changes were made to the mobile phase of HPLC procedure-1 where water replaced ammonium acetate and acetonitrile: methanol (1:2 v/v) replaced methanol.

The HPLC gradient (Table 3.3.2) was also modified to incorporate the new solvents. The mobile phase consisted of a step change gradient where the LC gradient initially consisted of 80% HPLC grade water and 20% ACN:MeOH (1: 2, v/v) gradually changing to 100 % ACN:MeOH (1: 2, v/v) over a period of 7.5 min. The mobile phase was maintained at 100% ACN:MeOH for 2.5 min. The column was then reconditioned before the next injection with 80% water and 20% ACN:MeOH (1: 2, v/v) for 4 min. The total analysis time between each injection was 14 min. The injection volume was 40 µl. All the other experimental procedures such as the column, SPE protocol and mass spectral parameters were retained from HPLC procedure-2.

Time	Water %	Acetonitrile:Methanol (1: 2, v/v) %	Flow ml/min
0.00	80	20	0.20
2.00	55	45	0.20
7.49	20	80	0.20
7.50	0	100	0.20
10.00	0	100	0.20
10.01	80	20	0.20
14.00	80	20	0.20

Table 3.3. 2 HPLC procedure-3

Using the new procedure both the internal standard d_4-8-iso-$PGF_{2\alpha}$ and metabolite 8-iso-$PGF_{2\alpha}$ could be detected (Fig.3.3.8). However, the chromatographic peaks were broad with tailing, and there was still a low signal to noise ratio resulting in a low sensitivity. It was decided that a change in the flow rate of the HPLC gradient might be a parameter that could improve the chromatography.

Fig.3.3. 8 Elution of d4-8-iso-PGF2α at 8.17 min (A) and 8-iso-PGF2α at 8.27 min (B) using HPLC procedure-3

3.3. 5 Evaluation of changes in flow rate

Five different flow rates of the HPLC were evaluated, ranging from 0.1 to 0.3 ml/min (0.1, 0.15, 0.20, 0.25, 0.3 ml/min). All the other variables such as the solid phase extraction, HPLC and mass spectral parameters were maintained as described in HPLC procedure-3. The effect of the different flow rates was assessed by measuring the peak areas of 8-iso-$PGF_{2\alpha}$ after adding 500 pmol/ d4-8-iso-$PGF_{2\alpha}$ 500 pmol added to urine. The experiment was conducted in triplicate.

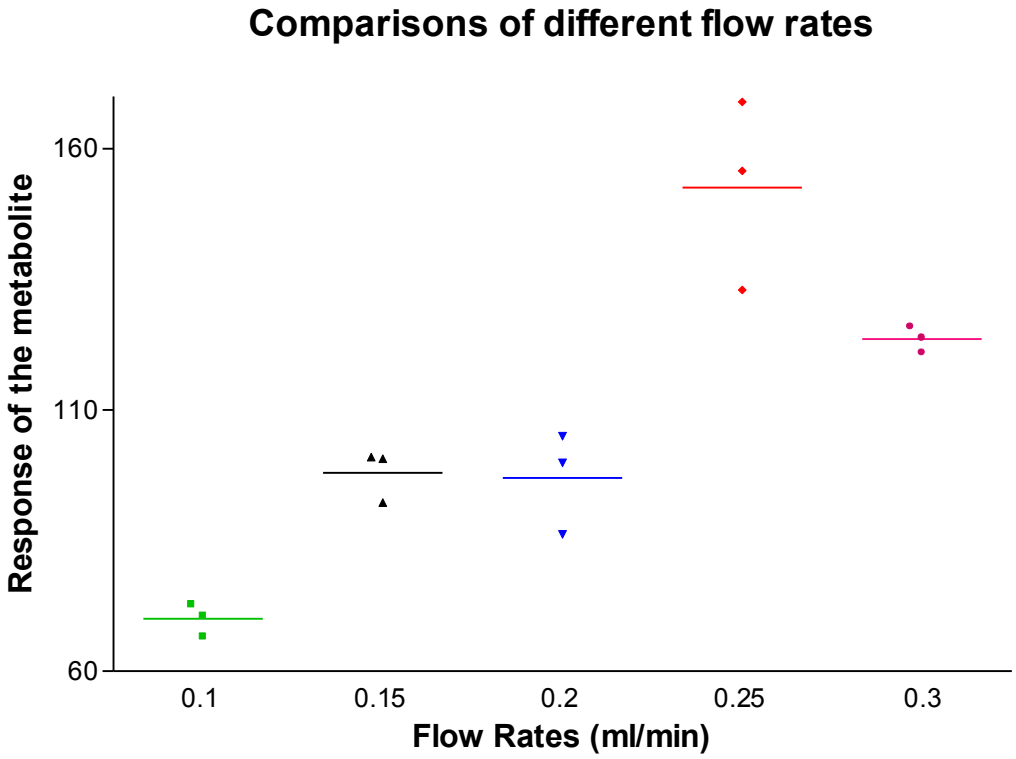

Fig.3.3. 9 Comparisons of different flow rates

From the results obtained (Fig. 3.3.9) it appeared that a flow rate increase from 0.20 to 0.25 ml/min increased the sensitivity of the procedure by approximately 64% compared to the original method.

To try and further increase the sensitivity of the method it was decided to optimise the extraction procedure of the assay and this is discussed in the following section.

3.3. 6 Comparing different SPE cartridges

After optimisation of the HPLC conditions, it was decided to try and improve the extraction of the metabolite during the solid phase extraction procedure. Two different cartridges C-18 and C-6 (100 mg) were compared. The SPE method described previously in section 2.3.3 (Fig. 3.3.6) was repeated using 1 ml of urine on the two cartridges. Each experiment was carried out in triplicate. The efficiency of the separation was evaluated by estimating the area under the curve after adding a known amount of the internal standard d_4-8-iso-$PGF_{2\alpha}$ (500 pmol) to the urine.

The results (mean/SEM) indicated that the C-6 stationary phase (3724±734.6) gave marginally better results than the C-18 cartridges (3065±44.5). Thus it was decided to continue the solid phase extraction using C-6 cartridges.

The benefits of using an extraction procedure was investigated by comparing the results with and without SPE as described below.

3.3. 7 Comparing recovery of 8-iso-PGF2α with and without SPE

To compare the inclusion of a SPE step versus no SPE a known amount (500 pmol) of the standard metabolite 8-iso-$PGF_{2\alpha}$ and 500 pmol of d_4-8-iso-$PGF_{2\alpha}$were added to 12 pooled urine samples, of which 6 were extracted on a SPE cartridge and 6 were not. The samples were then analysed using LC-MS/MS and the same HPLC parameters as HPLC procedure-

3. It was observed that the area under the curve (mean±SEM) from the urine that underwent SPE was 110±7 compared to 548±27 which approximates to an increase of 400% (Fig. 3.3.10).

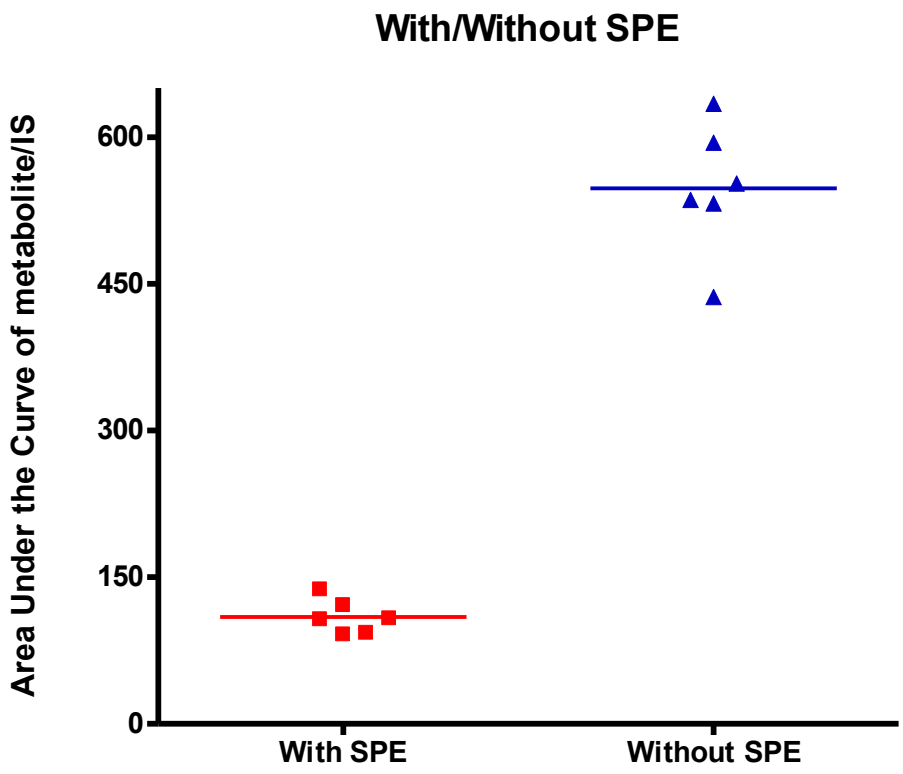

Fig.3.3. 10 Mean area under the curve of the metabolite following solid phase extraction was at 110±7 compared to 548±27 without solid phase extraction

The addition of the extraction procedure did initially appear to increase the sensitivity of the method as discussed in section 3.3.3 but the subsequent changes made to the HPLC solvents and gradients (section 3.3.4) and flow rate (section 3.3.5) and had resulted in an increase in the sensitivity of the method which meant that the SPE step was not necessary.

On the basis of the method developed outlined above, it was decided that the following method without SPE would be used for the urinary analysis of 8-iso-$PGF_{2\alpha}$.

3. 4 Analysis of control urines

Twelve laboratory control urine samples were run (in triplicates) on the same occasion to quantitate 8-iso-$PGF_{2\alpha}$ using the final method. The internal standard (d_4-8-iso-$PGF_{2\alpha}$) and the metabolite (Dworski 2001) could not be detected in 6 of the samples as the signal to noise ratio was too great (chromatograms not shown). The metabolite and the internal standard were however detected in the other six samples (Fig.3.4.1 and Fig.3.4.2). The elution times of the samples varied as shown in Table 3.4.1. They ranged from 4.04 min in control-6 to 7.60 min in control-4. Multiple peaks were observed in the chromatogram of the internal standard for control-2 where two peaks appeared at 3.98 and 5.56 respectively. Multiple peaks, possibly indicating the presence of regioisomers of the metabolite, were also observed at 5.56 and 8.79 min in control-2 and 7.58 and 7.92 min in control-4. The metabolite could not be detected in control-3.

	Elution times (min)
Laboratory control -1	**4.8**
Laboratory control-2	**5.8**
Laboratory control-3	**5.3**
Laboratory control-4	**7.6**
Laboratory control-5	**6.8**
Laboratory control-6	**4.04**

Table 3.4. 1 Elution times of metabolite and internal standard

Fig.3.4. 1 Elution of internal standard (green) and metabolite (red) in three laboratory controls. Elution of the metabolite was observed at 4.80 min in Lab control-1 and 5.50 min in Lab control-2 albeit with peak splitting of the internal standard and was not observed

Fig.3.4. 2 Elution of internal standard (green) and metabolite (red) in three laboratory controls. Peak splitting was observed in Lab control-4 at 7.78 and 7.97 min. The metabolite was observed to have eluted at 6.82 and 4.04 min in Lab control-5 and -6 respectively

3. 5 Discussion and conclusions

We aimed to develop a LC-MS/MS method for isoprostanes which involved minimum sample preparation to avoid artefact formation, which was more specific than previous methods and would increase the throughput compared to existing methods.

Preliminary studies (straight scans and fragmentation spectra) were conducted to identify the metabolites. The compounds detected had mass spectra consistent with the structures of the metabolite 8-iso-$PGF_{2\alpha}$ and the internal standard d_4-8-iso-$PGF_{2\alpha}$ (Fig. 3.3.2). The fragmentation studies produced progeny ions (Fig.3.3.3) which were identical to those reported by Liang *et al,.* (2003) and Zhang & Saku (2007).

Because F_2-isoprostanes are isomers of F_2-prostaglandins and consist of many stereoisomers, the prior separation of the metabolite of interest from its isomers is important for quantitation. The analysis of isoprostane in neat urine, using the initial HPLC method (procedure-1) was unsuccessful as shown in Fig. 3.3.5. At this point, it was theorised that in order to increase the sensitivity of the method the metabolite would need to be concentrated-and therefore an extraction procedure was introduced. It was additionally felt that the inclusion of a solid phase extraction step would purify i.e. remove the metabolite from the urine matrice. This theory was substantiated by the results obtained by (Li 1999) who observed that an extraction procedure (solid phase extraction) prior to HPLC analysis achieved a base line separation of 8-iso-$PGF_{2\alpha}$ and its isomers. Our method was further influenced by the assay of Zhang & Saku (2007), who observed that an unknown isomer coeluted with 8-iso-$PGF_{2\alpha}$ on a C18 column but separated with a better resolution when a C8 column was used. Modifications were therefore made to the method (section 3.3.3), by using a 5 µm C8 column 100 mm X 2.1 mm (which has a higher density

than the previously used HSF5 column) coupled with an extraction/concentration process using solid phase extraction procedure using a C18 cartridge. The results obtained were still not satisfactory (Fig. 3.3.7) and the HPLC gradient was therefore further modified (Table 3.3.2) by replacing ammonium acetate with water and acetonitrile:methanol (1:2 v/v) replaced methanol. It had been reported previously by Zhang & Saku (2007), that buffer solutions caused significant ion suppression of isoprostanes and after the change of the aqueous phase to water, a significant improvement in the sensitivity of the method was observed (Fig. 3.3.8). The sensitivity of the method was improved further by changing the flow rate of the HPLC gradient from 0.20 to 0.25 ml/min (section 3.3.5). Following the increase in the flow rate, the use of the SPE extraction procedure was re-evaluated. It was found that the SPE procedure resulted in a decrease in sensitivity (section 3.3.7). Even though the SPE would have enabled the purification and concentration of the metabolite and in theory should allow a higher concentration of the metabolite entering the MS, a drop in sensitivity could have been due to an "unknown matrix effect". (de Jong and Teerlink 2006) has suggested that the ion suppression can occur as a result of the presence of an unknown chemical contaminant in the SPE column.

The assay was then tested in laboratory control (section 3.4). Three major problems were observed:

- The internal standard and metabolite (section 3.4) could only be detected in half of the twelve samples tested, suggesting a very high and varied matrix effect between

the samples (Figs 3.4.1 and 2). (Liang 2003) reported that the metabolites were retained for a longer time on the column in urine samples and they referred to this observation as an "unknown matrix effect". They postulated that another unrelated compound co-eluted with the isoprostanes/metabolite of interest causing this effect. (Haschke 2007) have suggested another possible explanation for the matrix effect. They found that, electrospray ionisation (ESI), the technique used in the current study, was particularly susceptible to matrix effects and that an increase of signal intensity by an order of magnitude was obtained when the ionisation was switched to atmospheric pressure chemical ionisation (APCI).

- Another observation made in the current study was the peak splitting/presence of multiple peaks in some of the laboratory controls. An example of which has been enlarged in Fig 3.5.1. These numerous peaks have been postulated to be 8-iso-15 $PGF_{2\alpha}$, 11β- $PGF_{2\alpha}$, 15-$PGF_{2\alpha}$, 5-trans- $PGF_{2\alpha}$, $PGF_{2\alpha}$, 8-iso-$PGF_{2\beta}$, $PGF_{2\beta}$ and 5-trans- $PGF_{2\beta}$ identified and quantified by Li *et al.,* (1999) and Zhang & Saku (2007) as the eight regioisomers of 8-iso-$PGF_{2\alpha}$ (353>193). This raises the question of the specificity of this method, as there is the possibility of the coelution of the nine peaks quantified by Li *et al.,* (1999) and Zhang and Saku (2007), of which only a minor peak corresponded to that of the metabolite of interest. The presence of a minor unknown chromatographic peak was observed for d_4-8-iso-$PGF_{2\alpha}$. This finding is similar to that reported by Liang (2003), who ruled out the presence of possible interferences or artefacts in urine by extracting the samples with and without adding d_4-8-iso-$PGF_{2\alpha}$.

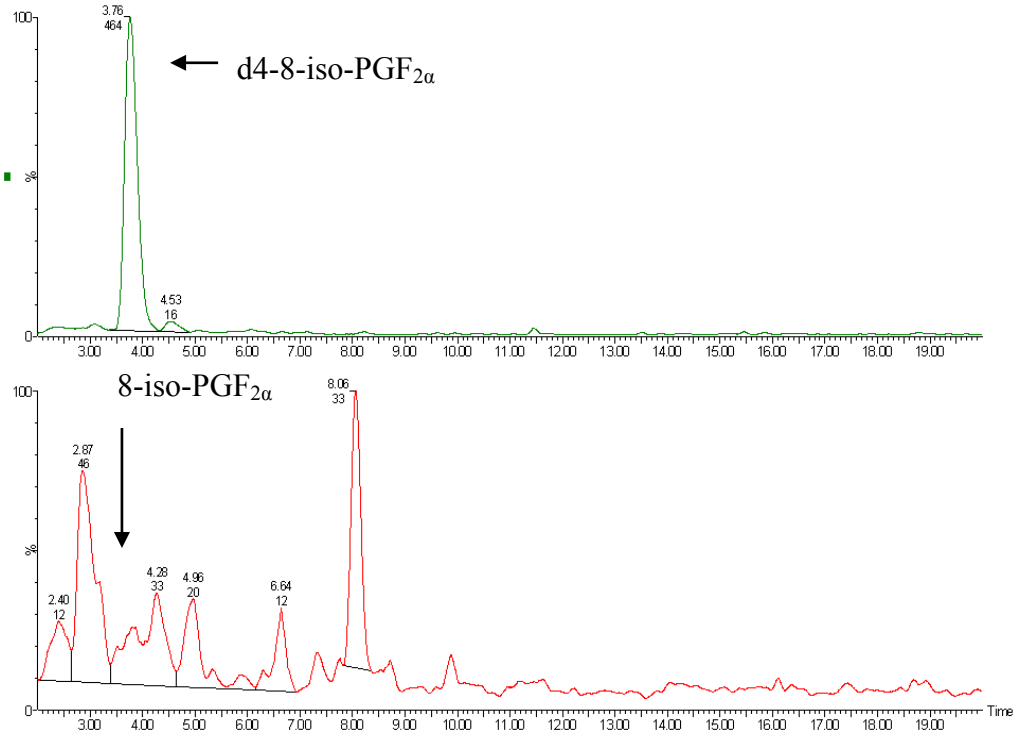

Fig.3.5. 1 Enlarged elution of internal standard (green) and metabolite (red) in laboratory control-2

- The third unexplained observation made in the current study was the inconsistency of the elution times (Fig. 3.4.1 and 3.4.2) of the metabolites and internal standard within a run, even though a blank was included between each sample was included to rule out "carry over" effects caused by the previous sample. This observation in the LC-MS/MS methodology has not been previously reported by other investigators but on close examination of the study of Saenger *et al.,* (2007) inconsistent elution times of the metabolite of interest (Dworski 2001) can be seen. In Fig. 3.5.2.A (taken from Saenger's study) the elution time of the internal standard and the metabolite was 6.32 min whereas in Fig. 3.5.2.B the same internal standard and metabolite eluted a minute earlier at 5.25 min (Saenger 2007). The reason for this inconsistency of retention times is not known. It can be postulated that as the method did not include solid phase extraction prior to LC-MS/MS analysis, the

column could have become overloaded with neat urine causing ion-pairing by unknown metabolites which could result in varied elution times.

Fig.3.5. 2 Inconsistency in the elution time of 8-iso-PGF2α taken from Saenger (2007)

The variation in the retention times could also result from the "unknown matrix effect" reported in 2006 by de Jong & Teerlink (discussed above) or from variations in the urinary concentration. The effect of the urinary concentration was investigated by examining whether there was a relationship between the urinary creatinine concentration and retention time, but no correlation was observed. Urine has also been reported to be more susceptible to matrix effects than any other biological fluid (Haschke 2007).

The conclusions drawn from the current study was that the method developed was neither sufficiently sensitive nor reproducible to be used for the accurate measurement of isoprostanes.

With the benefit of hindsight, if the study was to be repeated, the use of a one-step sample clean-up where the isoprostane is liberated using mild alkaline hydrolysis, followed by isolation of the metabolite using immunoaffinity chromatography (IAC) as described by Sircar & Subbaiah (2007) would be considered prior to quantitation (HPLC MS/MS). Another approach that can be considered either in isolation or in combination with the method of (Sircar & Subbaiah 2007) is the use of a semi-automated column switching technique and an on-line sample extraction procedure. The metabolite can be extracted online using an HPLC extraction column, after which the analyte from the extraction would then be back flushed on to an analytical column prior to MS/MS analysis (2007). The current study could not undertake such an experiment as the laboratory functions with as a shared facility and I could not change the machinery for the current experiment.

A common observation in the studies of Sircar *et al.,* (2007) and Haschke *et al.,* (2007) was that the methods were not as sensitive as the GC/MS method with the presence of multiple

peaks (isomers) and the peak of interest (8-iso-$PGF_{2\alpha}$) being minor. In addition, the absolute values of the metabolite reported by Sircar *et al.,* (2007) and Haschke *et al.,* (2007) were 5- to 10- fold lower respectively than those reported using GC/MS. Lastly, GC/MS probably measures all the sterioisomers together i.e. it measures the total isoprostanes present and also GC/MS has significantly greater chromatographic resolving power compared to HPLC which enables GC/MS to be more accurate and limits ion suppression effects.

In conclusion, the LC-MS/MS method which we attempted to develop proved to be unsatisfactory for measuring isoprostanes in small volumes of urine. It therefore appears that although most GC/MS methods require multiple steps and are labour intensive they remain at present the method of choice. Therefore, the use of other possible biomarkers of oxidative stress was investigated.

Chapter 4

Vitamin E metabolites

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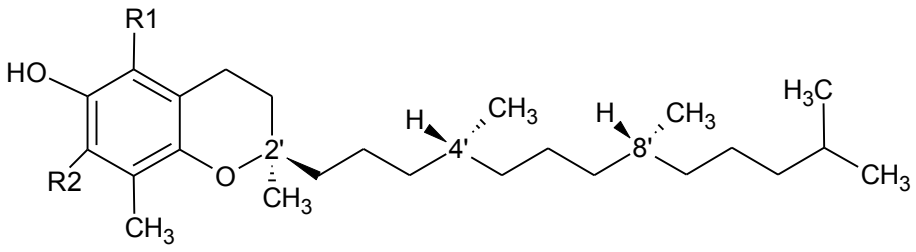
4 Vitamin E metabolites

4. 1 Background information

4.1. 1 History and nomenclature

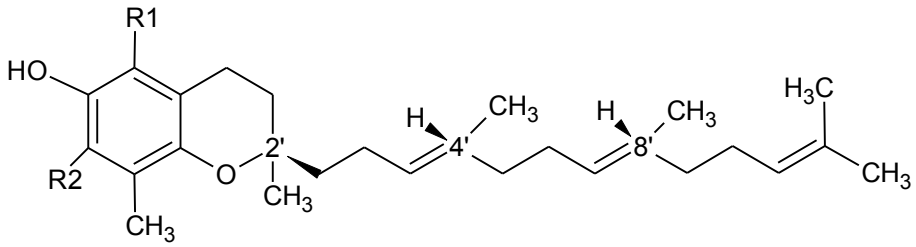
Vitamin E was first described by Evans and Bishop in the 1920s, as a dietary factor present in lettuce and wheat germ that prevented foetal resorption in rats fed a rancid lard diet (Evans and Bishop 1922;Evans and Burr 1925). A chemically pure compound with vitamin E activity was first isolated from wheat germ oil by (Evans 1936). It was partially characterised as an alcohol with the chemical formula $C_{29}H_{50}O_2$. The structural formula of α-tocopherol was published by (Fernholz 1938).

The term vitamin E refers to tocopherols and the tocotrienols, which have saturated and unsaturated phytyl side chains respectively. The α-, β-, γ- and δ- tocopherols and tocotrienols differ in the number and position of the methyl groups on the chromanol ring (Fig.4.1.1). In the case of α-tocopherol, three methyl groups are present on the chromanol ring, whereas δ- tocopherol has one a methyl group and β and γ –tocopherol contain two methyl groups and differ from each other with respect to the positioning of the methyl group as illustrated in Fig.4.1.1.



Tocopherol

α: R1=R2=CH_3
β: R1=CH_3,R2=H
γ: R1=H,R2=CH_3
δ: R1=R2=H



Tocotrienol

Fig.4.1. 1 Nomenclature of vitamin E

The key structural features of tocopherols and tocotrienols are the chromanol ring and the hydrocarbon side chain. The chromanol ring is responsible for the antioxidant properties of tocopherol and tocotrienols, while the side chain is responsible for their lipid-solubility. This combination of lipid-solubility and antioxidant activity makes vitamin E ideally suited to protect lipid molecules from oxidative damage. Vitamin E has been shown to account for the majority of the lipid soluble antioxidant activity in human blood (Burton 1983;Burton and Ingold 1986).

The phytyl side chain of the tocopherols has 3 chiral centres and can therefore exist as eight possible sterioisomers. Natural tocopherol is a single isomer, designated RRR, indicating it

has the same stereochemistry at every chiral centre (positions 2, 4' and 8' in Fig.4.1.1). Synthetic tocopherol made from trimethylhydroquinone and synthetic isophytol is an equimolar mixture of all eight different isomers and is designated all-rac-tocopherol.

The predominant form of vitamin E in human and animal diets differs around the world depending on the type of plant oils used. RRR-γ-tocopherol is the most abundant form (2-4 times in excess of RRR-α-tocopherol) in North American and European diets due to the widespread use of corn and soybean oil (Sheppard 1993). In contrast, South East Asian diets contain tocotrienols in much higher abundance than the North American diets due to the routine use of palm oil in cooking. However, in general over 90% of vitamin E in plasma and tissues is RRR-α-tocopherol (Traber 1998). The preferential enrichment with α-tocopherol *in vivo* results from the presence of an α-tocopherol transfer protein (α-TTP) (Traber 1998). α-TTP, is responsible for the preferential loading of nascent VLDL particles in the liver with α-tocopherol.

The relative affinities of α-TTP for the different vitamin E homologues were determined by evaluating competition between labeled and non-labeled compounds for transfer between membranes *in vitro,* and the following relative affinities were found: RRR- α-tocopherol taken as 100%; RRR-β-tocopherol 38%; RRR-γ-tocopherol 9%; RRR- δ-tocopherol 2%, SRR-α-tocopherol 11% and α-tocotrienol 12%. It appears from the data of (Hosomi 1997) and also from other data (Burton 1990;Burton 1995) that the position and number of methyl groups around the chromanol ring of the tocopherols and tocotrienols, as well as the stereochemistry at the C-2 position of the phytyl side chain are the most important determinants in the relative rate of transfer of tocopherol-type compounds by α-TTP. Thus, RRR- α-tocopherol has a higher activity than SRR- α-tocopherol because of the difference

in stereochemistry at the C-2 position and SRR- α-tocopherol has a higher activity than RRR-δ-tocopherol due to the number and position of methyl groups around the chroman ring, even though it does not have optimal stereochemistry at the C-2 position of the phytyl side chain. Comparison of the excretion rates of the metabolites of natural (RRR) versus synthetic (all-rac) α-TOH have also shown preferential retention of the natural stereoisomer (Traber 1998).

Besides the liver, the presence of low levels of mRNA for α-TTP has also been reported in the brain, spleen, lung and kidney of rats (Hosomi 1998). Low level expression in these organs may be essential for the transfer and retention of α-tocopherol within these organs.

4.1. 2 Metabolism

4.1.2. 1 Vitamin E metabolism

Although vitamin E was discovered in the 1920s relatively little is known about its metabolism. Vitamin E metabolites are likely to form two distinct groups. The first of these contains metabolites that are produced after reaction of vitamin E with oxidants, while the second contains metabolites that are produced by successive shortening of the side chain (Sato 1991).

Vitamin E metabolites are excreted as conjugates but virtually all of the research into the metabolism of vitamin E has investigated the free unconjugated urinary metabolites, after their enzymatic or acidic deconjugation. Urinary metabolites of vitamin E, were first reported by Simon *et al.,* (1956). They described two metabolites of α-tocopherol, 2-(3-hydroxy-3methyl-5-carboxypentyl)-3, 5, 6- trimethyl-1, 4-benzoquinone (α-tocopheronic acid, α-TA) and its γ-lactone (α-tocopheronolactone, α-TL), which were produced by both rabbits and humans following oxidation and opening of the chromanol ring (Fig.3.1.2). Enzymatic deconjugation of these metabolites with β-glucuronidase suggested that α-TA and α-TL were excreted as glucuronidate conjugates. Owing to their quinone structures, it was hypothesized that α-TA and α-TL were derived from the known α-tocopherol oxidation product, α- tocopherylquinone (α-THQ), following the β-oxidation and cyclisation of the phytyl side chain.

α-tocopherol can also undergo β-oxidation and shortening of its phytyl side chain without oxidation of the chromanol ring and this first results in the formation of α–carboxy-methyl-

hydroxy-butyl-hydroxy-chroman (α–CMBHC), further leading to the excretion of α-carboxy- ethyl- hydroxychroman (α-CEHC) after oxidation of α–CMBHC's side chain. α-CEHC excretion was found to increase when a certain plasma level of RRR-α-tocopherol was exceeded (Schultz 1995). It has also been suggested that there is a possibility of the artefactual oxidation of α-CEHC to α-TLHQ during the assay procedure for quantitation of the metabolites as observed by Pope *et al.,* (2001).

CEHC was first reported when it was found to be the major metabolite when rats were injected with radiolabelled vitamin E and when δ- tocopherol was injected, the δ form of CEHC was observed (Chiku et al. 1984).

The α-tocopherol homologue of δ-CEHC was first characterised in human urine by Schonfeld *et al.,* (1993) and a more detailed study was carried out by Schultz *et al.,* (1995) Enzymatic studies suggested α-CEHC was excreted as a sulphate conjugate and since this metabolite was only detected after a daily intake of 50-150 mg α-tocopherol, it was proposed to be an indicator of excess vitamin E (though this hypothesis has not been unequivocally proved in a clinical setting). They also proposed by that α-TL and α-TA were oxidation artefacts of the experimental procedure. This was shown to be plausible by the oxidative conversion of α-CEHC to α-TL in the presence of air and acid (Schonfeld 2006) and also by ultra-violet light (unpublished data Sharma et al.)

Fig.4.1. 2 Overview of vitamin E metabolism

Wechter et al. (1996) isolated a new endogenous natriuretic factor (a factor that controls the body's pool of extracellular fluid) which was characterized as unconjugated trimethyl-carboxyethyl-hydroxychroman (γ-CEHC) and was therefore presumed to be a metabolite of γ-tocopherol.

The urinary excretion of conjugated γ-CEHC was also reported by Traber *et al .,* (1998) and they proposed that CEHC metabolites were produced from excess vitamin E in the liver. The researchers also investigated the fate of differently deuterium labeled synthetic (all rac) and natural (RRR) α-tocopherol as α-CEHC. They found that α-CEHC derived from synthetic tocopherol was excreted in larger amounts than α-CEHC derived from natural tocopherol. They proposed that this resulted from the preferential loading of natural as opposed to synthetic α-tocopherol onto α-TTP and subsequently onto nascent VLDL in the liver thus reducing the likelihood of natural α-tocopherol being metabolised to α-CEHC.

Quantitative measurement of (human) urinary γ-CEHC using a deuterium labeled internal standard indicated that γ-tocopherol was inefficiently transferred onto α-TTP and VLDL and was consequently metabolised to γ-CEHC within a few days of ingestion (Swanson J.E. et al. 1999). Daily urinary γ-CEHC excretion was equivalent to about half the daily intake of γ-tocopherol. If other routes of excretion are taken into account this suggests that very little γ-tocopherol was retained in the body. In comparison only a small percentage of the daily intake of α-tocopherol was excreted as α-CEHC (Traber 1998). This explains why despite γ-tocopherol being the major tocopherol in western diets, α-tocopherol is the major tocopherol *in vivo.* This also emphasises the importance of α-TTP in vitamin E metabolism.

Vitamin E metabolites being lipophilic in nature would be expected to be excreted in the urine as conjugates, as detailed below in section 4.1.2.2. However, no definitive characterisation of the intact conjugates has been reported previously owing to a lack of suitable methods to analyse these polar metabolites directly.

4.1.2. 2 Excretion of vitamin E metabolites

In general, the metabolic breakdown of xenobiotics and lipophilic compounds (catabolism) occurs for two principal reasons. Firstly to reduce the activity or toxicity of compounds, which is especially important with regards to drugs, and secondly to increase the water solubility and thus the rate of excretion of waste compounds. The two most important routes of excretion of the waste products in the body are:

1. From the liver to the large intestine via bile
2. From the kidney via urine

Many biologically active molecules are lipophilic and remain un-ionised or only partially ionised at physiological pH. These molecules are often bound to plasma proteins and are not readily excreted in the bile or urine, or else are efficiently reabsorbed. Therefore in order to increase their excretion, hydrophobic compounds such as vitamin E undergo extensive metabolism in order to increase their water solubility. These metabolic reactions occur in a wide range of organs but the liver and the kidney are the primary sites of metabolism before excretion via bile or urine (Belanger 1985).

In general, the greater the lipophilicity of a substrate the more accessible it is to the sites of metabolism, particularly in the liver. Alkyl side-chains, as found in compounds such as vitamin E, are reduced in length by processes such as α, ω- and β-oxidation in the mitochondria or peroxisome. Reduction/oxidation reactions to introduce polar functionalities, including hydroxyl and carboxyl groups, mainly occur in the smooth endoplasmic reticulum (ER) and are catalysed by enzymes such as cytochrome P450/ cytochrome 450 reductase. These polar functionalities are then conjugated with groups such as sulphates or glucuronides to further increase their water solubility. Glucuronic acid is the most commonly attached to hydroxyl groups and these conjugation reactions are catalysed by a range of glucuronyl transferase enzymes, with broad specificity, in the ER (Meech and Mackenzie 1997). Sulphate conjugation (mainly of phenol groups), in contrast to glucuronidation, occurs in the cytosol. Once produced, these polar conjugates and either excreted in the bile directly from the liver or else are taken in the blood to the kidney where they are filtered in to the urine (Meech & Mackenzie 1997).

There is currently interest in measuring urinary metabolites of vitamin E, as it has been suggested that α-tocopheronolactone (α-TLHQ) with an oxidized chroman ring may be an indicator of oxidative stress. Although vitamin E metabolites are excreted in urine as the sulphate and glucuronide conjugates, virtually all studies to date have measured the free or enzymatically deconjugated metabolites by gas chromatography/ mass spectrometry. This involves a long complicated procedure with many preparatory steps and has a consequent risk of artefact formation. The aim of this study was to develop a method to measure the conjugated metabolites of vitamin E directly by liquid chromatography/ tandem mass spectrometry with minimum sample preparation to avoid artefact formation. The method was then validated for its precision, linearity and accuracy.

4.1. 3 Quantitation of vitamin E metabolites

In order to study vitamin E metabolism and its possible implications on oxidative stress it was necessary to measure vitamin E metabolites quantitatively in urine. A number of methods have been described in the literature; details are shown in Table 4.1.1.

Vitamin E metabolites are excreted in the urine as sulphate or glucuronide conjugates. The polar nature of these conjugates makes it difficult to analyse them directly using routine techniques such as high performance liquid chromatography (HPLC) or gas chromatography mass spectrometry (GC-MS). Therefore, in all the methods outlined, the metabolites were deconjugated either enzymatically or by acid hydrolysis prior to their analysis by HPLC or GC-MS.

These published analytical procedures can be divided into 4 main steps. These include 1) extraction of the metabolites from urine 2) deconjugation of these metabolites 3) extraction of the deconjugated metabolites and 4) analysis by HPLC or GC-MS. Each of these steps needs to be optimised to produce quantitative and reproducible data.

The complicated technical aspects and the problem of artefactual oxidation of the previous methodologies were the principal reasons to develop a new method to directly measure the conjugated vitamin E metabolites. Thus the method developed in the current study contains a simple sample preparation followed by a HPLC MS/MS run to minimise artefactual oxidation.

Table 4.1. 1 Past methodologies of measuring vitamin E metabolites

4. 2 Methodology

The development of the assay to quantitate vitamin E metabolites involved following the same general scheme as described for the development of the isoprostane assay (section 2.3) and this pattern is retained for all the method development assays in the thesis.

- Determination of molecular weight (using standards of metabolites)
- Determination of fragmentation ions (using standards of metabolites)
- Optimisation of HPLC parameters (using laboratory pooled urine)
- Tests of linearity, reproducibility and limit of detection of the developed assay (using laboratory pooled urine)

4.2. 1 Preliminary analysis

4.2.1. 1 Optimisation and determination of the vitamin E metabolites in scan mode

Fig. 4.2.1 shows the tandem mass spectral analysis of the vitamin E metabolites (100μmol/L) obtained in negative scan mode obtained over the mass range of 2-400 m/z [M-H$^-$] and 2-500 m/z [M-H$^-$] for the sulphate and glucuronide metabolites respectively. A mass was observed at m/z 356.9 [M-H$^-$] which corresponded to the theoretical mass of the sulphated vitamin E metabolites α-CEHC and α-TLHQ (Fig.4.2.1.A and B). The mass observed at m/z 453 [M-H$^-$] as illustrated in Fig.4.2.1.C and Fig.4.2.1.D corresponded to the theoretical masses of α-CEHC and α-TLHQ glucuronide. α-CEHC and α-TLHQ have the same molecular masses and are therefore isobaric.

Fig.4.2. 1 Parent ion scan of α –CEHC-sulphate (A) 356.9 m/z, α –TLHQ-sulphate (B) 356.9 m/z, α –CEHC-glucuronide (C) 453 m/z and α –TLHQ-glucuronide (D) 453.

4.2.1. 2 Optimisation of fragmentation of each vitamin E metabolite

Each compound (100µmol/L) was further characterised by fragmentation studies using the mass spectrometer operating in the product ion scan mode . The fragmentation analysis for the sulphate metabolites were observed in a scan mode of 2-400 m/z (Fig. 4.2.2.A&B). The fragmentation studies of α-CEHC sulphate led to the identification of a 'fingerprint' progeny ion at the intensity of 79.7 m/z. The characteristic daughter ion was identified as the sulphite ion (SO_3^-). Lesser intensity peaks were observed at 276.9 m/z, 241.3 m/z, 233.1 m/z and 163.1 m/z which were attributed to the fragmentation of the metabolite as shown in Fig.4.2.2.A. Fragmentation analysis of α-TLHQ sulphate revealed the presence of the same progeny ion and lesser intensity peaks similar to that of the α-CEHC sulphate (Fig.4.2.2.B), except that 243.1 mz (Fig. 4.2.2 A) was not observed.

The product ion analysis of the glucuronide metabolites was conducted in scan mode between 2-500 m/z. The highest intensity ion was observed at 112.8 m/z (Fig 4.2.3.A and B), which was formed by the loss of H_2O and CO_2 from the glucuronide moiety. Further fragmentation led to the formation of additional masses at 233.1 m/z and 276.6 m/z. The fragmentation studies of the α-CEHC and α-TLHQ glucuronides were very similar and thus the two metabolites could not be differentiated by mass spectral analysis alone.

Because of the isobaric nature of α-CEHC and α-TLHQ and their similar fragmentation patterns (except ion-243.1 m/z in the case of the sulphate metaboites), it was necessary to conduct separation studies using HPLC prior to mass spectrometry to differentiate the vitamin E metabolites according to their retention times on the column.

Fig.4.2. 2 Fragmentation patterns of α –CEHC-sulphate (A) and α –TLHQ-sulphate (B) with the progeny ion observed at 79.7 m/z and minor ions at 276.1 m/z, 233.1 m/z, 163.1 m/z in both the metabolites. In the case of α –CEHC-sulphate (A) ion 243.1 m/z can be used to differentiate the two sulphate metabolites

Fig.4.2. 3 Fragmentation patterns of α –CEHC-glucuronide (A) and α –TLHQ-glucuronide (B) with the Progeny ion observed at 112.80 m/z and minor ions at 276.6 m/z and 233.1m/z for both the metabolites. No differential ion was observed in the case of the glucuronide metabolites.

4.2.1. 3 Optimisation and determination of mass and fragmentation data of the internal standards

Unfortunately, no type 1 internal standards were available for CEHC and TLHQ glucuronides or sulphates and therefore androsterone glucuronide and lithocholic acid sulphate were used as internal standards in the current study. Both compounds contained a sterol structure and glucuronide or sulphate side chain and would therefore act as good type 2 internal standards. The response factor of the internal standards were found to be 1:1 i.e. the area under the curve for 5 nmol of the internal standards was found to be equal to the area under the curve for 5 nmol of the vitamin E metabolites. The tandem mass spectral analyses of the internal standards (100µmol/L) were obtained in a negative ion scan mode of 2-600 [M-H$^-$]. The masses observed at m/z 455.3 [M-H$^-$] and m/z 465.3 m/z [M-H$^-$] were that of the internal standards lithocholic acid sulphate (Fig.4.2.4.A) and androsterone glucuronide (Fig.4.2.4.B) respectively.

The internal standards were further characterised by fragmentation studies using the mass spectrometer operating in the product ion scan mode. The fragmentation analyses for the internal standards (100µmol/L) were observed in negative ion scan mode over the mass range of 2-550 m/z. The fragmentation study of lithocholic acid sulphate led to the identification of a daughter ion at the intensity of 97 m/z in Fig.4.2.4.C and was identified as the hydrogen sulphate ion (HSO_4^-). The product ion analysis of androsterone glucuronide led to the identification of a high intensity ion at 113.2 m/z (Fig.4.2.4.D), which was formed by the loss of H_2O and CO_2 from the glucuronide moiety.

Fig.4.2. 4 Scans 455.3 m/z (A) and 465.3 m/z (B) and fragmentation patterns 97 m/z (C)and 113.2 m/z (D) of the internal standards lithicholid acid sulphate (A and C) and androsterone glucuronide (B and D).

4.2.1. 4 LC-ESI-MS analysis of vitamin E metabolites

Because α-CEHC and α-TLHQ had identical masses and very similar fragmentation patterns, high performance liquid chromatography (HPLC) was used together with tandem mass spectrometry, to see if it was possible to distinguish each vitamin E metabolite by chromatographic retention time in addition to mass spectral analysis.

Two types of columns were tested for the separation of the vitamin E metabolites: C8 and HsF5. The C8 column was observed to give results that were 10- fold more sensitive than the HsF5 column and therefore was used in the method development (data not shown).

The chromatographic separations of the metabolites (100μmol/L) are shown in Fig. 4.2.5 and table 4.2.1 gives their retention times. α-CEHC sulphate eluted at 9.21 min and α-TLHQ sulphate at 8.46 min. The α-CEHC glucuronide eluted at 8.48 min. Two peaks were observed for the α-TLHQ glucuronide, a major peak eluted at 7.26 min and a minor one at 8.66 min. The minor peak was postulated to be an isomer with the structure as illustrated on chromatogram D Fig.3.2.5 and was first observed by Pope (2001). The major and minor peaks will be referred to as α-TLHQ glucuronide 1 and 2, respectively, in this study. The elution times meant that all the vitamin E metabolites could be separated in a single run. The internal standards glycolithocholic sulphate and androstane glucuronide eluted at 10.14 min and 10.82 min respectively. The mass spectrometric parameters have been presented in the materials and methods section 2.3 and are based on an in house methodology developed in our laboratory. Since the elution was satisfactory no changes were made to the method. The run was conducted initially in an organic solvent (ethanol) and then replicated in human urine (the chromatogram was observed to be identical).

	Retention time
α-CEHC sulphate	**9.21**
α-TLHQ sulphate	**8.46**
α-CEHC glucuronide	**8.48**
α-TLHQ glucuronide 1	**7.26**
α-TLHQ glucuronide 2	**8.66**
Androstane glucuronide (IS)	**10.14**
Lithocholic acid sulphate (IS)	**10.82**

Table 4.2. 1 Retention times of the vitamin E metabolites

Fig.4.2. 5 The LC-ESI-MS analysis of vitamin E metabolites where A- α-CEHC sulphate 9.21 min, B- α-TLHQ sulphate 8.46 min, C-α-CEHC glucuronide 8.48 min, D- α-TLHQ glucuronide 1 at 7.26 min and 8.66 min for 2, E-Androsterone glucuronide 10.14 min, and F-Glycolithicholic acid sulphate 10.82 min

4.2. 2 Validation of the method

To test the performance and robustness of the method, the assay was evaluated for its linearity, recovery and reproducibility.

4.2.2. 1 Linearity

In order to assess if the method gave a linear response, increasing amounts of known amounts of each of the vitamin E metabolites were added to a constant amount of internal standard and ethanol prior to analysis. Calibration curves with seven points of reference were plotted (0, 0.5, 1, 1.5, 2.5, 5 and 10 nmol equivalent to 0, 3.3, 6.6, 9.9, 16.6, 33.2 and 66.4 μmoles/l). The ratio of the areas for the vitamin E metabolites over the corresponding internal standard was calculated for each metabolite. The data obtained was analysed by linear regression where the best fit for the linear relationship was calculated using GraphPad Prism 4 software.

The correlation coefficients (r^2) were > 0.99 for all the metabolites (see Fig. 4.2.6 A-D). In a repeat experiment the linearity of the method ceased at 99.6 μmol/l.

The limit of detection and limit of quantitation (addressed in the following section 4.2.2.4 and have been tabulated in table 4.2.5) is on the lower end of the calibration curve but all the control samples analysed in the diabetic study were >2 folds above the limit of detection and quantitation.

Fig.4.2. 6 Calibration curves for (A) α-CEHC sulphate r2=0.9967 (B) α-TLHQ sulphate r2=0.9963(C) α-CEHC glucuronide r2=0.999and (D) α-TLHQ glucuronide r2=0.9987

4.2.2. 2 Recovery

Recovery studies involved analysis before and after known amounts of the metabolites (0.2, 0.5 and 2.5 nmol) were added to 150 µl laboratory control urine containing a constant amount of internal standard. The ratio of the areas for the vitamin E metabolites over the corresponding internal standard was calculated for each metabolite. From the amount of the metabolite present, the percentage recovery of the added analyte was then calculated using the following formula:

% Recovery = [(b-a)/c] X 100

where, a=amount found before addition, b=amount found after addition and c=actual amount of standard metabolite added

The mean percentage recoveries were determined using the three amounts of the metabolites added with triplicate injections of each. The recoveries were consistently greater than 90% and are summarised in the table below (Table 4.2.2).

Amount of Metabolite added (nmoles)	α-CEHC sulphate Recovery (%)	α-TLHQ sulphate Recovery (%)	α-CEHC glucuronide Recovery (%)	α-TLHQ glucuronide Recovery (%)
0.2	98	96	92	91
0.5	99	90	98	95
2.5	97	92	100	98

Table 4.2. 2 Percentage recoveries of vitamin E metabolites

4.2.2. 3 Reproducibility

To assess the reproducibility of the method, low, normal and high amounts of the α-CEHC and α-TLHQ glucuronide/sulphate metabolites (0.1, 0.5 and 2.5 nmol) were added to laboratory control urine. The intra-assay precision of the method was evaluated using twenty replicate injections of the three concentrations of the metabolites in the same run on a single day. Inter-assay reproducibility was assessed by running a single injection of the three concentrations on 20 separate occasions over a period of 60 days. The area under the peak for each metabolite and internal standard was calculated from the chromatogram using Mass Lynx software. The ratio of the areas for the vitamin E metabolites over the corresponding internal standard was calculated for each metabolite.

The intra- and inter-assay coefficients of variation for the metabolites are shown in Tables 3.2.3 and 3.2.4. The intra-assay coefficient of variation ranged from 0.60 to 3.73 % (Table 4.2.3) and the inter-assay coefficient of variation ranged from 1.18 to 4.32 % (Table 4.2.4).

	Concentration of metabolite 0.6 µmol/l (0.1 nmol) N=20 C.V.%	Concentration of metabolite 3.3 µmol/l (0.5 nmol) N=20 C.V.%	Concentration of metabolite 16.6 µmol/l (2.5 nmol) N=20 C.V.%
α-CEHC Sulphate	1.95	2.20	0.65
α-TLHQ Sulphate	2.63	0.60	1.42
α-CEHC Glucuronide	3.73	1.92	1.33
α-TLHQ Glucuronide	3.70	1.57	0.88

Table 4.2. 3 The intra-assay coefficients of variation for the vitamin E metabolites

	Concentration of metabolite 0.6 µmol/l (0.1 nmol) N=20 C.V.%	Concentration of metabolite 3.3 µmol/l (0.5 nmol) N=20 C.V.%	Concentration of metabolite 16.6 µmol/l (2.5 nmol) N=20 C.V.%
α-CEHC Sulphate	2.11	3.44	1.40
α-TLHQ Sulphate	3.06	1.18	3.60
α-CEHC Glucuronide	3.86	2.97	1.99
α-TLHQ Glucuronide	4.32	2.04	2.84

Table 4.2. 4 The inter-assay coefficient of variation of vitamin E metabolites

4.2.2. 4 Limit of detection

The limit of detection is a measure of the sensitivity of a method. There is both a functional and biological limit of detection. The functional limit indicates the cut off point for detection in a non-biological sample such as water or organic solvent, whereas the biological limit of detection indicates the cut off point for detection in a biological matrix such as urine. The limit of detection of the method was evaluated by diluting a known amount of metabolite sequentially and determining the amount at which detection was not possible. The lowest amount of the metabolites in urine and ethanol giving a signal to noise ratio of 5:1 were taken to be the biological and functional limits of detection in this study.

The functional and biological limits of detection for the four metabolites are tabulated below (Table.4.2.5). α-TLHQ glucuronide had the lowest limit of detection, with functional and biological limits of detection of 0.06 and 0.30 μmol/l respectively.

Metabolite	Functional Limit (µmol/l)	Biological Limit (µmol/l)
α-CEHC sulphate	0.10	0.50
α-TLHQ sulphate	0.20	0.60
α-CEHC glucuronide	1.30	5.10
α-TLHQ glucuronide	0.06	0.30

Table 4.2. 5 Functional and biological limits of detection for the vitamin E metabolites

4. 3 Discussion

Over the past 50 years a number of metabolites of vitamin E have been identified. Recently, however there has been a renewed interest in measuring urinary metabolites of vitamin E due to their proposed use as a biomarker of oxidative stress (Schultz, Cato, Corkeron, & Bryden 1995). Published studies to date on the metabolites have measured the free unconjugated compounds using GC/MS (Pope 2001) and HPLC-ECD (Lodge 2000). These methodologies require long deconjugation and extraction procedures with a high risk of artefact formation.

Schultz (1995) showed that α-CEHC could be almost totally converted to α-TLHQ after bubbling oxygen through a solution of 70 μM α-CEHC in 0.1 M HCL for 24 hours at room temperature. This was regarded as evidence that the presence of oxygen could cause artefactual oxidation and the possibility that some or all the lactone reported by others could have resulted from the artefactual oxidation of α-CEHC during the assay procedure. Pope (2000) observed poor reproducibility for α-TLHQ in their GC/MS assay, which they attributed to some artefactual conversion of α-CEHC to TLHQ. They found that despite trying to keep artefactual oxidation to a minimum by controlling parameters such as exposure to air, heat, acidity and reducing the number of solvents, 5-10 % of added d_9- α-CEHC was converted to d_9- α-TLHQ.

The aim of the current study was to establish, validate and use a rapid LC-MS/MS method to investigate the intact conjugates of vitamin E metabolites. The direct measurement of the conjugates has the great advantage that the conjugated group (glucuronide or sulphate) greatly reduces the risk of the artefactual formation of α-TLHQ from α-CEHC (Pope 2001).

The current study concentrated on metabolites of α-tocopherol as this is the most biologically active form of vitamin E.

Preliminary studies (straight scans and fragmentation spectra) were conducted to confirm the identity of the metabolites of interest. The molecular masses obtained were consistent with the theoretical masses of the metabolites and internal standards (Fig.4.2.1.A.B.C and D). However the α-CEHC and α-TLHQ metabolites had identical molecular weights and similar fragmentation patterns, except in the case of α-CEHC sulphate where a previously unreported peak at 241.3 m/z was observed (Fig.4.2.2.A). In theory this progeny ion at 241.3 m/z could have been used to distinguish the sulphate metabolites, but the glucuronide metabolites could not be differentiated due to their identical fragmentation patterns (Fig.4.2.3). It was therefore decided to separate the metabolites prior to their identification by mass spectrometry. High performance liquid chromatography (HPLC) was used prior to tandem mass spectrometry, which resulted in the successful separation of all of the vitamin E metabolites and the internal standards (Fig. 4.2.5). An additional unreported minor peak was also obtained for α-TLHQ glucuronide at 8.66 min (Fig.4.2.5.E), which was postulated to be an isomer. This isomer was also postulated to be present during the synthesis of α-TLHQ by Pope (2001).

An advantage of the new method was the speed of analysis. The established methodology of Pope (2000) included a long (eight hours) sample preparatory procedure which included extraction, deconjugation, desalting and derivatisation steps (Fig. 4.3.1) prior to each GC/MS run which took 30 min. The new method on the other hand measured vitamin E

metabolites in neat urine with virtually no sample preparation and a run time of 20 min (Fig.4.3.1).

The use of type II internal standards (androsterone glucuronide and lithocholic acid sulphate) in the current study (due to time constraints dueterated internal standards could not be synthesised) was substantiated by extensive validation procedures which were found to be satisfactory. The current method was found to be linear over the necessary working range, to be reproducible and having recovery of greater than 90%. The inter- and intra-assay coefficients of variation ranged from 0.60 to 4.32 % (Table. 4.2.3 and 4.2.4) in the current assay compared to 21.9 and 64.7 % (intra and inter) in the method using GC/MS developed by Pope and his co-workers (Pope, Clayton, & Muller 2000). These figures are well within the criteria for an LC-MS/MS method using type II internal standards. The limit of detection were observed to be 0.30-5.10 μmol/l in biological fluids (urine) which was well below the concentrations that control values could be accurately be detected and quantitated.

Fig.4.3. 1 Comparison between the existing GC/MS methodology and the new method (IS= Internal Standard)

Chapter 5

Amino Acids Associated with Nitric oxide ($NO^{\bullet}$)

TABLE OF CONTETS

5 Amino acids associated with Nitric oxide (NO•)

Nitric oxide ($NO^{\bullet}$) in its role as reactive nitrogen species (RNS) has been implicated as a source of oxidative stress in human physiology (section 1.1.4). It has particularly been studied in vascular integrity (Moncada and Higgs 2006). In the current study we have aimed to develop a method to quantitate the amino acids that are involved in the homeostasis of $NO^{\bullet}$ metabolism in the hope that they would indicate the oxidative stress status of the individual.

The following section will elaborate on the amino acids involved/ associated closely with $NO^{\bullet}$ in maintaining vascular equilibrium and the implications for oxidative stress.

5. 1 Background information

The endothelium plays an important role in the maintenance of vascular homeostasis, in part through the production of $NO^{\bullet}$ (Moncada & Higgs 2006). Vascular diseases, including atherosclerosis, hypertension and the complications of diabetes are characterised by impaired endothelium-derived $NO^{\bullet}$ bioactivity, and such impairment is thought to contribute to clinical events associated with vascular disease including myocardial infarction and stroke (Price 2008). Oxidative stress in the vascular wall is a prominent feature of vascular disease (Diaz 1997) and evidence indicates that impaired endothelium-derived $NO^{\bullet}$ bioactivity is due, in part, to increased oxidative stress (Cai and Harrison 2000).

$NO^{\bullet}$ is a gas, which is difficult to measure directly. In this study we aimed to develop a method to investigate $NO^{\bullet}$ metabolism by measuring a number of amino acids involved in

its metabolism and its synthesis by nitric oxide synthase (NOS) e.g L-arginine (precursor), L-citrulline (product), L-ADMA (inhibitor), L-homocysteine (implicated in $NO^{\bullet}$ bioavailability).

5.1. 1 History of nitric oxide

Nitric oxide is an inorganic free radical (formula N=O, abbreviated to $NO^{\bullet}$) and is considered to be one of the smallest and simplest biologically active molecules in nature. It was discovered in 1772 by Joseph Priestly as a clear, colorless gas and was thought to be merely an atmospheric pollutant (Moncada & Higgs 2006). However, in 1979 (Gruetter 1979) delivered a gaseous mixture of $NO^{\bullet}$ into an organ bath containing isolated pre-contracted strips of bovine coronary artery which resulted in the relaxing of the artery. This which led to the discovery that $NO^{\bullet}$ had vascular smooth muscle relaxant properties. (Furchgott and Zawadzki 1980), discovered that endothelial cells in vessels with an intact endothelium produce an endothelium-derived relaxing factor (EDRF) in response to stimulation by acetylcholine and in 1987, Palmer *et al.,* (1987) *et al.,* and Ignarro *et al.,* (1987) independently proved that EDRF was $NO^{\bullet}$. A year later, Moncada's group also demonstrated that $NO^{\bullet}$ was synthesized from the amino acid L-arginine (Palmer et al. 1988).

$NO^{\bullet}$ has not only been implicated in a range of conditions ranging from hypertension to septic shock and dementia (Moncada and Higgs 1993) but also in many areas of biomedicine, including cardiovascular function, neurotransmission, pain, wound healing and tissue repair, cancer, immune function, infection, respiratory function and eye disease (Yetik-Anacak and Catravas 2006). Today, it is hard to find a disease which is not associated with altered $NO^{\bullet}$ homeostasis. In fact because of its numerous endothelial

functions, the term “endothelial dysfunction”, originally coined in 1983 (Catravas 1983) has now become synonymous with reduced biological activity of NO• (Yetik-Anacak & Catravas 2006).

5.1. 2 Synthesis of NO•Synthesis of NO•

Many reports supported the idea that mammalian cells can synthesise nitric oxide (Ignarro 1987;Palmer 1987). In 1989 it was reported that endothelial cells contain a cytosolic enzyme which is either directly or indirectly regulated by calcium, which converts L-arginine to citrulline and a compound which behaves similarly to EDRF (Mayer 1989). It is now know that NO• is synthesised enzymatically from L-arginine by one of three nitric oxide synthases (NOS) with the production of L-citrulline as a by-product. The first isolation of NOS1 was reported from the brain (Bredt and Snyder 1990). This enzyme (nNOS) is found constitutively in a variety of cells, including endothelial cells and neurons (Griffith and Stuehr 1995). The second NOS, is inducible NOS (iNOS) or NOS2, which was isolated from macrophages by (Hevel 1991). Inducible NOS is expressed in numerous cells after several hours of exposure to cytokines and/or microbial products (Stuehr 1997). The last synthase to be identified was endothelial NOS (eNOS) or NOS3, which was isolated from bovine aortic endothelial cells (Palmer and Moncada 1989).

The activity of NOS is controlled by intracellular concentrations of calcium and calmodulin, NOS concentration is directly proportional to the production of NO• (Knowles and Moncada 1994). The main physiological stimulation contributing to the increase in NO• production in the endothelium is through the action of sheer stress on the blood vessels (Knowles & Moncada 1994).

5.1. 3 NO•, oxidative stress and endothelial dysfunction

Endothelial dysfunction refers to a loss of normal homeostatic functions (e.g., vasodilatation, platelet inhibition) often occurring early in the course of vascular diseases such as atherosclerosis, hypertension and complications of diabetes (Puddu 2005). One important manifestation of endothelial dysfunction is a reduction in endothelium-derived $NO^{\bullet}$ bioactivity which is an independent predictor of cardiovascular events in patients with coronary artery disease (Gokce 2002). In theory, such a decrease in $NO^{\bullet}$ bioactivity could result from reduced $NO^{\bullet}$ production or inactivation of $NO^{\bullet}$. There is considerable evidence for both of these situations in animal and human models of vascular disease.

Oxidative stress is a characteristic of many vascular diseases, including atherosclerosis, hypertension and complications of diabetes (Cai & Harrison 2000). Various stimuli have been proposed to promote vascular oxidative stress, including hypercholesterolaemia, hyperglycaemia, shear stress, angiotensin II, and proinflammatory cytokines (Cai & Harrison 2000;Griendling 2000).

Diseased blood vessels from hypercholesterolaemic rabbits produce substantial amounts of nitrogen oxides ($NO^{\bullet}$ oxidation products) despite the impairment in $NO^{\bullet}$-dependent vascular relaxation (Minor 1990). This finding suggested that $NO^{\bullet}$ production in vascular disease was not decreased, but that $NO^{\bullet}$ was inactivated before reaching its cellular target. Subsequent studies have established that oxidative inactivation of $NO^{\bullet}$ frequently involves the superoxide anion radical. For example, hypertension, hypercholesterolaemia and atherosclerosis are associated with an increase in the steady-state flux of superoxide in the vascular wall (Griendling 2000). Superoxide is then able to react with $NO^{\bullet}$ to produce the potent oxidant peroxynitrite (Kissner 1997). Peroxynitrite formation is kinetically favored

over other $NO^{\bullet}$ reactions and is likely to occur whenever both $NO^{\bullet}$ and superoxide are present (Radi 1996).

Various studies have provided evidence that direct inactivation of $NO^{\bullet}$ by superoxide is a mechanism for impaired $NO^{\bullet}$ bioactivity. For example, the addition of superoxide to vascular bioassay systems impairs $NO^{\bullet}$-dependent vessel relaxation (Gryglewski 1986). Conversely exogenous SOD improves the vascular relaxation response to endothelial-derived $NO^{\bullet}$ under both basal and acetylcholine stimulated conditions (Gryglewski 1986). It has also been shown that, blood vessels with decreased Cu/Zn-SOD activity exhibit enhanced vascular superoxide production and impaired $NO^{\bullet}$-mediated arterial relaxation (Lynch 1997). Finally, it has been shown that acute intra-arterial infusion of ascorbate at concentrations that effectively prevent superoxide interaction with $NO^{\bullet}$ (Jackson 1998) improves endothelium-dependent relaxation in patients with cardiovascular disease (Heitzer 2001b). In fact, patients that demonstrated the greatest improvement in $NO^{\bullet}$ bioactivity in response to ascorbate also exhibited the greatest reduction of cardiovascular events, which is consistent with the notion that oxidative stress induced endothelial dysfunction is clinically important (Heitzer 2000).

5.1. 4 Reduced bioavailability of $NO^{\bullet}$

Asymmetric dimethylarginine (ADMA) and homocysteine both affect $NO^{\bullet}$ bioavailability and will now be discussed.

5.1.4. 1 Assymetric dimethylarginine (ADMA) as an endogenous inhibitor of NOS

Asymmetric dimethylarginine (ADMA) is an endogenous molecule which can be detected in human blood and urine. It shows structural similarity to the amino acid L-arginine (Fig.5.1.1). Vallance *et al.,* (1992) were the first to describe substances that show similar structure to L-arginine but differ from it in that they contain one or two methyl groups and act as inhibitors of $NO^{\bullet}$ synthesis. These substances are found in human plasma and urine and Vallance *et al.,* (1992) also reported that asymmetric dimethylarginine was the one member of this group of substances that is present *in vivo* in sufficiently high concentrations to inhibit $NO^{\bullet}$ synthesis. They showed that after its isolation from human urine, ADMA induced a significant and concentration-dependent inhibition of $NO^{\bullet}$ production by cultured human macrophages (Vallance 1992). By contrast to ADMA, its structural isomer symmetric dimethylarginine (SDMA) (Fig.5.1.1) had no effect on $NO^{\bullet}$ production.

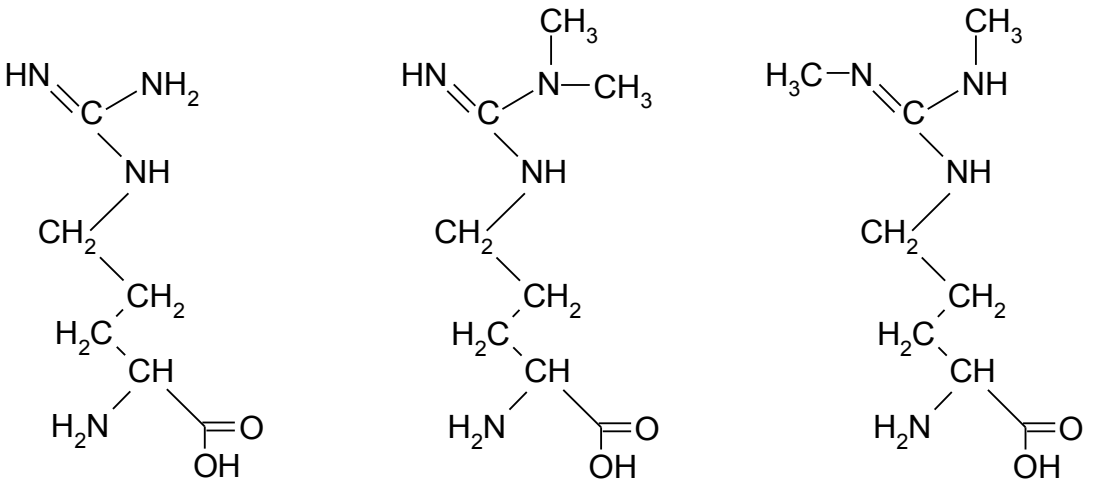


Fig. 5.1. 1 Chemical structures of L-arginine, asymmetric dimethylarginine (ADMA), and symmetric dimethylarginine (SDMA)

Other experimental studies in various laboratories have since shown that ADMA inhibits $NO^{\bullet}$ production *in vitro* within a concentration range that can be measured in plasma of patients with cardiovascular or metabolic diseases (Faraci 1995;Kurose 1995). Moreover, experiments with isolated, purified, cloned isoforms of NOS *in vitro* (Tsikas et al. 2000) and clinical studies in patients with varying plasma concentrations of ADMA have also demonstrated that ADMA inhibits $NO^{\bullet}$ production in a concentration dependent manner (Boger 1997;Boger 1998;Kielstein 2001).

Elevated ADMA concentrations are thus associated with a reduced systemic $NO^{\bullet}$ production. The latter can be assessed *in vivo* as a reduced urinary excretion of the stable $NO^{\bullet}$ metabolites, nitrite and nitrate and impaired endothelium-dependent vasodilation (Bode-Boger 1998;Boger, BodeBoger 1997). Taken together, these studies strongly suggest that ADMA could influence $NO^{\bullet}$ metabolism which could then precipitate endothelial dysfunction in humans.

5.1.4. 2 Homocysteine nitric oxide and oxidative stress

Homocysteine is a sulphur-containing amino acid which is not found in our daily diet. It is biosynthesised from methionine via a multi-step process including the formation of S-adenosyl methionine (SAM) and adenosyl homocysteine to finally yield homocysteine (Stryer 1981). It has been suggested that increased concentrations of plasma homocysteine (hyperhomocysteinaemia) may play a role in the pathogenesis of various diseases, especially at the cardiovascular level (Welch and Loscalzo 1998). It has also been suggested that hyperhomocysteinaemia may be an independent risk factor for cardiovascular disease (Perna 2003). Following the oxidation of homocysteine with nitric oxide, S-nitroso-homocysteine is produced, and it has therefore been suggested that increased homocysteine levels can reduce $NO^{\bullet}$ bioavailability (Perna 2003).

Homocysteine has been reported to reduce $NO^{\bullet}$ bioavailability by a number of additional mechanisms. Firstly, it has been reported that homocysteine can function as an antagonist of NOS as it triggers the accumulation of ADMA, but the exact mechanism is not clear (De Groote 1996). Secondly, electrochemical detection of $NO^{\bullet}$ released from endothelial cells exposed to homocysteine, showed that homocysteine produced an indirect suppression of endothelial nitric oxide synthase (eNOS) activity, and therefore $NO^{\bullet}$ production, without affecting NOS expression (Zhang 2000). Thirdly, evidence exists that homocysteine affects glutathione peroxidase activity, thus providing the environment for the propagation of ROS (Upchurch 1997). Endothelial glutathione peroxidase catalyses the reduction of hydrogen and lipid peroxides to the corresponding alcohol and also prevents the oxidative inactivation of $NO^{\bullet}$, however the exact mechanism of homocyteine's action on glutathione peroxidase is unknown. Finally, homocysteine has also been shown to reduce mRNA levels of glutathione peroxidase, indicating that the expression of the enzyme is inhibited or down

regulated. These findings were reported in mice and have yet to be confirmed in humans (Weiss 2009).

5.1. 5 Quantitation of amino acids associated with NO•

Analytical assays for monitoring NO• are complicated by NO•'s unique chemical and physical properties (Wink and Mitchell 1998) including its reactivity (Moller 2007), rapid diffusion (Williams 1996), and short half-life (Thomas 2006). The most commonly used technique that can detect free radicals directly is the spectroscopic technique (Barker and 1998;Toda 2007;Zhou and Arnold 1996) of electron spin resonance (ESR) otherwise also called as electron paramagnetic resonance (EPR) (Kleschyov 2000). For example, the reactive radical NO• is allowed to react with a trap (haemoglobin) to produce a long-lived radical (nitrosylhaemoglobin), which accumulates to a level that does permit detection by ESR. These methods for the detection of NO• benefit from simple, affordable instrumentation and conceptually straightforward analytical procedures, but their major drawback is that the techniques were not found to be accurate and have been reported to over estimate the oxidation of NO• (Zhang 1996). As such, even with these drawbacks the methods are widely used for detecting NO•, especially in biological systems *in vitro* (Hetrick & Schoenfisch 2009). Electrochemical sensors/ NO• electrodes are likely the most commonly employed analytical method for monitoring NO• in physiology/biology due to their specific inherent advantages, which include real-time monitoring (i.e. on site), amenability to miniaturization i.e. can be used in cells and tissues, and the ability to enhance selectivity and sensitivity via electrode modification i.e using porphyrin on the anode (Ciszewski & Milczarek 2003). Although electrochemical sensors are both sensitive and fast responding, questions with respect to accuracy remain unanswered as it has been

reported that the assays were unable to discriminate between NO• and carbon monoxide CO (Lee & Kim 2007) and the technique is known to be widely used in *in vitro* animal studies and has not known to be used to assess NO• metabolism *in vivo* involving human subjects in a clinical setting (Hetrick & Schoenfisch 2009).

NO• metabolism could also be monitored indirectly based on the spectrophotometric analysis by the Griess reaction (Green 1982) - which consists of nitrite's reaction under acidic conditions with aromatic amines generating a purple azo dye. This method has some limitations regarding detection limit and sensitivity, thus resulting unsuitable for nitrite detection in plasma (Giustarini 2004).

Another approach of understanding NO• metabolism could be by measuring amino acids associated with NO• metabolism; L-arginine (precursor of NO•), L- citrulline (product of NO• formation), L-ADMA (inhibitor of NOS) and L-homocysteine (decreases bioavailability of NO• by inhibiting NOS).

The most widely used analytical method for measuring amino acids in a clinical setting has been ion-exchange chromatography. It is a process that allows the separation of ions and polar molecules based on their charge. The procedure is fully automated, specific and accurate measuring 43 amino acids in biological fluids including plasma- 100 µl (Dickinson 1965), urine- 100 µl (Yokoyama 1991) and cerebrospinal fluid- 750 µl (Dickinson and Hamilton 1966) and follows the principle of the method established by (Hamilton 1963). The major drawback of this method is it is labour intensive requiring extensive sample preparation-including an extraction step, requiring upto 24 hours analytical time. Current methods based on ion exclusion chromatography Alpert (2007)

require elaborate sample preparation -including an extraction step, and requiring up to 24 hours analytical time.

Mass spectrometric approaches for quantitation of the amino acids associated with $NO^{\bullet}$ metabolism are limited to individual amino acids (detailed below) and no published study to date has quantified all the four amino acids associated with $NO^{\bullet}$ metabolism together.

Gas chromatographic-mass spectrometric methodologies for the quantitation of the amino acids are unsuitable unless all polar centres of the molecules are derivatised. A procedure for the quantitative determination of arginine and ADMA in human plasma and urine utilising a derivatisation protocol for subsequent GC–MS/MS analysis have been reported by (Tsikas 2003a). The sample underwent a clean-up step (ultrafiltration) and subsequent concentration by drying. Derivatisation of the amino acids required firstly esterification with acidic methanol and then the addition of pentafluoropropionic anhydride (PFPA) to form pentafluoropropionic acid amides. After derivatisation, the samples were suitable for GC separation. The major drawback of this method was the labour intensive sample preparation procedure which took approximately 2 hours prior to MS/MS analysis. A similar GC-MS/MS method was reported by Albsmeier and co-workers (2004), where sample clean-up consisted of protein precipitation with acetone followed by solid phase extraction prior to analysis. There were no significant advantages to the method of Tsikas *et al.,* (2003) and again the labour intensive sample preparation was the major drawback of the study.

Vishwanathan et al., (2000) reported the first LC–MS/MS method for L-arginine and L-ADMA. After protein precipitation with acetonitrile and solvent evaporation, the

underivatised amino acids were separated on a straight phase silica column. The chromatographic run time was about 15 min. However, the noise level of the chromatograms was high and the amino acids of interest were not always completely separated from endogenous interfering compounds, leading to doubts about specificity. *Quantitation* was carried out by ESI-MS as well as by ESI–MS/MS, although the MS/MS mode did not appear to substantially improves the selectivity.

Martens-Lobenhoffer and Bode-Böger (2003) subsequently developed an assay applicable to human plasma and urine, which derivatised the analytes with ortho-phthalaldehyde (OPA) and 2-mercaptoethanol prior to HPLC separation, following similar approaches developed for fluorescence detection by (Chen 1997). The OPA derivatives of arginine, ADMA and SDMA were analyzed by ESI–MS in the studies of (Martens-Lobenhoffer & Bode-Böger 2003). Because of the superior selectivity of the mass spectrometric detection, the laborious sample clean-up could be avoided, and sample preparation was reduced to protein precipitation for plasma and dilution for urine samples. In addition to L-Arginine and L-ADMA, the amino acid L-citrulline was quantified in the same run, using a C18 column with a run-time of 27 min.

Markowski *et al.,* (2007), reported a reversed phase-HPLC method for the quantitation of L-arginine, L-citrulline and L-ADMA in human urine. The sample preparation included a solid phase extraction procedure prior to drying and derivatisation using OPA prior to LC analysis on a C-18 column with a run-time of 60 min.

In the case of homocysteine, reported analytical studies have generally concentrated on measuring plasma concentrations of the amino acid. (Magera 1999), reported the first LC-

MS/MS study measuring urinary homocysteine, with a simple sample clean-up of protein precipitation by acetonitrile (0.05% containing formic acid). HPLC separation was achieved on a C-18 column with a run time of 2.5 min.

Rafii *et al.,* (2007) improved the method developed by Magera *et al.,* (1999) by including a derivatisation step using 7-fluorobenzofurazan-4-sulfonic acid ammonium salt which improved the chromatography.

We worked to develop an assay to measure all the amino acids associated with $NO^{\bullet}$ metabolism in a single run in order to gain a better understanding of the *in vivo* alteration of the $NO^{\bullet}$ metabolism in disease conditions such as diabetes. No study to date has looked into the metabolism of $NO^{\bullet}$ in its entirety i.e. its precursor (arginine) its inhibitors (ADMA, homocysteine) and the co-product of nitric oxide synthase (citrulline). It was with this aim that the current assay was developed to measure urinary concentrations of L-arginine, L-citrulline, L-ADMA and L-homocysteine.

5. 2 Method development

The development of the assay to quantitate the amino acids associated with $NO^{\bullet}$ metabolism involved the following steps

- Determination of molecular weight
- Determination of fragmentation ions
- Optimisation of HPLC parameters
- Tests of linearity, reproducibility and limit of detection of the developed assay

5.2. 1 Principles of 9-fluorenylmethyl chloroformate (FMOC) derivatisation

The quantitation of amino acids has traditionally been based on separation by ion-exchange chromatography, followed by post-column derivatisation (most commonly ninhydrin) for detection (Hearn & Grego 1984). In recent years rapid improvements in HPLC equipment and high-efficiency chromatographic columns have led to a significant change in approach to amino acid analysis. Thus many methods are frequently based on pre-column fluorescence derivatisation are simple, sensitive and without time-consuming manipulation (Hearn & Grego 1984).

One of the most promising pre-column derivatizing reagents is 9-fluorenylmethylchloroformate (FMOC) which has been used successfully for HPLC separations of amino acids by (Einarsson et al. 1983). The reagent reacts rapidly with the amine group of amino acids to form a highly fluorescent and stable carbamate derivative. A general reaction of FMOC with a primary amine is shown in Fig. 5.2.1

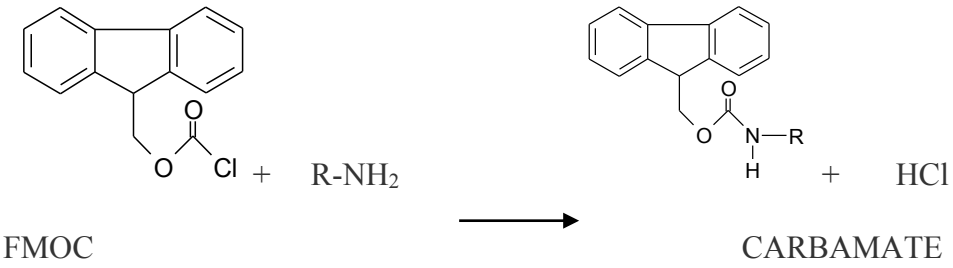


Fig.5.2. 1 Reaction of FMOC with an amino acid

The advantage of FMOC over other derivatising agents is its ability to react rapidly and quantitatively, under mild conditions, with amino acids forming in general a single stable derivative per amino acid. The derivatives can be detected with high sensitivity at low picomole levels (without being disturbed by sample matrix components such as salts) and the reagent does not interfere with the chromatographic separation of the amino acids (Einarsson 1983).

The major disadvantage of FMOC is its reactivity with water as the fluorescent alcohol - FMOC-OH, elutes in the middle of the chromatogram. At high concentrations, FMOC-OH overlaps with other amino acids in the chromatogram complicating their quantitation (Einarsson and Josefsson 1987). Other specific disadvantages of FMOC are the poor fluorescent properties of the cysteine and homocysteine derivative as well as the di-FMOC derivative of histidine.

The low fluorescent problem with cysteine and homocysteine was effectively solved by (Bank 1996) by derivatising the amino acids with FMOC in 0.1M borate buffer at pH 11.4 (as opposed to pH 7.7 used by Einarsson 1983) for a period of 40 min. This protocol was used in the current study. The method enabled the mass spectrometer to be used in negative ion mode, thus improving the chromatography of the amino acids (sharper/ narrower peaks) and increasing the signal to noise ratio thus enhancing the sensitivity of the assay.

5.2. 2 Preliminary Analysis

A method was initially developed to quantitate the amino acids associated with $NO^{\bullet}$ metabolism (L-arginine, L-citrulline, L-ADMA and L-homocysteine) in a single run, using an FMOC derivatising protocol. Subsequently, seven additional amino acids were also included in the analysis. They were;

L-glycine: non-polar uncharged side chain

L-serine: polar uncharged side chain

L-phenylalanine: aromatic hydrophobic side chain

L-glutamic acid: polar negatively charged side chain

L-taurine, L-cysteine and L-cystine: sulphur containing amino acids

The derivatisation and clean up procedures are presented sections 2.4.2 for the standards and 2.4.3 for urinary samples.

5.2.2. 1 Determination of the FMOC amino acids in scan mode

The tandem mass spectral analyses of the FMOC derivatives of the amino acids (100µmol/L) were obtained in scan mode over the mass range of 2-1000 [M-H^-] and are illustrated in Figs.5.2.2-5.2.4. The observed masses [M-H^-] corresponded to the theoretical mass of the FMOC amino acid except in the case of L-cysteine where the amino acid took on 2 FMOC moieties (Fig.5.2.3.C). The observed and theoretical mass of each FMOC amino acid is tabulated in Table 5.2.1.

Fig.5.2. 2 Parent scan of FMOC L -arginine 394.7 m/z (A), -citrulline: 396.1 m/z (B), -ADMA: 422.8 m/z (C) and -homocysteine: 356.1 m/z (D).

Fig.5.2. 3 Parent scan of FMOC L –taurine: 346.0 (A), -serine:326.1 (B), -cystine:683.5 (C) and –cysteine:342.0 (D).

Fig.5.2. 4 Parent scan of FMOC L –phenylalanine:386.6 m/z (A), -glutamic acid:368.4 m/z(B) and –glycine:296.5 m/z (C).

Figure	Amino acid	Observed mass [M-H$^-$]	Theoretical mass [M-H$^-$]
5.2.2.A	FMOC L-arginine	394.7	395
5.2.2.B	FMOC L-citrulline	396.1	396
5.2.2.C	FMOC L-ADMA	422.8	423
5.2.2.D	FMOC L-homocysteine	356.1	356
5.2.3.A	FMOC L-taurine	346.0	346
5.2.3.B	FMOC L-serine	326.1	326
5.2.3.C	2 (FMOC) L-cystine	683.5	684
5.2.3.D	FMOC L-cysteine	342.0	342
5.2.4.A	FMOC L-phenylalanine	386.6	386
5.2.4.B	FMOC L-glutamic acid	368.4	369
5.2.4.C	FMOC L-glycine	296.5	296

Table 5.2. 1 Observed and theoretical masses of the FMOC amino acids

5.2.2. 2 Optimisation of fragmentation of the FMOC amino acids

Each compound was further characterised by fragmentation studies using the mass spectrometer operating in the product ion scan mode. The fragmentation patterns of the amino acids (100μmol/L) were observed to give characteristic "fingerprint" progeny ions which corresponded to the molecular masses of the underivatised amino acids. The fragmentation pattern of each amino acid is shown in Figs.5.2.5-5.2.7 and the major progeny ions observed are tabulated in Table 5.2.2

Fig.5.2. 5 Product ion spectra of FMOC L –arginine 172.9 m/z, -citrulline: 173.9 m/z, -ADMA: 201.0 m/z, -homocysteine 159.8 m/z

Fig.5.2. 6 Product ion spectra of FMOC -L-taurine: 123.7 m/z (A), -serine m/z (B), -cystine m/z (C), -cysteine: 145.7 m/z (D)

Fig.5.2. 7 Product ion spectra of FMOC L-phenylalanine:190 m/z and minor ion: 164.1 m/z(A),-glutamic acid: 172.0 m/z and minor ions: 146 and 128 m/z (B) –glycine: 73.8 m/z minor ions: 99.9 and 117.9 m/z

Figure	Amino acid	Theoretical mass [M-H$^-$]	Major progeny ion used for MRM	Minor ions
5.2.5.A	FMOC L-arginine	395	172.9	N.D
5.2.5.B	FMOC L-citrulline	396	173.9	N.D
5.2.5.C	FMOC L-ADMA	423	201.0	N.D
5.2.5.D	FMOC L-homocysteine	356	159.8	N.D
5.2.6.A	FMOC L-taurine	346	123.7	N.D
5.2.6.B	FMOC L-serine	326	129.7	N.D
5.2.6.C	2 (FMOC) L-cystine	684	151.8	N.D
5.2.6.D	FMOC L-cysteine	342	145.7	N.D
5.2.7.A	FMOC L-phenylalanine	387	190.1	164.1
5.2.7.B	FMOC L-glutamic acid	368	172.0	146.0,128.0
5.2.7.C	FMOC L-glycine	297	73.8	99.9,117.9

Table 5.2. 2 Fragmentation patterns of FMOC amino acids (ND-not detected)

5.2.2. 3 Mass and fragmentation data for internal standards

In this study, $^{13}C_6$ L-arginine was used as an internal standard for the measurement of the amino acids. The response factor of the internal standards were found to be 1:1 i.e. the area under the curve for 5 nmol of the internal standards was found to be equal to the area under the curve for 5 nmol of all the amino acids studied. The tandem mass spectral analysis of the FMOC internal standard was obtained in scan mode over a mass range of 2-600 [M-H^-], and a mass was observed at m/z 400.6 [M-H^-] (Fig.5.2.8.A). The fragmentation analysis of the internal standard was observed in scan mode over a mass range of 2-400 m/z. This led to the identification of 'fingerprint' progeny ion at the intensity of 178.9 m/z (Fig.5.2.8.B) which corresponded to the molecular mass of the underivatised $^{13}C_6$ L-arginine.

Fig.5.2. 8 Parent ion scan: 400.6 m/z (A) and product ion spectrum: 178.95 (B) of FMOC L-13C6 arginine

5.2. 3 LC-ESI-MS analysis of amino acids

High performance liquid chromatography (HPLC) was used together with tandem mass spectrometry. A HyPURITY C8 column (100 mm X 2.1 mm with a 5 μ particle size) fitted with a guard column containing the same stationary phase (Phenomenex UK) was used. The use of HPLC prior to mass spectrometry had several additional advantages. Firstly, the use of the HPLC column enabled individual amino acids to be separated before entering the MS/MS, thus increasing the specificity of the method. Secondly there was a reduction of ion suppression and thirdly, the use of a solvent delay system allowed at least 300 analyses to be completed before routine maintenance of the mass spectrometer was necessary. Two gradients were evaluated to elucidate the best results. The first gradient (procedure-1) contained water and methanol: acetonitrile/MeOH: ACN (2:1). The use of methanol: acetonitrile (2:1) was used in case the isoprostane analysis was incorporated into the method at a later date. Details of the gradient are shown in Table.5.2.3. The mobile phase consisted of a step change gradient where the LC gradient started with 5% MeOH:ACN and 95% water for 2 min which was then increased to 45% MeOH:ACN and 55% water for 3 min. The gradient was further increased to 55% MeOH:ACN and 45% water for a further 2.49 min and was held at 100% MeOH:ACN for 8.5 min after which the gradient was reduced to 20% MeOH:ACN and 80% water for 4 min. The flow rate of the gradient varied from 0.25-0.50 ml/min.

Time (Min)	Water %	MeOH:ACN (2:1) %	Flow (ml/min)
0.00	95	5	0.50
2.00	55	45	0.25
5.00	45	55	0.25
7.49	20	80	0.25
7.50	0	100	0.50
16.00	80	20	0.50
20.00	80	20	0.50

Table 5.2. 3 HPLC procedure-1

The chromatographic elutions are shown for each of the FMOC compounds in Fig.5.2.9. An overlap of the chromatographic separations of L-cysteine and L-homocysteine were observed with a retention time of 8.78 min and 8.87 respectively. Also, a minor peak at 9.21 min was observed for both the amino acids. In addition no mass for glutamic acid could be observed.

Fig.5.2. 9 Elution using HPLC procedure-1. Absence of glutamic acid elution (G)

In order to try and elute the glutamic acid an acidic HPLC gradient was tried (procedure-2). Ammonium acetate containing 0.01% formic acid and acetonitrile (ACN) were used as the solvents. Details of the gradient used are shown in Table.5.2.4. The mobile phase consisted of a step change gradient which started with 5% ACN and 95% ammonium acetate (0.01% formic acid) for 2 min and was increased to 31% ACN and 69% ammonium acetate (0.01% formic acid) and held for 8 min. The gradient was then increased to 100% ACN and held for 5 min. The column was then reconditioned with 5% ACN and 95% ammonium acetate (0.01% formic acid) for 5 min.

Time (Min)	**Ammonium acetate + 0.01 % formic acid %**	**ACN %**	**Flow (ml/min)**
0.00	95	5	0.50
2.00	70	30	0.50
2.10	69	31	0.25
10.00	30	70	0.25
10.01	0	100	0.50
15.01	95	5	0.50
20.00	95	5	0.50

Table 5.2. 4 HPLC procedure-2

The chromatographic elutions are shown for each of the compounds in Fig.5.2.10 with glutamic acid eluting at 5.6 min. Procedure-2 gave better separation of the amino acids

including cysteine and homocysteine with elution taking place between 5.6 and 10.5 minutes. This compared to procedure-1 where the amino acids eluted between 5.8 and 8.9 minutes, with FMOC-L-cystine, L-ADMA, L-arginine and L-phenylalanine eluting between 7.35-7.57 min. The different retention times of procedure 1 and 2 have been tabulated in Table 5.2.5. Thus procedure 2 was used in future studies to validate the method.

The run was conducted initially in an organic solvent (ethanol) and then replicated in human urine (the chromatogram was observed to be identical).

	Procedure 1	Procedure 2
	Retention time	**Retention time**
L-Cystine	7.35	8.16
L-ADMA	7.57	6.79
IS/13C6L- Arginine	7.38	6.51
l-Citrulline	5.82	6.14
L-Arginine	7.38	6.51
L-Phenylalanine	7.57	8.25
L-Glutamic acid	ND	5.61
L-Homocysteine	8.87/9.21	10.49
L-Taurine	6.26	6.88
L-Cysteine	8.87/9.21	10.30
L-Serine	5.86	6.26
L-Glycine	6.07	6.57

Table 5.2. 5 Retention times of amino acids

Fig.5.2. 10 Elution using HPLC procedure-2, elutions of L-cystine:8.15 min,-ADMA: 6.79 min,-IS:6.51 min,-Citrulline:6.14 min,-Arginine:6.51 min,-Phenylalanine:8.25 min,-Glutamic acid:5.61 min,-Homocysteine:10.49 min,-Taurine:6.88 min,-Cysteine:10.30 min,-Serine:6.2

5.2. 4 Validation of method

To test the performance and robustness of the method, the assay was evaluated for its linearity, recovery, reproducibility and limit of detection.

5.2.4. 1 Linearity

In order to assess if the method gave a linear response, increasing amounts of each of the compounds and a constant amount (10 µl of 100 µmol/l) of the internal standard were added to 50 µl of pooled urine from laboratory volunteers and analysed as described in section 4.3.3. The amino acids could be divided into two groups according to their typical urinary concentration and so two ranges of concentrations were used in the linearity studies. The two calibration curves with seven points of reference were 0, 5, 10, 25, 50, 100 and 200 nmol (0, 33.2, 66.4, 166.0, 332.0, 664.0 and 1328 µmol/l) for ADMA, arginine, phenylalanine, glutamic acid, homocysteine, citrulline and 0, 10, 25, 50, 100, 250 and 500 nmol (0, 66.4, 166.0, 332.0, 664.0, 1328.0, 1660.0 and 3320.0 µmol/l) for taurine, serine, cysteine, cystine and glycine. The ratio of the areas for each metabolite over the internal standard was calculated for each metabolite. The data obtained was subjected to linear regression analysis where the best fit for the linear relationship was calculated using GraphPad Prism 4. The plots were linear up to 5000 and 9500 µmol/L respectively, for the two groups, which is approximately 4 times the highest concentration used (data not shown).

The correlation coefficients were greater than 0.99 for all the amino acids. Typical plots for the four amino acids related to $NO^{\bullet}$ metabolism are shown in Fig.5.2.12.

Fig.5.2. 11 Calibration curves for L-arginine, L-citrulline, L-ADMA, L-homocysteine

5.2.4. 2 Recovery

Recovery studies involved analysis before and after adding known amounts of the metabolites (0.2, 0.5 and 2.5 nmol) to 150 µl urine from laboratory controls containing a constant amount of internal standard. Injections at each concentration were performed in triplicate. The ratio of the areas for the amino acids over the corresponding internal standard was calculated for each amino acid. The percentage recovery of the added analyte was then calculated using the following formula:

% Recovery = [(b-a)/c] X 100

where, a=amount found before addition, b=amount found after addition and c=amount of standard metabolite added

The results are summarised in Table.5.2.6. The recoveries were consistently greater than 90%, with most being greater than 95%. (Recoveries above 85% are found to be acceptable by The Journal of Clinical Biochemistry)

	Amount of metabolite added (0.2 nmol) Recovery (%)	Amount of metabolite added (0.5 nmol) Recovery (%)	Amount of metabolite added (2.5 nmol) Recovery (%)
Arginine	95	97	98
Citrulline	97	99	100
ADMA	93	97	99
Homocysteine	98	97	99
Taurine	98	94	98
Serine	90	98	99
Glutamic acid	92	95	98
Glycine	99	97	100
Phenylalanine	92	94	95
Cysteine	95	93	99
Cystine	97	95	100

Table 5.2. 6 Percentage recoveries of the amino acids

5.2.4. 3 Reproducibility

To assess the reproducibility of the assay, repeated analysis were undertaken following the addition of low and high amounts of the amino acids (5 and 50 nmoles) to control urines. The intra- assay reproducibility was evaluated using fifteen replicate injections, of the two concentrations of the amino acids, within the same run on a single day. The inter-assay reproducibility was assessed by running a single injection of the two concentrations on fifteen separate occasions over a period of 45 days. The ratio of the areas for the amino acid over the corresponding internal standard was calculated for each metabolite.

The intra-assay coefficient of variation for the amino acids ranged from 0.07 to 1.23 % (Table 5.2.7) and the inter-assay C.V. of the amino acids ranged from 2.32 to 5.76 % (Table 5.2.8).

	Amount of metabolite added 5 nmol N=15 C.V. %	Amount of metabolite added 50 nmol N=15 C.V. %
Arginine	0.62	0.75
Citrulline	0.80	0.67
ADMA	0.61	0.59
Homocysteine	0.31	0.47
Taurine	0.32	0.27
Serine	0.11	0.19
Glutamic acid	0.41	0.39
Glycine	0.31	0.67
Phenylalanine	0.17	1.23
Cysteine	0.28	0.45
Cystine	0.07	0.7

Table 5.2. 7 The intra-assay coefficient of variation

	Amount of metabolite added 5 nmol N=15 C.V. %	**Amount of metabolite added 50 nmol N=15 C.V. %**
Arginine	2.67	3.45
Citrulline	2.32	2.89
ADMA	5.45	5.67
Homocysteine	5.45	4.76
Taurine	2.69	3.87
Serine	4.12	3.45
Glutamic acid	3.55	4.07
Glycine	2.86	3.12
Phenylalanine	4.57	5.12
Cysteine	5.46	5.76
Cystine	4.74	4.23

Table 5.2. 8 The inter-assay coefficient of variation

5.2.4. 4 Limit of detection

The limit of detection of the method was evaluated by diluting known amounts of the amino acids sequentially and determining the dilution at which detection was lost. The lowest amount of the metabolites in urine and ethanol giving a signal to noise ratio of 5:1 was considered to be the biological and functional limit of detection respectively.

The functional and biological limits of detection for the amino acids are tabulated below (Table 5.2.9) and vary between 0.01-0.63 and 0.02-1.25 μmol/l for the functional and biological limits respectively.

	Functional limit µmol/l	Biological limit µmol/l
Arginine	0.16	0.32
Citrulline	0.04	0.02
ADMA	0.32	0.63
Homocysteine	0.08	0.63
Taurine	0.02	0.16
Serine	0.16	0.63
Glutamic acid	0.01	0.16
Glycine	0.04	0.16
Phenylalanine	0.63	1.25
Cysteine	0.63	1.25
Cystine	0.04	0.16

Table 5.2. 9 The functional and biological limits of detection

5. 3 Discussion

Measuring $NO^{\bullet}$ metabolism directly *in vivo* is difficult (section 5.1.5) and thus indirect measurements of $NO^{\bullet}$ have been proposed. In this study we aimed to develop a methodology to investigate alterations in $NO^{\bullet}$ metabolism by measuring amino acids involved in $NO^{\bullet}$ metabolism; L-arginine (precursor of $NO^{\bullet}$), L- citrulline (product of $NO^{\bullet}$ formation), L-ADMA (inhibitor of NOS) and L-homocysteine (decreases bioavailability of $NO^{\bullet}$ by inhibiting NOS).

Traditionally, amino acid analysis is performed by ion-exchange chromatography with detection after post-column derivatisation. An inherent disadvantage of this technique is that long run times are required to obtain adequate resolution and a typical ion-exchange assay can take over 24-48 hours to complete (Teerlink 2007). GC/MS has also been used to quantitate amino acids but the assays require a sample clean-up and derivatising protocol resulting in the method being long and labour intensive (Vishwanathan 2000) as discussed in section 5.1.5. HPLC methods have been cited in the literature to be faster than the GC/MS methods but they also require extraction procedures using SPE (section 5.1.5). The present methodology used pre-column derivatisation and a separation protocol (HPLC) prior to MS/MS analysis and took only 20 min to analyse the amino acids of interest. Quantitation of individual amino acids using the FMOC derivatising protocol has been carried out (Bank 1996) but no previous study prior has looked at all the four amino acids associated with $NO^{\bullet}$ metabolism in a single run.

The quantitation of the amino acids associated with $NO^{\bullet}$ metabolism in biological fluids is also associated with analytical difficulties. Typically, concentrations of the amino acids associated with $NO^{\bullet}$ metabolism are two orders of magnitude lower than the typical amino

acids, requiring assays of high specificity and sensitivity (Macallister 1996). In this study HPLC was used together with tandem mass spectrometry, to separate all the amino acids of interest. The use of HPLC served two purposes, firstly it increased the specificity of the method and secondly it desalted the sample which helped to maintain the integrity of the mass spectrometer and increase the time between routine maintenance checks. In theory the method could have been developed using a shorter column and the use of a C8 column in this assay was retained to keep the instrumentation similar to the method developed for the analysis of vitamin E metabolites.

As amino acids are zwitter ions, thermally labile and non-volatile compounds, their analysis without derivatisation is difficult by means of reverse phase HPLC and impossible by GC (section 5.1.5). All other published HPLC methods have involved derivatisation using o-phthalaldehyde (OPA) to increase sensitivity and retention of these compounds on the HPLC columns as discussed in section 5.1.5. The inherent problems were the complex gradient systems used and an elution time of greater than seventy minutes (Blundell and Brydon 1987;Meyer 1997). Studies that did not utilise derivatisation techniques such as that of Vishwanthan *et al.,* (2000) where the sample was analysed after protein precipitation with acetonitrile and solvent evaporation, resulted in a high noise level and the amino acids of interest were not always completely separated from endogenous interfering compounds leading to doubts about their quantitation. (Weaving 2008) overcame this problem using an elaborate solid phase extraction (SPE) procedure but questions remained regarding the validation procedures, matrix effects caused by SPE, method selectivity and comparability issues as highlighted by Martins-Lobenhoffer (2009). The method of Weaving *et al.,* (2008) was validated using concentrations that were greater by 1000- fold compared to biological fluids (plasma and urine). They also failed to provide any data to evaluate the matrix effect

caused by the omission of the HPLC separation. Lastly, they did not account for the potential interference caused by SPE to the accuracy of their method. In addition, sample clean-up using SPE is labour intensive and 'blank' analyses have to be included to check for the presence of spurious peaks, because some batches of pre-packed columns may contain impurities, necessitating a conditioning step before use (de Jong & Teerlink 2006).

The methodology developed in this study for the analysis of amino acids involved a short derivatising protocol utilising 9-fluorenylmethyl chloroformate (FMOC) prior to LC-MS/MS analysis with minimum sample preparation. FMOC increases the detectability and quantitation as it improves the ionisation of amino acids and it also enables superior chromatographic resolution in the most frequently used mobile phase (acetonitrile, ammonium acetate, methanol and water) of reverse phase HPLC systems (Gartenmann and Kochhar 1999). The use of FMOC in the current assay also enabled us to quantitate seven additional amino acids in addition to those involved with $NO^{\bullet}$ metabolism in a single run.

The current method was found to be linear over the necessary working range (Table 5.3.1), to be reproducible and have recovery rates of greater than 95%. This compared to the recoveries of 84-95 % quoted by (Markowski, Baranowska, & Baranowski 2007) and his co-workers, using RP-HPLC. The coefficients of variation for inter- and intra-reproducibility varied from 0.07 to 5.76% in the current study which were better than the 8 % observed by (Martens-Lobenhoffer & Bode-Böger 2003) using LC-MS instrumentation and the 2.3-14.2% and 2.9-6.4% by Vishwanthan *et al.,* (2000) and (Rafii 2007) respectively using LC-MS/MS instrumentation.

The reference range of the urinary amino acids in healthy adults expressed per mmol/l creatinine compiled by (Tan and Gajra 2006) in Asian and Caucasian population, using ion-exchange chromatography is compared in Table.5.3.1 to the functional and biological limits of the developed assay.

It can be seen that the amino acids can be quantitated with confidence as the method developed in the current study is sufficiently sensitive to detect the low amounts present in urine.

In summary, we have developed a rapid method with minimal sample preparation to measure eleven amino acids in a single, multiplexed assay. In addition the utilisation of an isotopically labeled internal standard adds robustness to the measurements. The methodology developed to measure those amino acids associated with $NO^{\bullet}$ metabolism was used to investigate children with Type 1 diabetes in whom oxidative stress has been implicated (chapter 6).

	Functional Limit µmol/mmol creatinine	Functional Limit µmol/mmol creatinine	Functional Limit µmol/mmol creatinine	Functional Limit µmol/mmol creatinine
Arginine	0.83	1.66	2-13	ND-5
Citrulline	0.21	0.10	ND-3	ND-4
ADMA	1.66	3.28	ND	ND
Homocysteine	0.42	3.28	0-9	ND
Taurine	0.10	0.83	21-244	ND-180
Serine	0.83	3.28	21-133	ND-30
Glutamic acid	0.05	0.83	ND-7	ND-40
Glycine	0.21	0.83	37-690	ND-107
Phenylalanine	3.28	6.50	1-22	ND-19
Cysteine	3.28	6.50	NA	NA
Cystine	0.21	0.83	2-17	Nd-17

Reference Range I- Asian population-Tan and Gujara (2006)
Reference Range II- Caucasian population-Tan and Gujara (2006)
ND- Not detected
NA-Not available

Table 5.3. 1 Comparism of reference ranges to the limits of detection

Chapter 6

Diabetes

TABLE OF CONTETS

6 Diabetes

6.1 Background information

6.1. 1 Definition of diabetes

The term diabetes mellitus describes a metabolic disorder of multiple aetiology characterised by chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action or both. The underlying defects may result in the destruction of the beta cells of the pancreas with consequent insulin deficiency (Type 1), or result in resistance to insulin action (Type 2). The effects of diabetes include long-term damage, dysfunction and failure of various organs as discussed below (W.H.O 2006). Often the symptoms are not severe, or may be absent and consequently hyperglycaemia sufficient to cause pathological and functional changes may be present for a long time before the diagnosis is made. Subjects with diabetes are at a significantly increased risk of cardiovascular, peripheral vascular and cerebrovascular disease. Other long-term effects of diabetes mellitus include progressive development of retinophathy with potential blindness, nephropathy that may lead to renal failure and neuropathy. Several pathogenic processes have been implicated in the development of these long term effects of diabetes (Rother 2007).

Diabetes (Type-1 and 2) is one of the major metabolic disorders worldwide; the prevalence of which is estimated to rise from 6 % to over 10 % of the world population in this current decade (Rösen et al. 2001). According to the Health Survey of England, there are 2.35 million people with diabetes with 85 % (1.99 m) having Type-2 and 25 % (0.35 m) having Type-1 in England alone (2006). This is predicted to grow to more than 2.5 million by 2010 (Dinsdale 2008) and increase to 4 million by 2025 (Gould 2008). The national health

service (NHS) has been reported to be spending £1m an hour, 10% of its yearly budget, treating diabetes and its complications - equating to around 9 billion per year according to a report by the charity Diabetes UK (Gould 2008).

6.1. 2 Glycated haemoglobin (HbA1c%)

Glycosylated/glycated haemoglobin (HbA1c%) is used clinically as an indicator of diabetic control (Nathan, 1984). It is formed in a non-enzymatic reaction as a result of the exposure of haemoglobin to high plasma concentrations of glucose (Larsen 1990).

Once the haemoglobin molecule is glycated, it retains that chemical change and the accumulation of glycated haemoglobin within the red blood cells reflects the average level of glucose to which the cell has been exposed during its life cycle of approximately 120 days. HbA1c% is expressed as a percentage of total haemoglobin (Nathan, 1984).

The normal (non diabetic) value for HbA1c% is 4.0-6.5 and the NHS/NICE guide lines have advised an HbA1c% range of 6.5-7.5% as the target for diabetic patients in the U.K. An HbA1c% of >7.5% is considered to reflect poorly controlled diabetes mellitus and an increased risk factor for the complications associated with diabetes (Wild 2004).

6.1. 3 Diabetes and its complications

Long-term vascular complications represent a major cause of morbidity and mortality in patients with diabetes mellitus (Giuliano 1996). In addition, various biochemical disorders associated with vascular complications, such as hyperlipidemia and oxidative stress frequently co-exist with diabetes mellitus (Mezzetti 2000). Chronic hyperglycaemia is related to the pathophysiology of micro-vascular diseases. Even in a mild imbalance, glucose metabolism may directly affect endothelial function and several studies have demonstrated that hyperglycaemia plays an important role in the development of endothelial dysfunction and cardiovascular disease in type 1 diabetic patients (Pinkey 1998).

There seems to be a general agreement that the production of oxygen derived free radicals is increased in diabetic patients (Mezzetti 2000). Gopaul et al (1995) have reported that the mean concentration of esterified 8-iso-PGF2α in plasma from 39 patients with diabetes was approximately threefold higher than in healthy individuals. Davi et al (1999) reported that urinary 8-iso-$PGF_{2\alpha}$ was significantly higher in a group of 23 type 2 and type 1 patients than in age-matched control subjects. Finally, Flores and coworkers (2004) observed that even in patients on insulin therapy and with good diabetic control (6-7.5 Hba1c%), the urinary excretion of 8-iso-$PGF_{2\alpha}$ was significantly greater in diabetic patients compared with control subjects. Several clinical studies have shown increased levels of other markers of oxidative stress in type 1 and 2 diabetes, for instance 8-hydroxy-2′-deoxyguanosine (Krapfenbauer 1998) and oxidised LDL (oxLDL) (Leinonen et al. 1997; MacRury et al. 1993) in plasma. There is also evidence from experimental studies (see below) that the formation of reactive oxygen species (ROS) and disruption of $NO^{\bullet}$ metabolism is a direct consequence of hyperglycemia (Du et al. 1999). In conclusion, the above isoprostane and

biomarker studies indicate a general oxidative stress status (not site or tissue specific) in the diabetic cohorts and this could be the starting point for damage leading to the vascular complications seen in diabetes.

Diabetic nephropathy is another complication that is considered as a public health problem. At the onset of the disease, glomerular filtration rate (GFR) is high. It starts to decline approximately 5 years. The decline is sluggish, but over a time scale of approximately 20–25 years it inexorably leads to end-stage renal disorder (ESDR). The appearance of microalbuminuria, which may occur after 10 years, heralds early renal damage. The evolution to frank proteinuria is a critical phase because it accelerates renal damage, leading to ESRD within a few years (Locatelli 2003).

6.1. 4 Mechanisms for hyperglycaemia-induced oxidative damage

There is growing evidence that hyperglycaemia can induce ROS production and disrupt natural antioxidant defence mechanisms in patients with diabetes, thus leading to an increased oxidative stress in these individuals (Okazawa et al. 1996; Davi et al. 1999). Increased oxidative stress can elicit alterations in tissues that undergo insulin-dependent glucose uptake, thereby resulting in early tissue damage in target organs such as the occular lens, retina, peripheral nerves and renal glomerulus (Chung 2003). Possible mechanisms whereby hyperglycaemia can induce ROS production are detailed as follows:

6.1.4. 1 Polyol pathway flux (Fig.6.1.1)

One source of ROS is thought to be the polyol pathway. The first enzyme in the pathway is aldose reductase (AR) which reduces glucose to sorbitol utilizing NADPH. AR has a low affinity for glucose and at normal glucose concentrations found in non-diabetics, metabolism of glucose by this pathway is a very small percentage of total glucose use (Packer et al. 2001). In a hyperglycaemic environment (>10mmol/l), AR also reduces aldehydes generated by reactive oxygen species (ROS) to inactive alcohols and the increased intracellular glucose to sorbitol with a concomitant decrease in NADPH. In the polyol pathway, sorbitol is oxidised to fructose by the enzyme sorbitol dehydrogenase (SDH), with NAD^+ being reduced to NADH. The flux through this pathway during hyperglycaemia varies from 33% of total glucose use in the rabbit lens to 11% in human erythrocytes (Wilson 1992). Thus, the contribution of this pathway to diabetic complications is likely to be species, site and tissue dependent.

The reduction of glucose to sorbitol by AR consumes NADPH and as NADPH is required for regenerating reduced glutathione (GSH), this could induce or exacerbate intracellular oxidative stress (Brownlee 2001).

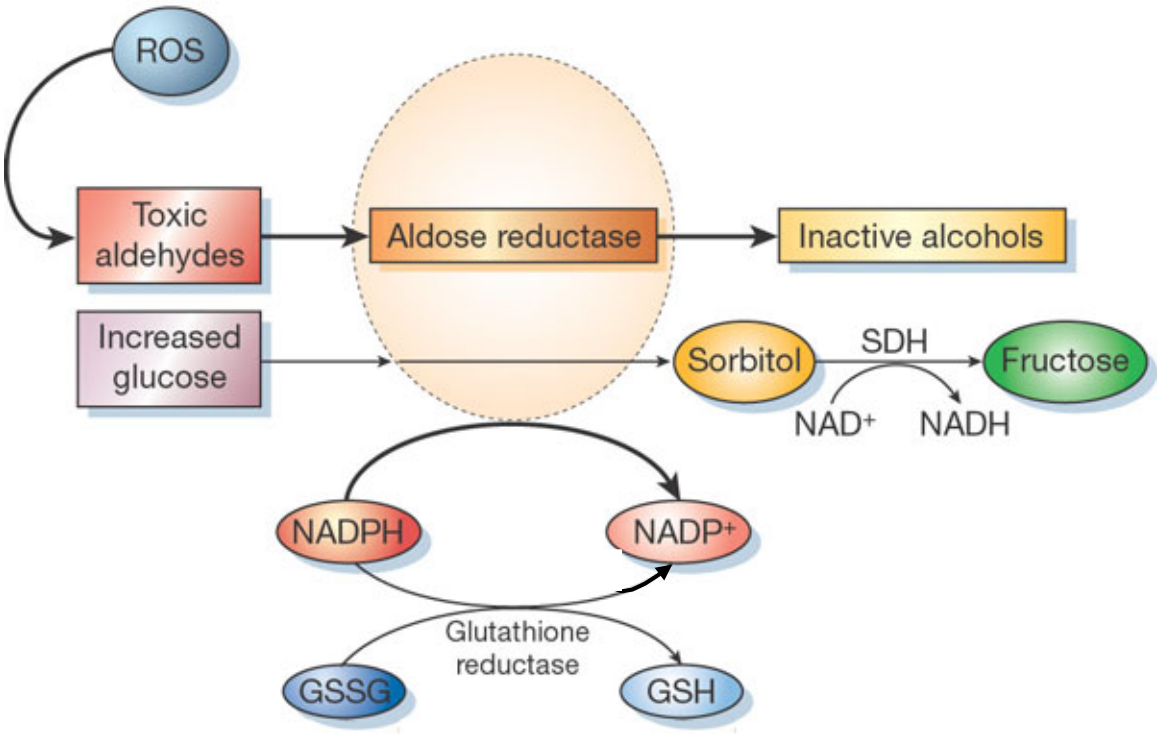


Fig.6.1. 1 Polyol Pathway (Brownlee 2001)
SDH- Sorbitol dehydrogenase, GSH- Reduced glutathione, GSSG- Oxidised glutathione

Decreased levels of GSH have in fact been found in the lenses of transgenic mice that overexpress aldose reductase, and this is the most likely mechanism by which increased flux through the polyol pathway has deleterious consequences (Lee 1999). This conclusion is further supported by experiments as detailed below.

Transgenic mice that overexpress AR specifically in their lenses showed a significant increase in oxidative stress when they became hyperglycemic, as indicated by a decrease in glutathione (GSH) and an increase in malondialdehyde in their lenses. Introducing an SDH-deficient mutation into these transgenic mice significantly normalised the GSH and malondialdehyde levels (Chung 2003). These results indicate that both enzymes of the polyol pathway contributed to hyperglycemia-induced oxidative stress in the lens.

Similar findings have been found in the nerves of wild-type mice which were made diabetic. Diabetes caused a significant decrease in GSH in their sciatic nerves. In the AR null mutant mice, diabetes did not lead to any decrease in the concentration of GSH in the nerve. These results indicate that AR is also a major contributor to hyperglycemia-induced oxidative stress in the nerve (Chung 2003).

6.1.4. 2 Increased formation of advanced glycation end products (AGE)

AGEs are a heterogeneous group of molecules formed from the nonenzymatic reaction of reducing sugars with free amino groups of intracellular and extracellular proteins (Brownlee 2001). AGEs are found in increased amounts in diabetic retinal vessels (Stitt 1997) and renal glomeruli (Horie 1997). Intracellular hyperglycaemia is the primary initiating event in the formation of both intracellular and extracellular AGEs (Degenhardt 1998).

The potential importance of AGEs in the pathogenesis of diabetic complications is indicated by the observation in animal models that two structurally unrelated AGE inhibitors (aminoguanidine and OPB-9195) partially prevented various functional and structural manifestations of diabetic microvascular disease in retina, nerve and kidney (Soulis-Liparota 1991, Hammes 1991 and Nakamura 1997). These findings were confirmed in a large randomized, double-blind, placebo-controlled, multi-centre trial of the AGE

inhibitor aminoguanidine in 690 patients with type-1 diabetes (Bolton 2004). In another study by Foiles and co-workers (2001) in type 1 diabetic patients with overt nephropathy, aminoguanidine was also observed to lower total urinary protein concentrations and slow the progression of the nephropathy, over and above the effects of existing optimal care. In addition, they also observed that aminoguanidine reduced the progression of diabetic retinopathy.

Production of intracellular AGE damages target cells by three general mechanisms. First, intracellular proteins modified by AGEs have altered function. Second, extracellular matrix components modified by AGE interact abnormally with other matrix components and with the receptors for matrix proteins (integrins) on cells. Third, plasma proteins modified by AGE bind to AGE receptors on endothelial cells, mesangial cells and macrophages, inducing receptor-mediated production of reactive oxygen species (Brownlee 2001).

6.1.4. 3 Activation of protein kinase C (PKC)

The PKC family comprises at least eleven isoforms, nine of which are activated by the lipid second messenger, diacylglycerol (DAG). Intracellular hyperglycaemia increases the amount of DAG in cultured microvascular cells and in the retina and renal glomeruli of diabetic animals (Brownlee 2001). Hyperglycaemia may also activate PKC isoforms indirectly through both ligation of AGE receptors (Portilla 2001) and increased activity of the polyol pathway (Keogh 1997) presumably by increasing reactive oxygen species (Brownlee 2001).

The activation of PKC has been shown to depress nitric oxide ($NO^{\bullet}$) production by inhibiting endothelial nitric oxide synthase (eNOS) (Brownlee 2001). In early experimental diabetes, the activation of the β isoform of PKC has been shown to result in abnormalities of retinal and renal blood flow possibly by supressing nitric oxide production (Ishi 1999). Abnormal activation of PKC-β has aslo been implicated in the decreased glomerular production of nitric oxide induced by experimental diabetes (Craven 1994) and in the decreased production of nitric oxide in smooth muscle cells that is induced by hyperglycaemia (Ganz 2000). Activation of PKC-β also inhibits insulin-stimulated expression of the messenger RNA for eNOS in cultured endothelial cells (Kuboki 2000).

In addition to affecting hyperglycaemia-induced abnormalities of blood flow and permeability, activation of PKC-β has also been shown to contribute to increased microvascular matrix protein accumulation by inducing expression of fibronectin and collagen both in cultured mesangial cells (Studer 1993) and in glomeruli of diabetic rats (Koya 1997). This effect also seems to be mediated through inhibition of nitric oxide production by PKC-β (Craven 1997)

Treatment with an inhibitor specific for PKC-β significantly reduced PKC-β activity in the retina and renal glomeruli of diabetic animals and at the same time significantly reduced diabetes-induced increases in retinal blood circulation, normalised increases in glomerular filtration rate and partially corrected urinary albumin excretion (Koya 2000).

6.1.4. 4 Mitochondrial dysfunction and insulin signaling

The pathways leading to insulin resistance may be synergistic and mitochondrial dysfunction can create a feedback loop, adding to the overall oxidative stress environment

(Fig.6.1.2). Studies have shown that fatty acid-induced insulin resistance can be caused by direct inhibition of insulin-stimulated glucose transport activity (Dresner 1999). A decrease in mitochondrial fatty acid oxidation, which is caused by mitochondrial dysfunction, results in increased levels of fatty acyl-CoA and DAG. These in turn activate stress-related Ser/Thr kinase activity and inhibit glucose transport by mechanisms (Lowell 2005). In relation to stress-activated kinases, oxidative stress also contributes to impaired insulin signaling by increased uncoupling protein-2 (UCP2) activity. Uncoupling proteins are mitochondrial transporters of the inner membrane that, when activated, cause protons to leak across the inner membrane, generating heat without contributing to ATP production (Rousset 2004 and Fisler 2006). UCP2 is thought to negatively regulate glucose-stimulated insulin secretion by reducing the amount of ATP produced (Rousset 2004). This idea is supported by studies that have demonstrated stimulation of UCP2 *in vitro and in vivo* by hyperglycemia and lipid fuels and in animal models of type 2 diabetes (Fisler 2006). Because ATP production is key to providing energy for almost all cellular processes, it is likely that decreased ATP production could affect insulin signaling in many different cell types. Further studies are needed to determine the exact effects of oxidative stress on the mitochondria and the link between the mitochondria and insulin resistance.

Fig.6.1. 2 Scheme of the putative pathways linking mitochondrial dysfunction and diabetes (Rains 2011)

6.1. 5 Diabetes, oxidative stress, nitric oxide and endothelial dysfunction

The mechanisms involved in the endothelial dysfunction seen in diabetes patients include a reduced bioavailability of nitric oxide ($NO^{\bullet}$), possibly due to the increased production of ROS as detailed in section 5.1 (Furchgott 1980, Cooke 1997, Cooke 2000). A deficiency of $NO^{\bullet}$ will tend to increase vascular resistance and promote atherogenesis (Cooke 1997). In addition to oxidative damage caused by conversion by conversion of $NO^{\bullet}$ to peroxynitrite, another possible mechanism for $NO^{\bullet}$ deficiency and cardiovascular morbidity is reduced $NO^{\bullet}$ synthesis caused by the endogenous arginine metabolite, asymmetric dimethylarginine (ADMA) (Harrison 1997) and have been discussed in section 5.1.4.1.

Plasma ADMA concentrations have been reported to be elevated in animal models of diabetes (Lin 2002, Xiong 2003) and in patients with impaired glucose tolerance (Miyazaki 1999), insulin resistance (Stuhlinger, 2002) and type 1 and 2 diabetes (Abbasi 2001,Tarnow 2004). It has also been reported that elevated plasma concentrations of ADMA are associated with increased risks of nonfatal stroke and myocardial infarction in type 1 diabetic patients with early nephropathy (Tarnow 2004). These findings suggest that elevated ADMA could contribute to an acceleration of atherosclerosis in the diabetic population. It is, therefore, tempting to speculate that ADMA might be a pathophysiological relevant factor for diabetes-associated complications.

Hyperhomocysteinaemia has been shown to be an independent risk factor for atherosclerosis in the general population (Welch 1998). The adverse effects of homocysteine on endothelial function may be mediated by reduced production and bioavailability of $NO^{\bullet}$ as a result of oxidative stress (Loscalzo 1996). The role of

homocysteine and its involvement in oxidative stress has been addressed in section 5.1.4.2. Studies in adult patients with type 1 diabetes have however demonstrated similar (Hultberg 1991, Chico 1999, Vaccaro 2000), lower (Robillon 1994, Cronin 1998) and higher (Hofmann 1998, Targher 2000) plasma homocysteine levels compared with non-diabetic controls. Thus, a direct link between homocysteine and diabetes has yet to be established.

Because of the evidence of increased oxidative stress in subjects with diabetes mellitus, the method developed for the measurement of urinary vitamin E metabolite and amino acids associated with nitric oxide metabolism were applied to 32 children with type 1 diabetes,

6. 2 Methodology

32 children and young people (16 male) with a mean age at study of 12.9 years (range 7.8-18.4) with DMI were studied. Their mean age at diagnosis was 10.8 years and the average duration of diabetes was 5 years. The patients were considered pre-syptomatic (i.e. showed no clinical complications associated with long-term diabetes including kidney disorder or cardiovascular disease with their microalbumin test result being well within the normal range of 3-30 g/L- Table 6.2). A urinary sample for measurement of vitamin E metabolites was obtained at a routine clinic visit and glycosylated haemoglobin (HbA1c) was measured as part of the standard clinic appointment using the DCCT aligned Bayer 2000+ system (Siemens Healthcare Diagnostics Inc, Deerfield, IL, USA).

32 aged matched healthy control subjects were drawn from the University College London Fetal Growth Study. This study consists of 1650 consecutive mothers who delivered a singleton, Caucasian baby free of pregnancy complications and whose offspring's growth has been followed for the last 10 yrs. As part of the follow-up of these children urine samples were obtained contemporaneously to the DMI study.

Ethical approval was obtained from the Ethics committees of University College London/University College London Hospitals Ethics Committee and the Great Ormond Street Hospital for Children/Institute of Child Health Ethics Committee. Written informed consent was obtained from the parents, and the children and young people when appropriate.

Each sample was analysed in triplicates and the mean of the three tests was taken as the final result.

Unless otherwise stated the results are expressed as mean ± 1SEM

The significance of differences of the mean values was determined using the Students t-test.

Table 6.2. 1 Clinical data of the diabetic patients including duration of diabetes in years, creatinine (umol/L) and Hba1c (%).

6. 3 Results

6.3. 1 Urinary vitamin E metabolites

Concentrations of the urinary vitamin E metabolites were quantified in 32 patients with type 1 diabetes mellitus and age-matched controls. Figures 6.3.1 to 6.3.5 illustrate the mean concentration per nmol/mmol creatinine (± 1 SEM) for each of the metabolites.

The mean concentration of α-TLHQ glucuronide 1 was 1098±279 in the diabetic subjects compared to 76±13 nmol/mmol creatinine in the sex and age-matched control group with $p<0.001$ (Fig.6.3.1)

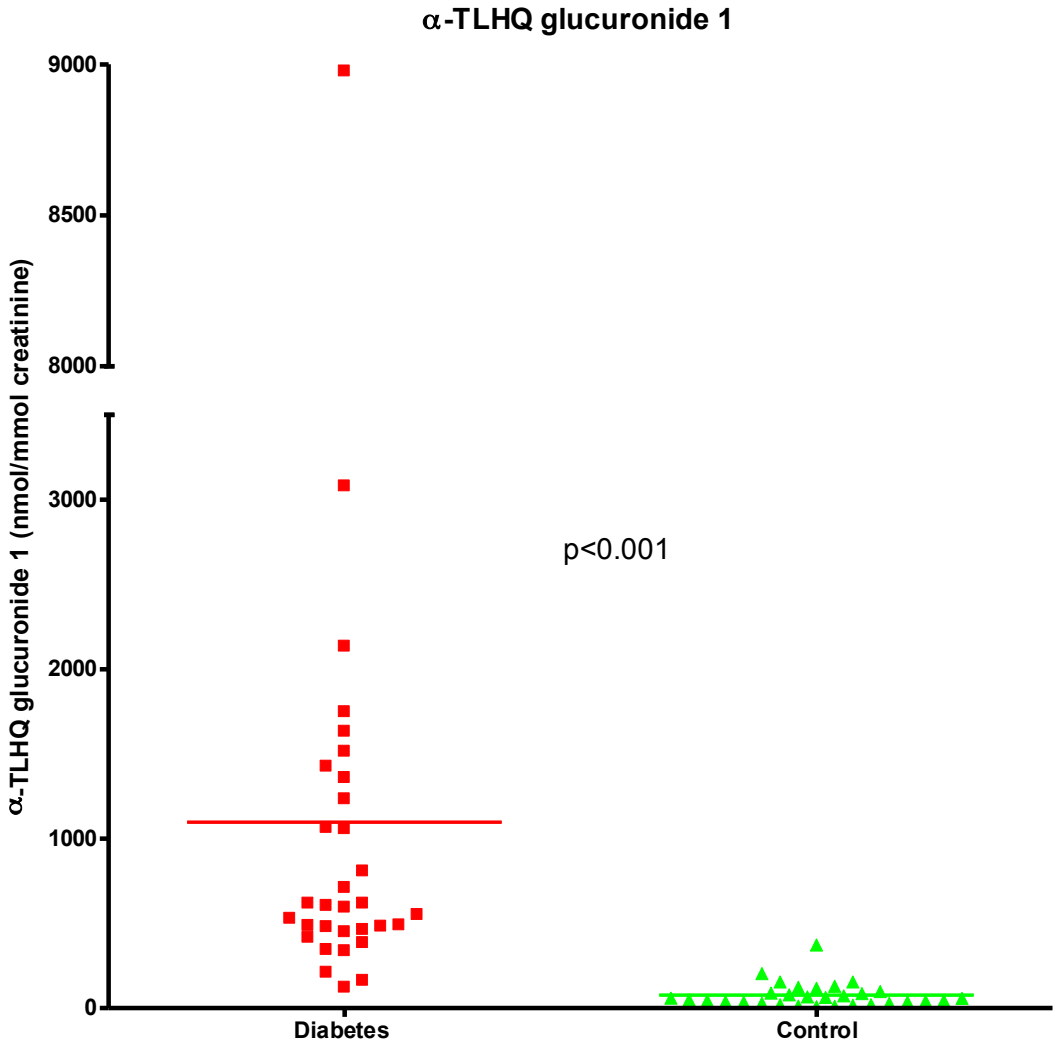


Fig.6.3. 1 α-TLHQ glucuronide 1 concentrations (nmol/mmol creatinine) in diabetic (1098±279) and control (76±13) urine, with p<0.001

The mean concentration of α-TLHQ glucuronide 2 was 562±166 in diabetic patients compared to 34±9 nmol/mmol creatinine in the age-matched controls with p<0.002 (Fig.6.3.2).

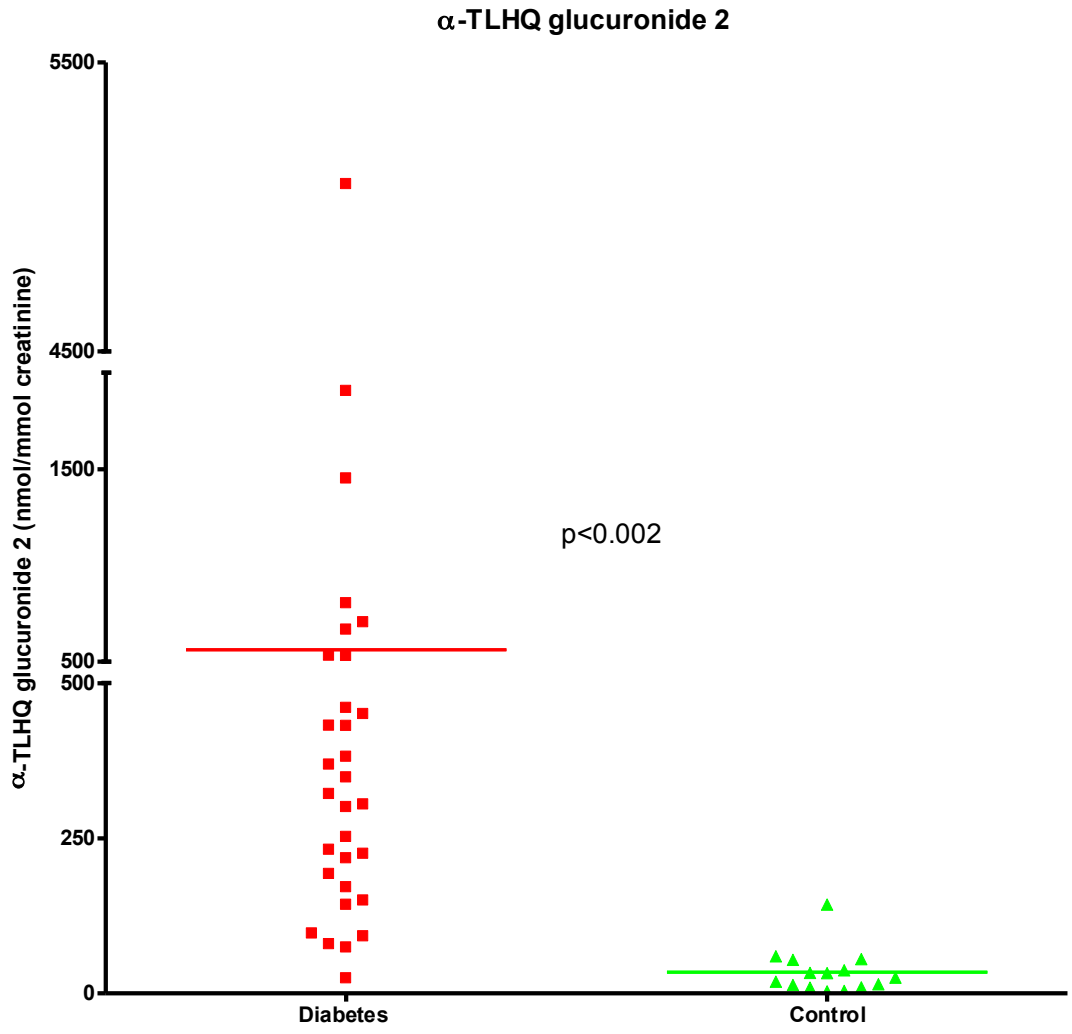


Fig.6.3. 2 α-TLHQ glucuronide 2 concentrations (nmol/mmol creatinine) in diabetic (562±166) and control (34±9) urine with p<0.002.

The mean concentration of α-TLHQ sulphate was 98±24 in diabetic patients compared to 10±2 nmol/mmol creatinine in the age-matched controls with p=0.001 (Fig.6.3.3).

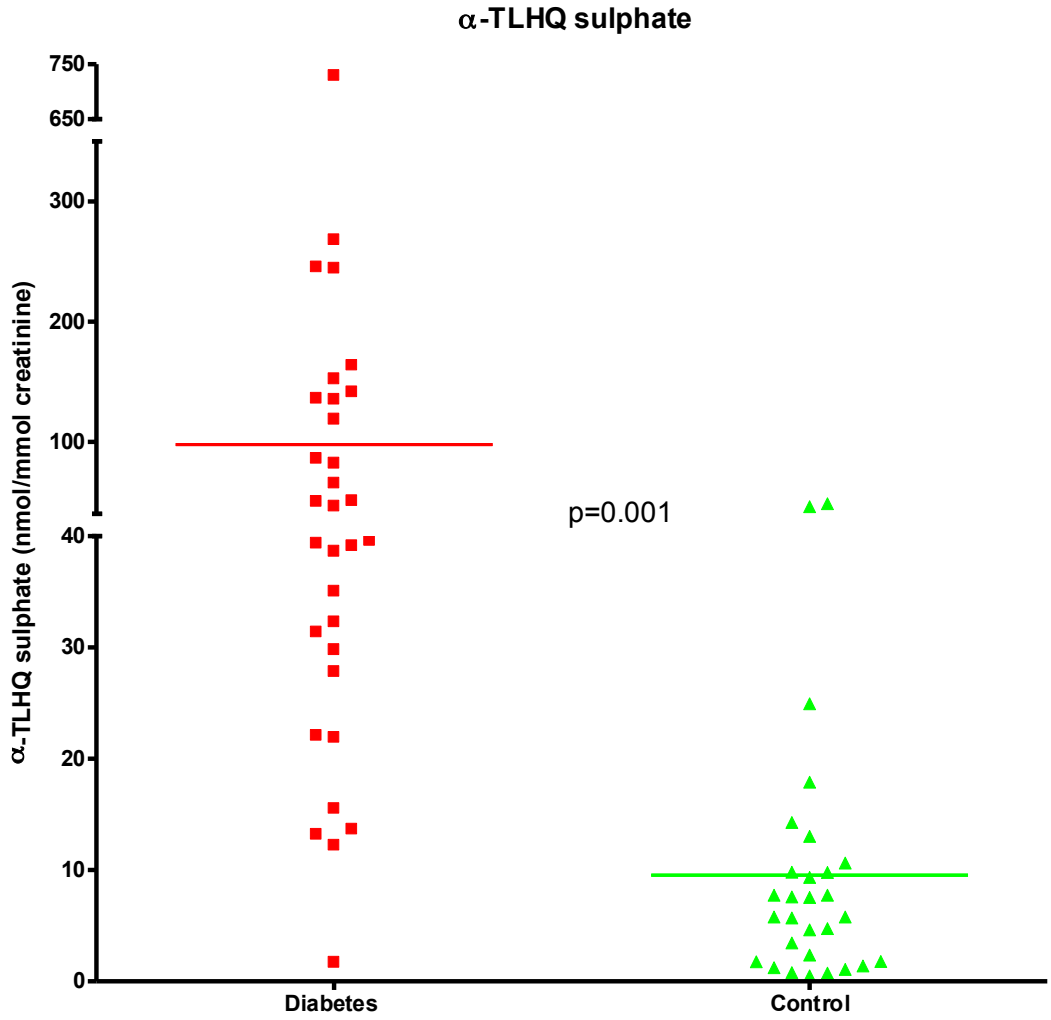


Fig.6.3. 3 α-TLHQ sulphate concentrations (nmol/mmol creatinine) in diabetic (98±24) and control (10±2) urine with p=0.001

The mean concentration of α-CEHC glucuronide was 126±16 in diabetic patients compared to 73±19 nmol/mmol creatinine in the age-matched controls with p<0.05 (Fig.6.3.4).

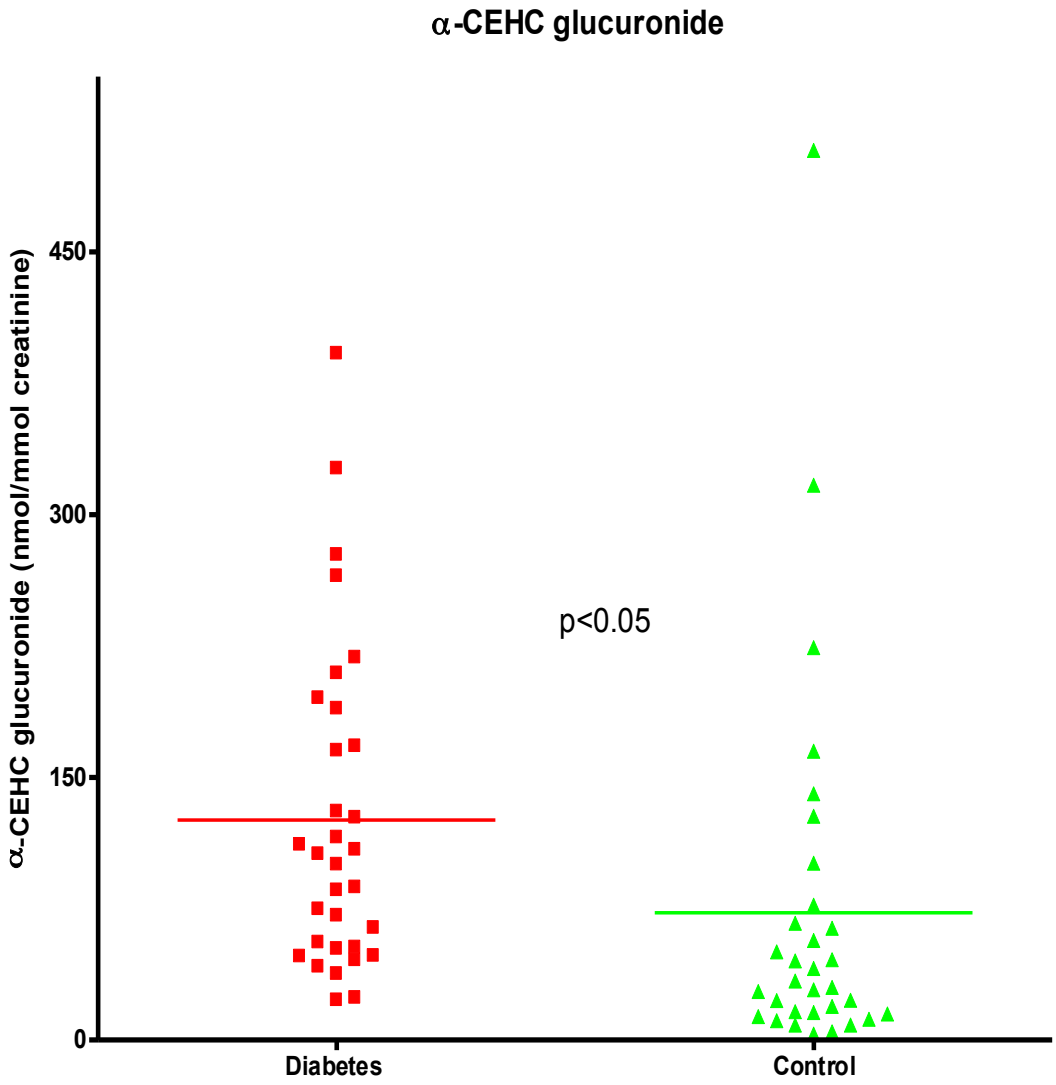


Fig.6.3. 4 α-CEHC glucuronide concentrations (nmol/mmol creatinine) in diabetic (126±16) and control (73±19) urine with p<0.05

The mean concentration of α-CEHC sulphate was 138±33 in diabetic patients compared to 57±12 nmol/mmol creatinine in the age-matched controls with $p<0.05$ (Fig.6.3.5).

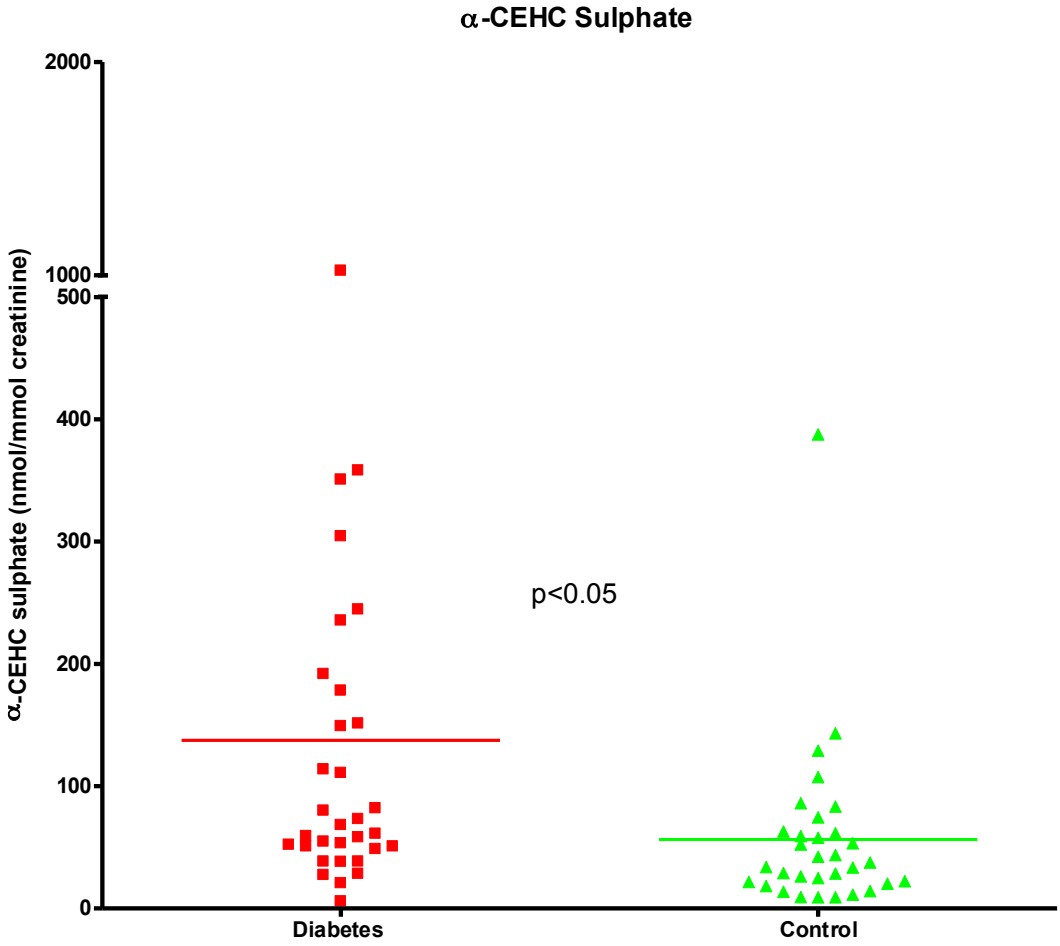


Fig.6.3. 5 α-CEHC sulphate (nmol/mmol creatinine) concentrations in diabetic (138±33) and control (57±12) urine with $p<0.05$

The urinary concentrations of the vitamin E metabolites are summarised in Table 6.3.1. The diabetic cohort excreted more vitamin E metabolites than the control subjects. The concentrations of the glucuronide metabolites were greater than the sulphates in both groups, but the concentrations of α-CEHC (glucuronide and sulphate) were similar within each cohort (Table 6.3.1), although there was more α-CEHC (glucuronide and sulphate) in diabetic group compared to controls. Lastly, the control subjects were also observed to excrete similar amounts of α-CEHC and α-TLHQ glucuronides. On the basis of these results it was decided to examine the concentrations of the metabolites expressed as ratios of their conjugates or total metabolites.

	Diabetic subjects N=32 nmol/mmol creatinine mean±SEM	Control subjects N=32 nmol/mmol creatinine mean±SEM	p value
α-TLHQ-glucuronide 1	1098±279	76±13	<0.001
α-TLHQ-glucuronide 2	562±166	34±9	<0.002
α-CEHC-glucuronide	126±16	73±19	<0.05
Total-glucuronide	1786	183	
α-TLHQ-sulphate	98±24	10±2	0.001
α-CEHC-sulphate	138±33	57±12	<0.05
Total-sulphate	236	67	

Table 6.3. 1 Summary of urinary concentrations of vitamin E metabolites

6.3. 2 Urinary vitamin E metabolites expressed as ratios

In an attempt to further compare the diabetic group with controls, vitamin E metabolites were also expressed in terms of either total sulphate or glucuronide conjugates (e.g. α-CEHC sulphate expressed as α-CEHC sulphate /α-CEHC sulphate + α-TLHQ sulphate) or total vitamin E metabolites (e.g. α-CEHC sulphate expressed as α-CEHC sulphate /α-CEHC sulphate + α-TLHQ sulphate + α-CEHC glucuronide + α-TLHQ glucuronide 1 + α-TLHQ glucuronide 2).

The results are summarised in Table 6.3.2. They show that all the ratios of the α-TLHQ metabolites were significantly increased in the diabetic subjects with the exception of α-TLHQ sulphate when expressed as ratios, where there was no significant difference. On the other hand all the ratios of the α-CEHC conjugates were significantly decreased in the diabetic subjects compared to the controls.

Variable	Diabetic subjects N=32 Ratio mean±SEM	Control subjects N=32 Ratio mean±SEM	p value
α-TLHQ glucuronide 1 / total glucuronide	0.61±0.03	0.49±0.04	0.02
α-TLHQ glucuronide 2 / total glucuronide	0.28±0.03	0.11±0.03	<0.0001
α-TLHQ glucuronide 1 / total vitamin E metabolites	0.52±0.03	0.36±0.03	<0.0005
α-TLHQ glucuronide 2 / total vitamin E metabolites	0.0002± 0.00003	0.00008± 0.00001	<0.0001
α-TLHQ sulphate/ total sulphate	0.40±0.03	0.15±0.02	<0.0001
α-TLHQ sulphate/ total vitamin E metabolites	0.06±0.01	0.04±0.01	NS
α-CEHC glucuronide/ total glucuronide	0.11±0.01	0.39±0.04	<0.0001
α-CEHC glucuronide/ total vitamin E metabolites	0.09±0.01	0.25±0.03	<0.0001
α-CEHC sulphate/ total sulphate	0.60±0.30	0.85±0.02	<0.0001
α-CEHC sulphate/ total vitamin E metabolites	0.08±0.02	0.26±0.03	<0.0001

Table 6.3. 2 The ratio of α-CEHC and α-TLHQ metabolites expressed as ratios of total sulphate/glucuronide and of total vitamin E metabolites in diabetic and age-matched controls.

6.3. 3 Urinary vitamin E metabolites in relation to glycated haemoglobin concentrations

The diabetic patients were divided into two groups according to their glycated haemoglobin concentrations, 6-7.5 HbA1c% (well controlled) and >7.5 HbA1c% (poorly controlled) respectively according to the NICE/NHS guidelines (section 6.1.2). A diagrammatic representation of the numbers in each group is shown in Fig. 6.3.6. The results for each vitamin E metabolite expressed per mmol creatinine are shown in Figs 6.3.7 to 6.3.11

The outliers could not be completely removed as the sample size is below 100 and the outliers were not the same sample in all the analysis (Barnett and Lewis 1994).

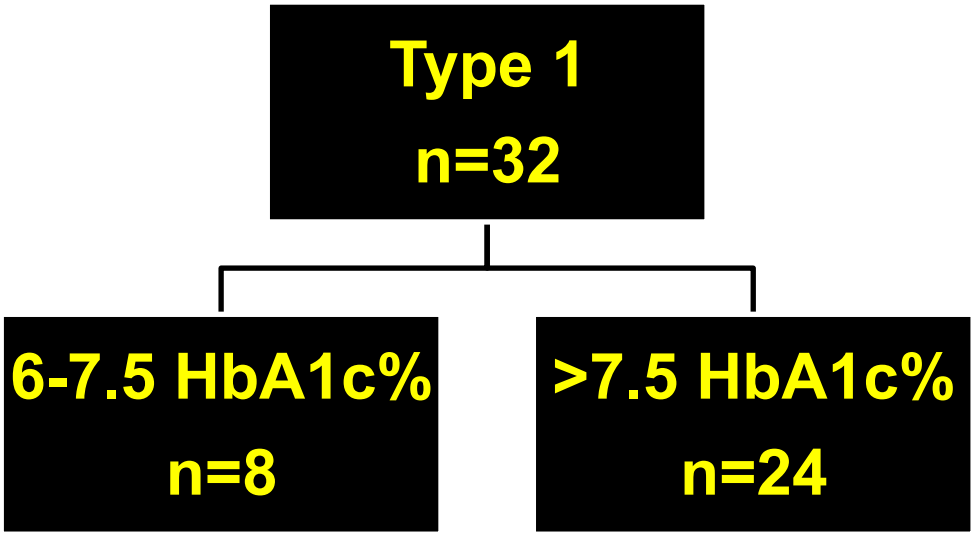


Fig.6.3. 6 Diabetic patients divided according to their glycated haemoglobin concentrations

The mean concentration (± 1 SEM) of α-TLHQ glucuronide 1 was 1119±356 in diabetic patients >7.5 HbA1c% and 1034±351 in patients with an HbA1c% of 6-7.5 compared to 76±13 nmol/mmol creatinine in the controls (Fig. 6.3.7) with p=001 and <0.001 respectively compared to the control group. There was no significant difference (NS) between the means of the two diabetic groups.

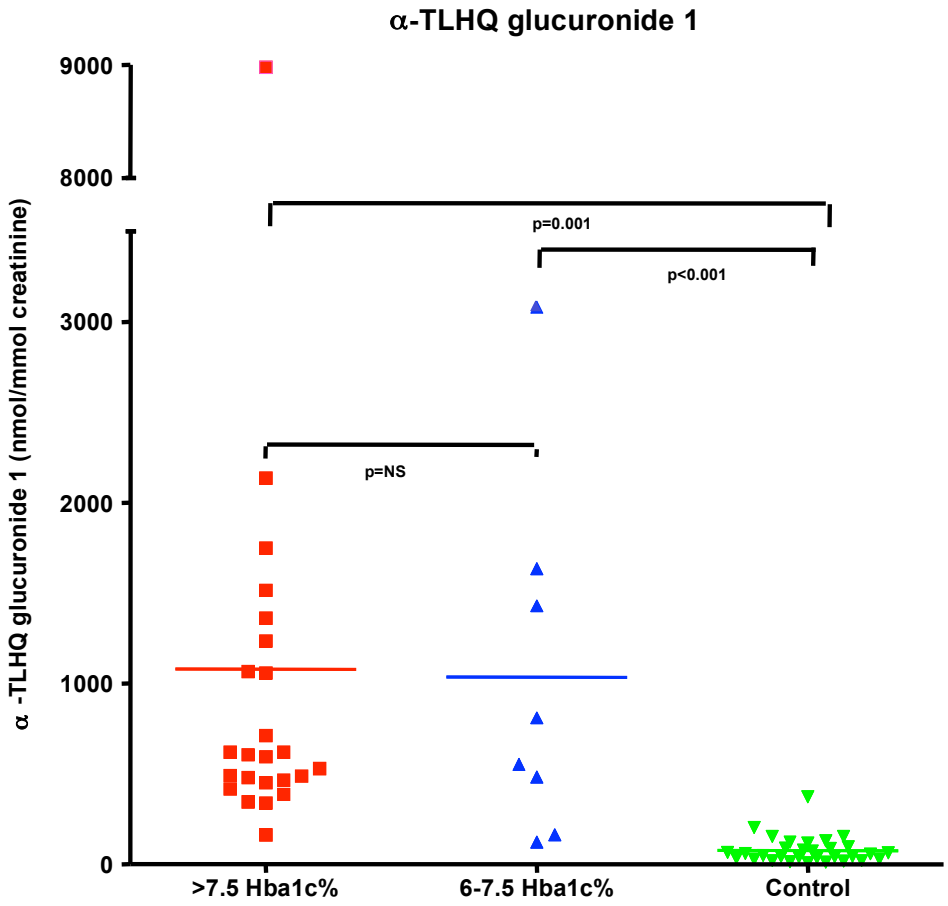


Fig.6.3. 7 Mean (± 1 SEM) α-TLHQ glucuronide 1 concentrations was 1119±356 in diabetic patients >7.5 HbA1c% and 1034±351 in patients with an HbA1c% of 6-7.5 and 76±13 nmol/mmol creatinine control urine with p=001 and <0.001 respectively compared to the control group. There was no significant difference (NS) between the means of the two diabetic groups

The mean concentration (± 1 SEM) of α-TLHQ glucuronide 2 was 443±91 in diabetic subjects >7.5 HbA1c % and 902±601 in subjects with an HbA1c% of 6-7.5 compared to 34±9 nmol/mmol creatinine in the controls (Fig. 6.3.8) with p=001 and p<0.002 respectively compared to the control group. There was no significant difference between the means of the two diabetic groups.

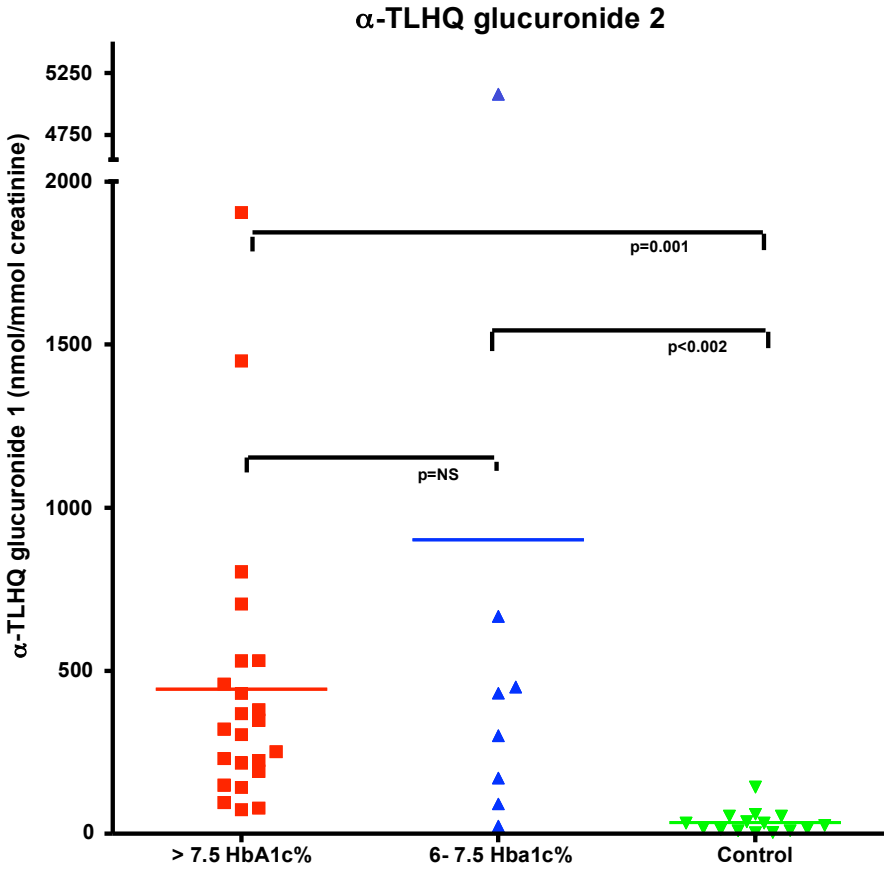


Fig.6.3. 8 Mean (± 1 SEM) α-TLHQ glucuronide 2 concentrations in diabetic 443±91 in diabetic subjects >7.5 HbA1c % and 902±601 in subjects with an HbA1c% of 6-7.5 compared to 34±9 nmol/mmol creatinine in control urine with p=001 and p<0.002 respectively compared to the control group. There was no significant difference between the means of the two diabetic groups

The mean concentration (± 1 SEM) of α-TLHQ sulphate was 67±57 in diabetic subjects >7.5 HbA1c% and 188±86 in patients with an HbA1c% 6-7.5 compared to 10±2 nmol/mmol creatinine in the age-matched controls (Fig. 6.3.9) with p<0.0001 in both the groups compared to the control group. Subjects with HbA1c% 6-7.5 were found to have a significantly higher concentration of the metabolite compared to patients with >7.5 HbA1c% (p<0.05).

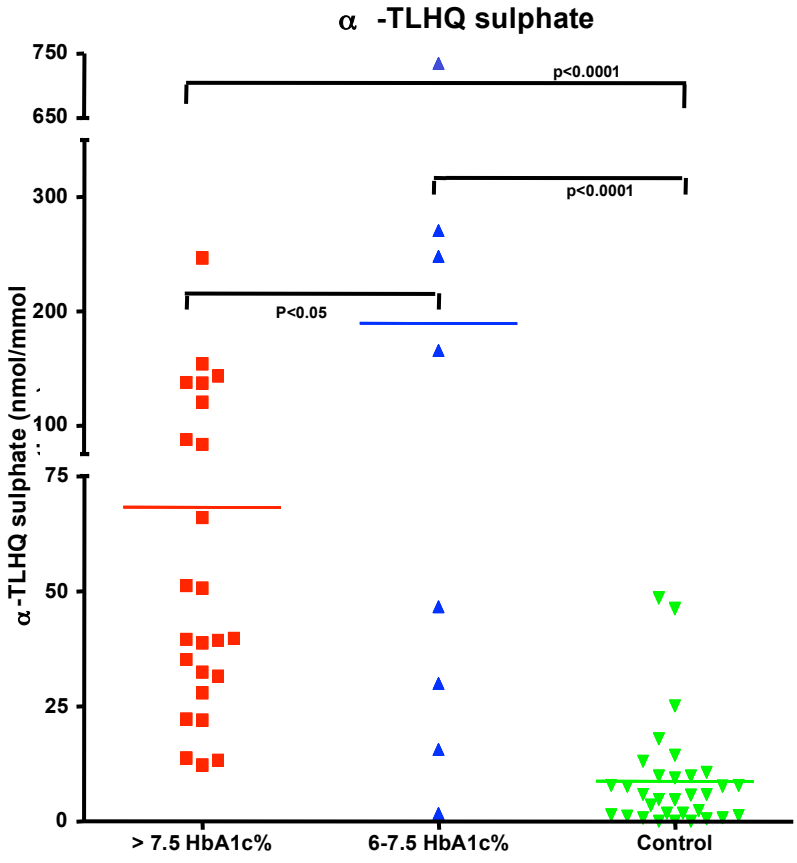


Fig.6.3. 9 Mean (± 1 SEM) α-TLHQ sulphate concentrations in diabetic was 67±57 in diabetic subjects >7.5 HbA1c% and 188±86 in patients with an HbA1c% 6-7.5 compared to 10±2 nmol/mmol creatinine in the age-matched controls with p<0.0001 in both the groups compared to the control group

The mean concentration (± 1 SEM) of α-CEHC glucuronide was 127±17 in diabetic patients >7.5 HbA1c % and 122±41 in patients with an HbA1c% 6-7.5 compared to 73±19 nmol/mmol creatinine in the age-matched controls (Fig. 6.3.10). There were no significant differences between any of the groups.

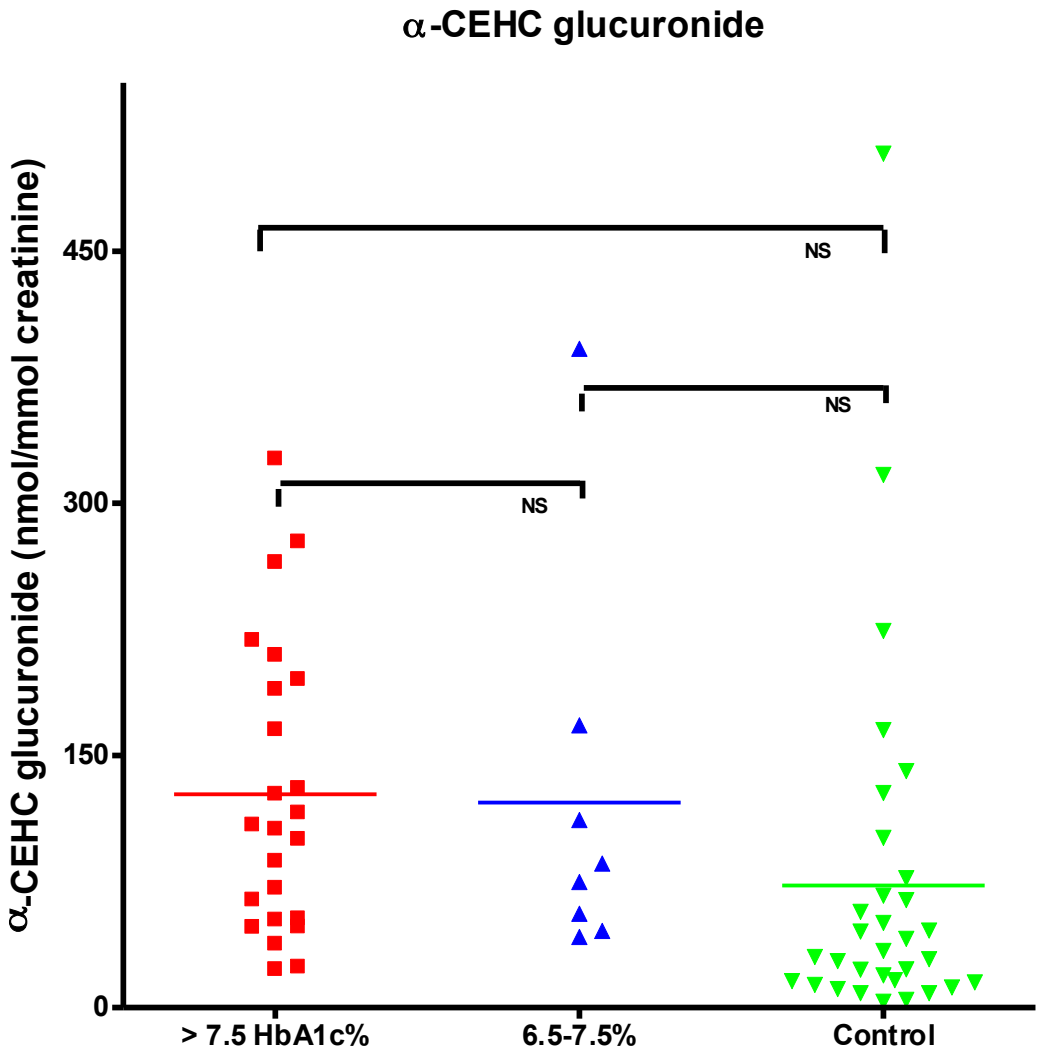


Fig.6.3. 10 Mean (± 1 SEM) α-CEHC glucuronide concentrations in127±17 in diabetic patients >7.5 HbA1c % and 122±41 in patients with an HbA1c% 6-7.5 compared to 73±19 nmol/mmol creatinine in control urine. There were no significant differences between any of the groups

The mean concentration (± 1 SEM) of α-CEHC sulphate was 90±13 in diabetic subjects >7.5 HbA1c% and 311±131 in patients with an HbA1c% of 6-7.5 compared to 57±12 nmol/mmol creatinine in the age-matched controls (Fig. 6.3.11). The difference between the subjects with HbA1c% of 6-7.5 was significantly increased ($p<0.0001$) compared to controls whereas there was no significant difference in the patient with >7.5 HbA1c%. Patients with HbA1c% of 6-7.5 were observed to have a significantly higher concentration of the metabolite compared to patients with >7.5 HbA1c% ($p<0.005$).

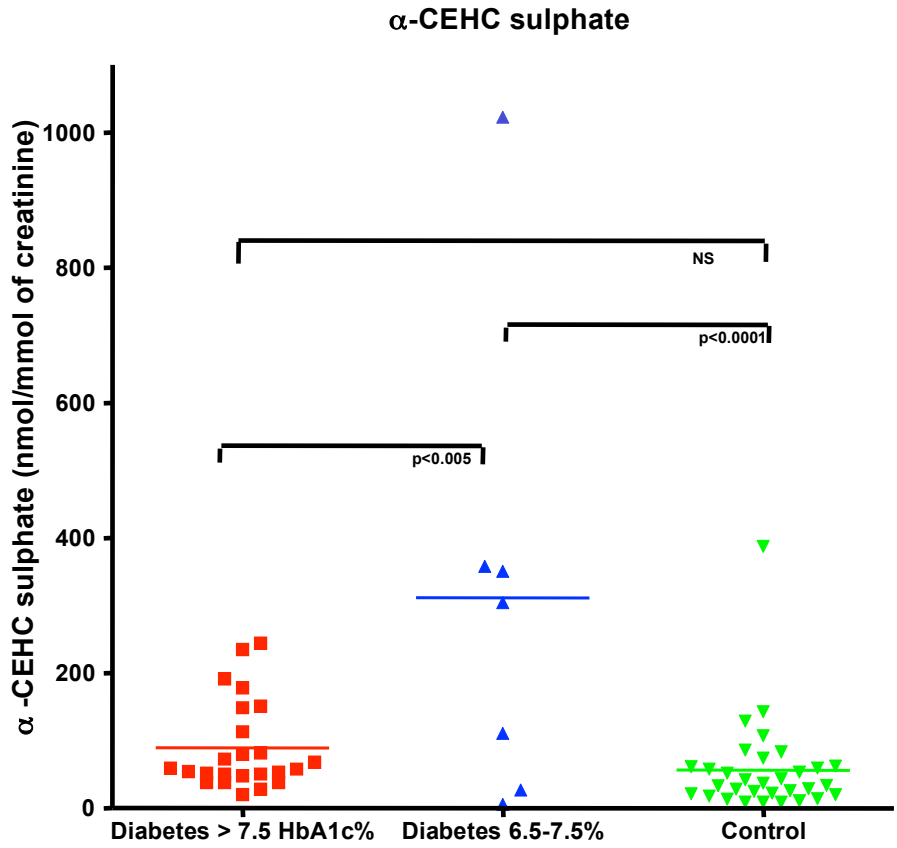


Fig.6.3. 11 Mean (± 1 SEM) α-CEHC sulphate concentrations in diabetic 90±13 in diabetic subjects >7.5 HbA1c% and 311±131 in patients with an HbA1c% of 6-7.5 compared to 57±12 nmol/mmol creatinine in the age-matched controls. Diabetics with HbA1c% of 6-7.5 were observed to have a significantly higher concentration of the metabolite compared to patients with >7.5 HbA1c% ($p<0.005$)

Table 6.3.3 summarises the statistical significance between the diabetic patients divided according to their HbA1c% and the control subjects. It also includes the statistical difference between the two diabetic groups. A point to be noted is that the outlier in the various analyses was not the same subject for all the metabolites.

Variable (nmol/mmol of creatinine)	**All Diabetics N=32 p value**	**6-7.5 HbA1c% N=8 p value**	**>7.5 HbA1c% N=24 p value**	**Inter-Diabetic analysis p value**	**Inter-Diabetic Analysis (-outlier) p value**
α-TLHQ glucuronide 1	<0.001	<0.001	0.001	NS	NS
α-TLHQ glucuronide 2	<0.002	<0.002	0.001	NS	NS
α-TLHQ sulphate	0.001	<0.0001	<0.0001	<0.05	NS
α-CEHC glucuronide	<0.05	NS	NS	NS	NS
α-CEHC sulphate	<0.05	<0.0001	NS	<0.005	<0.05

Table 6.3. 3 Significance of difference between the various groups of diabetic subjects and controls **(NS=not significant)**

6.3. 4 Quantitation of urinary amino acids involved in nitric oxide metabolism in diabetics and controls

The amino acids involved in nitric oxide ($NO^{\bullet}$) metabolism were quantitated per mmol creatinine in 32 patients with type 1 diabetes mellitus and 32 age- and sex-matched controls (Figures 6.3.12-15).

The diabetic patients were observed to have statistically significantly increased concentrations of all the amino acids as compared to the controls.

The mean concentration (± 1 SEM) of L-arginine was 6.68±1.22 μmol/mmol creatinine in diabetic patients compared to 0.25±0.03 μmol/mmol in age-matched controls $p<0.0001$ (Fig. 6.3.12). This equates to a greater than 26-fold increase of the mean urinary concentration of L-arginine between the two groups.

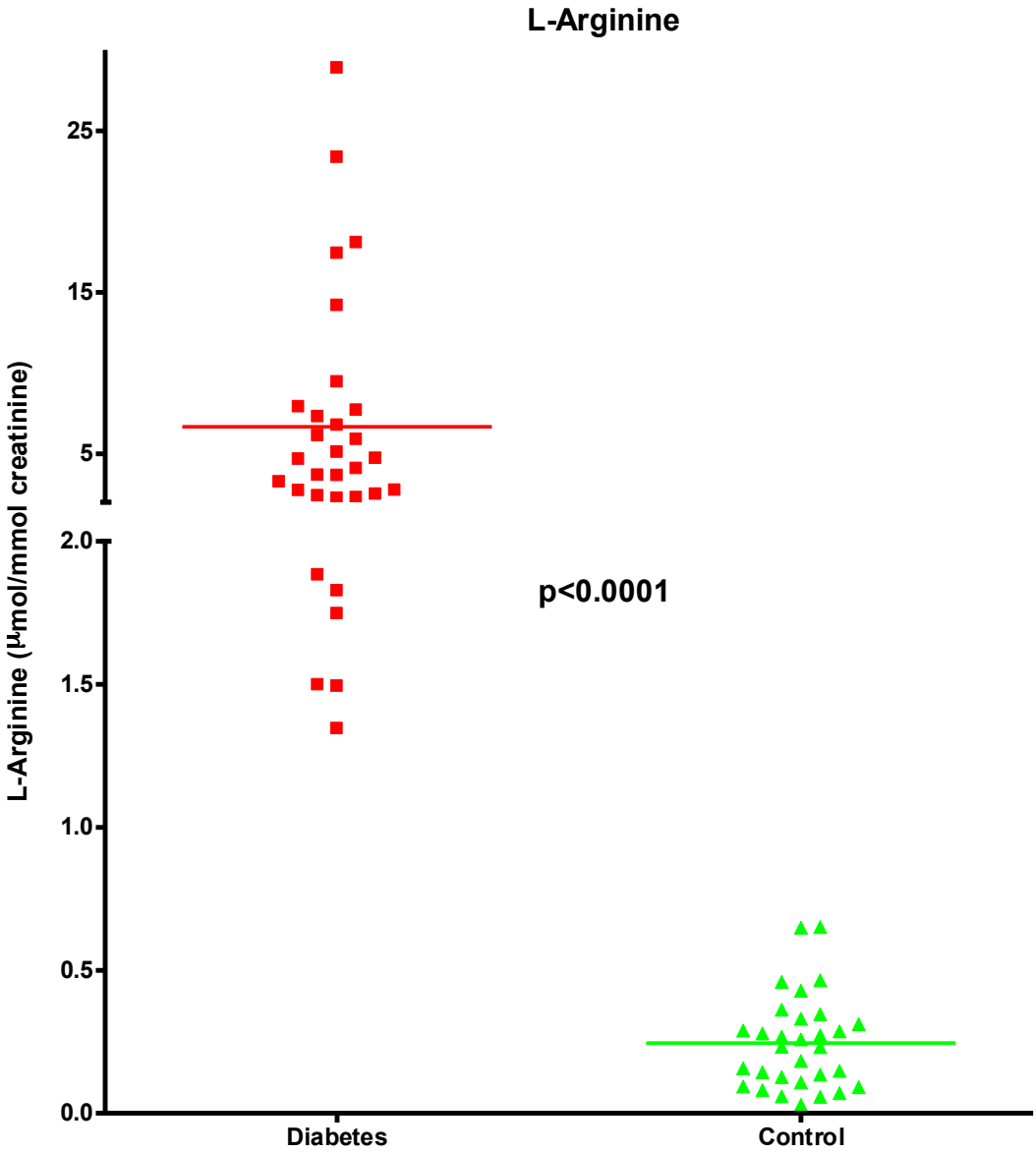


Fig.6.3. 12 Mean (± 1 SEM) L-arginine concentrations was 6.68±1.22 diabetic patients compared to 0.25±0.03 µmol/mmol in age-matched controls (p<0.0001)

In the case of L-citrulline, the diabetic patients had a mean concentration (± 1 SEM) of 1.83±0.24 compared to 0.06±0.01 µmol/mmol per creatinine in the age-matched controls with p<0.0001 (Fig. 6.3.13). This equates to a greater than 30- fold increase of the mean urine concentration of L-citrulline between the two groups.

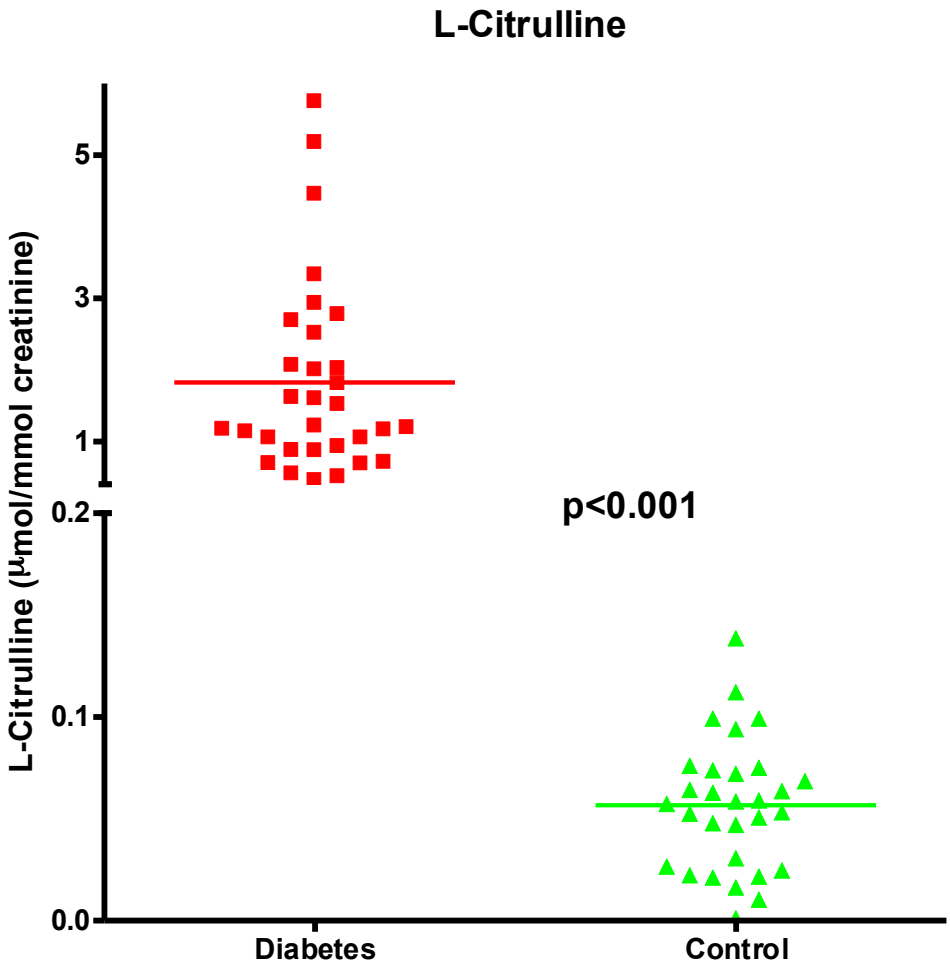


Fig.6.3. 13 Mean (± 1 SEM) L-citrulline concentratons 1.83±0.24 in diabetic compared to 0.06±0.01 µmol/mmol per creatinine in the age-matched controls with p<0.0001

The mean concentration (± 1 SEM) of L-ADMA (asymmetric dimethyl arginine) was 11.38±1.78 µmol/mmol per creatinine in diabetic patients and 0.39±0.05 in the age-matched controls, p<0.0001 (Fig. 6.3.14). This equates to almost a 30-fold increase of the mean urine concentration of L-ADMA between the diabetic and control groups.

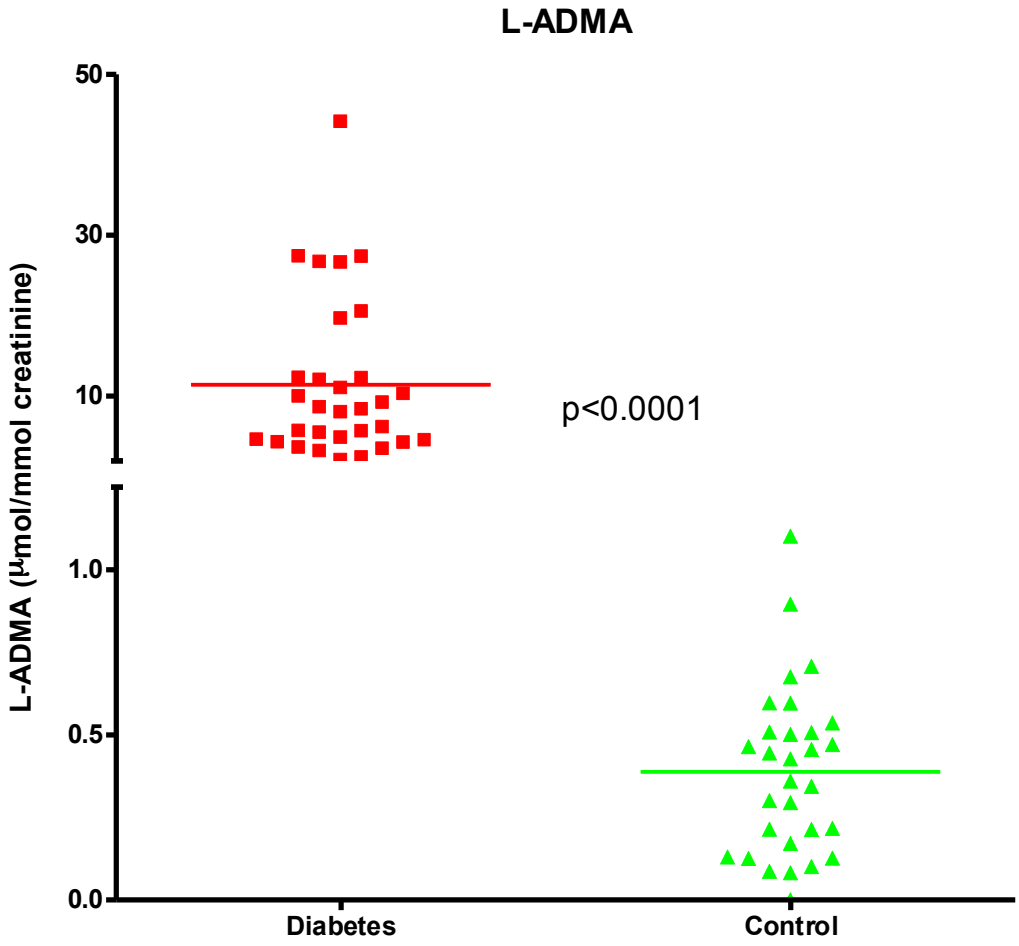


Fig.6.3. 14 Mean (± 1 SEM) L-ADMA concentrations 11.38±1.78 in diabetic patients and 0.39±0.05 µmol/mmol per creatinine in the age-matched controls with p<0.0001

In the diabetic patients the mean concentration (± 1 SEM) of homocysteine was 12.11±2.5 µmol/mmol per creatinine as compared to 0.24±0.05 in the age-matched controls, p<0.0001 (Fig. 6.3.15). This equates to a greater than 50-fold increase of the mean urine concentration of L-homocysteine between the diabetic and control groups.

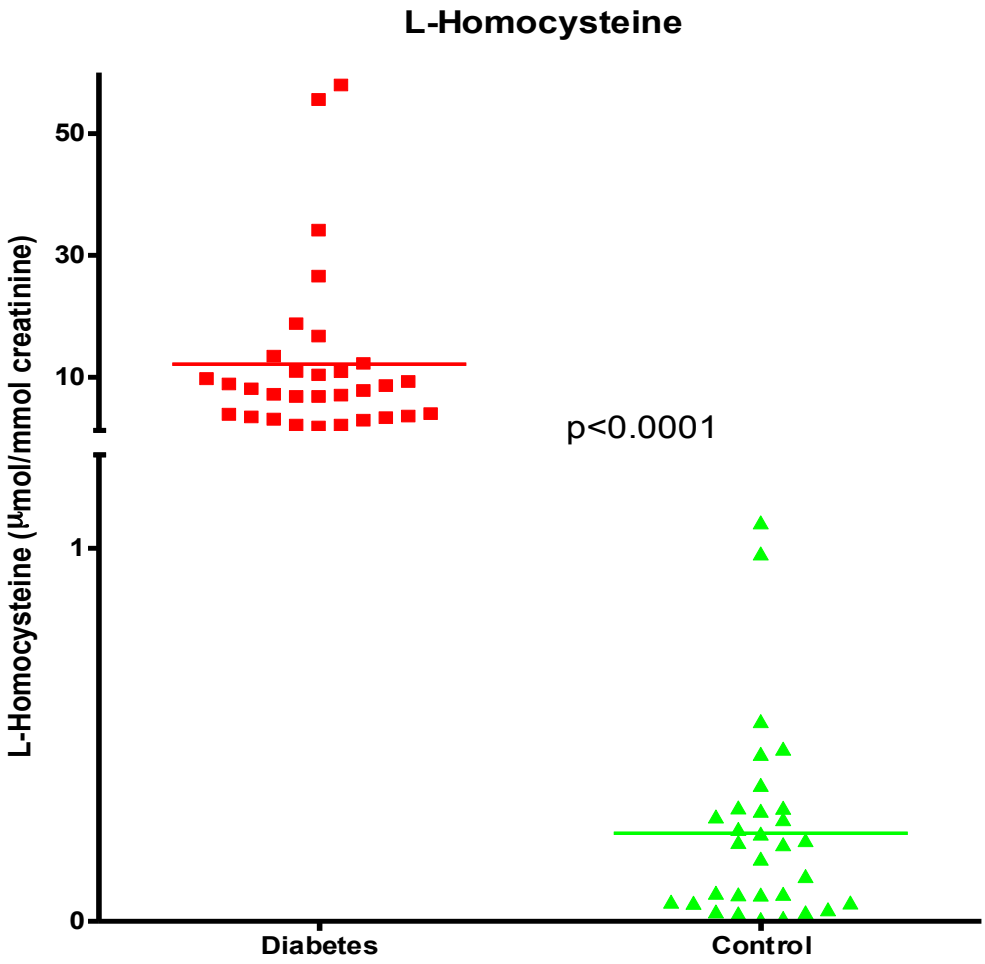


Fig.6.3. 15 Mean (± 1 SEM) L-homocysteine concentrations in was 12.11±2.5 and 0.24±0.05 µmol/mmol per creatinine in the age-matched controls with p<0.0001

6.3. 5 Quantitation of the other urinary amino acids in diabetic and control subjects

Seven additional amino acids, were also quantitated to evaluate their excretory pattern in type 1 diabetes mellitus. The results are summarised in Table 6.3.4 and illustrate that all the amino acids were significantly increased ($p<0.0001$) in the diabetic patients compared to the control subjects.

Amino acids (µmol/mmol of creatinine)	Diabetes (mean±SEM)	Control (mean±SEM)	p value
Cysteine	296±46	11±1	<0.0001
Cystine	161±20	4±0.5	<0.0001
Phenylalanine	46±10	0.8±0.1	<0.0001
Taurine	144±27	2±0.3	<0.0001
Serine	73±16	1±0.2	<0.0001
Glycine	32±6	0.8±0.1	<0.0001
Glutamic acid	9±1	0.3±0.04	<0.0001

Table 6.3. 4 The concentrations of amino acids in diabetic patients and age-matched controls.

6. 4 Discussion

Oxidative stress has been implicated in the pathogenesis of a number of clinical conditions including diabetes where there are reports of an increased production of reactive oxygen species and depleted concentrations of antioxidants (detailed in Section 6.1). There is, therefore a need for biomarkers of oxidative stress in conditions such as diabetes. Established biomarkers of oxidative stress such as malondialdehyde (MDA) have problems with specificity (Asakawa- Matsushita 1980) and accuracy i.e.over-estimation (Khoschsorur 2000), whereas isoprostanes, especially 8-iso-$PGF_{2\alpha;}$ which is considered the gold standard of measuring oxidative stress *in vivo* requires a laborious sample preparatory step and large volumes of sample to quantitate the metabolite (Sircar & Subbaiah 2007). In this study, we developed a rapid LC-MS/MS method to investigate if the urinary vitamin E metabolite, conjugated α-TLHQ could be used as a biomarker of oxidative stress in type 1 diabetes.

Diabetes induced endothelial dysfunction has been associated with reduced bioavailability of nitric oxide $NO^{\bullet}$ (discussed in Section 6.1.6), and therefore the second aim of the study was to measure urinary amino acids involved in $NO^{\bullet}$ metabolism. The method also allowed the measurement of seven additional amino acids- each representing a different group of amino acids.

Having established a simple, fast and reproducible assay for directly quantitating the conjugated vitamin E metabolites (chapter 4), we showed unequivocally for the first time that conjugates of α-TLHQ were real metabolites and not artefacts of the methodological procedure. Thus it was possible to investigate the principal aim of the current study –to see if conjugated α-TLHQ could be used as a biomarker of oxidative stress. The hypothesis put

forward in this study was that the concentrations of α-TLHQ (sulphate and glucuronide) would be higher in diabetic subjects compared to age-matched controls, with less change in the concentrations of conjugated α-CEHC.

The total urinary concentrations of all the metabolites (sulphate and glucuronide) were found to be greater by factor of approximately 8 in the diabetic children than their age-matched controls (Table 6.4.1), with the concentrations of α-TLHQ (glucuronide and sulphate) being approximately 15- times greater and the total α-CEHC (glucuronide and sulphate) being twice that of the control subjects (Table 6.4.1). The ratio of α-TLHQ: α-CEHC was approximately 7 in the diabetic cohort compared to 0.9 in the controls. When the individual metabolites were compared in the two groups, the mean concentrations of the conjugated α-TLHQ metabolites in the diabetic subjects were all highly significantly increased to the same degree of significance ($p<0.05$). This suggests that these results cannot be explained by a non-specific increase in urinary excretion of the conjugated metabolites. Although plasma concentrations α-tocopherol were not compared in the two groups, these results suggest an increased metabolism of vitamin E in diabetes compared to controls with oxidation of α-tocopherol being more prominent than chain shortening. This is compatible with an increase in oxidative stress in diabetes and suggests that conjugated α-TLHQ may be a useful biomarker.

Essentially similar results were obtained when the diabetic subjects were divided on the basis of their glycosylated haemoglobin concentrations (HbA1c%) into those who were poorly and well controlled. There was no evidence from this study that the poorly controlled subjects had an increased oxidative stress. To examine this further it will be necessary to follow diabetic subjects longitudinally and compare urinary concentrations of

conjugated α-TLHQ with other well recognised measures of diabetic control, such as HbA1c% and urinary albumin concentrations.

	Diabetic subjects mean nmol/mmol creatinine	**Control subjects mean nmol/mmol creatinine**	**Ratio Diabetic:Control**
α-TLHQ-glucuronide 1	1098	76	14.45
α-TLHQ-glucuronide	562	34	16.53
α-CEHC-glucuronide	126	73	9.8
α-TLHQ-sulphate	98	10	1.73
α-CEHC-sulphate	138	57	2.42
Total metabolites	2022	250	8.09
Total α-TLHQ	1758	120	14.65
Total α-CEHC	264	130	2.03
Total glucuronide	1786	183	9.76
Total sulphate	236	67	3.52

Table 6.4. 1 Summary of Vitamin E metabolites

The concentrations of the glucuronide metabolites were greater than the sulphates in both the cohorts, with the glucuronide:sulphate ratio being approximately 8 in diabetics and 3 in the controls. This was expected as humans have a higher capacity for glucuronidation than sulphation (Gibson & Skett 1994) due to a high activity of the enzyme UDP-glucuronyl

trasnsferase enzyme in the liver (Mulder 1992). Similar observations of preferential glucuronidation of the vitamin E metabolites were made by Pope when he conducted LC-MS/MS analysis on normal human urine (Pope 2001).

In summary, the results obtained in this study strengthen the hypothesis put forward by Liebler *et al.,* (1996) and Schonfeld *et al.,* (2006) that α-TLHQ could be an indicator/biomarker of oxidative stress, but the nature of the relationship between clinical severity of diabetes to oxidative stress status would require further investigation which was beyond the scope of this study.

Increases in the concentrations of other biomarkers of oxidative stress have been previously observed in subjects with diabetes by a number of researchers. Thus Dandona *et al.,* (1996) observed an approximately four- fold higher concentration of 8-hydroxy-2'-deoxyguanosine in mononuclear cells of diabetic patients compared to corresponding controls. This difference was statistically significant and demonstrated for the first time there was greater oxidative damage to DNA in diabetic patients. Davi *et al.,* (1999b) were the first group to demonstrate that the increased 8-epi-$PGF_{2\alpha}$ observed in both type 1 and type 2 diabetic patients could be normalised by vitamin E supplementation. Leonhardt *et al.,* (1996) had reported previously elevated levels of oxidised LDL and decreased concentrations of RRR-α- tocopherol in the plasma of diabetic patients when compared to healthy controls; making a case for investigating the role of vitamin E metabolites in diabetes. Studies of oxidative stress and vitamin E in diabetes have been detailed in section 6.1.5.

Patients with Type 1 diabetes have a two- to four- fold increased risk of vascular disease, and vasculopathy is the principal cause of death in these patients (Wotherspoon 2003). Vascular tone is influenced by a number of vasoactive substances produced by the endothelium, including vasodilators such as $NO^{\bullet}$ (Boger 1998b). In diabetes, endothelium dysfunction is thought to be primarily caused by a hyperglycaemia induced increase in reactive oxygen species production and a resultant reduction in $NO^{\bullet}$ formation (Devaraj 2006). This has been illustrated schematically in Fig.6.4.1. As a result of increased concentrations of reactive oxygen species, $NO^{\bullet}$ formation by NOS is inhibited by the increased concentrations of L-homocysteine and L-ADMA (section 5.1.5), which results in an increased concentration of L-arginine (substrate) and a lower concentration of citrulline (product).

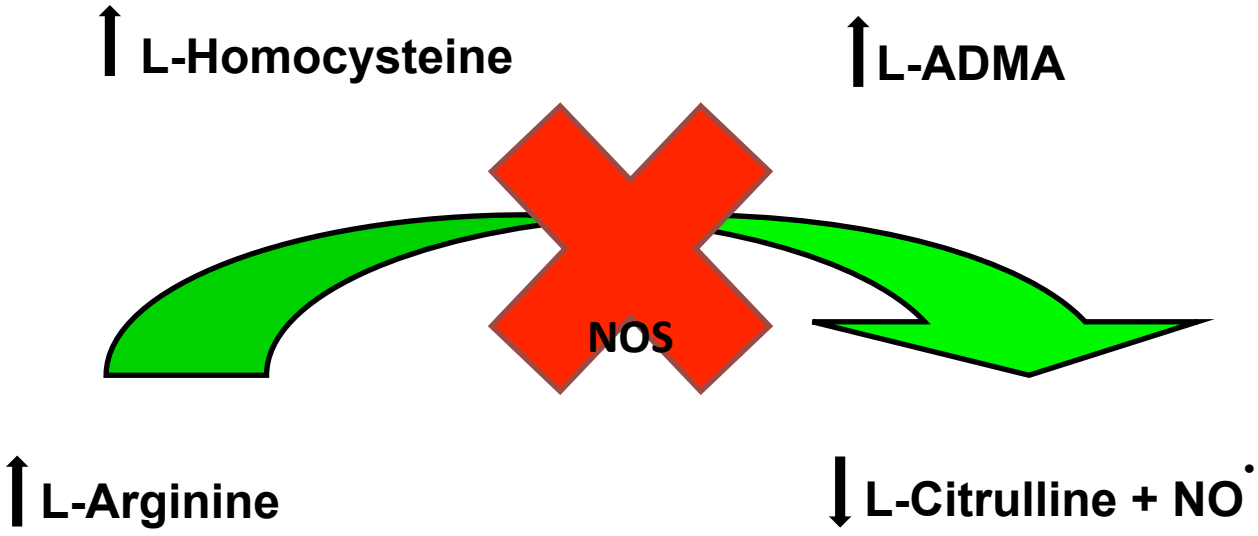


Fig. 6.4. 1 Inhibition of NO• formation by L-homocysteine and L-ADMA

Thus an assay was established (Chapter 5) to quantify the amino acids associated with $NO^{\bullet}$ metabolism (L-arginine, L-citrulline, L-ADMA and L-homocysteine). In addition seven other amino acids (each representing a different group of amino acids) were also quantified to evaluate their excretory pattern in type 1 diabetic patients. The urinary concentrations of the amino acids associated with $NO^{\bullet}$ metabolism were found to be highly significantly increased in diabetic patients compared to their age-matched controls (section 6.3.4). The increase in the urinary concentrations of L-arginine, L-ADMA and L-homocysteine in the diabetic patients was not an unexpected observation as it would follow the hypothesis of a decrease of $NO^{\bullet}$ formation by the action of L-ADMA and L-homocysteine on NOS. But the observation of an increased urinary concentration of L-citrulline and the seven additional amino acids (section 6.3.5) in the diabetic patients was unexpected.

The increase in the L-citrulline concentration could be attributed to the degradation of L-ADMA by dimethylarginine dimethylaminohydrolase (L-DDAH) to citrulline (Macallister 1996a), even though Lin *et al.,* (2002) observed *in vitro* that hyperglycaemic endothelial cells inhibit the action of L-DDAH. However, there is no current information of a human study that has documented the urinary or plasma concentration of citrulline in relation to the activity of L-DDAH.

Pereira *et al.,* (2008) observed that the plasma concentrations of L-arginine and L-ADMA were significantly higher in diabetic patients as compared to age- matched controls and suggested the use of plasma levels of L-arginine and L-ADMA as markers of oxidative stress and endothelial dysfunction in diabetes mellitus. Wotherspoon and co-workers (2006) reported a significant increase in plasma homocysteine levels in type 1 diabetic patients with microalbuminuria and suggested that homocysteine could act as an

independent marker of oxidative stress and endothelial dysfunction in type 1 diabetic patients.

The explanation for the generalised increased urinary excretion of the amino acids in the diabetic patients is likely to be multifactoral and there are at least three possible explanations for this observation. The most probable hypothesis for the loss of amino acids is due to the development of "early stage progression of nephritis" in the diabetic patients. Diabetic nephritis is characterised by thickening of the glomerulus and results in microalbuminuria, which is considered to be the first clinical marker of the condition, which increases as the disease progresses (American Diabetes Association 2009). The results of the present study suggest that prior to the microalbuminuria stage there is a loss of amino acids. This could result from an increased concentration of advanced glycated end products (AGE) or fluctuating blood pressure within the normal range (120-140/80-90 mmHg) in the diabetic cohort. The role of AGE in the progression of nephropathy is well documented in both animal (Hammes 1991;Nakamura 1997;Soulis-Liparota 1991) and human studies (Genuth 2005). We propose that AGE via production of reactive oxygen species (ROS), acts on integrins and causes structural modification of intracellular proteins (Brownlee 2001), thus causing structural changes to the glomerulus cells which may result in the loss of amino acids.

Drummond & Mauer (2002) suggested that variations of blood pressure in the normal range in a largely normoalbuminuric cohort of young type 1 diabetic patients caused diabetic glomerulopathy lesions. Studies in diabetic rats suggested that an increase in systemic blood pressure was associated with an increased width of the glomerular basement membrane (GBM) which was the principal morphometric abnormality in the kidney (Miller

1991). However, the patients in the study of Drummond and Mauer (2002) had a greater width of the glomerular basement membrane despite lower blood pressure. They proposed that systemic blood pressure may be linked causally to diabetic nephrotic lesions. They suggested this link could be direct, through effects of systemic blood pressure on glomerular hemodynamics, or indirect, through genes linked to the propensity to develop essential hypertension, but the exact explanation for these findings is still unkown.

The second hypothesis put forward is "proximal tubule damage". The amino acids are reabsorbed in the kidney in the proximal tubule (detailed later), and it is proposed that damage to the proximal tubule could explain the loss of amino acids in the urine of the diabetic children. Proximal tubule damage could be caused by the increased concentrations of AGE and the fluctuating blood pressure (as discussed above). Data to support this hypothesis has been obtained by urinary proteomic studies performed in our laboratory by Mills *et al.,* on the same sample set, which showed the loss of lysosomal proteins (unpublished observations). There are high levels of lysosomes in the proximal tubules and this could indicate damage to the proximal tubule in addition to the reduced filtration effect.

The third hypothesis to explain the high urinary excretion of the amino acids in the diabetic patients is based on the theory of "disturbed re-absorption of the amino acids in the proximal tubule in the kidney". Frohnert *et al.,* (1969) showed that the reabsorption of the amino acids occured in the brush border of the proximal tubule where the amino acids are bound specifically. Four amino acid transport systems have been described, which are neutral, basic, acidic and iminoglycine systems (Lewy & Windhager 1968). Young & Freedman (1971) observed that the renal transport of amino acids was influenced by the presence of glomerular filtrate and the renal capillary blood. Studies *in vivo* have

demonstrated that glucose infusions, impaired the renal absorption of amino acids (Drummond et al. 1964). Thier *et al.,* (1964) observed *in vitro* in the rat-kidney-cortex that the four transport systems were in a single site, which was the same site as that for glucose reabsorption. Glucose has also been shown to interact with amino acids in renal transport mechanisms in dogs and inhibit their absorption in the kidney (Webber 1961). The basis of the inhibition of amino acid re-absorption by glucose is due to their common dependence on a sodium-dependent ATPase in the proximal tubule that provides the energy for the transport systems (Reiser & Christiansen 1969).

To gain a better understanding for the unexpectedly increased urinary concentrations of all the amino acids measured it will be necessary to undertake longitudinal studies of plasma and urinary amino acid concentrations in diabetes and correlate the findings with routine measures such as Hba1c%, glomerular filtration rates and urinary albumin concentrations.

Chapter 7

Summary conclusions and limitations of the study

7 Summary conclusions and limitations

Oxidative stress has been implicated in a range of acute and chronic diseases but frequently it is unclear whether this is the cause or a result of the underlying condition. One of the problems in the field is a lack of reliable but relatively quick and simple methods to measure oxidative stress in man. In this study three potential assays of oxidative stress were investigated i.e. urinary prostaglandin, urinary α-tocopheronolactone (vitamin E metabolite) and urinary amino acids associated with nitric oxide metabolism. The aim of the project was to apply these assays to children with type 1 diabetes.

Initially, the aim was to establish and validate an LC-MS/MS method to measure concentrations of urinary isoprostanes (8-isoPGF$_{2\alpha}$), which are widely accepted as reliable indicators of oxidative stress (Milne & Morrow 2006). The most widely used method to measure isoprostanes uses gas chromatography–negative ion chemical ionization–mass spectrometry (GC-NICI-MS) with a stable isotope of d_4-8-iso-PGF$_{2\alpha}$ as an internal standard (Morrow, Minton, Badr, & Roberts 1994). The main advantage of this technique over other approaches is its high sensitivity, which yields quantitative results in the low picogram range. The method has been used to measure the metabolite in low quantities in urine (Obrosova, Van Huysen, Fathallah, Cao, Green, & Stevens 2002) and plasma (MacRury, Gordon, Wilson, Bradley, Gemmell, Paterson, Rumley, & MacCuish 1993). The major drawback of this method is that it is labour intensive as it requires an extraction, separation and derivatisation procedure, taking approximately 6-8 hours to analyse 12-15 samples. Enzyme immuno assays (EIA) and HPLC methods have also been developed with problems of overestimation in the former and sensitivity in the latter.

Thus it was described to try and develop a new HPLC MS-MS method with minimal sample preparation and a short run time to increase the sample throughput. However, the developed assay when tested on laboratory control samples could not reliably detect and quantify the metabolite. Three major problems were observed. The first was a lack of sensitivity presumably because of ion suppression (Figs.3.3.10 and 3.3.11); the second problem was that of specificity caused by the presence of multiple peaks (Fig.3.4.1). Finally, there were inconsistencies in the retention times of both 8-iso- $PGF_{2\alpha}$ and the deuterated internal standard (d4-8-iso- $PGF_{2\alpha}$).

It was then decided to develop a method to measure urinary vitamin E metabolites and investigate whether an oxidised vitamin E metabolite (α-TLHQ) could be used as a possible biomarker of oxidative stress.

Vitamin E metabolites are excreted in the urine as sulphate or glucuronide conjugates. The polar nature of these conjugates makes it difficult to analyse them directly using routine techniques such as high performance liquid chromatography (HPLC) or gas chromatography mass spectrometry (GC-MS). Therefore, in all previous methods, the metabolites were deconjugated either enzymatically or by acid hydrolysis prior to their analysis by HPLC or GC-MS. We aimed to establish and validate a method for measuring intact conjugates of urinary metabolites of vitamin E, as it has been suggested that α-tocopheronolactone (α-TLHQ) with an oxidised chromanol ring could be used as an indicator of oxidative stress. A primary objective of developing an LC-MS/MS assay to measure the intact conjugates was to reduce artefact formation, as the artefactual oxidation of α-CEHC to α-TLHQ cannot take place if the metabolites remain conjugated.

Using LC-MS/MS, conjugates of α-TLHQ were detected and quantified, thus proving unequivocally that it is a real metabolite produced *in vivo* and not a product of artefactual oxidation. The new method reduced the experimental time to 20 min compared to the established methodology of Pope et al; 2000 which took approximately 9 hours to prepare the vitamin E metabolites. The assay was also observed to be highly reproducible with the inter- and intra-assay coefficient of variation being less than 5 % (Table. 3.2.3) compared to 21.9 and 64.7 % for α-TLHQ (intra- and inter-assay) in the method using GC/MS developed by Pope and co-workers (2000).

When the conjugated urinary vitamin E metabolites were analysed in a group of children with type 1 diabetes, the mean concentration of the α-TLHQ metabolites were significantly increased in the patients with diabetes compared to controls ($p<0.001$), whereas the α-CEHC conjugates were not increased to the same degree of significance ($p<0.05$). This suggests that the results cannot be explained by a non-specific increase in urinary excretion of the conjugated metabolites.

Nitric oxide ($NO^{\bullet}$) has been implicated as a source of oxidative stress in human physiology (section 1.1.4). It has particularly been studied in its role in vascular integrity (Moncada & Higgs 2006). In the current study we aimed to develop a method to quantitate amino acids that are involved in the homeostasis of $NO^{\bullet}$ metabolism and might indicate the oxidative stress status of the individual.

A method was developed to measure in a single run amino acids involved in $NO^{\bullet}$ metabolism i.e. L-arginine (precursor of $NO^{\bullet}$), L- citrulline (product of $NO^{\bullet}$ formation), L-ADMA (inhibitor of NOS) and L-homocysteine (decreases bioavailability of $NO^{\bullet}$). The

method which used FMOC as the derivatising agent also enabled us to quantitate seven additional amino acids (each representing a different group) in a single run. The method was validated and it was observed that all 11 amino acids could be quantitated at concentrations agreeing with the reference ranges reported by Tan and Gajra (2006). The method was then applied to investigate the amino acids status in children with Type 1 diabetes mellitus and age -matched controls.

The urinary concentrations of all the amino acids were found to be significantly increased in the diabetic patients as compared to their age-matched controls (section 5.3.4). The reasons for the high urinary excretion of the amino acids in the diabetic patients is likely to be multifactoral including a) early stage progression of nephritis, b) proximal tubule damage and c) disturbed re-absorption of the amino acids in the proximal tubule in the kidney.

Diabetic nephropathy is a complication that is considered to be a public health problem. At the onset of the disease, the glomerular filtration rate (GFR) is high. It starts to decline after approximately 5 years. The decline is sluggish, but over a time scale of about 20–25 years it inexorably leads to end-stage renal disorder (ESDR). The appearance of microalbuminuria, which may occur after 10 years, heralds early renal damage (Locatelli 2003).

In this study the patients were non-symptomatic and had normal urinary microalbumin and creatinine concentrations. The result obtained in this study suggests that abnormal renal function occurs earlier than previously thought. It will therefore be interesting to follow amino acid excretion and kidney damage during the progression of diabetes. It would also

be interesting to speculate if these changes could be reversed/minimised by the prescription of anti-angiotensive drugs by clinicians to reduce the glomerular filtration rates.

To fully validate the use of urinary conjugates of α-TLHQ as a potential biomarker of *in vivo* oxidative stress and the use of amino acids associated with NO metabolism as indicators of early abnormal kidney function, it will be necessary to carry out the following studies:

- Follow urinary α-TLHQ concentrations longitudinally in diabetes subjects
- Investigate urinary α-TLHQ concentrations in other disease conditions where oxidative stress status is well documented such as cardiovascular diseases, Alzheimer's Disease, Parkinsons Disease, Down Syndrome, stroke, exercise and smokers
- Compare the α-TLHQ concentrations obtained in the above studies to the findings of existing biomarkers of oxidative stress such as isoprostanes, 8-hydroxy-2'-deoxyguanosine and TBARS which are well documented in these disease conditions
- Undertake longitudinal studies should be undertaken to measure the urinary amino acids concentrations during the course of diabetes and correlate the findings with existing clinical markers such as Hba1c%, urinary albumin concentrations and the plasma lipid profile.
- Correlate urinary concentrations of the amino acids should be correlated with plasma concentrations in longitudinal studies in diabetic subjects.

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